Mushroom Science XIX

Science and Cultivation Of Edible Fungi



Edited by J.J.P. Baars & A.S.M. Sonnenberg

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SCIENCE AND CULTIVATION OF EDIBLE AND MEDICINAL FUNGI

Edited by

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FOREWORD

The papers and posters published in these proceedings were all presented at the 19th Congress of the International Society for Mushroom Science in Amsterdam, the Netherlands. At total of 280 abstracts were submitted for either an oral presentation or a poster presentation. Abstracts were received from scientists and mushroom professionals from a total of 43 different countries. The abstracts were assessed by two independent reviewers chosen from the scientific committee. On basis of their judgements, a total of 118 submitted abstracts were selected for oral presentations. The other presentations were accepted as poster presentations.

Those scientists that were invited to give an oral presentation, were asked to send in a short paper, describing the main content of their presentation. Not all scientists chose to do so. Therefore not all oral presentations and posters are represented in these proceedings.

We have made a great effort to present all contributions in a uniform format. Please accept our apologies if there are any errors that have been overseen or caused by the editorial process.

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The XIXth Congress of the International Society for Mushroom Science (ISMS) was jointly organized by the Dutch Mushroom Days Foundation, the Southern Agriculture and Horticulture Organization (ZLTO), WageningenUR and CAOS in conjunction with the 34th edition of the the Dutch Mushroom Days.

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The International Society for Mushroom Science (ISMS) supports the development of the mushroom industry globally by facilitating the application and communication of science related to the mushroom business. It is non-political and non-profit making. ISMS seeks to further the cultivation of edible (including medicinal) macrofungi through the dissemination of information on new developments and the science of mushrooms and to stimulate the exchange of new ideas between growers and scientists around the world. The primary ISMS event is the International Mushroom Congress, which occurs every 3-5 years. Membership of ISMS is open to all and it aims to benefit all. More details can be found at its website; www.isms.biz.

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Mushroom Science

Year	*Location*	*Volume No.*		*Published*
1950	Peterborough	I		1951
1953	Gembloux	II		1954
1956	Paris	III		1957
1959	Copenhagen	IV		1960
1962	Philadelphia	V		1963
1965	Wageningen & Amsterdam	VI		1967
1968	Hamburg	VII		1989
1971	London	VIII		1972
1974	Tokyo & Taipei	IX	Part I & II	1976, 1977
1978	Bordeaux	X	Part I & II	1979
1981	Sydney	XI	Part I & II	1981
1987	Braunschweig	XII	Part I & II	1989
1991	Dublin	XIII	Part I & II	1991
1995	Oxford	XIV	Part I & II	1995
2000	Maastricht	XV	Part I & II	2000
2004	Miami	XVI		2004
2008	Cape Town	XVII		2008
2012	Beijing	XVIII		2012
2016	Amsterdam	XIX		2016

Welcome to an exciting week on mushrooms!

The Mushroom Days Foundation is privileged to welcome you to the 19th International Society for Mushroom Science (ISMS) conference. Together with the 34th edition of The Mushroom Days it forms a unique event that is not to be missed by those, involved in the global mushroom industry.

Mushrooms fascinate every one of us, and even after many years of research we are still eager to know more about them. We hope to have created the right atmosphere for you during the various items of this event, to experience a memorable week and to extend your knowledge and network within our industry.
And of course the city of Amsterdam is great fun to visit. I hope you have some time left for it.

Piet Lempens
Chairman Organising Committee



Welcome, Welcome, Welcome!

Welcome everyone to what will be another amazing ISMS Congress.

An ISMS Congress only comes around once every four years or so and is the “jewel in the crown” of the international mushroom calendar. The combination of scientists, students, and research workers getting together with growers, traders, and suppliers from all parts of the globe to learn about the latest discoveries and new technologies, has been a highly successful recipe for over half a century. This Amsterdam Congress will continue that fine tradition.

This year the 19th Congress will be extra special as it takes place in conjunction with the industry’s biggest trade show, Mushroom Days. These two mega events in the same week in the same geographic region will be a once in a lifetime occasion for our entire industry to come together. It is an excellent opportunity to not only see, hear, and touch the latest research and innovation, but also meet fellow mycophiles from all over the world.

No matter where you are from, how long you have been in the industry, or whether you are an ISMS veteran or a first time Congress delegate, you will have the opportunity to be part of a very special “family” during the week of the Congress – the ISMS Mushroom Family. It's a magnificent feeling that I welcome you to experience first hand, and share with your fellow delegates.

Experience tells me you will get the most out of your attendance if you actively participate in all aspects of the program day and night. Attend as many sessions as you can, visit the poster area during the breaks, say hello to as many of your fellow delegates as possible and celebrate the joy of being part of a unique industry.

An ISMS Congress also provides a brief but important opportunity for members and the wider industry community to engage with the Executive Committee and the Council about the Society. I encourage all delegates to attend the General Assembly on Monday evening. It will be a rare opportunity to discuss issues related to ISMS now and into the future.

An enormous amount of work goes into organizing an ISMS Congress. Thank you to the Amsterdam Organizing Committee for creating a tremendous platform for us all the enjoy this 19th Congress. I acknowledge the special efforts of Chair, Piet Lempens, and Eva and her team at CAOS. Thank you to the Scientific Committee headed up by Dr Anton Sonnenberg and Dr Johan Baars, and all those who submitted abstracts, papers, and posters, and those who reviewed those contributions.

Thank you to Peter Christiaens and our generous sponsors for helping make a professional event possible. Finally, a very big thank you to you, our delegates – without you there is no Congress. I have little doubt that this 19th ISMS Congress in Amsterdam will be a memorable and rewarding event for everyone. Please enjoy!

Greg Seymour

President, ISMS

I Keynote Lectures

Science and Cultivation of Edible Fungi, Baars & Sonnenberg (ed.)
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Summary of the keynote lectures.

Johan Baars and Anton Sonnenberg

WageningenUR Plant Breeding, Wageningen, NL

The 19th ISMS congress in Amsterdam offered a program with a lot of variation. The congress started with a keynote lecture by Günther Pauli, who is a gifted speaker. In his presentation "The economic, ecologic and social impact of community based mushroom farming" he urged the audience to pay more attention to implementation of basic mushroom growing skill in poor communities. This would be a way to alleviate poverty. He made a plea for a bigger social impact when introducing mushroom cultivation as a new crop to poor communities. As an example he talked about his adopted daughter who started her own mushroom growing company in Zimbabwe.

The second keynote lecture, "Future farming" given bij mrs. Elies Lemkes-Straver from the Dutch growers organisation ZLTO gave an overview of her vision on innovative trends in the mushroom industry.

Prof. Fred Brouns (Maastricht University) gave the third keynote lecture "Mushrooms: an ancient food source with gaining health interest", addressing the question whether science supports the benefit statements. His message was that much of the research that is published is only indicative of a possible health effect. Mushroom health science should be taken to a higher quality level in order to be able to convince the medical world. A more elaborate summary of his lecture is given elsewhere in these proceedings.

Prof. Willem de Vos (lecturing professor at the Wageningen and Helsinki University) gave an intriguing lecture on what is in our guts, some fungi but mainly bacteria. They play a very important role in our health and Prof de Vos made clear that many disease are related to the gut flora but solutions to some persistent disease may also lay within manipulating/changing the gut microbiome.

Prof Chen (Department of Cancer Biology, Beckman Research Institute of the City of Hope, Duarte, CA, gave an inspiring lecture on results of a phase 1 clinical trial of white button mushroom in patients with biochemically recurrent prostate cancer. Some details on this topic can also be found on YouTube (https://www.youtube.com/watch?v=8mmHJGRsOR0).

Prof Daniel Eastwood (Swansea University) gave an interesting key note lecture on the way in which study of a large number of genomes of wood decaying fungi can give insights on the evolution of fungal wood decay.

The final keynote speaker was Prof Han Wösten (University of Utrecht), who gave an overview of their work on the use of fungi for other purposes than consumption: As a source for hydrophobins with many industrial applications and the use of fungi as packing materials and even clothing.

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Science and Cultivation of Edible Fungi, Baars & Sonnenberg (ed.)
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Mushrooms: an ancient food source with gaining health interest.

Prof Dr Fred Brouns

Maastricht University, NUTRIM school of Nutrition and Translational Research in Metabolism
Scelta Institute, Venlo, Netherlands

Humans have been eating mushrooms most probably since mankind exists and trial and error will have learned what is edible and what not. There is not much documentation, though, on their consumption. Some historical reports mention their use for religious and spiritual sessions during which the use of specific mushrooms with brain function modifying properties helped to “open up for free en creative thinking” or “bring the Ghosts to the present” Anecdotal reports from japan and China mention their use for thousands of years in alternative medicine and natural healing practices where medicinal properties were thought to be induces by what later became known as specific oligo- and polysaccharides, proteins and bioactive peptides, tannins, lectins and polyphenols. These and other bioactive components are being claimed to be effective in reducing the risks of various cancers and also useful as adjunct therapy in treating disease. In Europe many of these claims, which are largely based on in vitro and animal studies, are not allowed in the market for lack of specificity of compounds and data from well-performed human intervention studies. Commercially widely available mushrooms such as white button mushrooms (WBM), are characterised by a favourable nutritional value. Having high water content and very low fat content they have a very low caloric value. Yet, WBM contain 3.5-4% protein (in comparison, milk 2.0-3.3%),with a relatively high presence of aspartate, glutamine and arginine. In addition WBM are high in dietary fibre, mainly as chitin and betaglucans which are are known to have favourable effects on gut microbiota and gut associated immune support. The sugar content in WBM is low (<5g/100g) and comprises mostly mannitol, trehalose and glucose. As such WBM fit very well in weight maintenance and cholesterol lowering diets. In general WBM contain about 30kcal/100g, comparable to broccoli, pepperoni and cauliflower. WBM is relatively rich in selenium, potassium and B vitamins and are the only non-animal food that contains significant amounts of Vit D2 after exposure to UV light. This review will highlight comparative aspects of common commercial mushrooms composition, along with health related interactions.

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II Research and innovations in the industry

Science and Cultivation of Edible Fungi, Baars & Sonnenberg (ed.)
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Incidence and fate of *Listeria monocytogenes* during mushroom growing, packing, and processing

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ABSTRACT

Listeria bacteria are widely found in agricultural environments including soils, surface water, decaying vegetation, and animal feces. Only one species, *Listeria monocytogenes*, is capable of causing human illness. *L. monocytogenes* is of particular concern in ready-to-eat fresh and minimally processed produce because it is able to grow in continuously wet and cool growing, packing, and processing environments and it is difficult to eradicate once it becomes established. Fresh commercially grown button mushrooms (*Agaricus bisporus*) have not been implicated in any cases of listeriosis to date. However, in North America, a succession of recalls of sliced mushrooms found to contain *L. monocytogenes* has occurred. Our food safety research program has demonstrated that *Listeria* spp. can be found in mushroom production and processing environments at levels similar to other food types. However, standard industry production practices include steps that decrease contamination risks. In a microbial survey of a small-scale mushroom research farm on the Penn State University campus, 29 of 184 samples (15.8%) were positive for non-pathogenic species of *Listeria*. *L. monocytogenes* was found at 3 sites (1.6%), each within the phase 1 ingredient receiving and compost preparation area. However, composted substrate is not a likely source of mushroom contamination since earlier work we conducted demonstrated that an industry standard 6-day phase 2 pasteurization and conditioning protocol was capable of achieving complete inactivation of inoculated (7 log/cfu/gm) *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7. The microbial ecology of casing soil, which is typically not heat treated, plays in a role in minimizing mushroom contamination risks. For instance, we showed that up to 4 log reductions of *L. monocytogenes* and *Salmonella* spp. inoculated into un-treated light peat-based casing soil occurred between casing and pinning, while no reductions occurred in previously autoclaved soil. Furthermore, when light peat soils were supplemented with up to 40% dark peat, which has lower levels of background microorganisms, the rate of pathogen destruction decreased slightly. In a year-long post-harvest microbial survey of 255 non-food contact surfaces in a mushroom packing and slicing facility, we showed that floors, walls, drains, and equipment surfaces in continuously wet areas can become harborage sites for persistent strains of *L. monocytogenes*. However, our mid-study recommendations for sanitation improvements dramatically decreased the incidence from a maximum of 31% of collected samples to 8.5%. Continuing education on cleaning, sanitizing, and facilities maintenance is therefore critical for maintaining the industry's excellent record for producing safe products.

Keywords: (Listeria, Salmonella, compost, casing)

INTRODUCTION

Concerns over contamination of fresh produce with *Listeria monocytogenes* have increased in recent years as a succession of recalls and listeriosis outbreaks have occurred. Although several recalls of North American grown mushrooms have occurred, to date, there have been no known outbreaks of listeriosis attributed to consumption of commercially grown *Agaricus bisporus* mushrooms. With the recent passage of the U.S. Food Safety Modernization Act (FSMA), a risk based approach toward proactive identification of potential hazards and development of science based control measures has become mandatory in most of the food industry. In this paper, a summary of research conducted at Penn State to identify sources of human pathogens in mushroom growing, packing, and slicing facilities and current industry practices to control them is presented.

1. Results & Discussion

1.1. Mushroom farm environment

Sources of *Listeria* spp. in the mushroom growing environment was studied by conducting a microbial survey on a small-scale mushroom research farm on the Pennsylvania State University campus *(4)*. A total of 184 swab samples were taken from floors, walls, and equipment within 5 production zones; 1) phase 1 composting area, 2) phase 2 composting area, 3) tray filling line, 4) growing rooms, and 5) a walk-in cooler. *Listeria* spp. were detected in 29 (15.8%) of samples. Non-pathogenic *L. innocua* was the most common species found with *L. monocytogenes* recovered only from 3 sites in Zone 1 where composting ingredients, including straw bedded horse manure, were mixed prior to phase 1 composting (Table 1).

These results are not unexpected given that many other studies have shown that *Listeria* spp. are commonly found in agricultural environments *(1)*. Interestingly, *L. monocytogenes* appears confined to animal manure areas and did not spread to other areas of the farm. It is speculated that non-pathogenic *Listeria* spp., that more efficiently utilized available nutrients, may have competitively excluded *L. monocytogenes*. Despite the absence of pathogenic *Listeria* in Zones 2-5, the presence of other *Listeria* spp. indicates environmental conditions that can support survival and growth of *L. monocytogenes* and that a review of sanitation practices is recommended.

TABLE 1. Incidence of *Listeria* spp. in a mushroom farm environment.

Zone	Number of samples	Number of isolates	*Listeria* spp.*					
			Lm	Lin	Lw	Lg	Ls	Liv
1	50	20	3	13	4	0	0	0
2	30	3	0	2	0	1	0	0
3	40	2	0	2	0	0	0	0
4	50	4	0	2	2	0	0	0
5	14	0	0	0	0	0	0	0
Total	184	29	3	19	6	1	0	0
% Prevalence		15.7	1.6	10.3	3.3	0.5	0	0

*Lm, *L. monocytogenes;* Lin, *L. innocua;* Lw, *L. welshimeri;* Lg, *L. grayi;* Ls, *L. seeligeri;* Liv, *L. ivanovii*

1.2. Mushroom composting

The fate of human pathogens during phase 2 composting was studied. Finished phase 1 substrate was inoculated with *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* spp., placed into sealed plastic bags, and heated in a controlled-temperature water bath at 48.8, 54.4, and 60°C. Initial pathogen inoculum values of 6-8 log/gm were reduced to non-detectable levels, determined by plating or after enrichment, after 36.0, 8.0, and 0.5 hours, respectively. Decimal reduction times (D-value) at 54.4°C demonstrated that *L. monocytogenes* and *E. coli* O157:H7 were the most heat tolerant followed by *E. coli*, total coliforms, *Enterobacteriaceae*, and *Salmonella* spp. *(5)* (Table 2).

TABLE 2. $D_{54.4}$ -values for thermal destruction of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* spp. in inoculated substrate and indicator organisms in uninoculated substrate.

Microorganism	$D_{54.4}$ (min)*
L. monocytogenes	27.0[a]
E. coli O157:H7	22.1[a]
Generic *E. Coli*	13.9[b]
Total coliforms	13.9[b]
Enterobacteriacae	13.5[b]
Salmonella spp.	10.8[b]

*Means values followed by the same letter are not significantly different (α = 0.05).

To simulate commercial phase 2 conditions, phase 1 substrate, inoculated with a 3-pathogen cocktail, was subjected to an industry standard 144-hour phase 2 pasteurization and conditioning treatment at the Penn State Mushroom Research Center (MRC) *(5)*. Phase 1 substrate (2.50 kg) and the 3-pathogen cocktail were mixed thoroughly and placed into polypropylene bags, each with a 3 X 6 cm perforated filter patch to allow air diffusion. Each bag was placed in the center of a standard wooden mushroom growing tray (61 X 61 X 30 cm) and uninoculated phase 1 substrate was filled around and above each bag until tray capacity was reached. Pasteurization was considered complete by the MRC operator when the temperature of the air and the lowest reading probe in the substrate reached 60°C for 2 h. Gradual reduction of temperature during the conditioning period continued until the end of the 6-day process, after which the bags were removed for laboratory analysis. Pathogen levels, initially between 7-8 log/gm, were not detectable by direct plating or enrichment. *E. coli*, coliforms, and *Enterobacteriaceae* were also below detectable levels. Aerobic plate count levels, initially at 9 log/gm, were reduced by only 2 log values, most likely due to survival of non-pathogenic thermophilic bacteria and fungi. The results of these studies show that the phase 2 composting process can eliminate risks associated with pathogens in raw animal manure. Growers are encouraged to verify complete destruction of pathogens, in particular *L. monocytogenes*, for their own composting processes through periodic compost testing and to conduct studies to assure uniform distribution of phase 2 substrate temperatures.

1.3. Mushroom casing

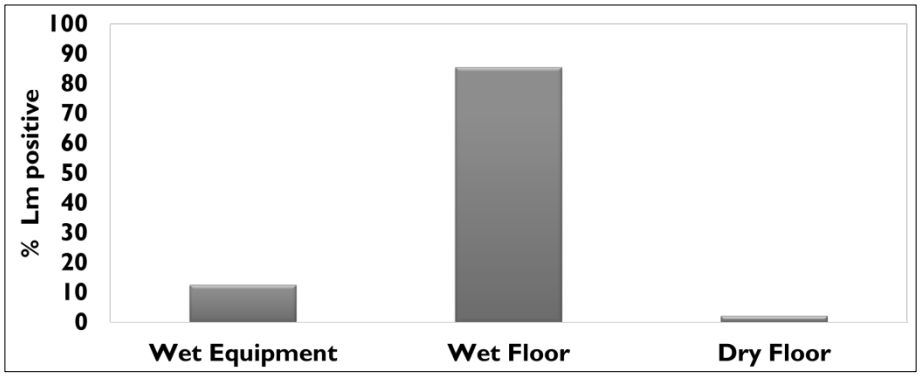
The fate of *L. monocytogenes* and *Salmonella* spp. in casing soil over the course of a complete spawn to harvest production cycle was studied by growing *Agaricus bisporus* mushrooms in 1-L plastic containers filled with casing soil overlaying phase 2 substrate *(3)*. Limestone neutralized casing soils were prepared at light:dark sphagnum peat ratios of 100:0, 80:20, and 60:40, mixed

with *Agaricus bisporus* spawn, and then inoculated with *L. monocytogenes* and *Salmonella* spp. at approximately 8 log/gm. Soil samples were periodically taken until the emergence of harvestable mushrooms. Background populations of aerobic bacteria, *Actinomycetes*, and yeasts + molds in the 100% light peat casing soil were on average 8.4, 7.8, 6.8 log CFU/gm, respectively, compared to either of the dark peat casing soils which averaged 6.6, 6.7, and 5.2 log CFU/gm. When control casing soils were heat-sterilized prior to pathogen inoculation, each at approximately7 log/gm, pathogen levels did not significantly change for up to 20 days. However, in non-heat treated soils, inoculated pathogen levels decreased by 3-4 logs after 20 days. Reductions for each of the pathogens was slightly, but significantly ($P \leq 0.05$), more rapid in 100% light peat casing soil suggesting that harmless background flora competitively inhibit human pathogens. Because pathogen reduction is not complete, growers should not accept peat and lime from suppliers unless absence of pathogens has first been verified. Furthermore, casing preparation and storage areas should be designed to prevent environmental contamination of casing ingredients and soil.

1.4. Mushroom packing and slicing environment

An *Agaricus* mushroom packing and slicing facility was surveyed for the incidence of *Listeria* spp. Of 255 non-food-contact environmental samples taken during 3 periods over 13 months *(2)*, 20.1% were positive for *L. monocytogenes*. The pathogen was found mainly in washing and slicing areas where floors were continuously wet and covered with mushroom debris (FIGURE 1). This level of contamination is consistent with studies conducted at other food production facilities *(1)*. Serotype 1/2c was the predominant (93.5%) serotype, followed by 1/2b (3.5%) and 1/2a (3%). Multi-virulence-locus sequence typing (MVLST) revealed all 1/2c isolates were Virulence Type 11 (VT 11) indicating that VT 11 had colonized the facility and thus was the persistent and predominant subtype. After period 2, efforts to repair floor cracks, review cleaning and sanitizing practices, and re-train the sanitation crew were conducted, the prevalence of *L. monocytogenes* decreased from 18.8% in Period 1 and 32.5% in Period 2 to only 9.2% in Period 3. This information can assist the industry design targeted intervention strategies to further reduce *L. monocytogenes* contamination in fresh mushroom packing and slicing facilities.

FIGURE 1. Frequency of *Listeria monocytogenes* postive samples sorted by location and environmental conditions.



From these studies, we have learned that *L. monocytogenes* can be found in mushroom growing, packing, and slicing environments. On the farm, phase 2 composting can completely eliminate this, and other manure-associated pathogens, from growth substrate. Pathogen reductions occur in un-

sterilized casing soil between casing and harvesting, although preventing peat and lime contamination is important to reduce mushroom contamination risks. *Listeria* spp. can colonize mushroom packing and slicing facilities. Continuous review and improvement of sanitation practices, along with regular environmental microbial verification testing, is essential to keep this pathogen under control.

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Comparison of Diffusive Gas Exchange Capacity of Filtered Spawn Bags

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ABSTRACT

In this experiment a comparison was made of 4 different spawn bags (SacO2, Bischof & Klein, Unicorn 3T and Unicorn 3B). Equal amounts of substrate were inoculated with equal amounts of *Lentinula edodes* spawn. Of every type 5 repetitions were executed resulting in a total of 25 bags. The gas distribution was measured daily for 15 days in all bags. Results showed that during the spawn run, the internal buildup of considerable CO_2 concentrations was observed in all bags. Vitality tests showed that mycelium grown in higher CO_2 concentrations showed more vigor than mycelium grown in lower CO_2 concentrations. Inside the bags however, these concentrations are highly dependent on the filter type and position. Most bags developed an increasing CO_2 gradient with the progression of the spawn run. Filter strips, located on the headspace (Bischof & Klein), induced the highest gradients (90% gradient), followed by bags with filter patches in the headspace: Unicorn 3T (67% gradient) and Unicorn 3B (35% gradient). The bags with filter strips touching the spawn ($SacO_2$) showed little or no CO_2 gradient (5% gradient). Leap-off test results showed that kernels grown in higher CO_2 concentrations were faster to gain a foothold on the petri dish than kernels which endured lower concentrations. Also, mycelium that showed suffocation symptoms showed little to no growth the first few days, but picked up speed after establishing first growth. Further testing in combination with a number of other parameters, like O_2 concentrations and temperature, will be conducted.

INTRODUCTION

Mycelium secretes exo-enzymes to decompose larger molecules in the substrate for which it needs oxygen. Without this electron acceptor, the mycelium is unable to release the energy from its nutrients and will stop growing or perish (Leatham & Leonard, 1989). Donoghue & Denison (1995) have shown this for impermeable bags where mycelial growth stopped after depletion of O_2. In breathing bags CO_2 concentrations will increase linearly with colony area until full colonization after which it will level off (Kinugawa & Tanesaka, 1990). Studies have shown that CO2 concentrations of up to 10% (Grabbe et al., 1984) and 20% (Zadrazil & Schliemann, 1975) are beneficial for mycelium growth. It must be noted that this was tested for only a limited number of lignicolous mushrooms (*Pleurotus ostreatus* and *Flammulina velutipes*).

Since it is essential in spawn and substrate production to obtain a pure culture after inoculation, the key is to allow optimal gas exchange without letting any contaminants enter the bag (Donoghue & Denison, 1995; Fehér, 1996; Sánchez, 2003). Moreover, the rate of mycelial development depends on the oxygen supply, achieved by air spaces present in the substrate and by gas exchange through permeability of the substrate and suitable micro-

porous filters (Leatham & Stahmann, 1987). Modern spawn bags are all so-called 'breathing bags' with filters sealed into place, either as filter patches or as filter strips crossing the bag's surface. In general, there are two types of filtration. Membrane filtration is the separation of 2 fractions by transportation through a permeable membrane. The success of membrane filtration relies entirely on the pore size of its material. The filter strips consist of non- woven, hydrophobic polypropylene material. This labyrinth of resilient fibers will trap any contaminant inside its fabric, equivalent to HEPA-filtration systems and is a form of depth filtration.

1. Materials and methods

In this experiment bags from different producers have been tested:

- $SacO_2$ PP75-BEU6-X32-57: 6 filter strips equally spaced over the substrate (S2)
- Bischof & Klein: 1 filter strip in the headspace (BK)
- Unicorn Type 3B: 1 filter patch in the headspace (UB)
- Unicorn Type 3T: 1 filter patch in the headspace (UT)
-



Fig. 1: from left to right: Bischof & Klein (BK), SacO2 (S2), Unicorn 3B (UB) and Unicorn 3T (UT)

The experiment was conducted on 4 bag types and was repeated 5 times resulting in a total of 25 bags tested. Each bag was fitted with 4 self-healing injection ports in a vertical line for samples to be taken in the headspace (Head) and the upper (High), middle (Mid) and lower (Low) layer of the spawn. Substrate recipe, volume and inoculant were kept the same for each bag. The bags were sterilized at 120°C for two hours, except for BK bags which were sterilized empty at 110°C for 30 minutes and filled with presterilized grain in front of a laminar flowhood. Inoculation was done in front of a laminar flowhood and after sealing and shaking, bags were taken to an incubation room at 25°C and 50% RH.

A Senseair K33% BLG CO2 sensor was used for daily sampling. To ensure the sterility of the spawn, the sample was not reinserted in the bag afterwards. The influence of this 100 ml loss of air per day on the total air volume of 7500 ml per bag on gradients inside the bags is considered negligible for this experiment.

After 15 days, the bags were placed in a refrigerator at 5° C for two days. From the center of each spawn layer a single grain kernel was picked and placed on a malt extract agar plate (1 l H_2O, 45g agar, 30g ME). The plates were incubated at 20° C in atmospheric CO_2 concentrations. The diameter of the colonies was measured after 3, 7, 10 and 14 days.

2. Results and discussion

Colonisation of the bags

The Bischof & Klein (BK) bags were fully incubated after 7-8 days, followed shortly by the $SacO_2$ (S2) spawn bags at 8 days. Both Unicorn bags were last to finish after 15 and 12 days for respective the B-filter (UB) and T-filter (UT). Differences were noticed in the colonies forming from the inoculum. S2 and BK bags both demonstrated healthy mycelium with vigorous growth and fuzzy edges. Both UB and UT bags were slower to kick-start and were characterized by sharply outlined edges of the colonies. It must be noted that only UB presented suffocation symptoms which were expressed by layered colonization. In the latter, the top layers of the spawn grew normally but there was slow colonization or none at all in the bottom layers. Eventually a crust was formed at the top, which reduced the gas exchange at the bottom of the bag even more.

CO_2 distribution

From the beginning, the increase in CO_2 is noticeable when measuring further away from the headspace. Bischof & Klein and both Unicorn models have significantly higher concentrations in the lower regions than in the headspace whereas S2 bags show more evenly distributed concentrations. Kinugawa & Tanesaka's claim (1990) of leveling CO_2 concentrations after full colonization did not take place or had not yet taken place in this experiment.

An interesting anomaly was observed in the UB bags as the CO_2 concentrations in the upper and middle regions of the spawn are 13,5% higher than in the lowest measuring location. Considering that the lower regions of the spawn appear suffocated. UT did not display the same symptoms as UB but did demonstrate slow colonization. Both S2 and BK bags show more vigorous growth which could explain the higher CO_2 concentrations.

To quantify and visualize the influence of the filter and more so the distance of the spawn to the filter, the increase in CO_2 concentration has been calculated relative to the headspace concentrations. The used absolute values are measured on the day the bag finished colonizing.

Table 3: Relative increase in CO_2 concentrations in function of increasing distance to headspace

	HEAD	HIGH	MID	LOW
S2	0	+4,80%	+5,12%	+4,07%
BK	0	+39,75%	+71,80%	+89,64%
UB	0	+25,96%	+41,66%	+35,43%
UT	0	+27,84%	+50,64%	+66,52%

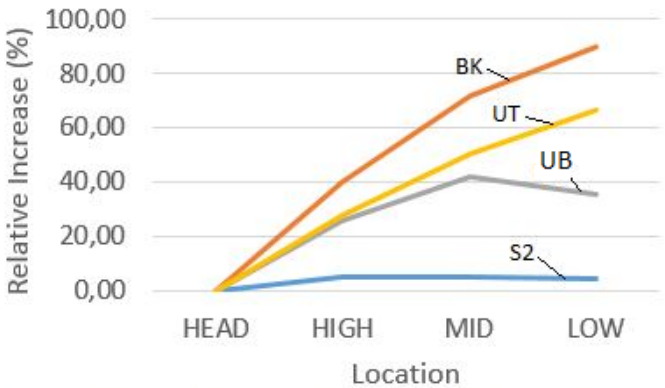
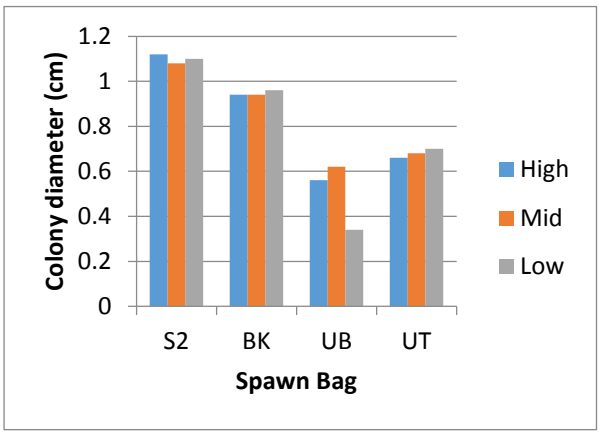


Fig. 2: Relative increase in CO_2 concentrations in function of increasing distance to headspace

Bischof & Klein spawn bags show the highest increase in CO_2 as bottom concentrations are nearly 90% higher than headspace values. For S2 bags the opposite is true. In these bags there is hardly any difference between the top and the bottom: 5%, 5% and 4% for respectively high, mid and low sections of the spawn compared to the headspace. The fact that there is no gas exchange in the headspace should be taken into account though. Nevertheless, the difference between the measured values in the spawn is almost non existing compared to the other brands. This is clear evidence that not only the filter material but also the filter position and distribution are of crucial importance for the CO_2 distribution.
The UT bags show the same graph as BK, but with a less steep inclination. The CO_2 concentrations increase with 28%, 51% and 67% with increasing distance to headspace.
The UB bags deviate from an upwards curve as the bottom concentrations are lower than the middle concentrations. Considering the suffocation symptoms, this could raise the idea of CO_2 being an indication for growth in these types of bags.

Spawn vitality

Kernels which had been exposed to higher CO_2 concentrations were faster to colonize the petri dish in the first 3 days. Hence, BK and S2 were the first to reach the rim of the plates, followed by UT and UB bags. No significant difference in average colony size was recorded from kernels harvested from the same bag. However, there were significant differences in colony size between S2 & BK and UB & UT. Previous studies (Zadrazil & Schliemann, 1975) have also shown a more vigorous growth in rising CO_2.



Even though leap-off was slower with UT and UB kernels, the colonies had no problem picking up speed over the following days. Only UB kernels collected from the bottom of the bags were significantly slower in the first 3 days and had an especially hard time gaining a foothold on the agar. When colonies reached 1 cm diameter, they caught up and the speed was again comparable to the other colonies.

Fig. 3: Colony diameter after 3 days of kernels harvested from 3 locations (high, mid and low) from different spawn bags

CONCLUSION

Evaluation of all collected data gave an interesting insight on gas distribution in spawn bags and its consequences for the mycelium. $SacO_2$ and Bischof & Klein displayed the highest CO_2 concentrations, up to 15,63% and 14,25% respectively, of the experiment. The bags were characterized by fast and thorough growth and 100% colonization after 8 and 7-8 days respectively. In the Unicorn 3B and 3T bags however lower concentrations, up to 13,72% and 12,09% respectively, were measured. These bags were significantly slower to colonize as they took almost twice as long to finish, 12 and 15 days for respectively 3T and 3B filters.

The mycelium in UB bags also showed suffocation symptoms near the bottom, characterized by pale mycelium and slow expansion of the colonies. These findings support the idea that CO_2 is an indication for growth and more so of colonization speed. The distribution of the CO_2 throughout the bag was examined and deemed crucial to obtain a uniform product.
Furthermore, the results from the leap-off test clearly indicated that high CO_2 concentrations during colonization resulted in faster leap-off from the kernels and vice versa. $SacO_2$ and BK samples showed more vigorous growth compared to the smaller colonies formed by the Unicorn bag samples. The kernels taken from the suffocated regions in the UB bags showed the slowest leap off time of the experiment. This clearly indicates that gas balance and position of the filters have a crucial effect on spawn health.
For more definite conclusions to be drawn, further testing is necessary and will include oxygen and temperature measurements.

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Technological and innovation needs of small scale mushroom growers in Europe

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ABSTRACT

Cultivation of grey Oyster (*Pleurotus ostreatus*), king Oyster (*Pleurotus eryngii*), Shiitake (*Lentinula edodes*), Enoki (*Flammulina velutipes*), Nameko (*Pholiota nameko*), Pioppino (*Agrocybe aegerita*) and Maitake (*Grifola frondosa*) is steadily increasing in Europe, however, the market remains small. Most of the exotic mushroom species are mainly produced by small scale- or even micro scale enterprises. The particular technological and innovation needs of the growers involved in small-scale production of exotic fungi were analyzed. The local mushroom producers in three different countries (the Netherlands, Germany and Greece) were interviewed using the standardized audit forms developed within the EU-Trafoon project (Traditional Food Network to improve the transfer of knowledge for innovation). The strengths and weaknesses as well as the opportunities and threats of the exotic mushroom industry were identified. Based on the results, new strategies and specific action points were initiated. For instance, issues related to substrate production, cultivation units (trays, bags or bottles), product development (new varieties of certain exotics), labeling (product-specific properties) and certification (organic cultivation) were prioritized. Although the production in Europe aims at the fresh market, application of the appropriate postharvest technology can reduce the losses associated with surplus production enhancing the income of the growers by value-addition. Marketing and business models were also developed to enable small enterprises to upgrade and up-skill through the networking and entrepreneurial training. Training activities for small scale mushroom growers were organized, aiming at the transfer of knowledge and application of existing research results of national and European projects in order to foster sustainable innovation and enhance competitiveness in the mushroom sector.
Keywords: cultivation, processing, quality, breeding, industry

INTRODUCTION

The well-established market acceptance of *Agaricus bisporus* species in Europe has been followed by a spectacular increase in the popularity of other cultivated mushroom species, which are often produced by small scale companies. The aim of the present study was to analyze the current situation in the mushroom sector and to assess the particular technological, innovation and entrepreneurship needs of the small and medium enterprises (SMEs) involved in the production of various exotic mushrooms.

Methodology

The first part of the survey was addressed to document the main cultivated exotic mushroom species in three different countries (the Netherlands, Germany and Greece) as well as to specify the local small scale mushroom growers of each country. In the second part, semi-structured interviews of stakeholders were carried out using the standardized innovation audit forms developed within the EU-Trafoon project. The third step focused on SWOT analysis with the aim to identify the strengths and weaknesses as well as the opportunities and threats in the exotic mushroom sector. The innovation audit forms were authorized by the growers. The information provided in this questionnaire was treated as confidential and used exclusively within the EU-Trafoon project for its intended purpose, the detection of technological and Research and Development (R&D) opportunities for small and medium enterprises (SMEs).

SWOT analysis

The SWOT method of analysis was carried out for each country separately in order to identify the particular parameters affecting the production of exotic fungi. The strengths and weaknesses are identified by an internal assessment of the sector and the opportunities and threats by an external evaluation. The internal assessment investigates all aspects of the production of exotic mushrooms, i.e. preparation of substrate, cultivation technology, growing facilities, equipment, products and services in order to determine the sector's strengths and weaknesses. The external evaluation focuses on the economic, social, technological and competitive environment in order to identify opportunities and threats. Based on the findings of the SWOT analysis, strategies and specific action points were stimulated with the target to indicate the strengths, eliminate the weaknesses, exploit the opportunities and hinder the threats.

1. Results & Discussion

Cultivation of exotic mushrooms in the three examined countries represents a small contribution in the mushroom industry, however, production of certain species such as grey Oyster (*Pleurotus ostreatus*), king Oyster (*Pleurotus eryngii*) and Shiitake (*Lentinula edodes*) is steadily increasing. Current production is limited to around 2.5 thousand tonnes per year and country. Other exotic mushroom species which are produced by small scale growers in the Netherlands and Germany are: Enoki (*Flammulina velutipes*), Nameko (*Pholiota nameko*), Pioppino (*Agrocybe aegerita*) and Maitake (*Grifola frondosa*). In Greece, the domestic production is mainly dominated by grey oyster. Some of the problems associated with cultivation of exotic mushrooms in Europe have been previously reported (Oei, 2005). The results of the national SWOT analyses were evaluated and an attempt was made to present a consolidated SWOT analysis integrating common factors influencing the production of exotic mushrooms in the three countries involved (Table 1).

1.1. Primary production

In the field of primary production the farms that are producing exotic mushroom species apply traditional and relatively old technology in comparison with the production of *A.*

bisporus. A very limited number of substrate producers supply with spawned sawdust and woodchips the small scale growers. Most of the exotic mushrooms are typically grown in bags using conventional raw materials as substrate. The bags are filled with substrate by means of auger filler with an opening spout. The sawdust-based substrate is commonly sterilized after filling in an autoclave at 121 °C or higher. Bulk sterilization, a highly energy-efficient and hygienic production method can be applied only if a certain quantity of substrate is produced per week. The choice of the appropriate bag also depends on the relevant sterilization technique used to prepare the substrate. In the Netherlands for example, a major part of the substrates is subjected to bulk pasteurization in a blender, followed by cooling, mixing with spawn and bag filling. Fruiting bodies are hand-picked. Manual harvesting helps extend the shelf life and also maintains the optimum quality of products for post-harvest processing. To improve hygiene and reduce production losses as well as labor costs, bottles can be used as alternative substrate containers. Regarding the filling technology, an automatic fill and seal machine (FFS) assisted conveyor belt can be employed to facilitate the process. Varying quantities of grain spawn are used by most of the growers. Spawning sterilized bags with solid spawn is time consuming and requires sterile conditions. Alternatively, liquid spawn can be applied in a comparably shorter time with a lower contamination rate. However, no standardized large-scale production system for liquid spawn exists in Europe. To increase production efficiency, varieties of which mycelium is optimally grown under similar climatic conditions could be cultivated in the same growing rooms using bottles placed on mobile trays or trolleys. To ensure stable and even higher prices, growers may produce exotic mushrooms organically. However, organic sawdust and wood chips are still available in limited quantities. For further development of organic cultivation of exotic mushrooms the amount of organic-certified sawdust should be enlarged.

1.2. Product development

With the availability of new breeding techniques it is possible to generate new types of varieties of exotic mushrooms. Until now, breeding has been mainly directed towards the needs of mushroom growers, traders and food processors in terms of yield, disease resistance and shelf life. A successful example in the field of exotic mushrooms is the sporeless oyster mushroom a few years ago. This innovation prevented growers to develop spore allergies. Breeding can also be directed to consumer demands by improving taste, nutritional values and other beneficial compounds in mushrooms (Sonnenberg and Baars, 2012). However, the genetic variation within each species with respect to these traits has rarely been investigated so far.

Table 1. SWOT analysis of the Dutch, German and Greek exotic mushroom sector

Strengths	**Weaknesses**
Desired quality Unique nutrient combinations Health images Well organized producer groups Acceptance from aware consumers	High perishability of fresh products Old growing facilities and technology Empirical small scale growers Production and market instability Lack of standardized substrate production Non systematic promotion of certain exotics
Opportunities	**Threats**
Use of modern cultivation technologies Organic mushroom production Adding value and enhance income by drying Labelling and packaging information Expansion of the market with new varieties	High production cost of substrate Limited amount of certified organic sawdust Competition by imported processed products Lack of education, research and extension Difficulties for subsidized investments

1.3. Processing and value addition

Although cultivated exotic mushrooms are mainly preferred in their fresh state in Europe, fresh mushrooms are highly perishable with short shelf life under ambient conditions of temperature and humidity and their commercialization becomes difficult in case of overproduction. Drying is a standard postharvest technology to prolong their commercial life and ensure long-term distribution (Argyropoulos et al., 2011). In addition, dehydration leads to the development of novel value-added products, which could also reduce the losses caused by surplus production enhancing the income of the growers. Accurate control of the air conditions during drying in cabinet dryers is required to avoid undesirable changes in quality of dried mushrooms. As drying is especially an energy intensive process, energy-efficient drying systems (heat pump dryer), or dryers using renewable energy sources to heat up the drying air (solar dryer) can be applied. In Greece, for example, a promising concept of forced convection type solar dryers represents the solar tunnel dryer developed at the University of Hohenheim. High-temperature dryers are mostly not applicable for small scale growers due to high capital, investment and operational costs. Therefore, introduction of low-cost and

locally manufactured solar dryers offers a promising alternative to reduce post-harvest losses. Another advantage of solar drying is the natural enrichment of mushrooms with vitamin D as ergosterol is converted to ergocalciferol under exposure to UV light, which may result in the development of new mushroom products and lead to market expansion.

1.4. Marketing and consumer awareness

Competition from imported, low quality fresh and processed exotic mushrooms from Asia causes price fluctuations and market instability. If consumers are informed about the health promoting effects of exotic mushrooms, the consumption of several exotic species cultivated in Europe is expected to increase and consequently the impact on the domestic production will be positive. Mixed exotic mushroom packages could be quite profitable. Innovative packaging made from water-resistant and renewable compostable material with more information on product-specific properties such as nutritional value, instructions for use, recipes and health aspects could draw the attention of consumers and may increase sales. Consumers also tend to be attracted to sustainable products that have been manufactured using local ingredients and cultivation processes which follow quality and safety standards. Promotion representatives with product demonstrations can play an important role in informing consumers on-site.

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The Use of a Respirometric Measurement System as a Valuable Tool for Optimizing Mushroom Substrates

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ABSTRACT

Oxygen Uptake Rate (OUR) is a tool to measure biological activity in a variety of mushroom substrates. Respirometric measurement is conducted within a closed vessel containing the inoculated substrate to be measured. The vessel is incubated under desired conditions over a set period of time. Oxygen is consumed and evolved CO_2 is captured allowing data to be plotted in real time. Examples of how this respirometric technology can be used for investigating the effects of various substrates and nutritional factors on growth characteristics of cultivated mushroom species will be discussed.
Keywords: Respirometric Measurement, OUR, Ergosterol

INTRODUCTION

Sylvan's goal is to continuously optimize the substrates we use to deliver our spawn products and adapt them to the needs of our customers. As a result, ongoing formulation work and testing of new substrates and nutritional factors are necessary to meet this goal. The purpose of this paper is to discuss how the use of a respirometric measurement technique can be used as a tool that is beneficial in the evaluation and selection of improved carriers for mushroom spawn as well as for other solid substrate fermentation processes.

Measuring the amount of biomass produced in a complex substrate, such as mushroom compost, or the rate of growth of a fungal culture on substrates, such as cereal grains (spawn) or synthetic carriers (ASR), can be particularly difficult. Methodologies utilizing ATP evolution are complex, time consuming and do not differentiate between fungal and other sources of biological growth (Manuela, 2004). Using the measurement of ergosterol (Gessner and Schmitt 1995) or chitin levels in a sample matrix can provide an excellent determination of total fungal biomass, but it does not differentiate between the living and non-living component. The use of pH change as a means of measuring biological activity in mixed matrices has also been used. However, due to variation in the buffering capacity of these materials, it is difficult to use the change in pH value over time as an accurate, independent measure of biological activity without first developing an accurate correlation methodology.

Oxygen Uptake Rate (OUR) measures the rate of oxygen consumption per mass of dry matter over time (mgO2/g DM/day) **(**Bechara *et al., 2011*). In our laboratory, we measure this type of

oxygen consumption using the Oxitop measuring system (WTW, Giessen, Germany) placed over a closed vessel (see figure 1). Aerobic biological activity within the vessel is measured as a function of oxygen consumption which in turn produces CO_2 and water. The CO_2 is absorbed by NaOH (added to the enclosed vessel) which creates a vacuum. The Oxitop unit contains a built in pressure sensor and infrared interface that communicates with a controller. The resulting data is plotted and oxygen uptake rates are calculated (Bechara 2007).

For OUR measurements a sample (fresh weight) is taken and transferred to the vessel. A small amount of NaOH is placed in the vessel and the Oxitop head is clamped on (see figure 1). The controller is used to activate the head to begin taking pressure readings. Vessels are then placed in a 25ºC incubator to ensure a stable temperature because variance in temperature could affect respiration and thus skew results. After 48 hours, the heads are deactivated and the data is downloaded and plotted in real time.

In addition to OUR values, pH and ergosterol (a component of the cell membrane in fungi) are also measured. By combining OUR data with ergosterol measurements, the amount of total (viable and nonviable) biomass present can be determined with accuracy because early in the fermentation process OUR values and ergosterol concentration are closely correlated. The correlation can be further verified as the fermentation progresses: a point is reached when OUR will plateau while ergosterol values continue to increase.

The quality of data can be further improved by the addition of pH measurements; pH represents a function of change induced by biological activity and can be used as a valuable quality assessment tool. Thus for our research purposes, all three measurements are used to get an accurate understanding of the dynamics within a particular solid state fermentation system.



Figure 1: OxiTop attached to enclosed vessel containing substrate with controller.

1. Results and Discussion

The development of new and innovative mushroom substrates begins on a small scale where nutrient sources and carriers are varied. Following inoculation, substrates are incubated at

varying temperatures over time until a desired endpoint is achieved. Throughout this fermentation period, mycelial growth is monitored by taking samples and testing for OUR, pH, and ergosterol. Figure 2 shows the results of a typical 17 day fermentation of four experimental materials. Ergosterol and OUR levels show a steady increase as expected due to the steady increase in mycelia biomass. Similarly, higher biomass results in increasing rates of oxygen uptake. The plateau observed in OUR on day 11 indicates that active mycelial colonization of substrate has slowed. As a general trend, the pH values of the substrates are measured as colonization progresses, decreases. This can be observed in Figure 2 where three of four substrates tested show a steady decline in pH values until day 11. Beyond this point the pH trend becomes variable confirming the hypothesis that pH measurements are unreliable when employed as independent metrics in substrate development. Based on these results, formulation 4 produced the greatest amount of fungal biomass as measured by ergosterol formation. However, the advantage in measuring OUR is seen in the observation that the majority of the growth occurred within the first 11 days.

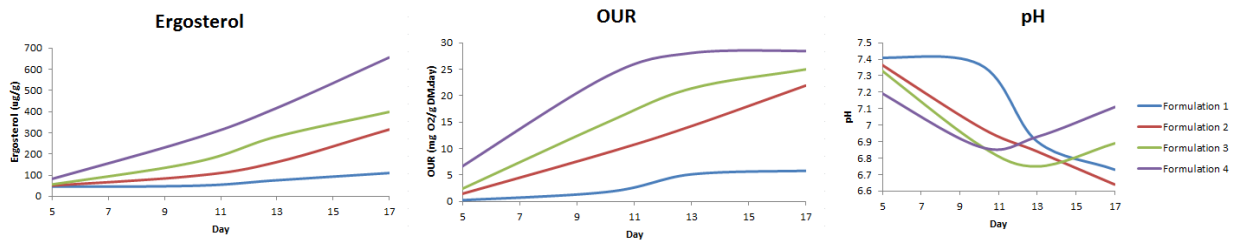


Figure 2: Graphs demonstrating the changes in growth related variables (ergosterol, OUR, and pH levels) over a 17 day fermentation period.

The improvement of new delivery systems for mushroom cultures and other solid substrate fermentation systems requires the analysis of complex substrates that utilize a variety of ingredients. In order to streamline the development of innovative new products, we have successfully employed a combination of analytical methods to rapidly screen formulations and achieve desired outcomes. Combining pH, Ergosterol measurement and OUR provides a powerful tool in our workshop allowing us to efficiently carry out these studies.

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From theory to practice - success in implementing drip irrigation in commercial mushroom *(A. bisporus*) cultivation

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ABSTRACT

In today's practice, mushroom watering is done by spraying systems. Watering has to be stopped to avoid quality damage, from the "pin setting" stage until all first flush mushrooms are picked. After the second flush, during the "No irrigation" periods, casing and compost humidity decreases, to a level that prevents development of first class mushrooms, which makes it unfeasible to pick the third flush. This is reducing the profitability of mushroom cultivation. Drip irrigation was developed to overcome these obstacles of the spray watering systems. Since water is applied below the mushrooms it does not cause any quality damage. In the last year experiments with the new watering system were carried out on three commercial mushroom farms, in three different countries with two different growing techniques: The one layer system and the six shelves (the standard cultivation way). The control was watering with a conventional spraying watering system. A special device was developed for the introduction of the drip tube lines into the casing and collection at the end of the growth cycle. With the use of this machinery, installing and collection drip irrigation in mushroom cultivation rooms became fully automatic. The drip lines can be used for few growing cycles. Drip irrigation succeeded to keep casing and compost humidity as needed even with 30% reduced thickness of the casing layer. Optimal compost and casing humidity is achieved by a newly developed computerized irrigation controller, with an algorithm that use input from compost and casing humidity sensors and the climate controller. Due to the "water shortage" of the spray irrigated rooms, the second flush mushrooms weight/size ratio was higher with drip irrigation, meaning: for the same diameter, less but heavier and better mushrooms. This situation also enables reduction of picking costs. The drip irrigated mushrooms quality of the third flush, was much higher (91% class A) than that of the spray irrigated mushrooms that were picked by machine (13% class A). The increased relative humidity, in the spray irrigated rooms, is avoided with drip irrigation, decreasing the energy needs for drying the rooms. Decreased bacterial blotch incidence, due to dryer mushrooms using drip irrigation, also enhanced mushrooms' quality. Total higher mushroom quality: 95.4% class A, as compared to only 74.6% class A, of the entire yield, using drip instead of spray irrigation increased the entire crop value. These results indicate that introduction of this new technology can improve profitability of mushroom growing.
Key words: Drip irrigation, Mushroom watering, yield, quality.

INTRODUCTION

Drip irrigation was invented in Israel, more than 60 years ago. Today, it is the most efficient way for irrigation of field and orchards crops worldwide. We have recently reported that drip irrigation overcomes the watering obstacles for mushroom cultivation[1]. Drip irrigation enables keeping of optimal water contents in compost and casing during entire crop cycle. The value of mushroom yields (quantity and quality) was found to be higher with drip irrigation, mainly due to the third picking flush[2]. The objective of the present study was to implement the former positive experiments results, in actual model farms.

1. Results & discussion

The study took place in three commercial mushroom farms, in three different countries (Germany, The Netherlands and Israel) with two different growing techniques: The one layer system and the six shelves (the standard cultivation way). The Israeli farm is practicing drip irrigation already three years, currently on the entire farm. The farm in the Netherlands is practicing drip on half of its rooms for a year. The one layer farm in Germany installed drip irrigation in three rooms few months ago. Mushroom yields of a growing cycle (three flushes), at the Netherlands farm, under the two irrigation technologies (drip and spray) are presented in figure 1.

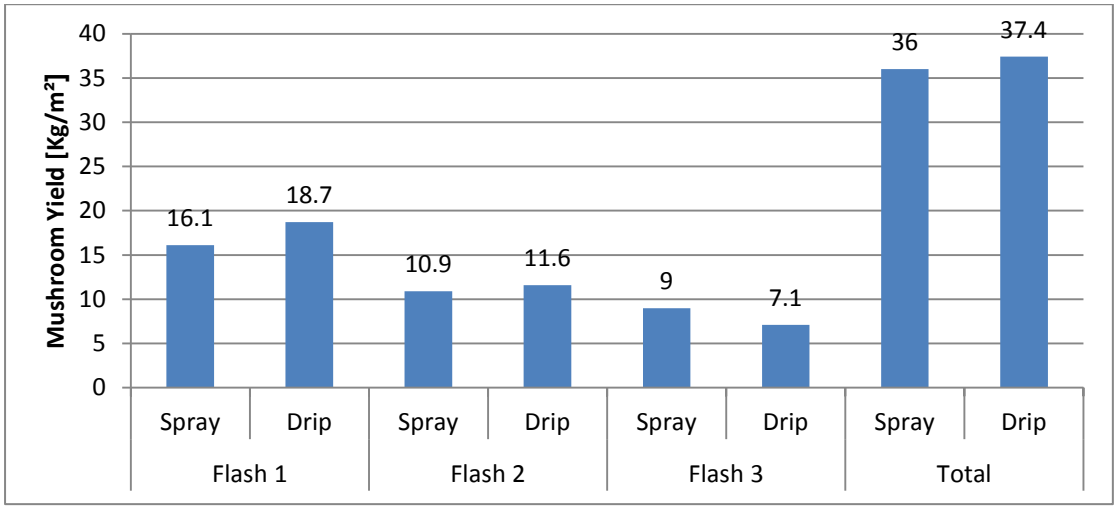


Fig 1. The impact of irrigation technology (drip and spray) on mushroom yields, during a crop cycle of three flushes

These results were achieved in conventional 280 m^2 growing rooms, on phase 3 compost, using 30% less casing quantities for the drip irrigation treatment. Mushroom quality, calculated as class A or B mushrooms percentage, of the same cultivation cycle is presented in figure 2.

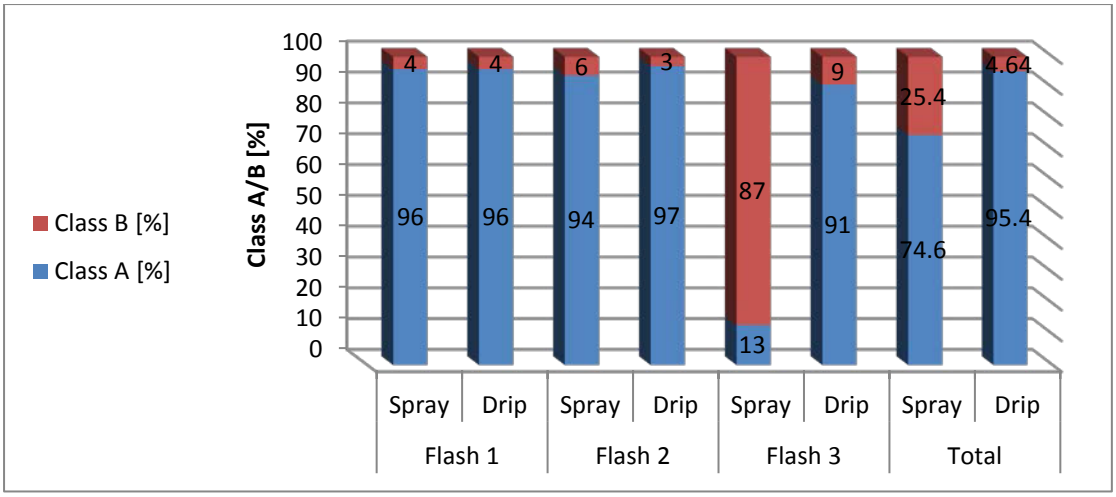


Fig 2. The impact of irrigation technology (drip and spray) on mushroom quality (as class A or B percentage) during a crop cycle of three flushes.

No mushroom quality differences were found during the first flush. The percentage of class B mushrooms was twice higher in the spray than in the drip irrigated rooms, at the second flush. At the third flush, on the other hand, class A drip irrigated mushrooms were 91% of the yield, compared to only 13% of spray irrigated mushrooms. Therefore, class A mushrooms were 95.4% with drip, compared to 74.6% with spry irrigation, for the entire cultivation cycle. These results are due to the facts that were presented in our former studies[2]: Drip irrigation keeps optimal compost and casing humidity during the entire mushroom cultivation cycle, allowing optimal flush three mushroom development. Class A mushrooms are hand-picked for the fresh market, during the third flush in drip rooms, compared to (mostly) class B mechanically harvested mushrooms, for industrial processing, in the spray irrigated rooms. At the drip irrigated rooms, as formally presented[2], most third flush mushrooms also develop to "Medium cup" size. This fact causes reduced picking costs and higher quality, enhancing the value of the drip irrigation technology. These results demonstrate again, on commercial scale dimensions, that the limiting factor for high quality and high yielding third picking flush, is mostly optimal water supply for the mushrooms development, rather than other factors like compost's nutrients availability. Drip irrigation implementation on commercial scale required the development of a unique irrigation control system, as well as machinery for drip pipelines application and removal for every cultivation cycle. These tasks were successfully achieved, and labor costs are the same as for the conventional (spray) cultivation technology. The presence of the drip pipelines in the mushroom cultivation shelves opens more options including: Their use for manipulating compost/casing temperature, and supply of nutrients and crop protection agents during mushroom cultivation. These options will be studied in the future.

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Drip Irrigation in Mushroom Cultivation should be Aligned with Common Practice

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ABSTRACT

By common practice, mushrooms (*A. bisporus)* grown on shelves are irrigated by movable spraying systems. After "pins" set, irrigation is restricted to avoid moisture on the fruit bodies, which can cause bacterial blotches. Irrigation restriction creates dehydration of the casing soil to an extent that the third flush is not justified. By using drip irrigation positioned **underneath the mushrooms**, we can keep soil moisture at optimum and even reduce the depth of casing soil, as less buffer is needed. Increased yield and quality on one hand, and a reduction in casing soil and energy for drying the mushrooms after survival irrigation, on the other hand, brings a significant economic advantage. Once we had shown proof of concept, we had to align the driplines with the ongoing agro-machinery cultivation practices. The challenge was to insert and collect the driplines without disturbing the flow of compost and casing soil. A mutual effort in mushroom agronomy produced higher yields, better quality, and alignment with the existing agro-machinery practices, resulting in a new feasible solution that can push the industry forward.
Key words: Drip Irrigation, Mushroom yield and quality, Alignment to Agro Machinery practices.

INTRODUCTION

The introduction of drip irrigation into conventional mushroom cultivation was challenging in 2 main aspects:

- The system must be cost-effective with an attractive ROI (return of investment).
- It should be easy to manage without extra labor.

My colleague, Dr. Ofer Danay will refer to the first challenge, explaining how we succeeded in increasing yield and quality in a way that the ROI in Israel was less than a year (due to high Mushrooms prices – 4 €/Kg). In Holland, (Paul Van Der Berg – Boekel) we expect it will take 1-1.5 years. I will refer mainly to:

- The simplicity of the hydraulic installation.
- The alignment to the existing agro-machinery.

The solution is combined of 3 main components:

Mush-line (dripper line)
Pressure-compensated non-leakage dripper
Installed subsurface at shallow depths
Remains constantly filled with water to enable rapid irrigation pulses
Broadens water distribution
Improves nutrient utilization
Delivers larger yields.

Controller
Designed as dedicated controller
Includes advanced algorithms
Allows remote access

Mechanical deployment system
Automatic laying and retrieving of dripper lines into and from the casing; Washing and disinfection unit.

Future features:
Circulating warm/cold water at low pressure to influence the casing soil temperature.
Flexible supplement supply as needed.

1. Results and discussion

1.1. Simplicity of hydraulic installation
The fact that the shelves concept is based on uniformity is helpful in creating simple, practical modular manifolds that fit the shelves' width and can be easily attached to the aluminium panel.

1.2. Alignment with existing Agro Machinery
We have today three model farms: Zarit in Israel; Paul Van Der Berg in Boekel, Holland, and Paul and associates in Wankum, Germany. All three farms, as modern mushroom farms, use 3 phase compost head fillers. The implementation is done either by the farm itself as in Israel, or by a contractor – CNC in Boekel and Wankum.



Picture 1: Inlet Manifold.

The concept was that while applying the compost and casing soil, the driplines will be inserted without disturbing or slowing down the head filler. **As for today, dripline insertion does not reduce the regular filler speed of 7-11 meters/ minute.**



Picture 2: Insertion unit on Thilot head filler.

In the picture we can see the insertion unit and the "Cassette". The "Cassette" is the basic unit that stays on the farm. All the rest of the agro- machinery system will be loaded on a truck and transported by the contractor from one farm to another, managed and controlled by the compost/ casing soil suppliers. The insertion unit will position the driplines at the depth of 3 cm !!! below the casing soil surface.

1.3. Re-Use of Driplines.

In order to re-use the driplines, we must guarantee 2 main points:

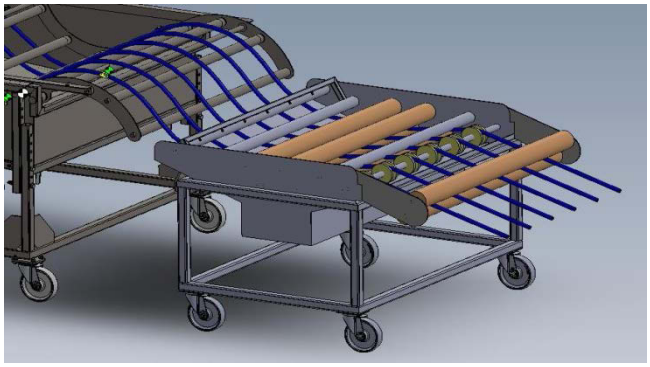
- No mechanical damage to the driplines.
- Disinfection of the driplines to avoid contamination.

While removing the compost and casing soil, we separate the driplines in a way that will not disturb the compost flow out. Later on, the driplines should be washed, disinfected, dried and rolled back on the cassettes.

We have 2 ways to make sure of effective disinfection:

(1) "Cooking" the room at 70^0c** and then injecting chlorine into the driplines;

(2) Driving the driplines through the disinfection unit.



Picture 3: Illustration of the disinfection unit

** As a precaution of the cooking effect on the driplines, we recommend to change the driplines twice a year.



Picture 4: Full agro-machinery system

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Multiflex Water Supply System (WSS) for controlling the water conditions of substrates and to improve mushroom production *(Agaricus bisporus)*

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ABSTRACT

Multiflex WSS: A new concept of supplying water for the production of mushrooms (*Agaricus bisporus*), in which an optimal water distribution is combined with measurement data and maintenance programs to achieve the best results and optimize the use of water.
Water sensors have been used to monitor the moisture in nine mushroom cultivation cycles. The sensors react to modifications in the casing soil when it is watered from above. If they are placed in the compost layer, they follow more or less the same pattern. Additionally, the 'compost and casing sensors' also react when the pins start absorbing water for evaporation and mushroom development.
The data of these measurements seem to be suitable for the automated Multiflex WSS. However, this has to be confirmed with further tests.
Keywords: mushroom production, automated watering, moisture sensor.

INTRODUCTION

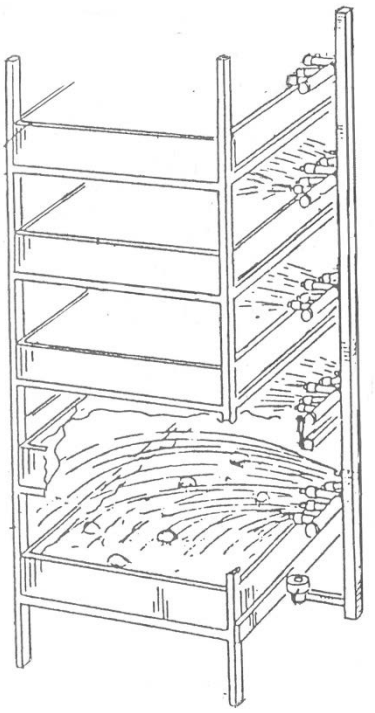
Water scarcity is a growing problem in the world. The majority of the available water, about 70%, is used in agriculture for the preparation of food (Mekonnen et al 2016). Therefore it is important to use the available water in a responsible way.

For the cultivation of mushrooms a lot of water is required, because mushrooms consist of more than 92% of water. One of the main goals of this research is to generate higher yields with less water.

In mushroom cultivation there are three different methods of supplying water: uncontrolled, controlled and results based water supply. At the moment, in many countries in the world mushroom cultivations are supplied with water manually by means of a single sprinkler or sprinkler head. This is an uncontrolled way of water supply in which a lot of water is wasted and it is person dependent how much water is used and where it ends up.

In order to achieve a more optimal water supply, the following should be taken into account: make sure the water ends up in a useful place (for the mushroom), avoid wastage, supply water in a reproducible way (person independent) and only supply water when necessary.

The Multiflex Water Supply System (WSS) covers the first three criteria. The specifically designed sprinkler arm creates an optimal water distribution over the full width of the mushroom beds (Figure 1). It can be manually moved by means of a sprinkler carriage, onto which the sprinkler arms are mounted.



For an optimal distribution over the length of the mushroom beds, the fully automated Multiflex WSS is a more appropriate method. Supplying water is always done in the same way (person independent) and all the mushroom beds get the same amount of water with a minimum of wastage. Another advantage is that it can be done at any desired time in the cultivation. This method is called controlled water supply.

The third method is the future and is called results based water supply. We decide when and how much water needs to be supplied, based on figures. For this, we must measure the moisture content in the mushroom beds with sensors and connect the sensors to the Multiflex WSS.

Figure 1**:** Optimal water distribution.

Around 90 litres of water per m^2 of growing surface is available directly after filling the growing room with full grown compost and casing soil. This seems enough for the production of 35 kg of mushrooms per m^2 in two flushes. Unfortunately this is not the case due to decreasing water potentials in casing soil and compost (Kalberer 2006). Another point of attention is that the casing soil has to be prevented from drying out. The reason for this is the irreversible character of the black peat. There is a linear relationship between the Water Holding Capacity (WHC) of unfrozen black peat and moisture content (Amsing, unpublished data). If the casing soil dries out, a maximum WHC is no longer possible. The casing soil and compost should have optimal water conditions during the entire mushroom cultivation cycle. Some research has been done regarding this subject, but there is not much recent information available and, in practice, it has not led to any automated water supply systems, based on water sensors, so far (Amsing 2009, Noble et al 2000). In order to make the step from controlled water supply to results based water supply, we started the research that is explained below.

Materials & Methods

The research is divided into three phases.

Phase 1

The Frequency Domain soil moisture sensor of the type GS3 (Decagon Devices) has been tested in the laboratory. This FD sensor measures the volumetric moisture level. The

measurements were done in mixtures of BVB Substrates casing soil, which vary in composition and gravimetric moisture level. The data have been analysed with a hand-held ProCheck meter.

Phase 2
Nine mushroom cultivation tests have been performed, four in a mushroom farm that produces mushrooms for the fresh market and five in a mushroom farm that cuts the mushrooms automatically for industrial use. Both the moisture sensors of the type GS3 and 10HS (Decagon Devices) were used. The 10HS sensor also measures the volumetric water content according to the Frequency Domain technology but has longer probes. The moisture data have been collected with an EM50 logger.

Phase 3
A literature study on moisture sensors, conducted by Jos Balendonck, Wageningen UR commissioned by BVB Substrates and Vullings Systemen B.V.

Results & Discussion

Phase 1
The BVB Excellent heavy RD casing soil was divided into six equal quantities of weight and every time more water was added. The GS3 moisture sensor generates reliable values of Volumetric Water Content (VWC) in casing soils with a high moisture content (Figure 2). The measured VWC is lower than the calculated VWC. This is also shown by measurements that were carried out in three different mixtures of casing soil with high moisture contents (Table 1). The difference between the calculated and measured VWC can be unacceptably high.

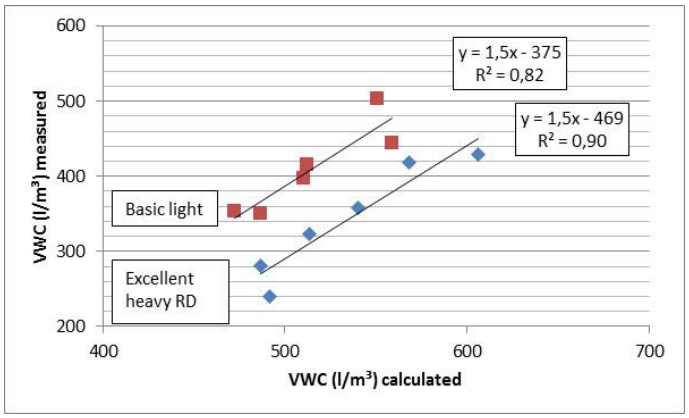


Figure 2: The relation between the calculated and measured VWC in casing soil (BVB Basic light and BVB Excellent heavy RD).

Casing soil	Basic heavy	Excellent light	Excellent heavy
Moisture (% w/w)	78,6	80,4	82,5
VWC calculated (l/m³)	666	635	646
VWC measured (l/m³)	491	475	535
VWC difference (%)	-26	-25	-17

Table 1: The gravimetric water content, the calculated and measured VWC in different types of casing soil of BVB Substrates.

Phase 2.
The technical part of the measurements with the sensors and data logger at the two mushroom farms went well. The sensors react to the watering of the casing soil (Figure 3). The sensor also reacts when water withdrawal from the compost takes place during the button outgrowth. We have noted that significant differences can be measured if the sensor is moved. However, the reproducibility of FD sensors is large and this offers possibilities.
The 10HS sensor has longer probes, but reacts less violently to changing water volumes than the GS3 and is therefore less suitable. This is partly because the casing soil layer is too thin, which means that air and compost are also measured.

The measured VWC in casing soil and compost does not reflect the actual situation. Furthermore, it is found that moisture signals from sensors of the same type, which are located at a short distance from each other, can vary widely. Therefore, the set-point can not be determined based on these signals and the water supply over the casing soil can not be controlled. The most important disturbing factor is the spatial variability of the pores in the substrate. The coarser the casing soil, the more spatial variation. In which case it is necessary to place more sensors in the mushroom bed in order to approximate a representative view of the moisture content.



Figure 3 The VWC measured with the GS3 and 10 HS sensors in casing soil and compost in a cultivation of mushrooms (15-Jul-2013 filling 02-Sept cutting harvest flush 1, 10-Sept cutting harvest flush 2).

The question, however, is whether accurate measurements are necessary. Sensors follow the increases and decreases of water during water supply and button outgrowth, and the resulting trend gives us the opportunity to control the water supply. The proposed approach for automating the time and amounts of water with the Multi-Flex WSS through the moisture content of the casing soil and compost when filling the cell is determined gravimetrically. This number is then taken as a reference, and together with the average trend measurement from multiple sensors used for controlling during cultivation. This research will take place in the coming period.

Phase 3.

The literature study concludes that there is a large number of techniques for measuring the moisture content in mushroom substrates. This also includes methods, such as nuclear techniques, which can be used for very accurate volumetric moisture measurements, but for practical reasons they can not be applied in the cultivation of mushrooms. Other suitable techniques, such as FD sensors, might be able to achieve a higher degree of accuracy after calibration.

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A review of microbiological and chemical safety-related research on mushroom cultivation and consumption in Ghana

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ABSTRACT

The mushroom industry in Ghana has seen some growth over the years, with various research carried out. These research have been largely focused on ethnomycological surveys and mushroom growth characteristics on various substrates. More research is being carried out on biochemical characteristics of some wild and cultivated mushrooms in recent times. With the increased cultivation and consumption of wild and cultivated mushrooms in the country, the need for safety measures in the industry from farm-to-fork cannot be overemphasized. This review gives an overview of the kind of microbiological and chemical safety- related research carried out on mushroom cultivation and consumption in Ghana, highlights some results obtained in these studies, and maps out some areas for consideration for research into safety-related issues on mushrooms in Ghana.
Keywords: Mushrooms, microbiological safety, chemical safety, Ghana

INTRODUCTION

Wild and cultivated mushrooms form a part of the Ghanaian diet, being largely consumed in soups and stews. *Volvariella spp.* (*V. volvacea* and *V. bombycina*), *Termitomyces spp.* (*T. globulos, T. schimperi, T. microcarpa, T. robustus* and *T. clypeatus*), *Coprinus disseminates*, *Auricularia auricular-judea*, and various *Pleurotus sp* are among the most consumed mushrooms in the country (Apetorgbor et al. 2008; Obodai et al. 2014a). Out of these mushrooms, *P. ostreatus* is the most commonly cultivated, being cultivated on various agro-industrial lignocellulosic materials found in Ghana following solid-state fermentation (Narh and Obodai 2013; Obodai et al. 2011). Most of the other consumed mushrooms have only been cultivated on experimental bases and are awaiting commercialization.

Collected wild mushrooms are either used fresh or preserved by sun-drying or smoking, while cultivated mushrooms are marketed fresh or sun-dried in transparent polythene or polypropylene bags, or powdered in transparent plastic bottles.

Due to this wide-spread consumption (Apetorgbor et al. 2008) and increasing cultivation of mushrooms in the country, it is prudent to conduct research into safety of collected and/or cultivated mushrooms and develop or adopt Good Agronomic Practices or other safety guidelines to ensure the safety of the produce from farm-to-fork.

In this report, an overview of microbiological and chemical safety-related studies on mushroom collection, cultivation and consumption in Ghana is given. Possibilities for further research in this area are also highlighted.

1. Discussions

There are various sources of microbiological and chemical-safety related hazards related to mushroom collection, cultivation and consumption. These include microbiological and chemical contaminants in the growth substrate before, during and after solid state fermentation (composting); in spawns in prepared compost bags or growing beds during incubation and growth; and on mushrooms during cropping, harvesting, packaging, storage and/or processing.

1.1. Microbiological safety

1.1.1. Studies related to growth substrates

Obodai and Odamtten (2012) and Obodai et al. (2010) have identified various contaminant microorganisms in growth substrates such as banana leaves, cocoa shells, maize stover, oil palm pericarp, rice straw and sawdust of *Triplochiton scleroxylon.* Identified fungi in these studies included *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus*, *Cladosporum herbarum*, *Mucor pusillus*, *Penicillum cyclopium*, *P. digitatum*, *Paeciliomyces varioti*, *Rhizopus oryzae* and *Trichoderma viride.* Individual bacteria were not characterized in these studies. However, it was reported that the bacterial populations in the various substrates exceeded that of the fungi.

1.1.2. Studies related to mushroom fruiting bodies

Studies on microbiological safety of mushroom fruiting bodies in Ghana are limited to the widely cultivated mushroom, *P. ostreatus,* and conducted with mushrooms cultivated under experimental conditions in Accra. These reports include studies on the microbial load of fresh and dried *P. ostreatus* packaged and stored under different conditions in Ghana. According to Kortei et al. (2014) microorganisms detected on both fresh and dried *P. ostreatus* stored in polythene and polypropylene storage packs at 20 ^{o}C with or without gamma irradiation at 1 and 2 kGy included aerobic mesophiles, *Bacillus cereus*, molds and yeasts. The D_{10}-values (decimal reduction dose) of *B. cereus* on mushrooms were related to the packaging material and the irradiation dose. According to Smith and Pillai (2004), the D_{10}-value is the radiation dose required to inactivate 90 % of a viable bacterial population or reduce the population by a factor of 10. Kortei et al. (2015) have reported that there is a notable effect on combining sun drying and irradiation on microbial loads on dried *P. ostreatus*.

1.2. Chemical safety

1.2.1. Metal content of mushrooms in Ghana

Metals and other chemical compounds are known to play some specific roles in various organisms. Copper and zinc are metals, which are essential for proper functioning of metalloenzymes including polyphenol oxidase (Tabner et al. 2003). However, these compounds may pose safety hazards when consumed in large quantities by humans (Al-Khlaifat and Al-Khashman 2007). Nutritional and other properties of mushrooms are also influenced by the chemical composition (nutrients and other chemicals) of the substrate on which the mushroom grows in the wild or is cultivated (Demirbaş 2000; Youri and Tano-Debrah 2005).

Studies on the metal content of mushrooms collected, cultivated and consumed in Ghana have been conducted on both wild and cultivated samples. The content and levels of some of these metals have been assessed in wild and cultivated mushrooms in Ghana using Atomic Absorption Spectroscopy

(AAS) (Obodai et al. 2014a; Obodai et al. 2014b; Quarcoo and Adotey 2013; Quarcoo et al. 2014). Detected levels of metals such as iron (Fe), sodium (Na), Potassium (K), Calcium (Ca), Chromium (Cr), Selenium (Se), Vanadium (V) and Zinc (Zn) in mushrooms (e.g. *A. auricula*, *L. squarrosulus*, *P. tuber-regium*, *T. clypeatus*, *T. robustus*) were generally below the accepted levels indicated by regulatory bodies such as European Commission and the WHO/FAO Expert Committee on Food Additives (Obodai et al. 2014a; Obodai et al. 2014b; Quarcoo and Adotey 2013; Quarcoo et al. 2014), and can therefore be considered to be safe.

Levels of heavy metals detected in mushrooms collected or cultivated in Ghana were usually either below or in the range of detected quantities reported in other literature and were not considered to be unsafe as the levels fell within the acceptable limits indicated by organizations such as the EU and WHO (Obodai et al. 2014a; Obodai et al. 2014b; Quarcoo and Adotey 2013). Lead (Pb), Arsenic (As), Cadmium (Cd) and Mercury (Hg) have been detected in *P. ostreatus* (a cultivated mushroom) and *T. clypeatus* (a wild mushroom) purchased from various markets in Accra (Quarcoo and Adotey 2013). The authors recorded heavy metal levels ranging from 0.35 – 0.57, 0.04 – 0.23 and 0.04 – 0.10 mg/kg for Cd, Pb and As respectively. The recorded Hg contents in both species (0.04 mg/kg) were higher than the recommended limits set by EC, WHO and FAO. However, the authors indicated that this was not a health risk due to the quantities of the mushroom fruiting bodies that were supposed to be consumed before the threat is posed. Obodai et al. (2014a) recorded a maximum of 0.60 mg/kg of Pb in wild fruiting bodies of *P. tuber-regium* and *T. robustus*. Pb contents of *P. ostreatus* strain EM-1 cultivated using composted and uncomposted cassava peels as substrate were 8.34 and 3.75 mg/kg respectively (Obodai et al. 2014b). The authors indicated the high capacity of *P. ostreatus* to bio-accumulate Pb.

1.2.2. Chemical residues in mushrooms

Mushrooms are known to have a high capacity for bio-accumulating many molecules including chemical residues from pesticides and fertilizers and metals from the growth substrate due to the structure of mycelia they possess (Demirbaş 2000; Youri and Tano-Debrah 2005).

Based on this present review, it is apparent that until presently, no studies have been conducted on chemical residues which may be present in mushrooms in Ghana. This is so although mushrooms are collected from various localities including forest areas (Apetorgbor et al. 2008) which may be close to farms or mining sites and may therefore be exposed to chemical residues.

1.2.3. Mycotoxin content of mushrooms in Ghana

Although yeast and molds including *Aspergillus flavus*, *A. fumigatus*, *A. niger* and *Mucor pusillus* have been identified in both fresh and dried samples of different species of mushrooms in Ghana (Kortei et al. 2014; Obodai et al. 2010; Obodai and Odamtten 2012), it appears that there has been no studies to determine the presence and quantities of various toxins including mycotoxins in mushroom samples in the country.

2. Considerations for further research

This review indicates that there are several areas related to microbiological and chemical safety of mushrooms which have not yet been (completely) covered in Ghana. To mention a few; the microbial phenology of growth substrates needs to be extended to more growth substrates and in a

broader study area where mushrooms and/or substrates are obtained; analysis of chemical (metal or chemical residue) content of various mushroom cultivation substrates; and both microbiological and chemical (metal, chemical residue and toxin) content analyses of various mushroom species cultivated on the various substrates or collected in the wild and processed in the country. Based on these studies, safety parameters must be identified and mitigation strategies developed or adopted to protect mushroom farmers, collectors, processors, consumers and other actors in the mushroom industry from farm-to-fork.

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Assessment of Primary Production of Horticultural Safety Management System of Mushroom Farms in South Africa

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Abstract

Growing global consumer concern over food safety compliance in the fresh produce industry requires producers to implement necessary quality assurance systems. Varying effectiveness has been noted in how countries and food companies interpret and implement food safety standards. A diagnostic instrument (DI) for global fresh produce industries was developed to measure the compliancy of companies with implemented food safety standards. The DI is comprised of indicators and descriptive grids for context factors, control and assurance activities to measure food safety output. The instrument can be used in primary production to assess food safety performance. In this study the DI was used to measure food safety standard compliancy of mushroom farming in South Africa. From 2013 to 2014, ten farms were independently assessed for their horticultural safety management system (HSMS) compliance via in-depth interviews with the quality assurance personnel at each farm. The data was processed using Microsoft Office Excel 2010 and represented in spider web diagrams. The diagnosis revealed that the mushroom farming industry had an average food safety output. The farms were implementing average to advanced HSMSs while they are operating in a moderate risk context. Insufficient performance areas in HSMSs noted included inadequate hazard analysis and analysis of control points, low specificity of pesticide assessment and inadequate control of suppliers and incoming materials. Recommendations to the industry and current short comings were suggested for realization of an improved industry wide food safety assurance system.

Keywords: Food safety diagnostic instrument, food safety output, food safety assurance, mushroom industry.

INTRODUCTION

There is a growing consumer concern over food safety of fresh produce (Van Boxstael *et al.*, 2013). Recently, there has been a reported occurrence of *Listeria* spp., including *L. monocytogenes*, at a mushroom production facility (Viswanath *et al.,*2013) and subsequent mushroom recalls from retail markets due to the presence of the food-borne pathogen contamination (www.inspection.gc.ca, www.fda.gov/Safety/Recalls). These food safety challenges are an indication of ineffective food safety systems. Therefore, retailers are expecting fresh produce growers to effectively implement and sustain food safety assurance systems (LaBorde, 2003). Food producers are pressured to continually improve their food safety performance. Careful diagnosis of the compliancy to implemented food safety standards is thus important as it provides the basis for improvement (Luning *etal.,* 2008).

Recently, diagnostic instruments were developed that allow food producers to assess effectiveness of implemented food safety management systems (Luning *et al., 2008*; Luning *et al.*, 2009; Luning et al., 2011). Based on previous developments, Kirezieva *et al.* (2013) tailored a diagnostic instrument particularly for global fresh produce chains and applied it in primary production in Belgium lettuce farms. The diagnostic instrument is a simple tool that can be applied at various organizational levels, i.e. at company, sector or governmental levels and in the food supply chains (Jacxsens *et al*., 2013*)*. The aim of this study was to apply the fresh produce diagnostic instrument (Kirezieva *et al.*, 2013) in enabling a comprehensive and differentiated assessment of implemented food safety systems/practices at South African mushroom farms. Such an evaluation is particularly because food safety compliance has become a *de facto* retailer requirement.

Results and Discussion

In this study, the generic picture of the context, horticultural safety management system (HSMS) performance and food safety output for the farms was produced (Fig. 1). In terms of product and process characteristics, all mushroom farms operated in low to medium (B, H and J) and medium (A, C-G and I) risk contexts with a generic score of 2 (Table 1). Lowest risk (assigned score 1) was recorded for the water samples (Table 2) as all farms use potable water for the mushroom watering processes. The highest risk observed for microbial contamination of the final product (Fig. 1a) was mainly because of the product's intrinsic properties (Lunning *et al.*, 2011). Although low to medium risk was attributed to the production system (Fig. 1a), studies have reported on the possibility of mushroom production systems supporting growth and survival of food borne pathogens (Viswanath *et al.,* 2013). Differences in organizational and chain characteristics (Fig. 1b) meant that companies offered different managerial support (Sampers *et al*., 2012). Farms D and F needed improvements in specific areas of their organizational and chain characteristics (Table 1). Highest risk was recorded for the requirements of stakeholders (Fig. 1b) meaning retailer specifications for mushroom specific quality assurance clauses (Luning *et al.*, 2011).

Generally, farms operated in a medium risk (score 2) context (Table 1). The medium risk context would require control activities in at least an average level (overall score 3) for realization of good food safety output (Lunning *et al.*, 2008; Luning *et al.*, 2011)*)*. The industry score in this respect should thus be maintained or improved since mushrooms are vulnerable to contamination and spoilage. Farms D, F and G had relatively low scores on control activities (Table 1) and therefore needed improvement. Specific areas of improvement included maintenance programs specificity, degree of incoming material control and supplier control, irrigation methods adequacy, intervention processes capability and availability and compliance to standard operating procedures (Fig 1c and d). Food safety assurance, as *de facto* required by retailers, may be achieved in practice when the food safety management system has been implemented on farms according to retailer specifications (Lunning *et al.*, 2009). Farms showed differences in the manner of their assurance activities (Table 1). The generic industry score for assurance activities was 3 (average level). Only one farm (B) conducted assurance activities at an advanced level (4) and another small farm (F) at a basic level (2) (Table 1). Farms H, I and J had

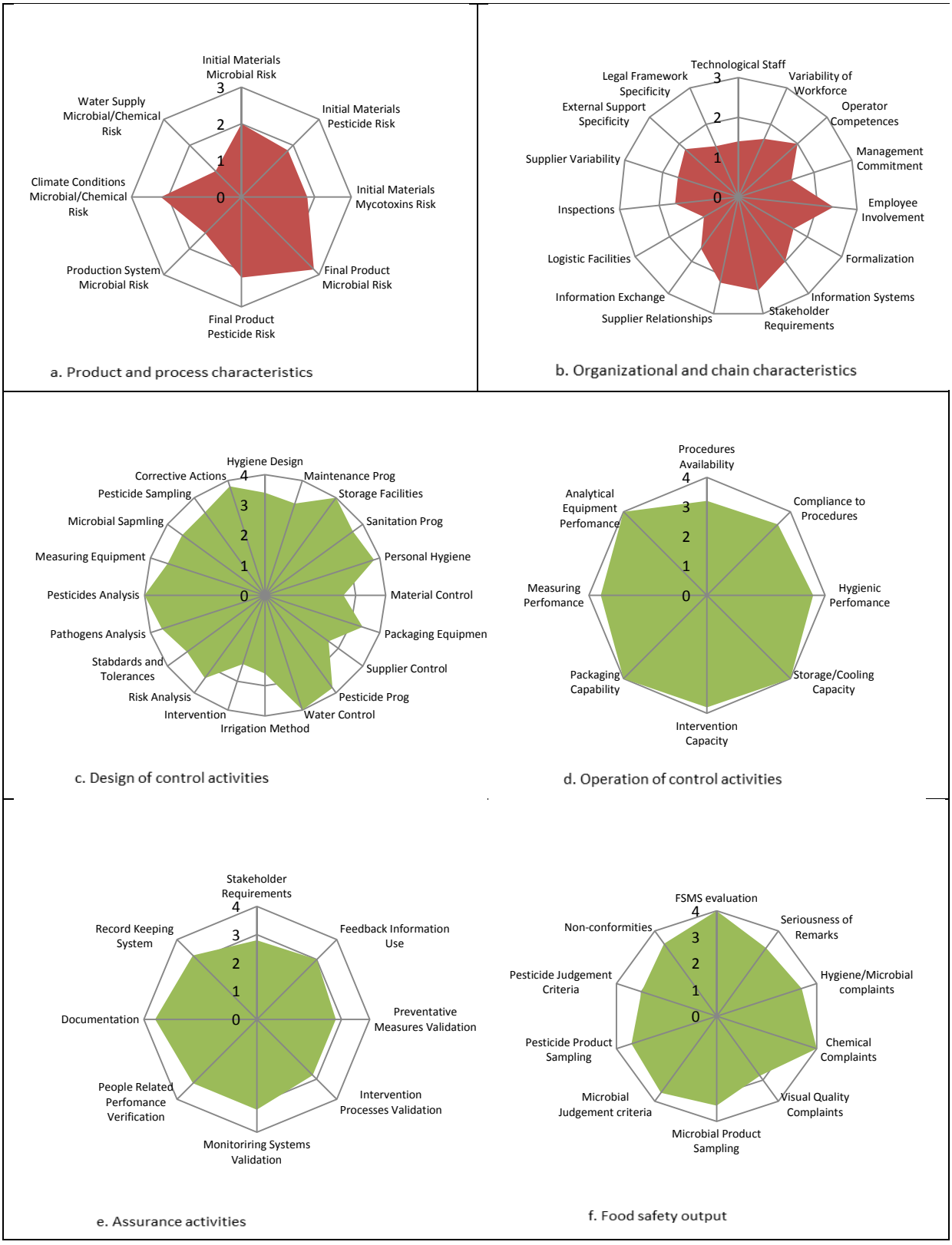


Figure 1. Overview of contextual factors, safety control and assurance activities and food safety output for ten mushroom farms in South Africa between 2013 and 2015. *For (a) and (b) the more coloured the pie charts are, the more risky context operated in. For (c) to (d) the more coloured the pie charts, the more compliant that aspect or system is in terms of horticultural safety management.*

Table 2. Assigned overall scores for contextual factors, safety control and assurance activities and food safety output for ten mushroom farms in South Africa

Aspect investigated	Farm A	B	C	D	E	F	G	H	I	J	Generic Score[a]
Product and process context	2	1-2	2	2	2	2	2	1-2	2	1-2	2
Organization and chain context	1-2	1	1-2	2-3	1-2	3	2	1-2	1-2	1-2	2
Design of control Activities	3-4	3-4	3-4	3	3-4	2	3	3-4	3-4	3-4	3
Operation of control activities	3-4	4	4	4	3-4	3	3	3-4	3-4	4	3-4
Assurance Activities	3	4	3	3	3	2	3	3-4	3-4	3-4	3
Food safety Output	3	4	3-4	3-4	3	2	2-3	3-4	3-4	3-4	3

[a] score assigned to the overall mean of all farms' respective indicators. Overall scores were assigned based on Luning *et al.* (2011)

assurance activities at average to advanced levels (3-4) while A, C, D and E conducted the activities at an average level. Maintenance and improvement of food safety systems is needed in all aspects (Fig. 1e).

In terms of food safety output (Fig. 1f), external and internal indicator assessment was done. Visual complaints were the most frequent type of complaint, and this may be because of the perishability nature of mushrooms. Based on the data and other reported findings of the diagnostic instrument (Luning *et al.*, 2011), an overall good food safety output (generic score 4) would have been expected in this study. To facilitate progress towards improved food safety outcomes, insufficient performance areas in the industry were highlighted as recommended areas for improvement. Based on this study the diagnostic instrument, developed for the fresh produce sector, can also be used in the mushroom industry. To develop a more improved HSMS, mushroom farms can carry independent diagnostic instrument assessments on the form of internal audits and studies of the trends of their food safety activities. Part of this conference paper has been published as Dzingirayi and korsten (2016).

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Impact of water extract of leached spent mushroom compost (LSMC) on cultivation of button mushroom *Agaricus bisporus*

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ABSTRACTS

One year old spent mushroom compost was leached with water for 24 days and treated with steam at 60 °C for 8 h to eradicate pathogenic organisms. The results of addition of leached spent mushroom compost (LSMC) extract in large scale confirmed a positive effect for total yield between treatments and controls. The results also indicated a significant difference on protein content of mushrooms as compared with controls. First flush was harvested 1.5 days earlier as compared to those controls.
Key words: Spent mushroom compost extract, mushroom cultivation, mushroom quality.

INTRODUCTION

Spent mush room compost (SMC) is mainly composed of a thermally treated cereal straw/animal manure mixture colonized by the fungal biomass (Ntougias, Zervakis et al. 2004). A vast volume of SMC, by-product of *Agaricus bisporus* cultivation, is produced in mushroom industries. Spent mushroom substrates are often allowed to weather but weathering is not enough to reduce salinity to a satisfactory level. Leaching is one of the possible options to remove the salts from SMC (Riahi and Arab 2004, Riahi and Azizi 2006, Riahi, et al. 2012). Previous studies suggested that compost and its aqueous extract can be used for suppression of plant diseases and enhance yield and quality of plants. Non aerated (NCT) and aerated compost tea (ACT) were tested for their effect on strawberry yield. The results showed that NCT had little effect on yield, while ACT improved yield as compared to the control (Welke 2005). Five different compost teas were examined on pack choi growth. The results showed that application of compost teas led to an increase in growth and mineral up take of this plant (Pant, et al. 2012). Little work has been done to assess the effect of spent mushroom compost tea on cultivation of button mushroom *A. bisporus*. However, recently Gea and his colleagues studied the effect of SMC tea on mycelia growth and yield of white button mushroom and reported that yield was not significantly affected by the spent mushroom compost tea. Another result of this study was a delay of 0.8-1.4 days in the onset of harvest of the first flush (Gea, et al. 2012). Application of compost tea on casing soil decreased mushroom production by 4–10 percent and delayed the mushroom production by one day (Navarro, et al. 2011). The present work was undertaken in order to investigate the usefulness and efficacy of LSMC tea on yield and quality of white button mushroom.

1. Materials and methods

1.1. Compost and Soil

The experiments were carried out at Malard Mushroom Company, the largest mushroom producer in Iran. This company used blocks of phase II compost spawned with A15. Casing soil is a mixture of %60 leached SMC + %20 local peat + %20 imported peat (BVB).

1.2. LSMC extracts preparation

One year old spent mushroom compost was leached with water for 24 days, and then treated with steam at 60 °C for 8 hrs to eradicate pathogenic organisms (Riahi and Arab 2004). For a large scale experiment, one third of a container of 200 liter capacity was filled with pasteurized spent mushroom compost, filled with water, stirred few times and left overnight. The next day the extract was pumped out, filtered and mixed with water v/v for irrigation. Physical and chemical properties of LSMC and LSMC extract was characterized (Table 1).

Table 1. Physical and chemical properties of SMC and LSMC extract

Parameter	LSMC	LSMC extract	unit
EC	2.2	1.78	µS/cm
Ca	1520	444	mg/L
Mg	576	55.2	mg/L
Cl	2200	360	mg/L
SO4	15300	1390	mg/L
K	8600	560	mg/L
NO3	86.91	171.65	mg/L
NaCl	3.7	0.6	%
P	267	13.75	mg/L
CaCO3	2800	170	mg/L
pH	5.96	7.14	

1.3. Microbial enumeration of LSMC extract

Microbial enumeration of LSMC extract was performed by using a serial dilution technique. The populations of total bacteria, Pseudomonas, fungi, yeast and actinomycetes were counted on different selective culture media. Extract was serially diluted (10^{-2}-10^{-6}) and 100 µl of each dilution was plated on the selective media. After an incubation period of 24-48 h at 25 °C, colony forming units (cfu) were counted to estimate the different microbial populations. Three replicates were made for each test.

1.4. Addition of LSMC extract on mushroom production in large scale experiment

Blocks of phase II compost spawned with A15 were placed in standard growing room at 25 °C. After a couple of weeks of growth of the mycelium, the surface of the compost was covered with 4 cm of casing soil. The climate conditions in all rooms were controlled by a Dalsem computer control system. The crop was aired for pinning, irrigation of casing started after initiation of pin heads. Treatment rooms (6 rooms with 1782.8 m^2 growing surface) were considered as a test and irrigated with LSMC extract. Rooms irrigated with tap water (6

rooms with 1782.7 m^2 growing surface) were used as controls. Mushroom yield was determined daily during three flushes. The impact of LSMC extract on yield was evaluated as productivity and calculated as the ratio of fresh weight of total mushroom yield to the weight of compost at the end of phase II (Andrade, et al. 2007).

1.5. Determination of mushroom quality

Dry matter was estimated according to Kalberer 1991. Dry matter percent (%) =100–weight difference. The crude protein content of mushrooms was determined by micro-Kjeldahl method using conversion factors (N x 6.25) (Crisan and Sands 1978). For ash and organic matter estimation, 10 grams of dried mushroom were burned in a furnace at 600 $^{\circ}$C. % OM = % DM - % ash content. Half of the organic matter was considered as carbon.

1.6. Statistical Analyses

All statistical analyses were performed using statistical Package for Social Scientists (SPSS) software (version 19) at $P \leq 0.05$. An Independent Sample t-Test and Mann-Whitney U test (for those data that not normally distributed) were used to test for significant differences in mushroom quality and production between controls and treatment.

2. Results and discussion

In this study pasteurized LSMC extract of *Agaricus bisporus* mushroom was made for testing its effect on quality and mushroom production. As the salinity of SMC is too high, leaching is one of the options that will reduce salinity of SMC (Riahi and Arab 2004). Chemical properties of LSMC and LSMC extract are shown in Table 1. An amount of nitrogen, potassium, sodium, calcium and phosphorus were presented in LSMC extract which will be available to mushroom by irrigation. The first flush was harvested 1.5 days earlier as compared to the controls. However the results showed that there was no significant difference in total yield between treatment with extract and the control (t = 0.71, P = 0.49).

Table 2. Impact of LSMC extract on yield and productivity

Treatment	Compost weight phase II (kg)	Compost weight ($kg.m^2$)	Growing area (m^2)	Room yield (kg)	Yield ($kg.m^2$)	productivity (%)	Growing cycle
T	27410	85.62	320.11	7174.1	22.41b[1]	26.1c*	56
T	26950	84.18	320.11	7582.5	23.68b	28.1c	60
T	27130	83.59	324.56	7232.3	22.28b	26.6c	61
T	28790	88.70	324.56	7176.2	22.11b	24.9c	58
T	24430	87.22	280.09	6525.4	23.29b	26.7c	54
T	18100	84.81	213.40	4164.8	19.51b	23.0c	58
C	27410	85.62	320.11	7223.1	22.56b	26.3c	60
C	27280	85.22	320.11	6853.6	21.41b	25.1c	60
C	27140	83.62	324.56	7290.3	22.46b	26.8c	61
C	28210	87.15	323.67	7374.7	22.78b	26.1c	60
C	22870	87.22	280.09	5970.3	21.31b	26.1c	56
C	18100	84.81	213.40	4127.4	19.34b	22.8c	60

* [1]Means within the same column followed by same letter do not differ significantly at ($P< 0.05$).

T= treatment and C= control.

In marketing of mushroom, quality speaks first. Non-significant differences were recorded between dry matter, carbon, ash and organic matter of the mushrooms of the treatment and the control. Irrigation of casing with LSMC extract increased total nitrogen (t=3.02, P=0.01) and protein content (t=2.66, P=0.02) of the mushrooms significantly (Table 3).

Table 3. Measurement of dry matter, total nitrogen, carbon, ash, organic matter and protein in picked mushrooms.

DM%	N%	C%	Ash%	OM%	Pr%	type
9.4	4.7c[1]	45.4	10	91.2	29.5a*	T
9.1	4.2c	45.4	9.2	91.2	26.4a	T
9.2	4.8c	45.6	9.8	91.2	30.1a	T
9	4.1c	45.6	9.2	90.8	25.8a	T
9.3	5.4c	45.6	9.2	90.8	34.1a	T
9.1	4.3c	45.4	8.8	90.8	26.9a	T
9.2	3.9d	45.3	9.4	90.6	24.9b	C
9.2	3.4d	45.9	9.2	91.8	21.6b	C
8.7	4.1d	45.9	8.6	90.8	26.1b	C
8.7	3.9d	45.7	9	91.4	24.9b	C
8.4	4.1d	45.5	9.6	91	26.2b	C
8.9	4 d	45.2	10	90.4	25.1b	C

*[1]Means within the same column followed by different letter are significant differences.
T=treatment and C=control

Total colony count of microflora including bacteria, fungi, actinomycetes, yeast and Pseudomonas spp. revealed a numerous microbial population (> 10^6 cfu/ml) in LSMC extract. Among them Pseudomonas spp. likely to play an important role in initiation of pin heads and development of mushroom fruiting bodies (Hayes and Nair 1976). High populations of bacteria especially Pseudomonas spp. in LSMC extract may be one of the reasons why mushroom yield was slightly higher and appeared faster than controls (Table 4).

Table 4. Bacterial populations of LSMC extract

Total bacteria (10^7 cfu ml^{-1})	Total fungi	Pseudomonas spp. (10^6 cfu ml^{-1})	Yeast (10^7 cfu ml^{-1})	Actinomyces
6.1	ND	1.2	1.05	ND

ND= not detected

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Drying as a method of adding value and reducing losses in the mushroom industry

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ABSTRACT

Although cultivated mushrooms are mainly consumed in their fresh state, drying is a standard postharvest technology to extend the shelf life of several wild mushroom species for off-season use by reducing water activity to a microbial safe level for storage. In particular, convective air drying of one of the most commercially important wild mushrooms (*Boletus* spp.) is performed in cabinet-, tunnel- and conveyor dryers at a temperature range between 50 and 70 °C. Correctly dried mushrooms with a maximum moisture content of 12% intensify their distinctive flavor without significant loss of their color and pleasant texture. Optimal final moisture content is also required to avoid spoilage of mushrooms due to insect infestation during storage. Experiments with a range of air conditions (temperature, humidity, velocity) were carried out using a high precision laboratory dryer in order to study the drying behavior and relevant quality aspects of some cultivated (*Agaricus bisporus*, *Pleurotus ostreatus*, *Lentinula edodes*) and wild (*Boletus edulis*, *Morchella vulgaris*, *Cantharellus cibarius*, *Craterellus cornucopioides*) mushrooms via mathematical modeling. Drying kinetics was described by a first-order reaction kinetics model, in which the drying constant was function of the air conditions and particle size. The dried samples were compared with those obtained by alternative drying methods such freeze drying in terms of color, texture and rehydration ratio. Air temperature and slice thickness were the most influential parameters on drying acceleration, whereas the effects of humidity and velocity of drying air on drying time were considered as lower than that of temperature. Different drying behaviors were also observed among the mushrooms due to differences in species geometry. Specifically, black trumpet was dried faster, followed by morel, whereas chanterelle indicated significantly longer drying periods. High drying temperatures (70 °C) in the beginning of the process caused dried mushrooms of hard texture with visible effects of browning. However, a stepwise increase of air temperature during the final stage of drying is recommended to ensure long-term stability of dried mushrooms. Freeze drying resulted in dried mushrooms of superior quality, preserving surface color, pleasant texture as well as greater rehydration ratio. Therefore, drying represents a fundamental processing step for the development of novel value-added products, which could also reduce the losses caused by surplus production enhancing the income of the mushroom growers.

Keywords: drying, processing, quality, mushroom, industry

INTRODUCTION

The objective of this study was to investigate the effect of drying air conditions (temperature, humidity and velocity) and slice thickness on drying behavior of some cultivated and wild mushrooms. These mushroom species are of particular interest in preparation of various traditional and gourmet products such as risotto, pasta, soups, etc. It was also intended to examine to which extent, convective drying had an impact on quality of mushrooms. In order to verify the influence of conventional drying on color, texture and rehydration ratio of mushrooms, an advanced drying technology i.e. freeze drying was applied.

Mushroom samples

Fresh cultivated mushrooms: shiitake (*Lentinula edodes*), oyster (*Pleurotus ostreatus*), white button and brown cremini (*Agaricus bisporus*) and wild mushrooms: cep (*Boletus edulis*), morel (*Morchella vulgaris*), chanterelle (*Cantharellus cibarius*), black trumpet (*Craterellus cornucopioides*), free of visual defect and characteristic pests, were purchased from the same supplier at the local market in Stuttgart, Germany. The mushroom samples were standardized according to size and cleaned thoroughly to remove adhering matter. For *A. bisporus*, slices of a varied thickness were obtained from the middle section of the fruiting bodies by cutting them vertically with an electrical slicer.

Drying experiments

The hot-air drying experiments were carried out using a high precision laboratory dryer designed at the Institute of Agricultural Engineering, University of Hohenheim (Stuttgart, Germany). A single layer of some cultivated and wild mushrooms was separately dried at different conditions of drying air (Temperature: 50-70 °C, specific humidity: 10-30 g/kg, air velocity: 0.5-0.9 m/s). The dryer is connected to an industrial computer, allowing pre-programming of the set conditions and continuous monitoring of temperature, relative humidity, air velocity and mass during the drying process (Argyropoulos et al., 2011a). Mass losses were recorded at regular intervals for the determination of drying curves and the estimation of total drying time. Another batch of samples was frozen at 25 °C for 24 h and then subjected to freeze drying (Model: Alpha 1-4, Christ, Osterode am Harz, Germany) at 0.28 mbar with a shelf temperature of 20 °C and condenser temperature of -55 °C. The dried mushrooms were packed in aluminum-coated polyethylene bags and stored at room temperature for analysis.

Quality evaluation

The moisture content of dried samples was measured by Karl Fisher titration (Riedel-de Haen, Seelze, Germany) in triplicate and expressed in gram per gram dry basis (g/g d.b.). Water activity of dried mushrooms was determined at 25 °C using a digital hygrometer (Rotronic AG, Bassersdorf, Switzerland). The color of fresh and dried mushrooms was measured by a reflectance Minolta colorimeter (model CR-400 Minolta Co, Ltd., Japan). The instrument was calibrated with a standard white plate at D_{65} illumination before taking measurements (Y=93.7, x=0.3158, y=0.3324). Three readings were performed per mushroom surface by placing the colorimeter head directly above the sample. The color parameters were

expressed as L^* describing lightness ($L^* = 0$ for black, $L^* = 100$ for white), a^* describing intensity in green-red ($a^* < 0$ for green, $a^* > 0$ for red), b^* describing intensity in blue-yellow ($b^* < 0$ for blue, $b^* > 0$ for yellow). The rehydration ratio of the dried mushroom samples was determined by soaking the samples in distilled water at 25 °C. The samples were removed, dried off with tissue paper and weighed. The water absorbed (g) divided by the dry sample weight (g) was defined as the rehydration ratio. Texture of rehydrated mushrooms was assessed using a texture analyzer (Instron Universal Testing Machine, Model 4301, Norwood, USA). A 5mm diameter needle probe was used to measure toughness as the force required to puncture an individual mushroom sample placed over a plate with an 8mm diameter hole. The crosshead speed was maintained at 100 mm/min.

Results & Discussion

The drying behavior of some cultivated and wild mushroom species dried at a temperature of 60 °C are shown in Figure 1. Mushrooms with different initial moisture contents were dried to an optimum moisture content range for safe storage (Argyropoulos et al., 2010). White and brown *A. bisporus* mushrooms exhibited similar drying characteristics and almost identical total drying times. On the other hand, more time was required for shiitake and oyster mushrooms to reach the target moisture content. In particular, shiitake mushrooms were dried at 60 °C in about 180 min whereas drying of oyster mushrooms took 140 min. As far as the wild species is concerned, trumpet was dried faster, followed by morel, whereas chanterelle indicated significantly longer drying periods.

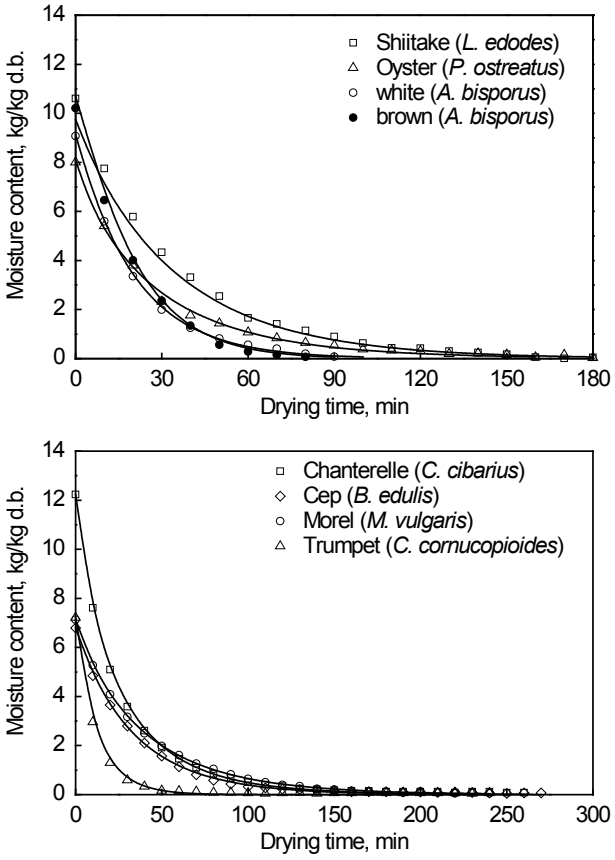


Figure 1. Single layer drying behavior of some cultivated (left) and wild (right) mushroom species.

This drying behavior can be explained by the different geometries and the structural buildup among the mushroom species. Figure 2 shows the results of single layer drying experiments of button *A. bisporus* mushrooms performed in a though-flow laboratory dryer. In general, the air temperature (left) and slice thickness (right) were the most influential parameters on drying acceleration, whereas the effects of humidity and velocity of air on the drying time were considered lower than that of temperature. The total drying time substantially decreased with a progressive increase of drying temperature. For instance, the drying time at 60 °C was about 42% shorter in comparison to the time required at 50 °C. An increase in temperature from 60 to 70 °C further reduced the drying time to about 80 min. This is an advantage of hot-air drying as the total operational time for freeze drying including freezing was more than 48 h.

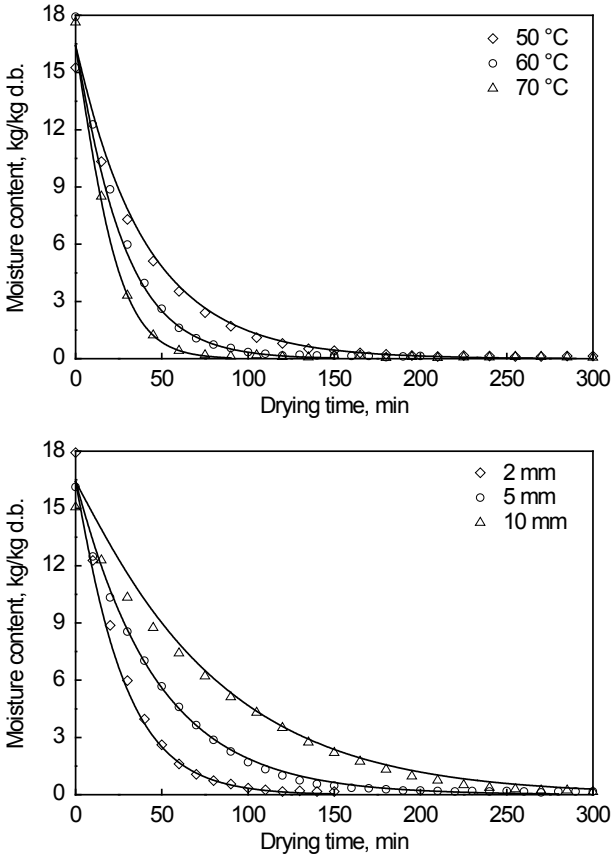


Figure 2. Single layer drying behavior of button *A. bisporus* mushrooms in a trough-flow dryer: effect of air temperature on drying time (left), effect of slice thickness on drying time (right).

The reduction of drying time is desired in practice, because the capacity of a dryer will be increased and allow for a considerable reduction of drying costs. Hence, thinner mushroom slices are preferable since thicker ones took longer time to reach a storable moisture content. However, extremely thin slices (d < 30 mm) become very fragile and difficult to handle them after drying. The effect of air temperature during convective drying on lightness (left) and rehydration ratio (right) of *A. bisporus* mushroom is shown exemplarily in Figure 3. Hot-air

drying caused moderate to intensive color changes in white button mushrooms. In the beginning of the process, the high-moisture mushroom surface along with the enzymatic activity occurred in mushroom tissues can cause rapid tissue darkening (Aryropoulos et al., 2011b). Enzymatic browning is induced by the polyphenol oxidase group of enzymes including tyrosinase, which is abundant and active in mushroom tissues. Mushroom tissue browning is also certain to happen by the interaction of amino acids with carbohydrates at higher temperatures of drying air. Drying of mushrooms at 50 °C resulted in some tissue darkening, which was more substantial at 60 °C. Remarkable color deterioration as a result of tissue darkening was observed at 70 °C. Moreover, the structural damage and shrinkage noticed in mushrooms dried at 70 °C slightly hindered moisture adsorption during rehydration. On the contrary, freeze drying preserved the color of mushrooms sufficiently. This can be explained by the reduced operating pressure in the freeze dryer, which presumably retarded the action of tyrosinase. The freeze dried mushrooms were also characterized by less collapsed structure, higher porosity and consequently greater rehydration ratio. Freezing prior to drying along with the low temperature during freeze drying protected the primary structure and prevented the thermal stress on mushroom tissues associated with negative impacts on quality.

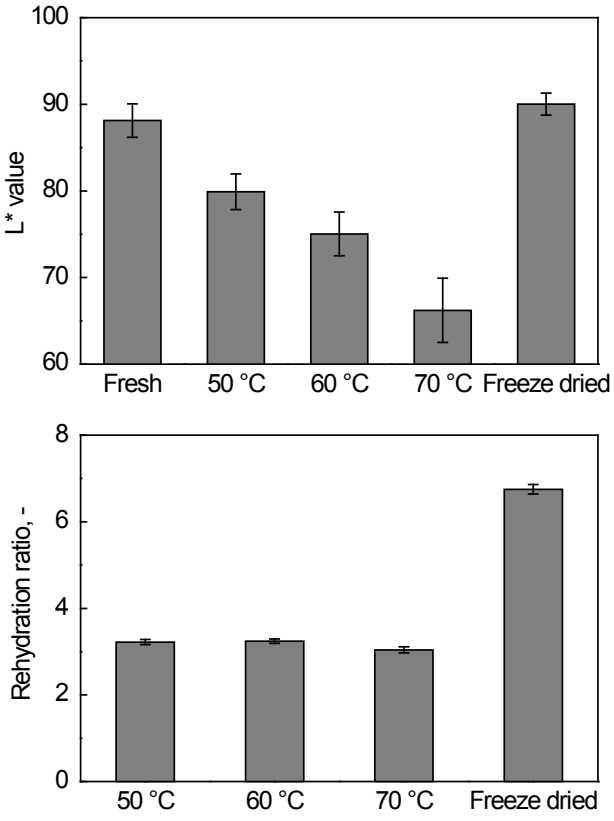


Figure 3. Lightness (left) and rehydration ratio (right) of dried button *A. bisporus* mushrooms.

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Establishing an International *Flammulina* Community.

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Knowledge and utilization of mushrooms have remained underdeveloped compared to other microorganisms, plants and animals, and a necessary catch-up is hindered by scattering of mushroom research world-wide. Existing communication structures (e.g. international meetings, journals, fungal and other communities) inadequately address the special problems that divide the mushroom field. To improve utilization and application of mushrooms there is an urgent need for communities that overcome the most prevalent boundaries.

The mushroom field in its current shape is very young. While mushrooms have been in use for thousands of years, modern large-scale cultivation has only existed for a few decades. The sudden rise of this high-tech industry for mushrooms and related products outgrew the practical application of academic mushroom research. Consequently, producers and manufacturers have assembled in separate, highly specialized communities. The availability of advanced molecular technology for mushroom research is even newer than modern large-scale cultivation. Genome sequencing, various molecular markers, controlled gene manipulation and several other tools are just existing or are still under development for mushroom research. Although modern research would certainly be beneficial for the mushroom industry, there has been little time to establish connections.

Another problem is the different perception of mushrooms in various cultures, and the resulting large numbers of mushroom model species. Western mushroom production is dominated by a single species (*Agaricus* sp.), while other species are not very popular or even distrusted as food. Possibly as a consequence, Western research has adopted mostly 'non-gourmet' model species (e.g. *Schizophyllum commun*e, *Coprinopsis cinerea*, *Laccaria bicolor*) or non mushroom forming basidiomycetes (e.g Corticioid species). This is considerably different from Asia, where cultivated and wild mushrooms are thoroughly integrated in food and medicine, and a wide range of edible mushroom model species is being studied (to name a few; *Flammulina velutipes*, *Lentinula edodis*, *Pleurotus osteratus*, *Tremella fuciformis*, *Auricularia judea*, *Volvariella volvacea*). In addition, a low priority of mushroom research and a corresponding decrease of funding in the West does not support new implementation of Asian model mushrooms. Similarly, less importance and thus less promotion of Western model species does not help implementation in Asia. Meanwhile, the near simultaneous availability of new technology for different model species diminished a central focus and selection of a leading model. These differences are also not only important between 'East' and 'West', but equally valid between 'North' and 'South' or between 'developed countries' and 'developing countries', all adding to the division of the field.

Related to the recent changes in the mushroom field is the fact that the majority of groups that perform mushroom research do so on an entirely different level than that of advanced molecular genetics. With mainly agricultural, application oriented backgrounds and practical tasks like identification of new resources, strain related taxonomy, strain improvement, pest-control, and basic genetics for breeding, the need for advanced biochemical or molecular genetics research is not directly obvious. And even if it is understood, actually making this transition is very, very difficult without having the proper infrastructure or academic background.

Finally, a lot of advanced research that is affiliated with mushroom forming fungi involves isolated components of mushrooms without a biological context. Genes and enzymes that are of interest for industrial purposes are frequently transferred, optimized and produced in host expression systems. Similarly, bioactive compounds are extracted, purified, and then studied in highly specific medical settings. And for food studies, nutritional values of mushrooms are determined on biochemically purified extracts. These disciplines are far apart, making exchange or connection of this information unlikely.

Opposite to strongly specialized communities, existing 'broad' communities (those focusing on all medicinal mushrooms, the genetics of the fungal Kingdom, production of all edible fungi, or even the total of fungi in general) do bring together thousands of species. Here the problem exists in the extreme number of addressed species, which allows little in depth information on individuals. In both cases, knowledge and expertise on any given mushroom species remains fragmented.

This situation could be improved with the establishment of international, species specific mushroom communities that provide central, online platforms where any information on a single mushroom species can be accumulated. Such a platform will traverse all different groups and industries affiliated to a species, and thus form a simple way of connecting them. The cross disciplinary nature will promote identification of topics that are of interest to multiple participants and provide easier starting points for collaborations. Platforms could also coordinate unifying activities like satellite meetings, help the publication of data from groups with limited English capabilities, or the exchange of resources ranging from employees to strains to technology.

The crucial question is if founding such communities is feasible. We have put the idea to the test for *Flammulina velutipes*, a relatively well established mushroom. *F. velutipes* is supported by a long research history, a large industry for edible and some medicinal purposes, is suitable for laboratory cultivation and is genetically accessible. At the same time, involved but different disciplines never really connected.

Starting with a screen of recent literature and invitations to join our project for an International *Flammulina* Community, a preliminary list of fifteen interested persons was generated. Further enquiries through newly collected members and professional social networks expanded the group to almost forty persons. With this number, we approached the ISMS to see if we could organize the very first International *Flammulina* Community meeting and mark its official establishment. The aim of the meeting was to introduce and connect people from diverse disciplines working with *Flammulina* species and create a first interdisciplinary and international network. The meeting, supported by the ISMS and the Chinese Edible Fungi Association (CEFA) was held in November 2015 at Fujian Agriculture and Forestry University (Fuzhou, China), and attracted over twenty-five participants from four different countries. Including FAFU students and group members, the meeting exceeded forty participants of six different countries. Participants represented geneticists, molecular biologists, breeders, factory managers, national agricultural institutes, local research institutes and biochemical biologists. Most of the participants were new to each other, irrespective of the origin of country. Presentations of the different disciplines discussed sometimes remarkably overlapping topics on mushroom development, substrate, colour, medicinal compounds, biotechnology, and crosses, yet from very different viewpoints. This demonstrated the potential for collaboration and it was unanimously decided to continue and expand the International *Flammulina* Community.

For the near future we are planning a follow up meeting and the start of a website that includes a member section, a multilingual literature collection, representatives of *Flammulina* factories,

a section for breeders and traders, a research section, and a centrally organized strain library. Any person that is interested in *Flammulina*, regardless of profession, is strongly invited to join our community.

It is my sincere hope that our example of starting a community for *F. velutipes* will stimulate people to start their own communities for other mushroom species. This is very well possible and the interest is larger than you think.

III Pests & diseases

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Identifying *Trichoderma aggressivum* within the European mushroom industry

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ABSTRACT

As part of an EU FP7 funded research project 'MushTV', work was conducted to identify locations on compost facilities and mushroom growing facilities across Europe where infective material of *Trichoderma aggressivum* might be detected. Five phase 3 compost facilities and four growers were surveyed and any *Trichoderma* isolates detected were isolated and identified using microbiological and molecular methods. Compost samples were taken from compost facilities and swab samples were taken at grower facilities. A case study was also carried out on an experimental growing unit where experimental crops, artificially infected with *T. aggressivum,* were grown. The experimental unit was surveyed throughout the course of the trial to track the presence of *T. aggressivum* on the facility.

No *T. aggressivum* was identified at the Phase 3 compost facilities during the course of the work but occasionally other *Trichoderma* spp. were detected. All growing facilities included in the survey were experiencing, or had recently experienced issues with *Trichoderma* green mould. *T. aggressivum* was detected on three of the four growing facilities while a different *Trichoderma* species was detected on the fourth site. The case study on the experimental growing unit detected *T. aggressivum* at many locations when trials with *T. aggressivum*-inoculated crops were in progress, despite the adoption of a high level of attention to hygiene. Once this information was known, and an even more stringent and targeted hygiene regime was put in place, the level of *T. aggressivum* detected decreased. The conclusion from this work is that *Trichoderma*, if present in a crop, is easily transmitted from an infected crop to diverse locations on the facility, even when there is a high level of attention to hygiene. However, effective steam cook-out and thorough cleaning and disinfection can eliminate *T. aggressivum* from the facility.

Keywords: *Agaricus bisporus*, green mould, survey, Phase 3

INTRODUCTION

Over the past twenty years the mushroom industry in Europe has been hit by sporadic, but economically damaging, outbreaks of compost green mould caused by *Trichoderma aggressivum*. This organism has the potential to cause crop losses of up to 100%. In the late 2000's Phase 3 production in several European countries was hit by *T. aggressivum* and

outbreaks of this aggressive compost green mould still affect European and international mushroom industries.

Trichoderma aggressivum can be present in Phase 3 compost as a result of an earlier contamination incident at the compost facility. Low level presence in Phase 3 compost is likely to go undetected at tunnel emptying as a small amount of *Trichoderma*-infected compost will go unnoticed due to the high level of automation. O'Brien et al. (2011) showed that infection of Phase 3 compost with as little as 0.01% *Trichoderma*-infected compost could cause up to 50% yield loss. In view of the high cost of crop failures when *T. aggressivum* is present in mushroom crops, the objective of this work was to survey compost facilities and grower facilities to identify the most likely locations to detect it, if present, and to gather information on how widespread it is. A case study was conducted on an experimental growing unit where *T. aggressivum* trials were in progress.

1. Results & Discussion

1.1. Phase 3 Compost Facilities

Samples were taken at 14 different locations at each of five Phase 3 compost facilities and tested for the presence of *T. aggressivum* between October and December 2012. Sample types and locations included fresh Phase 3 off the emptying conveyor, compost debris on the conveyor structure, compost debris in the dispatch area and compost debris on the rear of transport vehicle (Figure 1). No *T. aggressivum* was detected in any sample. Moulds such as *Mucor* sp., *Penicillium* spp. and *Aspergillus* spp. were found in many of the debris samples but not in any of the fresh Phase 3 samples during the sampling period. No Phase 3 facility was experiencing a *T. aggressivum* problem at the time and these results support that observation.



Figure 1. Compost debris on the emptying conveyor during Phase 3 tunnel emptying (left) and in the dispatch area (right).

1.2. Phase 3 Compost

Samples of fresh Phase 3 were taken from up to 30 tunnels for each of five Phase 3 compost facilities. This represented a full cycle of all Phase 3 tunnels (or tunnel complex) at each facility. A total of 142 Phase 3 compost samples were taken between June 2012 and July 2013. *Trichoderma* spp. were detected in 8/142 samples but none of these were *T. aggressivum* as confirmed by PCR (Mills, 1996). Other moulds (*Mucor, Penicillium,*

Aspergillus) were detected in 22/142 samples. The fact that some *Trichoderma* spp. were detected in Phase 3 compost samples means that the detection methods were effective, and had any *T. aggressivum* been present, the methods used should have detected it. Thus, Phase 3 compost producers should routinely test for presence of moulds, and have any *Trichoderma* spp. tested by PCR to alert them to the possibility that *T. aggressivum* may be present, requiring further action to be taken.

1.3. Mushroom Growing Facilities

Two Phase 2 block farms, 1 Phase 3 block farm and 1 Phase 3 bulk farm, all with some history of *Trichoderma* problems were sampled intensively between April and September 2013. At the time of sampling, none of the four farms was 'cooking-out' the growing rooms with steam at the end of the crop, including the Phase 3 bulk farm where the steam boiler was broken. Samples were taken at up to 36 locations on each farm and included swabs of surfaces (growing room doors, handles, floors, shelving, picking trolleys, compost debris, canteen areas, office areas and outdoor areas) as well as compost/casing debris samples. *Trichoderma* spp. were detected on all farms in 30-70% of samples. *Trichoderma aggressivum* was confirmed by PCR for 26-42% of the samples on three farms but no *T. aggressivum* was detected on the Phase 3 block farm (Table 1).

Table 1. Detection of *Trichoderma* spp. and *T. aggressivum* (by PCR) at four mushroom growing units (Sites A-D) with different growing systems. Site B was sampled twice.

	Site A	Site Bi	Site Bii	Site C	Site D
Growing System:	Phase 2 blocks	Phase 2 blocks	Phase 2 blocks	Phase 3 bulk	Phase 3 blocks
Steam cook out:	No	No	No	Yes*	No
Total No. of samples	36	24	35	34	24
Trichoderma positive	25	12	23	10	10
T. aggressivum (by PCR) (% of samples)	14 (39%)	10 (42%)	14 (40%)	9 (26%)	0

* Steam boiler was broken for several months prior to sampling so there was no end of crop steaming at the time of sampling.

1.4. Case Study on an Experimental Growing Unit

Experimental trials were conducted at AFBI, Loughgall, Northern Ireland involving Phase 3 compost that had been inoculated at spawning time with a point infection of *T. aggressivum*. At the end of the spawn run in small scale bulk incubation tunnels, the substrate was subsequently bulk handled, transported to a growing room, filled onto shelves and cropped. Thus *T. aggressivum* infected compost was present in the bulk incubation facility as well as the growing facility for the duration of the trials until incubation tunnels and growing rooms were steam sterilized, waste compost removed and rooms and facilities cleaned and re-steamed/disinfected before reuse.

The trials at AFBI provided an ideal opportunity to test a Phase 3 compost producing facility and a mushroom growing facility for the presence of *T. aggressivum* at key stages during the

progression of T. *aggressivum* cropping trials. The locations that were sampled were similar to those sampled on commercial farms and compost facilities and included growing room floors, doors, shelves, central corridor, canteen areas, office area and outdoor areas as well as areas associated with Phase 2 spawning, Phase 3 incubation and compost handling.

At the end of the first crop (Crop A), where *T. aggressivum* had been present, 75% of samples from around the facility were positive for *T. aggressivum* (Table 2), despite the fact that staff were fully cognisant of the difficulties in eliminating *T. aggressivum* propagules and routine daily cleaning and disinfection procedures were strictly followed. After steam cookout of Crop A, an intensive cleaning and disinfection procedure followed. Samples that were taken following the end of Crop A cleanup were all negative, as were samples taken after spawning and filling of Phase 3 incubation tunnels with Crop B compost, indicating the effectiveness of thorough meticulous cleaning and disinfection in eliminating *T. aggressivum* contamination.

Samples that were taken after emptying compost from the *T. aggressivum* inoculated Phase 3 tunnel and its transportation to the growing room (Crop B) were again positive for *T. aggressivum* (30%). This increased to 40 and 50% of samples being positive as the crop progressed, highlighting how easy it is for the environs of a mushroom facility to become contaminated with *T. aggressivum* propagules once there is a contaminated crop in progress.

Table 2. Detection of *Trichoderma* spp. and *T. aggressivum* (by PCR) at different crop stages during experimental trials with *T. aggressivum* infected Phase 3 compost at an experimental mushroom facility.

	End of Crop A	After steaming, cleaning and disinfection	After Phase 2 spawning of Crop B	After emptying Phase 3 and filling of Crop B	After 1st Flush of Crop B	End of Crop B
Total No. of samples	20	30	9	10	10	12
Trichoderma positive	15	0	2	4	4	6
T. aggressivum	15	0	0	3	4	6
% of samples	75%	0%	0%	30%	40%	50%

These results help to explain why growers often find it difficult to get rid of a *T. aggressivum* infection as they may not be aware of how widespread *T. aggressivum* is on their facility. There is a high likelihood that farms with *T. aggressivum* problems are cross-contaminating fresh compost (Phase 2 or Phase 3) coming on to the farm. Disease surveys, such as outlined in Table 1, have helped many growers to identify weaknesses in their cleaning and disinfection regimes. This is particularly important for growers who do not use steam to cook out their crops at the end of the cycle, as there will be a massive load of *T. aggressivum* in the spent compost from an infected crop that can be easily distributed around the farm during house emptying and clean up. However, some growers without steam can

still do an excellent job at cleaning and disinfecting their facilities which will be reflected by an absence of disease on the farm.

The use of steam to cook out infected crops is no guarantee in itself that *T. aggressivum* problems will not occur. Once a green mould infection is present in a crop, *T. aggressivum* propagules (in compost/casing debris and spores) are readily spread around the facility to places that are not steamed, and not always disinfected (offices, canteen, corridors, trolleys, benches *etc.*) thus it is important to disinfect all surfaces on a regular basis to eliminate potential background contamination.

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Growth, dispersal and impact on yield of *Agaricus bisporus* by *Trichoderma aggressivum* during bulk spawn run.

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ABSTRACT

A long and complex association between *Trichoderma* and *Agaricus* mushroom cultivation in the Irish mushroom industry began in 1985 when 'compost green mould' reached epidemic proportions in the Phase II bag cultivation system. The causative agent, identified as *Trichoderma aggressivum f. europaeum* was initially associated only with outbreaks in *in-situ* growing systems (bags, trays, or shelves) until spring 2006 when the disease reached the technologically advanced bulk spawn-run (Phase III) systems in Europe with devastating consequences. Sporadic outbreaks since then highlighted a knowledge gap of how *T. aggressivum* behaves within the Phase III system.

This work focussed on characterising the growth, dispersal and impact on yield of *T. aggressivum* in bulk-incubation tunnels. Experimental mini composting tunnels were subdivided along vertical and horizontal planes with *Trichoderma* infected spawn grains positioned in the back, lower quadrant. When compost was removed from the mini-tunnels in an artificial unmixed procedure, distances travelled by *T. aggressivum* within the bulk spawn-run were generally limited to just *ca* 0.5 -1.0 m. *T. aggressivum* was not generally visible at the end of bulk spawn-run. The severity of the subsequent mushroom crop yield losses was highly correlated with the position of the compost in the tunnel relative to the point of inoculation. These initial compost trials increased our understanding of how far and in what direction *Trichoderma* can grow during the 17 day bulk spawn-run period.

A subsequent series of trials incorporated bulk handling operations similar to those carried out at commercial premises. Yield losses were highly correlated with the degree of compost mixing and indicated that *T. aggressivum* can readily infect and colonise otherwise healthy, productive mushroom compost evidenced by yields of 25 - 34 kg m^{2-1} in the control compost. Further, the use of equipment that had handled *T. aggressivum* infected compost was shown to infect clean compost from adjacent newly opened tunnels, spreading the infection further. Crucially, studies have now confirmed that fully colonised phase III compost is susceptible to *T. aggressivum* infection. Results indicate that the bulk spawn-run process potentially increases the risk of a small compost infection becoming a serious disease outbreak, however not as a result of significant growth within the compost during the 17 day spawn-run but as a direct consequence of the bulk handling and mixing operations that disperse and spread small infections through much larger compost masses.

Keywords: Compost Green Mould, *Trichoderma aggressivum, Agaricus bisporus,* Bulk spawn-run (Phase III) compost.

INTRODUCTION

Late in 1985, 'compost green mould' reached epidemic proportions in the Phase II bag cultivation system in the Northern Ireland mushroom industry. The disease was characterised by a rapid infestation of the compost by *Trichoderma* and subsequent inhibition of *Agaricus* fructification. *Trichoderma aggressivum f. europaeum* was identified as the causative agent. Compost green mould subsequently appeared throughout the British Isles and across Europe, Canada and the U.S.A with all initial outbreaks associated with *in-situ* growing in bags, trays, or shelves. Then in spring 2006, the disease reached the technologically advanced bulk spawn-run (Phase III) systems in the Dutch mushroom industry resulting in multi-million Euro losses (Hermans, 2006). Since then, sporadic outbreaks of *T. aggressivum* have been consistently reported by Phase III producers across Europe, Canada and South Africa. In the bulk spawn-run system, large volumes of re-circulated air are forced through an unsegregated compost mass so theoretically the potential to infect all compost within the tunnel could be high. Yet, observations of *T. aggressivum* outbreaks on grower facilities showed seemingly conflicting evidence: several growers getting compost from the same tunnel reporting completely different infection levels ranging from none to total crop wipe-out (Lemmers, 2010). Recent research suggested that bulk Phase III compost may not be as immune to *T. aggressivum* infection as was shown to be the case for *in-situ* Phase II in the 1990's (Grogan *et al* 2012, Baars *et al*, 2012). However, there was still a dearth of information on how *T. aggressivum* grew inside a Bulk Phase III tunnel, how it was subsequently distributed in compost during tunnel emptying and compost transportation, and how infected propagules might be distributed during the bulk handling operations. The aim of this EU FP7 funded "MushTV" research was to gain a better understanding of the epidemiology of *T. aggressivum* in Phase III compost, in particular how the organism exploits this bulk system of mushroom compost production to its advantage.

1. Results & Discussion

Growth of T. aggressivum within a bulk incubation tunnel

Bulk compost experimental mini tunnels 2.0 x 1.0 x 2.5 m were each filled with *ca* 1.8 t spawned compost to a height of *ca* 1.9 m. A *Trichoderma* inoculum was established by inserting 48 grains of *T. aggressivum* infected *A. bisporus* A15 (Sylvan Inc.) spawn in the bottom left hand corner of the mini tunnel *ca* 15 cm from the floor and two perpendicular side walls. 1 ml sterile water mixed with 5 g spawn grains was used as an uninoculated control in an adjacent tunnel. The compost temperature was maintained as close to 25^{O}C as possible for 17 days according to standard bulk spawn-run protocols. At emptying, each mini-tunnel was spatially differentiated along four vertical



Figure 1. Experimental mini composting tunnel subdivided along vertical and horizontal planes then cropped individually to quantify T. aggressivum growth within the bulk spawn-run tunnel.

and three horizontal planes (Figure 1); compost was artificially removed undisturbed and filled at 86 kg m^{2-1} onto shelves to identified positioned plots in the growing room.
After 17 days bulk spawn run *Trichoderma* was not readily visible on emptying the experimental mini tunnels. On very careful inspection, two small sporulating pockets were just discernible and *Agaricus* mycelium appeared retarded close to the inoculation point but as this would certainly have gone undetected during automated commercial operations composters would be oblivious to any compost infection. Results indicated that distances travelled by *T. aggressivum* within the bulk spawn-run were generally limited to just *ca* 0.5 -1.0 m; growth rates in broad agreement with previous *in vitro* and *in vivo* studies. The severity of the subsequent mushroom crop production yield losses was highly correlated with the position of the compost in the tunnel relative to the growth of the *Trichoderma.* Whilst crop yield was largely unaffected in compost from the front two or three compost slices, average yields from the back slice closest to the inoculation point were generally reduced by *ca* 50%. Sub-dividing this back slice further into top, middle and bottom fractions indicated yield losses were 24%, 42% and 100% respectively. These initial compost trials have been essential in increasing our understanding of how far and in what direction *Trichoderma* can grow during the 17 day bulk spawn-run period.

Dispersal & spread of T. aggressivum during bulk handling operations at tunnel emptying
A second series of four trials studied the effect of three simulated mixing processes (action of the compost tunnel winch, horizontal layered filling of transportation vehicles and vertical lorry emptying for shelf growing at grower holdings) on dispersal and spread of *T. aggressivum* during three differentiated handling operations (compost emptied unmixed, compost emptied mixed within each of 4 individual tunnel slices, compost emptied mixed and all 4 tunnel slices layered on top of each other). The latter most closely simulating bulk handling operations as carried out at commercial premises (Figure 2).

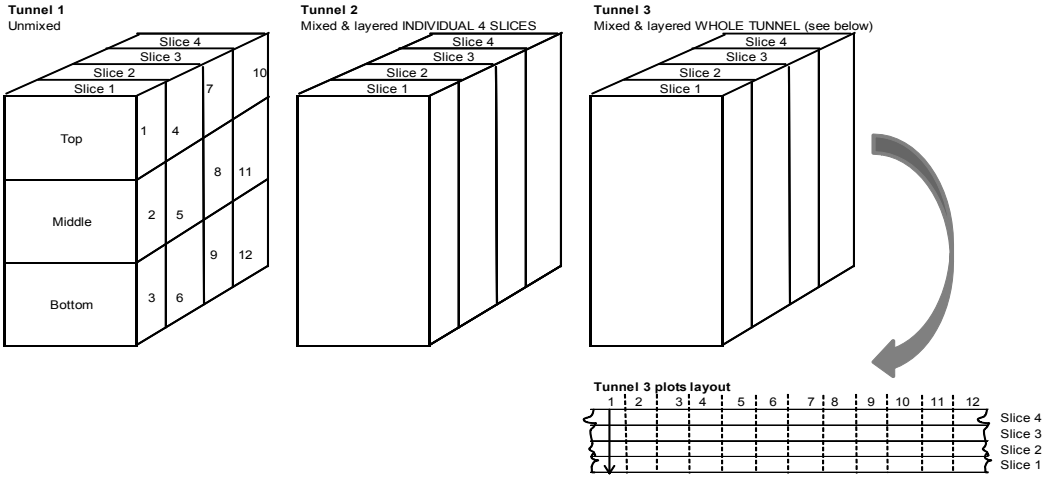


Figure 2**.** Compost Tunnels sub-division and post spawn-run mixing treatments applied
Consistently, no dispersal from the point of inoculation was evident in compost from unmixed slices; the infection was dispersed through the inoculated slice only in compost from individually mixed slices, and compost fully mixed across all 4 tunnel slices dispersed the initial localised *T. aggressivum* infection throughout all compost in that tunnel. The severity of the yield losses was highly correlated with the degree of mixing – essentially 0% yield loss from unmixed compost except in the inoculated quadrant where it was 100%; *ca* 100% yield loss in the inoculated mixed

slice but close to 0% yield loss from the remaining 3 slices in that tunnel and *ca* 100% yield loss across all compost from the tunnel in the fully mixed treatments. These trials further indicated that *T. aggressivum* can readily infect and colonise otherwise healthy, productive mushroom compost as evidenced by typical yields of 25 - 34 kg m^{2-1} in control plots and uninfected compost.

Effect of contaminated equipment on T. aggressivum dispersal during tunnel emptying.

In light of the this new information, two final trials investigated if conveyor equipment previously used to handle compost inoculated with *T. aggressivum* could cross contaminate uninoculated compost when emptied from two adjacent newly opened tunnels. To differentiate between possible aerial contamination from the emptying hall and contamination from equipment, in the first trial, no cleaning took place between the emptying of the three tunnels, while in the second trial, a stringent hygiene programme was implemented over night before the third tunnel was emptied. Further, the first slice of this third tunnel was emptied manually without using the contaminated conveyor.

Consequently, compost emptied from all three tunnels in the first trial was heavily contaminated with *Trichoderma aggressivum* and crop yield effectively reduced to zero. In contrast, during the second trial the *Trichoderma* infection was significantly reduced. Hygiene verification protocols indicated that cleaning the spawning hall to minimise "aerial" cross contamination had been effective. Subsequently, the manually emptied first slice of the third tunnel showed no *Trichoderma* infection during cropping producing a normal crop yield of 26 kg m^{2-1}. In comparison, the % yield loss relative to the control was >85% for compost from the inoculated tunnel, 26% for compost emptied from the second uninoculated tunnel using contaminated equipment and 55% from the remainder of the third tunnel emptied the following day with the same contaminated equipment. Elapsed time may have facilitated growth of the *Trichoderma* propagules on the contaminated conveyor and trailer.

Thus where the infection occurs in a tunnel is critical; if it is near the front, all subsequent compost will come in contact with now infected machinery; if it is near the back then a large proportion of compost from the front of the tunnel should yield normally. This new knowledge could explain previous observations where several growers getting compost from the same tunnel reported completely different levels of infection ranging from none to total crop wipe-out.

Crucially, these studies have now confirmed that fully colonised Phase III compost is susceptible to *T. aggressivum* infection. Results clearly indicate that the bulk spawn-run process potentially increases the risk of a small compost infection becoming a serious disease outbreak, however not as a result of significant growth within the compost during the 17 day spawn-run but as a direct consequence of the bulk handling and mixing operations that disperse and spread small infections through much larger compost masses. This has significant ramifications for best practice guidelines at both compost yards and growing units further discussed in MushTV Factsheet 03/15.

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A sensitive and quantitative test for >31 viral RNAs associated with the Mushroom Virus X diseases in both compost and mushrooms.

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ABSTRACT

Mushroom Virus X (MVX) disease is a syndrome affecting the commercial fungal crop *Agaricus bisporus* (the common white mushroom). The disease has been widespread across Europe for the last 25 years causing a number of symptoms: (1) brown discolouration of the mushroom cap, (2) delayed fruit body development and (3) misshapen fruit bodies. The symptoms have been found to coincide with an increased number of double stranded (ds)RNA elements which vary in size between ~20kb and ~600bp

Double-stranded (ds)RNA was purified from the fruit bodies of one symptomless sample and nine diseased samples and sequenced by Illumina MiSeq. The resulting sequences were compared to known sequence information for the MVX dsRNA banding patterns. Primers were designed and validated for all of the viral RNAs for qPCR detection.

RNA extraction from mushroom compost combined with a quantitative RT-qPCR technique were used to develop a test to measure relative levels of each of the novel viral RNAs, to be used by the growers and to aid epidemiological studies of the disease.

Keywords: Mycovirus, MVX, Sequencing, Testing, Virome

INTRODUCTION

The Mushroom Virus X (MVX) disease first became a problem in the mushroom industry in the mid to late 1990s and caused an epidemic in Europe in the early 2000s. In the UK alone it is estimated to have cost the UK mushroom industry £50M in 2000 (NAO 2003). A range of symptoms are linked to the disease including brown discolouration of the mushroom cap, delay of mushroom formation and malformation of the mushrooms. These symptoms are occasionally seen together but more often seen individually indicating that the different symptoms are caused by different agents of disease. MVX disease is associated with the appearance of a whole plethora of dsRNAs which have been recently proven to be viral in origin (Deakin and Dobbs et al 2016). Diagnosis of MVX has traditionally been done by measuring the size of dsRNAs present in mushroom tissue and the browning symptom (aka Brown Cap Mushroom Disease or BCMD) has been linked to a series of dsRNAs of low molecular weight by independent studies (Grogan et al 2003, Sonnenberg and Lavrijssen 2004). New research from Eastwood et al (2015) has found that it is not just the presence of

these low molecular weight viral RNAs that is important for symptom expression but in fact the titre of the RNAs is vitally important.
A recent study by Deakin and Dobbs et al (2016) has identified 19 viruses and 8 ORFans associated with the MVX disease using deep sequencing. This sequence data has been used to develop Reverse Transcription quantitative (RT-q)PCR tests for each of the viral RNAs, allowing the quantification of >31 viral RNAs. This breakthrough, along with the development of a sensitive RNA extraction method for mushroom compost, means that there are now powerful tools available to characterise the MVX disease and BCMD.
Here we discuss how the novel viruses fit into the current knowledge of MVX and how the virus tests can be used effectively.

Results & Discussion

Partial sequence data for MVX bands was made available to us by Mike Challen (formerly HRI, UK), Anton Sonnenberg (APR, Netherlands) and Pete Romaine (Penn State, USA). All of the viral sequences were compared using BLASTn in order to identify homologous sequences and the matches are highlighted in grey in Table 1. In addition to these matches it was also found that ORFan 2 is homologous to a 1400bp fragment of the S-RNA sequence which interestingly hybridizes to three RNAs of 2.4kb (S-RNA), 5.2kb (M-RNA) and >13kb (L-RNA) (Kuang et al 2004). The full length sequence of the four low molecular weight RNAs associated with BCMD have been named Agaricus bisporus Virus 16 (AbV16) by Deakin and Dobbs et al (2016) and are hypothesized to be a single multipartite virus (Table 1).
The new multi-virus RT-qPCR test was used in order to quantify the entire RNA virome of both compost and mushroom samples taken from industry, which were identified as either healthy or suffering from BCMD (Table 2). The samples were taken at first or second flush and the crop was determined to be (1) non-symptomatic, (2) Displaying off-white mushrooms or (3) Displaying brown mushrooms. The viral RNAs have been categorized into three categories based on the frequency that the different RNAs have appeared in all of the previous RT-qPCR test samples. The ubiquitous viral RNAs have been detected in the vast majority of both healthy and BCMD samples, often at very high levels and they include AbV2 (aka H1, VXXL3) and ORFan 2 (aka H3, VX3, S-RNA) as well as two circular or repetitive RNAs which have a unit size of approximately 1kb. The non-symptomatic viral RNAs are a large group of viruses which have been detected in both healthy and BCMD infected samples but are not seen as frequently as the ubiquitous viral RNAs. The disease-associated viral RNAs are a large group of RNAs which are commonly seen in disease samples at very high titre but are less frequently seen in healthy samples and they include the AbV16 RNAs as well as the AbV6 RNAs (aka band 15 and H2) amongst others. It is worth noting that although AbV6 RNA1 matches the partial sequence designated the name H2, due to the large number of diverse viruses that were sequenced in the size range, it is possible that the H2 band on gels may have been due to infection by different viruses. The same partial sequence of H2 was used in a hybridization study by Adie et al (2004) where it was found to hybridise to four different sized bands (9.4kb, 8.6kb, 7.8kb and 7.0kb).

There are extremely high levels of AbV16 RNAs in both the compost and the mushroom sample which come from the crops in which brown mushrooms are seen and high but comparatively lower levels in the samples which have only off white mushrooms.

Table 1. A summary of the dsRNA molecules associated with the Mushroom Virus X disease. The HRI and PRI dsRNA bands higlighted in grey have strong evidence to suggest that they are synonymous with the fully sequenced viral RNAs (eg matching sequence data) whilst the potential identity of other viral RNAs are inferred by size alone.

[1] East Malling Research (Deakin and Dobbs et al Manuscript), [2] Horticultural Research Institute (Grogan et al 2003), [3] Plant Research International (Sonnenberg and Lavrijssen 2004) * Due to the large number of diverse viruses found between 8kb and 9kb in size it is difficult to know if the appearance of H2 and b7 on gels was consistently a single virus.

Name[1]	**Short name**		**Contig**	**Length**	**√/?**	**Name[2]**	**Length**	**Name[3]**	**Length**
					?	b1	20200	VXXL1	20000
					?	b2	18300	VXXL2	18000
Agaricus bisporus virus 2	AbV2		C19-C23-C1	16525	√	H1	16200	VXXL3	17000
Agaricus bisporus endornavirus 1	AbEV1		C40	12730	√	b3	14400	VXL1	14000
					?	b4	13600		
					?	b5	12600		
Agaricus bisporus virus 3	AbV3		C3	9340	?	b6	10250	VXL2	9900
Agaricus bisporus virus 6-2990	AbV6-2990	RNA 1	C2a	8921	?				
Agaricus bisporus virus 6	AbV6	RNA 1	C2	8848	√	H2*	9400		
Agaricus bisporus virus 7	AbV7		C4	8759	√	b9	7000		
Agaricus bisporus spherical virus	AbSV		C5	8540	?	b7*	8600		
Agaricus bisporus virus 5	AbV5		C6	8371	?				
Agaricus bisporus virus 8	AbV8		C8	8280	?				
Agaricus bisporus virus 9	AbV9		C13-C18-C14	7713	?	b8	7800	VXL3	7800
Agaricus bisporus virus 10	AbV10		C7	7033	?				
Agaricus bisporus virus 11	AbV11		C10	6981	?				
Agaricus bisporus virus 12	AbV12		C9	6893	?	b10	6600		
Agaricus bisporus virus 13	AbV13		C15	6202	?	b11	5900	VXL4	6300
					?	b12	5400		
ORFan 1			C34	5078	?	b13	4800	VX1	5100
Mushroom bacilliform virus	MBV		C11	4009	?	b14	4100	VX2	4000
Agaricus bisporus virus 6	AbV6	RNA 2	C12	3559	√	b15	3600		
Agaricus bisporus mitovirus 1	AbMV1		C41	3439	?				
Agaricus bisporus virus 14	AbV14		C21	3482	?	b16	3300		
Agaricus bisporus virus 15	AbV15		C21a	3405	?				
ORFan 2			C17	2311	√	H3	2400	VX3	2600
ORFan 3			C23	>1742	√	b17	2200		
Agaricus bisporus virus 16	AbV16	RNA 2	C20	1946	√	b18	2000	VX4	2200
ORFan 4			C28	1935	?				
Agaricus bisporus virus 16	AbV16	RNA 1	C22	1826	√	b19	1800	VX5	2100

					?	b20	1600		
ORFan 5			C27	1110	?	b21	1100		
ORFan 6			C38	1089	?				
ORFan 7			C24	927	?				
Agaricus bisporus virus 16	AbV16	RNA 3	C29	781	√	b22	800	VX6	1000
ORFan 8			C31	703	√			VX7	950
Agaricus bisporus virus 16	AbV16	RNA 4	C33	558	√	b23	600	VX8	800

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				Mushroom			Compost		
		Bands	**Length**	Non-symptomatic	BCMD-Infected Off White	BCMD-Infected Brown	Non-symptomatic	BCMD-Infected Off White	BCMD-Infected Brown
Disease-associated viral RNAs	AbV16 RNA1	18, VX4	1826	5	22	97,396	1	463	127,256
	AbV16 RNA2	19, VX5	1946	6	90	147,117	5	3,245	55,070
	AbV16 RNA3	22, VX6	781	5	64	225,669	10	1,323	233,421
	AbV16 RNA4	23, VX8	558	3	633	318,277	4	1,207	295,533
	ORFan 8	VX7	703	1	2	24,756	2	259	1,363,290
	AbV6 RNA1	H2	8848	6,031	0	7,640	12	0	37,923
	AbSV		8540	23	5	4,175	6	2	37,672
	AbV10		7033	698	3	759	13,958	4	2,697
	AbV12		6893	6	2	38,368	7	5	9,486
	MBV		4009	24,878	92,767	13,566	1,471	3,731	1,165
	AbV6 RNA2	15	3559	50,402	0	36,166	28	2	32,837
Non-symptomatic viral RNAs	AbEV1	3	12730	33	707	17	42	95	18
	AbV3		9340	5	0	1	0	0	1
	AbV6-2990		8921	14,270	24,414	79,526	2,175	12,207	1,456
	AbV7		8759	4,621	749	3	3,333	114	4,642
	AbV5		8371	6	2	1	0	2	3
	AbV8		8280	6	2	7	4	6	3
	AbV9 (C13)		7713	46	0	0	5	0	14
	AbV9 (C18)		7713	41	13	26	18	2	1
	AbV11		6981	21,136	0	0	4	0	1
	AbV13		6202	6	13	1	1	0	2
	AbV14		3482	35	2	190	4	18	116
	AbV15		3405	13	0	0	1	1	102
	AbMV1		3439	5	2	6	1	0	3
	ORFan 4		1935	6	0	6	32	2	10
Ubiquitous Viral RNAs	AbV2 (C1)	H1, VXXL3	16525	112	58	83	385	29	82
	AbV2 (C23)	H1, VXXL3, 17	16525	639	106	66	767	115	294
	AbV2 (C19)	H1, VXXL3	16525	114,982	2,558	1,562	13,502	1,037	2,366
	ORFan 2	H3	2311	218,033	20,092	8,171	99,264	5,996	37,968
	ORFan 5		1110	11,944	20,321	24,160	1,135,490	25,959	592,097
	ORFan 6		1089	14,186	13,313	24,317	28,631	14,298	37,149

No Virus	Low Virus	High Virus

Figure 1. The viral profiles of BCMD-infected and uninfected compost and mushrooms, measured by RT-qPCR. The viral RNAs have been categorized based on the frequency of their appearance in healthy crops and those suffering BCMD. The Ct values for each viral RNA were normalized to the host 18S gene to produce the ΔCt values ($\Delta Ct = Ct_{test}-Ct_{18S}$). The ΔCt values were then transformed to make interpretation more intuitive; so that the larger values denote higher RNA titre ($Value = 2^{\wedge(-\Delta Ct)*100000000}$)

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This is the same pattern that was recently identified by Eastwood et al (2015) in their comparison of brown infected mushrooms and white-infected mushrooms. This is the first indication though that the mycelia in compost may also be host to the proposed persistent (off-white symptoms) and acute (brown symptoms) phases of the BCMD lifestyle. While the RT-qPCR method for quantifying AbV16 in mushroom samples is a good test for diagnosing suspected BCMD cases, testing the level of AbV16 RNAs in compost can be used to determine the potential for BCMD symptoms before the compost is used. Further study is required to determine if any of the other viruses, so commonly found coinfecting with AbV16, have a synergistic or antagonistic role to play in infection and BCMD symptom expression. Observational study using the RT-qPCR testing of mushrooms has begun this kind of viral interactions investigation (Dobbs and Deakin et al 2016) however studies involving manipulation of the viral complement as well as titre will be required to get a full understanding of which viruses are key to disease symptom progression in the fluctuating and dynamic complex of viruses associated with the MVX diseases.
The RT-qPCR tests have many advantages over the traditional gel tests previously used to study the dsRNAs associated with the MVX diseases (Table 2).

Table 2. Comparison of different virus detection methods for *Agaricus bisporus*.

	dsRNA banding pattern	RT-qPCR	Deep Sequencing
Detection in compost	-	+++	+
Fungal tissue required	+ (10s of grams)	+++ (milligrams)	+ (10s of grams)
Quantification	+/-	+++	+++
Identification accuracy	+	+++	+++
Sensitivity	+/-	++	+++
Time	+ (1 week)	+ (1 week)	- (>4 weeks)
Cost	+ (£100s)	+ (£100s)	- (£1000s)
Detect unknown viruses	+	-	+++

The ability to test compost for viruses is a massive step forward in being able to trace the movement of MVX disease and to be able to predict outbreaks. RT-qPCR testing is very specific which means that virus identification is much more accurate than using size information alone. The use of small amounts of fungal material for the new tests gives it the potential to be scaled up for high-throughput robotic screening. This could reduce the cost and time of testing even further, making routine testing of compost and mushrooms even more attractive to the industry.

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Evaluation of Metrafenone against *Verticillium* and *Cladobotryum* spp. – Causal Agents of Dry Bubble and Cobweb Disease

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ABSTRACT

The continuously decreasing number of pesticides that are allowed in mushroom cultivation highlights the need to further develop novel fungicides in this sector. For this purpose the efficacy of metrafenone against the fungal mushroom pathogens *Verticillium fungicola* var. *fungicola* and *Cladobotryum mycophilum* was evaluated. To obtain good level of disease optimum, inoculation rates of several *Verticillium* and *Cladobotryum* spp. strains, together with optimal time of inoculation, were specified in pilot trials. Next, experimental cropping experiments were conducted under controlled conditions to evaluate the control of both fungal pathogens using metrafenone and prochloraz-manganese, the commercially relatively widely available fungicide. Experiments were carried out according standard practices on mushroom farms and each disease trial was executed in duplicate. Different application treatments for metrafenone were evaluated, together with different treatments and doses of prochloraz-manganese. Cropping experiments showed promising disease control of metrafenone against cobweb and dry bubble disease. Residue analyses indicated that metrafenone residue levels in harvested mushrooms were conform the MRL values.
Keywords: *Agaricus*, fungicide, metrafenone, dry bubble disease, cobweb disease

INTRODUCTION

Fungal diseases are a major problem in commercial mushroom farms and can cause severe crop losses. Two important pathogens are *Verticillium fungicola* and *Cladobotryum* spp., causal agents from respectively dry bubble disease and cobweb disease. Disease control must now be based on a sustainable integrated pest management system (IPM). IPM focuses amongst others on combining improved hygiene procedures and optimum use of available crop protection agents to control and reduce the incidence of disease. In most European countries prochloraz-manganese is the only fungicide which is available for the mushroom sector [1].

This paper describes the use of metrafenone [2] as possible crop protection agent for the control of dry bubble and cobweb disease. Effects of this agent on mushroom yield are measured and residue analyses are performed. These experiments are part of the MushTV project (FP7-SME-2011-286736).

1. Material and methods

Cropping experiments to evaluate the effect of pesticides against selected pathogens were carried out on the test farm of the Belgian mushroom research center Inagro. Mushrooms (Sylvan A15) were cultivated in controlled-climate (GICOM NL) growing chambers (0.2m² containers) according to standard protocols; cac-ing technique was followed. Steaming out was done after each growing cycle. Trials were performed following the agreement for Good Experimental Practices (GEP). Based on a number of preliminary trails to establish the optimal level of disease, fungal strains *Verticillium fungicola* var. *fungicola* (V279; $1x10^6$ spores/m²) and *Cladobotryum mycophilum* (192B1; $3x10^6$ spores/m²) were selected to infect the crop by spraying the casing soil.

To evaluate the efficacy of Vivando (active compound metrafenone, 500g/l) two different treatments were assessed: 1ml in 150ml water/m² at aeration (manufacturer's recommended rates; treatment X) and 1ml in 1l water/m² at aeration (treatment Y). Infected control plots were treated with Sporgon (prochloraz-manganese 46%) at the manufacturer's recommended rates (3g/m² at casing). In addition, efficacy of Sporgon was evaluated at a lower concentration of 1g/m² applied at aeration. Infected containers without treatment with crop protection agents were used as a positive control on the success of fungal infection. In each efficacy evaluation experiment ten parallels of each treatment were positioned according to a randomized block design. For efficacy against *Verticillium* as well as *Cladobotryum*, each trial was repeated. Number of disease symptoms was evaluated for three flushes, except in the second trial.

To test for phytotoxic effects, cropping experiments were performed in 1.5m² containers (90kg/m² phase III compost); cac-ing material was added. Metrafenone was applied following treatment X and treatment Y as mentioned above; both standard dose and double dose were evaluated, next to a negative control object with no treatment. Metrafenone residue levels were analysed using the GC-MS/MS method (Primoris, Belgium).

2. Results & Discussion

2.1. Control of dry bubble symptoms

In the first trial, results of Agrova indicated that dry bubble control by Vivando treatment X as well as treatment Y was significantly better than the infected control (see table 1). The treatment of Sporgon 3g/m² at casing did not significantly lower the number of dry bubbles. Given the relatively high level of disease in third flush in the non-infected control plots, a certain level of cross-contamination/secondary infections may have occurred.

Table 1. Number of dry bubble infections/m² observed in 1st, 2nd and 3rd flush

	1st fl	2nd fl	3rd fl	total	
No *Verticillium* – control object	0	1.5	42	43.5	cd
Verticillium – control object	8	83.5	472	563.5	a
No *Verticillium*, Vivando treatment X	0	0.5	69	69.5	cd
No *Verticillium*, Vivando treatment Y	0	0	43.5	43.5	d
Verticillium, Vivando treatment X	1	14.5	142.5	158	b
Verticillium, Vivando treatment Y	1	16.5	135	152.5	bc
Verticillium, Sporgon 3g/m²	3.5	43	260.5	307	ab
Verticillium, Sporgon 1g/m²	0	28	234.5	262.5	b

Values followed by the same character are not significantly different (Tukey P 0.05); coefficient of variation: 52.85; LSD (least significant difference) 5%: 29.642

A second trial was set up to evaluate previous obtained results. In this repeat trial it was decided to terminate the cropping cycle after second flush because too high level of disease was observed. Again, treatment of Vivando did significantly lower the number of dry bubble disease symptoms. Compared to the first dry bubble trial, treatment of Sporgon gave less control.

2.2. Control of cobweb symptoms

Similar to the dry bubble efficacy trials, results of Agrova of the cobweb trial showed that control against cobweb was significantly better with Vivando. This was the case for treatment X as well as treatment Y. Also Sporgon application of 3g/m² showed optimal control of infection for the three flushes. Treatment of 1g/m² Sporgon gave lower efficacy than the standard Sporgon application but no significantly more cobweb symptoms were noted (see figure 1).

Again, a repeat trial was performed. Same results as in the first cobweb trial were obtained.

2.3. Effect on yield and residue level

To evaluate phytotoxic effects mushroom yield was measured in cropping experiments. For this purpose a number of parameters was assessed: height of mycelium in casing at cooling down, the number of pinnings and speed of pinning after cooling down. The overall yield of the three flushes of the non-infected control was 32.7kg/m² (i.e. 363.3kg/ton phase III compost). Both metrafenone treatments led to a statistically significant higher yield, ranging from 34.6kg/m² (384.4kg/ton phase III compost) till 35.5kg/m² (394.4kg/ton phase III compost).

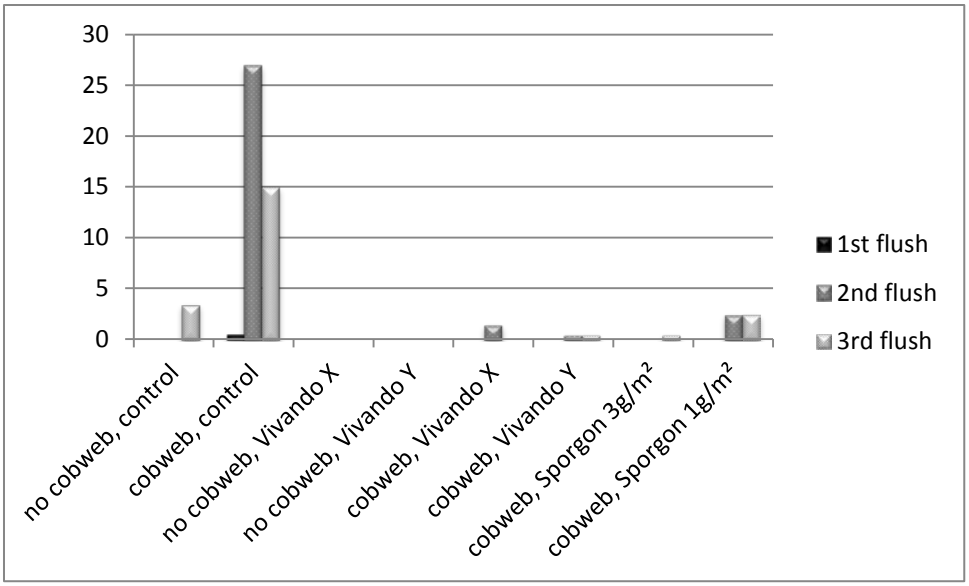


Figure 1. Number of cobweb infections/m² observed in 1st, 2nd and 3rd flush

Residue data from mushrooms harvested in first, second and third flush for all treatments showed all metrafenone residue levels conform the MRL values.

2.4. Conclusion

Using two different applications, Vivando was tested against *Verticillium* and *Cladobotryum* in efficacy cropping trials. Compared to the commercially available fungicide prochloraz-manganese, disease control of this product was very promising for efficacy against both pathogens.

No phytotoxic effects of metrafenone on yield were observed. Metrafenone residue levels in harvested mushrooms were conform the MRL values.

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Evaluation of bio-fungicides for the control of fungal diseases on *Agaricus bisporus*

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ABSTRACT

Two prominent fungal pathogens *Trichoderma aggressivum* and *Lecanicillium fungicola* currently cause the most widespread diseases, Green Mold and Dry Bubble respectively, of the cultivated *Agaricus bisporus* mushroom production worldwide. With the decrease in number of registered and effective chemical fungicides available to growers, the industry needs to look for more environmentally friendly alternatives. Cropping experiments were conducted at the Pennsylvania State University Mushroom Research Center to determine if several novel bio-fungicides and essential oils were effective in controlling these fungal pathogens. It has been reported that the degree to which *T. aggressivum* develops on mushroom grain spawn, following inoculation with *T. aggressivum,* is positively correlated with disease severity in actual mushroom production trials. Therefore, the extent to which a fungicide protects the spawn from green mold would potentially be predictive of the level of disease control achieved in cropping trials. Commercial spawn of an off-white hybrid mushroom strain was treated with calcium sulphate as the bio-fungicide carrier (control) and a mixture of calcium sulphate and commercially available biological fungicides and compared to synthetic non-grain spawn. Cropping experiments were used to evaluate combinations of Mertect®, Bravo®, JAZZ® and Trilogy® for efficacy against *T. aggressivum. L. fungicola* was inoculated with a spore suspension several days after casing. The trials consisted of combinations of un-inoculated and inoculated-untreated controls compared to a treatment of registered chemicals. Different rates of application and combinations of bio-fungicides were tested. The most efficient method and timing for these chemicals is reported here and efficacy was determined by fresh mushroom yields and quantification of diseased mushrooms, both bubbles and spotted mushrooms. Our results suggest that most bio-fungicides tested were not effective against either pathogen. However, a couple of essential oils were effective and a naturally occurring bacterium, *Streptomyces griseoviridis*, a commercially available biological fungicide was as effective as the registered chemical treatment in reducing *L. fungicola* disease development.

INTRODUCTION

When considering control of diseases caused by living organisms, three requirements for a disease to develop should be considered. A susceptible host, a pathogen and a favorable

environment for disease development, therefore, control of the disease has to focus on one of these factors. Controlling fungal diseases of mushrooms is difficult on commercial farms because the environmental and nutritional needs for mushroom production similar also encourage the development of three main fungal diseases: *Trichoderma* green mold, *Lecanicillium fungicola,* dry bubble and *Cladobotryum,* cobweb. Ecological controls such as a reduction in temperature or humidity are impractical, since these factors are crucial to the mushroom's yield and quality. Management of these diseases in mushroom houses consists primarily of the use of registered fungicides and sanitation practices on the farm. Only two fungicides are registered in the United States for use against these pathogens: Mertect® (Thiabendazole) and Bravo® (Chlorothalonil). Most US farmers were using Topsin®-treated spawn for control of *Trichoderma* until the registration was discontinued in 2009. Recently, farmers have questioned the effectiveness of these fungicides and rely of farm sanitation and hygiene for fungal disease control. The application of essential oils to control diseases affecting the production of edible mushroom has been reported by several researchers (Glamoclija et al. 2009; Regnier and Combrinck 2010). The objective of this study was to determine the efficacy of several bio-fungicides as mycobiocides against the two most important mushroom pathogens and specific isolates native to North America; *L. fungicola* and *T. aggressivum*.

Material and Methods

Three experiments were conducted using commercial compost filled into trays for the Phase II at the Mushroom Research Center with computer controlled environmental conditions for air and bed temperatures. In all experiments at spawning, the Phase II compost was observed to be of medium length, chocolate brown in color, with no detectable odor of ammonia. A commercial, off-white hybrid strain of *Agaricus* (J.E. Lange) Imbach was added to the Phase II compost at a rate of 3% and a commercial delayed-release supplement was added at a rate of 4% (dry weight basis). After 18 days of spawn growth at 24-25^{o} C, the trays were cased with a 4.0 cm layer of pasteurized sphagnum peat moss and limestone mix. On the day of casing, commercial Casing Inoculum (CI) was added to the casing, just before its application, at the rate of 500 g of CI to 1 m^{2} of growing area. After casing compost temperature was held at 24-25^{o} C after which time it was lowered to 19^{o} C for flushing. As the pins formed and began to enlarge, the air temperature was maintained between 17-18^{o} C and held at that temperature throughout the remainder of the crop. Three to four such breaks were harvested during the picking period that started about 18 d after casing, with the mushroom weight and size recorded during the harvest period.

These cropping trials were done using environmentally controlled production rooms at the Mushroom Research Center at Penn State University. Cropping experiments using *L. fungicola* as a pathogen were conducted to evaluate the efficacy of several bio-fungicides for control of disease development and influence on mushroom yield. A conidial suspension (1 $\times$ 10^{5} conidia ml-1) of *L. fungicola* was prepared just prior to the experimentation. Thiabendazole (Mertect 340 F®) applied day 7 after casing and Chlorothalonil (Bravo Weatherstik® 720) applied day 9 after casing were used as commercial fungicides treatment. While Sesame Oil (Organocide®), *Coniothyrium minitans* (Contans WG®), *Gliocladium*

catenulatum (Prestop®), *Streptomyces griseoviridis* (Mycostop®) and Saponins of *Chenopodium quinoa* (Heads-Up Plant Protectant®) were used as alternative bio-fungicides. The bio-fungicides applications were either applied according the label's Active Ingredient rate or at a 2x rate. The timing of the applications for the first cropping experiment was 9 days after casing and for the second cropping experiment day 7, 9 and 15 days after casing. The casing was sprayed with the *L. fungicola* spore suspension 10 days after casing. First break harvest began 17-18 days after casing. Watering and temperature programs were followed according to the standard method.

For the third cropping experiment, inoculation using *Trichoderma aggressivum aggressivum* (Ta2) occurred the day after spawning. A spore suspension of 1.6 x 10^8 was made using standard methods. Inoculation was a modified procedure used by Rinker and Alm (1998). The tubs were prepared to receive *T. aggressivum* by creating a 5 cm hole in the center of each tub and then delivering 0.100 ml of the spore suspension. The point of inoculation was marked with a sterilized tooth pick. After the spores were delivered, several grains of the spawn and/or supplement were placed in the hole. This method was repeated for the tubs not to be inoculated with the exception that the spore suspension was replaced with water. Spawn grains were treated with either *Streptomyces lydicus* (Actinovate®) or *Bacillus subtilis* QST 713 strain (JAZZ®) and untreated spawn was used as the control. In addition to treated spawn we tested commercially prepared "grain-less" synthetic spawn from three different suppliers. These synthetic spawns are proprietary mixtures of materials used to replace the traditional grain spawn.

Mushrooms were harvested for 3 or 4 growing cycles, or breaks, depending on disease severity. Yield data was statistically analyzed for a completely randomized design using the Waller Duncan K-ratio t-test at a significance level of 0.05 to separate the means. The number of symptomatic mushrooms (spotting, bubble or split stems) or *T. aggressivum* surface coverage was counted daily and totaled for each break (7-day total) and reported as percentage of mushrooms infected.

Results and Discussion

The first cropping experiment results suggested that *S. griseoviridis* demonstrated significantly better control of *L. fungicola* than the other bio-fungicides tested, Table 1. The level of control was not significantly different than the un-inoculated control and the Thiabendazole/Chlorothalonil chemical treatments. The three break total yields were not significantly different apart from the *Streptomyces* treatment that had significantly lower disease control compared to the chemical control, *G. catenulatum* and *C. minitans* treatments.

Table 1. Total fresh mushroom yield for three breaks and incidence of *L. fungicola* disease by the end of cropping for the un-inoculated control, the inoculated untreated, and the chemical or bio-fungicide treatments. Numbers in columns followed by the same letter are not significantly different from one another (P, 0.05).

Treatment	Total Yield Kg/m2		Disease Incidence	
Control Uninoculated	20.36	bcd	0.00	c
Control Inoculated	19.83	cd	15.5	a
Thiabendazole/Chlorothalonil Inoculated	21.99	a	1.0	c
Gliocladium catenulatum Inoculated	21.84	ab	15.0	a
Streptomyces griseoviridis Inoculated	19.07	d	5.3	bc
Coniothyrium minitans Inoculated	21.11	abc	13.0	a
Sesame Oil Inoculated	20.34	cd	13.3	a
Saponins of *Chenopodium quinoa* Inoculated	20.00	cd	10.0	a

The second cropping experiment was conducted to investigate the optimum rates of *S. griseoviridis* and timing of its application, Table 2. The results suggest that there was no significant difference in total four break yields between treatments however, the inoculated untreated control and the *S. griseoviridis* application on day 9 after casing were significantly higher in incidence of *L. fungicola* disease when compared to the un-inoculated control and the 2x rate and three applications of *S. griseoviridis.*

Table 2. Total fresh mushroom yield for four breaks and incidence of *L. fungicola* disease by the end of cropping for the un-inoculated control and the inoculated untreated, chemical and *Streptomyces griseoviridis* at different rates and timing of applications treatments. Numbers in columns followed by the same letter are not significantly different from one another (P, 0.05).

Treatment	Total Yield Kg/m2		Disease Incidence	
Control Uninoculated	29.11	a	0.0	b
Control Inoculated	26.93	a	24.8	a
Thiabendazole/Chlorothalonil Inoculated	27.28	a	1.0	b
Streptomyces griseoviridis day 9 Inoculated	28.45	a	25.3	a
2X *Streptomyces griseoviridis* day 9 Inoculated	26.67	a	9.5	b
Streptomyces griseoviridis day 7, 9,15 Inoculated	28.45	a	3.0	b

The third cropping experiment results suggest that synthetic spawn was effective in controlling *T. aggressivum* disease, Table 3. The yields results show that the all three synthetic spawns and the un-inoculated control yields were significantly higher than the inoculated untreated control and the *Streptomyces lydicus* and *Bacillus subtilis* treatments. The incidence of *T. aggressivum* disease was also significantly less for the untreated control and synthetic spawns when compared to the other treatments.

Table 3. Total fresh mushroom yield for three breaks and incidence of *T. aggressivum* disease by the end of cropping for the un-inoculated control and the inoculated control, chemical or bio-fungicide and the three different synthetic spawn treatments. Numbers in columns followed by the same letter are not significantly different from one another (P, 0.05).

Treatment	Total Yield Kg/m2		Disease (Ta2) Incidence	
Control - Uninoculated	21.23	a	0.0	c
Control - Inoculated	12.20	bc	36.3	abc
Synthetic Spawn A - Inoculated	21.70	a	0.0	c
Synthetic Spawn B - Inoculated	18.05	ab	6.3	c
Synthetic Spawn C - Inoculated	18.31	ab	0.0	c
Streptomyces lydicus 1X - Inoculated	10.05	c	47.5	ab
Streptomyces lydicu 2X - Inoculated	9.17	c	50.0	ab
Streptomyces lydicu 3X - Inoculated	7.39	c	61.3	a
Bacillus subtilis QST 713 strain - Inoculated	7.35	c	16.7	bc

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Mushroom disease detection, surveilance and farm health

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ABSTRACT

Mushrooms are susceptible to various diseases of which the causal agent may be bacterial, fungal or viral. Four main mycoparasitic fungal diseases are of economic importance on white button mushrooms and include; compost green mould (*Trichoderma aggressivum*), dry bubble (*Lecanicillium fungicola*), cobweb (*Cladobotryum dendroides*) and wet bubble (*Mycogone perniciosa*). A series of test methods have been developed, optimized and used in the industry to detect the presence of these fungal pathogens prior to symptom expression. The use of standard microbiological methods, advanced identification techniques and molecular methods provides a diagnostic platform for accurate and reliable monitoring as well as disease diagnosis. Molecular methods currently include conventional PCR and real-time PCR. This paper explores the possibility of using a quantitative PCR (qPCR) method in the form of droplet digital PCR (ddPCR) for more accurate detection of these pathogens. Once optimized, quantification using ddPCR is possible without the reliance on a DNA quantitative standard, as required by other qPCR methods. Commercial mushroom farms in South Africa were monitored for mycoparasitic fungal disease presence over a period of four years, using conventional and real-time PCR. Samples (n = 912) were collected from three areas on each farm (n = 13) *i.e.* composting area, preparation area and production area, presence or absence where determined using the novel ddPCR, with the future vision of pathogenic load quantification. Analysis showed that *Trichoderma* spp. and *Cladobotryum* spp. are the most frequently detected pathogens in all three production areas. Isolation trends per annum for all four pathogens show an increased pathogen/disease presence in the months of January to March and again from May to July as well as November to December, with fluctuations throughout the rest of the year. The development and application of the described detection methods have proven to be an invaluable tool in assisting farmers to control disease more effectively through an early warning system.
Keywords: Mushroom pathogens; disease monitoring; droplet digital PCR; farm health checks; mushroom diseases

INTRODUCTION

White button mushrooms (*Agaricus* sp.) are susceptible to a wide range of bacterial, fungal and viral diseases (Fletcher *et al*., 1989). A few mycoparasitic species of fungal pathogens are of economic importance to the cultivation of *Agaricus* mushrooms. These pathogens are of importance due to their effect on the production yield and the quality of the mushrooms

produced. The four main fungal diseases under consideration include, green mould (*Trichoderma aggressivum*), dry bubble (*Lecanicillium fungicola*), cobweb (*Cladobotryum dendroides*) and wet bubble (*Mycogone perniciosa*), which are annually responsible for considerable disease outbreaks worldwide (Largeteau & Savoie, 2010). A diagnostic platform including a series of test methods was developed, optimized and used in the South African mushroom industry to detect the presence of all the fungal pathogens prior to disease expression. The diagnostic platform includes the use of standard microbiological methods, advanced identification techniques (MALDI-TOF) and molecular methods [Real-time PCR and droplet digital PCR (ddPCR)] for accurate as well as reliable monitoring and diagnostic systems. The aim of this paper is to test the proposed improvement of the current diagnostic platform to include ddPCR as the molecular method for detection by testing test strains as well as environmental samples.

Results & Discussion

Droplet digital PCR is a direct measurement of target molecules which eliminates the need for calibration and standard curves (Henrich *et al*., 2012; Sanders *et al*., 2013; Sedlak & Jerome, 2013), which will enable future disease monitoring and diagnosis to easily and accurately quantify pathogenic loads. Droplet digital PCR allows for partitioning of the sample, enabling a greater precision and sensitivity in comparison with conventional or real-time PCR (Hayden *et al*., 2013). In addition, partitioning of individual reactions into a picolitre droplet scale, allows ddPCR to have reduced interference with PCR inhibitors present due to the environmental nature of the sample. The technique also proves itself as a promising platform of analysis for pathogen monitoring, even at low inoculum concentrations (Sanders *et al*., 2013; Lui & Tan, 2014).

To investigate if ddPCR is a comparable platform for detection and quantitation of fungal pathogens, ddPCR reactions were performed with fungal pathogens from the International Mushroom Fungal Pathogen collection. Primers used for amplification of *Trichoderma* only allow for positive species amplification of *Trichoderma aggressivum*, whereas primer sets for *Cladobotryum*, *Mycogone* and *Lecanicillium* are genus specific and will allow amplification of various species within the respective pathogen group. Representatives from each fungal group were chosen, after which isolated genomic DNA was amplified with the ddPCR platform. A visual representation of positive and negative droplet amplification for the chosen fungal isolates may be observed in Figure 1.

Implementation of detection was initiated with a Farm Heath Check (FHC), performed on 13 commercial and smallholder mushroom farms in South Africa (Figure 2). The new proposed diagnostic platform was used for accurate and reliable monitoring over the time period. Sampling areas on farms were divided into three broad thematic categories i.e. composting area, preparation area and production area. On-farm functions such as casing, spawning, mushroom growing etc. were then classified into their representative functional groups and representative samples were collected in each respective category. The most frequently detected and isolated pathogens were *Trichoderma* spp. and *Cladobotryum* spp., prevelant in

all three production areas. Isolation trends per annum for all four pathogens reflected increased pathogen presence in the months of January to March, from May to July, and again during November to December, with fluctuations throughout the rest of the year. The development and application of the described detection methods have proven to be an invaluable tool in assisting farmers to control disease more effectively through an early warning system provided by the diagnostic services developed.

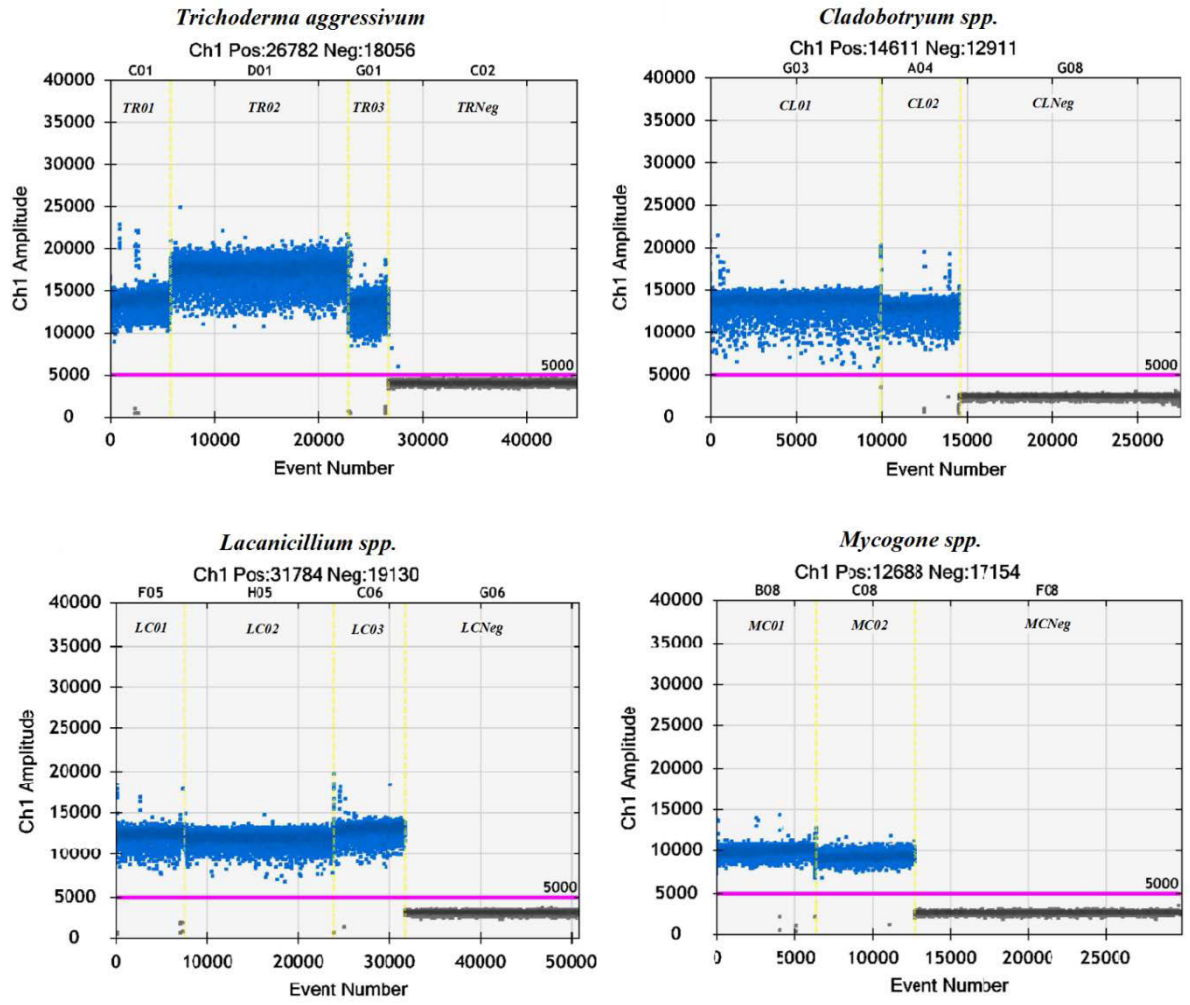


Figure 1. A visual representation of positive and negative droplets following ddPCR reactions for mycoparasitic pathogens of *Agaricus* mushrooms, ***Trichoderma aggressivum*** (top left panel), **TR01** - *Trichoderma aggressivum var. europaeum*, MES13004; **TR02** - *Trichoderma aggressivum var. aggressivum,* CBS 100528; **TR03** - *Trichoderma aggressivum var. europaeum*, MES13067, ***Cladobotryum*** **species** (top right panel), **CL01** - *Cladobotryum* spp., ACC314; **CL02** - *Cladobotryum mycophilum*, AMC00107, ***Lecanicillium*** **species** (bottom left panel), **LC01** - *Lecanicillium fungicola var. Aleophilum*, ACC123; **LC02** - *Lecanicillium fungicola var. Aleophilum,* DAR43407; **LC03** - *Lecanicillium fungicola var. Aleophilum,* DAR42124 and ***Mycogone*** **species** (bottom right panel), **MC01** - *Mycogone perniciosa*, ACC176; **MC02** - *Mycogone perniciosa*, MES11240. Minimum threshold for droplet positivity is shown with a pink line. Positive droplets in blue and negative droplets in gray-scale are shown for all pathogen reactions, blank reactions without any DNA were used as negative controls.

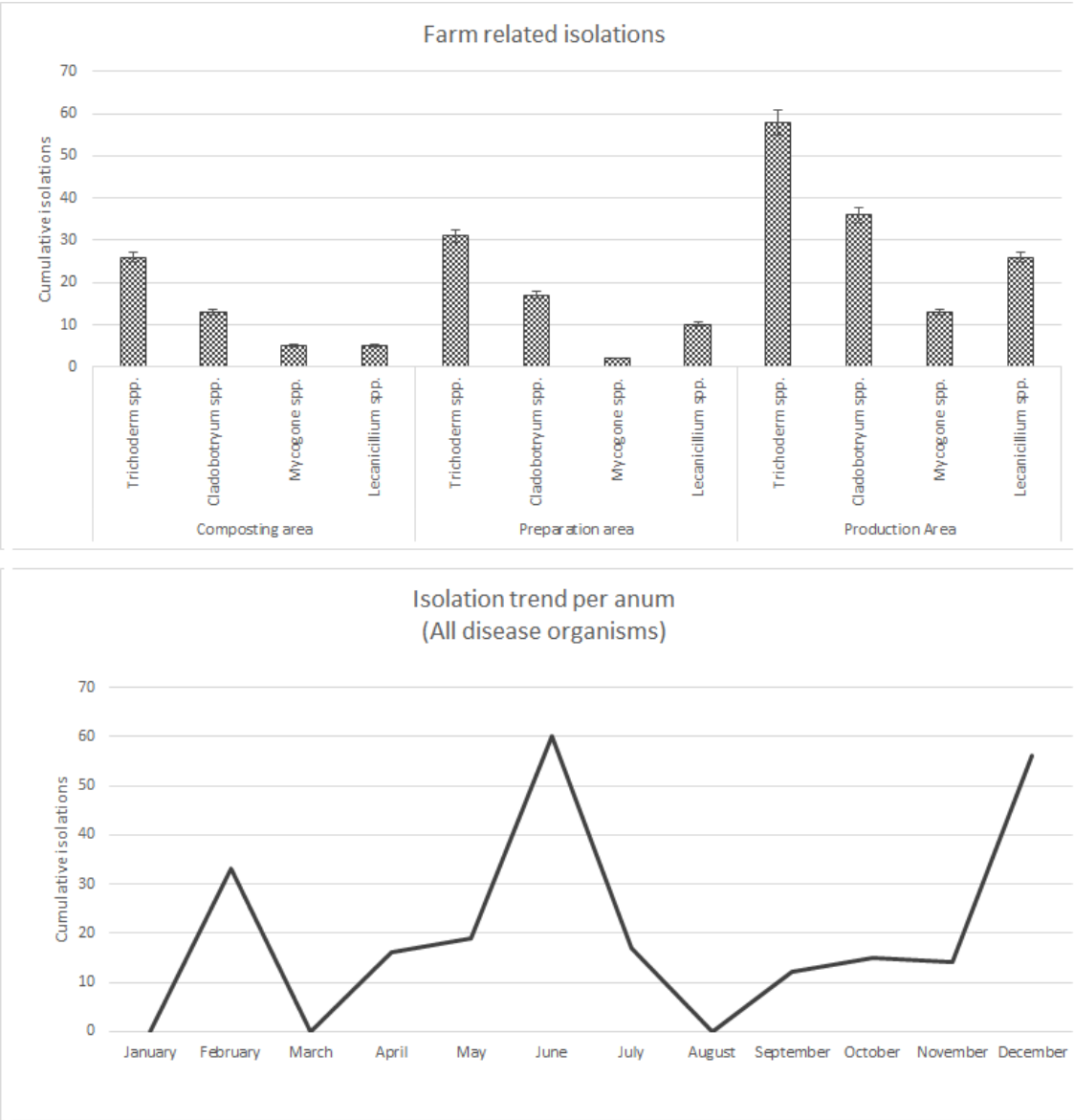


Figure 2. Top panel depicts positive detection, isolation and identification of mushroom fungal pathogens in three main categories on commercial and smallholder farms in South Africa (N = 13 farms; n = 912). Bottom panel depicts disease and isolation trends for the four main mycoparasitic mushroom pathogens, *Trichoderma aggressivum*, *Mycogone* spp., *Cladobotryum* spp., and *Lecanicillium* spp. over four (yearly) monitoring cycles.

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Sampling and detection of Dry Bubble (Lecanicillium sp.) on mushroom farms

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ABSTRACT

Major advances have been achieved in sampling for the detection of *Lecanicillium*. The key development is the use of a broad roller for detection of spores and mycelium over a large area (eg floor, wall), and then either extracting the inoculum as either elutions from the sampling roller, swabs from the roller, or direct transfer of the roller samples to agar "plates". Key achievements using the broad roller method include:

- *Lecanicillium* detected in 90% of sites on a dry bubble-infected mushroom farms by sampling floors with large, short-napped paint roller sleeves using a combination of direct inoculation and swabbing
- The connection between dirty floors and crop infection strengthened by the detection of *Lecanicillium* on the soles of the shoes of harvester staff, supervisors and on the steps of picking trolleys.

Validation of the techniques is continuing on tray, shelf and bag farms, and the sampling techniques will be assessed for the detection of *Cladobotryum* and *Trichoderma* with a focus on roller sampling, touch plates and flies.

The diagnostics team have successfully developed PCR-based methods for the detection of *Lecanicillium fungicola* and generic *Lecanicillium* sp. The *Lecanicillium fungicola* probe is detecting L. *fungicola* with high accuracy (>90%) compared to conventional microbiological identification, and the *Lecanicillium* sp. probe is detecting a range of *Lecanicillium* spp. Amplicons are identified by sequencing. The Lecanicillium primers are part of a multiplex primer panel which is also detecting *Trichoderma* and *Cladobotryum* sp. The three fungal qPCR primers are working with accuracies in the high 90% range.

Methods to discriminate between alive and dead *Lecanicillium* sp. are being evaluated using a growth enrichment approach. Cultures are either heat-treated or not (control samples), incubated and quantified using qPCR. Live cells showed 43-fold increase in signal after incubation.

Keywords: (Lecanicillium, Cladobotryum, Trichoderma, sampling, qPCR)

INTRODUCTION

The objective of the project is to develop and test a reliable sampling protocol and molecular detection of Dry Bubble (*Lecanicillium* sp.), on commercial mushroom farms in Australia. The research will support the development of a cost-effective, rapid and reliable disease diagnostic service to the mushroom industry is the aim of the project. The project teams are focussing their efforts on developing and testing a reliable farm sampling methodology and on molecular detection methods.

1. Results & Discussion

1.1. Sampling and detection

Large paint roller sleeves (270mm wide) with a short 6mm nap were used to sample: two grow room floors from each of three flushes, early and late; two sections of utility corridor floor outside two third flush rooms (symptomatic); tea room floor; and, floor of a mid-first flush room in which dry bubble had been reported on the bed.

1.1.1. Large roller sampling and parallel sampling

Of the ten sites sampled with the large rollers, *Lecanicillium* was isolated by direct transfer to PLSA in large-format agar dishes from eight sites (Table 1). One of the sites which failed to yield *Lecanicillium* from direct inoculation of the roller was from the floor of a first flush room on day one of picking, a site that would not be expected to have *Lecanicillium* present. On the other hand, the second site which failed to show *Lecanicillium* by direct inoculation of PLSA was the corridor floor outside a third flush room, a site from which *Lecanicillium* has consistently been isolated previously on farms with a dry bubble-affected third flush.

Of the ten rollers, seven were shown to be positive for *Lecanicillium* from swabs and three negative, one of which again was the roller from the first flush room floor on day one of picking. The other two negative swabs were from the floor in a mid-first flush room in which dry bubble had been reported (sample no. 3) and from the floor in a second flush room on the first day of picking (sample no. 4).

There was 70% concurrence between the two different isolation techniques – direct plating of the roller and swabbing from roller (Fletcher and Gaze 2008). The results were divergent for sample no. 3 – mid-1st flush room in which bubble had been reported; sample no. 4 – floor of early 2nd flush room; and sample no. 8 – corridor floor outside symptomatic 3rd flush room.

Considering both techniques in combination, 90% of the samples proved positive; the only concurrent negative sample being no. 1, the floor of the first flush room on day one of pick, where *Lecanicillium* would be very unlikely to be present.

1.1.2. Small rollers sampling

More hits were obtained from swabs off the rollers than were obtained by direct inoculation to medium-format PLSA dishes (Table 2; Figure 2) suggesting *Lecanicillium* propagules are retained within the nap of the roller sleeve and are not contacting the agar surface. There was 63% concurrence between the two methods and if considered together, the methods accounted for a 72% positive return for *Lecanicillium*. The two methods recorded concurrent

negative results for: sample numbers 11, 21 and 22. The results were divergent for samples 12, 13, 25 and 26 however this was due to the PCR technique being more sensitive than agar-based detection. Sequencing of *Lecanicillium* amplicons has shown that the PCR primers are accurately detecting this species.
In terms of on-farm Micro detection of *Lecanicillium*, these results demonstrate that the floor rollers are a very efficient and effective method of sampling large areas of both hard porous and non-porous surfaces – there is a high number of positive detections from the rollers which represents an aggregate sample of a large surface. Coupled with swabbing of the rollers onto PLSA, a 90% positive detection rate was achieved from the floors of a dry bubble-affected mushroom farm.

1.2. Molecular detection
The diagnostics team have successfully developed PCR-based methods for the detection of *Lecanicillium fungicola* and generic *Lecanicillium* sp. The *Lecanicillium fungicola* probe is detecting *L. fungicola* with high accuracy (>90%) compared to conventional microbiological identification, and the *Lecanicillium* sp. probe is detecting a range of *Lecanicillium* spp. Amplicons are identified by sequencing. The Lecanicillium primers are part of a multiplex primer panel which is also detecting *Trichoderma* and *Cladobotryum* sp. The three fungal qPCR primers are working with accuracies in the high 90% range.

1.3. Dead and alive discrimination
Duplicate swab samples were taken from old plate cultures of *T. harzianum*, *C .mycophilum*, *L. fungicola* and a clean PDA plate (sham) and sealed in 1.5 ml Eppendorf tubes for control (no treatment) and the following treatments:

- Expt 1 - 15 min 90°C and 15 min -20°C heat-freeze cycle repeated twice
- Expt 2 – 25 min at 70°C
- Expt 3 - 15 min at 55°C

The pathogens grew unevenly in liquid cultures: *C. mycophilum* mycelium was visible after 24 h, *L. fungicola* mycelium – after 48 hours; and the cultures were not homogenous with mycelia forming distinct clumps. No growth was observed in the controls.
The 90°C heat-thaw and 70°C heat-only treatments resulted in no increases in the amounts of DNA extracted from the enrichment cultures even after 72 hrs of incubation. This suggested efficient killing of the mycelia and spores; however, in many cases the amount of DNA was below the detection limit, especially in the treated samples or samples immediately after inoculation (most likely due to dilution).
Treatment at 55°C reduced the viability of the inocula which resulted in growth delayed for about 24 h (*L. fungicola)* or 48 h (*C. mycophilum)*.
Enrichment technique allows several-fold amplification of the pathogen signal within the first 24-28 hrs of culture (depending of species) and is sufficient to detect lack of growth (dead spores/mycelium). Whether enrichments from casing-containing samples to determine the effect of co-cultures would confirm the preliminary results remains to be determined. If confirmed, this technique could be used in routine testing to amplify a signal that may

otherwise be below detection limit. As this would delay sample processing, the enrichment procotol would require optimization.

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Tables and Figures (Maximal 2 figures and/or 2 tables)

Table 1. Large paint roller swab

No	Room	Flush	Comment	Agar	PCR - Roller swabs		
					1	2	3
1	13	1	First day pick	—	—	—	—
2	9	1	Late first flush	*Lecanicillium*	—	—	+
3	12	1	Mid flush, bubble	*Lecanicillium*	—	—	—
4	5	2	Early second flush	*Lecanicillium*	—	—	—
5	7	2	Late second flush	*Lecanicillium*	+	—	—
6	20	3	Early third flush	*Lecanicillium*	+	+	+
7	21	3	Late third flush	*Lecanicillium*	+	—	+
8	Corridor		Outside third flush	—	—	+	—
9	Corridor		Outside third flush	*Lecanicillium*	+	+	+
10	Tea			*Lecanicillium*	+	—	—

Table 2 Small paint roller swab results

No	Sample	Agar	PCR- Roller swabs		
			1	2	3
11	Tea room tables, far row	—	—	—	—
12	Tea room tables, middle	—	—	+	—
13	Tea room tables, near	—	—	+	—
17	Step of picking trolley 1	*Lecanicillium*	+	+	+
19	Step of picking trolley 2	*Lecanicillium*	+	+	+
21	Feet of picker A	—	—	—	—
22	Feet of picker B	—	—	—	—
23	Feet of picker C	*Lecanicillium*	—	+	+
24	Feet of picker D	*Lecanicillium*	—	+	—
25	Feet of picker E	—	—	—	+
26	Feet of Supervisor	—	—	—	+

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Multiplexed Detection and Relative Quantitation of Bacterial And Fungal Pathogens of Mushrooms

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ABSTRACT

PCR-based methods for pathogen detection are fast, sensitive and specific. However, given the diversity of pathogens capable of infecting mushroom crops and affecting crop value, the capacity to combine or multiplex multiple PCR tests is essential to allow the implementation of these methods within the mushroom cultivation industry. Using the Multiplex-Tandem PCR technology, we have developed systems for the rapid detection of up to 8 different pathogens (including pathogenic *Trichoderma*, *Lecanicilium*, *Cladobotryum*, and *Pseudomonas tolaasii*) with concurrent differentiation of *Trichoderma aggressivum* (v. *aggressivum* and v. *europaeum*) and *Lecanicillium fungicola* (v. *aleophilum* and v. *fungicola*) variants. These assays enable relative quantitation of each pathogen and can be applied to compost and casing in addition to environmental swabs. Each assay within the multiplex has been validated by nucleic acid sequencing and trials on mushroom swabs. The assay does not necessarily require extraction, and the results on diseased mushrooms produce the expected result. The utility of this multiplex assay to mushroom farms will be discussed.
Keywords: PCR, Multiplexed assays

INTRODUCTION

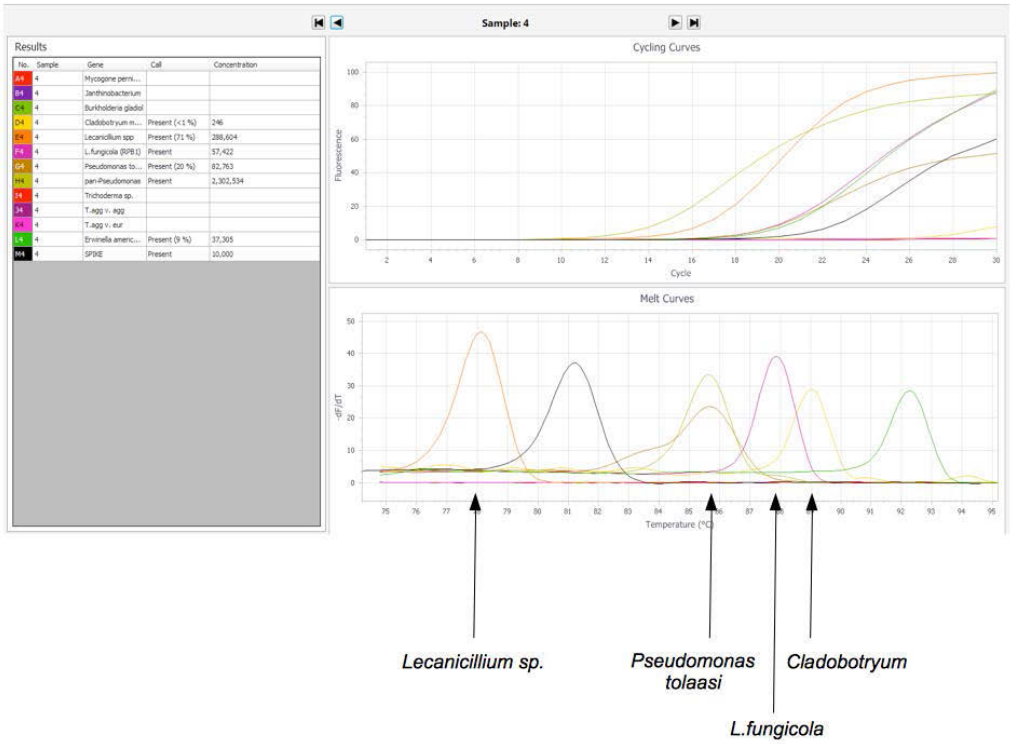
It is essential that mushroom growers know what diseases are present on their farms and where any disease reservoirs are located. they must also be able to determine the efficacy of any disease control measures. Unfortunately, culture based diagnostic services are expensive and slow, which reduces their usefulness to growers. PCR methods for disease diagnosis are fast and sensitive but may be complex to develop, be prone to contamination depending on the competency of the operator, and require specific assays for each pathogen to be investigated. Multiplexed Tandem PCR is a 2-step process, allowing the simultaneous detection of multiple pathogens. In the first step assays for all pathogens are performed simultaneously in a multiplexed PCR reaction, but this is only carried out for a small number of cycles. Because very little dNTP is consumed in this number of cycles each PCR can proceed independently of other reactions, thus preserving the quantitative relation between pathogens. The Step 1 product is then diluted and divided into a number of real time PCR reactions, one for each of the pre-amplified targets. Since these reactions use primers that are nested inside the primers used for Step 1, non-specific amplicons synthesised in Step 1 are not amplified in Step 2. This technology is automated, and is widely used in the medical and

veterinary fields (Stanley and Szewczuk, 2005; Perera et. al; 2015; Lau, Stanley and Sorrell, 2013; Szewczuk et. al; 2010).
AusDiagnostics have developed a panel of assays to identify and quantitate up to 8 different mushroom pathogens, with 24 samples being analysed in less than 3 hours.

Results & Discussion

All assays were initially validated using samples from reference collections, with amplicons being sequenced to confirm assay specificity.

Figure 1. Example of results from mushroom pathogens panel.



Mushrooms exhibiting visible signs of disease were obtained from a local grower. The tops and stems of each mushroom were swabbed, and eluted into 1ml sampling buffer. The eluate was then frozen and subsequently heated to 90^{O}C for 5 minutes. Ten microlitres was used directly in the PCR (samples of eluate were also extracted, but results were very similar). The system was run using 10 cycles in the Step 1 reaction. Positive samples were identified based on amplification curve and amplicon melt temperature, and each pathogen identified was quantitated relative to the internal control, and also expressed as a percentage of total pathogen content.
Based on farm ID of disease, the assay panel can correctly identify the expected pathogens. Interestingly the multiplex PCR was able to detect multiple pathogens on each sample. Whilst disease could be correlated with the presence of the fungal pathogens *Lecanicillium* and *Trichoderma*, *Pseudomonas tolaasi* was identified at low levels on most samples with visible disease correlating only with high concentrations of this pathogen.

Table1 Detection of multiple pathogens from mushrooms by MT-PCR. Pathogens are quantitated relative to the internal control, and expressed as log10 DNA copy number.

	Farm ID	Pathogen 1	Pathogen 2	Pathogen 3
1	***Dry Bubble***	*Lecanicillium fungicola* +++++	*Pseudomonas tolaasi* +++++	
2	***Bacterial Blotch***	*Pseudomonas tolaasi* +++		
3	***Bacterial Blotch***	*Pseudomonas tolaasi* +++++		
4	***Dry Bubble***	*Lecanicillium fungicola* +++++	*Pseudomonas tolaasi* ++++	*Ewingella americana* ++++
5	***Vert or Trich***	*Ewingella americana* +++++	*Trichoderma* sp. ++	
6	***Vert or Trich***	*Ewingella americana* +++++	*Pseudomonas tolaasi* +++	*Trichoderma* sp. ++
7	***Clean 3rd flush***	*Pseudomonas tolaasi* ++		
8	***Clean 2nd flush***	*Pseudomonas tolaasi* ++		
9	***Clean 1st flush***	Nothing Detected		

Subsequently, samples were obtained from the same grower on sequential days of the second flush of growth. These samples had no visible signs of disease, and upon testing no fungal pathogens were found. Low levels of P. tolaasi were seen, with increasing concentrations on sequential days (data not shown). No disease was seen on the subsequent third flush of growth, confirming the negative results from flush two.

CONCLUSION

Multiplexed molecular diagnostics offers the potential to detect disease on crops before it is visible, in growing rooms and equipment, or in raw materials, and save the cost of diseased crops.

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Characterization of Pseudomonas species causing brown blotch of Agaricus bisporus

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ABSTRACT

Bacterial blotch is occasionally causing damage in the production of common mushroom (*Agaricus bisporus*). The disease is found worldwide and can be caused by different fluorescent Pseudomonas species present in casing material. For identification of the causative agents of blotch in the Netherlands and Belgium, fluorescent *Pseudomonas* species were isolated from mushrooms with mild or severe brown blotch symptoms collected at six growers . Strains were tested for pathogenicity by streaking the mushroom head with bacterial slime using a cotton swab and incubation of mushrooms for eight days at 15^{o}C. In total 16 isolates were collected varying in virulence. These, together with 14 other *Pseudomonas* strains present in different culture collections (LMG and at WUR), including the type strains of *P. gingeri*, *P. tolaasii*, *P. agaricus* and *P. reactans* were characterized and tentatively identified by multilocus sequence analysis based on four genes (*rpoD, rpoB, gyrB, glyA*) and by average nucleotide identity using whole genome sequences . We found three species, *P. tolaasii, P. gingeri* and an unnamed species able to cause severe symptoms whereas other unnamed species were able to cause mild symptoms. The information can be used for the development of diagnostic tools and in studies on epidemiology and disease management.
Keywords: *Agaricus bisporus*, bacterial blotch, *Pseudomonas*.

INTRODUCTION

Bacterial blotch is causing worldwide damage in the production of common mushroom (*Agaricus bisporus*). The disease is recognized by a rapid browning of the cap due to fungal production of tyrosinases and phenols (Brennan et al., 2000). The disease is caused by various fluorescent Pseudomonas species present in casing material, under which *P. tolaasii*, *P. gingeri*, *P. constantinii*, *P. fluorescens*, *P. 'reactans'* and yet unnamed *Pseudomonas* species (Godfrey, 2003). The fast growth of the mushrooms and the high water content make mushrooms vulnerable for infections with the bacterial pathogens. All blotch-causing Pseudomonas species described so far are genetically and phenotypically heterogeneous (Abou-Zeid, 2012; Munsch et al., 2000). Control of the blotch is mainly based on hygiene, a decrease of the temperature and relative humidity (Elphinstone, 2012). Use of compounds to

disinfect mushrooms were studied such as biocides(Geels, 1995), antibiotics (Geels, 1995), bacteriophages (Kim et al., 2011), antagonists (Nair & Fahy, 1972) and essential oils (Soković & Van Griensven, 2006), but control effects were often limited and in Europe the compounds are not registered as crop protection agents. Some mushroom genotypes may be less susceptible for bacterial blotch, but scientific evidence is lacking.
Knowledge on the diversity of the pathogen is indispensable, not only for the development of diagnostic assays but also to understand plant pathogen interactions and to select representative strains for studies on epidemiology, control and breeding. The aim of our study was to characterize *Pseudomonas* species that cause blotch in Dutch and Belgium mushroom production crops. For this, fluorescent Pseudomonas bacteria were isolated from mushrooms showing differences in blotch severity after which they were tested on mushrooms for virulence. Whole genome sequence (WGS) analysis was conducted on a selection of the virulent strains. For comparison, also WGS analysis was performed on a panel of reference strains present in culture collections. The sequence information was used to characterize strains with multilocus sequence analysis (MLSA) to study phylogenetic relationships, and with average nucleotide identity (ANI) to delineate different species. ANI represents a mean to determine similarity values between homologous genomic regions shared by two genomes. It is accepted that ANI values of 95–96% can be used as a boundary for species delineation (Konstantinidis et al., 2006).

1. Materials and methods

For identification of the causative agents of blotch in the Netherlands and Belgium, fluorescent *Pseudomonas* species were isolated from mushrooms with mild or severe brown blotch symptoms collected at six growers in the Netherlands and one in Belgium. Symptomatic cap material was homogenized in a buffer and dilution plated on King's B medium. Fluorescent pigment producing colonies of in total 105 isolates were plated to pure cultures. Strains were tested for pathogenicity by streaking the mushroom cap with bacterial slime using a cotton swab and incubation of mushrooms for eight days at 15^{o}C. Sixteen isolates varying in virulence were selected for further analysis. The whole genome sequences (WGS) were determined using HiSeq sequencing of these, together with 14 other *Pseudomonas* strains present in culture collections at LMG (Ghent) and WUR (Wageningen), including the type strains of *P. gingeri*, *P. tolaasii*, *P. agaricus* and *P. reactans.* Strains were characterized on the basis of the WGS by multilocus sequence analysis using four house-keeping genes (*rpoD, rpoB, gyrB, glyA*), and also by average nucleotide identity (ANI) using whole genome sequences.

Results

Results are summarized in Fig. 1. De ANI en MLSA analysis resulted in largely the same grouping. On the basis of ANI, the 30 isolates sequenced could be grouped in 10 tentative genomic species. The 16 isolates from symptomatic mushrooms could be distributed over six genomic species. Eight isolates causing severe symptoms were grouped in three tentative

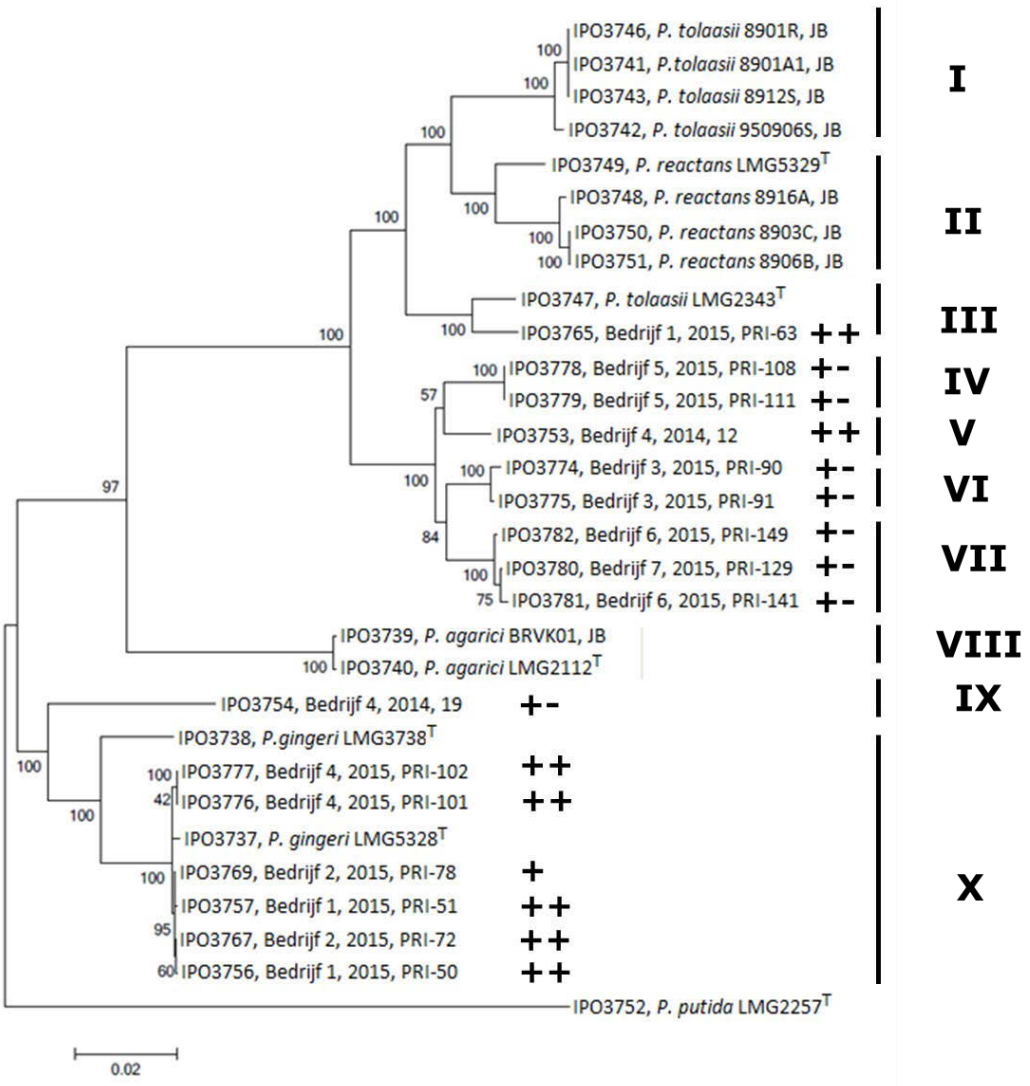


Figure 1. Multilocus sequence analysis of Pseudomonas species causing different diseases on mushrooms, based on *rpoD, rpoB, gyrB, glyA* sequences. The relatedness between isolates was determined using a maximum parsimony phylogenetic analysis. Branch lengths are proportional to the number of changes on a given branch, and bootstrap values are given for each node. *P. putida* LMG2257 was used as an outgroup. Strains indicated with a species name were isolated in the past and deposited in the WUR (IPO-) collection. Type strains indicated with a superscript T were derived from the LMG bacterial collection in Ghent. Strains indicated with "Bedrijf" (= company) were isolated in 2014 and 2015 from blotch-infected mushrooms grown in the Netherlands or Belgium. The virulence of the recent strains was determined and indicated with a +/– (mild symptoms), + (strong symptoms) or a ++ (very strong symptoms) (see Figure 2). The Roman figures indicate the 10 tentative genomic species that could be distinguished on the basis of average nucleotide identity analysis.

species, one strain grouped with the type strain of *P. tolaasii*, one strain with an unnamed species and six grouped with the type strain of *P. gingeri* (LMG3738). The other weakly virulent strains were distributed over four tentative species. In mushrooms from one grower

strains belonging to both *P. tolaasii* and *P. gingerii* were isolated (results not shown). In mushrooms from other growers only one species was found. Examples of mushrooms showing symptoms in the different severity classes (-, +/-, + and ++) are provided in Fig. 2.
In several cases, Pseudomonas isolates present in the WUR collections were misidentified. Four so called "*P. tolaasii*" isolates (IPO3764, IPO3741, IPO3743 en IPO3742) did not group with the type strain of *P. tolaasii*. Three *P. reactans* isolates present at WUR and one *P. agarici* were correctly identified.

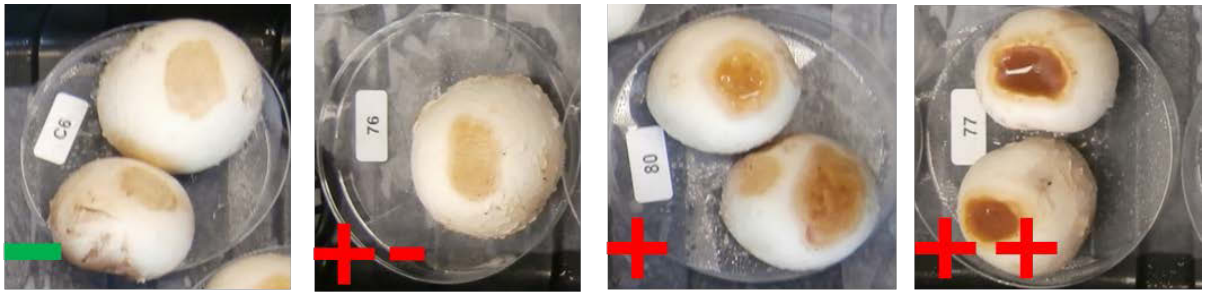


Figure 2. Examples of mushrooms in the different severity classes: - (no symptoms, water control), +/– (mild symptoms), + (strong symptoms) or a ++ (very strong symptoms).

Discussion

Bacterial blotch in mushroom production in the Netherlands is caused by various Pseudomonas species. Severe symptoms are caused by *P. gingeri*, *P. tolaasii* and an unnamed Pseudomonas genomic species, whereas several other unnamed species were able to cause mild symptoms. The incidence with the individual species is unknown and consequently also the importance in blotch outbreaks. The results give cause to extend the survey to further investigate the diversity of *Pseudomonas* species causing bacterial blotch in Europe. Analysis of strains deposited in culture collections again indicated the risks for misidentification ; information provided with strains should be handled with caution.The whole genome sequences will be used to develop diagnostic tools, in particular TaqMan assays, for diagnosis of blotch-diseased mushrooms. Diagnostic assays will also be used for studies on infection sources and the effect of control measures, to detect the pathogens in casing material, breeding material, air, water and materials used in mushroom production.

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Characterisation of the viruses associated with Mushroom Virus X (MVX) and detecting MVX-associated viruses within the European mushroom industry

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ABSTRACT

Mushroom Virus X (MVX) is a syndrome of symptoms which were first observed in crops of *Agaricus bisporus* in England in1990. One of the main symptoms has been off-colour or brown mushrooms in white strain crops. MVX symptoms have been correlated with the presence of a complex of up to 30viral dsRNAs, ranging in size from 0.6 kb to 20.2 kb. As part of an EU FP7-funded project 'MushTV', all the MVX viruses have been characterised and industry surveys have been undertake. Nineteen viruses have now been identified, of which 16 are new to science. We now know that the brown cap symptom is largely caused by the newly identified virus AbV16, (***A**garicus **b**isporus* **V**irus **16**) or 'Brown Cap Mushroom Virus' (BCMV). Other viruses, AbV6 and MBV may also contribute to the symptoms. Each virus can contain several distinct elements, for example AbV16 contains four RNA molecules with distinct sequences, AbV16-1, AbV16-2, AbV16-3 and AbV16-4.

A survey of compost producers was conducted to determine prevalence of the viruses in industry samples. Selected mushroom growing facilities, including some that had recent or on-going experience of brown mushroom symptoms were also targeted for sampling. All samples were tested for the presence of AbV16-1 and AbV6-2. Out of a total of 249 Phase 3 compost samples tested from across Europe, the AbV16 virus was detected in 8 samples (3%) and AbV6 was detected in six samples (2%), indicating a low level of presence in European Phase 3 mushroom compost. However the AbV16 virus was found in approximately 11% of the samples taken from the mushroom growing facilities and AbV6 was found in 16%.

Some of the samples taken from industry were also used in epidemiological bioassay cropping experiments to determine if infected compost would induce symptoms in subsequent crops. Brown mushroom symptoms in these cropping experiments were determined using a chromameter to quantify the colour of the mushrooms. Compost samples from compost and grower facilities, which were positive for AbV16 and/or AbV6, were found to transmit viruses into the healthy compost and resulting mushrooms, however, brown mushroom symptoms only developed in the cases where the infected compost had been taken from underneath a crop displaying brown mushroom symptoms. Thus, MVX viruses AbV16 and AbV6 can be present in both compost and mushrooms that do not exhibit any brown mushroom symptoms. Further work is required to understand what triggers symptom expression.

Keywords: *Agaricus bisporus*, fungal viruses, Phase 3 compost, epidemiology,

INTRODUCTION

Over the past twenty years the mushroom industry in Europe has been hit by economically damaging outbreaks of a new fungal virus disease which became known as 'Mushroom Virus X' (MVX). MVX symptoms included: bare patches in crops; malformed or poor quality mushrooms; off-colour or brown mushrooms in white strain crops; and mushrooms which deteriorate quickly post-harvest (Gaze *et al.*, 2000; Grogan *et al.*, 2003). MVX-associated brown mushroom symptoms have continued to be reported at various times in several EU and international countries.

MVX emerged against a background of increased mechanisation and expansion of bulk Phase 3 production across Europe. At the same time there has been increased movement of bulk compost within and between countries, increasing the opportunity for the spread of diseases from one growing or composting facility or country to another. Mushroom growing facilities have also expanded and become more industrial and automated with many operations now carried out by machinery. Compost handling equipment becomes heavily laden with compost debris and must be well cleaned before a new batch of compost is handled, to prevent cross contamination of compost batches. This is especially important when there are outbreaks of virus disease as the transmission of fungal viruses occurs when virus-infected mycelium gets an opportunity to anastomose with a healthy, non-infected mycelium, as can potentially happen during bulk handling operations.

This work aims to provide information on the key viral elements linked to MVX associated brown mushroom symptoms. We present results from surveys of Phase 3 compost facilities and growers across Europe in 2012-2014. Epidemiological experiments were also carried out to characterise how the viral elements were transmitted and expressed in crops.

1. Results & Discussion

1.1 MVX virus characterisation

Nineteen viruses have now been identified from the many viral elements originally associated with MVX, of which 16 are new to science (Dobbs *et al*. 2016). We now know that the brown mushroom symptom is largely caused by the newly identified virus AbV16, (***A**garicus **b**isporus* **V**irus **16**) or 'Brown Cap Mushroom Virus' (BCMV). Other viruses, AbV6 and MBV (Mushroom bacilliform virus) may also be involved.

1.2 Phase 3 Compost Facilities

Phase 3 compost debris samples were taken at various locations at a number of Phase 3 compost facilities across Europe between 2012 and 2014. All were tested for the presence of the mushroom viruses AbV16 and AbV6 using the PCR method described by Fleming-Archibald *et al.* (2015). Sample types and locations included Phase 3 compost debris off the emptying conveyor structure, the dispatch area and on the rear of transport vehicles. Out of a total of 52 samples, AbV16 was detected in one debris sample in the dispatch area of one facility while AbV6 was detected in one debris sample in the dispatch area of another facility. This raises an interesting question. Did the positive samples originate from the compost facility (where more positive results would have been expected) or were they carried back to the compost facility on the transport vehicle following delivery of compost to a farm experiencing virus problems? There is some evidence to suggest that transport vehicles can become contaminated when delivering compost to farms with live virus

issues that are not using steam to kill off crops before emptying. This highlights the need for Phase 3 compost producers to ensure that (1) they have effective tunnel cleaning/dispatch area clean up procedures in place and (2) that transport vehicles are thoroughly cleaned and disinfected after deliveries to farms, especially when there is a live virus problem.

1.3 Phase 3 Compost

Samples of fresh Phase 3 compost (249 in total), taken directly from tunnels at emptying, were obtained from a number of Phase 3 compost facilities across Europe in 2013 and 2014 and tested for presence of AbV16 and AbV6. Nine samples were positive. AbV16 was detected in 8/249 samples (3.2%) and AbV6 was detected in 6/249 samples (2.4%); 3 samples had both. These viruses were not detected in 96% of Phase 3 samples during this routine testing program across a range of compost producers indicating a low level of presence. The fact that the viruses were detected means that the detection methods were effective and that Phase 3 can become infected at compost facilities. Thus, Phase 3 compost producers should routinely test for presence of viruses to alert them to the possibility of a potential virus outbreak and the need to review hygiene procedures with a view to identifying and eliminating any weaknesses where cross contamination may occur.

1.4 Mushroom Growing Facilities

Mushroom, compost and some debris samples were obtained from 16 grower facilities across Europe in 2013 and 2014 at a time when virus symptoms were an issue for some growers. Eleven of the growers had a current or recent issue of virus symptoms (off/brown colour, poor quality mushrooms, crop delay) while five growers had no virus related issues. Many of the samples from the 11 growers with a history of virus related symptoms tested positive for AbV16 and/or AbV6 while samples from the 5 growers reporting no virus related problems were all negative. A total of 165 samples were tested, of which 11% were positive for AbV16 and 16% were positive for AbV6. Thus there was a higher incidence of virus detection on farms reporting virus symptoms.

1.5 Bioassay experiments with compost samples

Bioassay experiments were conducted with Phase 3 compost samples coming from industry sources to see what symptoms, if any, developed when such compost samples were introduced into virus-free Phase 3 compost under experimental conditions (Teagasc experimental mushroom unit). One Phase 3 compost facility and one grower facility were selected for study. The grower used only one Phase 3 compost source and had only recently started to experience off-coloured and brown mushroom symptoms in his crops, so it was suggested that the compost source must have been infected with BCMV. The composter supplied several growers, some of whom had ongoing virus issues. Phase 3 compost samples were taken from a single batch of compost at different locations and times - from tunnel emptying at the Phase 3 facility to end of 1st Flush at the grower facility - and used as inoculum in the bioassay experiment. All inoculum samples were tested for presence of BCMV (AbV16) and AbV6. The mushrooms derived from the inoculated bioassay crops were tested for virus presence and mushroom colour (expressed as ΔE) was measured using a Minolta CR-300 chromameter. Table 1 indicates that ABV6 was detected in three of the compost samples taken before cropping but both AbV16 and AbV6 were detected in the compost taken from beneath a symptomatic crop. Interestingly, this was the only inoculum that produced visible symptoms in terms of off coloured brown mushrooms as the colour quality of mushrooms from the other

inoculum sources was normal. AbV16 was detected in mushrooms grown with the two inocula that originated at the growing facility while the inoculum that came from the Phase 3 tunnel produced mushrooms that were negative for AbV16. A repeat experiment confirmed these results.

Table 1. Detection of the mushroom viruses AbV16 and AbV6 in compost inoculum and in mushrooms from a bioassay experiment, inoculated with the same compost. Mushroom colour data for 1st and 2nd flush mushrooms is also given.

Source of Compost Inoculum	Virus presence in Inoculum		Virus presence in 1st flush Mushrooms from bioassay		Colour of mushrooms (mean ΔE)*	
	AbV16	AbV6	AbV16	AbV6	1st Flush	2nd flush
Batch A from Phase 3 tunnel at emptying	-	+	-	+	8.12	6.20
Batch A off the truck at delivery to the grower	-	+	Weak +	+	8.03	6.05
Batch A from freshly filled shelf	-	+	Weak +	+	8.12	5.93
Batch A at the end of 1st flush from under symptomatic mushrooms	+	+	+	+	**15.84**	9.20

* High quality white mushrooms have a ΔE of <10.

There are three possible interpretations of these results. The first is that the AbV16 virus was present in the Phase 3 compost coming from the tunnel but at a level that was not detectable by the virus detection method used (PCR). The second possible interpretation is that the truck delivering the Phase 3 compost had become contaminated while delivering compost to another farm where virus problems were occurring. The third possibility is that infected compost debris at the grower's own facility, where there was an on-going issue with virus, was contaminating the fresh Phase 3 compost arriving on his farm. Even though the grower had a steam cook out in place, there are still opportunities for infected compost debris, and mushroom spores from virus-infected crops, to exit the cropping house before the end of crop thereby potentially contaminating Phase 3 filling equipment and shelf-filling operations.

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Finding a Suitable Biocide for Use in the Mushroom Industry

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ABSTRACT

As part of the EU financed project "MushTV" a variety of biocides was tested for efficacy against *Agaricus bisporus* (a model system for MVX infected *A. bisporus*) and *Trichoderma aggressivum*. In laboratory tests, efficacy of 12 biocides, each based on different active ingredients, was tested both at the recommended dose and time (mostly 15 minutes) and at a double dose and prolonged time (mostly 1 hour), and compared with the effectiveness of formalin (at recommended doses), the industry standard disinfectant being replaced). Disinfectants were tested against basidiospores or mycelium of *A. bisporus* and compost particles that were colonised by *A. bisporus* mycelium.

In laboratory tests, many of the disinfectants were able to kill the basidiospores and mycelium of *A. bisporus* at the concentrations and disinfection times tested. However, when present in compost particles, *A. bisporus* could only be killed effectively with formalin, and a few other disinfectants.

With respect to *T. aggressivum*, conidiospores were killed in laboratory tests by all disinfectants, except gaseous ozone. Mycelium of *T. aggressivum* could be killed by formalin, and a few other disinfectants. Neither gaseous ozone nor electrochemically activated water had any effect on mycelium. None of the disinfectants was able to kill *T. aggressivum* in infected compost particles.

Attempts were made to extrapolate the laboratory results to the practical conditions in commercial mushroom cultivation. Various types of surface that may occur in the mushroom industry (rubber mats, stainless steel, mats from growing rooms and tunnels, aluminium, concrete, isolation panel and coated steel) were contaminated with either basidiospores of *A. bisporus* or conidiospores of *T. aggressivum*. Based on efficacy and the lack of leaving a residue, electrochemically activated water (ECAS), a product based on hypochlorite and a product based in a combination of peracetic acid and hydrogen peroxide were selected for testing. ECAS was not very effective under these semi-commercial conditions. The products based on hypochlorite or on peracetic acid/hydrogen peroxide however, showed good efficacy. Next to this, residues of these products disappeared within hours after application.

Compost debris from infected crops holds a severe risk of spreading disease that can be transmitted by compost, such as *Trichoderma* and MVX. It is not possible to completely kill mycelium in compost with disinfectants. Disinfectants offer no real alternative to steam cook out to control mushroom diseases.

Keywords: biocide, disinfectant, Agaricus bisporus, Trichoderma aggressivum.

INTRODUCTION

Mushroom cultivation is a crop in which very little pesticides are used, either because of a low number of pesticides registered for use in mushroom cultivation or because of a low consumer acceptance of pesticide residues on the produce. As pests and diseases may be difficult to control, the mushroom industry has to take all kind of precautions to work as hygienically as possible.
Hygienic precautions in the mushroom industry are aimed at exclusion of harmful organisms, containment of those that could not be excluded and elimination of those that remain. Means of exclusion include absolute filtration of air, well-sealed and over-pressurized rooms, dust and fly filters on cropping houses, the use of disinfectants and a strict observance of hygiene measures aimed at prevention of spread by personnel. Next to this efforts are made to separate activities that are likely to harbour risks of contamination.
Disinfectants or "Biocides" are used on mushroom facilities in many ways to kill any undesirable pests and pathogens that may be present and that could contaminate the next batch of compost or crop of mushrooms. The efficacy of a disinfectant depends on many factors including active ingredient, concentration, persistence, exposure time, type of surface treated (rough or smooth), presence of organic matter (i.e. dirty surfaces) and pH. Most efficacy tests are conducted under laboratory conditions - and for medical or veterinary pathogens rather than horticultural ones. In some cases mushroom-specific tests have been commissioned by the manufacturer for products that registered for use in the mushroom industry although these were usually laboratory based too. As part of the MushTV project (www.mushtv.eu, grant Agreement No: FP7-SME-2011-286836) the project aimed at developing testing methodologies that can reflect conditions on mushroom farms and finding a suitable biocide for use in the mushroom industry. For this *Trichoderma aggressivum* was be used as a test organism. Next to this *Agaricus bisporus* will be used as model organism for MVX infected *A. bisporus*.

1. Results and discussion

The growers organisations participating in the MushTV project were asked to provide names of commercially available products they would like to have tested. This resulted in a list of 45 product names. These products were grouped according to the active principle involved. Formalin is currently the industry standard disinfectant in the Netherlands but it is not approved in many European countries. From each group a disinfectant product was chosen to represent the group (Table 1).
For all disinfectants in Table 1, efficacy was tested *in vitro* against *Agaricus bisporus* (mycelium on cellophane discs, basidiospores and *A. bisporus* present in compost particles) and against *Trichoderma aggressivum* (mycelium on cellophane discs, conidiospores and *T. aggressivum*
present in compost particles. Disinfectants were tested both at the recommended dose and twice the recommended dose. Next to this disinfectants were tested at the recommended disinfection period and an extended disinfection period.

Table 1. Disinfectant products tested in the MushTV project.

Disinfectant	Active principle
1. Formalin	Formaldehyde
2. Jet 5	Peracetic acid & H_2O_2
3. Eco Des	Quaternary ammonium
4. Eco Chlor	Chlorine
5. Virocid	Aldehydes / quaternary ammonium, glutaaraldehyde
6. Neuthox 75	Disinfectant generators produce liquids called NEUTHOX or NOW
7. Prophyl	Phenolics
8. Ozone (gaseous)	O_3
9. Menno Clean	Benzoic acid
10. Tersan	A blend of amphoteric and quaternary ammonium.
11. Food Clean CIP 720	NaOH and NaClO
12. Thyme oil	Thymus vulgaris
13. TriFog	A blend of quaternary ammonium, alcohols and phenolics

In case of *A. bisporus*, most disinfectants are effective in killing basidiospores and vegetatively growing mycelium. With respect to disinfection of basidiospores, Eco-Des gave counter-intuitive results in this experiment. Longer disinfection periods appear to be less effective than shorter ones. Also higher concentrations of disinfectant appeared less effective than lower concentrations. At all concentrations and disinfection periods, Tersan seemed to be less effective than most other disinfectants in killing basidiospores. Gaseous ozone (O_3) performed even worse. At low levels of O_3 there even seemed to be a stimulation of spore germination. As compared to basidiospores, Tersan and ozone were more effective in killing vegetative growing mycelium of *A. bisporus*. When comparing efficacy of the different disinfectants for killing vegetative growing mycelium, all performed rather well, except Neuthox 75.

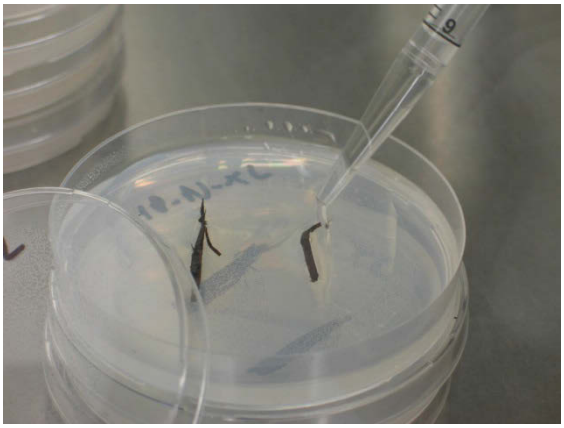


Figure 1. Sprinkling compost particles with disinfectant solution.

When present in compost particles, mycelium of *A. bisporus* is less easily killed by disinfectants. The mycelium appears to be protected to a certain extent by the organic matter in the compost particles. Only formalin, is able to kill all mushroom mycelium in compost particles. Also Eco-Des, Prophyl and Menno Clean reached a high level of efficacy on Agaricus mycelium in compost.

With respect to *T. aggressivum*, its conidiospores were killed rather effectively by all disinfectants tested, except gaseous ozone. The vegetatively growing mycelium of *T. aggressivum* is killed less easily. Formalin, Virocid and Menno Clean were among the most potent disinfectants for mycelium. The other disinfectants are less effective and the worst performing disinfectants were Neuthox and ozone, showing no disinfecting action on mycelium. None of the disinfectants was able to kill *T. aggressivum*, when present in

compost particles, even when used for extended periods of time. As no disinfection product was able to kill *Trichoderma aggressivum* and only a very limited number had success in killing *A. bisporus* under laboratory conditions, it is very clear that cleaning away compost from machinery, nets and tunnels and on compost yards and farms is critical for good hygiene. Mushroom farms are advised to apply a proper cook-out! Heat can kill the pathogens when present in compost.

Choosing a biocide for use in the mushroom industry does not only depend on efficacy. Also aspects such as corrosivity, method of application (washing, fogging), safety to the user and environment, costs, risk of accumulating residues upon unintended contamination of mushrooms, commercial availability in the future play a role in making a choice. The growers organisations involved in the project have put a lot of emphasis on the risk of accumulating residues of disinfectants in mushrooms.

Next, attempts were made to extrapolate the laboratory results to the practical conditions in commercial mushroom cultivation. For such extrapolations, various types of surface that may occur in the mushroom industry (rubber mats, stainless steel, mats from growing rooms and tunnels, aluminium, concrete, insulation panel and coated steel) were contaminated with either basidiospores of *Agaricus bisporus* or conidiospores of *Trichoderma aggressivum*. Based on efficacy and the lack of leaving a residue, three disinfectants were chosen for testing under "semi-commercial" conditions; ECAS (electrochemically activated water), a product based on hypochlorite and a product based in a combination of peracetic acid and hydrogen peroxide. Results showed that ECAS was not very effective under these semi-commercial conditions. The products based on hypochlorite or on peracetic acid/hydrogen peroxide however, showed good efficacy. Both these products however are corrosive.

One of the compost companies was using a peracetic acid/hydrogen peroxide based product for disinfection of the spawning hall. Their experiences with the product were good, but they have been taking care of protecting the construction of their facilities with a special coating. Within the project the compost company has started to use swabs for monitoring the presence of yeasts and moulds on their facilities. Own research by the compost company has shown to them that in their facilities, the peracetic acid/hydrogen peroxide based product has good efficacy in killing the yeasts and moulds.

As disinfection needs to be preceded by thorough cleaning, a number of Dutch and Belgian growers have started to experiment with the use of industrial foam based detergents. For these foam based detergents to work properly, they need to be applied with specialised equipment and high pressure supply of water and air. As this equipment is quite expensive, the farmers have experimented with cheaper solutions with varying degrees of success.

This project produced a technical factsheet entitled 'Use of chemical disinfectants in mushroomproduction' which is available in three languages (English, Dutch and Polish) from the project website (www.mushtv.eu). A video summarizing the results of the project can be found at YouTube (https://www.youtube.com/watch?v=0_sssfgajg0#t=30).

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Mycoparasites isolated from *Agaricus macrosporus* in nature

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ABSTRACT

White button mushroom industry is threatened by mycoparasites such as *Lecanicillium fungicola* causing dry bubble disease and *Cladobotyrum dendroides* causing cobweb disease. The pathogens infect stages in mushroom development. They induce various symptoms on the host, such as bubbles, split stipes and spotty caps and destroy their host´s hyphae. Observations on these mycopathogens in nature are however rare, possibly because they are rather overlooked. Here, we describe mycopathogens found on *Agaricus macrosporus* in nature. Strains were isolated and we tested their behavior with different basidiomycetes in the laboratory.

Drumstick-like closed young fruiting bodies of *A. macrosporus* appear regularly each year in August to November underneath a Douglas fir on the North Campus of the University of Göttingen at sunny days of pleasant temperature. The young mushrooms open within 3 days by perforating the veil at the edges of the cap. In the next 2 days, the caps flatten in order to stretch out their pinkish-brownish gills for spore release and to then degenerate. Deformed fruiting bodies of irregular stipe and cap shapes appeared in September 2015. Fluffy mycelium covered the surface of the mis-shaped parts of the fruiting bodies. Microscopic analysis revealed ascomycetous types of conidiophores producing multiple 1-3 celled conidia. Similar structures were observed on decaying stipes of overturned mushrooms. The mycopathogens were isolated from infested mushroom tissues and used to infect in the lab commercially produced *Agaricus bisporus* and *Pleurotus ostreatus* mushrooms and mycelial cultures of basidiomycetes. The pathogens were very aggressive towards *A. bisporus* and produced huge amounts of conidiospores on the mushrooms. *P. ostreatus* in contrast was highly resistant. Slight infections were observed only with one of the isolates at stipe regions of only a few *P. ostreatus* mushrooms. Mycelium of lab strains of *Coprinopsis cinerea* was attacked by the mycoparasites but not mycelium of *P. ostreatus.* Conidiophores, conidiospores and ITS sequencing are used to determine the identity of the isolates.

Keywords: *Agaricus macrosporus*, mycopathogen, disease, ascomycete, conidia

INTRODUCTION

Commercially cultivated mushrooms such as the white button mushroom *Agaricus bisporus* can be spoiled by several pathogenic agents such as viruses, bacteria and fungi. Fungal pathogens such as *Lecanicillium fungicola* causing dry bubble disease (Berendsen et al., 2010), *Cladobotryum dendroides* causing cobweb disease (McKay et al., 1999), and *Mycogone pericosa* causing web bubble disease (Kouser and Shah, 2013) attack at any stage

of the mushroom development. Thereby, they can cause great losses to the white mushroom industry. When fruiting bodies are infected at an early stage of development (primordia or young mushrooms), they may develop into deformed mushrooms with for example split stipes and caps. Several mycopathogenic strains were previously isolated from commercially cultivated mushrooms, such as from different *Agaricus* and also *Pleurotus* species (Gray and Mogan-Jones, 1981; McKay et al., 1999). In contrast, descriptions of such infections in nature are rare. However, there are some reports on incidences of mycopathogens on mushrooms in fields and forests from species in the orders Agaricales (including *Agaricus bitorquis*), Boletales, Hymenochaetales, Russuales, and Thelephorales (Zare and Gams, 2008). Here, we observed mycopathogens attacking another *Agaricus* species in the nature and isolated the responsible strains for characterization in the laboratory.

1. Results & Discussion

1.1. Agaricus macrosporus *fruiting bodies in nature*

Drumstick-like young mushrooms of *Agaricus macrosporus* were yearly observed in 2 to 3 flushes during August to November in the needle and cone litter underneath a Douglas fir (*Pseudotsuga menziesii*) in a meadow on the North Campus of the University of Göttingen, under suitable conditions of weather, i.e. usually on sunny days with temperatures of 15-23 ^{o}C which followed after raining days with humidity levels of 50-80 % (day phase) and 80-95

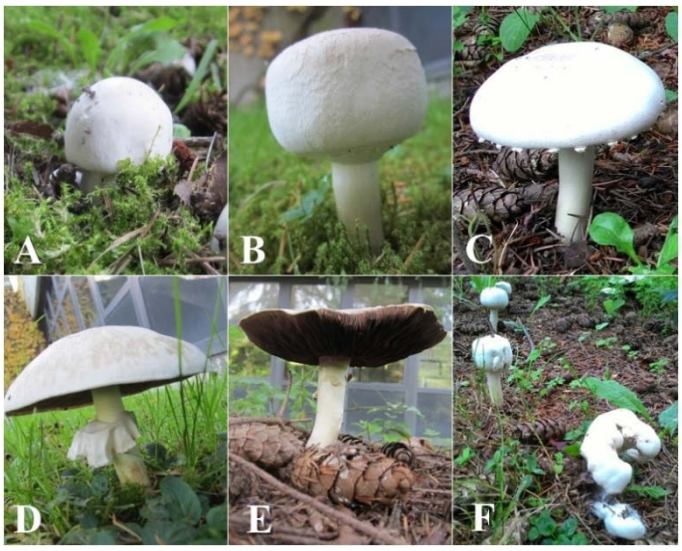


Figure 1. Different stages of fruiting body development of *Agaricus macrosporus* under a Douglas fir tree at the North Campus of the University of Göttingen. A. Young mushroom coming out from the ground which has an ovate shape of the cap. B. Young still closed mushroom with stipe elongated and the upper cap surface flattened. C. and D. Convex freshly opened caps with veil remnants hanging at the edge of the cap (C.) and the main partial veil staying as an annulus around the stipe (D.). E. Fully opened flattened cap with brown gills and degenerated partial veil. F. A deformed mushroom infected by a mycopathogen found among other healthy young mushrooms.

% (night phase). In September 2015, the mushrooms came in two flushes, with a period interval of two weeks. The mushrooms come out solely or in loose groups. The mushrooms at the beginning are silky white in color and have an ovate cap shape when they are coming out of the ground (Fig. 1A). Later with growth, they get flattened on the top of the cap along with the elongation of the cylindrical stipe, giving then a drumstick-like shape (Fig. 1B,F). Within three further days, the young mushrooms open and the caps become convex with remnants of the partial veils hanging at the edge of the caps (Fig. 1C). The main parts of the partial veils will stay as an annulus around the stipes (Fig. 1D). The caps then flatten in the next two days in order to stretch out their first pinkish, then brownish gills for spore release and to afterwards degenerate (Fig. 1E).

1.2. A diseased fruiting body from nature

In September 2015, we observed in the first flush of fruiting bodies among many healthy mushrooms a nearly matured but deformed fruiting body of *A. macrosporus* which had the cap and stipe split (Fig. 1F). Foreign white fluffy mycelia covered parts of the mushroom suggesting the individual to be infected by a mycopathogen. In the second flush of mushrooms, many more structures at different stages of development were also infested by such fungal disease. Mycelia from the infestations were isolated from mushrooms, decaying tissues of overturned fruiting bodies and also from turf in the surrounding lawn. The mycopathogenic isolates were cultivated on 2% malt extract agar (MEA) and YMG/T

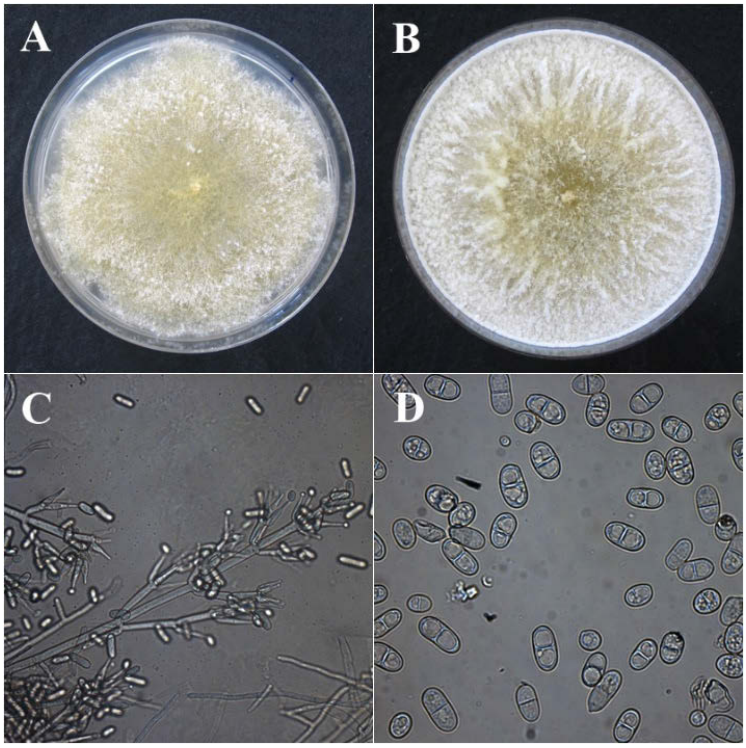


Figure 2. Morphology of a mycopathogenic fungus isolated from an *A. macrosporus* mushroom. A. Colony on 2% MEA and B. on YMG/T agar medium grown at 22 ^{o}C. C. and D. Photos of conidiophores and conidia as observed under a light microscope.

(Granado et al., 1997) on which they show different growth morphologies and colors. Initially, colonies on both media were white in color before they turned yellow (Fig. 2A,B) and on YMG/T with age pinkish. On YMG/T medium, mycelia were denser and produced more aerial hyphae and spores than on 2% MEA. Microscopic analysis revealed ascomycetous types of conidiophores which produced on the tips of phialides septated conidia with up to four cells (Fig. 2C,D). The conidia are rod-shaped with rounded edges (Fig. 2D). The morphology of isolated fungi resembles descriptions of some ascomycetes fungi belonging to the order of Hypocreales which include many known mycopathogens, among the cobweb disease causing *C. dendroides* from the family of *Cordycipitaceae* and *L. fungicola* and *M. perniciosa* from the family of Hypocreaceae causing dry bubble and wet bubble disease, respectively (McKay et al., 1999; Berendsen et al., 2010; Kouser and Shah, 2013). In order to identify the species of our fungal isolates, we are analyzing their morphologic characters in detail and we are isolating genomic DNA of the strains for ITS-sequence determination.

1.3. Infection of basidiomycetes by isolated ascomycetous strains

In order to proof that our isolates are indeed mycopathogens, we need to fulfill Koch´s postulate that the strains must cause the disease as observed in nature when inoculated onto healthy, susceptible mushrooms in the laboratory. Commercially produced *A. bisporus* and *P. ostreatus* mushrooms bought from supermarkets were therefore used to test their susceptibility to the disease in the lab when confronted with the mycopathogenic isolates. In general, the isolates were very hostile towards *A. bisporus* and produced enormous amounts of conidia on infected mushrooms. Mushrooms of *P. ostreatus,* in contrast, were better resistant to the disease. We are also testing the behavior of the isolates against mycelial cultures of a range of basidiomycetes. For example, mycelium of lab strains of *Coprinopsis cinerea* was easily colonized and attacked by the mycoparasites in contrast to those of some other species.

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Olfactory mechanism of preferable host-*Flammulina velutipes* selection by the stored food mite Tyrophagus putrescentiae

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ABSTRACT

Tyrophagus putrescentiae is one of the cosmopolitan storage mites, commonly occurring in China, and also is an important pest in mushroom cultivation. Efficacy of chemical pesticides in controlling *T. putrescentiae* is low due to the fact that the mite can burrow into inner tissues and hide in. Chemosensory genes play very important roles in host location or sex pheromone detection, and therefore they were effectively used in pest control. In our study, odor molecules of the preferred host-*Flammulina velutipes* were verified by behavioral responses of *T. putrescentia* using an insect olfactory apparatus and identified with GC-MS. Among these substances, β-Caryophyllene (1000-10000 ppm) has a significant attractive effect on the mite. In order to associate the olfactory related genes with host location or sex pheromone detection, chemosensory proteins were identified for the first time in *T. putrescentiae*, and their 3D structures were predicted by homology modelling to further understand physiological functions, specifically in host recognition. Based on a docking study, we found that (-) -Alloaromadendrene, 2 -Methylnaphthalene and Cyclopentadecane had high binding affinities to TputCSP1. TputCSP1, which may mediate the host recognition.
Keywords: chemosensory proteins; acarid mite; olfactory system; chemosensory recognition.

INTRODUCTION

Many animals use semiochemicals or pheromones to communicate, and then perform a series of behavioral responses, such as feeding, mating, avoiding, marking territory, finding prey and host recognition[1-3]. In this process, several important chemosensory gene families play a role, coding for odorant binding proteins (OBPs), chemosensory proteins (CSPs), pheromone binding protein (PBPs), odorant degrading enzymes (ODEs) and chemical receptors, such as odorant (ORs), ionotropic (IRs) and gustatory receptors (GRs).

Arthropod chemosensory proteins (CSPs) are a group of small, water-soluble proteins (10 to

30 kDa), which are meant to transport hydrophobic chemicals to the corresponding receptors on sensory neurons in a wide variety of Arthropod species[4-5]. The CSP gene family is much more conserved across insects, where CSPs have the typical conserved four-cysteine signature (C1-X_6-C2-X_{6-18}-C3-X_2-C4). They are expressed in various tissues, especially in insect antennae, suggesting their involvement in physiological processes beyond chemoreception, such as female survival and reproduction, limb regeneration and embryo development[6-8]. It is confirmed that CSPs are capable of binding a range of aliphatic compounds, esters and other long chain compounds that are typical components of pheromonal blends[9, 10] .

Tyrophagus putrescentiae Schrank is an important economic pest in the world with a wide range of habitats, including stored products, crop seeds, mushrooms, domestic animals and pets, and house dust[11, 12]. In order to associate the olfactory related genes with host location or sex pheromone detection, CSPs were identified to further understand physiological functions, specifically in host recognition. We also analyze host volatiles of *Flammulina velutipes* by headspace solid phase micro extractions, GC-MS and olfactometer to screen strong phobotaxis on *T. putrescentiae*.. This study may provide some molecular targets for control and insight into mite olfaction research, thus providing an essential foundation for the development of efficient, simple and sustainable pest control strategies.

1. Results & Discussion

1.1. Behavioral responses of T. putrescentiae to the host volatiles

Volatiles were collected from the preferred host *Flammulina velutipes* by headspace solid phase micro extractions and GC-MS under normal conditions and mite stress. Compounds under normal condition were Hexanal, Benzaldehyde, 2,4,6-Octatrienal, 3-Octanone, Benzeneacetaldehyde, 2-pentyl-2-Amylfuran, Isoledene, a-Guaiene, b-Himachalene, Longipinene, 7-methyl-6-Tridecene, decahydro-2,6-dimethyl- Naphthalene, 1-methyl-Naphthalene, b-Chamigrene, Cuparene, Neoisolongifolene, Himachala-2,4-diene, β-Caryophyllene, β-Vatirenene, d-Cadinene, a-Cadinene, Butyl isohexyl ester phthalic acid, (3aS,3bR,4S,7R,-7aR)-7-methyl-3-methylidene-4-(propan-2-yl)octahydro-1H-cyclopenta[1,3]cyclopropa[1,2]benzene, 1,1,7-Trimethyl -4-methylenedecahydro-1H-cyclopropaazulene and 1,2,4a,5,6,8a-hexahydro-4,7- dimethyl -1-(1-methylethyl)- Naphthalene. Compounds under mite stress were 2-methyl-5-(1-methylethyl)-Phenol, 2-methyl-Dodecane, (1S)-(+)-3-Carene, 2-methyl-Naphthalene, Tridecane, 2-methyl-Tridecane, 3-methyl-Tridecane, (E)-5-Tetradecene, Tetradecane, 2-methyl-Tetradecane, 7-methyl-3,4-Octadiene, 1-Tridecene, Cyclopentadecane, Pentadecane, (Z)-7-Hexadecene, Hexadecane, Cedrol, Octacosane, Heptadecane and 2,6,10,14-tetramethyl-Pentadecane.

The 4 of 10 compounds that elicited negative behavioural responses from the mite, *T.*

putrescentiae, were Cedrol, Tetradecane, Cyclopentadecane and 3-Octanone. 3-Octanone elicited the greatest negative behavioural response. β-Caryophyllene (1000-10000 ppm) has asignificant attractive effect on the mite.

1.2. Identification of chemosensory protein genes from T. putrescentiae transcriptome

Two distinct unigenes were identified encoding putative CSPs with four conserved cysteine residues, coinciding with the characteristics of the CSP family. We named the CSP genes as TputCSP1 and TputCSP2 following the nomenclature established for *T. putrescentiae* under the accession numbers KP776981 and KP776982 in GenBank. The calculated molecular mass of TputCSP1-2 were 14.9 kDa and 12.1 kDa, respectively. The theoretical pI of the two CSPs were 7.63 and 8.62, respectively.

1.3. Homology modeling and Docking of the CSPs in T. putrescentiae

In order to select suitable templates for TputCSP1-2, we used the "first approach mode" of the SWISS-MODEL server to search the best models. Six templates had between 44.34% to 57.69% identity with TputCSP1. The greatest sequence identity of the six templates was CSPsg4 from *Schistocerca gregaria* (PDB: 2GVS). The protein structures of TputCSP1-2 predicted from the target-templates (PDB: 2GVS) with 100% using the two molecular modeling server.

The potential binding characteristics of the two TputCSPs with 17 odorants were predicted using AutoDock. The compounds (-)- Alloaromadendrene, 2 -Methylnaphthalene and Cyclopentadecane had lower inhibition constants (kl < 10 μm) and lower binding energies with TputCSP1; -6.84, -6.88, and -7.80, respectively.

However, 17 compounds had higher inhibition constants, ranging from 37.39 μm to 619.56 μm with TputCSP2, except for 2 -Methylnaphthalene, which had the lowest inhibit constant, 13.24 μm. The compounds 5 (*Z*) 1 , 5 -Octadien -3 -ol had the highest inhibit constant and the highest binding energy (kl = 922.78 μm, 619.56 μm, respectively) both with TputCSP1 and TputCSP2. TputCSP1 showed medium-affinity binding to Neoisolongifolane, β -himachalane, (*E*) -Citral, and Rosefuran. Moreover, TputCSP2 showed medium-affinity binding to Tridecane, Pentadecane, and Tetradecane. These results suggest that the two proteins have different communication functions in *T. putrescentiae*. Three alarm pheromones of *T. putrescentiae* had no strong binding properties.

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Mushroom mites in cultures of *Coprinopsis cinerea*

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ABSTRACT

Mushroom mites (Acari, Acaridida: *Tyrophagus putrescentiae*, syn. *Tyrophagus lintneri*, syn. *Povelsenia neotropicus*; other common names: storage mites, mold mites, house dust mites) are feared pests in mushroom beds as well as in fungal laboratory cultures. These cosmopolitan tyroglyphid mites are generalists that prefer high-fat and high-protein feeds. They infest stored grains, flour, vegetables, cheese, meat and ham, straw stacks, bird nests and other habitats and are usually associated with moist and dark conditions. The mites are attracted to feedstuff by C8 compounds typical for mushroom odors. In fungal cultures, they consume the vegetative mycelium and possibly developmental structures and they are vectors of bacteria and ascomycetous molds. Presence of mites causes allergic reactions in humans due to their fecal pellets and due to transferred molds and their spores.

An incidence of mite infestations of *Coprinopsis cinerea* cultures in our laboratory was used to observe their behavior with the fungus. Mites were reared on *C. cinerea* mycelium in a standardized technique to breed animals for further experiments. In piles of Petri dishes, they actively enter any fungal cultures and graze on monokaryons and dikaryons. Observations on reproduction, development and death of the animals in fungal cultures are presented. The mites are well adapted to temperature conditions of 25-28°C at which *C. cinerea* fruits. Further, they develop well at 60% humidity in dark. At higher temperature, lower humidity and under too much light, the mites quickly die. The mites prefer to graze on freshly grown mycelium as compared to senescent mycelium and avoid multicellular structures such as primordia and sclerotia. They can distribute both the asexual oidia generated abundantly on monokaryons and the sexual basidiospores formed in fruiting bodies on dikaryons to new locations. On the other hand, they bring bacteria into the fungal cultures which inhibit mycelial growth. The fungus reacts with laccase production on bacterial contamination. Slimy growing bacteria can kill the mites when these get stuck in the slimy colonies.

Keywords: mites, larvae, eggs, mycelial grazing, spores

INTRODUCTION

Commercial mushroom cultivation is in constant danger to fail by various kinds of pests, microbial infections and viruses that might infest the fungal substrate, the fungal mycelium and/or the fruiting

bodies on the substrates. Among pest animals, larvae and adults of certain flies and gnats, mites, springtails and nematodes might be named. Highest hygienic standards are required to avoid any of such problems (Kües and Rühl, 2007). The ubiquitous house dust mites, also called mushroom mites and with current Latin name *Tyrophagus putrescentiae* (syn. *Povelsenia neotropicus*), are among the pests reported to occur in mushroom beds (Davis, 1944; Fan and Zhang, 2007). In the laboratory, ubiquitous house dust mites might get attracted to old fungal cultures standing for longer time on the benches close to potential entry places of the animals. Such places of origin can be rarely used and therefore easily dried out sinks installed within laboratory benches. Within 30 years of active research, infections by mites through neglected sinks have been witnessed in microbial cultures by the senior author (UK) on occasions in three different institutes (at the FU Berlin, the ETH Zurich and in Göttingen). However, infestations through sinks can easily be controlled by regularly letting fresh water into the sinks because mites avoid water and by daily cleaning the benches. Old drafty, since imperfectly shutting wooden laboratory windows (in the meantime replaced) were noticed in Göttingen as another potential infection source of mites from which they wandered into fungal cultures which were placed for some time on attached laboratory benches. Other more difficult to handle places of concern are larger climate chambers in which larger fruiting studies with multiple cultures are performed and which are difficult to clean in all small gaps present in walls, floors and furnishing where mites with their eggs might hide. In addition to thorough room cleaning, in order to avoid outbreaks of mites we therefore incubate fungal cultures on spacers laid onto flat trays with water to block unwanted migration of mites into plates and we follow up the strict standard that no culture should be taken back from the culture room uncontrolled into the laboratory.

To avoid loss of valuable fungal cultures, fast measures of removal, cleaning and disinfection must be taken in cases of unintended mite incidences. Due to these fast must-actions, observations on behavior of mites in fungal cultures are generally very limited. Since we wanted to learn more about mites in fungal cultures, we used an incident of mite infection in *Coprinopsis cinerea* cultures to take the animals in culture for targeted research.

1. Results & Discussion

1.1. Identification of mite infestations in cultures

Dust mites are very small translucent animals (300-600 μM) difficult to detect by eye within vigorous aerial mycelium (Fig. 1). A first indication for a mite in a mycelial culture is when tiny sharp holes in the mycelium become visible which look like as made by a stitch with a needle. Once such a sharp pinhole is discovered, one or more mites are detected at the places with highest probability when the pinholes are observed under a binocular (Fig. 1). By their translucent body and by a habit to dive beneath an aerial mycelium through self-made tunnels generated through grazing of hyphae, up- and down-focusing with the micrometer screw of a binocular and possibly an opening of the covering mycelial layer by a needle can help to detect a hiding animal.

1.2. Grazing by mites in cultures

Once within a fungal culture, mites graze on the mycelium, thereby enlarging the first tiny holes within the mycelium (Fig. 1). Notably, used up agar beneath the mycelium is not eaten by the mites (Fig. 1 and 2). Mites seem first not to move much within the fluffy mycelial colonies. With time



Fig. 1 House dust mites *Tyrophagus putrescentiae* in cultures of the fungus *Coprinopsis cinerea*. Yellow circles mark pinholes in the mycelia where mites will be detected under a binocular. First, there are only single or few mites in the tiny mycelial holes but with raising diameters of grazed mycelium, there are also eggs and larvae among fecal pellets.

however, they proliferate and raising numbers of adults as well as eggs and larvae can be found within the cultures in the enlarging mycelium-free areas (Fig. 1). Further with time, small holes within the aerial mycelium are observed at many more places within a colony, indicating that mites were spreading. Mycelia holes enlarge and fuse to larger mycelium-free areas until finally most of the aerial mycelium has been consumed. In contrast to vegetative hyphae, most of the multicellular structures are avoided for comsumption (Fig. 2) but the inner cap tissues of mature fruiting bodies with basidiospores (not shown here). Both vegetative spores (oidia) as well as basidiospores can be transferred by the animals to fresh substrate (not shown). Also, the mites were found to carry different types of bacteria with them which can grow when transmitted by mites onto fresh media. Bacteria may thereby hinder outgrowth of the fungus. The fungus on the other hand tries to fight the bacteria by production of laccase (see Fig. 4 in Rühl et al., 2007). Bacteria were however rarely observed to grow on fully grazed fungal plates, possibly by the fact that the fungus previously used up the nutrients (Fig. 1). Bacteria which grew on the grazed agar plates had typically a slimy appearance and mites can get stuck in the slimy bacterial masses (not shown).

1.3. An easy culture and propagation technique of mites

Mites for experiments are reared on mycelial cultures of *C. cinerea*. We regularly grow the fungus (usually *C. cinerea* homokaryon AmutBmut but any monokaryon or dikaryon is possible) at its optimal temperature at 37°C in the dark (Granado et al., 1997). Originally for feeding the mites, we took three small agar pieces (4 x 4 mm) with mites and eggs on top from older cultures and laid these three pieces in even distances onto freshly grown mycelium. Inoculated plates were sealed with Parafilm (mites can percolate Parafilm but it was used to keep good moisture conditions as long as possible in the cultures), piled and wrapped with aluminium foil in order to exclude light (mites are shade-loving). The wrapped piles were positioned onto metal rags in larger containers with a level of water at the bottom in which the rags were standing. The latter was done to avoid that the hydrophobic mites escaped from the piles into the environment and, also, to hinder that not new types of mites came into the cultures. Piles with mites were kept apart from the laboratory in a greenhouse at 25-28% and 40-70% humidity (in average 60%) humidity. The mycelium on a plate sufficed for consumption by mites for three to four weeks, upon which a new round of inoculated cultures was started. Mites can however be kept also longer on the grazed plates. Up to two month



Fig. 2 House dust mites *Tyrophagus putrescentiae* in cultures of the fungus *Coprinopis cinerea* with different levels of grazing of aerial mycelium.

is no problem and there will be still sufficient eggs and living mites that might be used for new inoculations.

With more experience over the time, we simplified the system (i) by not closing the plates anymore with Parafilm, (ii) by piling open four-weeks-old grazed agar plates with raised mites, their larvae and eggs in between freshly grown sterile *C. cinerea* cultures, (iii) by putting the piles of Petri dishes onto rags in boxes with some water at their bottoms, (iv) by closing the boxes with a loose lid to avoid light and allow aeration, and (v) by incubating the boxes at room temperature in a dark room in another building away from the laboratory. We found that under such conditions, the agar medium will keep sufficient humid, offering the mites for at least four weeks plenty mycelium in order to proliferate. The mites are attracted to the freshly grown cultures and migrate from the formerly grazed cultures into the above or below fresh cultures. Again, there is no problem to store grazed plates for up to 2 month under such conditions and there will be still enough living animals to newly infest formerly sterile fresh fungal cultures. Thus, sufficient animals can easily be raised for any specific studies in their interaction with the fungus. In such a manner, we have kept our mite population now for more than five years going.

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Cob web and dry bubble diseases in *Lentinula edodes* cultivation –A new report

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ABSTRACT

Lentinula edodes (Berk.) Pegler, commonly known as Shiitake mushroom, is a wood decaying basidiomycete cultivated in Asia, Europe, North America and Australia using either cut oak logs or saw dust as the substrate. It ranks third among the most cultivated mushrooms globally. Like other mushrooms, shiitake is also attacked by several fungal pathogens during its bag cultivation. Fungal competitors of shiitake logs are always been an issue even with the best management practices. Some most common fungal competitors of shiitake mushroom are e.g. *Trichoderma longibrachiatum*, Blue mold (*Penicillium* sp.), Red bread mold (*Neurospora sitophila*), *Mucor* spp., *Aspergillus* spp. Black Bulgur, Bachelor's Buttons or Rubber Buttons, Black Jelly Drops or Poor Man's Licorice *(Bulgaria inquinans), Hypoxlyon truncatum*, Turkey Tail (*Coriolus versicolor*) and Split Gill fungus (*Schizophyllum commune*), causing yield reduction and subsequently economic losses. In present study, the two fungal diseases Cobweb and Dry bubble were encountered on shiitake fruiting bodies during its saw dust cultivation. During the present study the causal organisms of the two diseases were isolated and identified as *Cladobotryum* and *Lecanicillium* or *Verticillium* based on morphological and microscopical characters. The identification was also confirmed using ITS 5.8S rDNA sequences comparisons against NCBI databases. Different botanicals were used to control the cob web and dry bubble diseases on shiitake mushroom. To the best of our knowledge and available literature, this is the first report of *Cladobotryum* and *Verticillium* causing cob web and dry bubble diseases on shiitake mushroom.

Keywords: *Lentinula edodes*, diseases, Cob web, dry bubble, *Cladobotryum, Verticillium*

INTRODUCTION

Lentinula edodes (Berk.) Pegler, or Shiitake mushroom, is commonly cultivated in Australasian, European, North American countries and using either cut oak logs or saw dust as the substrate (Tsuneda, 1994). At present, shiitake is one of the five most cultivated edible mushrooms in the world (Chang, 1999; Royse, 1997; Wasser and Weis, 1997 and Stamets 2000). This mushroom is gregarious on fallen wood of a wide variety of deciduous trees, especially shii, oak, chestnut, beech, maple, sweet gum, poplar (aspen, cottonwood), alder, hornbeam, ironwood, chinquapin, mulberry in a warm, moist climate. Natural occurrence of this mushroom has been reported from

China, Japan, Korea, Vietnam, Thailand, Burma, North Borneo, Philippines, Taiwan, and Papua New Guinea (Wasser and Weis, 1997 and Stamets, 2000).

Many fungal diseases occur during cultivation of shiitake, because fungal pathogens also thrive in warm and humid conditions. There are some of the most common fungal competitors e.g. green mould (*Trichoderma longibrachiatum*), Blue mold (*Penicillium* sp.), Red bread mold (*Neurospora sitophila*), *Mucor* spp., *Aspergillus* spp. Black Bulgur, Bachelor's Buttons or Rubber Buttons, Black Jelly Drops or Poor Man's Licorice *(Bulgaria Inquinans), Hypoxlyon truncatum*, Turkey Tail (*Coriolus versicolor*) and Split Gill fungus (*Schizophyllum commune*) (Leifa Fan *et al.,* 2000).

Cobweb and Dry bubble is a common disease of button mushroom in every country that causes yield reduction and subsequently economic losses. In present study, these two fungal diseases were encountered on shiitake fruiting body. Therefore, the study was aimed to identify and characterize the pathogens responsible for cobweb disease and dry bubble on shiitake fruit bodies for development of the efficient management of the diseases.

1. Results & Discussion

Traditionally shiitake mushroom has been cultivated on natural logs of various tree species. Later, environment-friendly shiitake cultivation technology was developed using synthetic log where sawdust is the main ingredient. In this technology, sawdust is supplemented with some starchy substance like cereal bran, maize and some chemicals like calcium carbonate, calcium sulfate (gypsum) and sugar. The sawdust based technology is based on complete steam sterilization of the substrate. The presence of sugars and starch along with sterilization makes it vulnerable towards the attack of sugar loving competitor/pathogenic fungi. During the present study, symptoms of diseases were observed during the cultivation of shiitake mushroom causing severe crop losses.

2.1. Disease symptoms

Cob web: Cob web disease was observed as white silky mycelium which grows over surface of sawdust shiitake log. It climbs up and covers mushrooms in its path (Fig 1).

Dry Bubble: Dry bubble symptoms were observed as Muddy brown, often sunken spots on the cap of shiitake and grayish white moldy growth seen on pileus. At later stages the mushroom becomes dry and leathery. Initially infected ones did not develop or remained small (Fig 2).





Fig.1. Cobweb symptoms on diseased crops of Shiitake (Fluffy mycelium over the logs and dense spore mass on the diseased fruit bodies





Fig.2. Dry bubble disease symptoms on Shiitake fruiting bodies caused

The fungal pathogens were isolated from fruit body and wood logs using standard practice and identified as isolates of *Cladobotryum and Verticillium* (Fig. 3). *Cladobotryum* spp. produced conidia on erect hyaline conidiophore often arising from aerial mycelium, branching irregularly. Conidia (phialospores) were hyaline, mostly 2-celled, ovoid to oblong, held together in irregular or tangled chains. *Lecanicillium* or *Verticillium* spp. produced conidia on slender branched conidiophores. Conidia were ovoid to ellipsoid, hyaline, one celled, borne singly or in small moist clusters apically.



Fig. 3. Growth pattern and microphotographs of *Cladobotryum* spp. (right)

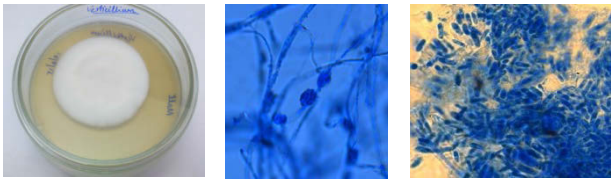


Fig. 4. Growth pattern and microphotographs of *Verticlium* spp. (right)

2.2. Molecular characterization

The identification of isolated pathogenic fungi was confirmed using DNA sequencing of the ITS 5.8s rDNA region and blasting the sequences against NCBI databases. The sequences confirmed the identification of the pathogens as *Cladobotryum mycophilum*, *C. dendroides* and *Verticillium* spp..

2.3. Management of diseases with botanicals

Different plant extracts *viz*, *Dhatura strumonium, Tagetus erecta, Nyctanthes arortristis, Parthenium spp, Callistomon lanceolatus, Cannabis sativa, Gadenia spp., Thooja campacta, Ricinus cummunis* and *Eucalyptus* spp. were evaluated (both dry, hot water and alcoholic extract) against *Cladobotryum* and *Verticillium.* The maximum inhibition in mycelium growth was recorded in *Cannabis sativa* (32.02%) and the least inhibition was recorded in case of *Parthenium* spp. (16.52%) in all the extracts.

Table1. Evaluation of botanicals against *Cladobotryum* spp

Botanicals	**Growth inhibition (%) against control**		
	Dry extract	**Boiled extract**	**Alcoholic extract**
Dhatura Strumonium	24.26	7.87	15.26
Tagetus erecta	27.34	3.81	14.48
Nyctanthes arortristis	20.76	6.32	12.6
Parthenium spp.	16.52	9.66	-
Callistomon lanceolatus	23.25	4.77	20.8
Cannabis sativa	32.02	17.81	56.3
Gadenia spp	21.05	12.41	-
Thooja campacta	23.54	15.03	31.37
Ricinus cummunis	16.66	1.7	17.64
Eucalyptus spp.	24.42	3.22	30.81
CD (0.05)	1.26	2.82	1.06

Cladobotryum dendroides (= *Dactylium dendroides*) has been regarded as the major causal agent of cobweb disease of the cultivated mushrooms (Grogan & Gaze 2000, McKay *et al*. 1999) and is found in all mushroom-growing countries worldwide (Fletcher *et al*. 1989, Geels *et al*. 1988). In the United Kingdom and Ireland, crop losses of up to 40% were recorded due to this pathogen (Gams 1980, Groppe *et al.* 1995). A strong presence of the cobweb disease was observed in white button and king oyster mushroom crops in Spain (Gea et al. 2014). Sharma et al. (2015) also reported the presence of different *Cladobotryum* spp. from *A. bisporus*, *Pleurotus* and *Calocybe indica* crops in India.

Dry bubble disease was first reported by Constantin and Dufour (1892), who described all bubble diseases then known, and referred to them as 'la môle' disease. Ware (1933) also described dry bubble disease and named the causal agent *Verticillium malthousei*. The parasitic fungus has been isolated from *Pleurotus ostreatus* also. On inoculation of healthy *P. ostreatus*, *A. bitorquis* and *A. bisporus*, these isolates caused disease and could be re-isolated, thereby fulfilling Koch's postulates (Gea et al., 2003; Marlowe, 1982). *Lecanicillium fungicola* has also been mentioned as a pathogen of *Coltricha perennis* and *Pleurotus sapidus* (Marlowe, 1982). In addition, *L. fungicola* has been isolated from the basidiomycetes *Marasmiellus ramealis*, *Thelephora terrestris*, *Henningsomyces candidus*, *Hypholoma capnoides* and *Laccaria laccata* (Gams et al., 2004; Zare and Gams, 2008).

To the best of our knowledge and available literature, this is the first report of *Cladobotryum* and *Verticillium* causing cob web and dry bubble diseases on shiitake mushroom.

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Investigation on Mushroom Pests and Diseases in Kenya. A Case Study of Nairobi Environs and Neighboring Counties

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ABSTRACT

Mushroom cultivation in Kenya is an emerging industry, which is rapidly gaining popularity among the small scale farmers. The two main mushrooms grown for nutritional and economic value are *Agaricus* and *Pleurotus species*. Other species include *Lentinula edodes* and *Ganoderma lucidum*. However, pests and diseases are a major challenge to mushroom production in Kenya resulting to yields losses coupled with abandonment to mushroom farming. An investigation was carried out to document the pest and disease occurrence in Nairobi environs and neighboring counties .Focus of the study majored on *Pleurotus* and *Agaricus* sp. This was achieved through farm visits, a well-structured questionnaire and retrieval of information from research organization where farmers consult for disease /pest identification. The investigation captured information such as pests/ disease occurrence, stage of mushroom infestation, symptoms, possible pathogen source, and process followed during cultivation and pest /disease damage. The most common pests and diseases documented included; moulds such as green mould (*Tricoderma species*), brown mould and black mould, wet bubble, mites, beetles, flies, sciarid larvae ,slugs and a competitor mushroom (*Coprinus sps*). Disease occurrence was noted during spawn run, mycelial development, on casing soil and fruit bodies. 80% of the farmers sourced for already prepared substrate and compost a situation where due cultivation process could have been compromised. The moulds especially green mould and mites caused significant yield loss (over 50%) among the oyster and button mushroom farmers. The pest burrowed the fruit bodies of *Pleurotus* especially under the gills and the stipes thus lowering the quality of the mushrooms. Fruit bodies abnormalities were also noted. Limited technological knowhow coupled with poor extension services among the mushroom farmers on pest disease management, are a serious draw back to mushroom production in Kenya. Therefore thorough training on cultivation techniques pertaining to proper pasteurization of substrate and compost preparation and general hygiene should be emphasized. Proper documentation on pests and diseases affecting mushrooms should also be conducted.

Key words: Pests, Diseases, *Agaricus*, *Pleurotus*, Investigation

INTRODUCTION

Mushroom cultivation in Kenya is an emerging industry which is rapidly gaining popularity among the small scale farmers. However majority of these farmers are lacking adequate compost preparation, pasteurization and proper environmental control facilities leading to development of diseases and pests attributed to yield loss and even abandonment of mushroom farming. To date, no research in Kenya has documented pest and disease affecting cultivated mushroom and corresponding yield losses, yet the information is very important for successful and profitably mushroom business among the mushroom growers. The aim of the study was to document pest and disease occurrence affecting mushroom (button, *Agaricus* sp. and Oyster, *Pleurotus species)* cultivation in Nairobi environs and neighboring counties. Other species include *Lentinula edodes* and *Ganoderma lucidum*. This was achieved through farm visits, a well-structured questionnaire and retrieval of information from research organization where farmers consult for disease /pest identification.

Results and Discussion

Our preliminary results conducted from 11 button and 9 oyster mushroom farmers, show over 75% mushroom (Figure 1) yield losses are associated with pest and diseases. Button mushroom farmers were the most affected with more than half of the farmers interviewed, recording 76-100% losses. This is a significant loss which could be avoided through adequate compost preparation, pasteurization and proper environmental control facilities. However most of the farmers interviewed experienced contamination through poor methods of pasteurization, lack of proper environmental control facilities and poor hygiene.

We recorded 7 mushroom diseases namely; dry bubble, wet bubble, Bacterial Blotch, green mould caused by *Trichoderma species*, White, brown and black mould. Fruiting bodies abnormalities and browning of the caps were also noted. All the diseases affected both oyster and button mushrooms, with all farmers interviewed documenting over 50% yield losses. Diseases were prevalent in all stage of mushroom growth; spawn run, mycelial development, casing soil and fruiting. Moulds, in particular green mould occurred during spawn run and pinning causing significant yield loss (over 50%) among the oyster and button mushroom farmers by affecting mycelial development. The possible causes suggested, included poor pasteurization of the substrates. Although most of farmers (over 70 %) used a wide range of chemicals during their sterilization phase, diseases infestations were still high (Table 1). Over 80% of button small scale farmers sourced for already prepared substrate. Most farmers lack the proper skills of compost preparation and therefore prefer purchasing already prepared compost, a situation where due cultivation process could be compromised.

Pests prevalence was also high, and affected both oyster and button farmers resulting to more than 50% losses. A total of 10 mushroom pests (mites, beetles, fruit flies, sciarid larvae, Phorid larvae, nematodes, eelworms, slugs, maize stalk borer and *Coprinus* species, a competitor mushroom) were recorded. Mites were the most prevalent pest causing significant yield loss (over 50%) among the oyster and button mushroom farmers. The mites (pest) burrowed under the gills and stipe lowering the quality of the mushrooms. Poor hygiene and

poor housing could be associated with high numbers of pests among farmers (Sharma et al., 2007).

Conclusions and Recommendations

Limited technological knowhow coupled with poor extension services among the mushroom farmers on pest disease management, are a serious draw back to mushroom production in Kenya. Therefore thorough training on cultivation techniques pertaining to proper pasteurization of substrate and compost preparation and general hygiene should be emphasized.

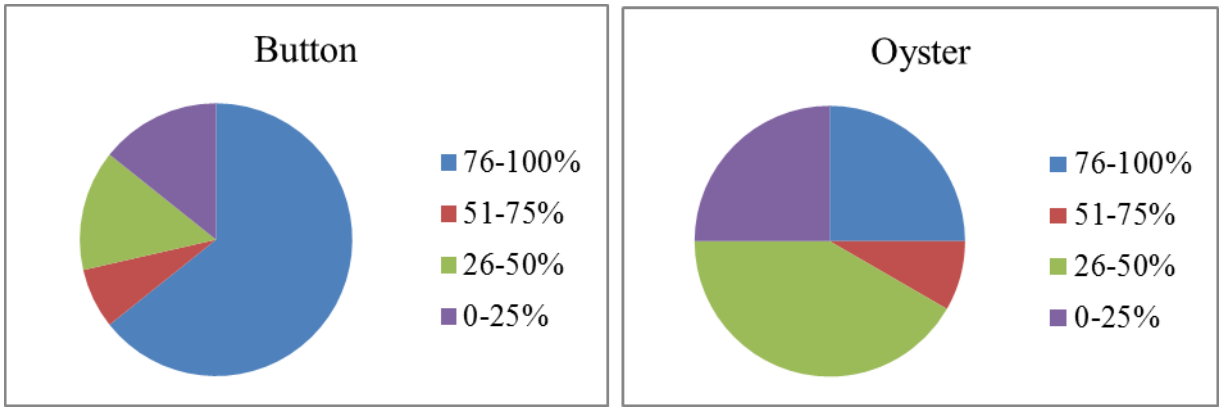


Figure 1: Proportion of farmers and corresponding percentage mushroom losses caused by pest and diseases in button and oyster cultivation

Table 1: Some common pathogens and pest of mushrooms in Kenya.

Pathogen/pest	**Causal organism**	**Symptoms**	**Possible control method**
Diseases			
Wet bubble	*Mycogone* sp	Characterized by development of irregular and undifferentiated mass of mushroom tissues.	Hygiene procedures/practices should be reviewed to make sure that they are being followed.
Dry Bubble	*Lecanicillium* sp	During the early stages of pinning the infected pins forms small bubbles of tissue. Partial stipe split and malformations	Hygiene procedures/practices should be reviewed to make sure that they are being followed.

Table 1 (cont'd): Some common pathogens and pest of mushrooms in Kenya.

Pathogen/pest	**Causal organism**	**Symptoms**	**Possible control method**
Green Mould	*Trichoderma* sp.	Usually characterized by extensive mat like mycelia that forms green patches on casing and in the compost. Normally starts as white mycelial growth.	Properly pasteurized casing and compost is required. Mites and flies are carriers of the *Trichoderma* spore and should be controlled.
White Mould	*Scopulariopsis* sp	Thick, fluffy plaster mold spots usually white in color. Appears on casing .Middle of spots appears as flecks of plaster.	Over humid and sticky grains should not be used for spawning.
Brown Mould	*Papulaspora byssina*	A spot appears as granular mass (like fie sand) hazel in colour.	Spay mould nidus with the superphosphate.
Browning caps	Excessively chlorinated water, excess formalin etc.	Browning of pin heads.	Avoid use of highly chlorinated water or excessive use of formalin for spraying.
Slugs		Glistening slime trails and irregular cavities on the caps and stalks.	Use of molluscide baits at a distance of 2-3 m apart around entire mushroom beds.
Sciarid fly	*Lycoriella* sp	Its larvae feed on the mycelium and the developing mushrooms.	Control mushroom flies. Proper pasteurization of substrate.
Mites	*Tyrophagus* sp	Causes unpleasant sensation to mushroom workers. The mites feed on the mycelium.	Workers should ensure strict hygiene by wearing clean clothes and regularly dipping their shoes in disinfectants.
Stalk borer	*Busseola fusca*	Burrows the mushroom stalk	Proper pasteurization of the substrate material.
Phorid Larvae	*Megaselia* sp	Forms tunnels along the stipe. The stipe turns brown along the tunnel.	Sanitation that focuses on pest elimination or destruction.
Ink caps	*Coprinus* sp	Slender, delicate bell shaped mushrooms.	Proper pasteurization of the substrate/compost is necessary.

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The Influence of the Pleurotus Substrate Microbial Colonisation on the Growth of *Trichoderma pleuroti*

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ABSTRACT

Currently, the biggest problem in *Pleurotus ostreatus* growing in the Czech Republic is an adverse effect of *Trichoderma pleuroti* infection. The purpose of this work was (i) to identify possible sources of the contamination in a compost yard and a mushroom farm, (ii) to uncover the influence of the selected factors on the growth of *T. pleuroti* and (iii) to prepare the substrate resistant to the *T. pleuroti* infection. We used various combinations of the treatment (the natural outside fermentation of the straw, the temperature treatment of the substrate, and the addition of a lime component) and studied their effect on the growth of *P. ostreatus* and *T. pleuroti* and the changes in the microbiome of the substrate.

Surprisingly, we didn't identify *T. pleuroti* in the air of the compost yard and the oyster mushroom farm and therefore we focused our attention on the substrate. We observed that *T. pleuroti* developed faster in the sterilized substrate than in the substrate subjected to heat treatment of 60-70 °C. Moreover, sa straw infected with *T. pleuroti,* subsequently fermented (30 °C, 36 hours) and then steamed (60, 65 or 70 °C) was completely colonized with mycelium of *P. ostreatus*, whereas, on the straw infected and steamed without the fermentation procedure, we observed the development of *T. pleuroti.*

We also compared the *Pleurotus* substrate samples subjected to the short straw fermentation at different temperatures which were steamed for 24 hours at 70 °C with and without the addition of the lime component. In these samples, bacterial and fungal operational taxonomic units (OTUs) were identified on a DNA level using the 545 pyrosequencing. The majority of the bacterial sequences belong to OTUs corresponding to *Paenibacillus* and *Bacillus* species and the most frequent fungal OTUs correspond to *Wallemia* and *Verticillium* spp. All these OTUs were more abundant in the samples after steaming (70 °C) than without steaming. Contrary to the temperature of the fermentation, the addition of the lime component had no significant effect on the composition of the microbiome.

We can conclude that the pre-fermentation of the straw can inhibit the growth of *T. pleuroti*. We also found that the substrate treatment at 70 °C does not cause the decrease in microbial growth; right the opposite: it can support particular bacterial and fungal species development, which subsequently leads to the higher resistance of the substrate against *T. pleuroti* infection.

Key words: *Pleurotus ostreatus*, *Trichoderma pleuroti*, pyrosequencing, substrate

INTRODUCTION

Trichoderma species are competitive fungi of oyster mushroom (*Pleurotus ostreatus*) culture that are known for almost thirty years. Later, the species was specified as *Trichoderma pleuroti* (Park et al. 2006). The occurrence of this fungus was observed in many countries such as Spain (Gea 2009) and Poland, where it causes significant damage (Kommon-Zelazovska et al. 2007) and was also confirmed in the Czech Republic (Novotny et al. 2016). The incidence of *T. pleuroti* can be eliminated by appropriate sanitary conditions and heat-treatment of the substrate.

Here, we focused on the selected compost yard and a mushroom farm, in which *T. pleuroti* has been detected for many years. We searched for a source of the contamination and tried to eliminate this contaminating fungus in the substrate.

Results & Discussion

In the selected compost yard and the mushroom farm monitored during three-year period, we tried to identify the source of *T. pleuroti.* Forty Petri dishes with nutrient agar were placed over the compost yard and the mushroom farm and spores collected by air sampling were subsequently incubated. Various fungal genera were detected even in the clean room of the mushroom farm. Spores of *T. pleuroti* were detected only in two cases. Other fungi detected in the air did not grow on the substrate and did not compete with *P. ostreatus*. Therefore, it seems that *T. pleuroti* does not spread by the air and for this reason, we focused our attention to the substrate.

We studied the ability of *P. ostreatus* to compete with *T. pleuroti* in the substrate at laboratory conditions. The straw was enriched with 10 % of the contaminated substrate with *T. pleuroti* and was subjected to natural aerobic fermentation at 30 °C and subsequently heat-treated at 70 °C for 24 hours. Samples were then inoculated with grain spawn of *P. ostreatus* and after spawn-running, individual straws were collected from the colonised as well as non-colonised parts of the substrate and placed on Petri dishes with nutrient agar. After seven days, mycelium of *P. ostreatus* was observed in the samples from the colonised substrate and, surprisingly, *Scytalidium* prevailed in the non-colonised samples (its presence was also confirmed by pyrosequencing, see below) over *T. pleuroti*.

It was described that particular bacteria can support the growth of *P. ostreatus* mycelium (Nagy et al, 2012). Therefore we studied the influence of *Bacillus subtilis* and *B. macerans* on the growth of *P. ostreatus* and *T. pleuroti* on the Petri dish with nutrient agar. In this experiment, we neither observed any inhibition effect of *Bacillus* species on *T. pleuroti* nor any positive effect of *Bacillus* species on *P. ostreatus*. We also studied this phenomenon in operating conditions. The influence of the filtrates from the culture of *B. subtilis, B. macerans,* substrate prepared on the bulk and processed in tunnel and substrate prepared on the bulk and not treated in tunnel on growth of two strains of *T. pleuroti*. The tested *Trichoderma* strains created smaller colonies when they were influenced by filtrate obtained from two substrates for cultivation of oyster mushroom. We assume that in this particular case, *Bacillus* species did not play any significant role in supporting the growth of *P.*

ostreatus mycelium. Metabolites produced by bacteria in substrate suppress the growth of *T. pleuroti*.

To optimise the substrate preparation process, we first studied the influence of the temperature on the spore survival in the laboratory conditions using two strains of *T. pleuroti* (CPPF 416 and CPPF 432). Spore suspension of used strains did not survive heat treatment above 65 °C, lethal dose was 20 min at 55°C and 10 min at 65°C for the strain CPPF 416 and CPPF 432, respectively. However, in operating conditions, even the temperature of 70°C for 24 hours did not inhibit the development of *T. pleuroti* in some cases. It could indicate that the fungus grew into the bulk straw and was protected from destroying by the heat-treatment. On the other hand, when we implemented the fermentation at 30 °C followed by steaming, *T. pleuroti* was not present in majority of cases.

Subsequently, we compared the relationship between mycelia of *P. ostreatus* and *T. pleuroti* on the steamed (70 °C) as well as sterilised (121 °C) straw with spawn mixed into the whole bulk of the substrate. Steamed straw was completely colonised by *P. ostreatus* mycelium, whereas on sterilised straw, *T. pleuroti* suppressed the growth of *P. ostreatus* mycelium. We presumed that it was caused by the absence of microbial population in the sterilised substrate, which normally inhibits the development of *T. pleuroti.* In other words, sterilisation of the substrate prevented the bacterial growth and it was consequently prone to contamination by *T. pleuroti*.

To confirm our hypothesis and to identify particular bacterial genera, we conducted the metagenomic study. We compared samples of the straw after the short-term fermentation at various temperatures and after steaming at 70 °C for 24 h with and without the addition of the lime component. We analysed eight individual samples (Table 1) in one 454-sequencing run as described in Baldrian et al (2012) and we obtained, after the removal of low quality, short

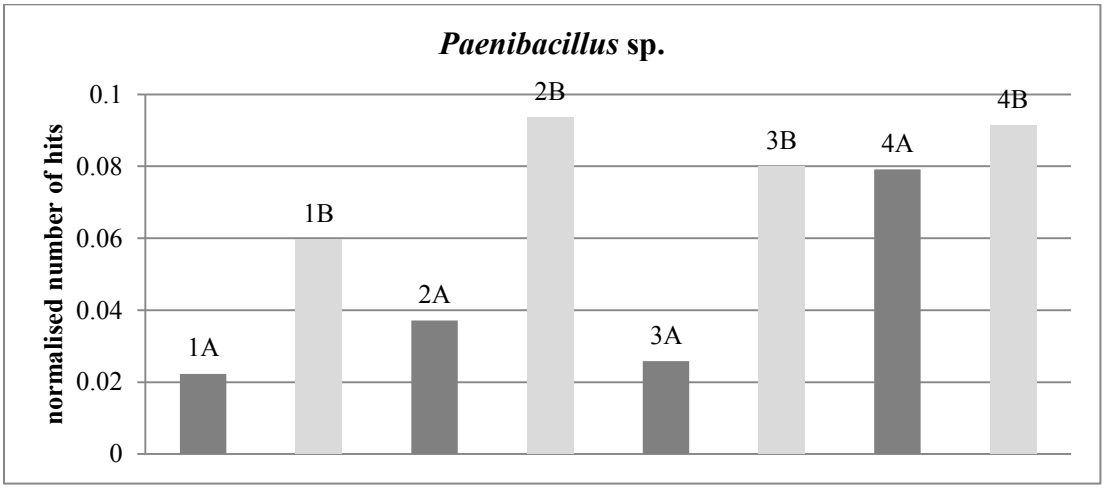


Fig. 1 Relative occurrence of *Paenibacillus* OTUs in different substrates. A - no steaming, B - after steaming (70 °C). Samples differed also in the temperature of fermentation and in the addition of Ca component Detailed description of substrate types is in Table 1.

Thus, we identified the combination of three-day fermentation (30 °C) and heat-treatment (70 °C) as a major factor for reduction of *T. pleuroti* in substrate of *P. ostreatus*.

and chimaeric sequences, 54 507 sequences. In the samples, 3 445 bacterial operational taxonomic units (OTUs) and 387 fungal OTUs were identified at the sequence similarity level of 95 and 97%, respectively. The genera of bacteria and fungi corresponding to largest numbers of OTUs are listed in table 2. Fig. 1 shows that the dominating bacterial genus *Paenibacillus* was more abundant in the samples after steaming (70 °C). Conversely, the addition of the lime component did not have any influence on the composition of microbiota.

Table 1 Detailed description of substrate conditions in the samples

	Temperature of fermentation	Steaming (70° C)	Addition of Ca component
1A	10° C	No	Yes
1B	10° C	Yes	Yes
2A	30° C	No	Yes
2B	30° C	Yes	Yes
3A	30° C	No	No
3B	30° C	Yes	No
4A	40° C	No	No
4B	40° C	Yes	No

Table 2. Main identified genera of bacteria and fungi

Bacterial genus.	Number of corresponding bacterial OTUs	Fungal genus.	Number of corresponding fungal OTUs
Paenibacillus	230	*Wallemia*	59
Bacillus	135	*Scytalidium*	19
Thermobacillus	96	*Aspergillus*	18
Thermobispora	57	*Verticillium*	15
		Penicillium	15

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IV Substrate and composting

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A comprehensive mathematical model to simulate the composting process for production of substrate for *Agaricus bisporus*

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ABSTRACT

The use of mathematical models to simulate chemical and biological processes can help industries to gain a better understanding of their processes and improving them. For a complex process such as composting, where the activities of several microbiological communities change the biochemistry of the surrounding environment, a mathematical model helps to identify which of the mechanisms have the highest impact on the process and on the final characteristics of the compost. A simulation based on the mathematical model predicts the outcome of design changes to the process, and accelerates research and development efforts.

A comprehensive mathematical model was developed, gathering state-of-the-art knowledge about the microbiological, chemical and physical mechanisms taking place in composting. The aim of the model is to simulate the trends in temperature, compaction and composition (in water, dry matter, carbohydrates and organic nitrogen) of compost piles in tunnels, throughout three phases of the process for *A. bisporus* substrate production.

In the model is included the growth and death kinetics of important microbiological groups under the limitation of moisture content, temperature and substrate availability. Heat and mass transport between the compost and the gas phase, compaction and porosity changes throughout time and height of the pile are modelled as well. The aeration scheme is also included in the simulation, as it plays an important role in controlling the process in tunnels.

The model developed can successfully predict the temperature trends of the process as it happens at CNC Grondstoffen. It brought important insights to the process, such as the importance of the different microbiological communities throughout time and across the height of the composting pile. Because the aeration control is included in the simulations, different flowrates, temperatures and composition could be simulated and their effect on the characteristics of the compost could be estimated. We are currently expanding the model to include other microbiological communities and proceed to experimentally validate it.

The presented model systematically aggregates the current knowledge for the production of substrate for *A. bisporus*, and can be used as a tool to synthesize and study process design improvements.

Keywords: model, simulation, composting, tunnel, *Agaricus bisprous*

INTRODUCTION

The composting process to produce substrate for the cultivation of *A. bisporus* is traditionally

divided in three phases: fermentation, pasteurization & conditioning and spawn-run. In Europe, the last two phases are done in large tunnels with controlled aeration, and at CNC Grondstoffen, in particular, the first phase is also processed in tunnels (IVC®).
To drive process improvements, mathematical models to describe and simulate a process can be used as a tool for process design. Van Lier et al. (1994) developed and validated a mathematical model for the composting process for mushroom substrate in a tunnel, which provided good predictions of key process parameters such as temperature and dry matter. The increased knowledge of the microbial and chemical composition of composting material led to the development of general composting models that included different microbial groups and substrates (Sole-Mauri, 2007), while others focused on the physical characteristics of the composting pile and the gas flow (Richard et al., 2004).
The model herein presented combines published knowledge from these different fields, and adapts them to describe the most important microbiological, chemical and physical processes playing a role in the three phases of the composting process for mushroom substrate production.
The physical system, where each process phase takes place, is similar: a rectangular pile of organic material inside tunnels where air is fed from the bottom by a ventilation system. Therefore, the core of the model are the physical-chemical processes that occur in a compost pile, which are described below. What differentiates each phase are the initial composition, the microbial species involved and the process control strategy, achieved by means of ventilation.

1. Model description

Changes in the physical and chemical characteristics of the compost pile are mostly due to the activities of microorganisms. Therefore, microbial growth, decay and lysis are at the centre of the model. The microbial species found to be of relevance to the process were thermophilic and mesophilic bacteria, and the fungal species *A. bisporus* and *Scytalidium thermophilum*. The growth of these microorganisms depends on temperature, substrate availability (C, N and O sources) and water availability. Microorganisms are also involved in the hydrolysis of solid substrates, such as cellulose. As some of the substrates and products are gases, the transport of molecules between the gas and the liquid phases (O_2, CO_2, H_2O and NH_3) has to be accounted for. In the liquid phase, ionic equilibria are described for the dissociation of various species, with the pH as key variable.
Microbial growth and maintenance processes generate heat, which is transported through the pile via the gas flow through the pores. Due to the large size of the compost pile, heat losses through the walls are considered negligible, compared to heat removal by convection. The gas flow is driven by the pressure drop across the pile and depends on porosity. The accompanying water removal also affects compaction and porosity, which are taken into account.
The model is built to determine the changes in time and space (across the height of the pile) of the composition of the gas, the liquid, and the biodegradable fraction of the solid phase of the composting pile (figure 1). The pile is divided in horizontal layers with homogeneous composition, which are connected by the gas flow between them.

In tunnels, temperature control is done by aeration. Therefore, the model includes the control system used in the tunnels, which regulates the amount of fresh and recycled air fed to the pile.

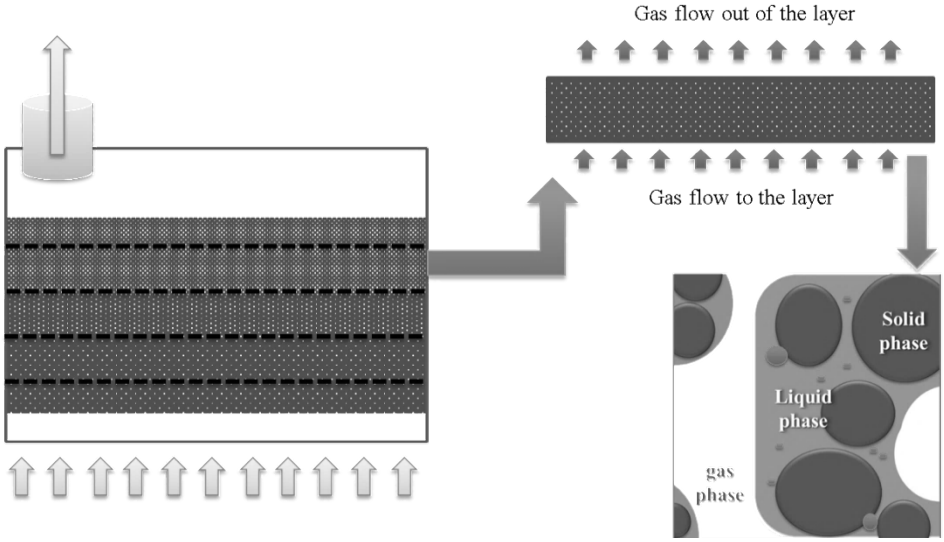


Figure 1. Visual summary of the different levels modelled. Each layer consists of a gas, a liquid and a solid phase, with homogeneous properties. Microorganisms are part of the liquid phase.

2. **Results & Discussion**

The mathematical model successfully predicts the temperature curves of the three process phases as they occur in the tunnels at CNC Grondstoffen, as well as simulate the response of the ventilation control system currently used in the tunnels.

In the first version of the model, where only the described organisms and their growth on glucose and protein as carbon and nitrogen sources were included, the composition of the compost pile at the end of each phase could not be properly predicted. Some questions were hence: both in phase 1 and phase 2, the activity of the microorganisms modelled produced enough energy for the temperature trends to be met, but not enough to simulate dry matter degradation to the extent that it occurs in the tunnels. Simulations have helped to direct research attention to hypotheses involving multiple substrate take up and to expand the number of microorganisms included in the model. Future developments are to incorporate knowledge from experiments and tune parameters that cannot be currently determined (e.g. buffering capacity), to make the model a better approximation of reality.
The model is very flexible and – despite the complexity and uncertainty – it is realistic enough to bring out trends related to different initial compositions, control schemes and even different weather conditions. For example, compost porosity is associated with compost composition and pile height. The effect of different loads of compost per m^2 of tunnel floor on the porosity of compost, gas flow and temperature homogeneity throughout the pile can be estimated. These are examples of factors that can influence process and operation design.

A more concrete example of a design study is given in figure 2. To test the effect of changing ventilator speed on the operation cost and final product quality, two scenarios were simulated with the model: one where the ventilation speed was increased by 20% and another where it was decreased by 20%. These scenarios were compared to the standard process simulation for phase 2, with respect to temperature, electricity consumption by ventilators and end *S. thermophilum* concentration, a fungi whose presence has been proven to stimulate *A. bisporus*

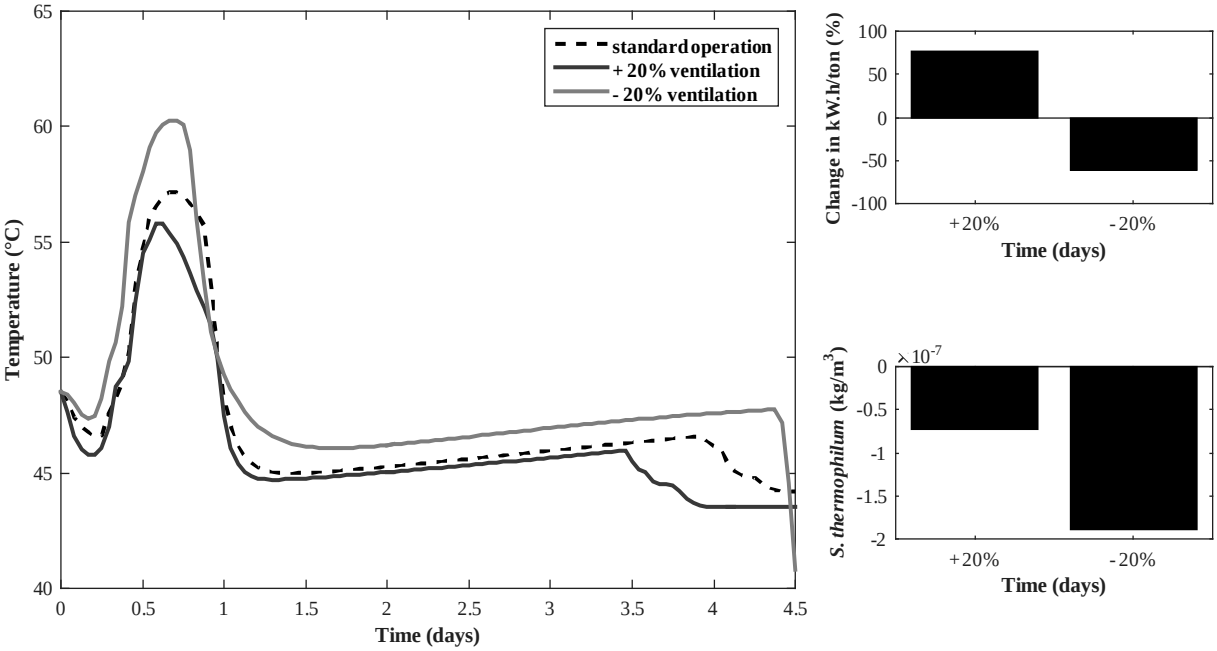


Figure 2. Example of a design study for phase 2.The model was run with a 20 % increase and a 20 % decrease in aeration speed and compared to the simulation of the standard operation.

colonization of compost in phase 3 (Straatsma et al., 1994). The model simulations indicate that, even though a decrease in ventilation could bring savings in terms of electricity usage, it would probably affect negatively the concentration of *S. thermophilum*, compromising compost quality. It is important to note that the results from these two scenarios were not experimentally validated and they are presented to serve as an indication of the capabilities of the model when used as a tool to make a preliminary evaluation of the effect of changes to the process.

The developed model combines several models from literature on the biochemical and physical changes in compost throughout time, with the control system for aeration in place in real tunnels. This tool has given insight into the uncertainties in the present knowledge and has directed further R&D. The model offers also the possibility to test the effect of different operation conditions on the quality of the compost, *in silico* before implementation.

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Figure 2. Example of a design study by in phase 2 using the model. The model was run with a 20 % increase in aeration speed and a 20 % decrease in aeration speed compared to standard

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Varieties in bacterial communities in compost during composting and cultivation

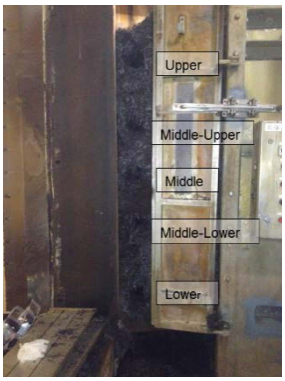
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ABSTRACT

In the production of substrate for Agaricus bisporus CNC grondstoffen uses straw based horse manure from over 2000 horse stables in Europe. In order to obtain a homogenous substrate, over the years CNC grondstoffen has developed a process in which mixing is an essential part in order to achieve a constant high quality compost. Compost quality is intensely monitored during production by sampling. Among other parameters pH, Ash, Nitrogen and total solids are determined. But how representative is a sample from a tunnel of 4m height*6m wide*30 m long and how homogeneous is compost in a tunnel in reality? Since composting is mainly a decomposition process in which bacteria and fungi play a major role the environmental conditions create the community of microbial decomposers. In other words when we measure the composition of bacterial communities at different locations (horizontal and vertical) in tunnels and during cultivation, differences or shifts in microbial community indicate the variation and/or changes in the system.
In this study the composition of bacteria and fungi in compost used for mushroom cultivation was determined. The substrate has been examined from end phase 2 (end of thermophilic phase before inoculation with *Agaricus*) till the end of 2nd flush of mushrooms.

Figure 1. Phase 3 tunnel (left) horizontal sampling and vertical sampling (right)





The analysis of the bacterial and fungal communities has been done on DNA extracted from sampled material using Ion Torrent sequencing technology of partial fragments of ribosomal genes of bacteria (16S rDNA).
These techniques give a better view on the bacterial community then methods that are based on plate cultures. Not all bacteria in compost can be isolated using traditional plate methods.

Results bacterial community during composting process in phase 2 and 3 (figures 2 and 3).

- A low variation in composition between samples within and between tunnels was observed (standard deviation ranged from 0.01 to 0.10). The 4 most abundant bacterial phyla (cumulative relative abundance > 90%) were Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria

From this we can conclude that the conditions for growth of bacteria in phase 2 and phase 3 tunnels is rather homogeneous and therefore we can conclude that the compost as it is produced at CNC grondstoffen as a substrate for growth of Agaricus bisporus is homogeneous.

Figure 2: Relative abundance of bacterial phyla at the end of 2nd Phase and 3rd Phase compost (end). Average of 8 samples taken horizontally (4 meters distance horizontally between samples).

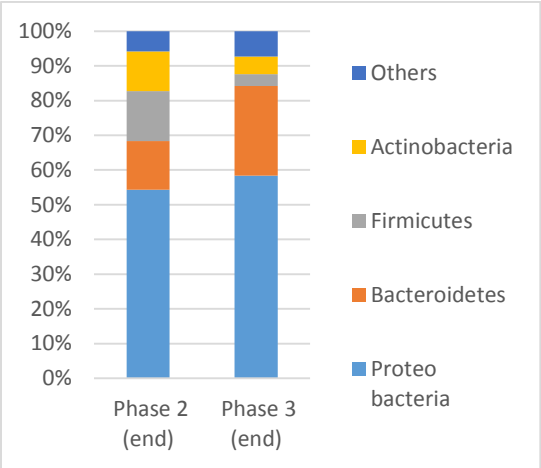
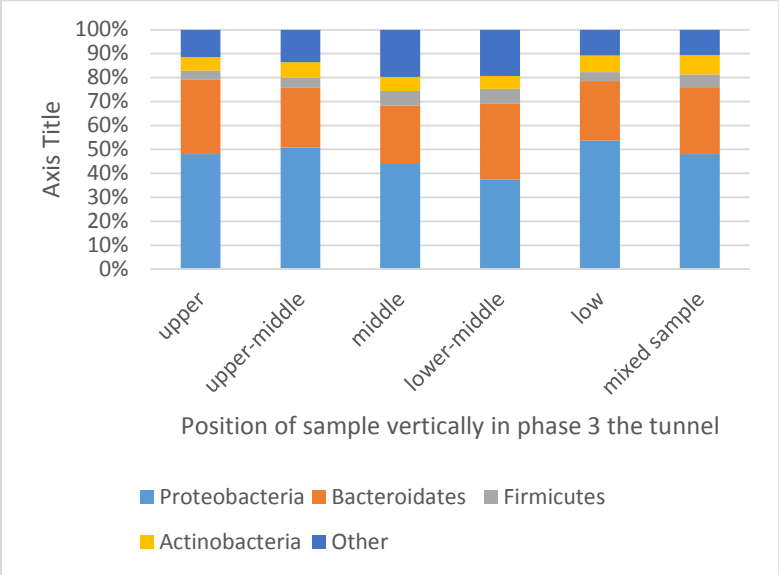


Figure 3: Relative abundance of bacterial phyla in 3rd phase compost (end). Vertical distribution. 5 Samples and 1 mixed sample



Results bacterial community during cultivation of mushrooms

- During cultivation of mushrooms changes in bacterial community composition are observed in the substrate samples taken from the mushroom beds. In particular the increasing

importance of Firmicutes (*Bacilli* and *Paenibacilli*) and *Planctomycetes* (unclassified, part of "other") is evident. Also local variety increases (upper, middle and low) (figure 4). So we see that we start with a homogeneous compost that after filling and during cultivation changes. The compost at different levels in the bed changes and becomes less homogeneous. This may be caused by the way of filling, caccing and/or watering. It is known from other studies (personal communication A. Sonnenberg) that the consumption of nutrients in the different (height) levels of the compost also varies. We do not know whether these observations are connected. During cultivation we see a significant shift in composition. This is particular evident when we compare samples before and after 1st flush (figure 5). During cultivation the conditions in the bed change such as pH, mineral, cellulose and hemicellulose becomes partly consumed, production of amino acids and other exudates of Agaricus all leading to a shift in bacterial population. We do not know yet what impact is of these shifts in population. We speculate that a more homogeneous compost in the mushroom bed will give a better performance with respect to yield, quality and incidence of diseases. The techniques used in this study for elucidating the bacterial community in compost can be used in intervention studies.

Figure 4. Vertical distribution of bacterial phyla during mushroom cultivation after ventilation before buttoning (left) and at the end of 2nd flush (right). Standard deviation varied from 0.02 to 0.14)

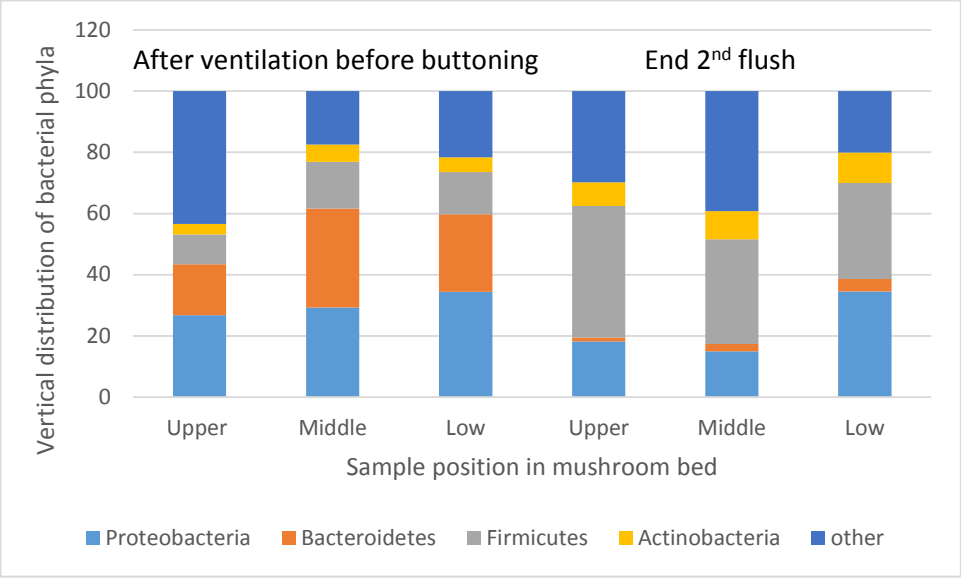
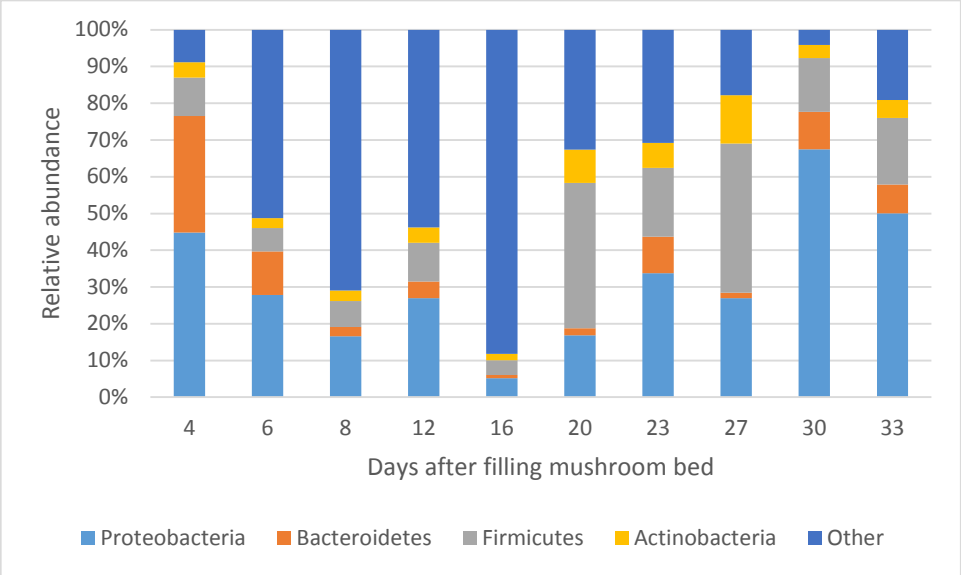


Figure 5. Relative abundance of bacterial phyla in compost during mushroom cultivation.



Concluding: compost from a phase 3 process as performed at CNC grondstoffen is homogeneous. The compost changes during cultivation as a result of filling, watering, cultivation and change in local conditions during cultivation. It is speculated that focus on more homogeneous conditions during cultivation may lead to a better performance.

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Use of Cold or Frozen Phase III Compost for Mushroom Cultivation

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ABSTRACT

An alternative to preparing one's own compost for *Agaricus bisporus* production is to purchase the substrate from a third party. A limiting factor in the growth of Latin American mushroom production is compost preparation. Central composting facilities supplying satellite growers are rare. Production of one's own compost is limited to the economics and scale of production. An alternative for Panama and Costa Rica has been to receive spawn-run compost from offshore. The spawn-run compost arrives by container cold or frozen and after warm up to 20^{o}C it is cased. Production in three breaks averages 28 kg per square meter. This paper will discuss the problems, challenges, procedures, successes, failures, and economics encountered in mushroom production from cold or frozen blocks of Phase III compost.
Keywords: *Agaricus bisporus,* Panama, Costa Rica, cultivation, spawn-run

INTRODUCTION

Mushroom production in Latin America began in Argentina in 1941 (Lahmann and Rinker, 1995). Since those humble beginnings, overall production has steadily increased, with at least 65 thousand tonnes of *Agaricus bisporus, A. subrufrescens, Lentinula edodes* and *Pleurotus* sp. produced in 2002, an increase of nearly 50% from 1994 (Lahmann and Rinker, 2004). Yet, in the same period, the number of producers decreased by about 55%.

The investment to construct a stand-alone mushroom farm with both production facilities and supportive compost preparation is economically staggering in Latin America. A small mushroom farm, supplying a small and low per capita consumption market, would over capitalize in the composting component, even if Phase II or III tunnels were not installed. And, the production of good quality compost is more challenging on a small scale.

The Latin American mushroom industry is a fledging one, especially the smaller Central American countries. A unique adaption of centralized composting that is common in North America and Europe is that compost companies from off-shore are supplying spawn-run (Phase III) compost in cold or frozen blocks to Central America. This paper will highlight the development of mushroom production in Panama and Costa Rica utilizing this recent technology.

product is prepared for shipment to Central America. The spawn-run compost is cooled to shipping temperatures with vacuum cooling, liquid nitrogen, dry ice or ice to about 0^{o}C. Blocks are filled with 18- 22 kg wet weight with the desired strains.
The bagged compost is loaded typically into 12.2m x 2.9m (40 ft x 9.5 ft) refrigerated shipping containers either on steam-treated pallets or stacked by-hand and separated vertically by steam-treated wooden studs to facilitate management of temperature during shipment. Upwards of 1250 bags with a total weight approximating 25 metric tonnes are loaded per reefer. Reefer temperature is set for as low -3^{o}C. A thermograph is placed into each container to monitor temperature during shipment. After two to three weeks the container arrives at port and is transported to the farm.

2. Farm description

The Costa Rican farm (near Cartago), illustrated in the paper, was built in 1970 using hollow concrete blocks and wooden shelving. The eight growing rooms, 240 m^2 of growing surface each, are equipped with air conditioning but not with heating capabilities. Ambient air (16 to 28^{o}C; night/day) is used instead. This farm has been receiving compost from Europe since 2012. There are two other farms in the country that are importing spawn-run compost.
The Panama farm (La Mitra, distrito la Chorrera, Panama Province), discussed in this paper, was constructed in 2013 by Champion Group Poland (Warynskiego 32-36, Grudziadz 86-300, Poland) with six aluminum-shelved 265 m^2 growing rooms, equipped with cooling, heating, humidification, automatic watering and computer control. The farm uses a head filling machine, nets and puller and conveyors for compost and casing. There is another farm in the western part of Panama (Sector Buena Vista, Bugava, Chiriqui Province) that has imported spawn-run compost since 2015.

3. Management at the farm

The spawn-run compost arrives at the farm -3 to 0^{o}C and is sometimes partial frozen. Upon arrival the compost is kept cold until needed. At the Costa Rican farm the holding area is a cold storage building and in Panama, a cooled shipping container.
At the Costa Rican farm, the bags are arranged directly onto the wooden shelves. The top of the block's plastic is cut but not removed. The substrate is allowed to warm-up until 'soft', using ambient fresh air. Then, the top plastic is removed and the compost is broken up by-hand and leveled. Fill weight approximates 90 kg m^{-2}. Two applications of water are applied before pressing by-hand, 3 L m^{-2} and then 2 L m^{-2} containing an insecticide. The day following the watering, the compost is cased by-hand 4.5-5 cm thick using imported Baltic peat. Five hundred grams of spawn-run compost per square meter from each shipment is added to the casing to prepare a CAC (Compost Added at Casing) material. After casing and when all the water is applied, the casing surface is covered with plastic. Using an air temperature of 22-24^{0}C the compost temperature is maintained at 28-29^{0}C until flushing 5 to 6 days after casing. Pinning is initiated by removing the plastic, setting the compost temperature at 19-20^{o}C with air 16-17^{o}C. (Both white and brown strains are placed in the

same room.) First harvest begins 10-11 days after flushing with whites picking one day earlier than browns.

At the Panama farm, the spawn-run compost is filled and cased at the same time. Because the substrate must be pliable to be used in the machinery, the substrate must warm up for at least 16 hours prior to filling. After warm-up, the bags are completely emptied into a filling hopper, and shelves are filled with 90 kg m^{-2}, supplemented, CACed (450 to 500 g m^{-2}) and cased (4.5-5 cm) with peat from the Baltics or the Netherlands. After casing, the substrate heats up rapidly requiring at least one day to bring it under control. Once the compost temperature is stable at 28°C, the CO_2 is maintained greater than 5000 ppm with humidity over 99%. In about five days from casing, the room is flushed by lowering the compost temperature over four days to 19-20°C, arriving at air temperature of 16-17°C. Carbon dioxide drops to 1000 ppm and humidity to 89-90%. Production commences 10-11 days after flushing.

Mushrooms on both farm systems are harvested manually with approximate 28 kg m^{-2} and 30–32 kg m^{-2} harvested on the Costa Rican and Panama farms, respectively. Mushrooms are harvested for two or three flushes. All the mushrooms from each farm go to fresh market sales.

4. Economics

The costs of the spawn-run compost include the cost of substrate directly, transportation costs from site of production to the farm, custom's charges and possibly storage at the port of arrival.

The production of one's own compost includes material costs, equipment to handle and mix substrate, infrastructure costs for compost wharf and its services of electricity, water and maintenance, and labour. Additionally, depending on the system for production of spawn-run material, there may be bunkers, and pasteurization and conditioning as well as spawn-run tunnels. If bulk tunnels are not used and a single-zone system is utilized the number of crops per year decreases substantially with substantial difference in sales per square meter per year. For example, the Costa Rican farm, switched from a single-zone system to imported spawn-run compost in 2012. Since then, mushroom production has increased three and half times with annual costs of compost slightly more than doubling.

5. Challenges, successes and lessons learned

5.1 Shipment problems

Intrinsic challenges exist from the spawn-run production facilities in Europe to the mushroom farms in Central America. The spawn-run compost must traverse over 9000 km from port-to-port, excluding transportation to the port of departure and from port of arrival to the farm sight. There may be up to three weeks on the ocean and additional days on land awaiting shipment and pick up at port of arrival. There are custom and port clearances to be met. All of these can create delays which ripple into supply of mushrooms to buyers, quality of the spawn-run compost and scheduling of farm activities and employees.

The transportation of spawn-run product is in the control of the land and sea transportation companies. Cooling equipment must function properly for the entire time that the container is in transit. Malfunctioning or careless management of the cooling equipment in transit may result in overheated substrate which affects substrate quality. The presence of *Trichoderma* sp has been correlated to poor temperature management during shipment. The containers themselves must be packed properly to withstand the handling by the shippers. Poorly packed containers or roughly handled containers will result in bags shifting and damaged, and increased difficulty in maintaining the desired temperature. Shipping delays are frequent and even some containers are lost. Some growers maintain an inventory of spawn-compost for emergencies to balance any variation in shipping volumes.

5.2 Quality of substrate

Spawn-run suppliers vacuum cool, use liquid nitrogen, dry ice (CO_2) or ice to cool the substrate. The vacuum cooling tends to dry the substrate; whereas, the use of dry ice and ice add moisture. The quality of the substrate is not influenced greatly by any process. Addition of moisture to the substrate requires careful attention. Watering compost too cold ($<10^oC$) will slow down and inhibit the re-knitting of the mycelia. However, watering the compost once the substrate has re-knit will result in little absorption since the mushroom mycelium is hydrophobic.

5.3 Insect and disease problems associated with the systems

Costa Rican farm has problems with phorid and sciarid flies and wet bubble disease, likely vectored by the flies. The warm-up period prior to casing with its increased mycelial growth is attractive to phorids. And, in combination with older facilities that are looser in structure, the crops are more easily infested. The modernly constructed farms in Panama are experiencing challenges with cecids and sciarids. Occasionally, *Trichoderma* sp. appear in the compost late in the crop. Its origin has yet to be determined.

CONCLUSIONS

Decentralized mushroom production systems separate and concentrate specialization in growing media preparation and production. Shipment of spawn-run compost by cargo ship has become an option for growers in Central America where farms are small and the economics of compost preparation for small area is disproportionate. This rather unique cross oceanic system is a viable one for stimulating mushroom production.

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New insights into the microbial communities and biological activities that define mushroom compost

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ABSTRACT

The yield and quality of a mushroom crop is vitally dependent on maintaining consistent, reliable quality in the mushroom compost used as a growth substrate. Achieving this consistency requires an in-depth understanding of the biology of the composting process. Detailed chemical and biological data were obtained for a standard composting run and mushroom production run under conditions typical for the Australian mushroom industry. Activities of eleven different compost enzymes were determined, along with physicochemical measurements and analysis of the microbial community (T-RFLP and Illumina sequencing) at 37 timepoints from raw materials through composting to the end of the mushroom production process, examining both casing and compost during three flushes of mushroom cropping. Enzymatic activities in compost were highest during the pre-wet phase, and decreased by the end of rick turn, sharply increased during Phase 2 (after pasteurization), only to decrease again throughout spawn run. Near infrared fingerprints, a technique used for characterizing organic samples, revealed clear differences between casing and compost at various stages of composting and mushroom production, suggesting this may be a useful methodology in characterizing compost and casing in future research. Over 30,000 different microbes were identified in compost with highest diversity at the end of Phase 1 and an obvious biological succession throughout the whole process. The bacterial community in Phase 1 was dominated successively by an *Acinetobacter* followed by *Bacillus*, an unidentified *Proteobacterium* and *Thermus*. In Phase 2 and Phase 3, the main bacteria were a *Pseudoxanthomonas* strain together with *Steroidobacter* and *Chitinophaga*. The fungal community also showed a strong biological succession during pre-wet and Phase 1, with a strain of *Lewia* dominating initially, followed by *Myceliophora*, an unidentified fungus, a strain of *Penicillium,* and *Scytalidium thermophilum*. *Scytalidium thermophilum* dominated throughout Phase 2 and Phase 3 until it was overgrown by *Agaricus* during the spawn run. The data obtained represent the most detailed biochemical and molecular study yet undertaken of the mushroom composting process, and have revealed the complexity of microbial populations present in high yielding compost, many of which have never been identified before. More work is required to confirm which of these are essential for composting, and which could potentially be used as supplements to promote composting efficiency.

Keywords: compost microbial ecology, enzyme activities, DNA sequencing, *Pseudoxanthomonas*, *Scytalidium*

INTRODUCTION

Preparation of mushroom compost is a solid-state fermentation process that relies on the activity of complex and largely unknown communities of microorganisms. The microbial and biochemical characteristics of the compost are critical in converting cellulose and hemicellulose feedstock components into compounds that support both high yielding growth of the *Agaricus* mycelium, and selectivity for *Agaricus* over other fungi. Under Australian conditions, the feedstocks used are usually wheat straw and poultry manure, though other feedstocks are used internationally. The composition and activity of the microbial community varies with the materials used in composting and on the conditions during the compost preparation and mushroom growing processes. The key microbes involved in these processes have been studied extensively during the past 50 years, and many key bacteria and fungi have been identified by cultivating them under laboratory conditions (comprehensively reviewed by Ryckeboer *et al.* (2003)). The bacterial strains that have been isolated from mushroom compost are predominantly *Bacillus/Paenibacillus*, *Pseudomonas*, and thermotolerant actinomycetes, while the fungal isolates include many strains of *Aspergillus*, *Mucor*, *Penicillium* and *Scytalidium*, amongst a broad range of other genera (Ryckeboer *et al.*, 2003). However, in recent years it has become clear that since >99% of microbes in soils and composts are very difficult or impossible to isolate under laboratory conditions (Rappe & Giovannoni, 2003, Pham & Kim, 2012), many highly important microbes for the composting process may well remain undetected. In this study, we have identified and quantified compost bacteria and fungi using modern molecular techniques that analyse total DNA isolated directly from compost and avoid the cultivation step entirely. This has provided a multi-faceted and highly detailed view of biological changes in compost and casing during compost production and mushroom cropping under Australian conditions.

1. Results and Discussion

Samples were collected (5-fold or greater replication) from wheat straw and chicken manure feedstocks from a commercially-produced compost at 37 time points throughout the composting process (bale break, pre-wet windrows, Phase 1, Phase 2, spawn run), and from compost and casing during production (before, during and after three flushes of harvesting). Mushroom yield from a small-scale production system was 47.8 kg m^{-2} (430 g kg^{-1} compost) from three flushes. Biological activity was monitored throughout the process by measuring (a) activities of eleven different microbial enzymes in compost, (b) fungal and bacterial diversity, and (c) carbon profile using infrared spectral fingerprints.

1.1. Pre-wet windrows

Biological activity was high during bale break and pre-wet. High enzymatic activities of invertase, glucosidase, chitinase were measured together with some xylanase and cellulase, corresponding to rapid breakdown of easily accessible plant products. Total extractable carbon fell by 60% as these compounds were assimilated into microbial biomass. Microbial diversity varied strongly during this treatment phase with detection of many uncharacterized bacterial and fungal species. Bacterial diversity changed rapidly with dominance at specific

times of typical soil and plant-related bacteria like *Acinetobacter* and *Bacillus*, but also many other species (Fig. 1). Fungal diversity also varied with initial dominance of *Lewia* (probably from the wheat straw, as it is commonly known from wheat grain and stubble), followed by unknown genera, and then *Myceliophthora*, a cellulose-degrading genus (Fig. 2). Considerable variation was found due to differences in aeration and mixing and to lack of homogeneity of the substrate. Although there is some evidence for the presence of thermophilic microbes in the pre-wet phase, most of the key populations appear to derive from the wheat straw. At the start of the pre-wet phase, there is also evidence for organisms derived from poultry manure or goody water with a substantial population of a species of *Arcobacter* that is related to poultry pathogens. However, this organism had disappeared entirely by the end of the pre-wet phase, and there is no evidence for persistence into later phases of composting.

1.2. Phase 1

Phase 1 composting was done in a commercial-scale enclosed bunker, allowing consistent temperature and aeration control. Cellulase, xylanase, glucosidase and protease activities were high at the start and decreased during Phase 1. The bacterial community became more diverse, and this diversity reached a peak at the end of Phase 1 (Fig. 1). The dominant bacteria were a previously uncharacterized *Proteobacterium*, followed in succession by the thermophilic genus *Thermus*. Interestingly, this *Thermus* strain was also found in a high proportion in the goody water, suggesting that use of goody water may be important for inoculation of compost. The thermophilic fungus *Scytalidium* was dominant early in Phase 1, but was overgrown by other species later in Phase 1.

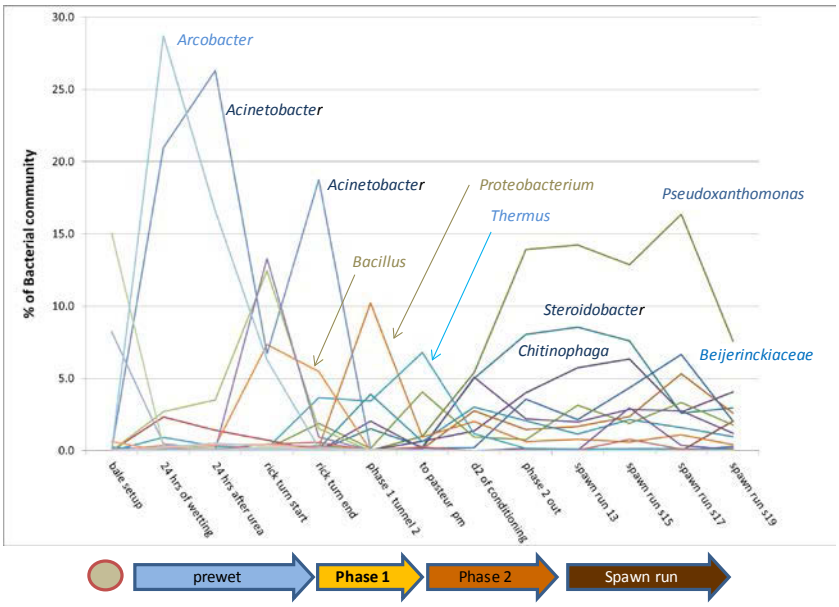


Figure 1. Bacterial succession during composting and spawn run determined by Illumina Miseq sequencing of the 16S rRNA gene.

1.3. Phase 2

Pasteurization and conditioning in Phase 2 leads to extensive chemical and biological changes in the compost. Peak activities of glucosidase, cellulose and xylanase were measured, together with dramatic changes in microbial composition. The dominant fungal component was *Scytalidium thermophilum*, in agreement with previous studies (Straatsma *et al.*, 1989), and it seems likely that much of the enzyme activity is mediated by this species. *Scytalidium thermophilum* dominated the molecular fungal profile from pasteurization through to spawn run, until it was overgrown by *Agaricus* mycelium, ending the rapid succession of species found in composting (Fig. 2). This was also the case for the bacterial community in Phase 2, with three bacterial strains dominating the population throughout this period. *Steroidobacter* and *Chitinophaga* are not well characterized genera, but *Chitinobacter* may be associated with fungal mycelia, since it is chitinolytic but not cellulolytic, and *Steroidobacter* may degrade fungal byproducts. This suggests that they may be associated with the dominant *Scytalidium* population which may have relevance in design of *Scytalidium* inoculants. The dominant bacterium in Phase 2 was a species of *Pseudoxanthomonas*. This genus is also poorly characterized, but is thought to comprise bacteria that use ammonia as a source of energy, therefore not respiring organic carbon to CO_2. The dominance of uncharacterized bacteria in Phase 2 highlights the need for further work on these microorganisms to better understand their role and importance in composting.

1.4. Spawn run and cropping

Enzyme activities during spawn run were dominated by those associated with the *Agaricus* mycelium, such as peroxidase and laccase, with enhanced hydrolase activities found in the compost phase during cropping. No significant enzymatic activity was measured in casing. *Scytalidium* continued to dominate the compost fungal population until overgrown by *Agaricus*. The *Pseudoxanthomonas* bacterial strain identified in Phase 2 persisted at high levels in the compost throughout spawn run and cropping, but soil related genera also appeared in the compost after application of casing. The dominant bacteria in the casing layer were soil bacteria (*Pedobacter* and *Caulobacter*), with an increasing proportion of *Pseudomonas*. These *Pseudomonas* species are of a greater diversity than those previously reported to be important for fruiting (Noble *et al.*, 2009), and it is possible that interaction of these organisms with the mycelium may play a role in reduced yields in later harvest flushes. However, no cyclical correlation of specific strains with flushes was found in this study.

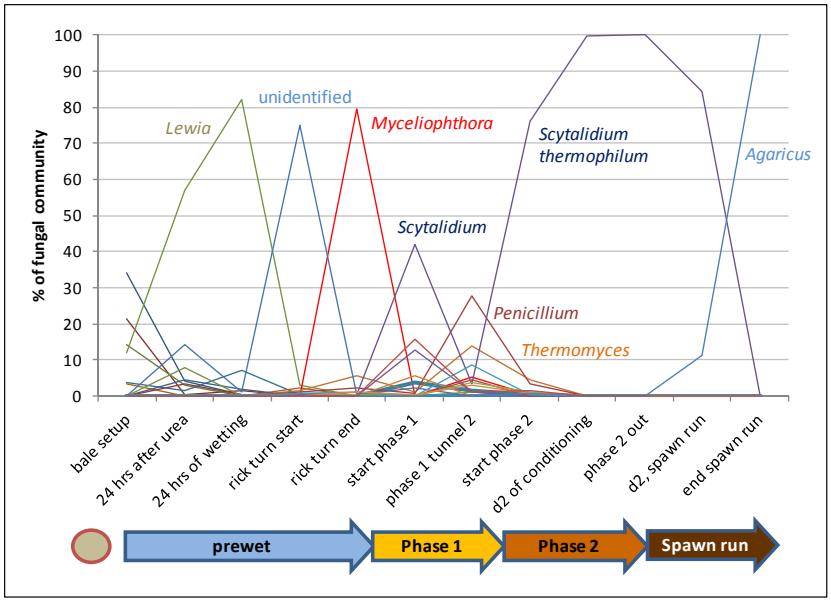


Figure 2. Fungal succession during composting and spawn run determined by Illumina Miseq sequencing of the ITS gene region.

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Development of an NGS data analysis strategy to identify the microbial population present in mushroom compost

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ABSTRACT

Mushroom yield is supposed to be related to the quality of the compost that is used for the commercial cultivation. Little is known about the composition of the microflora during the various stages of compost preparation and the effect it has on quality. In this study we have developed and applied a reliable strategy to isolate microbial DNA from compost. We have employed Next Generation Sequencing technology to generate 16S rRNA sequence data for the isolated DNA and we have developed an effective data analysis strategy to characterize the data functionally. Analysis of the 16S rRNA data confirmed expected shifts in the microbial taxonomic composition during compost production. The presented strategy can in principle be more broadly used for the characterization of the microbial population in any kind of (soil related) environment.

INTRODUCTION

Button mushrooms (*Agaricus bisporus*) are commercially cultivated on compost that has been specifically prepared for the intended use. In general, *A. bisporus* compost mainly consists of wheat straw, horse and chicken manure, and gypsum [1]. Before mushroom mycelium is introduced in the third phase of the preparation process two composting stages, having different controlled conditions with respect to aeration, mixing and temperature, are applied to make the nutrients accessible for mycelium growth. During the two first phases the nutrients are liberated from the raw input materials by the action of the microflora that is present. During phase I aerobic fermentation commences as microbial growth and reproduction naturally occur in the bulk ingredients. Heat, ammonia, and carbon dioxide are released as by-products during this process. Due to the released heat (~70 ºC) and increasing [2]. The conditions applied at the start of phase II selectively kill undesired (micro-)organisms, fungi and/or ammonia, microorganisms

cease growing and the liberated carbohydrates start to chemically caramelize insects that will compete for food or directly attack the mushroom. Whereas at the end of phase II the composting process is completed by the remaining microflora which have eliminated the simple soluble sugars and gaseous and soluble ammonia created during phase I [2]. The composition of the microflora is supposed to shift dramatically during phase II from thermophile and manure-related to a more soil-like diverse population. Until now detailed studies of the transition in microbial population during phase II are sparse [3,4]. We decided to use Next Generation Sequencing to map the population shifts that occur during this phase in more detail.

Results & Discussion

Compost samples were taken at the start and at the end of phase 2 of the composting process. We isolated the microbial DNA using a dedicated commercial 'kit' that was reported to yield high quality DNA in the extraction of protist DNA from soil [5]. Yet we found considerable degradation of the DNA during isolation (smear in Figure 1a). Compost contains humic acids, which are known to affect the isolation efficiency [6]. We therefore modified the isolation protocol by early addition of DNA stabilizing buffer and by decreasing the handling time. Furthermore, we decreased the end volume to increase DNA concentration. The modifications resulted in a reproducible isolation procedure that yields DNA of high quality (see Figure 1b).

To determine the extent of the shift in the prokaryotic microbiome at the end of phase 2 with respect to the start, the isolated DNA was sent out for sequencing and annotation of the 16S rRNA content. We obtained raw data files containing ~200 000 pseudoreads of 300 bases per sample. Based on the alignment of the pseudoreads against the GreenGenes database [7], a taxonomic analysis was performed (see Figure 2). The Taxonomic classification was calculated by assigning each read to a specific operational taxonomic unit (OTU) using the sequence alignment score. The taxonomic depth of the lineage was based on the identity threshold of the rank; for Phylum a threshold of 80% was used. As expected we found a clear shift in the bacterial composition during phase II of the composting. At the start the population was dominated by thermophilic organisms from the phylum Thermus and the Firmicutes (Bacillus and Clostridia), whereas at the end of phase II the population was dominated by Actinobacteria, Bacteriodetes and Gammaproteobacteria. This observation, although more detailed, is in line with the report by He et al. [3]. At the same time the diversity had clearly increased as more phyla were substantially represented at the end of phase II, making the composition more soil-like [8].

It appeared that only less than 1% of the sequences was identical to one of the sequences deposited in the reference database, indicating that the bacteria that are present have not been isolated and sequenced before and that phase II compost could provide a potential source for as

yet unknown gene functions. Summarizing, the analysis of the 16S rRNA data confirmed expected shifts in the microbial taxonomic composition during compost production and these shifts could be the basis for further development of quality-assays. The presented strategy can in principle be more broadly used for the characterization of the microbial population in any kind of (soil related) environment.

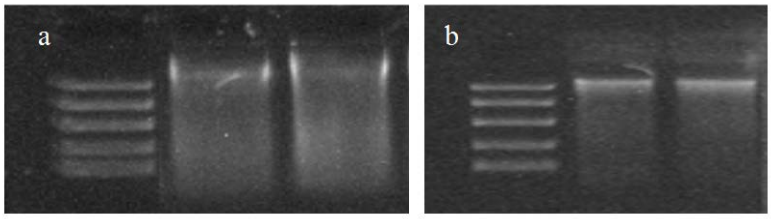


Figure 1. DNA gel profiles for various isolations from compost. a & b) lane 1: gene ruler high range; lane 2: phase 2 start; lane 3: phase 2 end. Compost samples were provided by CNC. They were stored at -20℃ directly after they were taken. a) DNA was isolated from the compost using the Powermax DNA isolation kit (Mo Bio Laboratories Inc.). b) To increase the stability of the DNA the following adaptations were made: a buffer containing sodium citrate, EDTA and ammonium sulfate was added to the frozen compost, and then the compost was transferred to buffer C1; the amount of elution buffer was decreased from 5ml to 2ml to increase the concentration of the DNA.

(sub)-phylum	start	end
Acidobacteria	0.0%	2.0%
Actinobacteria	2.7%	10.3%
Bacteroidetes	2.0%	18.8%
Bacilli	28.8%	2.6%
Clostridia	12.9%	5.3%
Chloroflexi	0.1%	3.9%
Gemmatimonadetes	0.0%	1.9%
Planctomycetes	0.0%	2.5%
Alphaproteobacteria	0.5%	6.4%
Betaproteobacteria	0.4%	1.8%
Deltaproteobacteria	0.1%	4.4%
Gammaproteobacteria	1.8%	25.2%
Spirochaetes	0.0%	0.4%
Tenericutes	1.2%	0.3%
Thermi	35.5%	1.5%
Thermotogae	3.0%	0.0%
Unclassified	11.90%	14%

Figure 2. Taxonomic diversity at the phylum level at the start and end of phase 2 during composting. Only the phyla that cover more than 1% of the reads in one of the two or in both cases are shown. The isolated DNA was sequenced by Baseclear. Paired-end sequence reads were generated using the Illumina MiSeq system. FASTQ sequence files were generated using the Illumina Casava pipeline version 1.8.3. Initial quality assessment was based on data passing

the Illumina Chastity filtering. Subsequently, reads containing adapters and/or PhiX control signal were removed by Baseclear using an in-house filtering protocol. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0. The pseudoreads (merged paired end reads) were aligned against the GreenGenes database version 13_5 [8] using the "Map reads to reference" option of the CLC Genomics Workbench version 7.5.1. The taxonomic analysis was performed based on the alignment of the pseudoreads against the GreenGenes database.

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The use of corn stover to replace straw in compost formulations for the production of *Agaricus bisporus*

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ABSTRACT

The first step in the production of the button mushroom, *Agaricus bisporus*, is Phase I composting. Phase I composting is a high-temperature biological and chemical process needed to prepare and modify the raw materials for the introduction of *A. bisporus* mycelium, in the form of spawn. The main objectives of Phase I composting are to prepare a homogenous, high moisture mixture that forms a complex lignocellulosic and selective substrate that is used as a food source for *A. bisporus*. The raw materials that are utilized in formulating this mix vary throughout the world, based on availability and costs. The basic formula that is most widely adopted in Western production systems consists, at a minimum, of wheat straw or wheat straw-bedded horse manure, poultry manure and gypsum (calcium sulfate). In the eastern part of North America, mulch hay is used in large quantities to replace straw due to its availability and relatively low costs. Other nitrogen sources (meals and inorganic fertilizers) can be used in place of or as a supplement to poultry manure based on costs and availability. Other carbohydrate materials (corn cobs, cottonseed hulls, leaves, etc.) can also be supplemented to these straw or hay-based formulations. Corn stover, the plant material left in the field after the harvest of the grain kernel, is a raw material that is relatively abundant in regions that produce corn and can be used to supplement the formulation as well. Previous, unpublished work has demonstrated that corn stover can be used up to 20 percent of the formulation without any adverse impact on mushroom yield. A challenge to producing affordable mushroom substrate in some regions is a limited supply of straw, whether wheat or rice straw. Cropping experiments were conducted at the Pennsylvania State University Mushroom Research Center to determine if corn stover could be used at 50% and 100% of the bulk raw material in the Phase I compost formulation to produce a high yielding *A. bisporus* crop. Results showed that, when composted properly, mushroom yields grown on both 50 and 100% corn stover formulations yielded as high as traditional straw-based compost formulations. These results suggest that corn stover can be used in place of straw to produce *A. bisporus* compost in regions that have limited or no economically affordable straw supplies.

Keywords: (*Agaricus bisporus,* Phase I, Compost, Corn Stover)

INTRODUCTION

There are many different compost formulations used through the world with a straw-based formula probably being the most widely adopted. A wheat straw, or wheat straw-bedded horse manure formula is commonly used in most of the leading *Agaricus* producing countries. In the eastern region of North America many farms also use mulch hay or a mulch hay/straw based formulas. However, supplies of these materials can often be inconsistent due to the weather effects on agronomic yields or due to increasing demands for grains or plant materials used in the production of bio-based fuels. Additionally, there are regions, such as northern China, that do not have an economically, readily available straw supply. For these reasons, experiments were set up to test the effectiveness of using corn stover as the base for an *Agaricus bisporus* formulation. Corn stover is the plant residue left in the field after the harvesting of the grain. Corn is a crop that is grown all over the world and is also increasing in planted acreage both in the United States as well as globally (faostat.org), thereby possibly providing an alternative raw material for mushroom substrate production.

1. Methods and Materials

The control compost was prepared at the Mushroom Research Center (MRC) at the Pennsylvania State University, University Park campus following a standard University formula consisting of: wheat straw-bedded horse manure, dehydrated poultry manure, dried distiller's grain and gypsum. The raw materials, excluding the dried distiller's grain, were mixed with water in a Jaylor ® feed mixer to reach approximately a 60% starting moisture on day 0. The mixed ingredients were then placed into an aerated bunker for 3 days, with air supplied to the mix based on a timer to prevent anaerobic conditions from occurring. Compost temperatures reached approximately 80ºC during the first three days of Phase I composting. On day 3, the material was removed from the bunker, dried distiller's grain added and mixed in the feed mixer with the addition of supplemental water and again placed into the aerated bunker for three more days with temperatures again reaching approximately 80C. On day 6, the material was removed from the bunker, mixed with additional water again and filled into 1.5 m^2 wooden trays. Treatment 2 (50:50 corn stover) consisted of a formulation in which ½ of the straw-bedded horse manure was replaced with corn stover. The corn stover and ½ of the formulated horse manure and ½ of the formulated poultry manure were mixed together in the feed mixer with the addition of water and allowed to passively compost for a 5 day prewet period. Following the 5 day prewet period, the prewet pile was added to the feed mixer along with the remaining horse manure, poultry manure and gypsum with the addition of more water prior to be placed in the aerated Phase I bunker at day 0 of Phase I. Treatment 2 was then removed from the bunker on day 3, added to the feed mixer along with the dried distiller's grain and additional water and placed back into the aerated bunker until day 6 at which time the compost was mixed again with additional water prior to filling the wooden trays to begin Phase II.

Treatment 3 (100 % corn stover) consisted of a formulation in which corn stover was used to replace all of the wheat-straw bedded horse manure. The corn stover and ½ of the formulated poultry manure were mixed in the feed mixer with the addition of water and allowed to passively compost for a 5 day prewet period. Following the prewet period, the remaining poultry manure, gypsum, and water were added into the feed mixer and then placed into an aerated bunker. The Phase I composting procedure followed the same timeline as previously described.

The wooden trays, filled with Phase I compost, were then placed into an environmentally controlled room at the MRC for an 8 day Phase II composting period where pasteurization was achieved at 60-65°C for 2 hours, followed by conditioning at which the compost was held at 48°C to eliminate free ammonia after which the compost was cooled to a temperature below 28ºC to prepare for spawning. Compost from each treatment was sent to the Agricultural Analytical Lab at Penn State University for chemical analyses on the day of spawning (Table 1). A commercial, off-white hybrid strain of Agaricus (J.E. Lange) Imbach was added to the Phase II compost at a rate of 3% and a commercial delayed-release supplement was added at a rate of 4% (dry weight basis). The spawn and supplement were mixed into the compost by hand and 22.7 kg of the spawned compost (wet weight) was filled into 0.25m^2 tubs (12 tubs per treatment). The tubs were placed on stainless steel racks, covered with plastic to increase carbon dioxide concentration in the substrate and to promote vegetative mycelial growth and then placed in environmentally controlled growth chambers at the MRC for a 16 day spawn run. During spawn run, room humidity was maintained near 98% and substrate temperatures were maintained between 23 and 25°C. On day 14 the plastic was removed from the tubs to allow free moisture that formed on the substrate surface to evaporate prior to the addition of a sphagnum-based casing layer. On day 16, agricultural lime-amended peat moss was mixed with water to achieve approximately an 82% moisture mixture. A commercially provided casing inoculum (CI) was added to the peat mixture at a rate of approximately 500g/m^2 growing area. After the casing was applied, room humidity was maintained at approximately 98% until day 11 at which point humidity was reduced to approximately 90% for the remainder of the cropping period. Substrate temperatures were maintained near 25ºC until day 5 when the room was cooled to 21-22°C to induce mushroom fruiting body formation. Mushrooms were harvested, counted and weighed daily when the mushrooms were mature (prior to veil stretching and once the stipe was elongated and straight). Harvesting was initiated on day 15 and continued for 3 weeks (3 flushes). Water was applied from the day of casing through the end of the cropping cycle *as* needed based on the experience of the *author*. Mushrooms were counted and weighed daily, as needed, for three flushes to determine yields. Mushroom yield data was analyzed for yield by break and by total yield. Statistical analyses were done using SAS Ver. 9.4 and treatment means were compared using the Waller-Duncan K ratio test (Table 2).

2. Results

Analytical results for the compost samples at spawning show that the moisture, total nitrogen, pH and C:N ratios were similar for all three treatments. Soluble salt concentration was lower in the 100% corn stover formula compared to the standard wheat-straw bedded horse manure formula.

Table 1. Compost chemical analyses at spawning.

Treatment	pH	Moisture (%)	Total N (%) Dry Wt	C:N	Soluble Salts (mmhos/cm)
Control	7.4	66.6	2.4	15.7	10.44
50% Corn Stover	7.5	64.2	2.5	15.9	9.09
100% Corn Stover	7.5	66.1	2.4	15.8	6.96

Both the 50% and 100% corn stover formula composts yielded as well as the standard control formula for each break as well as the total yield (Table 2). Each corn stover-based formula yielded significantly higher than the control formula. However, the Biological Efficiency (BE) (total yield wet weight/dry weight of compost) for the control formula was only 78%, lower than typical yields at the MRC. Typical BE's average between 90 – 95% at the PSU MRC. Based on BE's of 89 and 90% for the 50 and 100% corn stover formulas (respectively), these compost formulations yielded as well as our standard formula.

Table 2. Yield comparisons by break. Numbers in columns followed by the same letter are not significantly different from one another (P, 0.05).

Treatment	kg/m^2								% Bio-Efficiency
	Break 1		Break 2		Break 3		Total		
Control	10.68	b	8.83	B	4.04	b	23.56	b	78.00
50% Corn stover	11.82	a	11.53	A	5.25	a	28.60	a	89.00
100 % Corn stover	12.26	a	11.36	A	4.15	b	27.78	a	91.00

CONCLUSIONS

This study demonstrates the ability to either supplement a straw-bedded horse manure formula with corn stover or to utilize a "straight" corn stover formula for the production of *Agaricus bisporus* in regions where other carbohydrate based raw materials are either not available or more costly. A corn stover based formula may require a longer composting duration than a

straw-based formula, as was done for this study in which we incorporated a 5 day prewet to better prepare the higher lignin based carbohydrate source for mushroom production. The exact changes that would be needed to adapt to a 100% corn stover formula will depend on the nature of the existing composting system that a farm is utilizing. The use of corn stover will allow a much more economical approach to substrate preparation in regions that are lacking in wheat straw or straw-based horse manure.

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Aerated composting. A silent practical breakthrough.

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ABSTRACT

This paper is a summary of experiences and observations at many different composting yards in Europe and Japan with aerated composting for *Agaricus bisporus* since 1987.
The process of balancing between increasing temperatures and absorbing water.
The practical importance of forming brown substances by Maillard reactions is expressed.
Explaining the sequence of algae, th. fungi, th. bacteria, oxidations at Maillard reactions and chemical reactions at specific temperature ranges with the effect on selectivity and structure.
The present opinion of optimal figures of pH value, moisture content, NH_4 content, Nitrogen content, ash content, EC value and C/N ratio at the moment of spawning are evaluated.
The relevant pattern of on/off settings at different temperature levels will be described in this paper.
Keywords: heating, temp. sequence, Maillard reactions, protective coating, selectivity.

INTRODUCTION

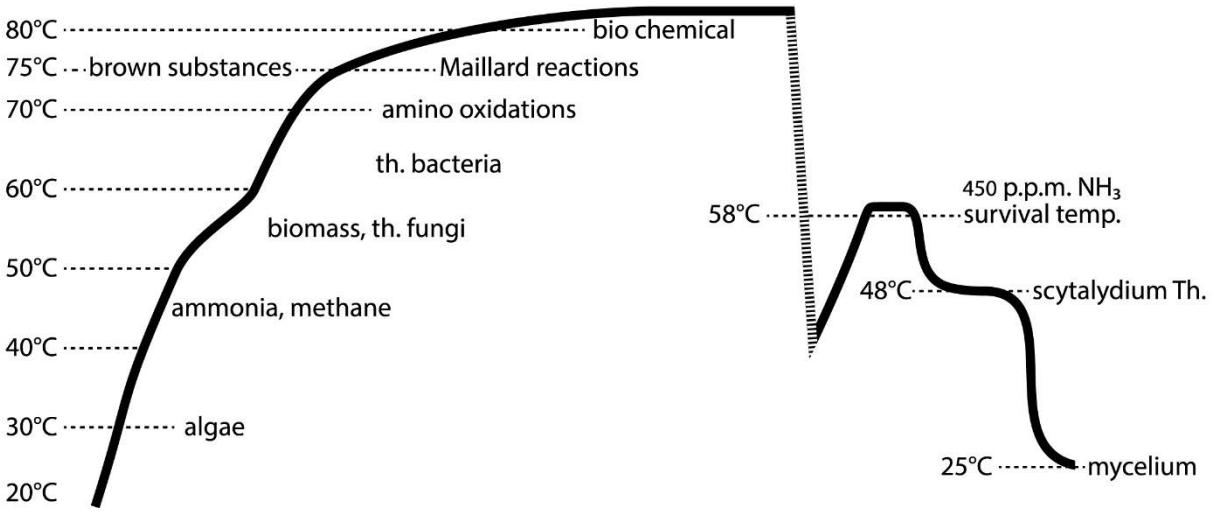
The great inspirers in research of aerated composting in Europe were Mr. Jean Laborde and Mr. Alex Overstijns. The first modern system of aerated composting was carried out at Kuhn, Full in Switzerland at February 1987 and is still in operation. The main technical breakthrough was at Tolson's, Australia in 1992 a new type of bunker with a spigot floor and in Europe at Walkro Belgium in October 1993 with an open aerated spigot floor and a conveyor belt on top. C.N.C at Moerdijk NL. started in April 1995 with an unique Phase I system in tunnels and an industrial pre wetting factory. Afterwards mainly concrete bunkers with spigot floors and an on/off aeration system were build, filled by bunker fillers or overhead conveyor systems for filling in layers.
At this moment aerated composting is **the** common solution for producing more stable Phase I composts on a smaller area with less odor problems. The type of compost did in general not change much since 50 years but the structure improved by more homogeneous, less greasy, less over fermented composts. This is important for better results at peak-heating in tunnels and consequently at incubation and cropping.

1. Temperature sequence

For a consistently high yielding substrate, a high temperature Phase I seems to be required and thermal destruction of microbial populations is an essential part of the succession process. The nitrogenous complex with brown substances forms a protective coating around the remaining carbohydrates, like cellulose and hemi-cellulose. This limits their utilization by

competitor molds. The enzymes of *A. bisporus* mycelium have the possibility to degrade such a coating and creates access to the underlying nutrients by the production of hydrogen peroxide and oxalic acid.

Table 1.



* < 50^0 C. At the first heating, ammonia plays an important role in destroying the cutine layer of the straw as the first opening. Adding urea or sulfate of ammonia in an early stage does accelerate this process. Ammonia softens the straw chemically, making more organic matter available for converting nitrogen in other more complex forms.
The first start, being loose water in the matrix, is a more anaerobic process of methanogenic bacteria and methane-gas production accompanied with mal-odor. This can be reduced by several flips and adding not too much water at the start. This part takes much oxygen.
* Between 50^0 and 60^0 C. The further thermophilic biomass composed of fungi, bacteria and yeasts increases rapidly and converts easy degradable carbohydrates into sugars, proteins, CO_2, ammonia and amino-acids. This part seems very important to build a firm biomass. It could be positive to have a not very fast temp. increase at this stage.
* Microbiological activity in composting at temp. under 65^0 C. burns more organic material and it mainly reduces the available excess of nutrition and also can have a negative effect on a more soft structure. At temp. over 65^0 C. the heating process is mainly conducted by Th. bacteria and does take less oxygen.
Typical brown substances are formed between 70^0 and 80^0 C. in the presence of ammonia.
The change in darkening of the straw is important for the nutrient availability for *A. bisporus* besides the more stabilized selectivity.
* From 70^0 till 75^0 C. mainly a biological process by creating poly phenols out of therm. fungi, using soluble sugars and starch. Forming brown substances completed with lignin degradation by enzymatic reactions and amino oxidations. These reactions do integrate Nitrogen and protect it.
* Over 75^0 till 80^0 C. more formation of brown protective substances by bio-chemical activity (Maillard-reactions). Production of caramellanoidins from sugars and amino-acids by which

nitrogen products are protected to many molds. Giving a compost at emptying a bunker a specific caramellious smell.

2. Optimal figures

Apart from visional inspection of a compost at the moment of spawning you need to have reliable chemical analyses. Herewith some general average figures:

* pH-value 7.3, which is lower by the use of sulfate of ammonia at Phase I. Goal is pH 6.2 after 15 days of spawn-running.
* NH_4 or ammonium content 0,02 %. High pH combined with high NH_4 is negative.
* Moisture content 68 %, Always related to the structure. In general never water is added at spawning but always at filling Phase I compost in tunnels. Optimal 63 % of Phase III.
* Nitrogen content dry material 2,1 % for horse manure composts and 2,2 % for straw composts.
* Ash content about 25 % to have sufficient organic material and not too much pollution by silicates, sand or other not producing minerals.
* C/N-ratio dry material 17 with which there are empirically the best yields.

Besides just figures, mainly the structure of the compost is very important for an optimal yielding capacity. It is hard to bring structure into figures, the same like color and smell.
There are some quality monitoring procedures in use to give a figure at certain stages of the preparation of compost for color, structure and smell.
Compost can be too homogeneous. In the past in the traditional rick system, mixing and more mixing was necessary to create more homogeneity and many times the result was poor. Nowadays with the modern mixing stations in combination aerated composting in bunkers too much homogeneity can be created and a soft, sticky compost can be the result. We feel that some parts of less degraded straw can create a framework and give more volume of the Phase III compost.
This results in more activity and temp. difference at growing and a better temp. increase at the end of the first flush. Even if these less fermented straw-parts do not contribute to the direct nutrition of the mushroom. A strong general opinion is also that over fermentation is much more negative than under composting.

3. Practical

Temperature increase seems by far the most important item in aerated composting. After filling the bunker, at day 0, the temp. should reach 80 ^{0} C. in about 12 hrs. Therefore also at pre wetting, outside or in a bunker, the last 3 days before filling the bunker, the temp. should be at least 75^0 C. to give a good start of Phase I.
The process of fermentation is basically a **balance** between absorbing water and increasing temperatures. The first watering of the straw should not create anaerobic conditions but an temp. increase. This temp. increase, at ammoniatical conditions, creates softening of the straw. This dewaxing creates more absorption and makes other carbohydrates free for heating

activity. This creates brown substances by so called Maillard-reactions and more softening and burning carbohydrates for activity. By more watering at high temp. the chemical reactions at 80^0 C. take place to create a chemical burning effect without losing structure in order to make a not greasy dark compost every week. In this respect composting means burning carbohydrates at aerobic conditions and for this in every step not too much water should be added. Because it is difficult to give water for more than 2 days in advance and if the straw is opened after high temp. than in a later stage more water can be absorbed.
Measuring the oxygen content in the compost fails in practice regularly and it can be used to find the optimal timer settings in order to get an optimal temp. increase towards 80^0 C.
This because an optimal temperature increase is always a sign for optimal aeration.

Here follows a general insight of different optimal oxygen levels. Info Thed Vestjes.

	Part one	Part two	Part three
	Start till 65^0 C.	65^0 C. till 80^0	80^0 C. till evt. higher.
oxygen in compost	10 %	Min. oxygen 9 %	Min. oxygen 8 %
Need for fresh-air,	10 m^3 /ton/hr.	7-8 m^3 /ton/hr.	3-4 m^3 /ton/hr.
Eventual setting:	25 min. on	15 min on	5 min. on
	5 min. off	15 min. off.	15 min. off

In closed bunkers it is important to ventilate above the compost in order to reduce the temperature lower than 50^0 C. and create the survival of *Scytalidium th.* If the fan capacity at a certain pressure is known, than the fresh air capacity per minute can be calculated.

Goal is to produce week by week a black and not greasy type of compost, which can be finished well at peak heating. The ammonia, NH_3-reading at the end of pasteurization, plays a more important role in this than the nitrogen content. By giving more or less ammonia in the form about 20 kg. of poultry manure, or about 2 kg. of sulfate of ammonia or about 50 ltr. of goody water per ton of compost at the end of fermentation or filling the tunnel, the level can be changed. This in order to calibrate the ammonia-level at the end of pasteurizing at an optimal figure of 400 till 500 ppm for creating more selectivity against weed molds.

CONCLUSION

Though conflicting discussions between rival camps of scientists in the eighties till early 1992 regarding low temp. fermentation against high temp. aerobic fermentation were carried out. Different practical composters proved conclusively the supremacy of high temp. aerobic fermentation by the use of bunkers with spigot floors. Since Mr. Jean Laborde did set the puzzle together is composting less scientific. With a good pre wetting, by intensive mixing of the ingredients and a mechanical shredding of the straw, this system can successfully be used in big and small operations all over the world.

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A technology for early fruiting in *Lentinula edodes* cultivation (Shiitake mushroom)

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ABSTRACT

Traditionally shiitake mushroom has been grown on natural logs of various trees species. Generally, logs of 7-15 cm diameter., cut into 1m lengths, with holes drilled 2.5 cm of log diameter and are evenly spaced length-wise every 15 cm along the row. Holes are plugged with wood piece spawn or sawdust spawns, and then finally sealed with hot wax. Yields obtained from log system may be as high as 33%. This traditional method takes 8-18 months. Another environment-friendly shiitake cultivation technology is the synthetic log production system in which sawdust is the main ingredient; although, straw and corncobs are also used. Moisture level is maintained to a level of 60 per cent. The bags are sterilized for 2 h at 121°C in large autoclaves and after cooling, seeded with spawn. This method takes around 80-90 days time using different strains and is the most recent method for shiitake cultivation all through the world. The biological efficiency achieved in the method is 75 to 100%. In India, the production of this mushroom did not pick up due to its long cropping cycle. During the present study, a new strain DMRO-Shiitake-338S has been developed. Using this strain of *Lentinula edodes,* with some changes in substrate combinations, supplementations and agronomic practices, the fruiting period of the mushroom has been reduced to 42-45 days with an average biological efficiency of 125-130 per cent in a total cropping period of four months.
Keywords: *Lentinula edodes*, shiitake, cultivation technology, early fruiting

INTRODUCTION

Shiitake mushrooms are a treasured and traditional delicacy at dinner tables of China, Japan and Korea. These favorite mushrooms from the Orient are not only delicious and nutritious food with great flavor and an pleasant aroma, but they also contain a material well known for its medicinal benefits. Lentinan (β-1, 3 glucan with β-1, 6 and β-1, 3 glucopyranoside branching), a water soluble polysaccharide produced and extracted from shiitake, is an approved anti-cancer drug in Japan.

Shiitake is one of the most popular mushrooms both as food and medicine. At global level it has third position and contributes 24% to total mushroom production. Traditionally shiitake has been

grown on natural logs of various species of trees but currently oak (*Quercus*) logs are most popular. Generally, logs of 7-15 cm dia are cut into 1m lengths; holes are drilled; plugged with wood piece spawn and finally sealed with hot wax. Spawn run may take 6-18 months which depends upon many factors. To induce fruiting, the logs are shifted to cooler and humid area and shock treatment is given (Chang and Miles 1989). Yields obtained from log system may be as high as 33%; best production occurs in second and third years.

Later, to make the shiitake cultivation more environment-friendly, synthetic log production system was developed where sawdust is the main ingredient supplemented with some starchy substance like cereal bran, maize and some chemicals like calcium carbonate, calcium sulfate (gypsum) and sugar keeping the moisture content to a level of 60 per cent. Substrate filled polypropylene bags (2.5 Kg) are sterilized and seeded with spawn. The total production cycle on synthetic logs is 3-4 months and biological efficiency achieved is 75 to 125 per cent (Royse 2001).

In India, the production of this mushroom did not pick up due to its long cropping cycle. During the present study, we have tried to reduce the cultivation period with high biological efficiency so that the cultivation this mushroom should be taken up a faster pace.

1. Results & Discussion

1.1 Strain used

A new strain (named as DMRO-Shiitake-338S) was developed and used in the present study. This strain gave the fruiting in 42-45 days with an average biological efficiency of 125-130 per cent in a total cropping period of four months.

1.2. Substrate preparation:

Sawdust of selected trees was used as substrate and 100kg of saw dust was added with 20 kg wheat bran, 1 kg of calcium carbonate ($CaCO_3$) and 0.5 kg calcium sulphate ($CaSO_4$). The mixed substrate was wetted up to 60% moisture and filled in Poly Propylene bags (1.2- 1.5kg wet/ bag). Bags were sterilized in an autoclave at 22 psi for 90 min.

1.3. Spawning, Spawn running, mycelial coat and bump formation:

Grain spawn was inoculated @3% (wet wt basis, top spawning) and bags incubated at 4 hr/20 hr light / dark cycles at 23-25°C. Spawn run took 36-40 days including bump formation and browning.

1.4. Fruiting:

The spawn run bags were given cold water shock treatment by immersing in cold water (6-8°C) for 10-15 minutes after removing polypropylene bags. The room temperature was brought down to 20-22°C while RH (85-90%) and light for 10-12 hours/ day were maintained. After 2-3 days

of the cold water treatment small primordia developed, which mature into full grown fruit bodies in next 3-4 days. The complete process is depicted in Fig 1.

There are several reports of cultivation of shiitake mushroom on different substrate and different strains with varying biological efficiency. In Mexico and other Latin American countries, experimental cultivation of shiitake has been carried out using wood shavings, e.g. *Alnus, Bursera, Carpinus, Eucalyptus, Heliocarpus* and *Nothofagus* (Mata et al., 1990; Morales and Martínez-Carrera, 1991; Morales et al., 1991; Pire et al., 2001). Coffee pulp, sugar cane bagasse, straw from sev eral cereals and vineyard pruning has also been employed (Mata and Gaitán-Hernández, 1992, 1994; Salmones et al., 1999; Gaitán-Hernández and Mata, 2004; Gaitán-Hernán dez et al., 2006; Gaitán-Hernández et al., 2011).

Mata et al. (1998) used wheat straw pasteurized at 65°C and supplemented with BE up to 38%-44%. However, Savoie et al. (2000) obtained a BE of approximately 60% using pasteurized wheat straw and Gaitán-Hernández and Mata (2004) reported a BE of 46.9% and 50% when they used two strains of shiitake on wheat straw, in this case pasteurizing through hot water immersion. Gaitán-Hernández et al. (2006) used sterilized wheat and barley straw in the cultivation of four shiitake strains. In that study, BE of 49% in wheat straw and 77% in barley straw were obtained. The BE values reported in previous studies vary according to the strain, substrate, supplements and treatments used (Mata and Gaitán-Hernández, 1994; Salmones et al., 1999; Leifa et al., 2000; Morais et al., 2000; Pire et al., 2001; Philippoussis et al., 2003, 2007; Rossi et al., 2003; Fung et al., 2005).



Spawn Run	Bag Opening stage	40rd day (Pinning stage)
43th day (Maturation stage)	45th Day (Mature crop)	45th day (Close view of mature crop)

Fig 1. Stages in short duration cultivation technology of shiitake mushroom (*Lentinula edodes*)

In the present study also, we have cultivated a newly developed strain on a particular saw dust, which gave early fruiting and high biological efficiency. The results are in consensus with the earlier studies, where different substrate and strain gave different BE of this mushroom. During the study a higher yield in lesser time can be achieved while in traditionally natural log cultivation method of shiitake, the complete cycle of cropping takes 5-6 years with an average yield of 33% and in synthetic log cultivation system also takes 3-4 months in initiation of fruiting body 75 to 125 per cent (Royse 2001).

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Utilization of locally available biomass for cultivation of *Calocybe indica* mushroom.

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ABSTRACT

Calocybe indica is an indigenous mushroom commonly known as milky mushroom that can be cultivated throughout the year, except December and January months in the entire plains of India. Cultivation of this variety of mushroom has been studied on various substrates like paddy straw, sugarcane trash, cotton waste and coconut coir pith. But utilization of wastes such as shredded leaves for the cultivation of this mushroom has not been done so far. The objective of the present study was to investigate the potential and utilization of the locally available forage wastes i.e *Syzygium cumini* (Jamun), *Cassia fistula* (Amaltas), *Bauhinia variegata* (Kachnar) and *Azadirachta indica* (Neem). These were taken in five different ratios i.e., 0:100, 75:25, 50:50, 25:75 and 100:0. Results indicated that all the leaves used in the study supported the growth of *Calocybe indica*. The highest biological efficiency was obtained from the mushroom cultivated on 100% leaves of *Bauhinia variegata* i.e. Biological efficiency up to 120% followed by the combination of the above leaves with 50% of Triticum aestivum straw (B.E 102%). Similar results were obtained at 50:50 ratios in case of *Triticum aestivum* (Wheat) straw mixed with Azadirachta indica leaves. Lowest yield (8%) was recorded from the 100% leaves of *Cassia fistula*. The results revealed the potential usage of leaves as one of the substrate for the cultivation of edible and medicinal mushrooms.
Key words: *Calocybe indica*, Milky mushroom, leaves of medicinal plants, Neem.

INTRODUCTION

Mushroom are being used from ancient times as food but now consumption has increased due to changing perception and health consciousness. It is a cheapest protein source and contains abundant nutritional and medicinal constituents. *Calocybe indica* commonly known as 'Milky mushroom' or 'Dhudhiya Khumb' or 'Dhudh Chatta' in India. It is an edible fruiting body of fungi and being cultivated commercially in India. This mushroom is native to India and was first introduced by R.P. Purkayastha and A. Chandra in India during 1974. A substrate is an important substance for cultivation of mushroom. Wide range of substrate are used for its cultivation like rice straw (*Oryza sativa*), wheat straw (*Triticum aestivum*), soya bean straw (*Glycine max*), sugarcane baggase (*Saccharum officinarum*), coconut coir pith (*Cocos nucifera*) as well as cotton waste (*Gossypium species*) [1,2]. Among them rice straw (*Oryza sativa*) is the best suitable lingo-cellulosic substrate for growing *C.indica* mushroom [3]. Other substrates like stalks of maize (*Zea mays*), sorghum stalks (*Sorghum bicolor*) and

straw of vetiver grass (*Chrysopogon zizanioides*) were also found to be suitable for growing *Calocybe indica.* Forage waste is easily available in India like shredded leaves of medicinally important plant. Medicinally important leaves include *Syzygium cumini*, *Cassia fistula*, *Bauhinia variegata* and *Azadirachta indica. Syzygium cumini* leaves are loaded with antioxidants and flavonoids essential for a healthy body and mind. Leaves of *Cassia fistula* contain organics like rhein, rheinglucoside, sennoside and glycosides which are useful for wide range of skin infection. Leaves of *Bauhinia variegata* rich in phenol content and provide a source of dietary antioxidants and have a wide medicinal potential especially in constipation and cardiovascular disease [4, 5]. Active constituents of *Azadirachta indica* leaf extract include isomeldenin, nimbin, nimbinene, 6-desacetyllnimbinene, nimbandiol, immobile, nimocinol, quercetin, and beta-sitosterol, which is used for treatment of leprosy, eye disorders, bloody nose, intestinal worms, stomach upset etc.[6, 7]. Utilization of these shredded leaves for the cultivation of this mushroom has not been done so far. The objective of the present study was to investigate the potential and utilization of the locally available leaves of medicinal plants. As these leaves are blend with medicinally important constituents, so it's use in combination with mushroom substrate might helpful in translocation of important compounds to the mushroom grown on it.

1. Results and Discussion

The mycelium of *C.indica* has been found compatible on various types of substrates. It was observed that rate of colonization by *C.indica* mycelium was faster on leaves of *Bauhinia variegata* and *Triticum aestivum* straw as compared to *Azadirachta indica*, *Cassia fistula* and *Syzygium cumini*. The mycelium took 30 days to complete colonization of *Bauhinia variegata* leaves and *Triticum aestivum* straw whereas it took 41 to 48 days in colonization of other leaves. The shortest time for primordial initiation was on 100% leaves of *Bauhinia variegata* substrate (30 days) followed by 50% leaves of *Bauhinia variegata* (36 days) and *Azadirachta indica* (37 days) with *Triticum aestivum* straw (Table 1). The longest time for primordial initiation was on 100% leaves of *Cassia fistula* (49 days). Mycelium took 48 days on 100% *Syzygium cumini* leaves followed by 48.5 days on 75% leaves of *Cassia fistula* + 25% *Triticum aestivum* to form primordia. Among leaves maximum numbers of fruiting bodies (19) were formed on 25% *Bauhinia variegata* leaves in combination with 75% *Triticum aestivum*. Minimum numbers of fruiting bodies (3) were obtained on 100% leaves of *Cassia fistula* whereas on *Triticum aestivum* straw it was 24. When we compared average weight of fruiting bodies we found that average weight of fruiting bodies was highest on only leaves of *Bauhinia variegata* (900gm) followed by substrate comprising of 50% *Bauhinia variegata* leaves + 50% *Triticum aestivum* straw (765gm). *C.indica* shown its compatibility on all types of leaves studied, but productivity was highest on *Triticum aestivum* straw. Among leaves, highest biological efficiency was observed on 100% leaves of *Bauhinia variegata* i.e 120 % followed by the combination of the above leaves with 50% of *Triticum aestivum* straw (B.E 102%). Similar results were obtained at 50:50 ratios in case of *Triticum aestivum* straw mixed with *Azadirachta indica* leaves. Biological yield was found highest on 100% leaves of *Bauhinia variegata* i.e 60% followed by leaves of *Bauhinia variegata* (50%) and *Azadirachta indica* used in combination with *Triticum aestivum* straw (50%) whereas lowest B.Y obtained from 100% leaves of *Cassia fistula* (8%). Among leaves used highest

number of fruiting bodies as well as maximum biological efficiency of *Calocybe indica* mushroom obtained on 100% use of *Bauhinia variegata* leaves in comparison to all other treatment level of different leaves used.

In the present study, among leaves 100 % *Cassia fistula* leaves proved as best substrate for cultivation of *C.indica* mushroom as there was fastest colonization of mycelium on it and gave highest biological efficiency along with robust yield-contributing characteristics of *Calocybe indica* mushroom. This study has been well supported from the work of John et al. 2014, who investigated different substrates for the cultivation of *C.indica* mushroom. Lee et al. 2010 reported that different substrates affect the growth and yield of *Calocybe indica*. They recommend rice straw is the best suitable lingo-cellulosic substrate for growing *C.indica* white mushroom Moreover, it was found from above study that use of leaves are highly beneficial because of their medicinally important constituent present in them and there is every possibility of the translocation of bio-molecules.

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Table 1: Effect of substrates on growth and yield of *Calocybe indica.*

No. of Sets	*Triticum aestivum* straw supplemented with different amount of distinct medicinally important leaves Treatment		Time required from inoculation to primordial initiation (days)	Avg. No. of fruiting bodies	Total Yield (gm)	Biological Yield (%)	Biological Efficiency (%)
	Leaves	Level					
1.	*Azadirachta indica* (Neem)	250gm leaves +750gm WS	46	9	300	20	40
		500gm leaves +500gm WS	37	18	763.5	50.9	101.8
		750gm leaves +250gm WS	41	15	600	40	80
		1000gm leaves	47	10	390	26	52
2.	*Cassia fistula* (Amaltas)	250gm leaves +750gm WS	42	16	600	40	80
		500gm leaves +500gm WS	46	11	300	20	40
		750gm leaves +250gm WS	48.5	6	165	11	22
		1000gm leaves	49	3	120	8	16
3.	*Syzgium cumini* (Jamun)	250gm leaves +750gm WS	40.5	17	615	41	82
		500gm leaves +500gm WS	41	6	225	15	30
		750gm leaves +250gm WS	44	9	315	21	42
		1000gm leaves	48	6	210	14	28
4.	*Bauhinia variegata* (Kachnar)	250gm leaves +750gm WS	40	19	600	40	80
		500gm leaves +500gm WS	36	17	765	51	102
		750gm leaves +250gm WS	38	15	750	50	100
		1000gm leaves	30	18	900	60	120
5.	CONTROL - 1000gm *Triticum aestivum* Straw		30	24	930	62	124

***WS= *Triticum aestivum* straw**

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Impact of compost supplemented with waste paper and anaerobically digested food waste on cultivation of edible mushroom *Coprinus comatus* (O.F. Müll.) Pers.

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ABSTRACT

Commercial cultivation of edible mushroom *Coprinus comatus* is usually performed on bed cultures or in bags on the composted substrate from agricultural waste as wheat straw, cotton waste or rice straw with addition of chicken manure as a main source of nitrogen. The latest improvement of cereal production leads to reduction of the straw length in order to lower waste production therefore there is of lack of this kind of waste as a main compound of the mushroom cultivation substrate. However, recent attention on improving utilisation of such a waste as pulp and office paper brings it as interesting alternative for mushroom compost compounds. As renewable energy production become more popular, anaerobically digested (AD) organic waste becomes easily accessible agricultural waste material. AD digestate contain resistant fibrous waste fractions, anaerobic bacteria, is rich source of nitrogen, phosphorus and sulfur, with favorable for mushroom substrate preparation pH of 8.0-8.5.

The objective of this study was to investigate impact of composted Straw-Paper-Digestate substrate on yield, dry matter content and morphological features of *Coprinus comatus.*

Mushrooms were cultivated on four composts with different amount of digestate, straw and paper. The control was conventional straw-chicken manure compost. The substrate composition was based on dry matter of substrate before processing, evened to obtain 40% of compost DM. Dewatered digestate was added at the start of *phase I* composting. Cultivation was performed according to Stoknes *et al.* (2013).

The addition of anaerobically digested organic food waste and composted office paper waste positively affected yield and dry matter of investigated species in comparison to control compost. The best yield was obtained on the substrate with the highest amount of digestate and lowest paper content. In addition, dry matter content of mushrooms harvested from Straw-Paper-Digestate substrates ranged from 8 to 12%. Outcome of presented study implies the feasibility of composted Straw-Paper-Digestate as a substrate in *Coprinus comatus* cultivation.

Keywords: substrate, yield, dry matter, shaggy mane, mushrooms

INTRODUCTION

Commercial compost preparation is conducted in three phases: phase I, mixing and thermophilic composting (temp. up to 80ºC); phase II (pasteurisation - 60ºC) and conditioning (45-50ºC); phase

III – spawning. This is long, water demanding and high CO_2 footprint leaving process which demands improvement. Anaerobic digestion (AD) is a cost effective method for treating bio wastes which produce biogas and the remaining effluent can be reused as a fertilizer (Haraldsen et al. 2010). Study performed by Stoknes et al. (2013) shows that digestate from AD can be used as addition to substrate in cultivation of *Agaricus* mushroom. In Poland there are over 90 operating sewage treatment plants and ca. 60 working agricultural biogas plants which can provide the appropriate amount of digestate for potential commercial cultivation of *Coprinus comatus*. The species in nature is common in Europe and North America (Park and Lee 2005), however mostly cultivated in China, Taiwan, Korea (Ho and Peng 2006, Cui et al. 2008).

In following experiment five cultivation composts were used. Conventional compost (purchased from the commercial compost producer HAJDUK, Poland), was used as a control along with four composts with different levels of digestate, straw and paper. The composition was based on dry matter of substrate before processing, and was evened to obtain 40% of DM of substrate (Table 1). The digestate from AD of source separated household food waste was separated using a decanter centrifuge. The resulting digestate solid was added to *phase I* substrate, and further prepared according to Stoknes *et al.* (2013). Cultivation was performed with use of polypropylene 50 micron, autoclavable plastic bags (capacity of 7 liters, flat size 38 cm wide x 57 cm high), with four linear ventilation filters were applied. Bags were filled with approximately 3 kg of composted and pasteurized compost and left for cooling to approx. 22ºC. After cooling down compost was inoculated with granular spawn on wheat grain, obtained from the mushroom spawn laboratory MYCELIA bvba, Belgium in amount of 3% of fresh substrate weight. The bags were sealed with adhesive tape and shaken by hand until the spawn appeared evenly distributed. The spawning was performed in stable controlled conditions, at 25ºC. After 14 days, when the spawn had overgrown the substrate, bags were opened and a 5 cm layer of casing was applied and moved into the growing chamber for additional 10 days. The temperature inside the cultivation chamber was lowered and held at approx. 18-20ºC. The air humidity for fruit body development was kept at 85-95%. The cultivation chambers received indirect natural light from the sides. The cultivation room was aired not to allow CO_2 concentration to exceed 1000 ppm. The first flush was harvested 2 weeks after casing was applied. Three flushes were harvested and yields were determined as weight of harvested fresh mushrooms from the whole cropping period per fresh weight of substrate at inoculation. Experiments were established in fully randomized design, in 3 replications and 2 cultivation cycles. When comparing the experimental results, the analysis of variance for randomized block with 5 composts treatments was applied (level of significance $\alpha = 0.05$). The results of cultivation experiments were discussed based on mean values from cultivation cycles.

Table 1 Composted substrate composition [% of dry matter]

Substrate	Straw	Paper	Chicken manure	Digestate	Gypsum	C:N ratio
COM0	82	0	15	0	3	20:1
SPDG10	28	56	3	10	3	60:1
SPDG20	25	50	3	20	3	40:1
SPDG30	21	42	3	30	3	30:1
SPDG40	18	36	3	40	3	20:1

The aim of the presented study was to assess the possibility to use waste from anaerobic biogas production mixed with straw and recycled office paper as a component in mushroom compost for cultivation of *Coprinus comatus*.

Results & Discussion

The response in the yield of *Coprinus comatus* differed depending on the amount of the digestate, paper and straw in the cultivation compost. Yield of fruiting bodies was influenced by the increasing amount of digestate and was significantly higher on substrate with the highest addition of digestate (40% of DW), when compared with control substrate and other examined substrates. Substrates with addition of 20 and 30% (SPDG20 and SPDG30 respectively) of digestate did not differ significantly between themselves and the yield on that substrate was lower than the one obtained on SPDG40. The lowest yield was obtained on the substrate SPDG10 – with only 10% of digestate addition and control substrate – COM0, above yields did not differ significantly (Fig. 1). The dry matter of fruiting bodies ranged from 8 to 12%, and was highest on substrates with 30 and 40% of digestate (11,77 and 12,13 respectively) and it is comparable with data from literature (Vaz et al. 2001).
All investigated composts supported growth of mycelium of *C. comatus* and produced mushrooms. However for the substrates with addition of digestate, yields of fruiting bodies depended highly on the substrate composition. Yields increased on the substrates with higher amount of digestate, but the amount of straw and paper in those substrates was decreasing. *Coprinus comatus* is reported to yield 102-381 g $kg^{-1}$of substrate (Chaiyama at al. 2007, Mshandete, and Cuff 2008, Mwita et al. 2011, Raymond et al. 2012).

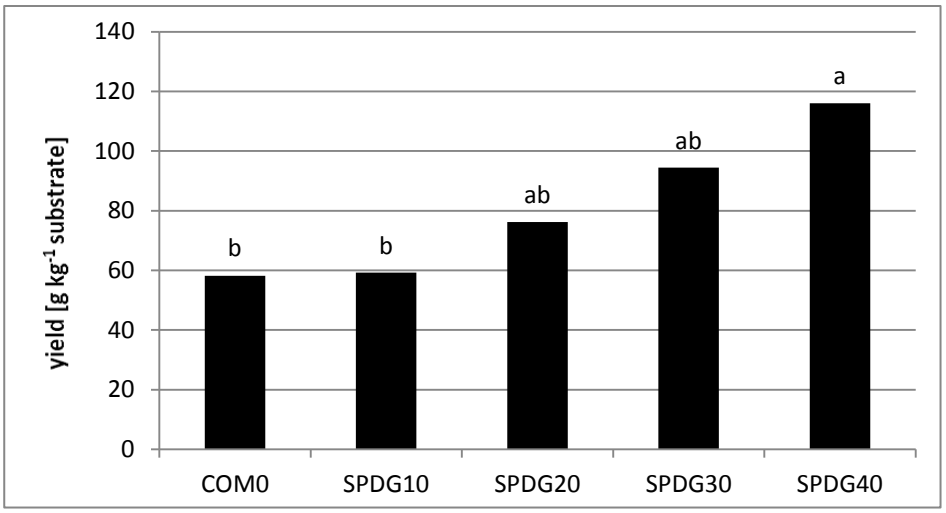


Fig 1. Yield *Coprinus comatus,* on different compost types [g/kg FM substrate]
Different letters indicate significant differences by Duncan's test (α=0.05)

In the study performed by Siqueira et al. (2011), sufficient addition of nitrogen to the carbon rich cultivation substrate highly improves growth of fruiting bodies. However, not only the amount but also the selection of nitrogen source is important, since *Basidiomycetes* mushroom, do not produce nitrate reducing enzymes (Gerrits 1998), the best for *C. comatus* is organic nitrogen (Jang et al. 2009). Optimal ratio of C:N in the substrate varies for different mushroom species, however the

20:1 is suitable for most mushroom to perform successful mycelial growth (Chang and Miles 1989). According to this ratio the commercial cultivation substrates are composed to perform the best growth of mycelium and the best yielding. This ratio was also applicable for commercial compost used in this study and for the SPDG40 substrate which gave the best yield (Fig.1). However there are very limited data on the cultivation of *C. comatus* on digestate/paper supplemented compost. The study of Stoknes et al. (2013) shows successful cultivation of *Agaricus subrufescens* and *A. bisporus* with the best yields for both species on high digestate supplemented compost (36-47% DW).

Therefore additional studies on this species and commercial use digestate and waste paper for the substrate composition should be performed. As high nitrogen demanding cultivation there is a potential of reusing the effluent from biogas stations, which number is increasing yearly.

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Yield and economic viability of two indigenous strains of *Lentinus squarrosulus* cultivated on composted sawdust of various tropical woods

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ABSTRACT

Lentinus squarrosulus is an indigenous edible mushroom with medicinal properties, which is presently not biotechnologically exploited for consumption and commercial purposes in Ghana. The cultivability of *L. squarrosulus* was assessed on composted sawdust of mixed tropical woods for its yield and economic viability of the harvested fruiting bodies. Wild fruiting bodies of the mushroom were collected from the Greater Accra and Volta Regions of Ghana. Prepared spawns of the two strains (Lsf and Sqw) were separately inoculated on composted sawdust supplemented with rice bran and quick lime. The weight and number of fruiting bodies harvested per flush, the flush number and the intervals between flushes were among the parameters recorded during the 52 days cropping period. The biological efficiency (BE) was also calculated. Mycelia and basidiocarp macromorphologies of the strains were also compared. Insignificant differences ($p>0.05$) were largely observed among the yield parameters for the two strains. Total yields and BEs recorded were 327g and 36.27 %, and 376 g and 41.78 % for strains Lsf and Sqw respectively. Commercial cultivation of the mushroom could possibly accrue reasonably high profit margins (12 – 24 %) and profit percentages (13 – 31 %) based on activity-based costing calculated using the mean yields per compost bag within the cropping period. This is the first report on cultivation of *L. squarrosulus* from Ghana and estimation of the possible profit margins of the mushroom's commercial cultivation.
Keywords: Biotechnology, Cultivability, Economic viability, *Lentinus squarrosulus*, Yield

INTRODUCTION

The mushroom industry in Ghana has seen some steady growth over the years with more mushroom consumers buying into the idea of consuming cultivated mushrooms, although it has been the trend in the recent past to pick mushrooms from the wild when in season either for domestic use or economic gains. The importance of variety in food cannot be overemphasized. As such, though cultivation techniques of *Pleurotus ostreatus* strain EM-1 has extensively been studied in the country leading to the mushroom being the most widely cultivated in Ghana (Obodai et al. 2011) and its immediate neighbouring countries, it is

prudent to explore possibilities for domesticating other indigenous wild mushrooms like *Lentinus squarrosulus* for consumption and biotechnological exploitation.

L. squarrosulus is an edible mushroom which has various organoleptic, nutritional and medicinal attributes (Ayodele et al. 2007; Mhd Omar et al. 2011). As part of studies on the mushrooms' domestication in Ghana, studies on the mycelial growth on solid media of the two indigenous strains of *L. squarrosulus* (Lsf and Sqw) collected from the Greater Accra and Volta Regions of Ghana respectively have been conducted (Narh Mensah and Obodai 2014).

The present study aimed at unravelling the growth and yield patterns, on composted sawdust, of these two indigenous strains of *L. squarrosulus* to assess the mushrooms' growth characteristics, yield patterns, and potential economic viability on composted sawdust of mixed tropical woods using facilities currently used for *Pleurotus ostreatus* production in Ghana. This is for the goal of onward technology transfer to mushroom farmers and other interested persons.

1. Results and Discussions

1.1. Mycelia growth in compost bags

Mycelia of both strains fully colonized the substrate (composted sawdust of *Triplochiton scleroxylon* supplemented with rice bran, 12 %, and quick lime, 0.5 %, *w/w* on dry weight basis) in the compost bags by the fourth week of incubation. There were observed variations in mycelia coloration in individual compost bags irrespective of the strain. This coloration ranged from dark brown/black at the top (solid green arrows; Figure 1a, b and c) of the bags to whitish closer to the bottom of the bags (red arrows; Figure 1b and c). Mycelia at the top of the bags (closer to the neck of the compost bag) were relatively older than the mycelia at the bottom of the bags as mycelia colonized the substrate from the top of the compost bags, where inoculation was done, and extended downwards. Undefined sectors of mycelia with different coloration were also observed on the compost bags during incubation (dashed green arrows; **Error! Reference source not found.**b and c). The sectors were persistent throughout the cropping period. These results corroborate the report that the mycelia of *L. squarrosulus* show different morphology (color and presence of sectors) with age on various growth media (Narh Mensah and Obodai 2014). The observed colors and sectors could be mistaken for contaminants which often occur in compost bags during incubation and cropping/fruiting in mushroom production.



Figure 1: Pictures showing the mycelia morphology on the compost bags (a) after incubation and (b and c) during cropping. (a) Shows the mycelia (green arrow) and pinheads (blue arrow) on the surface of the compost bags immediately after incubation. (b) and (c) show the

compost bags with the sectors of differently colored mycelia (red arrows) even with emergence of the first flush. Solid green and red arrows point to brown-black mycelia at the top of the compost bags and to whitish mycelia closer to the bottom of the compost bags respectively.
NB: Compost bags were placed vertically only for the purpose of taking the pictures.
There were challenges in visually estimating the mycelia density of both strains on the substrate in the compost bags due to the brown color of the sawdust and the change in mycelia coloration from whitish to brownish-black with time on the substrate (solid green arrows; Figure 1a, b and c).
Color differences and sectoring observed in "old" mycelia compared to "young" mycelia may be a result of senescence (Stamets and Chilton 1983), or products of enzymatic activity between enzymes released (Tripathi et al. 2012) and compounds (e.g. phenolic compounds) in the substrate and/or mycelia (Mhd Omar et al. 2011). Analysis of the chemical composition of fruiting bodies of these two *L. squarrosulus* strains cultivated as described herein has shown that, in addition to other differences in their chemical composition, strain Lsf has significantly lower phenolic acid content than strain Sqw (Obodai et al. 2014). Further investigations of these hypotheses may open channels for some biotechnological applications of *L. squarrosulus*.

1.2. Yield characteristics of strains on composted sawdust
There were wide variations in the yield recorded per strain per flush. Yields of both strains decreased steadily with each flush. Although strain Sqw recorded higher yields in the first (59.5±7.1 g) and second flushes (42.7±12.6 g) than Lsf (46.8±20.5 g and 33.2±15.7 g for the first and second flushes respectively), the recorded yields were not significantly different ($p>0.05$) between the strains and across the flushes. The trend of reduction in yield for subsequent flushes has been reported for various species/ strains of mushrooms including *P. ostreatus* and has been attributed to reduction in nutritional content and structure of the substrate with each harvest (Obodai et al. 2011).
Some variations in other yield characteristics of the two strains within the 52-day cropping period was also observed (Table 1). The day of bag opening to first flush for strain Sqw (13 days) was about twice that recorded for Lsf (6 days). Weight and number of Lsf fruiting bodies per bag were averagely 108 g and 43 whereas that for Sqw were averagely 125 g and 33 respectively.
The recorded flush number, yield and Biological efficiencies (BEs) obtained for the two strains in this study exceeds those in other literature. Ayodele et al. (2007) recorded flush numbers and yields of 1-2 flushes and 7-32 g of fresh mushroom, and BEs ranging from 0.87-4.27 %. Yields ranging from 20-40 g were reported by Oghenekaro et al. (2009). The disparity in the reported yields in these studies may be multifactorial. These include the differences in the substrate formulations used and/or differences in abiotic conditions such as temperature and humidity at the time of experimentation. Abiotic factors influence various growth parameters of mushrooms including their yield, nutritional composition and fruit body morphology (Jae-Soon 2004; Stamets 2000).

Table 1: Yield characteristics of *L. squarrosulus* strains on composted sawdust

Parameter	*L. squarrosulus* strain	
	Lsf	Sqw
Day of bag opening to 1st flush (days)	6±2.041[a]	13±1.871[b]
Flush number	3	4
Interval between flushes (days)	11±7.145[a]	11±5.428[a]
Weight of fruiting bodies per flush (g)	36.8±6.643[*b]	31.33±17.451[**a]
Weight of fruiting bodies per bag (g)	108±37.55[a]	125±38.38[a]
Number of fruiting bodies per flush	14±5.15[*b]	8±4.084[**a]
Number of fruiting bodies per bag	43±10.701[a]	33±9.192[a]
Total yield (g) [***]	327	376
Biological efficiency (%) [****]	36.27	41.78

Values recorded are mean ± standard error. n=3; Values in the same row followed by different letters are significantly different; [*]: Averaged from the three flushes obtained; [**]: Averaged from the four flushes obtained; [***]: Total weight of fruiting bodies obtained from the three replicates from each strain within the cropping period; [****]: (fresh weight of mushroom/dry weight of substrate at spawning) X 100.

1.3. Fruiting body macromorphology

Both the young and mature fruiting bodies of both of the cultivated strains were mostly funnel-shaped and whitish cream (Figure 2). Pictures of the fruiting bodies of the wild mushroom strains in their original habitats (growing on decaying wood) have been presented by Narh Mensah and Obodai (2014). Like the wild strains, the fruiting bodies mostly had leathery caps with smooth, slightly wavy margins and hairy surfaces. The caps of both strains were deeply cyathiform – a feature shared with fruiting bodies of *L. roseus* (Karunarathna et al. 2011) – and caps of both strains felt fleshy to the touch.

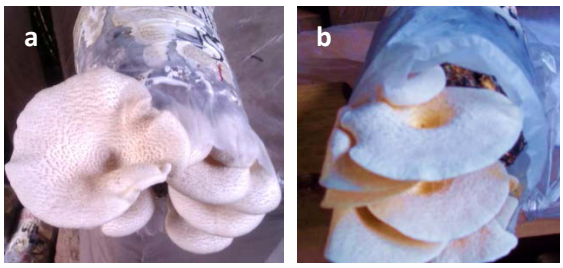


Figure 2: Picture showing L. squarrosulus strain (a) Lsf and (b) Sqw flushing on composted sawdust.

1.4. Estimated economic viability of commercial cultivation

The economic viability estimated according to activity based costing indicated that commercial cultivation of these two strains of *L. squarrosulus* could possibly accrue reasonably high profit margins and profit percentages. However, despite the largely insignificant differences in the yield characteristics of the two strains, the profit margins and

profit percentages of *L. squarrosulus* strain Lsf was considerably lower (12 and 13 % respectively) than that of *L. squarrosulus* strain Sqw (24 and 31 % respectively).

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Cultivation Potential of *Lentinus giganteus* (Urupaha): A Wild Edible Mushroom of Sri Lanka

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ABSTRACT

A study was undertaken with the objective of investigating artificial cultivation potential of naturally grown *Lentinus giganteus* (Urupaha): the biggest wild edible mushroom of Sri Lanka using the locally adapted technologies used for oyster muswhroom cultivation. The mushroom was obtained from its natural habitat and the tissue culture technique was employed to isolate the fungus on potato dextrose agar (PDA) medium. Finger millets, paddy grains and saw dust alone or mixed with each other at different combinations along with the other standard ingredients were used to find out the best substrates for spawn production. Changing compactness of the substrate by increasing weight of the compost filled per standard 13" size bag, changing bag sizes ranging from 8" to 13", making 1" diameter size holes of different numbers in bags after spawn run, and different sawdust media were tested in separate experiments to understand their effect on mushroom yields in 3 flushes. *L. giganteus* was able to successfully cultivate on sawdust based growth medium supplemented with other ingredients. Fast spawn production was achieved when paddy alone or combinations of, paddy and sawdust (1:1), paddy and finger millet (1:1) and saw dust and finger millet (1:1) were used as the spawn substrate. Compactness of the bags when prepared as 0.98-1.07 kg substrate per bag, and standard 13" size polypropelene bags recorded significantly high yield compared to other treatments. No significant difference was observed among treatments for the yield of mushroom on mango (*Mangifera indica*), rubber (*Hevea brasiliensis*), Alstoniya (*Alstoniya macrophylla*) and Albizzia (*Albizzia odorotissima*) used as the compost medium. Results revealed the potential of commercial cultivation of *L. giganteus* on saw dust based compost media under ambient tropical conditions. The findings will be the first report in investigating the commercial cultivation potential of *L. giganteus* using locally adapted technologies.

Keywords: Growth media*, Lentinus giganteus,* mushrooms, sawdust substrates.

INTRODUCTION

Although a large number of mushroom species showing varying degree of edibility, only a few number of species have been commercially cultivated around the world. There may be still a number of unexplored species of edible mushrooms grown naturaly and can be artificially cultivated. Sri Lanka's rich mushroom biodiversity and its tropical agro climatic conditions offer greater scope for cultivation of a variety of mushrooms at a comercial level.

Although there is a potential, limited or no attempts were made in Sri Lanka to exploite the potential of cultivating wild edible mushrooms under artificial conditions and to develop low cost technologies using locally available materials such as timber and agricultural wastes. *Lentinus giganteus* Berk. (locally called "Urupaha") (Pegler, 1986) is a wild edible mushroom which can be seen on the grounds of grass which buried wood of 20 cm or more below surface. An attempt has been made to cultivate this mushroom previously only at experimental levels (Udugama, 1995) however, no further reports are available on the commercial cultivation aspects of this mushroom in Sri Lanka. In line with the above, the current research was conducted to find out fast spawn production and commercial cultivation potential of *Lentinus giganteus* using locally adoptod technologies.

1. Results & Discussion

Spawn production of *L. Giganteus* was tested on different substrates with various combinations of saw dust, fingermillets and paddy as the principle spawn medium. Fast spawn production was achieved when paddy alone (substrate colonization of 93%), paddy and saw dust at 1:1 (90%), paddy and finger millet at1:1(85%) and saw dust and finger millet at 1:1 (87%) ratios were used as the spawn substrate and were significantly different (P>0.05) from spawn media prepared with finger millet or saw dust as the sole spawn substrate (data not shown). The light requirements for fast colonization of the fungus was also measured under 4 light conditions (Ambient light, 24 hours darkness, 12 hrs dark and 12 hrs light, and under complete dark). Results revealed that significant spawn colonization was achieved under complete darkness compared to other treatments (data not shown).

The impact of the lenght of the prolypreopelene bag, compactness of the bags after filling composts, and number of 1" diameter holes made in the bag, were also tested on saw dust based compost medium and results are presented in the table 1. Significantly high mushroom

Table 1. Total yield (g) of *Lentinus giganteus* when grown on different length size bags, compactness ranges, and bags prepared with varying number of 1" diameter size holes.

	Length (inches) of the 7" wide polypropylene bag					
	8"	9"	10"	11"	12"	13"*
+Total yield (g)	-	-	5.96[b]	12.19[b]	0.37[b]	59.77[a]
	Compactness range (compost kg/bag)					
	0.98-1.07	1.08-1.10	1.11-1.13	1.14-1.16	1.17-1.19	
Total yield (g)	125.11[a]	51.99[b]	52.23[b]	59.40[b]	66.86[b]	
	Number of 1" diameter size holes per bag					
	6	10	14	20	Control**	
Total yield (g)	20.80	13.75	11.75	17.38	13.84	ns***

* Standard size bags used by farmers ** Control = Bags with no additional holes

*** ns = not significant

+ Means with same letters in a row do not differ significantly with DMRT test at p>0.05

yield was recorded on standard 13" long polypropelene bags (mean = 59.8 g) compared to the other bag lengths. This indicates that the currently used 13" size polypropelene bags for cultivating other oyster mushrooms in Sri Lanka can still be used as the standard bag size for *L. giganteus* cultivation. None of the bags of both 8" and 9" length size produced fruitings during the observation period either because all those were destroyed or contaminated. Total mushroom yield when bags prepared with different compactness, was significant (P>0.05) among treatments (table 1) and the bags with weight range of 0.98-1.07 kg recorded significantly high mushroom yield (mean = 125.1 g). The filled bags need to be properly compressed for fast mycelial colanization and that would affect the mushroom yield, thereafter (Kwon and Kim, 2004), and loosely or very tightly compressed bags may not provide optimum condition for the growth of the fungus.

The number of 1" dimeter holes made on the compost bag surface allowing mushroom fruiting and it's effect on mushroom yield was found not significant (table 1). However, better mushroom yield could achived when 6 holes were made on different places of the compost bags (mean = 20.8 g) compared to 10 (mean = 13.75g) , 14 (mean = 11.75g) , 20 (mean = 17.38g) and no holes (mean = 13.84g) per bag in additoin to the opening of the mouth of the bag after the incubation period.

Different saw dust types, different combinations of saw dust types, and different combinations of saw dusts (Alstonia) and rice straw were used as the basic medium along with other ingredients in 3 experiments to test their effect on mushroom yield and results are presented in the figure 1.

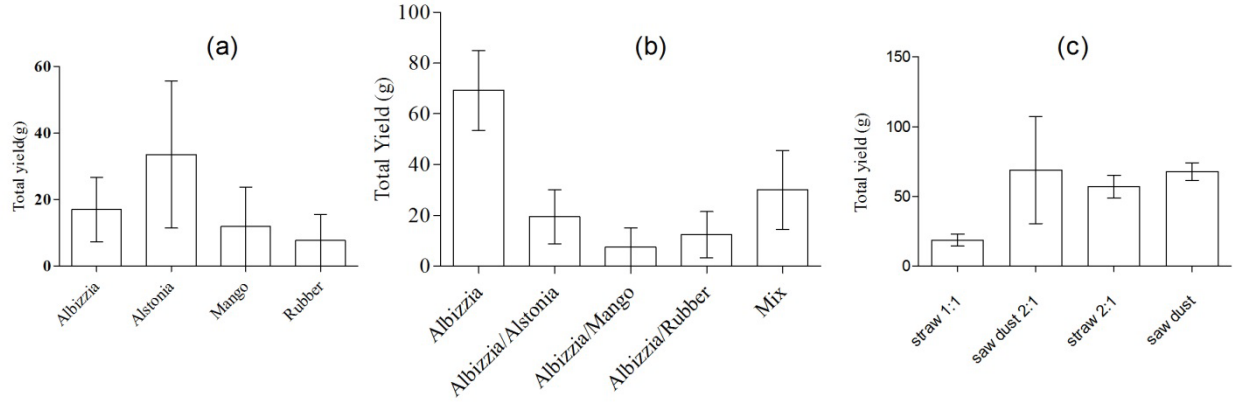


Figure 1. Yield (g) of *Lentinus giganteus* on (a) different sawdust based substrates, (b) mixtures of sawdust based substrates and (c) Saw dusts : Paddy straw at different v/v combinations.

No significant differences could observed on mushroom yields when different saw dust types such as Albezzia (*Albizia odorotissima)*, Alstonia (*Alstonia macrophylla*), Mango (*Mangifera indica*) and Rubber (*Hevea brasiliensis*) were used. However, Alstoniya provided comparatively higher mushroom yield (average= 33.6 g) in 3 flushes compared to rest of the treatments (fig. 1a). In the second experiment, treatment with Albizia alone produced significantly higher yield compared with the other treatments of saw dust mixtures at different combinations (fig. 1b). The combinations of straw and saw dust as the basic compost medium did not affect the yield of *L. giganteus* significantly (figure 1c).

The study has national significance as this wild edible mushroom is shown to be cultivated under farmers conditions with similar cultivation techniques practiced for oyster mushroom cultivation, at present. Further, the consumers get benefitted from the outcome that different varieties of mushrooms are avilable in the market yearround. However, different compost media which can provide optimum growth of *L. giganteus* enabling increased yields, need to be investigated in detail in future experiments in order to commercialize this mushroom at farmers levels.

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Technology Transfer of Yanagi matsutake *(Agrocybe Cylindracea)* Production to Thai Crofters

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ABSTRACT

Since Yanagi matsutake mushroom (*Agrocybe cylindracea*) contains high-value nutrients and tastes very good, it is popular amongst consumers in Thailand. However, at present the supply of mushroom in Thailand does not meet the demand of consumers as the country is located in the tropical area where regular temperature is higher than the optimal temperature for growing Yanagi. To address the problem, this research was divided into three parts. The first part, the optimal temperature and moisture for growing Yanagi matsutake in Thailand were studied. Based on the studied optimal conditions, a low-cost Yanagi farm was then designed for crofters. Finally, knowledge regarding how to design a Yanagi farm forshowed that optimal temperature and moisture for growing Yanagi in Thailand were about 25°C - 28°C and higher than 80%, respectively. The design of a Yanagi farm was completed and the implementation of knowledge transfer has been studying. It is hoped that the transferred knowledge can help crofters improve the productivity of Yanagi matsutake and subsequently, their standards of living will be improved.
Keywords: Yanagi matsutake, optimal conditions, knowledge transfer

INTRODUCTION

The objective of this work is to design an effective farming system for Yanagi matsutake production to help improve the standard of living of crofters in Thailand. Yanagi matsutake (*Agrocybe cylindracea*) mushroom is popular amongst consumers as it contains high-value nutrients and tastes very good. However, Thai crofters who grow Yanagi matsutake are facing two major problems relating to optimal cultivating temperature and optimal farming system.

A literature review indicates that the suitable temperature for growing Yanagi matsutake is about 25°C-28°C while the average temperature in Thailand is around 32°C-36°C. As a result, the inconsistency of Yanagi productivity has been found. Farming system is another problem. Most Thai crofters use the open farming system to grow Yanagi due to low investment cost. However, the open farm allows the mushroom to be exposed and easily contaminated by pollutants in the air.

To address the problems mentioned earlier, King Mongkut's University of Technology Thonburi (KMUTT) has built up a team to work with communities of crofters growing Yanangi. This work is divided into three parts. The first part, the study of optimal conditions for growing Yanagi matsutake in Thailand were conducted. From the studied optimal conditions, a low-cost Yanagi farm which produces high and consistent productivity was then designed. Finally, the KMUTT staff has transferred the information and knowledge derived from the first two parts to crofter communities.

1. Results & Discussion

1.1. Study on optimal condition for Yanagi matsutake production in Thailand

Experiments conducted in laboratory was divided into two parts. In the first part, the optimal temperature for mycelium growth was studied, and the second part, the optimal condition for mycelium growth was studied. In the first part, the testing temperature varied from 24°C - 30°C and it was found that the optimal temperature for mushroom fruiting mycelium growth was about 28°C - 29°C and the duration to develop full mycelium growth in each substrate bag is about 45 – 50 days.

In the second part, the testing temperature and the moisture in the air varied from 24°C - 30°C and 80% – 95%, respectively. Results show that the optimal temperature for fruiting stage of Yanagi in Thailand is about 25°C - 27°C and the optimal moisture in the air is about 85% - 95%.

1.2. Designing and constructing an open farm for Yanagi matsutake

According to the optimal conditions studied from the previous part, the specification of farm that has been designed by is followed;

- Dimension: 6m × 12m × 3m
- Capacity: 13,000 substrate bags
- Wall: 5-cm thick, made from polystyrene
- Evaporation system: 7.56-m^2 cooling pad, 124-cm diameter of fan, high pressure sprinkle, automatic temperature and moisture controller
- Testing conditions: 26°C ± 1°C and 90% of moisture in the air

Testing results are shown in Figure 1.

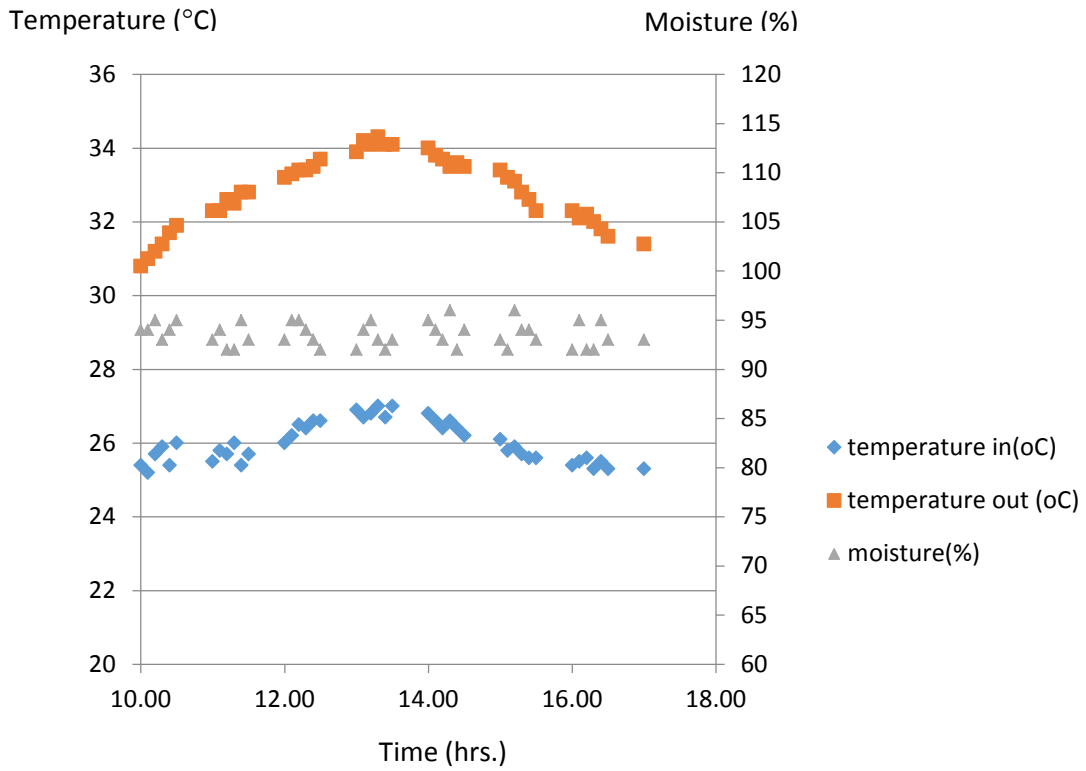


Figure 1. Testing results of temperature and moisture in the designed farm

Figure 1 shows that the designed farm can control temperature and moisture as expected. The capacity of designed farm is 13,000 substrate bags of mushroom. The temperature and moisture of operating condition are at 28°C- 29°C and 60%- 65% RH (relative humidity), respectively. The duration of filament mushroom growing fully in each mushroom bag is about 50 – 52 days.

With respect to the growth of mushroom, results show that there were 80% - 85% of chunks of mushroom that could produce mushroom at the setting temperature, 25°C- 27 °C, and moisture in the air, 85-95%. Based on the testing data and designed Yanagni farm, estimated investment cost and payback periods can be shown in Table 1.

Table 1. Investment cost and return of operating Yanagi farm (Based on 1 farm)

Detail	Baht
Cost of mushroom chunks (Per year)	364,000.00
Electricity	40,000.00
Labor cost (2 people x 300 baht x300 days)	180,000.00
Depreciation (5 years)	80,000.00
Production lost (2%)	20,592.00
Interest rate (9%)	36,000.00
Investment cost (Per year)	**720,592.00**
Income of the sale of mushroom	**1,029,600.00**
Profit of the sale of mushroom	**309,008**
Construction cost	**400,000**
Years of return	**1.3 (Years)**

Table 1 shows that investment cost for the designed farm is about 400,000 baht and the crofter can return their investment cost within 1.3 years.

1.3. Transferring knowledge to crofters

One of the university's missions is to deploy knowledge related to science and technology to help improve the standard of living of people in rural areas. With respect to this project, workshops on how to construct and operate the designed Yanangi farm have been organized to transfer knowledge to crofters. So far, three workshops have been organized for 185 crofters who are interested in growing this kind of mushroom.

V Marketing & consumers research

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A study of 10 years Brazilian Mushroom market development

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ABSTRACT

The mushroom production in Brazil has started in 1952. Brazil has a population of about 205 million people. Mushroom market has been developed slowly. Few people in Brazil consume mushrooms, so there is a huge market to be developed. The country has an interesting melting-pot culture made with the influence of immigrants from many different countries. This characteristic gives Brazilian people a mind always opened to new ideas, flexibility and creativity to adapt to different situations and to received people from all around the world. Also the different dishes with mushrooms and each particular way of grow can be well accepted.

Brazilian mushroom industry has changed during the last ten years, button mushroom was used be the first production. However, although consumption is increasing, the production of *A.bisporus* is decreasing in Brazil because of high cost of production and the country imports candy mushrooms from China. The most growers started to produce *Pleurotus ostreatus* instead of button with the same technology. Currently, *Pleurotus* species is the main mushroom being consumed in the country. Simple and modern technologies are used to grow, process and market mushrooms. It can be grown all over the year without cold rooms and low cost, what will determine a grower profit. The variation of mushroom prices was a major factor influencing consumption. Actually, prices are considered very or moderately expensive.

We carried out a study (2005-2015) to understand the patterns of mushroom production and consumption in Brazil. Data were analyzed variables studied. Basic data to carry out production cost and further marketing research and mushroom cultivation methods are discussed as well as an integral strategy to increase mushroom consumption and profit.

INTRODUCTION

The Southeast and South regions of Brazil, holds more than 66% of the market for total consumption goods, 70% of the gross national product (GNP), 20% of the territory surface, and 100% of the mushroom production in Brazil.

Due to the Taiwanese influence, 99% of the white mushroom in Brazil is canned. In 1995, the importation of canned mushrooms was opened and the price dropped from R$ 10.00 (USD $ 10.00) to R$ 3.20 (USD $ 3.50). In consequence, the mushroom market changed, demand increased, and mushroom consumption started reaching diverse social classes (Bueno, 2010).

From 1997 to 1998, 80% of the canned mushrooms imported to Brazil came from China and Vietnam. Mushroom growers organized The Mogi das Cruzes and Suzano Mushroom Growers Association to require an antidumping law to protect the canned mushroom

produced in Brazil. In 1998, a law passed by the Government law protected national canned mushroom against dumping imports from China (AFESP, 2003). After 1998, imports of canned mushroom from China were reduced by 86.8%. However, the participation of imported mushroom in the national consumption had increased from 51.9% to 59.7%, because other countries started exporting to Brazil (Bueno, 2010).

In 2002, when the Brazilian market was still opened to imports and China exported canned mushrooms to Brazil, the exchange rate was USD $ 1.00= R$ 4.00, the market became more stable, and mushroom growers had clear motivation to keep improving their business. In consequence, the mushroom production increased by 63% from 1997 to 2002 (AFESP, 2008).

Total mushroom sales in Brazil went up by 85.5% in one year (2002). Domestic sales increased 97.3% and exports were reduced by 95.5%. One of the reasons was that the mushroom demand was higher than the supply (Furlan, 2003). Another reason could have been that, after 2003, Chile became a strong competitor while the U.S. dollar-Brazilian real exchange rate dropped, so that the internal market became more interesting than the international market (AFESP, 2008).

During 2003 to 2008 a research was carried out with 123 growers around Mogi das Cruzes, of which 92% were *Agaricus bisporus* grower and 8% grow others mushroom. Production volumes were estimated to be 1.255.170kg/year of commercialized mushrooms in 2003, and 4.455.247 kg/year in 2008. It means an increase of 254% commercialized mushrooms in this period (SEBRAE, 2008).

However since 2012 the most part of *Agaricus bisporus* Brazilian growers became to grow Hiratake (*Pleurotus ostreatus*) instead of *A.bisporus.* The present research carried out study of production cost variables in 2005 and 2015 and proposes to analyze monthly data in prices traded over 10 years in order to find answers about the change in the mushroom industry.

1. Material and Methods

Mogi das Cruzes, the State of São Paulo, were selected for the study from 2005 to 2015 with 424.300 people (IBGE, 2016). It used to be the main mushroom production region in Brazil, with almost 200 mushroom growers (AFESP, 2002).

The first Exploratory research was done on the average *A.bisporus* prices paid for the grower over the period of 2005.

The second exploratory study was done with eight growers that answered questions about their fixed and variable costs, involving labor, materials and indirect manufacturing costs. The interview protocols were applied individually by formal interviews.

Descriptive analyses with the ton cost information of compost and sold mushroom was calculated. The composition of cost structure of mushroom compost was showed. The fixed costs were based on spending on investments, in infrastructure and equipment. The analyses procedure follows Brazilian law and considering these expenditures allocated for 10 years with regard to equipment and vehicles. Thus, it has calculated the rate of 10% per annum of the value of these investments to the composition of the production cost.

The data of 2005 related to amounts of raw materials, labor and costs reported by growers have been updated by the cost values of 2016. Therefore, it was possible to measure the impact of changes in costs today.
Another analysis performed was the gross profit margin that producers obtained in 2005 and what is the profit margin in 2016.

2. Results and Discussion

Brazilian mushroom growers were very heterogeneous in terms of technology used, however small growers studied in this experiment using the same traditional technology as the most part of Brazilian growers.

Table 1: *A. bisporus* production costs during 2005

	A	B	C	D	E	F	G	H	*Mediana*
Total investments in infrastructure and equipment	83.660	24.070	169.400	112.070	148.380	84.378	207.000	249.000	*130.225*
Variable costs (per batch)	4.964	1.843	3.935	3.480	4.308	6.264	3.697	4.875	*4.122*
Cost of labor (per batch)	3.217	514	2.405	1.645	3.904	6.299	3.445	4.020	*3.331*
Other costs (per batch)	1.240	405	101	480	2.275	650	1.600	444	*565*
Number of batches of compost per year	11	8	30	11	11	15	11	30	*11*
Quantity tons per batch compost	33	8	20	18	25	35	20	30	*23*
Number tons of compost per year	360	64	600	198	275	525	220	900	*317*
Quantity tons *A.bisporus* production (conversion=15%)	54	10	90	30	41	79	33	135	*48*
Cost of compost (ton)	544	646	1.169	934	1.013	619	1.472	1.141	*974*
A.bisporus ton (ton)	3.626	4.308	7.794	6.227	6.753	4.124	9.814	7.609	*6.490*
Cost of compost kg	0,54	0,65	1,17	0,93	1,01	0,62	1,47	1,14	*0,97*
A.bisporus kg	3,63	4,31	7,79	6,23	6,75	4,12	9,81	7,61	*6,49*

Data about these growers, which had been working with mushrooms for more than 10 years (SEBRAE, 2008), showed very low value of investment with a median of R$ 130.225 or US$55.67[1]US$ (Bacen, 2016). The median compost production in 2005 was 317 tons per year (Table 1) and the mushroom production was 48 tons per year per grower results, assuming an average rate of 15% conversion for each compound ton. The median cost of R $ 0.97 (US$0.41[1]) per kg of compost and the average price received by growers for mushrooms was R$ 6.49 per kg. (US$2.7747[1]) It is worth remembering that for the calculation of costs; it has considered investment in infrastructure, which already indicates that some growers were operating at a loss in 2005, with the average price of R$ 4.00 (US$1.7101[1]) mushroom sales for per kilo. Between eight sampled growers, only one operated with gross profit in 2005 and in 2015 only two growers were profitable. The gross profit, which has been mentioned; the

Table 2: *A. bisporus* production costs of 2005 with the costs values updated to 2016

	A	B	C	D	E	F	G	H	*Mediana*
Total investments in infrastructure and equipment	83.660	24.070	169.400	112.070	148.380	84.378	207.000	249.000	*130.225*
Variable costs (per batch)	11.050	5.777	7.274	7.596	10.059	11.212	6.182	8.829	*8.213*
Cost of labor (per batch)	7.630	1.444	5.175	4.950	8.050	5.568	6.155	9.388	*5.862*
Other costs (per batch)	1.827	655	174	455	3.923	1.096	1.433	623	*875*
Number of batches of compost per year	11	8	30	11	11	15	11	30	*11*
Quantity tons per batch compost	33	8	20	18	25	35	20	30	*23*
Number tons of compost per year	360	64	600	198	275	525	220	900	*317*
Quantity tons *A.bisporus* production (conversion=15%)	54	10	90	30	41	79	33	135	*48*
Cost of compost (ton)	883	1.285	1.478	1.345	1.475	752	1.724	1.458	*1.401*
A.bisporus ton (ton)	5.886	8.569	9.854	8.966	9.832	5.012	11.490	9.720	*9.343*
Cost of compost kg	0,88	1,29	1,48	1,34	1,47	0,75	1,72	1,46	*1,40*
A.bisporus ton kg	5,89	8,57	9,85	8,97	9,83	5,01	11,49	9,72	*9,34*

net income is considered (even deducting selling and administrative expenses) in 2015 any grower could maintain production at an average price of $ 6.50 [2]($1,6648). Although labor costs are not high (the average was R$5.862 or US$2.506)[1] and the variable cost R$ 8.213 or US$3.511[1].

[1] The values of Real were converted by the price of a dollar in 2005 at R$ 2,339 (Bacen, 2016

Table 3: Descriptive statistics of *Agaricus bisporus* production costs in 2015

	Average	Median	Standard Deviation	Coefficient of Variation
Total investments in infrastructure and equipment	132.137,56	112.070,00	68.975,46	0,52
Variable costs	8.733,02	8.829,00	2.106,61	0,24
Cost of labor	6.834,06	6.155,00	3.277,04	0,48
Other costs	1.366,03	1.095,75	1.155,84	0,85
Values (unit of measurement) per batch	16,89	11,00	8,88	0,53
	23,74	25,00	8,35	0,35
Quantity of compost tone per year	418,52	359,70	263,75	0,63
Quantity tons per batch compost	54,11	50,00	30,94	0,57
Cost of compost tone	784,86	778,90	182,96	0,23
Quantity tons *A.bisporus* production	6.191,36	6.429,68	2.172,97	0,35
Cost of compost kg	0,78	0,78	0,18	0,23
A.bisporus kg Cost	6,19	6,43	2,17	0,35

Table 4: Variation of the median costs for the years 2005 and 2015

	A	B	C	D	E	F	H	Mediana
Variable costs	122,59%	213,48%	84,85%	118,28%	133,49%	78,99%	81,11%	118,97%
Cost of labor	137,19%	180,69%	115,18%	200,91%	106,20%	-11,61%	133,53%	123,16%
Other costs	47,36%	61,74%	72,10%	-5,14%	72,44%	68,58%	40,35%	51,06%
Cost of compost ton	62,33%	98,92%	26,44%	43,99%	45,59%	21,54%	27,75%	46,65%

The value for kg compost produced has increased in the period 2005 – 2015 to R$ 1.40 [2](US$0,3585) (Table 1) and mushroom price/kg to R$ 9.34 [2](US$2,3923) Thus, there was an increase of 46.65% compost ton cost (table 3). This reflects a variation in variable costs 118.97%. The average cost of labor was 123.16% more expensive at 2015 and other costs composed primarily of fuel and electricity had growth of 51.06%.

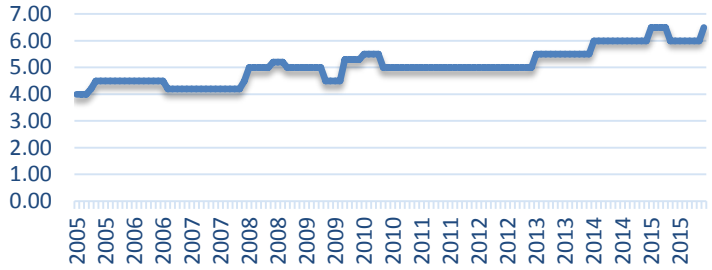


Figure 1: *Agaricus bisporus* kg evolution price from 2005 to 2015.
Source: Agaricus bisporus distributor in Mogi das Cruzes city, SP state.

[2] The values of Real were converted by the price of a dollar in 2015 at R$ 3,9042 (Bacen, 2016)

A comparison of gross margins, Figure 1 indicates that the selling price of 1 kg of mushrooms at 2005 was R$ 4.00 [1] (U$1.710)[1]and Table 1 shows the average unit cost of R $ 6.49. [1] (US$2,7747).This results in gross loss because many growers do not consider to be calculate the cost initial investments.

Taking the same data of 2005 growers and upgrade costs with the 2015 cost values, the average selling price of 1kg mushrooms of R$ 6.50 [2] (US$1,6648) and the average unit cost of mushroom production of R$ 9.34[2](US$2,3923) The new gross profit margin is even more unfavorable to the producers of the sample. This margin does not yet contain expenses such as marketing and business administration. If these latter costs are included in the production and sale of *A.bisporus*, the growers actually suffer losses.

The selling price of mushrooms has increased 62.5% over the 10 years examined. Even being greater than the cost variation (44%), they were already higher than recommended.

Between 8 growers studied, after 10 years, one still growing only *A.bisporus,* another one grows a small amount of *A.bisporus* with other mushrooms. Three growers had started to grow "White Pleurotus" and Hiratake instead *A.bisporus* and 4 growers had changed activity to different business out of the land.

Typical Brazilian tropical climates, with the maximum temperature range between summer and winter is about 25 degrees Celsius is very sustainable to grow tropical weather mushrooms as Hiratake without cold rooms and low cost.

[1] The lack of technology in Brazil is the main reason why there is a slow growth of the national mushroom business. When the price of mushrooms was high it justified low yields, but after the impact of globalized markets, mushroom growers need to increase their yields to be competitive and to keep their business profitable The values of Real were converted by the price of a dollar in 2005 at R$ 2,339 (Bacen, 2016)

[2] [2] The values of Real were converted by the price of a dollar in 2015 at R$ 3,9042 (Bacen, 2016)

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New Approach to Introduce Oyster Mushroom Farming to Potential Growers in South Africa

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ABSTRACT

Since 2014 a demand to grow oyster mushrooms was created as result of a documentary shown on National Television Channel SABC2. Emerging farmers were encouraged to venture into mushroom cultivation. To date more than a 160 requests for training were received. In 2015 learners from five Provinces in South Africa were selected for training. A new participatory approach was followed over a three day period. Currently the City of Johannesburg as part of their Blue Economy Programme, plan to erect 100 mushroom production units within the city boundaries and 16 officials responsible for implementation received training using the new course layout. Theory and practical sessions were alternated. Learners were introduced to mushrooms in general with a tasting session. Both small-scale and large-scale oyster mushroom production farms and techniques were discussed, with great emphasis on the use of quality spawn and general hygiene. Other aspects that were dealt with were potential substrates and substrate preparation, mushroom growth room structures as well as atmospheric control within these. Pests and diseases and the prevention thereof were discussed in detail. Farm management, food health and safety, and nutrition and health were addressed. Packaging options and the drying of mushrooms were demonstrated. Different legislations regarding finance, labour and environment, and different farm certification schemes were mentioned. As part of the course an analysis of the current smallholder farmer situation scenario was conducted. Here, learners also had to draw village/area maps to familiarise themselves with distances from substrate sources, spawn producers and potential markets. An introduction towards starting your own business which addressed recordkeeping, buying basics, costing, stock control, marketing and business planning were put into practice. As homework the potential farmers were asked to start preparing a business plan which will enable them to source start-up financial support. The new course in oyster mushroom farming was well received by learners. They appreciated that questions and participation were encouraged and they felt strongly that the training would aid them in future ventures. As part of mentorship an email group was formed and a news letter established to encourage two-way communication between these potential farmers and central mentors, and to keep them up-to-date with relevant information needed to develop their own farms.

Keywords: oyster mushrooms, training, business planning

INTRODUCTION

In the early 1990's Emeritus Prof Albert Eicker then from the University of Pretoria started to promote oyster mushrooms as an alternative source of protein as a dietary requirement for the South African population. When Dr Yolisa Pakela-Jezile of the Smallholder Agricultural Development Unit joined the team in the early 2000's the programme became active and the word started to spread across the country. Training started with humble training material and mostly relied on on-site demonstrations for a few hours due to the long travelling distances.
Different governmental organization bought into the project, such as the Department of Science and Technology, Transnet, Gauteng Department Agriculture and Rural Development and a couple of Municipalities, but not the Department of Agriculture, Forestry and Fisheries. There was also no organized selection process to identify beneficiaries, other than interest. At that stage government's main aim of measurement was the number of trainees and the number of cooperatives established. Although they invested in infrastructure, limited links on the ground with government officials on the implementation and support of these projects were developed and the growers received little input from local and national departments such as the Department of Trade and Industry. Not with standing a number of successful projects got of the ground, but soon stopped due to conflict within groups as well as vandalism. Another factor was that when government stop supplying spawn for free to their door steps and no plan was made by the growers to continue. That was mainly due to the lack of business planning.
Due to increased demand for training in oyster mushroom production and the above mentioned factors the team had to rethink the whole process of selecting beneficiaries and what training modules they had to work into a three to five day centralized course for start-up mushroom growers.

Results & Discussion

From 2014 the demand from the general public for information and training regarding oyster mushroom farming increased dramatically. This was mainly due to a documentary that is regularly broadcasted on National Television Channel SABC2. The programme Living Land encourages emerging farmers from rural, peri-urban and urban areas to venture into oyster mushroom cultivation. Since that time more than 160 requests for training were received.
After discussions between the Mayor of the City of Johannesburg (CoJ) and Gunter Pauli, an economist, from the Zero Emissions Research Initiative (ZERI) Foundation, CoJ decided to venture into Blue Economy projects. Oyster mushroom production was identified as a health promoting and profitable option to implement and CoJ is in the process of erecting 100 production units. These units will form part of seven agricultural-parks close to needy areas within the city borders and training of both officials and participants is needed.
Previously funding agencies paid in full for the training of beneficiaries, which meant transport, board and lodging as well as the course fees and course material. A start-up packet which included 4 kg of spawn and growing bags were included. Due to the demand since 2014 it has been difficult to make a pre-selection of real interested parties. It was decided that learners had to pay a registration fee and own transport. The Agricultural Research Council

only paid for board and lodging as well as course material. In the end a total of 22 learners representing Gauteng, Limpopo, Mpumalanga, Kwazulu-Natal and Eastern Cape Provinces of South Africa attended.

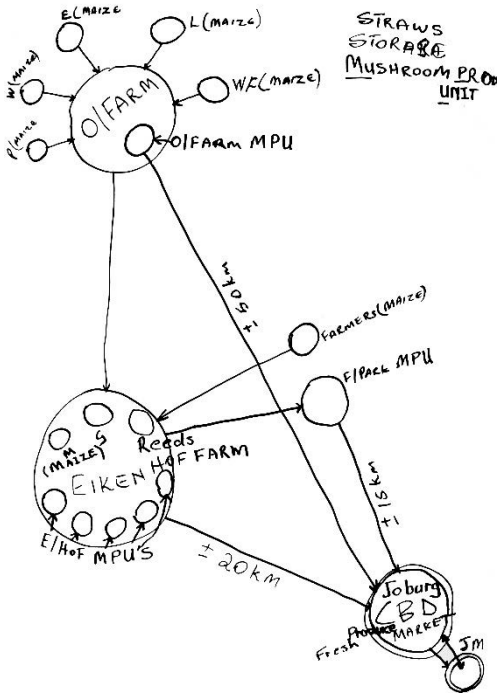
A new participatory approach was followed over a three day period for the first group and the officials from the City of Johannesburg received a five day training course. Training was conducted centrally at the ARC-PPRI, Roodeplaat East Campus due to the difficulty to transport training material to other venues. Theory (Koch, 2009) in the normal form of lectures and practical participatory sessions were alternated. Learners were introduced to mushrooms in general with a tasting session which included button, oyster and black mushrooms. Both small-scale and large-scale oyster mushroom production farms and techniques were discussed, with great emphasis on the use of high quality spawn and strict hygiene. As the country is experiencing the worst drought and record summer temperatures in decades learners were introduced to potential substrates other than wheat straw or combinations thereof. Substrate preparation, both open air fermentation and hot water treatment, spawning and bagging options were demonstrated. As conditions and resources available varies between farmers, options for mushroom growth room structures were discussed and the management of atmospheric conditions within these were stressed. Pests such as mushroom gnats and diseases, and the prevention thereof were discussed in detail as chemical control in oyster mushroom production is difficult to administer (MushWorld, 2004). Farm management, food health and safety, and nutrition and health were addressed. Packaging options, label information and drying of oyster mushrooms were demonstrated. Different legislations regarding finance, labour and environment, and different farm certification schemes were mentioned.

As part of the course an analysis of the current smallholder farmer situation scenario was conducted. Here, learners also had to draw village/area maps to familiarise themselves with distances from substrate sources, spawn producers and potential markets. An introduction towards starting your own business which addressed recordkeeping, buying basics, costing, stock control, marketing and business planning were put into practice (Mutio, 1999). As homework, the potential farmers were asked to start preparing a business plan which will enable them to source start-up financial support. To date two business plans were received from two farmer groups that attended the training in late November 2015. The two groups have two and three members respectively. The one group has already started with production and a marketing campaign. The City of Johannesburg will use the above mentioned technologies to screen potential beneficiaries before the planned production units will be issued to them.

The introduction course in oyster mushroom farming was well received by the potential farmers and the officials from the City of Johannesburg. Both groups appreciated that questions and participation were encouraged and felt strongly that the new approach in training will aid them in the future. See testimonial from Zac. The farmer group requested that in future, funders/donors be invited to present opportunities and that real South African success stories, applicable to their living environment, be presented. As part of continuous mentorship an email group was formed to encourage two-way communication between these potential farmers and central mentors. To keep every body up-to-date with relevant

information needed to develop their own farms, the Ngowa News, a two monthly newsletter was established.

Figure 2 A village/area map linking three production areas namely Eikenhof, Orange Farm and Freedom Park with the fresh produce market near the CBD of Johannesburg.



Zac's Testimonial

In November 2015 I went for three days training at the ARC under the leadership of Dr Susan Koch. On 5 February 2016 I started growing my first 20 bags using wheat straw which was difficult to find due to the drought. On 26 February the first mushrooms started to fruit. Training with the ARC gave me insight and now I understand that I can also train other beginners. Due to lack of capital I raised funds from other freelance jobs. Every two weeks I cultivate 20 bags. To date I have had no contamination on my bags because I make sure everything is clean and boil my substrate well to make sure that other fungi do not compete with my spawn. All surfaces/floors, hands, feet and equipment are cleaned with bleach. A basin of bleach is always with me. I recommend ARC for the wisdom given to me. I feel that I chased poverty out of my family with Agriculture. If I could get help from any funding institution, I will make a big difference in my community and help people that are not working, especially woman and the disabled. For now I am still operating in my house which is too small to produce more.

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The effectiveness of the oyster mushroom growing on the locally available substrates in rural regions of Africa and Europe

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ABSTRACT

The oyster mushroom *Pleurotus ostreatus* (Jacq.) P.Kumm. is a saprophytic fungus speeding up wood decomposition and producing the healthy and nutritious fruiting bodies. This ability could be used for biodegradation of waste wood unusable in the forest management or wood technology and a variety of other agricultural wastes. This ability could also be used for a very important purpose: to provide an opportunity to work as well as livelihood for the people in rural and poor areas.

The Mushroom Training and Resource Centre (MTRC) aims to improve the livelihoods for present and future generations within rural communities in Kabale, Kanungu and Kisoro districts of Uganda through increased mushroom production and sales. Mushrooms are grown on agricultural wastes, converting them into valuable food. The production of the oyster fruiting bodies runs for 3 months. Production reached approximately 50% of the biological efficiency. Evaluating the production and Cash Flow shows the farmer breaks even in the 6th week or at the half of the second month of production.

The economic evaluation of the oyster mushroom production inoculated into 40 beech (*Fagus sylvatica* L.) wood blocks during a five year period was made under forest condition of Slovak Republic. The biological efficiency reached almost 30 %. Economic return has been measured by dynamic indicators of the investment projects evaluation. Results of the net present value (NPV=798.80€), as well as the profitability index (PI= 2.52), and discounted payback period (DPP≈ 2years) point at the high profitability of such project type in given conditions.

Keywords: oyster mushroom, mushroom growing, substratum, costs and income

INTRODUCTION

The current paper presents a comparison between results of the oyster mushroom cultivation in two completely different regions - agricultural area of tropical Africa in a developing Republic of Uganda and a temperate forest area of Slovak Republic in Central Europe. However, different areas have multiple things in common: **cultivation of the oyster mushroom**, utilization of the **local natural plant products for a substratum**, **both simple and environmentally friendly method of cultivation**, to strive for the most efficient production and the **economic profit**.

The oyster mushroom is a common saprophytic fungus in deciduous forests. Its ability to speeding up wood decomposition could be used for biodegradation of waste wood unserviceable in forest management as well as decomposition of various vegetable wastes in agriculture (Pavlík, Pavlík 2013). The genus *Pleurotus* (oyster mushroom) comprises some of the most popular edible mushrooms due to their favourable organoleptic and medicinal properties, vigorous growth and undemanding cultivation conditions (Gregori et al. 2007).
They are rich in vitamins, amino acids, and minerals, besides being significantly involved in human detoxification processes. Recent research has revealed the important role they play in reducing blood cholesterol (Alam et al. 2011). The polysaccharides found in their fruiting bodies have revealed important biological activities (Vamanu 2012).
Using the local raw materials that are available at low cost enables a rural based farmer to under-take the business level at medium or large commercial enterprise. It can provide employment to all the family members with less labour force and capital investment in the business. The cultivation of mushrooms is environmentally beneficial. Mushrooms are grown on agricultural wastes, converting them into valuable food. Also, once used for mushroom cultivation the wastes are transformed into "mushroom compost", a valuable natural fertilizer. The ability of oyster mushroom to degrade wood cell walls and carry out the cooperative processes with other fungi and bacteria plays a key role in the successful decomposition of organic substances, formation of simple organic products, establishment of the soil community and formation of fertile soil (Heilmann-Clausen, Boddy 2005; Piškur et al. 2011).
At the example of oyster mushroom can be presented, that cultivation of certain wood-destroying fungi can also bring production quantities of tasty and healthy fruiting bodies of fungi and ultimately a financial profit.

Results & Discussion

The cultivation of oyster mushrooms in Uganda as an income generating activity for rural farmers gained prominence in the 1990s. The oyster mushroom is by far the easiest and least expensive mushroom to grow. The uniqueness of mushrooms growing is that it can be done with low capital investment, it requires minimal space and it is a home based activity which favours especially women to engage in. The agricultural wastes of sorghum, millet, beans, peas, wheat, sawdust and maize can be used as substrates to grow mushrooms. The use of locally available materials thus ensures the ecological sustainability.
Over the last 7 years since the Mushroom Training and Resource Centre was constructed in Kabale (Uganda), over 10,000 farmers have been introduced to mushroom cultivation and acquired training at different levels. Working from the grass-roots and using meagre resources, MTRC has built a well-run effective centre for the production of mushroom and provision of mushroom-training to local and regional communities. MTRC has strong social priorities; aiming to empower and bring economic development to the farmer groups they work with.
Several strains of *Pleurotus ostreatus* are produced at MTRC: White, Brown, Grey, SHBE, 2191, 2125. The growing medium – substrates - are prepared differently, according to species and quality. After soaking (12-24 hours), drying, pasteurisation and inoculation is 4 kilograms of substrate filling into polyethylene bags and they are placed in incubating rooms to

overgrowing. After 3-4 weeks of incubation fruiting starts which goes on producing mushrooms flushes for 3-4 months. Spawn produced at MTRC yields up to 4 kilograms of fresh mushrooms per growing bag. The largest harvests are picked in the early stages of each bag (-approx. 50% in 1st month, 37,5 % in 2nd month) as the flushes gradually diminish in size and frequency until the bag gets depleted losing weight and yields (- maximally 12,5% in 3rd month). 70% of the total production is usually sold as fresh mushrooms and 30% as dried.

For startup small sale farmers (up to 60 bags) have been trained in the use of cheaply available alternatives in cases where they cannot afford the stipulated equipment. The starting costs or investment into the enterprise (- grow room, fermentation basin, solar dryer, labor, production material) for small farmer are approximately 250 USD, for medium scale farmer (500 bags) approx. 1400 USD and for large scale farmer (5000 bags) approx. 14000USD. Costs are increasing even more by the operational and re-investment ones. The small farmer breaks even in the 6th week, and the medium and large farmer in the 5th week or at the beginning of the second month of production. The average production of fruiting bodies reaches approximately 4 kg per one bag, i.e. the biological efficiency (B.E. in accordance to Stamets 2000) in this case is approximately 50%.

Wood mass is the most important source of income for the Slovak forest management. Therefore, efforts aimed at the most effective evaluation of the wood mass is the primary interest of the forest managers and forest owners. Relatively large quantity of wood remains in the forest stand and is the basis for the formation of soil and nutrients in soil. There is possibility for an unconventional ways of ecological processing and use of wood materials especially those of low quality. One of these approaches is the use of decomposing activity of wood destroying fungi in targeted decomposition of low quality wood and at different size fractions. Cultivation of certain wood-destroying fungi can also bring production quantities of tasty and healthy fruiting bodies of fungi and ultimately a financial profit (Pavlík et al. 2012).

During 5 years of research, a process of fruiting body formation has been analysed in detail on 40 beech logs directly in the conditions of forest stand. Logs 20-40 cm in diameter and 45-55 cm long were inoculated with the oyster mushroom inoculum in the form of grain spawn. The biological efficiency was calculated based on actual weight and humidity of logs at the beginning of the experiment, and actual weight of fresh fruit bodies growing up on the logs during 5 years. *Pleurotus ostreatus* var columbinus isolate MTFCCA019 was obtained from the Mykoforest Type Culture Collection of the Mykoforest Company from Velčice, Slovakia.

During the research period, fruiting bodies of the oyster mushroom have been formed on all inoculated logs. Total yield of fruiting bodies from 40 beech logs was 353 695 g, and the most productive beech log produced 12 560 g of fruit bodies during 5 years. Maximal production was reached in the third year, but the second year was very good for production as well. In the fourth and especially in the fifth year the production of fruit bodies was very low. The mean value of B.E. during the research period was 29.05%, maximal value of B.E. on particular log reached 47.75% and minimal value of B.E. was 17.51% on beech logs (Hraško et al. 2014).

To assess the profitability of mushrooms production we have followed individual costs items resulting from the practical experiment (- wood price, cutting, inoculation, overgrowing, stabilisation in forest, controlling, fruiting bodies picking and packaging). The production costs for the oyster mushroom in the first year presents the amount of 5.93€/log. The length of the experiment was given by the fruiting period of the oyster mushroom on the beech saw log

which presents five years as mentioned before. The maintenance of the logs during the next each year presents only the small costs in amount of 40 €/m^3/year (1 €/log/year). These are the only expected costs taken into the consideration in the following 4 years.

Following the results we can point out that maximal biological production of fruit bodies was reached in the third year of production. However, from the economical point of view the highest net present value (NPV) was reached in the fourth year as the difference between cumulative discounted revenues and costs. The maximal increase of the revenues was in the second year of the production due to increase of the biological efficiency of the mushroom production but also mainly by marked decrease of the production costs in further years of the project.

Following the investment rules we can point out that the project of oyster mushroom production is profitable under given conditions when NPV reaches plus value in the amount of 797.94€ and the profitability of the investments is less than 2 years of the whole project period (DPP < 5 years). Under given conditions, the project reaches high profitability index in the amount of 2.51 (Hraško et al. 2014).

The cultivation of oyster mushrooms on the example of Uganda and Slovakia, as well as the method of evaluating its effectiveness is completely different. However, it is simple and economically profitable for a given countries. Each helps to people and to nature. Benefits for nature is not financially quantified and both production processes could be shortened by one stage and would be more profitable. Therefore, growing of the oyster mushroom using a local agro or wood wastes, has great perspective - for people and for nature.

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VI Nutrition, Bioactive Compounds, Medicinal Aspects

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Retail *Agaricus bisporus* mushrooms good source of vitamin D in Australian food supply

Glenn Cardwell
Nutrition Impact Pty Ltd, Perth, Western Australia

Conflict of interest: Research done on behalf of the Australian Mushroom Growers

INTRODUCTION

Vitamin D deficiency is a public health concern as one-third of adult Australians are vitamin D deficient and one million suffer osteoporosis. Previous estimates are that Australians consume 5-10% of the Adequate Intake (AI) of vitamin D. Food Standards Australia New Zealand (FSANZ) wants to increase the online database of vitamin D containing foods, at industry's cost, to help the public choose foods high in biologically available vitamin D.

Agricultural studies show that *Agaricus bisporus*, the common button mushroom, exposed to ultraviolet (UV) light, generate and retain high levels of vitamin D2. Some farmers intentionally expose mushrooms to UV light post-harvest and market them as "Vitamin D Mushrooms", as they meet the adult Adequate Intake (AI) of 10 mcg in a 100g serve (3 medium mushrooms). What is not known is the vitamin D content of regular retail mushrooms when compared to the vitamin D levels of intentionally UV-exposed mushrooms. Independent market surveys reveal that four in five Australians (83%) eat mushrooms. If regular retail mushrooms can provide more than 10% AI vitamin D, Australian consumers will benefit in cost and convenience.

Methods

We randomly selected metropolitan supermarkets (Woolworths, Coles, Foodland) in five capital cities to ensure the analysis covered a variety of mushroom suppliers, and randomly purchased 28 x 200-400g retail mushroom samples for measurement, including regular whole and sliced mushrooms, and sliced UV-exposed mushrooms.

Results

Mushrooms were analysed by an independent government laboratory, the National Measurement Institute, Melbourne. The results revealed that retail raw mushrooms have high levels of vitamin D (average 2.3 mcg or 23% AI per 100g serve; range 1-5 mcg/100g). This is higher than previously reported for commercial retail mushrooms. UV-exposed raw mushrooms provide at

least the AI of vitamin D (average 26 mcg/100g). Three samples (sliced and whole regular mushrooms, and sliced UV-exposed mushrooms) were fried for 5 minutes before analysis, revealing no loss of vitamin D2 content.

CONCLUSION

Regular retail mushrooms provide 23% of the AI of vitamin D in a 100g serve. Mushrooms exposed to UV light post-harvest provide 100% of the AI of vitamin D in 100g serve. Both regular and UV-exposed mushrooms are good sources of vitamin D in Australia.

Keywords: Vitamin D, osteoporosis, ultra-violet light, supermarket, Australian

INTRODUCTION

In Australia, according to Osteoporosis Australia, 5% of the population (1.2 million/24 million) have osteoporosis, with a further 6.3 million, or a total of 31% of the population, having low bone mass (http://www.osteoporosis.org.au/about-osteoporosis). The main nutritional causes of poor bone mass are insufficient calcium from the diet, and insufficient vitamin D from the diet and from sun exposure. Australians get only 10% of their required vitamin D from food (Nowson 2012), while sun exposure and supplements rarely make up the difference as vitamin D deficiency is commonplace in Australia with 23% of adults having insufficient levels (ABS 2014). Deficiency is more common during winter and in people living in the southern part of the country.

Very few Australian foods have been analysed for vitamin D using current methodology. Recent figures for some foods, such as meat, eggs and canned fish, have been provided by the food industry. To help the public choose foods high in biologically available vitamin D, Food Standards Australia New Zealand (FSANZ) aims to increase the online vitamin D database, recently analysing a selected group of vitamin D-containing foods.

Agricultural studies show that *Agaricus bisporus*, the common button mushroom, exposed to sources of ultraviolet (UV) light, such as sunlight, generate and retain high levels of vitamin D2 (Koyyalamudi 2009; Simon 2011; Phillips 2013). Wild mushrooms in Europe commonly have 2-40 mcg vitamin D2/100g (Mattila 2002; Teichmann 2007). Some farmers intentionally expose mushrooms to UV light post-harvest and market them as "Vitamin D Mushrooms", since they meet the adult Adequate Intake (AI) of 10 mcg in a 100g serve (3 medium mushrooms).

What is not known is the vitamin D content of regular retail mushrooms when compared to the vitamin D levels of intentionally UV-exposed mushrooms. Independent market surveys reveal that four in five Australians (83%) eat mushrooms. If regular retail mushrooms can provide more than 10% AI vitamin D, Australian consumers will benefit in cost and convenience.

1. Methods

In April 2015, randomly selected major metropolitan supermarkets (Woolworths, Coles, Foodland) in five state capital cities in Australia were sampled for retail mushrooms to ensure the analysis covered a variety of mushroom suppliers, and the samples came from high turnover stores. Trained samplers randomly purchased 10 x UV-exposed mushrooms (300g punnets labelled as 'Vitamin D Mushrooms'), 12 x regular whole mushrooms (200-400g punnets), and 6 x regular sliced mushrooms (200g punnets). The samples were taken at the time of peak consumer demand (2-6pm) on a weekday, immediately placed into light-proof plastic wrap, then placed in an insulated box with pre-frozen ice bricks and couriered to the independent government laboratory, the National Measurement Institute (NMI) in Melbourne, to arrive that evening or the next morning. Three samples were cooked on-site at the NMI by frying, without oil, for five minutes. The samples underwent alkaline saponification followed by solvent extraction. The resultant extracts were fractionated by normal phase High Performance Liquid Chromatography (HPLC), then analysed by reverse phase HPLC with Photodiode Array detection (PDA). Vitamin D3 was used as an internal standard and quantitation was against known standards of Vitamin D2

2. Results & Discussion

The results revealed that retail raw mushrooms provide significant levels of vitamin D (average 2.3 mcg or 23% AI per 100g serve; range 1-5 mcg/100g), with similar figures for sliced and whole mushrooms. This is higher than previously reported for commercial retail mushrooms, with figures commonly being <1.0 mcg/100g fresh weight (Phillips 2013; Urbain 2015). Retail UV-exposed raw mushrooms provide at least the AI of vitamin D (average 26 mcg/100g; range 17-37 mcg/100g). Three samples (sliced and whole regular mushrooms, and sliced UV-exposed mushrooms) showed there was no loss of vitamin D through cooking.

Table 1. Average vitamin D content of retail mushrooms

Mushroom type			No. of samples	Moisture g/100g	Ergocalciferol (vitamin D2) mcg/100g
Regular	sliced	raw	6	93.2	2.2
		cooked	1	87.7	3.1
	whole	raw	12	93.1	2.3
		cooked	1	91.6	2.3
Vitamin D	sliced	raw	10	92.6	26.6
		cooked	1	87.1	52.0

The results were submitted to Food Standards Australia New Zealand (FSANZ) and accepted in June 2015 as official figures for publication on their website:

(https://www.foodstandards.gov.au/science/monitoringnutrients/nutrientables/Documents/FINAL%20-%20Vitamin%20D%20Mushrooms%20report.pdf). This is the first time that there have been official vitamin D levels for mushrooms in Australia.

In conclusion, regular retail mushrooms from popular Australian supermarkets provide 23% of the AI of vitamin D in a 100g serve as measured by HPLC at the Australian government laboratory, the National Measurement Institute. This is higher than expected and requires further investigation to determine why Australian retail mushrooms have higher vitamin D levels than previously reported in other countries. As expected, mushrooms intentionally exposed to UV light post-harvest provide at least 100% of the AI of vitamin D in a 100g serve. Both regular and UV-exposed mushrooms are good sources of vitamin D in Australia.

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Increasing vitamin D_2 content of cultivated mushrooms by processing and drying

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ABSTRACT

Cultivated mushrooms contain little (0.005-0.080 µg vitamin D_2 per g dry matter) to no vitamin D_2 due to the absence of sunlight in growing rooms. However, vitamin D_2 in mushrooms can be significantly increased by irradiation with natural or artificial UV-light. Drying is a standard method for the postharvest preservation of mushrooms on industrial scale. Therefore, experiments using different drying methods namely conventional hot-air drying in a high precision laboratory dryer (T=60 °C, x=10 g/kg, v=0.6 m/s), sun drying, solar drying and freeze drying (T=20 °C, p=0.28 mbar) were carried out and their effects on drying behavior and vitamin D_2 content of cremini (brown) *Agaricus bisporus* mushrooms were investigated. Prior to drying, samples were divided into three batches: The first batch of samples was sliced to approx. 30 mm thickness and then dried under the sun or in a forced convection-type solar drying system. The second batch of mushroom slices was irradiated with an UVB dose of 1.5 J/cm^2 at 25 °C for 20 min before hot-air drying and freeze drying, while a batch of uncut UVB-treated fruiting bodies was used for control. For the determination of vitamin D_2, all dried samples were subjected to saponification, the analytes were extracted with n-Hexane and injected into the HPLC. Mushroom slices subjected to 60 °C were dried significantly faster than those dehydrated by other methods. Also, solar drying enhanced the drying rate of samples considerably resulting in a shorter total drying time in comparison with sun drying. Freeze drying took about 22 h to reach optimum final moisture content for safe storage. Slicing of mushrooms prior to UVB exposure induced a ten-fold increase in vitamin D_2 content. In particular, UVB irradiation of whole mushrooms resulted in 44.50 µg/g dry matter, while sliced mushrooms produced 406.26 µg/g. Vitamin D_2 content of sun- and solar dried mushroom slices was about 36.40 and 38.86 µg/g dry matter, respectively. Apart from the irradiation dose or the exposure time, slicing of samples before UVB treatment is an essential processing step to maximize vitamin D_2 in mushrooms. Furthermore, drying in natural or forced convection type solar dryers can be an effective approach for increasing vitamin D_2 content in mushrooms.
Keywords: solar drying, processing, quality, mushroom, industry

INTRODUCTION

The objective of this study was to investigate the impacts of processing and drying on vitamin D_2 enrichment of cultivated mushrooms. In particular, the effect of the increased exposure surface area via slicing of mushrooms prior to a controlled period of UVB irradiation was examined. Also, the influence of sunlight exposure on the vitamin D_2 content of untreated mushrooms during natural

sun drying and drying in a forced convection type solar dryer was evaluated. Dried UVB-treated mushrooms obtained by either hot-air drying in a lab dryer or freeze drying were used for control.

1.1. Irradiation of fresh mushrooms

Fresh cultivated mushrooms brown *Agaricus bisporus* (Lange) Sing. were supplied from a local market in Stuttgart, Germany. UVB irradiation of fresh mushrooms was performed in a UV irradiation chamber equipped with UVB-lamps (Dr. Groebel GmbH, Ettlingen, Germany). Whole and sliced (30 ± 0.2 mm thickness) fruiting bodies were evenly distributed on a tray and samples exposed on both sides were irradiated at 1.5 J/cm^2, for 20 min at 25 °C.

1.2. Drying experiments

A batch of untreated mushroom slices was subjected to either natural sun drying or solar drying in a solar bubble dryer (SBD^{TM}). A single layer of UVB-irradiated slices was dried in a high precision trough-flow laboratory dryer at temperature of 60 °C, specific humidity of 10 g/kg and air velocity of 0.6 m/s. Another batch of UVB-irradiated mushroom samples was frozen at 25 °C for 24 h and then subjected to freeze drying (Alpha 1-4, Christ, Osterode am Harz, Germany). The operating pressure was set at 0.28 mbar with a shelf temperature of 20 °C and condenser temperature of -55 °C. After drying the moisture content of all samples was determined by Karl Fisher titration (Riedel-de Haen, Seelze, Germany) in triplicate and expressed in gram per gram dry basis (g/g d.b.). Dried mushrooms were vacuum-sealed in aluminum-coated polyethylene bags and stored at room temperature for analysis of vitamin D_2.

1.3. Extraction procedure

The extraction of vitamin D_2 was performed following the method described in detail in previous relevant studies with some minor modifications. Prior to extraction the dried mushrooms were ground to a fine powder. Samples of 2.0 g ± 0.005 g were weighted into a 50 ml Falcon tube, 1.0 ml internal standard (vitamin D_3) with a concentration of 100 µg/ml (Serva Electrophoresis Gmbh, Heidleberg, Deutschland) was added and the sample was then mixed with 1.333 ml sodium ascorbate solution (1.75 g ascorbic acid in 10 ml NaOH) (AppliChem GmbH, Darmstadt, Deutschland), 19 ml of 99.7 % ethanol (VWR Chemicals, Leuven, Belgien) and 4 ml of 50% potassium hydroxide (Merck, Darmstadt, Deutschland). The mixture was saponified in a water bath at 80 °C for 60 min. The mixture was then cooled down to room temperature. The extraction of vitamin D_2 was carried out with 15 ml of n-hexane (ROTH, Karlsruhe, Deutschland). Next, the mixture was centrifuged for 8 min at 4500 g (Heraeus, Hanau, Deutschland). The hexane phase was transferred into a new falcon tube and the procedure was repeated twice, however for the last extraction step only 10 ml were added. The pooled organic layers were washed with deionised water until neutralized. The organic layer was then rotary evaporated and immediately redissolved in 1 ml of tetrahydrofuran (Carl Roth GmbH, Karlsruhe, Deutschland). Then the samples were centrifuged for 5 min at 16000 g at 20 °C to remove solid impurities and used for subsequent analyses by HPLC. In all irradiated samples the internal standard coeluted with an unknown substance. Hence, samples without internal standard were prepared to calculate the amount of the interfering substance.

1.4. HPLC analysis

Vitamin D_2 analysis was carried out using a Spherisorb ODS II analytical column, 4.6 x 150 mm, 3 µm particle size (Bischhoff, Leonberg, Deutschland) in a HPLC-system (Shimadzu GmbH, Duisburg, Germany). The isocratic eluent was composed of 77% acetonitrile (ROTH, Karlsruhe, Deutschland), 14% deionised water (Merck Millipore, Darmstadt, Deutschland) and 9% tetrahydrofuran (ROTH, Karlsruhe, Deutschland), the flow rate was 2 ml/min and the analysis time was 30 min. The injection volume was 150µl. The UV-signal was registered at 265 nm. Standards for the quantitative analysis of Vitamin D_2 were made through the dilution of vitamin D_2 and D_3 stock solutions (both 0.6 mg/ml). A set of seven calibration standards with different concentrations of vitamin D_2 and D_3 were prepared with these stock solutions. The concentrations of vitamin D_2 and D_3 were 10, 20, 100, 200 and 300, 400, 600 µg/ml, respectively. Vitamin D_2 detection limit was 0.5 µg/ml.

2. Results & Discussion

2.1. Irradiation of whole and sliced mushrooms

No vitamin D_2 was detected in non-irradiated-freeze-dried mushrooms, whereas the UVB treated samples generated significant amounts of vitamin D_2 independently of drying method employed. Other studies reported small amounts of vitamin D_2 in untreated mushrooms ranging from 0.005 to 0.080 µg/g dry matter presumably due to accidental exposure of mushroom samples to sunlight before analysis (Urbain et al., 2011). The vitamin D_2 contents of whole and sliced UVB irradiated-freeze-dried mushrooms are shown in Figure 1. In particular, UVB irradiation of whole mushrooms generated 44.50 µg/g dry matter, while sliced mushrooms produced 406.26 µg/g dry matter. Hence, slicing of mushrooms prior to UVB exposure induced a ten-fold increase in vitamin D_2 content. Urbain et al. (2015) also found that thinner slices yielded significantly higher amounts since a larger surface area of mushroom's gills has been exposed to UVB irradiation (Kalaras et al., 2012). As

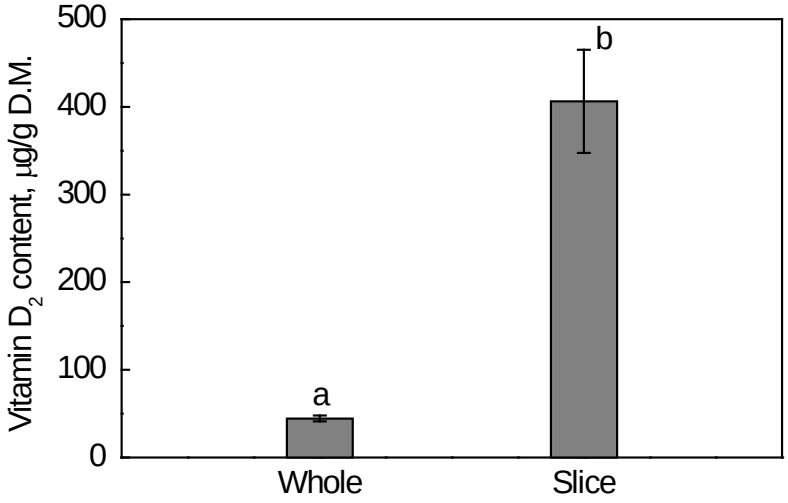


Figure 1. Effect of slicing on vitamin D_2 content of UVB irradiated mushrooms. Whole and sliced (30 ± 0.2 mm) samples were irradiated on both sides. Mean values (n = 9) with different lower case letters are significantly different ($p < 0.05$). Error bars represent standard deviations.

gills contain most of the ergosterol in fruiting bodies (Jasinghe and Perera, 2005), the degree of cap opening may influence vitamin D_2 formation when irradiating whole mushrooms. However, the conversion rate of ergosterol to vitamin D_2 greatly depends on irradiation dose, irradiation time and mushroom's orientation towards the UV source.

2.2. Effect of drying method

Mushroom slices dried to 60 °C were dried significantly faster than those dehydrated by other methods. Also, the solar bubble dryer remarkably enhanced the drying rate of mushrooms resulting in a shorter drying time in comparison with natural sun drying. Freeze drying took about 22 h to reach the target final moisture content for safe storage. Figure 2 shows the results of vitamin D_2 content of mushrooms subjected to different processing methods. Vitamin D_2 content of sun-dried and solar-dried mushroom slices was about 36.40 and 38.86 µg/g dry matter, respectively. Moreover, exposure of samples to controlled conditions of artificial UV light followed by either hot-air drying or freeze drying generated significantly higher amounts of vitamin D_2. It should be noted that the vitamin D_2 content of the freeze dried and air dried samples was not significantly different. Based on the results, UVB-irradiated or sun-dried mushrooms are an excellent source of dietary vitamin D. This is of special importance as only few food products such as salmon (12.4 µg/g), herring (15.4 µg/g) and egg yolk (7.8 µg/g) are naturally rich in vitamin D (Lamberg-Allardt, 2006) but in remarkably lower amounts. Hence, the recommended daily intake of 600-800 IU vitamin D for adults (German Nutrition Society, 2012) can be ensured by mushroom consumption.

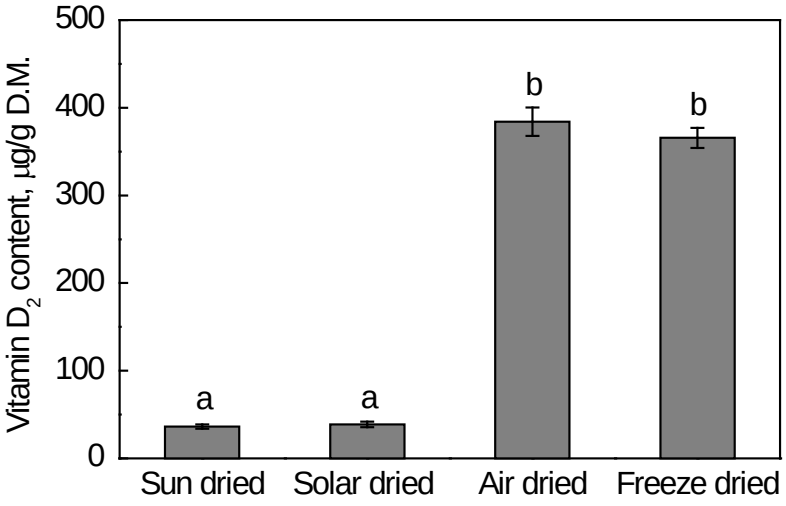


Figure 2. Vitamin D_2 content of mushroom slices (30 ± 0.2 mm) dried by different methods. Air dried and freeze dried samples were previously UVB-irradiated at 1.5 J/cm^2 for 20 min. Mean values (n = 9) with different lower case letters are significantly different ($p < 0.05$). Error bars represent standard deviations.

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Extracts of vitamin D_2 enriched *Lentinula edodes* (Shiitake) mushrooms as anti-inflammatory, immune-modulatory agents

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ABSTRACT

Mushrooms, like *Lentinula edodes*, are an abundant source of ergosterol, which is the precursor of vitamin D_2**.** Additionally, they contain immunomodulating polysaccharides, such as Glucans (α Glucans and β Glucans)**.** Vitamin D has well-established anti-inflammatory properties. Vitamin D supplement improves the sensitivity to anti-viral therapy for chronic Hepatitis C Virus infection. In a former study we have demonstrated that extracts derived from *Lentinula edodes* (Shiitake) mushrooms exert an anti-inflammatory effect[1] . Therefore vitamin D_2 enriched mushrooms were studied in order to verify the impact of both ingredients. At the first stage, fruit bodies of *L. edodes* at different development stages were picked. Immediately after picking the fruit bodies were exposed to UV-B irradiation in order to raise their vitamin D_2 content. The vitamin content increased dramatically, to 1600 IU in 1.0 gram dry weight, compared to no vitamin in the un-irradiated mushrooms. The highest vitamin content was found in fruit bodies that were picked at the "flat" development stage, two days after the optimal marketing picking time (day +2). Four groups of C57B1/6 mice were orally treated with vitamin D_2 enriched or non-enriched mushrooms extract prior and during Concanavalin A - immune-mediated liver injury. Following feeding of the Vitamin D-enriched mushrooms extracts to immune-mediated hepatitis harboring mice, ALT serum levels decreased from 6238 U/L for the control (untreated) group to 2802, 3183 and 2872 U/L for Vitamin D, non-enriched and enriched mushrooms extracts treated groups, respectively ($p< 0.019$, < 0.036 and < 0.022, respectively). Proportion of severe liver injury was decreased from 83% in control group to 60%, 50% and 40% at the Vitamin D, non-enriched and enriched mushrooms extracts treated groups, respectively. Pathological examination of liver biopsies revealed a corresponding improvement of the immune mediated liver injury. Therefore it was demonstrated that oral administration of *Lentinula edodes* edible-mushroom and/or Vitamin D supplements, exert an anti-inflammatory effect in the immune-mediated hepatitis model. The data supports their potential use as safe immune-modulatory agents and as an adjuvant therapy for Hepatitis C Virus infection and Non-Alcoholic Steatohepatitis.
Key words: vitamin D_2, mushrooms, immunomodulation, anti-inflammation

INTRODUCTION

Vitamin D status in humans is an important factor for human health. The classic bone diseases associated with vitamin D deficiency are well known, but these days, modern research have found a putative link between vitamin D status and chronic diseases including Non-Alcoholic Fatty Liver Disease (NAFLD). The main source for vitamin D is exposure to sunlight. But in modern society, individuals spend less time exposed to sunlight due to various factors. Therefore, the use of foods containing vitamin D is an important source. However the main vitamin D food sources are: meat, poultry, eggs and milk products, a problematic issue when it comes to vegetarians and vegans. Mushrooms however are an abundant source of ergosterol, which is a vitamin precursor and is transformed to vitamin D_2 following UV-B irradiation. In a former study we have demonstrated the anti-inflammatory effect of *Lentinula edodes* (Shiitake) extracts[1]. Therefore the objective of this research is to develop vitamin D_2 containing Shiitake mushrooms and to examine this product's anti-inflammatory, immunomodulatory impact of in fatty liver rodent model.

Results & Discussion

UV-B irradiation of *L. edodes* and *P. ostreatus* mushrooms was used to raise their vitamin D_2 content. The study included those two species as potential source of fungal vitamin D. This vitamin content increased dramatically, compared to un-irradiated mushrooms. The results are presented in table.1.

Table.1. The impact of UVB irradiation on Vitamin D_2 content (µg/g DM) of *Lentinula edodes* (Shiitake) and *Pleurotus ostreatus* (Oyster) mushrooms

	Shiitake (Control)	Shiitake irradiated	Oyster (Control)	Oyster irradiated
Vitamin D_2	0	68.901	0	76.5
Standard Deviation.		7.767		6.488

The impact of mushroom physiological age at picking on vitamin D_2 content (following UVB irradiation) was studied. It was demonstrated that Shiitake mushrooms highest vitamin D content were achieved when picking took place, one or two days later than the regular picking time. The results are demonstrated in figure .1.

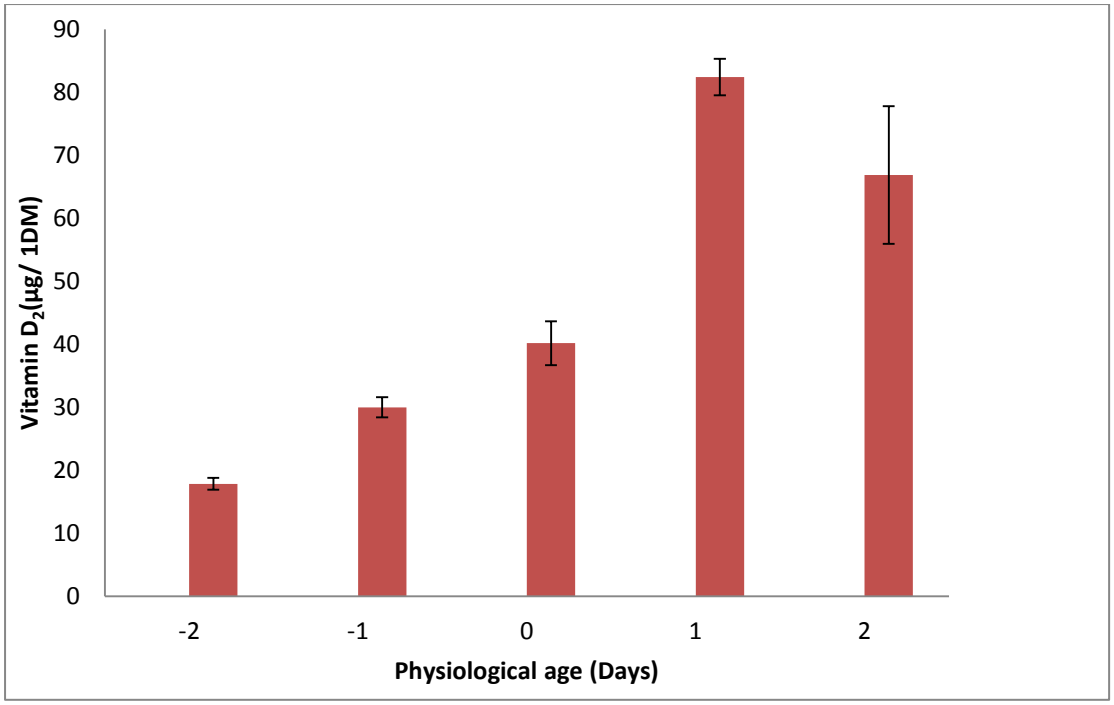


Figure 1. The impact of picking stage (physiological age) on vitamin D_2 content of UVB irradiated Shiitake mushrooms. The regular harvest stage is named 0.

A possible explanation for these results is in the dimensions and the "flatness" of the mushroom's cup. Therefore, the weight ratio between each mushroom's cup and stalk, at each physiological age (picking stage), was measured and the results are presented in figure 2. It was demonstrated that cup/stalk ratio increased with the mushrooms physiological age, representing increased flatness of the mushrooms fruiting bodies. The flatter mushrooms represent larger area exposed to UVB irradiation leading to increased ergosterol amounts of transformed to vitamin D_2

Preliminary studies on the impact of vitamin D2 enriched Shiitake mushrooms in a liver disease mouse model were made. It was demonstrated that: following feeding of the vitamin D-enriched mushroom extracts to immune-mediated hepatitis harboring mice, induced by injection of concanavalin A, alanine aminotransferase (ALT) serum levels decreased from 6238 U/L for the control (untreated) group to 2802, 3183 and 2872 U/L for Vitamin D, non-enriched and enriched mushrooms extracts treated groups, respectively. Proportion of severe liver injury was decreased from 83% in control group to 60%, 50% and 40% at the Vitamin D, non-enriched and enriched mushrooms extracts treated groups, respectively. Pathological examination of liver biopsies revealed a corresponding improvement of the immune mediated liver injury. In summary, it was demonstrated that oral administration of vitamin D2 enriched Shiitake mushrooms and/or vitamin D supplements, exert an anti-inflammatory effect in the immune-mediated hepatitis model. The data supports their potential use as safe immune-modulatory agents and as an adjuvant therapy for Hepatitis C Virus infection and Non-Alcoholic Steatohepatitis.

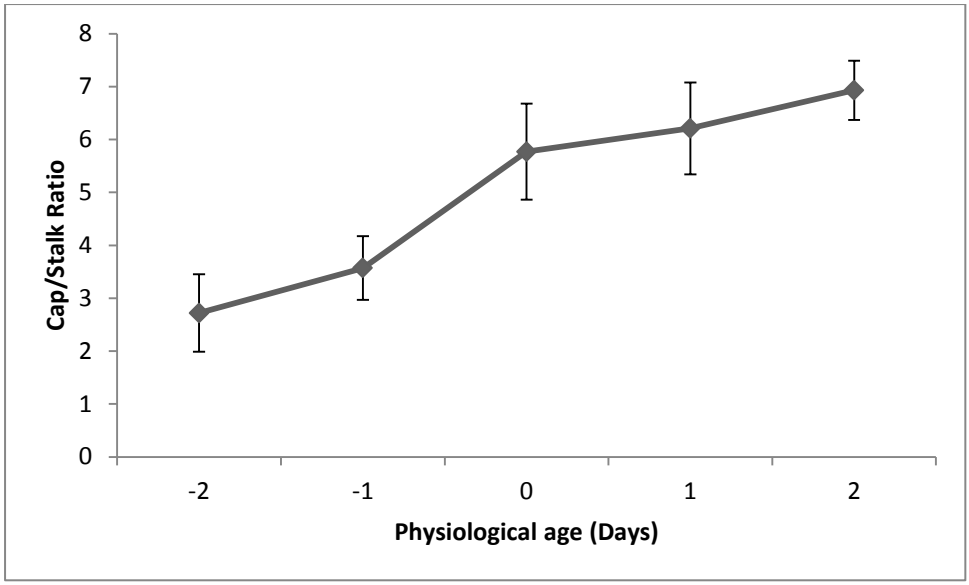


Figure 2. The impact of picking stage (physiological age) on Shiitake mushrooms cup/stalk weight ratio. The regular harvest stage is named 0.

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Mushrooms and Health Global Initiative: Communicating nutrition research

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ABSTRACT

1. Background

Consumers seem to have an insatiable appetite for nutrition and health news. A Google search on "nutrition and health trends" returns 128M results; a search on "consumer interest in nutrition" returns 52M results. In this information age, there is also misinformation as a survey of US dietitians reported that most (67%) nutrition information is based on half-truths rather than published peer-reviewed research. The Mushrooms and Health Global Initiative (MHGI or Initiative) focuses on collecting, evaluating, and communicating scientific findings on mushrooms' health and nutrition benefits to establish the mushroom industry as the credible and reliable source of nutrition research information related to fresh mushrooms.

2. Purpose

A primary objective of the Initiative is to base informational materials, customized for health influencers and media so that they can accurately and efficiently communicate mushrooms' benefits, on research findings. Credible scientific information, translated into easy to understand articles about mushrooms appearing regularly in various communication channels, can be instrumental in changing consumers' purchase patterns from "nice to have" to "must have" and make mushrooms an essential, easy, and tasty way to better health.

3. Methods

- Utilizing the *2014 Mushrooms and Health Report* as the basis for globally communicating the science linking mushrooms and health
- Sharing health-based informational activities in the *Bulletin* to demonstrate how various countries customize and interpret research results locally
- Facilitating exchange of ideas and resources via social media websites and blogs for Australia, Canada, Italy, Netherlands, South Africa, United Kingdom and Ireland and the United States
- Maintaining the Initiative website – accessed by visitors from all continents except Antarctica – as the repository of the *Bulletin* and research reports
- Utilizing the expertise of an international directory of mushroom researchers and sharing research-supported public relations efforts among interested individuals, mushroom organizations, scientists, academic institutions and media.

4. Results

Since 2008, 32 issues of the *Bulletin* have been e-distributed to interested individuals, mushroom industry organizations for posting on their website and sharing with science and health researchers. A survey of readers indicated that:

- 78% read the *Bulletin* regularly
- 61% share the *Bulletin* with colleagues
- 13% post/share the *Bulletin* on websites
- 70% find the research abstracts very useful
- 74% find the reports from different countries on how to communicate nutrition research very useful.

5. Conclusion

Since its beginning about a decade ago, the Initiative has focused on being recognized as the global science and communications site for credible information on mushrooms' health benefits. Converting the *Bulletin* to a "news" format and making website information mobile friendly can enhance these efforts going forward.

Keywords: Mushrooms and Health Global Initiative

6. Health as a Driver of Mushroom Promotion – Introduction

Consumer research in the United States (U.S.) and Australia has identified that credible information about the health benefits of mushrooms can increase consumers' perception of these benefits and persuade them to increase their per capita consumption of mushrooms (Rose Research "2011 Fresh Mushrooms Attitude and Usage Study" available at www.mushroomcouncil.org/research accessed Feb. 20, 2016; Seymour G. September 2007 "Mushrooms and Health Global PR Initiative" presentation at the International Medicinal Mushrooms Conference Ljubljana, Slovenia). The International Society for Mushroom Science (ISMS) has provided a unique global platform for the mushroom industry to leverage these important research findings at a national, regional, and individual producer/marketer level. ISMS provides an "umbrella" for several corporate member countries to jointly fund a project now known as The Mushrooms and Health Global Initiative (Initiative). These investors make significant savings by sharing the cost of the identification and communication of compelling scientific reasons for consumers to eat more mushrooms; and the frequent dissemination of consistent messages about mushrooms, nutrition and health into global channels through the Initiative's asses (Mushrooms and Health Report, Bulletin and website.

7. Results

The Scientific Platform for Promotion Programs

When the Initiative began there was a plethora of approximately 11,000 published papers on mushrooms and health. The need was to organize, evaluate and broadly communicate the scientifically validated benefits of mushroom consumption. This was accomplished through the Initiative's *Mushrooms and Health Report* (Commonwealth Scientific and Industrial Research Organisation, 2008 updated biennially through 2014), which remains the underpinning resource

document for responding to media inquiries or preparing releases to key influencers. "The role of edible mushrooms in health: Evaluation of the evidence" (Roupas P, Keogh J, Noakes, M et al 2012, Journal of Functional Foods, 4, 687-709) a review manuscript based on the *Mushrooms and Health Report*, was among that journal's Top 20 most often downloaded articles during the last full quarter of 2014 and was the 15th most downloaded paper for the entire year. The review still was being downloaded 2.5 years after it was published and was among the 8 most cited published articles demonstrating the continued interest in mushroom health and nutrition and the need for a core reference document.

The Bulletin Interprets Scientifically-based Communications

The quarterly newsletter/*Bulletin* includes summarized abstracts of recent research and practical examples of how those in public relations/marketing interpret and customize research results for their local markets. Approximately three-fourths (74 percent) of *Bulletin* readers found country specific examples very useful. These include participating in health professionals' conferences and exhibits, sponsoring tastings at events and food blogger campaigns, partnerships with health organizations, promoting the role of mushrooms in weight management and tips on blending mushrooms with meat to lower calories.

The *Bulletin* provides a strong communications platform to help assure frequent and consistent dissemination of new ideas so mushrooms stay top of mind. This facilitates ongoing mushroom publicity around the globe, critical to increase worldwide demand. Greater sharing of information and communication is further facilitated by listing countries' social media websites and blogs. The collective media attention generated by individual country activity then spills into all markets globally via media outlets and the internet thus leveraging the messages and reach of the Initiative.

8. Discussion and Significance

Promotion Programs Return Results

An initial and continuing user of the information generated by the Initiative, the Australian Mushroom Growers Association (AMGA) revised its marketing strategy to reposition mushrooms in the minds of consumers through a series of messages based on scientific findings about the nutrition and health consequences of regularly incorporating mushrooms into meals. According to a test-market research project 2010/11, there was a significant increase in consumer demand for mushrooms, reflected in both an increase in the number of units sold and an increase in the average unit value. Qualitative and quantitative market research suggests that this change in consumer behavior was in response to a change in consumer perceptions of the health consequences of eating mushrooms, the primary message communicated in the advertising campaign (Alston, J.M. and Parks, J.C. 2012 "The returns to promotion of healthy choices in Tasmania: Are you in the dark about the power of mushrooms?" *AJARE* 56, 347-365).

A subsequent evaluation of AMGA's generic promotion programs discovered for the first time that health aspects have trumped taste as the major perceived benefit of mushrooms. Ease of use, versatility and flexibility of preparation round out the top five most salient reasons for liking mushrooms suggesting that a bundle of benefits be re-enforced. Although there is increasing awareness of the health benefits such as heart health of eating mushrooms, fewer than half of

purchasers are unaware of specific benefits – suggesting more communication work needs to be done (Mobium Group, *Mushroom Monitor* 2014, 140-145; 154-158).
Responding to a query on the benefit of health messages on attracting media attention and sustaining consumer purchase behavior, the South African Mushroom Farmers Association (SAMFA) commented: "We get more enquiries from media for more information since we shifted our focus onto the health aspects and increased consumer interest when we post health information on our Facebook page. The health message is a more sustainable behavioural driver for increasing consumption than just a recipe because many food products offer recipes but not many food products have the health attributes of mushrooms so that creates a real lasting unique selling proposition (USP)."

Health Research Leads to Product Development and Global Interest

Capitalizing on mushrooms' nutrient profile, health and wellness benefits, the U.S. Mushroom Council (Council) integrated its nutrition research program with foodservice efforts. Research based on mushrooms' nutrient composition demonstrated that consumers could lower the energy density of their meals by replacing higher calorie ingredients/foods with mushrooms. Subsequent research focused on mushrooms' functional ability to 'blend' with other foods thereby integrating taste, nutrient composition, functional characteristics, price and cost efficiencies. A culinary technique known as the Blend involves adding chopped fresh mushrooms, which look, taste and perform like meat, to ground meat to make common recipes Americans love (burgers, meatballs, and tacos) more healthful and flavorful. From initial research through pilot programs, influencer communication and direct-to-consumer initiatives, the Blend escalated into a fully integrated, new business strategy to open new mushroom applications. Other countries communicated the 'blend trend' to consumers through cooking demonstrations and food blogger campaigns resulting in further media coverage.

9. Conclusion

The Initiative was founded on the premise that science-based health messages can drive consumer behavior. Formal market research suggests that health messages do alter consumer behavior. Informal comments from Initiative participants suggest that health messages can help create a more lasting USP. Progress has been made over the past decade, yet there is still work to be done to increase communication effectiveness and to make the Initiative the recognized global science and communications site for the media, news bureaus, researchers, health professionals, and the industry seeking credible information on mushrooms' health and nutrient benefits. This requires greater participation from mushroom producing countries, enhancing the technology that supports the Initiative outputs and capitalizing on the global reach of social media.

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Taste components of *Lentinula edodes*

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ABSTRACT

Lentinula edodes had become the second most popular edible mushroom and been cultivated widely in the world for its nutritional, medicinal properties and delicate flavor. Its taste compounds, including soluble sugars, organic acids, amino acids and 5′-nucleotides, could contribute to the palatable taste of *L. edodes*. In this research, the effects of mushroom strains, culture substrates and harvested stages on *L. edodes* taste compounds contents were studied, and taste components in *L. edodes* fruiting bodies were identified and analyzed by HPAEC and HPLC. The research results revealed that two identified *L. edodes* strains Shenxiang No.10 and L605 fruiting bodies contained high levels of umami components, strain L868 fruiting bodies contained high levels of mannitol, while strain L241 fruiting bodies contained high levels of succinic acid. The effects of culture substrates on taste components of *L. edodes* showed that single carbon and nitrogen sources were beneficial to soluble sugars and polyols, organic acids and sweet amino acids production, single carbon source was beneficial to essential amino acids production, mixed carbon source was beneficial to umami 5′-nucleotides and high mushroom yields production, mixed carbon source or mixed nitrogen source were more beneficial to produce *L. edodes* fruit bodies with high EUC values. High C/N values were beneficial to trehalose, arabitol, malic acid and succinic acid production, while low C/N values were beneficial to mannitol and citric acid production. Fruiting bodies harvested at different growth stages revealed that mannitol contents were higher in the pileus and increased at mature growth stages, while arabitol contents were higher in the stipe and peaked at growth stage 5. Succinic acid contents peaked at stage 5 in the pileus and stipe during mature growth stages. Fruiting bodies harvested at stage 5 contained higher levels of umami components during mature growth stages.
L. edodes strains, culture substrates and harvested stages had big effects on taste compounds production in different degrees. Results in this research could provide information for cultivation of *L. edodes*, which would contain more taste components, and mushroom yields would arrive at relatively high levels to produce more economic benefits.
Keywords: *Lentinula edodes*, taste components, mushroom strains, culture substrates, growth stages

INTRODUCTION

Lentinula edodes (Berk.), usually called Xianggu in China, shiitake in Japan, is one of the most important traditional edible and medicinal fungus in China and other East Asian

countries for its desirable taste flavor and great medicinal properties. The taste components in edible mushrooms are primarily influenced by several water-soluble substances, including soluble sugars and polyols, free amino acids, 5'-nucleotides and organic acids. Variation in concentrations of these compounds affects the taste, such as sweet, umami, sour and so on. Soluble sugars and polyols in mushrooms contribute to a sweet taste, but not to the typical mushroom taste. Organic acids, related to the metabolic processes of synthetic phenols, amino acids, esters and aromatic substances, playan important role in the flavor of edible mushrooms. Umami-taste is recognized as a basic taste, typified by glutamic acid and its salt monosodium glutamate (MSG), which give a savory meat soups or rich meaty taste sensation. Aspartic acid, glutamic acid and some 5'-nucleotides were also considered as MSG-like components, and values of equivalent umami concentration (EUC) were used to evaluate umami of mushrooms. Mushroom strains, culture substrates and harvested stages had big effects on these taste compounds production in different degrees. The aims of this study were to investigate the effects of mushroom strains, culture substrates and harvested stages on taste components contents of *L. edodes*, which had important significance to *L. edodes* cultivation and its condiment industry.

Results & Discussion

The research results revealed that two identified *L. edodes* strains Shenxiang No.10 and L605 fruiting bodies contained high levels of umami components, strain L868 fruiting bodies contained high levels of mannitol, while strain L241 fruiting bodies contained high levels of succinic acid. The effects of culture substrates on taste components of *L. edodes* showed that single carbon and nitrogen sources were beneficial to soluble sugars and polyols, organic acids and sweet amino acids production, single carbon source was beneficial to essential amino acids production, mixed carbon source was beneficial to umami 5′-nucleotides and high mushroom yields production, mixed carbon source or mixed nitrogen source were more beneficial to produce *L. edodes* fruit bodies with high EUC values. High C/N values were beneficial to trehalose, arabitol, malic acid and succinic acid production, while low C/N values were beneficial to mannitol and citric acid production. Fruiting bodies harvested at different growth stages revealed that mannitol contents were higher in the pileus and increased at mature growth stages, while arabitol contents were higher in the stipe and peaked at growth stage 5. Succinic acid contents peaked at stage 5 in the pileus and stipe during mature growth stages. Fruiting bodies harvested at stage 5 contained higher levels of umami components during mature growth stages.

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Tables and Figures

Figure 1. EUCvalues of *L. edodes* harvested at different stages of maturity in the pileus and stipe

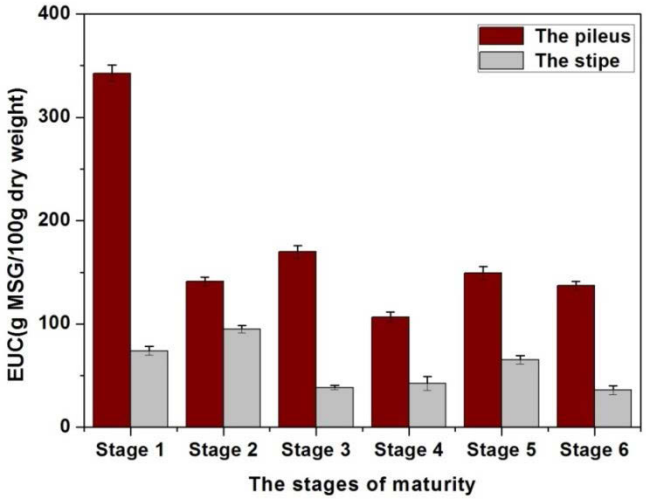
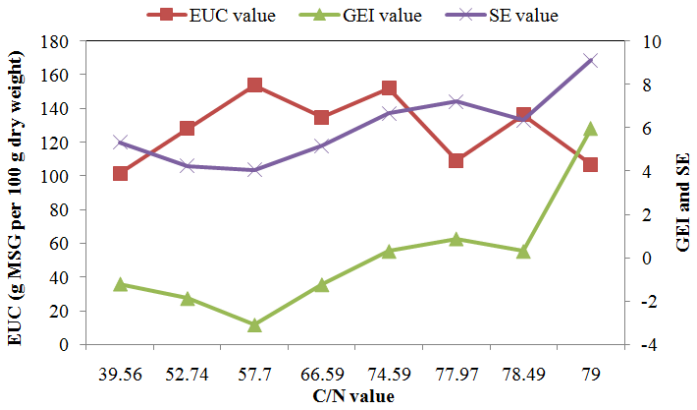


Figure 2. EUC, GEI and SE values of *L. edodes* at different C/N values of culture substrates



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Identification of key taste compounds for *A. bisporus* mushrooms (Forestiere®, Heirloom and XXX)

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ABSTRACT

Market research indicates that "taste" is the number one category motivator for usage of mushrooms. Taste is the perception of salt, sweet, sour, bitter and umami. The water soluble substances in the mouth responsible for each of these tastes include 5'-nucleotides, amino acids, organic acids, soluble sugars and polyols. These taste metabolites give key information to consumers to allow them to identify and appreciate a food as tasty, nutritious and a source of energy. The overall aim of this industry based project is to obtain new research knowledge of the relationship between the taste composition of industrially produced mushrooms and their contribution to the sensory taste perception.

High Performance Anion Exchange-Pulsed Amperometric Detection (HPAE-PAD) was used for analysis of sugars/polyols and amino acids. High Pressure Liquid Chromatography-Diode Array Detection (HPLC-DAD) for the analysis of 5'-nucleotides and organic acids. ^{1}H-nuclear magnetic resonance (^{1}H-NMR) was also incorporated as a potential rapid method for the overall identification of taste metabolites in comparison to lengthy chromatography methods. Sensory analysis included panel development and training (11 females and 7 males). Determination of recognition and detection threshold values for mushroom taste metabolites followed ISO Standard (3972:2011). Preparation of artificial mushroom taste recombinants. Omission studies to determine the key compounds for *A. bisporus* (Forestiere®, Heirloom and XXX) mushrooms.[1] Similar work was previously completed for one white *A. bisporus* mushroom.[2]

Triangle tests indicated very highly significant taste difference between the aqueous mushroom extract for Forestiere® and the white XXX mushroom. A significant taste difference ($p<0.001$) was also observed between the Forestiere® mushroom and Heirloom chestnut mushroom. No significant taste difference was seen between the white XXX mushroom and Heirloom chestnut mushroom. The results show that the key metabolites are different for each of the *A. bisporus* mushroom varieties studied. The key taste compounds for Forestiere® mushrooms were bitter tasting amino acids and 5'-nucleotides. For Heirloom, 5'-nucleotides, umami tasting amino acids and bitter tasting amino acids were found to be significantly important for this mushroom variety. Interestingly, for the white XXX mushroom, the taste panel couldn't detect a significant taste difference for the omission of 5'-nucleotides. Important key taste compounds contributing to XXX white mushroom were umami, bitter and sweet tasting amino acids. Sour tasting organic acids were a significantly important ($p<0.05$) group of taste compounds to all three mushroom varieties studied.

This is the most extensive sensory study ever completed for mushrooms to date. The key taste compounds were identified for each mushroom variety. The concentrations whereby the human tongue can detect and describe each of the important taste metabolites were found.
Keywords: *A. bisporus*; Taste; Chromatography; ^{1}H-NMR; Sensory Analysis;

INTRODUCTION

Mushrooms belong to the Third Kingdom Fungi. Nutritiously beneficial, and among one of the most powerful properties is their taste. Mushroom taste is recognised as being unique, versatile and natural.
Some popular *A. bisporus* mushroom varieties grown commercially include XXX, Heirloom and Forestiere®. These varieties are significantly different in appearance and their taste attributes are often discussed among mushrooms growers and spawn suppliers. However, little research in terms of analytical chemistry of taste metabolites and/or sensory science comparing consumer perception of different mushroom strains has been reported.
The aim of this study was to address key knowledge gaps in the area of mushroom research. This was done in two steps: (1) Development and validation of analytical methods to profile the taste metabolites; (2) Specific sensory tests (following ISO guidelines) to determine perceived taste difference with the trained taste panel. This sensomic approach has been successfully applied for the identification of key taste enhancers in many food types for example, cooked crustaceans, rapeseed oil, sherry, roasted malt and beef bouillion.
In the past, research investigating mushroom taste was based on the calculation of the Equivalent Umami Concentration (EUC). The EUC is a concept developed by Yamaguchi (1971) to describe the intensity of umami taste in different food products. This EUC is non-food specific and was developed empirically taking into account the umami contribution of free amino acids (glutamic and aspartic acids) and 5'-nucleotides (5'-XMP, 5'-IMP, 5'-AMP, 5'-GMP). Although the EUC might provide some guidance of the umami intensity in complex food matrices, its use in foods as a predictive tool is limited. EUC does not take into account other key metabolites that might contribute significantly to taste. Previous studies have also shown no correlation between EUC and perceived umami taste in food products containing shiitake mushrooms.[4]
The aim of this research was to identify the key compounds responsible for mushroom taste using a systematic sensomics approach rather than EUC calculation. The chemical taste differences between the three *A. bisporus* varieties will be a tool used to investigate further the biochemical pathways responsible for the formation of taste compounds. Improve mushroom taste through genetic or cropping practices. Finally this research has been used to educate consumers/retailers on the taste difference between mushroom varieties.

1. Results & Discussion

1.1. Chemical Taste Analysis

Thirty two taste metabolites from three *A. bisporus* varieties (XXX, Heirloom and Forestiere®) at the physiological closed cup stage were identified and quantified at three flushes. Principal Component Analysis (PCA) of the results showed which compounds

differentiate between the three varieties studied. Mushrooms varieties clustered in function of their taste metabolites. The first principal component (PC1) explained 37.2% of the total separation while PC2 explained 13.7%. White XXX mushrooms are correlated with umami amino acids, 5'-nucleotides and trehalose. In general, Forestiere® is correlated with sweeter tasting metabolites (mannitol, glycine and valine, lysine and ribose). Taste metabolite composition for Heirloom and Forestiere® varieties varied with flush stage. Forestiere® Flush 1 had higher levels of mannitol than Flush 2 and Flush 3. Heirloom was found to be more correlated with bitter tasting amino acids. Findings suggest that concentrations of these bitter tasting compounds decrease towards the end of crop, such as Flush 3. Calculation of the dose over threshold (DoT) value for each taste metabolite identified and quantified determine what compounds should have an impact on human taste perception.

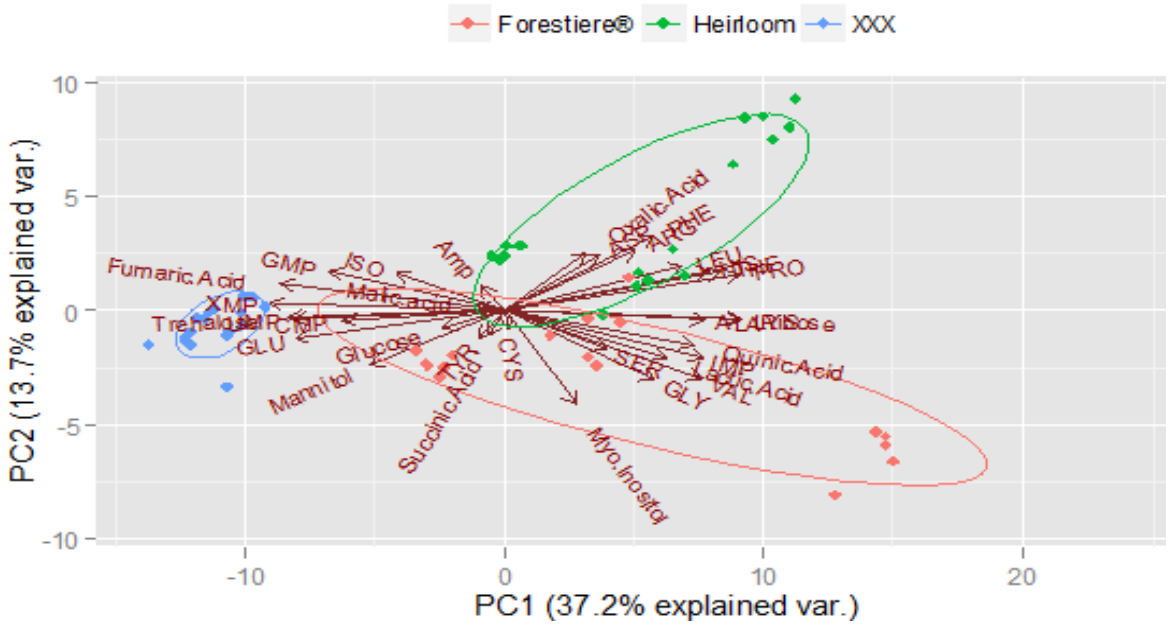


Figure 1. PCA plots to show all taste metabolites for three *A. bisporus* varieties (XXX, Heirloom and Forestiere®)

1.2. Sensory Analysis

The trained taste panel was exposed to the *A. bisporus* aqueous mushroom extracts for XXX, Heirloom and Forestiere®. Triangle tests determined significant taste differences between (Forestiere® and XXX) and between (Forestiere® and Heirloom). Interestingly, no significant taste difference was found between (XXX and Heirloom). This highlighted that Forestiere® was significantly different in terms of taste from the other two varieties. In general the panel responded well to the taste of *A. bisporus* aqueous mushroom extracts. Heirloom was the most accepted mushroom extract with 93.8% acceptability (n=146). XXX was the second most accepted mushroom extracts at 89.6% (n=154) and Forestiere® was the least accepted mushroom with 85.5% (n=158). In terms of taste acceptability, the three strains performed satisfactorily. Results highlight that consumers might have greater acceptance for mushroom varieties that they are more exposed to on a regular basis such as *A. bisporus* varieties (this was confirmed with exotic mushrooms, data not shown).

In order to better understand the relationship between chemical and sensory analyses, artificial mushroom reconstitution and sensory omission studies were carried out.

1.2.1. Artificial mushroom reconstitution studies

The trained sensory panel determined detection and recognition concentrations for all 23 taste metabolites with a Dose Over Threshold (DoT) >1. Based on the chemical profile for each *A. bisporus* mushroom variety, artificial mushroom extracts were prepared using food grade standards. The artificial mushroom extracts were rated by the panel in terms of salt, sour, sweet, bitter, umami and mouth dry/astringent on a scale from 0 (taste not detected) to 3 (very intense). Umami was the most intensely rated taste for both the aqueous mushroom extract and the artificial mushroom extract for all three *A. bisporus* mushroom varieties (Figure 2).

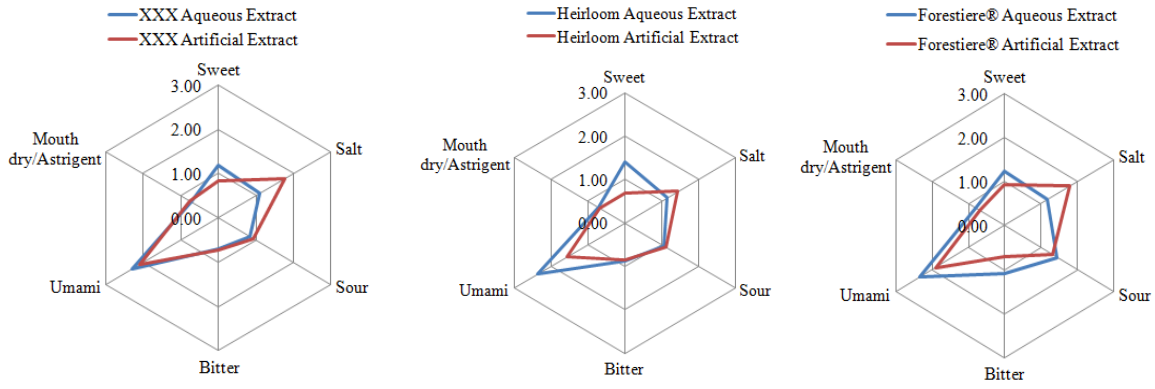


Figure 2. Taste profile for three *A. bisporus* mushroom varieties

1.2.2. Sensory Omission Studies

In order to determine the taste contribution of each individual / group of metabolites, artificial mushroom extracts were prepared specifically omitting these metabolites. These solutions were then presented to the taste panel by means of the triangle test. This was done for the 3 different *Agaricus* mushrooms strains.

The results confirmed what compounds contribute significantly to the general overall *Agaricus* mushrooms taste: aspartic acid, fumaric acid and 1-octen-3-ol. This is the first time that fumaric acid has been identified as a key taste metabolite in *Agaricus* mushrooms.

Other taste compounds contribute significantly to the taste for brown strains (5'-nucleotides). Glutamic acid, valine, isoleucine and leucine were significantly important to XXX and Heirloom varieties. Arginine was significant to XXX and Forestiere®. The omission studies suggest that some compounds such as glutamic acid contribute to the differentiation between some strains (Forestiere versus XXX and Forestiere versus Heirloom).

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Nutritional and Sensory attributes of cookies as influenced by addition of *L.edodes* powder

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ABSTRACT

L.edodes commonly known as Shiitake mushroom is the second largest cultivated edible mushroom in the world. Apart from its excellent nutritional qualities, it contains a large number of bioactive molecules having great therapeutic implications. Despite sporadic research, *L.edodes* is not consumed as part of the regular diet in India. Hence, the study was carried out with the objective of using *L.edodes* powder as a functional food ingredient in cookies. Studies on the effect of substitution of *L.edodes* powder (LP) on the nutritional as well as the sensory parameters of cookies at 5, 10, 15, 20 and 25% levels were carried out. Sensory attributes were studied on nine point Hedonic scale through a panel of nine semi trained judges for standardization of food samples. Proximate composition revealed that mushroom enriched cookies had higher protein, ash and mineral (iron, calcium & phosphorus) content as compared to control. Physical characteristics of cookies showed that the diameter of control and mushroom enriched cookies did not vary markedly. The protein content was found to be increased from 2.2 gm to maximum 2.9 gm/100 gm in the cookies. Also, addition of *L.edodes* powder increased the overall mineral content in them. The sensory analysis indicated that there was no significant difference between the cookies produced even when compared to the control 100% refined wheat flour cookies. The overall acceptance of the cookies was ranked to 10% *L.edodes* powder substitute.
Keywords: *L.edodes,* supplementation, nutritional, sensory, cookies

INTRODUCTION

Mushrooms are usually eaten cooked or raw and as garnishing to a meal. They have also been used in medicine for centuries in the orient but their potential as 'Health potentiators' and 'Elicitors of immune system' have been recognised recently. They are the major source of potent new pharmaceutical products and have become attractive as a functional food and a source for the development of drugs and nutraceuticals. The active constituents found in mushrooms are polysaccharides (β-glucans), dietary fibres, oligosaccharides, triterpenoids, peptides and glycoproteins, proteins, alcohols , phenols, mineral elements[1] such as zinc, copper, iodine, iron, Ca, P ,K, Se, vitamins, amino acids. *Lentinus edodes* (Shiitake) is the second most important cultivated mushroom in the world after *Agaricus bisporus* [2] and is known to have significant pharmacological effects and physiological properties like bioregulation (immune enhancement), maintenance of homeostasis and regulation of

biorhythm, cure and prevention from life threatening diseases such as cancer, cerebral stroke and heart diseases[3]. It also has active bio molecules such as beta glucans and polysaccharides that exhibits antifungal, anti-inflammatory, antitumor, antiviral, antibacterial, hepatoprotective, antidiabetic, hypolipedemic, antithrombotic and hypotensive activities[4]. Since these mushrooms are consumed mainly in Japan and China, their promotion as a health food needs equal attention in India. Moreover, due to their perishable nature and limited savour of people, there is very less consumption of fresh shiitake mushrooms in India. Value addition is one novel way by which there is scope to increase its popularity and acceptance amongst the people as well as to ensure its proper post harvest management. Ready to eat (RTE) foods are increasingly popular with the consumer predominantly due to their convenience of consumption and ease of preparation and storage. Dehydrated mushrooms are used as an important ingredient in several food formulations including instant soups, pasta, snack seasonings, casseroles, and meat and rice dishes in many countries. Keeping all these facts in mind, the present study was undertaken to study the scope of value addition by *L.edodes* in cookies and also its overall acceptability.

1. Results & Discussion

1.1. Proximate analysis of Mushroom fortified Cookies

The crude protein content of cookies prepared from composite flour of wheat and mushroom flour ranged from 6.54 to 9.64%. The protein content increased proportionally with increased mushroom level used to replace wheat flour in cookies formulations. Cookies containing 25 % mushroom flour level significantly recorded the highest protein content (9.67%). These findings are in agreement with previous studies conducted on supplementation of bakery foods with high protein peanut flour [5]. High protein content was reported on the incorporation of defatted soya bean flour for the preparation of cookies[6]. Similar findings were obtained when pigeon pea flour was incorporated with Barnyard millet flour[7.] Our findings indicated that the protein content of the cookies improved with the incorporation of mushroom flour and in line with previous study. Other result showed that the addition of 5%-25% mushroom flour slightly affected the fat content (i.e an increase in its content from 21.67% - 24.23%) compared with plain Wheat flour cookies (20.12%). However, the addition of mushroom flour up to 25% slightly increased the fat content (24.23%) in the cookies (Table 1). The moisture percentage showed a steady increase due to increase in the mushroom flour addition. Meanwhile, even though total ash content was found to increase in line with the mushroom level in cookies formulations, it was not significant. All cookies recorded total ash content ranging from 0.66% - 0.83%. An increase in the total ash content of cookies by incorporation of fenugreek flour has also been observed[8]. On the other hand, Carbohydrate content was reduced as a result of *L.edodes* powder addition.

1.2. Organoleptic evaluation of mushroom fortified cookies

The effects of mushroom supplementation on the sensory characteristics of cookies are presented in Table 2. With the increase in the level of mushroom in the formulation, the sensory scores for shape, colour, texture and odour of cookies decreased and increased sharply. Similar results have been reported where the cookies were enriched with mushroom

powder by replacing refined wheat flour (maida) at 0, 5, 10 and 15% and evaluated for sensory quality characteristics[9]. Result from the work done by others also showed that the percentage of moisture, ash, fat and protein of cookies increased in line with the levels of mushroom powder used except for carbohydrate[10]. Sensory evaluation Sensory evaluation for the color, texture, taste, aroma and overall acceptability were done in order to determine consumer acceptability. A numerical hedonic scale ranging from 1 to 9 (1 is Excellent and 9 for Very poor) was used for sensory evaluation [11.] It was observed that the cookies enriched with 10% of mushroom powder scored highest for all sensory quality attributes as compared to other enrichment levels.

Table1. Nutritional composition of mushroom based cookies*

Treatments (%)	Moisture	Ash	Protein	Fat	Carbohydrate
0	3.38±0.16	0.66±0.03	6.54±0.44	20.12±0.25	69.30±3.01
5	3.42±0.17	0.72±0.03	7.81±0.44	21.67±0.65	66.38±3.01
10	3.45±0.17	0.72±0.03	8.25±0.47	22.98±0.67	64.60±3.08
15	3.50±0.17	0.75±0.03	9.33±0.50	23.01±0.82	63.41±3.13
20	3.52±0.18	0.78±0.38	9.56±0.51	23.98±0.91	62.16±3.17
25	3.58±0.18	0.83±0.38	9.67±0.51	24.23±1.02	61.69±3.18

*(g/100gm)

Table2. Sensory attributes of biscuits incorporated with different levels of *L.edodes* powder

Treatments (%)	Colour	Texture	Taste	Aroma	Overall Acceptability	Total Score
0	8.12±0.78	8.07±1.00	8.05±1.00	7.89±0.93	8.12±0.93	40.25
5	7.87±1.41	6.09±1.48	6.01±1.79	6.38±1.39	6.15±1.48	32.50
10	7.58±1.48	6.69±1.41	6.28±1.51	6.89±1.27	6.25±1.45	33.69
15	6.73±1.58	5.98±1.30	6.23±1.50	5.88±1.14	6.04±1.41	30.86
20	6.64±1.67	5.74±1.23	5.78±1.50	5.71±1.17	5.75±1.37	29.62
25	6.67±1.71	5.21±1.34	5.56±1.52	5.55±1.45	5.15±1.34	28.14

CONCLUSIONS

Cookies made from mushroom flour with 10% addition were high in protein and acceptable. The protein composition of these samples showed that protein, which plays a very important role in improving sensory properties of baking products, could be used for enriching the protein content of cookies. These studies have shown the potential for developing protein-

rich cookies from edible fungi such as shiitake. Also, the flavour and aroma can be enhanced by utilizing certain essence and addition of dried fruits.

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Food for the poorest, a case of applied ecology on the African equator.

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ABSTRACT

Local food production in the Democratic republic of Congo is a key issue for an ever-increasing population. The country lies in different climatic zones, one of which is the lowland rain forest on the equator. Local transport is strongly hampered by failing route maintenance. Slash and burn agriculture, bush meat extraction and subsidence agriculture are common practice. Mushrooms are strongly appreciated as food by the locals. However, up to now, local markets only sell wild or naturally occurring mushrooms, the offer furthermore being limited by the growing season.

A programme has been set up to select local species that are highly appreciated and easy to grow. Tests have been developed to produce spawn and a programme is set up to spread the growing technology to the local population. The technology has to be easy, cheap, using local resources, and has to be adapted to a very hot and humid climate. Contrary to most places, labour is cheap, and optimising yield is not yet a preoccupation. Selected species have to belong to the local food habits in order to avoid promotion for a locally unknown resource. In the region the main energy resource in the countryside still is charcoal. Avoiding further woodland and tree felling in order to sterilise or pasteurise substrates has to be overcome by selecting aggressive growing fungal species with a very large ecological amplitude, even developing on non sterilised or pasteurized substrates. Attention has been paid in minimising culture waste that cannot naturally composted and disposed of.

The project is still in development. Starting from the inventory of mushrooms on sale on the local markets, it became obvious that one of the best candidates for growing is Schizophyllum commune. S. commune has the enormous advantage of being a species largely used in laboratory experiments, of which the genetic code is well-known, with strong enzymatic capacities, fast growing on a wide variety of substrates, easily dried, , conserved and handled , and, most important, a very palatable species to the local taste, making a good price on the local market. However, taste seems to vary according to the isolate. The set-up of the project, the construction of the spawn laboratory, the construction of a primitive mushroom house, the possible use of bamboo containers for growing, and the local culinary skills in preparing and

using Schizophyllum are presented. It became clear that S. commune has a future as an edible species, at least in certain regions of the globe.

1. Results and discussion

Working near the equator, a challenge: physical data.

The mushroom growing project is developed at the University of Kisangani (0°31′0″N 25°12′0″E), Democratic republic of Congo. The city is situated at an altitude of 447 m and lies in a zone characterized by a tropical monsoon climate. The humidity is very high throughout the year. The average rainfall during the driest month (January) is 53 mm; the wettest month (October) has an average rainfall of 218 mm, the average yearly rainfall being 1620 mm. The daily mean temperature during the year varies only slightly and is about 25°C, the average highest temperatures are about 30°C, but peaks up to 36 °C and slightly more, occur; the average lowest temperatures are about 20 °C

Working in the Kisangani town, a challenge: population data and agriculture

The population of Kisangani varies according to the political stability (peace and war). Figures (estimates) over the last decade vary between 600.000 and more than 1,2 million people. The population in the whole of the Congo republic (U.N. estimate 68 million people) is fast growing (currently 2.8 % yearly), 46 % of the population being younger than 15 years, and 41 % of the children are in a serious or critical nutritional situation (Linard et al.,2014; Paluku Bahwere J.-L. & Donnen, P., 2014).

The available land for agriculture very often lies far away from the house: walking one or two hours (single route) to reach the field is not exceptional. Subsistence agriculture through slash and burn technique is still the rule. Industrial agriculture is replaced by small-scale agriculture in an urban or peri-urban setting, mostly deserving local needs.

Mycology in Kisangani: collaboration between Belgian universities and the Kisangani University

In order to support the university curriculum in biology and agriculture, collaboration was set up between the Flemish interuniversity Council (VLIR) and the Kisangani University. In the beginning teaching focused on the background, mainly ecology, and as far is meant necessary, systematics. Afterwards, within the mycological part, mushroom growing and its backgrounds was explained (in French), mainly based on the training program organized by the Belgian spawn producing company Mycelia (Group training on the production of sterilized substrates and cultivation of lignicolous mushrooms; www.mycelia.be), complemented with the experiences in third world countries as the Philippines, using mainly FAO documentation (Mushroom cultivation by people with disabilities - a guide, fao.org) and other literature resources.

The selection of a species to grow

Students had the task to visit the local markets during a year in order to make an inventory of the offer. It turned out that only carpophores picked in the wild are offered for sale.

To our astonishment the "top" species turned out to be *Schizophyllum commune* L. Everywhere on local markets in Central Africa this species can be found. However, as far as we are aware, this species is more appreciated in the tropical rain forest zone than in the miombo woodland zone, were the species is mainly consumed if no other mushrooms are available.
In some other regions of the world *Schizophyllum* is a largely eaten species as well (Ruan Soto F. et al., 2009; Quinonez-Martinez M. et al., 2014).

Advantages and disadvantages to grow *Schizophyllum commune*
Seen from the developed world, *Schizophyllum commune* is very tough, not palatable at all. Furthermore it certainly needs a lot of work to collect the carpophores in order to make a dish. The proof of the pudding, however, is in the eating. *Schizophyllum* has a very good and quite strong taste, but remains tough. In Africa the carpophores are pounded and than used as a side dish or as an ingredient of a cooked meal (Milinganyo Thombo, M.N., 2011). Furthermore polypores or very tough carpophores can be prepared by cooking them with a liquid obtained from a preparation of ashes (generally obtained from baked banana skins, corncobs or palm inflorescence dried and burned) in order to render them soft and palatable. Picking needs a lot of time, but probably less if the carpophores are cultivated instead of being picked in nature. It is tradition that small children, and certainly girls, help the mother in picking. Picking in a mushroom house, near the home, must be less energy demanding than collecting in the wild. Furthermore the harvest is easy to dry and to be conserved until market circumstances (out of the mushroom season) generate a better price.
There are several advantages of growing *Schizophyllum* instead of other species. *Schizophyllum* has a huge ecological amplitude; it is a laboratory "Guinea pig" with a worldwide distribution. It means that its biology is very well known, that it is fast growing, that numerous isolates can be purchased.
A disadvantage is that *Schizophyllum commune* can be a disease agent to humans, responsible for the infection of the respiratory system and even the cause of meningitis (Premamalini, T. et al., 2011).

Spawn production
Using rice without bran was not a success, the whole becoming too sticky, possibly because of the numerous broken grains; using rice grains with their bran (paddy rice) gave good results however.

Substrate inoculation
On logs: on the local market wood logs (up to10 cm diam.), used for constructing huts, were bought and cut in pieces of about 120 cm. Holes were drilled and inoculation took place with the spawn plugs. Logs were covered with palm leaves, kept moist, and after about three and a half month the first carpophores appeared.
In bags: fresh saw dust and shoveling are easy to obtain. Mixed with rice bran and rice kernels they make a very good substrate to be put in bags and to be inoculated. Young, but fully grown stems of a giant bamboo (*Phyllostachys* spec.) are cut just under each internodium, each piece forming a natural container with a diameter of about 10-12 cm. Holes are drilled in

these containers or slits are made with the machete in order to permit oxygen exchange avoiding suffocation of the mycelium and substrate. These containers are filled with substrate and inoculated. Sterilizing the containers had a very negative effect. After sterilization the containers are prone to colonization with numerous fungi during the incubation period, whereas the unsterilized containers remained unaffected on the outside. The substrate has been sterilized or has been inoculated without any treatment (sterilization or pasteurization). Trials growing mushrooms on not heat-treated substrates are only possible with very aggressive mycelia (as those of *Schizophyllum*) and, in order to overgrow the substrate very fast, the amount of inoculum used was higher than normal.
Pasteurization of substrate can also be done under the climatic conditions of Kisangani by using a solar dryer. Furthermore the solar dryer can be used to dry the harvested carpophores in order to conserve them. Up to now drying of carpophores is simply done in the sun, on a cloth on the soil, on a metal roof of a hut, or even along the roadside on the black tarmac, conditions which are certainly not ideal.

Results

After 3,5-4,5 months after inoculation carpophores can be harvested, also on not sterilized or pasteurized substrate. It is now the time to improve and to optimize the culture, having the local circumstances in mind.

The future

In the coming year we will set up a demonstration and training plot, and select woman to train and to set up a cultivation trial within their own agricultural activities, using the local substrates they have at hand. We will not try to grow other species for the time being because *Schizophyllum* is one of the easiest, most aggressive species, which few substrate preferences, and strongly liked by the locals. We hope that some people can make a living or a partial living out of it.
Because of its good taste it is not excluded that *Schizophyllum* may have a future as edible species, in other regions and with other uses than in the tropics. Local recipes in the Kisangani region, belonging to the oral tradition, have been noted; a cooking book with mushroom recipes however, does not exist.
Being aware that under certain circumstances *Schizophyllum* can be used to recycle some waste material, or to produce nutriceuticals, we think it is a species with a great future
It is evident that growing *Schizophyllum* can only have a marginal effect on the food situation, but as it is a fast growing species, as the technology to grow it is very easy, it can be a help for a poor population prone to social instability and even war situations.

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Antiproliferative Activity of Protein Extracts from *Lignosus tigris* (Chon S. Tan) on MCF7 cell line

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ABSTRACT

Mushrooms have received considerable attention for its bioactive compounds with immunomodulatory and anticancer effects. The aim of this study was to investigate the anticancer activity of the fractions and protein components isolated from cold water extract (CWE) of *L. tigris* sclerotium in breast cancer (MCF7) cells. Breast cancer is one of the most common cancers affecting women. Conventional chemotherapy is often limited by the development of drug resistance and various side effects throughout the body. CWE fractionation was carried out on a Sephadex G-50 gel and three fractions of high (HMW), medium (MMW) and low molecular weight (LMW) were collected. High molecular weight (HMW) fraction exhibited greater cytotoxicity compared to other fractions on MCF7 cells. Proteins of the CWE-HMW were isolated by ammonium sulphate precipitation. The CWE-HMW protein was further purified using anion exchange chromatography and protein compounds were identified by LC-MS/MS analysis. Ammonium sulphate precipitated proteins from HMW fraction showed high antiproliferative activity against the MCF7 cells with IC_{50} of 1.17µg/ml. No activity was observed in non-protein fraction of the HMW. Anion exchange chromatographic fractions H1, H2, H3 and H10 showed potent cytotoxicity activity against MCF7 cells with IC_{50} values of 0.79 ± 0.05 µg/mL, 0.72 ± 0.02 µg/mL, 0.78 ± 0.14 µg/mL and 4.17 ± 0.25 µg/mL, respectively and less cytotoxic against breast normal cell lines. LC-MS/MS analysis revealed that lectin was identified as its major component in fraction H10. Mushroom lectins have been shown to possess potential immunomodulatory, antiproliferative and antitumor activities.
Keywords: *L. tigris*, protein, anticancer, MCF7, lectin

INTRODUCTION

Mushrooms have received considerable attention as an edible and medical resource. *Lignosus* spp. also known as the "Tiger Milk mushroom" is a popular medicinal mushroom consumed by

the indigenous people in Southeast Asia and China. The sclerotium is the part of the mushroom with medicinal value and is utilized by the local communities as traditional medicine. In Malaysia, *L. rhinocerotis* has been traditionally utilised by the indigenous communities as general tonic to maintain health and to treat asthma, cough, fever, food poisoning, wounds and breast cancer (Lee et al., 2009).

Studies have reported that the *L. rhinocerotis* sclerotial extracts possess potent antioxidant activity, considerable antiproliferative activity against human breast carcinoma (MCF-7) and human lung carcinoma (A549) cells, immunomodulatory and anti-acute inflammatory properties (Wong et al., 2011; Lee et al., 2012; Yap et al., 2013; Lee et al., 2014). Recently, *L. tigris* (Chon S. Tan), one of the species of the Tiger Milk mushroom has been discovered in the tropical forest located in Pahang, Malaysia (Tan et al., 2013). Recent study has reported that the sclerotium of the *L. tigris* mushroom contains high nutritional value and possesses potent superoxide anion scavenging activity (Kong et al., 2016).

In this study, we aim to investigate the antiproliferative activity of the *L. tigris* sclerotia. Mushroom proteins have been reported to be potential pharmaceutically active components. (Wong et al., 2010). Therefore, in this study, the bioactive proteins that are responsible for anti-proliferative action are isolated and investigated using nano-ESI-LC-MS/MS analysis.

1. Results and Discussion

Cold water extraction of the *L. tigris* sclerotial powder yielded 240 g/kg dry weight of sclerotia with a composition of 40.6% carbohydrate and 7.3% protein. Using the MTT assay, the CWE showed potent antiproliferative activity (IC_{50} = 28.93 ± 7.74 µg/mL) and induced cell death against MCF7 cells in a dose-dependent manner. CWE of the *L. tigris* showed more potent antiproliferative activity against MCF7 cells compared to *L. rhinocerotis* (Lee et al., 2012; Yap et al., 2013).

To further determine the components with antiproliferative properties, CWE was fractionated by Sephadex G-50 gel filtration and three pooled fractions, high (HMW), medium (MMW) and low molecular weight (LMW) were collected. CWE-HMW fraction was the most cytotoxic against MCF7 with IC_{50} values of 4.23 ± 0.08 µg/mL compared to CWE-MMW and CWE-LMW fractions with IC_{50} values of ~35 µg/mL and > 900 µg/mL, respectively (Table 1). Protein in CWE-HMW was precipitated with ammonium sulphate at 100 % saturation. Cytotoxicity of the protein and non-protein parts was evaluated on MCF7 cells. The CWE-HMW protein fraction showed potent cytotoxic activity on MCF7 cells with IC_{50} value of 1.17 ± 0.47 µg/mL, while the non-protein part was not cytotoxic to the tested cell line (IC_{50} = > 1000 µg/mL). HMW proteins were then purified by Resource Q anion exchange chromatography. A total of eleven chromatographic fractions (H1-H11) were collected from the elution with 0.02 M Tris–HCl buffer (pH 8.0) using a linear NaCl gradient. The cytotoxicity activities of the 11 anion exchange chromatographic fractions against MCF7 cells were evaluated using MTT assay. Three eluted fractions, H1, H2 and H3 showed highest cytotoxicity activity against MCF7 cells with IC_{50}

values of 0.79 ± 0.05 μg/mL, 0.72 ± 0.02 μg/mL and 0.78 ± 0.14 μg/mL, respectively, and less cytotoxic against 184B5 cells (breast normal cell line) with selectivity index of 6.14, 3.46 and 2.59, respectively. Fraction H10 showed higher IC_{50} value (4.17 ± 0.25 μg/mL) on MCF7 cells and moderate cytotoxic selectivity to 184B5 cells.

Table 1: Cytotoxic activities (IC_{50}) of *L. tigris* sclerotial extracts and anion exchange chromatographic fractions on breast cancer (MCF7) and normal cell lines (184B5).

Extracts	Cytotoxicity (IC_{50}) (μg/mL)	
	MCF7	184B5
CWE	28.9 ± 7.7	ND
CWE-HMW	4.23 ± 0.08	ND
CWE-MMW	34.75 ± 1.77	ND
CWE-LMW	931 ± 19.8	ND
CWE-HMW Protein	1.17 ± 0.47	ND
CWE-HMW Non-protein	>1000	ND
Anion exchange chromatographic fractions		
H1	0.79 ± 0.05	4.85 ± 0.08
H2	0.72 ± 0.02	2.49 ± 0.40
H3	0.78 ± 0.14	2.02 ± 0.32
H4	4.73 ± 0.22	1.39 ± 0.01
H5	5.66 ± 0.22	6.74 ± 1.15
H6	6.73 ± 0.83	9.56 ± 0.86
H7	4.80 ± 0.24	6.90 ± 0.71
H8	2.21 ± 0.22	2.94 ± 0.09
H9	2.76 ± 0.44	2.48 ± 0.18
H10	4.17 ± 0.25	9.18 ± 0.77
H11	2.44 ± 0.08	2.16 ± 0.25

Abbreviation: CWE, cold water extract; HMW high molecular weight; MMW, medium molecular weight; LMW, low molecular weight; ND, not determined.

Protein composition in the fractions H1, H2, H3 and H10 were further analyzed using LC-MS/MS. LC-MS/MS analysis, using the *L. rhinocerotis* TM02 genome as the matching database. The results revealed that hypothetical protein 876NA and 230NA are the major proteins in fraction H1, H2 and H3. Serine protease was present in the 4 fractions, H1 (11.35%), H2 (9.77%), H3 (7.05%) and H10 (3.80%) and lectin was identified mainly in fraction H10 (44.74%). Yap et al. (2015) has reported that serine proteases isolated from *L. rhinocerotis* sclerotia showed potent antiproliferative activity on MCF7 cells. Lectins from various species of

mushrooms have also been shown to possess antitumor, antiproliferative, and immunomodulatory activities (Xu et al., 2011). This study suggests that serine protease and lectins from the *L. tigris* sclerotia may be the potential bioactive proteins that play an important role in the antiproliferative effect on breast cancer MCF7 cells.

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A New Analytical Fingerprinting Method for Quality Control of Medicinal Mushroom Products

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ABSTRACT

A systematic scientific testing methodology is proposed for medicinal mushroom products. Sixty samples representing 6 species of basidiomycetes and ascomycetes were evaluated for β-(1–3)(1–6)-glucans, α-glucans, and ergosterol. Using a fruit body for a baseline, test results demonstrate that β-glucans and ergosterol provide a value range that permits the development of a fingerprint and enables accurate quality control over a range of medicinal mushroom morphological stages. Testing for α-glucan reveals levels of starch-based adulterants and carrier materials.
Keywords: Medicinal Mushrooms, Mycelium, Alpha-Glucan, Beta-Glucan, Ergosterol

INTRODUCTION

Medicinal mushrooms (MM) is the common name given to certain basidiomycetes and ascomycetes marketed as health foods or dietary supplements. MM are part of an extensive range of health products in Asia where Chinese traditional medicine has utilized fungi and plant preparations for thousands of years. Today, different morphological stages of MM, some traditional and others more modern, are marketed in Asia and internationally. These stages include the fruit body (mushroom), sclerotium, mycelium, and spores. Mushrooms and spores are produced by standard industrial cultivation methods whereas mycelium is produced by fermentation or as a solid state grain spawn. Thousands of branded products made from the various fungal stages are sold worldwide, even though no systematic scientific verification of active or marker compounds has been established. Despite 50 years of research that has identified and characterized the primary medicinal constituents of MM, few pure analytical standards are available with which to measure or quantify them.

To provide a qualitative and quantitative measurement of known active compounds in a MM product, scientifically validated and commercially available methodologies for practical use in quality control were identified, in addition to a method for common product adulterants and carrier materials used in fungal extract products. The fruit body and sclerotium are utilized as a baseline for fingerprinting since those are the stages of MM historically utilized in traditional medicine and upon which most MM research is based. The fruit body is also the standard used for product identity. MM products were evaluated (Tables 1 & 2) on the basis of two primary active compounds of fruit bodies: β-(1–3)(1–6)-glucans and ergosterol. α-Glucan analysis is utilized as a means of identifying starches, which are common carrier materials. Most β-glucan testing methods are designed specifically for grains. The use of these grain-specific tests is not accurate for mushrooms. The presence of starch in a mushroom product can artificially elevate quantitative test results for

polysaccharides and give a false-positive value for the medicinal β-D-glucans. For that reason, it is important to utilize a test method specific to mushroom β-glucans which will also separate the α-glucans from the total.

Table 1. β-Glucan, α-Glucan, and Ergosterol Values of Chaga (*Inonotus obliquus*), *Cordyceps militaris*, *Cordyceps* Anamorphs, and *Trametes* (*T. versicolor*)

Sample	Description	β-D-Glucan	α-Glucan	Ergosterol
M1	Chaga sclerotia*	7.44	0.11	
M2	Chaga sclerotia*	6.79	1.05	0.040
ME1	Chaga sclerotia extract	11.95	1.11	
ME2	Chaga sclerotia extract	11.93	0.80	0.030
GS1	Chaga mycelium - rice	0.0	70.0	
GS2	Chaga mycelium - sorghum	4.18	28.22	0.007
GS3	Chaga mycelium - rice	7.36	44.14	0.000
GS4	Chaga mycelium - rice	6.20	40.17	
M3	*Cordyceps militaris* fruit body*	37.0	2.0	
M4	*C. militaris* fruit body*	34.4	1.7	
ME3	*C. militaris* fruit body extract	33.0	3.7	0.31**
GS5	*Cordyceps* mycelium - oats	1.0	45.1	
GS6	*Cordyceps* mycelium - sorghum	5.2	24.1	
GS7	*Cordyceps* mycelium - sorghum	10.9	53.9	
GS8	*Cordyceps* mycelium - sorghum	1.5	64.0	
GS9	*Cordyceps* mycelium - rice	2.3	46.3	0.006**
PM1	*Cordyceps* mycelium Cs-4	10.6	4.6	0.709**
PM2	*Cordyceps* mycelium Cs-4	7.85	1.71	0.030
M5	*Trametes* fruit body	57.3	0.1	
M6	*Trametes* fruit body	65.9	0.2	
ME4	*Trametes* fruit body extract	55.4	0.4	0.000
ME5	*Trametes* fruit body extract	55.8	0.2	
GS10	*Trametes* mycelium - sorghum	6.70	24.55	0.012
GS11	*Trametes* mycelium - rice	9.06	44.78	0.010
	Brown rice - cooked	0.85	73.67	0.000
	Sorghum grain - cooked	1.89	63.52	

Cordyceps mycelium products = uncharacterized anamorphs labeled as *Cordyceps sinensis*. Cs-4 is produced by fermentation. *Samples tested at SGS Laboratories and Megazyme International Ireland. **Analyses by BioProfile Laboratories. All data are expressed as % dry weight. Empty cell = not tested.

A method specifically designed for β-D-glucans in mushrooms and yeast was developed in 2004 by Megazyme International Ireland (Megazyme, 2016). The Megazyme method detects and quantifies soluble and insoluble β-(1–3)(1–6)-glucans. Using the Megazyme method, all stages of MM products can be tested, whether fruit bodies, fruit body extracts, mycelium, or even grain spawn mycelium. β-Glucan testing was performed by SGS Laboratories, Vancouver, B.C. A subset of sample results was validated and confirmed by the laboratory of Megazyme International Ireland.

Ergosterol, the major fungal sterol, is a definitive marker compound that can be used to assess the amount of fungal material in a given product (Dalla-Santa et al., 2012; Ng et al., 2008). Ergosterol testing was performed by Dr. Katherine Phillips, Virginia Tech, Blacksburg, VA. Dr. Phillips

conducts ongoing research on ergosterol and vitamin D2 (Simon et al., 2011). Ergosterol tests were also performed by BioProfile Laboratories, St. Paul, MN.

The presence of starch in dietary supplements, especially as a carrier for extract products, is an ongoing industry issue. MM grain spawn products present a similar issue, since the grain is not separated from the mycelium. Testing for α-glucans will determine the level of grain residue present in grain-based mycelium. It will also identify starch carriers used in mushroom extracts. Mushrooms contain only minor amounts of starch; on average, less than 3% dry weight (Kalač, 2013). The Megazyme β-glucan test kit also measures α-glucan (Megazyme, 2016).

Table 2. β-Glucan, α-Glucan, and Ergosterol Values of Maitake (*Grifola frondosa*), Reishi (*Ganoderma lucidum*), and Shiitake (*Lentinula edodes*)

Sample	Description	β-D-Glucan	α-Glucan	Ergosterol
M7	Maitake fruit body	36.7	1.2	
M8	Maitake fruit body*	36.0	1.8	
M9	Maitake fruit body*	40.58	2.02	0.490
M10	Maitake fruit body	46.2	5.7	0.391**
ME6	Maitake fruit body extract	37.95	5.23	0.510**
ME7	Maitake fruit body extract	40.80	3.33	0.330
GS12	Maitake mycelium - sorghum	3.46	39.91	0.007
GS13	Maitake mycelium - rice	6.38	44.47	0.032
M11	Reishi fruit body*	33.1	0.1	
M12	Reishi fruit body	29.8	0.7	
M13	Reishi fruit body*	31.9	0.2	
M14	Reishi fruit body	25.40	0.46	0.120
ME8	Reishi fruit body extract	43.47	0.66	0.090
ME9	Reishi fruit body extract	40.77	2.35	0.080**
GS14	Reishi mycelium - sorghum	5.96	29.61	0.019
GS15	Reishi mycelium - sorghum	10.01	31.51	0.006
GS16	Reishi mycelium - rice	4.48	48.88	0.006
GS17	Reishi mycelium - rice	9.76	49.13	
GS18	Reishi mycelium - sorghum	7.3	45.2	
GS19	Reishi mycelium - sorghum	12.9	31.5	
GS20	Reishi mycelium - oats	1.0	38.9	
GS21	Reishi mycelium - rice	1.1	51.4	
M15	Shiitake fruit body	35.39	3.46	0.230
M16	Shiitake fruit body	31.3	0.78	
M17	Shiitake fruit body	34.0	2.10	
M18	Shiitake fruit body	25.60	3.06	0.520
ME10	Shiitake fruit body extract	35.92	2.62	
ME11	Shiitake fruit body extract	30.41	3.77	0.150
GS22	Shiitake mycelium - sorghum	7.20	38.88	0.016

*Identical samples tested at SGS and Megazyme International. **Analyses by BioProfile Laboratories.
All data are expressed as % dry weight. Empty cell = not tested.

Samples of MM were selected from 6 species of fungi in 3 major groupings: fruit bodies, fruit body extracts, and grain spawn mycelium. Commercial mycelium products (grain spawn) manufactured in the U.S. were purchased from online resellers. In total, 22 samples were obtained from 13 companies. Two samples are pure *Cordyceps* mycelium from China. Dried mushrooms (n = 18)

were selected from retail packaged products from China and identified by the author. A second set of mushrooms was purchased fresh in the markets of Vancouver, B.C. by mycologist Paul Kroeger, who then dried them. *Trametes versicolor* was harvested wild in British Columbia. All mushroom extracts (n = 11) were manufactured in China for Nammex. Extracts were prepared by cooking coarsely ground mushrooms for 3 hours in hot water (80 °C), evaporating the fluid and drying it on the mushroom marc. The extraction ratio was 1:1 (1 kg dry raw material = 1 kg dried extract).

Results & Discussion

β-Glucan results show a consistent pattern in fruit bodies and fruit body extracts with a quantitative range of 25.4–65.9% (Tables 1 & 2). It has been reported that β-glucan levels can be affected by the specific cultivar and available nutrients of the substrate (Bak et al., 2015). This may account for some values outside of the range of results. β-Glucan in grain spawn mycelium products is significantly lower and consistently below 10%. This may reflect the dominance of grain, which would dilute the mycelial mass. In some grain spawn products, β-glucan is nearly absent.

Levels of α-glucan are low in the mushrooms and mushroom extracts, which is consistent with historical nutritional data showing little to no starch in mushrooms (Kalač, 2013). This is also an indication that a MM product has not been adulterated by starch. The grain spawn mycelium products contain α-glucan in the range of 24.1–70% (Tables 1 & 2). This provides further evidence that grain is the dominant component of these products and the likely reason for lower β-glucan levels compared to those of fruit bodies and fruit body extracts.

Ergosterol levels are highest in fruit bodies—especially the fleshy fungi—and lower in the polypores and chaga sclerotia. In general, the grain spawn mycelium products contain significantly less ergosterol (Tables 1 & 2). These results indicate that grain spawn mycelium products contain consistently low amounts of fungal tissue.

Using the three specific compounds and the test methods outlined in this study provides a primary means for a comprehensive fingerprint of MM products for the purpose of quality control. Additional compounds specific to individual species, such as triterpenoids in reishi and chaga and cordycepin in *Cordyceps militaris*, will expand and enrich the scope of this fingerprinting method.

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Enhancement of nutritional and nutraceutical properties of *Calocybe indica* by UVB irradiation

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ABSTRACT

Mushrooms have always been appreciated by the consumers not only because of their unique flavour and texture but also due to the presence of immense nutritional and nutraceutical power. Numerous active substances like phenolics, triterpenoids, polysaccharides and ergosterol makes these edible fungi a key source of nutraceuticals. An indigenous mushroom *Calocybe indica* is a very popular speciality mushroom of India. It is known to possess high content of nutraceutical compounds including ergosterol. This ergosterol is a plant steroid which gets converted to vitamin D_2 in the presence of UV rays by a photochemical reaction. In wake of the above facts the current study was carried out to enhance the vitamin D_2 content by UV-B light treatment and its effect on the other nutraceutical properties of *Calocybe indica.* In the present study a UV-B light source having intensity 5.3w/m^2 was used to expose mushrooms for the different time duration of 0 min, 30 min, 60 min and 90 min. Results indicated that the vitamin D_2 content got enhanced with the time duration under UVB light source i.e. 85±0.15, 182±1.6, 187±0.4 and 182 ±0.8 µg/g (dry weight) for 0, 30, 60 and 90min respectively. Highest concentration of vitamin D_2 was found at 60 min duration. Major antioxidant compounds like total phenol contents estimated were also found to be increased after irradiating under UVB light from 7.36± 0.07mg/gm to 47.14mg/g after 60min exposure. Flavonoid content (1.5µg/g in 0min and 1.86µg/g in 60min exposed samples) also showed the similar trend as TPC contents. Results revealed that the irradiation for 60min under UVB light is a cheap and convenient method to enhance the nutritional and nutraceutical contents of *C. indica*.

Key words: Nutraceutical, Antioxidants, Phenolics, Ergosterol

INTRODUCTION

Mushrooms are considered to be healthy due to the presence of great nutritional, nutraceutical and functional properties. High contents of physiologically active components like ergosterol, phenolic compounds, triterpenoids and steroids make them a key source of nutraceutical[1]. Out of these compounds vitamin D is essential not only for the strong bones and teeths but also for cell division and strengthening the immune system. Its deficiency or insufficiency leads to major health problems like rickets, octeomalacia and osteoporosis in children, women and elderly populations. Basically vitamin D_3 (cholecalciferol) is the compound

synthesized in the human skin on exposure to UV rays coming from the sunlight[2]. Similarly mushrooms are the excellent source of ergosterol (Vitamin D_2 / provitamin D) which on exposure to UV light produces vitamin D by a photochemical reaction[3].
Calocybe indica is a popular medicinal mushroom of India. It was first cultivated in 1976 in India[4]. The medicinal properties of this mushroom help to prevent the occurrence of diseases like carcinogenesis, ageing, physical injury, infection and cardiovascular disease[5].

Ergosterol contents have been analysed in *Agaricus bisporus*, *Lentinus edodes*, *Flammulina veluptipes, Grifola frondosa, Pleurotus ostreatus*[6]. *Calocybe indica* also contains ergosterol, however there is no information regarding its content till now. To our knowledge no literature provides information regarding its nutrient content as well as nutraceutical composition. Present paper is an attempt to study ergosterol content present in *Calocybe indica* mushroom and the enhancement of its vitamin D2 content by UV-B at different exposure times (dose-response)$_{çççç}$. The paper also emphasizes the effect of the UV-B light exposure on the other nutraceutical components such as phenolic compounds.

1. Material and methods

1.1 Mushroom sample preparation

Mushrooms (*Calocybe indica*) were obtained from the local market INA, Delhi and were treated on the same day. They were washed, cleaned to remove dirt, chopped lengthwise, placed on shelves and exposed to the UV-B (ultraviolet-B) radiation, at intensity of 5.3w/m2 under a UV-B lamp (Philips UV-B narrowband TL-20W) in a chamber for the time period of 30, 60 and 90 minutes. The irradiated samples were separately freeze-dried/lyophilized and ground using a mortar pestle for further analysis.

1.2 Ergosterol assay

Mushrooms were analyzed according to the method described by Sundar et al[7], modified as given below. Freeze dried mushroom sample powders (0.5 g) were accurately weighed and mixed with 4 ml of sodium ascorbate solution (17.5 g of sodium ascorbate in 100 ml of 1 M sodium hydroxide), 50 ml of ethanol (95% pure), 10 ml of 50% potassium hydroxide (85% pure, Merck Chemicals). The mixture was saponified under reflux at 80 ˚C for 1h. The mixture was extracted with 15 ml de-ionized water, followed by 15 ml ethanol. The pooled organic layers were washed three times with 50 ml of 3% KOH in 5% ethanol. The organic layer was transferred into a round bottom flask, rotary evaporated to dryness at 40 C, and immediately re-dissolved in ethanol. Then HPLC was carried for the quantification of vitamin D using calibration curve.

1.3 Determination of nutraceutical components

1.3.1 Extraction of antioxidant components

Two gram freeze-dried mushroom sample was extracted with 40 mL methanol at room temperature in shaking condition for 48 h in the dark. The extracts was centrifuged and the residues were re-extracted with 10 mL methanol for 2 h. Extracts were combined and

methanol was evaporated to dryness at 40 °C under vacuum using rota vapour re-dissolved in 1mL of methanol.

1.3.2 Determination of total phenolic and flavonoid content

Total phenolic content was measured at 20 mg/ml concentration of the extracts. To 0.1 ml of mushroom extracts in 13% hydrochloric acid/ methanol (60:40, v/v), 2 ml of 2% sodium carbonate followed by 0.1 ml Folin ciocalteu reagents were added. After 30 min absorbance was measured at 750 nm[8]. Gallic acid was used as standard. The results were expressed as mg of Gallic acid equivalents (GAEs) per gram of mushroom extract. The flavonoid content was estimated at concentration of 20 mg/ml. To 1 ml of extracts, 1 ml of 10% Aluminium chloride, potassium acetate (1 M, 0.1 ml) and 3.8 ml of Methanol were added and the mixture was incubated for 40 min at room temperature. Then absorbance was measured at 415 nm[8]. The results were expressed as µg of quercetin equivalents (CEs) per gram of mushroom extract.

2. Results and Discussion

2.1 Vitamin D_2 concentration in Calocybe indica mushroom exposed to UV-B light.

The vitamin D_2 concentrations of *Calocybe indica* mushroom exposed under UV-B for different time duration are shown in Table I. The concentration of vitamin D_2 in unexposed sample was found 85.3±0.15 μg/g (dry weight). Interestingly vitamin D enhanced with the time duration i.e. from 85±0.15 (0 min) to 182±1.6 (30 min), 187±0.4 (60 min) µg/g (dry weight). The highest concentration was found at 60 min 187±0.4 µg/g. whereas at 90 min the content found to be decreased to 182 ±0.8 µg/g. It has been seen that prolonged irradiation does not increase vitamin D2 content, the reason being that prolonged irradiation could produce irreversible ‘‘over-irradiation products’’ by dimerization and ring cleavage.

Table 1: Vitamin D_2 concentrations in *Calocybe indica* after exposing under UVB light at different time duration

Time duration for exposure under UVB light (in minutes)	0 min	30 min	60min	90min
Vitamin D_2 (µg/g/dw)	85.3± 0.15	182.8± 1.68	187.0 ± 0.41	182.8± 0.81

Each value is expressed as mean ± standard deviation (n=3).

2.2 Nutraceutical composition of Calocybe indica

The methanolic extracts of *Calocybe indica* showed a great potential of total phenol content. The content of total phenols and flavonoids is reported in Table 2. The highest content of total phenols was found in samples which were exposed to UVB light for 60 min i.e 47.14mg/g. Babu & Rao[9] have reported similar results in *Calocybe indica* (cap) analyzed for TPC content which was 18.53±0.02 mg/g. In our study flavonoid content also showed the similar trend as TPC contents. The content enhanced from 1.5- 1.86 µg/g, flavonoid content showed the same pattern as the phenolics.

Table 2: Total phenol and flavonoid content of methanolic extracts

Time of exposure	Total Phenols (mg/g)	Total Flavonoid (µg/g)
0min	7.36± 0.07d	1.5±0.07b
30min	18.45±0.39c	1.3±0.1d
60min	47.14±0.17a	1.86±0.05a
90min	30.14±0.34b	1.4±0.05c

Each value is expressed as mean ± standard deviation (n=3). Means with different letters within a row are significantly different ($P<0.05$) using DMRT

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The Medicinal Properties and Efficacy of Cultivated Tiger Milk Mushroom (*Lignosus rhinocerotis* TM02)

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ABSTRACT

Lignosus rhinocerotis, commonly known as Tiger Milk Mushroom, has been used for more than 400 years by South East Asian communities for its healing properties on more than 15 types of medical ailments including asthma, fever, cough, cold, sinusitis, cancer, food poisoning, joint pain, and wound healing. It has also been consumed as general health tonic for improving vitality. However, research and development of this rare mushroom was hindered by its limited supply from the wild. Recent successful commercial cultivation and mass production of *L. rhinocerotis* TM02 has enabled numerous scientific studies to be conducted to verify claims of its bioactivity and, the efficacy and safety profile of *L .rhinocerotis* TM02. Scientific investigations revealed that *L. rhinocerotis* TM02 contains various active components such as the polysaccharides-protein complexes, β-glucan, protein, immune-modulatory proteins, all of which are biologically active in rendering significant medicinal properties such as anti-inflammatory, immuno-modulatory, anti-proliferative, antioxidative, neurite outgrowth stimulation etc. Genomic study has shown the presence of a novel fungal immuno-modulatory protein (FIP) in this mushroom. The gene sequence and three dimensional model of this novel fungal immune-modulatory protein have been recently elucidated. Pre-clinical toxicology evaluations showed that *L.rhinocerotis* TM02 did not induce any adverse effect on fertility, teratogenicity and genotoxicity. The no-observed-adverse-effect level (NOAEL) dose of the *L.rhinocerotis* TM02 in a 180-day chronic toxicity study is more than 1000 mg/kg which is equivalent to 162 mg/kg human dose whilst the anticipated dose for a 60 kg adult is only ~10g. The effective dosage of *L.rhinocerotis* TM02 is 0.5g per day for an adult, therefore there is a wide safety margin for consumption. Based on users' experiences and feedback, *L.rhinocerotis* TM02 has been shown to be effective in a broad spectrum of respiratory health and immune health conditions, maintaining the health of joints, improving vitality and stamina, and improving the quality of life of cancer patients.

Keywords: anti-inflammatory, immuno-modulatory, anti-proliferative, antioxidative.

INTRODUCTION

Lignosus rhinocerotis (Cooke) Ryvarden, locally referred to as Tiger Milk Mushroom, is one of the most popular medicinal mushrooms consumed by the various communities in South East Asia from time immemorial. It belongs to the family of *Polyporaceae* (Basidiomycota) and consists of sclerotium , fruiting body with pileus and stipe (Cooke, 1879). The sclerotium is the part containing valuable medicinal value (Chang and Lee, 2004). Its history of usage can be traced back to 1664 when it was obtained from the local indigenous people and was described as "*Lac tygridis*" (in Latin) and was recorded to be used for treating diseases (Everlyn, 1664). Both Ridley (1899) and Burkill (1966) described a fungi species, *Polyporus rhinocerus* (synonym of *Lignosus rhinocerotis*) (in Malay '*susu rimau*' translating to "tiger's milk") and recorded its use to treat congestion and cough. A survey by Lee *et al* (2009) showed that the *Lignosus* species ('*susu rimau*') is the most common macrofungi used by majority of the indigenous communities in Peninsular Malaysia. These indigenous communities usually use it to treat cough, asthma, fever, cancer, food poisoning, wound healing, and as a general tonic. For postpartum, the traditional way of preparation is to boil sliced sclerotium with other herbs such as "tongkat ali" (*Eurycoma longifolia*) or with *Polyalthia bullata* (black tongkat). For other purposes, it is orally consumed as a decoction (Chang and Lee, 2004; Lee *et al*, 2009). In China, *Polyporus rhinocerus*, or '*hurulingzhi*' in Chinese, is a common traditional medicine to treat liver cancer, chronic hepatitis and gastric ulcer (Huang, 1999). Despite its various traditional claims, there are limited scientific studies of the mushroom mainly due to its scarcity and difficulty in cultivation. It was not until 2009 that the cultivation of *L. rhinocerotis* TM02 was successful and subsequently commercialized. The unlimited supply from its successful cultivation enabled biopharmacological studies, and made therapeutic use possible (Tan, 2009).

1. Results & Discussion

1.1. Identification and Authentication

In Malaysia, three species of *Lignosus* were discovered. Besides the commonly known *Lignosus rhinocerotis*, two other species, *L. cameronensis* and *L. tigris* were identified (Tan *et al*, 2013). They are similar in morphology, and their identification and differentiation is largely based on pileus pore size. However this identification criteria can be imprecise and unreliable. Identification by genetic markers is more precise and hence, genetic markers were developed in 2010 to allow identification of *L.rhinocerotis* in mycelia culture and the sclerotia where pileuses are absent (Tan *et al*, 2010). The nucleotides identity in internal transcribed spacer (ITS) gene region of rRNA of known *Lignosus* sp are sequenced, compared and subsequently, a specific primer pair based on the DNA sequences within the ITS region is designed as the specific genetic marker for *L.rhinocerotis* (Tan *et al*, 2010).

1.2. Healthful composition

This list below are the content of certain important healthful composition found in *L. rhinocerotic* TM02 : β-1,3/1,6-glucan = 286.8 mg/g, α-glucan = 29.91 mg/g, Adenosine (mw 264.24) = 0.282 mg/g, Cordycepin (mw 251.24) = 0.873 mg/g, Adenine (mw 135.13) = 0.024

mg/g, Hydroxyethyl-adenosine (mw 311.12) = 0.268 mg/g, Ethyl-Adenosine (mw 295.12) = 0.399 mg/g, Glycoprotein = 186.1 mg/g, and Total Polysaccharides = 401.4 mg/g.

1.3. Safety Evaluation

As *L. rhinocerotis* became increasingly popular in recent years as a nutraceutical, safety evaluation of the sclerotial powder likewise, has become an important prerequisite. The *Lignosus rhinocerotis* TM02 cultivar were selected for production into sclerotial powder, and the recommended dosage is 5–10 mg/kg/day. Pre-clinical studies (using *Sprague Dawley* rats) were carried out to ascertain its safety profile. Acute (7 days), subacute (28 days) and chronic (180 days) toxicity study revealed no adverse effect with doses up to 2000mg/kg/day (for acute), 1000mg/kg/day (subacute and chronic) respectively (Lee *et al*, 2013; Lee *et al*, 2011). There were no adverse change or toxic effect on body weight, haematological and clinical biochemistry parameters and histological examination . The toxicity studies' dosage is equivalent to 162 mg/kg human dose and the anticipated dose for a 60 kg adult is only ~10g which translates to 0.5g per day for an adult, thus there is wide safety margin. In terms of impact on fertility and teratogenicity, oral administration of 100mg/kg/day sclerotial powder for 7-8 weeks did not cause any adverse effect in rats. *In vitro* assessment of genotoxicity was conducted using Ames test and the results revealed no gene mutations. In conclusion, the no-observed-adverse-effect level (NOAEL) dose for fertility, teratogenicity and genotoxicity studies was determined to be higher than 1000mg/kg (Lee *et al*, 2013). The establishment of safety profile of *L.rhinocerotis* TM02 ensures safe consumption of the mushroom as a nutraceutical to promote general well-being and a multitude of medicinal purposes.

1.4 Biopharmacological Efficacy Assessment Based on Scientific Studies

Research on the pharmaco-properties of *L.rhinocerotis* is essential to generate evidence-based proofs to substantiate the ethno-botanical claims of *L.rhinocerotis*.

Anti-inflammatory: The present study investigates the anti-inflammatory activity of the sclerotia of the *L.rhinocerotis* TM02 using carrageenan induced anti-inflammatory model (rat paw edema). The anti-inflammatory effect of methanol extract, cold water extract and hot water extract was measured via carrageenan-induced paw oedema test in *Sprague Dawley* rat. All three extracts exhibited significant anti-inflammatory effect *in vivo*, with highest potency in cold water extract (Lee *et al*, 2011). There was an unpublished *in vivo* study on ovalbumin-sensitized asthmatic *Sprague Dawley* rats treated with *L.rhinocerotis*. The study showed the ability of *L.rhinocerotis* treatment to reduce total immunoglobulin E in serum, cytokines e.g. IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid, and also reducing lung eosinophil infiltration

Immuno-modulating: Extracts of *L.rhinocerotis*, and particularly the sclerotial polysaccharide constituents exhibit stimulatory effects on human innate immune cells. The constituents have also been associated with immune modulation in preclinical study, and are hypothesised to exert anti-tumor effects as a result of these immune properties (Wong *et al*, 2010; Guo *et al*, 2011). Genomic and proteomic studies showed that *L.rhinocerotis* contains fungal immunomodulating protein (FIP) that may be attributed to this activity (Yap et al, 2014).

Anti-Oxidant : *L.rhinocerotis* TM02 exhibited ORAC 6.0 value of 1,480 umol TE/g. A recent *in vitro* study (Yap *et al*, 2013) suggested that the antioxidant capacity of the mushroom sclerotium is comparable to many other medicinal mushrooms, which is generally moderately low. The water extracts, however, exhibited strong superoxide anion scavenging activity, indicating that the extract might be helpful in preventing certain type of oxidative stress. Taken as a whole, the *L.rhinocerotis* sclerotial powder offers a promising source of functional ingredient . Its efficacy in this function is attributed to its antioxidant capacity, specifically the superoxide anion scavenging activity.

Anti-proliferative : Lai *et al* (2008) was the first to investigate the anti-proliferative effects of the sclerotial polysaccharides of the mushroom. They reported that the hot water extract of *P. rhinocerotis* exhibited anti-proliferative activity against different kinds of leukemic cells. Recently, Lee *et al* (2012) reported that the cold water extract of *L.rhinocerotis* TM02 exhibited direct specific cytotoxicity on human breast carcinoma (MCF-7) and human lung carcinoma (A549) cell lines with IC50 of 96.7µg/ml and 466.7µg/ml. Their results also showed that the cold water extract was essentially not cytotoxic against the normal breast and lung cells. Its cytotoxic action is attributed to a high molecular weight fraction isolated from the cold water extract, and that the cytotoxic action is mediated via apoptosis. The anti-proliferative action against MCF-7 cells provides a plausible scientific basis for the traditional use of *L.rhinocerotis* sclerotia in breast cancer treatment by indigenous communities in Malaysia.

1.5. Efficacy Assessment Based on Functional Observation

A survey was conducted among the volunteers who had taken Tiger Milk Mushroom for various health concerns. The volunteers were given 0.5g of Tiger Milk Mushroom per day continuously for 1-2 weeks and response were collected after the completion of treatment. The volunteers' testimonials can be categorized as follows:

Relief of respiratory-related illness: Tiger Milk Mushroom improves breathing in patients with respiratory health concerns. It also enables them to expel phlegm with ease, especially for volunteers who smoke.

Relief of asthmatic symptoms: One of the most prominent benefits of Tiger Milk Mushroom is its ability to relieve asthmatic symptoms. It improves the patients' breathing and also reduces the frequency of inhaler usage. For those suffering from asthmatic attacks, consumption of Tiger Milk Mushroom was able to shorten the recovery period and decreases the recurrence of subsequent asthmatic attacks.

Treatment of chronic cough: Tiger Milk Mushroom had successfully cured many chronic cough cases, and the testimony of Malaysia's former Prime Minister, Tun Dr. Mahathir (who is a medical doctor by training) was one of the most prominent report.

Relief of allergy: Tiger Milk Mushroom helps to relief various allergy symptoms including respiratory allergy such as nasal and sinus symptoms. In cases of skin allergy such as eczema and allergy to food or chemicals, it has been reported that rashes subsided within few days of tropical application.

Treatment of joint pains: Tiger Milk Mushroom was found to be effective in the treatment of joint pains (ie as a result of dengue fever), one week after consumption. It is also effective

in relieving joint pains in the elderly, rheumatoid arthritis and osteoarthritis patients. This may be attributed to its anti-inflammatory activity.

Improvement of stamina: Tiger Milk Mushroom was also found to improve stamina and alertness in healthy volunteers. It also significantly prolongs the stamina of athletes (unpublished survey).

Anti-tumor: Traditionally, tiger milk mushroom was used to heal cancer, especially breast cancer. Cancer patients who consumed Tiger Milk Mushroom for more than a week showed greatly improved the quality of life and were more energetic.

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Mushroom immunomodulation and germplasm: variation between and within mushroom species and perspectives for application.

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ABSTRACT

The fact that mushrooms modulate the immune system is well documented. Few studies compared the efficacy of different mushrooms species to modulate the immune system, whereas, an intraspecies comparison of strains has hardly been described. Our **aim** was to make this comparison between seven species and within the species *Agaricus bisporus*. The *in vitro* effect on dendritic cells (DCs) as key antigen presenting cells and on cocultures of DCs and T cells was assessed. Thereto, freeze dried pulverized mushrooms were added to *ex vivo* DCs and DC-T cell cocultures. In addition the effect of five *A. bisporus* strains on the development of allergy was assessed *in* vivo. Mice were fed a diet containing 5% freeze dried *A. bisporus* followed by a sensitization and finally a challenge with egg allergen. In both experiments the effect of cytokine secretion by immune cells was measured. In the *in vivo* experiment the acute allergen-specific skin response was measured as well as serum IgE and mast cell protease. The **results** revealed a large difference in cytokine secretion by immune cells as induced by different mushrooms species. Similar results were obtained when comparing different strains of *A. bisporus*. In the *in vivo* experiment it was shown that some *A. bisporus* strains alleviated the onset of egg allergy. This was shown using the acute allergen-specific skin response as well as through the measurement of various immune parameters using isolated immune cells. Taken together it was **concluded** that different mushroom species as well as different strains of one species differentially modulate the immune system. Some *A. bisporus* strains are promising candidates to alleviate the onset of allergy.
Keywords: immunomodulation, *Agaricus bisporus*

INTRODUCTION

The incidence of hypersensitive disorders, i.e. allergies, autoimmune disorders and chronic inflammations, in industrialized countries has dramatically increased over the past decades. Currently 40-50% of the population suffers from at least one of these disorders varying from a mild hay fever to a chronic inflammatory bowel disorder requiring hospitalization. Mushrooms have been shown to modulate the immune system and to remedy infectious diseases, cancer, allergies, autoimmune and inflammatory disorders (Chan, Chan et al. 2009,

Rop, Mlcek et al. 2009). The role of mushroom polysaccharides, notably β-glucans, as immunomodulators has been well documented although other macromolecules and low molecular weight compounds may also play a role (Borchers, Krishnamurthy et al. 2008). In immunomodulation, mushroom macromolecules function as pathogen associated molecular patterns (PAMPs) to be recognized by pattern recognition receptors (PRRs). The PRRs reside on innate immune cells that interact with cells of the adaptive immune system. Although edible mushrooms are non-invasive fungi, mushroom PAMPs are recognized by dendritic cells (DCs), notably in the gut, and may be activated and mature. The mature DCs may stimulate the adaptive immune system through interaction with T cells. Although, stimulation of DCs by mushroom compounds has been described, little comparative data do exist on the immunomodulatory capacity of different mushrooms species or of varieties of the same mushroom species. Here we describe the effect of seven mushroom species on DCs alone and in co-culture with T-cells. Because genetic diversity within a species may also lead to differences in immune modulation we also compared the effect of 16 *Agaricus bisporus* strains on DCs and in DC-T cell cocultures. Five strains were chosen and tested *in vivo* to assess their effect on the development of egg allergy in a mouse model.

Results & Discussion

To assess the effect of mushrooms on dendritic cells (DCs) and on DCs cocultured with T-cells we lyophilized mushrooms and ground them to a fine powder. The powder was used as such or was extracted in boiling water with a subsequent precipitation of the extract with alcohol. Next, 100 µl (500 µg/ml) of the pulverized mushrooms were added to 100 µl murine DCs (1-5x10^5 cells/ml). After 24 hrs the cell culture medium was harvested and cytokines measured or T cells (5x10^5 cells/ml) were added. In the latter case the cells were cultured for another seven days where after cell culture medium was harvested and cytokines measured. In figures 1 and 2 the results of these experiments are displayed. It was revealed that secretion of tumor necrosis factor α (TNF-α) a proinflammatory cytokine was induced by all mushroom species and all strains of *Agaricus bisporus* (figures 1A and 2A). However, the level to which TNF-α was secreted differed between mushroom species and strains. Also, the complete homogenates (CH) and alcohol precipitates (AP) differed in the levels to which they induced TNF-α secretion. Alcohol precipitation usually leads to an enrichment of the β-glucan fraction. Although β-glucans are appreciated for their immunomodulatory properties, the combination with other compounds present in CH preparations may still induce stronger cytokine responses as is seen for several mushroom species. Similar results were observed for interleukin (IL)-6 and IL-12 although the response for most mushroom species lagged behind that of *Agaricus subrufescence* and *Grifola frondosa* (figures 1C and D). The anti-inflammatory cytokine IL-10 was only induced by *Agaricus subrufescence*, *Grifola frondosa* and, to a minor extent, by *Pleurotus ostreatus* (Figure 1C). For the *A. bisporus* strains a differential pattern of cytokine secretion was observed for TNF-α, IL-6 and -10 (Figure 2A-C).

In the DC-T cell coculture experiments it was observed that only the AP and CH preparations of *A. subrufescence* induced both interferon (IFN)-γ and IL-17A. The CH of three other mushrooms did induce the secretion of IFN-γ albeit to a low level (figures 1 E and F). For the

A. bisporus strains it was observed that six of the 16 strains stimulated the secretion of both IFN-γ and IL-17A. The cytokines IFN-γ and IL-17A are the signature cytokines for respectively a T helper (Th)1 and Th17 response. A Th1 response is usually induced upon an infection with intracellular pathogens (viruses, bacteria) whereas a Th17 is induced by extracellular pathogens (bacteria, fungi). However, this is never a black and white situation and often both responses are to some extent simultaneously induced.

The induction of Th1 and Th17 responses by six *A. bisporus* strains urged us to investigate their effect on the development of allergy *in vivo*. Thereto five strains were selected, two good, two medium and one none inducer of IFN-γ/IL17A in the *in vitro* experiment. Mice were fed a diet containing 5% freeze dried *A. bisporus* for two weeks followed by a sensitization and finally a challenge with egg allergen. The effect on cytokine secretion by immune cells ,the acute allergen-specific skin response , as well as serum IgE and mast cell protease were assessed. Two of the five selected *A. bisporus* strains alleviated the onset of egg allergy revealed by the skin response. IL-10 secretion by immune cells from the sensitized mice corroborated these results.

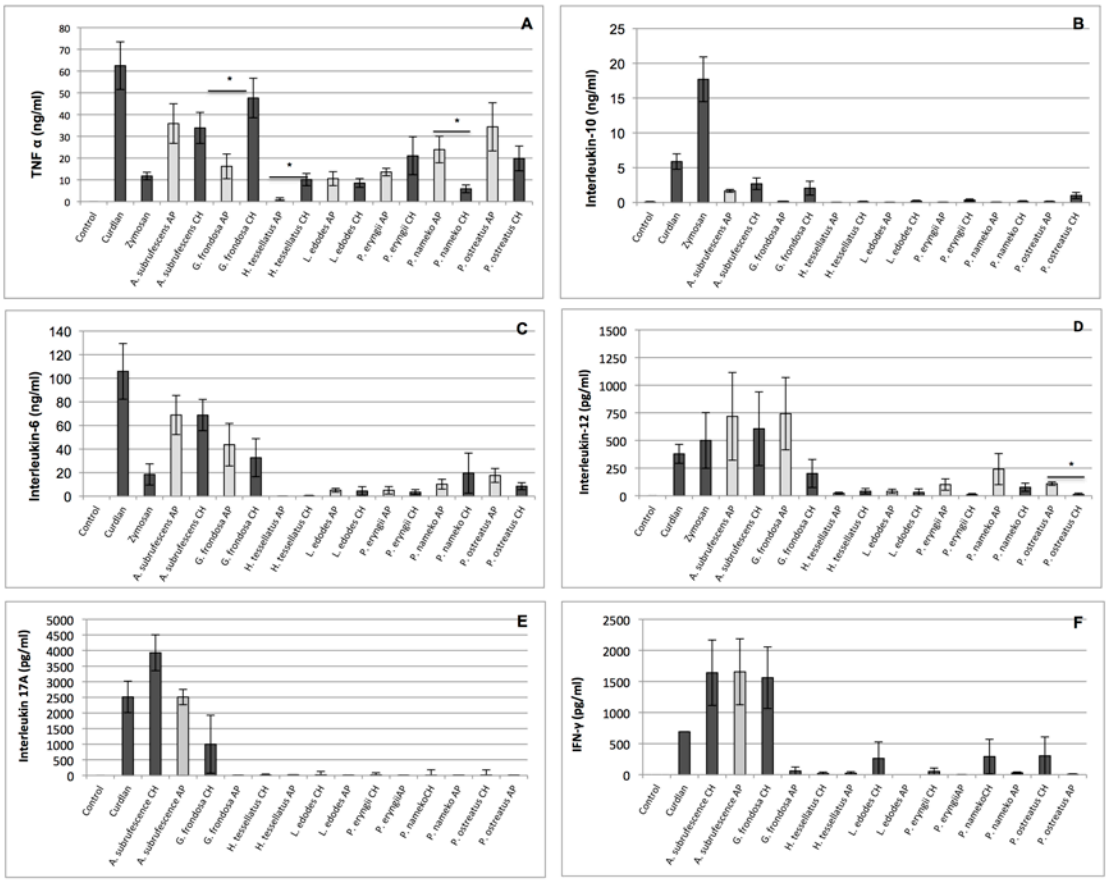


Figure 1. The effect of seven mushroom species on dendritic cells (DCs) (panels A-D) and DC-T cell cocultures (panels E &F). Both complete homogenates (CH) as well as alcohol precipitates (AP) comprising predominantly the β-glucan fraction were evaluated. **A-D**. The results obtained for the cytokines TNF-α, interleukin (IL)-10, IL-6 and IL-12. **E&F**. The results obtained for the cytokines IL-17A and interferon-γ.

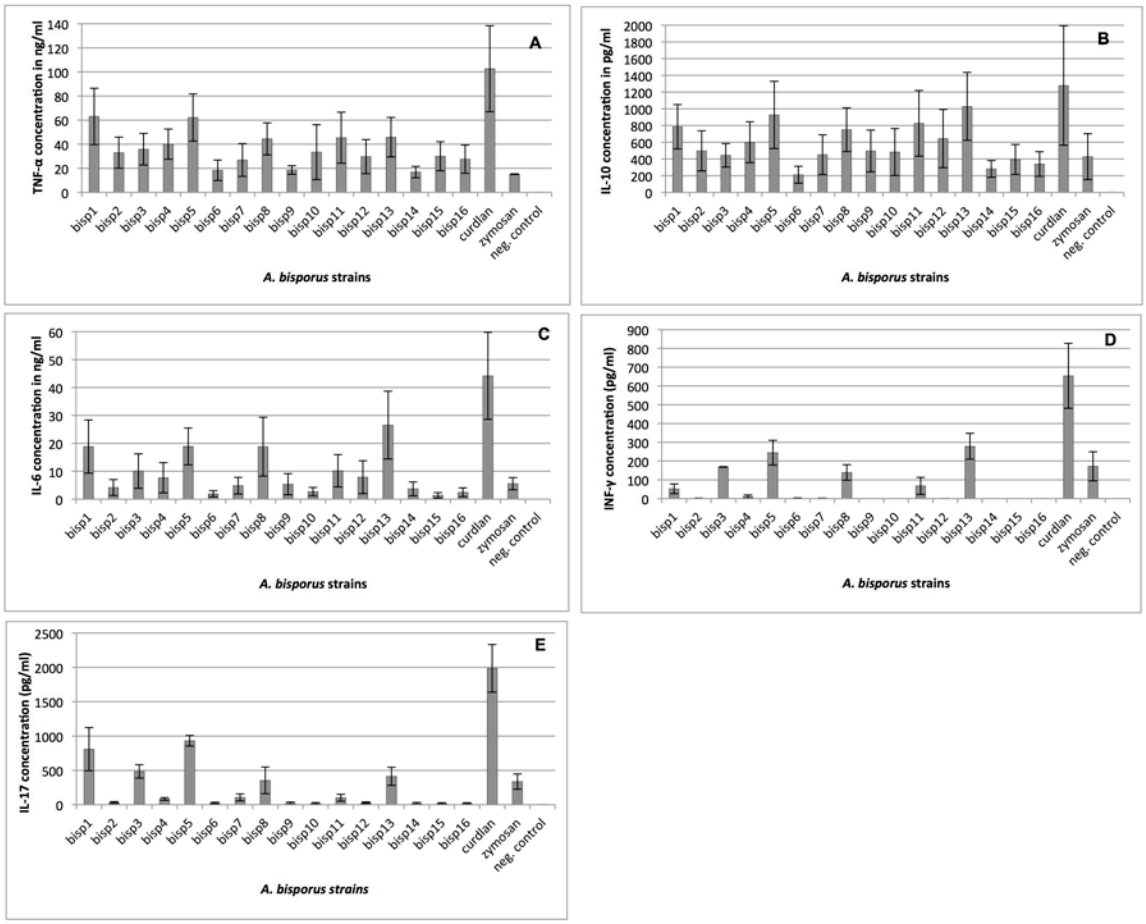


Figure 2. The effect of 16 strains of Agaricus bisporus on dendritic cells (DCs) (panels A-C) and DC-T cell cocultures (panels D & E). Complete homogenates of lyophilized mushrooms were added to DCs and DC-T cell cocultures. The effect on the secretion of TNF-α, IL-6 and IL-10 by DCs (panels A-C) and IFN-γ and IL-17 by T cells (panels D and E) is shown.

In conclusion, both different mushrooms species as well as strains from one species differentially modulate the immune system. In a mouse egg allergy model it was demonstrated that some *A. bisporus* strains are promising candidates to alleviate the onset of allergy.

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Water-Soluble Polysaccharides from *Pleurotus pulmonarius* are resistant to human digestion system

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ABSTRACT

Water-soluble polysaccharides were extracted from *Pleurotus pulmonarius* which will be formulated for food ingredient with prebiotic functionality. To enable food or food component to act as prebiotic, it must first be resistant to acid hydrolysis in the stomach and remain non-digestible by human digestive enzymes. In this study, the in vitro digestibility of the water-soluble polysaccharides (WSP) from *Pleurotus pulmonarius* was investigated against mammalian gastric and pancreatic juices. The WSP was prepared by hot water extraction method and was partially purified via protein removal process. The digestibility of the WSP was determined by calculating the degree of its hydrolysis (based on amount of released reducing sugars) when subjected to artificial human gastric and porcine pancreatic juices. The WSP contained 54.0 % of polysaccharides in which total glucan constituted 31.3 % and non-glucan polysaccharides constituted the remaining 22.7 %. The total glucan was found consisted of 23.5 % of β-glucan and 7.8 % of α-glucan. The WSP was found contaminated with minimal amount of protein (1.94%) and the moisture content of WSP was relatively low (8.70 %). The FT-IR spectra of the WSP was also identical to the FT-IR spectra of the purified β-glucan. Compared to the commercial reference prebiotics [Fructo-oligosaccharide (FOS) and Inulin], which were less stable in the strong acidic environment; the mushroom water-soluble polysaccharides showed much higher degree of resistance to artificial human gastric juice. WSP was highly resistant to both weak (pH 3-5) and strong acidic environment (pH 1-2) and its resistance was not affected by pH of the artificial human gastric juice. In contrast, the resistance of both FOS and Inulin substrates were significantly lower in strong acidic environment (pH 1 and pH 2) than in weak acidic environment (pH 3, pH 4 and pH 5). Under small intestine environment, the corresponding order for hydrolysis by porcine pancreatic juice was WSP > FOS > Inulin. In conclusion, the mushroom WSP is resistant to acid hydrolysis in the stomach and almost non-digestible by human digestive enzymes. The resistance of the tested substrates towards artificial human digestion system followed the order: WSP > Inulin > FOS. The finding also suggests that the WSP from *Pleurotus pulmonarius* fulfil the first prebiotic criteria.
Keywords: *Pleurotus pulmonarius*; Mushroom polysaccharides; Hydrolysis; Glucan; Human digestion system

INTRODUCTION

To enable food or food component to act as prebiotic; Roberfroid (2007) suggest that the following criteria must be fulfilled; 1) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; 2) fermentation by intestinal microflora; and 3) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being. In

this study, as an attempt to evaluate if the water-soluble polysaccharides extracted from *Pleurotus pulmonarius* could fulfil the first prebiotic criteria; we measured the in vitro digestibility of the water-soluble polysaccharides extracted from *Pleurotus pulmonarius* in human digestive tract. If the mushroom water-soluble polysaccharides remained intact after these digestion processes, this indicate that they would be available for subsequent microbial fermentation in the colon and justified to be tested against the second and third prebiotic criteria as mentioned previously.

1. Results & Discussion

Composition and identity of extracted mushroom crude water-soluble polysaccharides (WSP)

The WSP contained 54.0 % of polysaccharides in which total glucan constituted 31.3 % and non-glucan polysaccharides constituted the remaining 22.7 %. The total glucan was found consisted of 23.5 % of β-glucan and 7.8 % of α-glucan. The WSP was found contaminated with minimal amount of protein (1.94%) and the moisture content of WSP was relatively low (8.70 %)

The non-glucan polysaccharides might composed of xylan and cellulose, which were extracted along with glucan in the crude mushroom polysaccharide of P. pulmonarius by hot water treatment (Satitmanwiwat et al., 2012). The results indicated that the hot water extraction procedure executed in our study had successfully produced significant amount of β-glucan rich polysaccharides with minimal contamination of reducing sugars and protein.

Apart from using a mushroom β-glucan kit to determine the presence of β-glucan in a mushroom sample, FT-IR spectroscopy could be applied to achieve the similar purpose. The spectra in Figure 1 was identical to the FT-IR spectrum of the purified β-glucan (90.2%, dry weight basis) generated by Satitmanwiwat et al. (2012).

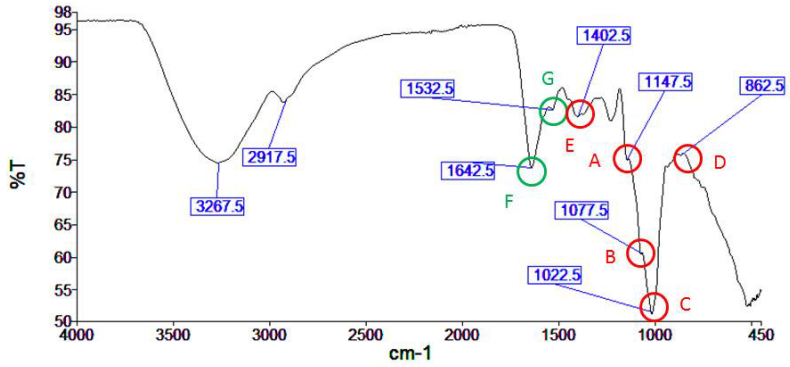


Figure 1. FT-IR spectrum of the crude water-soluble polysaccharide extracted from *Pleurotus pulmonarius*. The peaks at A, B, C and D indicated glucose residues (Gutiérrez et al., 1996). The peaks at E (1402 cm−1) and D (862 cm-1) showed the presence of β-glucan (Gutiérrez et al., 1996). The peaks at F and G might also indicated the presence of protein (Jantaramanant et al., 2014).

Resistance of Pleurotus pulmonarius WSP, FOS and Inulin against gastric and pancreatic juices

As shown in Figure 2, compared to the commercial reference prebiotics [Fructo-oligosaccharide (FOS) and Inulin], which were less stable in the strong acidic environment; the mushroom water-soluble polysaccharides showed much higher degree of resistance to artificial human gastric juice. WSP was highly resistant to both weak (pH 3-5) and strong acidic environment (pH 1-2) and its resistance was not affected by pH of the artificial human gastric juice. In contrast, the resistance of

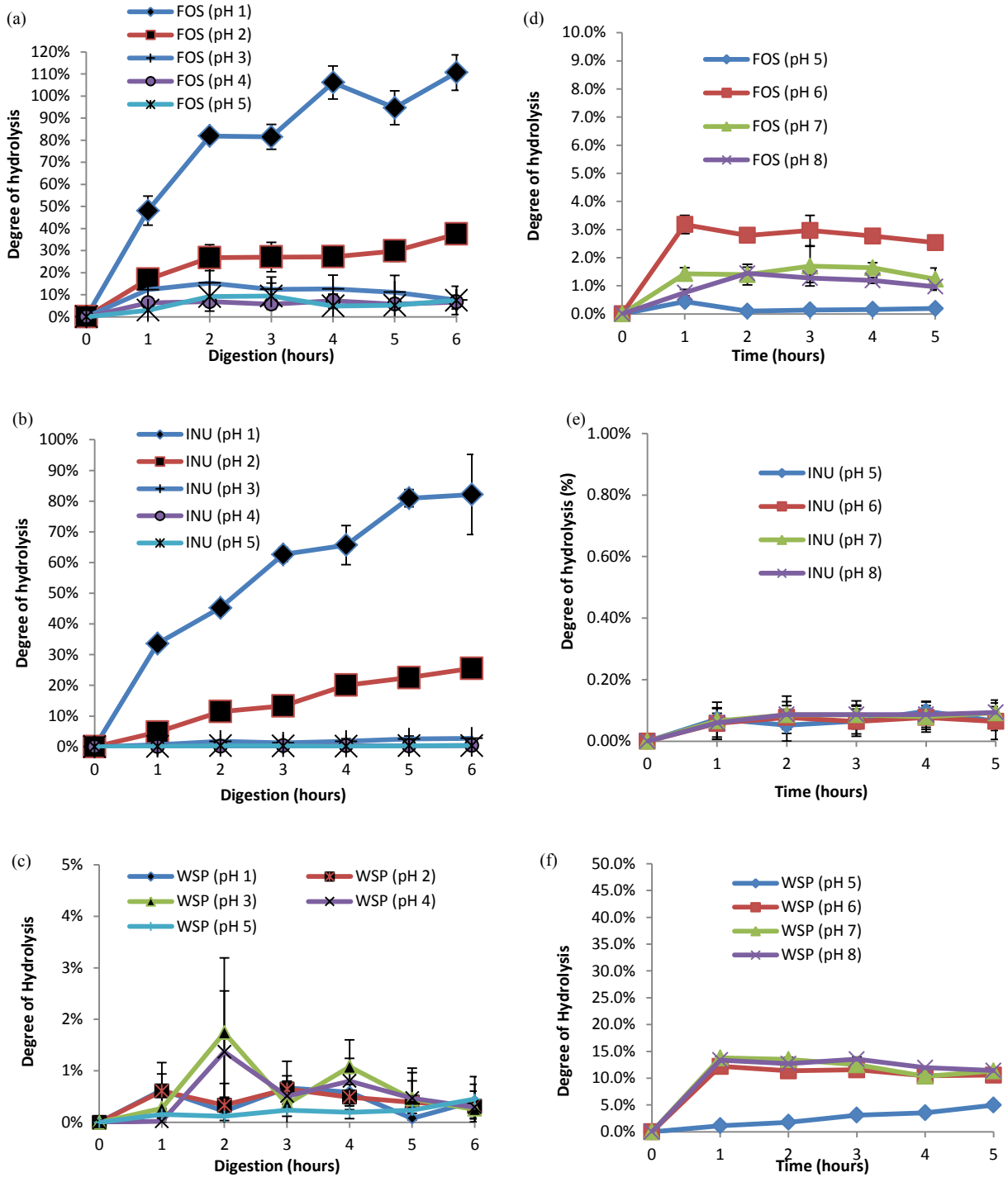


Figure 2. Hydrolysis (%) of Fructo-oligosaccharide (FOS) (a), Inulin (INU) (b), and mushroom water soluble polysaccharides (WSP) (c) by artificial human gastric juice; incubated at 37°C for 6 h. Hydrolysis (%) of Fructo-oligosaccharide (FOS) (d), Inulin (INU) (e), and mushroom water soluble polysaccharides (WSP) (f) by pancreatic juice at various pH values, incubated at 37°C for 5 h. Each data point is the average of three independently replicated experiments. Error bar represent standard deviation of mean, n=3.

both FOS and Inulin substrates were significantly lower in strong acidic environment (pH 1 and pH 2) than in weak acidic environment (pH 3, pH 4 and pH 5). Under small intestine environment, the corresponding order for hydrolysis by porcine pancreatic juice was WSP > FOS > Inulin ($p<0.05$).

Table 1. Estimates of indigestibility (%)* of FOS, Inulin & WSP after digestion by gastric (pH 1, 2, 3, 4 & 5) and pancreatic (pH 6-8) juices

Treatments	pH of gastric juice				
	1	2	3	4	5
FOS	$0.0\pm0.00^{a,A}$	$61.3\pm0.50^{a,B}$	$86.4\pm0.71^{a,C}$	$91.8\pm0.75^{b,D}$	$91.7\pm0.75^{b,D}$
Inulin	$17.8\pm0.00^{b,A}$	$74.4\pm0.01^{b,B}$	$97.9\pm0.01^{b,C}$	$99.6\pm0.01^{c,C}$	$99.7\pm0.01^{c,C}$
WSP	$86.50\pm0.80^{c,A}$	$86.4\pm0.80^{c,A}$	$86.2\pm0.80^{a,A}$	$86.3\pm0.80^{a,A}$	$86.6\pm0.80^{a,A}$

* $I = \frac{R(PANC)}{B} \times 100$, where I was indigestibility (%), R(PANC) was residue of substrate after first digestion by artificial human gastric juice at pH 1, pH 2, pH 3, pH 4 and pH 5, respectively and subsequent digestion by porcine pancreatic juice at near neutral environment (pH 6, pH 7, pH 8) (g); and B was initial amount of substrate before digestion by gastric and pancreatic juices (g). Each data point is the average of three independently value. ^{abcd}Means in the same column followed by different superscript lower case letters are significantly different ($p<0.05$). ^{ABCD}Means in the same row followed by different superscript upper case letters are significantly different ($p<0.05$).

Indigestibility of Pleurotus pulmonarius WSP, FOS and Inulin

As shown in Table 1, the mushroom WSP was resistant (more than 85 %) to both weak (pH 3, pH 4, pH 5) and strong acidic environment (pH 1, pH 2). In contrast, FOS and inulin showed a different trend in both environments. The indigestibility (%) of both substrates were significantly higher (more than 85 %) in weak acidic environment (pH 3, pH 4, pH 5) than in strong acidic environment (pH 1, pH 2) ($p<0.05$). At strong acidic environment (pH 1, pH 2), except WSP, a large percentage of FOS and inulin(more than 25 %) were hydrolysed by artificial human digestion system. In fact, at pH 1, all the FOS was totally hydrolysed while there was 17.8 % of inulin still remained intact.

In conclusion, the mushroom WSP is resistant to acid hydrolysis in the stomach and almost non-digestible by human digestive enzymes. The resistance of the tested substrates towards artificial human digestion system followed the order: WSP > Inulin > FOS.

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VII Genetics & Breeding

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Development of a Revised UPOV Guideline for New Agaricus Mushroom Varieties

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ABSTRACT

Several breeding programmes exist throughout the world for the creation of new Agaricus mushroom varieties. Due to the easy nature in which Agaricus mushroom varieties can be propagated and plagiarised, an efficient system of intellectual property protection is required to safeguard the investment of any breeder creating a new variety. The International Union for the Protection of New Varieties of Plants (UPOV) provides and promotes an effective system of plant variety protection, with the aim of encouraging the development of new varieties of plants, for the benefit of society. At present, 74 countries and Intergovernmental Organisations, such as the European Union, are parties to the UPOV Convention. Although Agaricus mushrooms belong to the Fungi kingdom, UPOV does allow its system to be utilised for the protection of this and other mushroom genera such as Pleurotus and Shiitake. Before any variety can be awarded a grant of protection, it has to undergo a technical examination to assess whether it is distinct, uniform and stable (DUS). For this purpose, UPOV has developed in collaboration with its Member States, numerous Test Guidelines to provide technical guidance on how to test new candidate varieties in individual species or genera. In 2010, UPOV created an initial Test Guideline for Agaricus mushroom (TG/259/1) based upon the knowledge of a limited number of varieties in the marketplace. This guideline contains a table of 21 morphological and physiological characteristics, which have to be express themselves phenotypically and which need to be observed visually. After five years of working with TG/259/1, examination authorities and breeders of Agarics mushroom verities expressed a need to improve the existing Test Guideline to take into account new traits and to identify varieties more clearly. The Community Plant Variety Office (CPVO) of the European Union was appointed as the Leading Expert for the revision of the Agaricus Test Guideline, with the assistance of interested experts from Hungary, the Netherlands, France, Japan, South Korea, as well as individual breeders and breeders' organisations. Brainstorming meetings and practical sessions manipulating Agaricus fruit bodies have been held between the experts throughout 2015 detecting enhancements to the Test Guideline. Up to a dozen new characteristics have been proposed, including those on mycelium, pin setting, and the opening of the cap. The collaborative work amongst experts will continue into 2016, with the aim to have a revised Agaricus Test Guideline adopted by UPOV in 2017.
Keywords: UPOV, Test Guideline, DUS, TWV, Leading/interested experts

INTRODUCTION

Agaricus mushrooms are a popular food crop in several countries throughout the world which can be eaten roar or cooked. To the consumer, Agaricus mushroom varieties look more or less the same, the main differentiating trait being whether they are white or brown mushrooms. In reality, there are numerous Agaricus varieties of common knowledge; some have been around for some time, others are no longer available, and a smaller number have been created recently. For this latter group of varieties the challenge is twofold: how to differentiate a new variety from already existing varieties, and how to ensure that it will not be plagiarized.

1. Results & Discussion

1.1. The UPOV System

There are about half a dozen major breeding programmes on Agaricus mushrooms in the world. These strive for important economic traits: good shelf life, attractive appearance, firmness, eating quality, etc. The Agaricus mushroom breeders expressed a decade ago the need to have an effective intellectual property rights (IPR) system to ensure that new varieties can be clearly identified and protected. The International Union for the Protection of New Plant Varieties (UPOV) is a *sui generis* IPR system which has been specifically adapted for the process of plant breeding and has been developed with the aim of encouraging breeders to develop new varieties of plants. The UPOV system is also open to the protection of new varieties from the Fungus kingdom, including Agaricus. At present there are 74 Members to UPOV, with new members joining each year. Apart from individual countries, the UPOV system is also open to intergovernmental organisations; the European Union has been a Member since 2005.

1.2. UPOV Test Guidelines

Under UPOV principles a variety can be afforded plant breeders' rights if it is: distinct, uniform, stable (DUS), novel, and has an acceptable variety denomination. An examination authority carries scientific trials in the open, greenhouse, or under laboratory conditions, to judge whether a new variety is DUS. The examination authority determines this by observing a series of phenotypic characteristics which found within any particular botanical taxon. UPOV has developed test guidelines for varieties of the most popular botanical taxa being applied for protection. Each of these test guidelines includes a section on: (i) the subject of the test guideline, (ii) the material required, (iii) the method of examination, (iv) the assessment of DUS, (v) the grouping of varieties and organization of the growing trial, (vi) an introduction to the table of characteristics, (vii) the table of characteristics, (viii) explanations on the table of characteristics (ix) a literature review, (x) the technical questionnaire. At present there are 312 UPOV test guidelines, which are updated periodically in conjunction with its Members to reflect the breeding advances within the botanical taxon. The UPOV Technical Working party for Vegetables (TWV) has been responsible for developing test guidelines in Fungi. A first version of a test guideline for Agaricus mushrooms – TG/259/1 – was approved by UPOV in 2010. Test guidelines were also created for Shiitake mushrooms

in 2012, and for Pleurotus mushrooms in 2013. All 312 UPOV test guidelines are openly accessible via the UPOV website.

1.3. The Current UPOV Test Guideline for Agaricus mushroom: TG/259/1

The original version test guideline for Agaricus was conceived by the TWV in 2007 - 2009 and approved by the UPOV Technical Committee in March 2010. It consists of 21 morphological and physiological characteristics, the most emblematic of which are: the longitudinal shape of the stipe; the longitudinal shape of the cap; the colour of the cap; the average number of spores of the basidium; the shape of the central part of the upper side of the open cap; the time of first day of harvest. These six characteristics are used for grouping in the DUS trial by the examination authority, to select the most similar varieties of common knowledge to grow alongside the candidate variety. The scope of TG/259/1 covers the whole of the Agaricus genus; to date, four varieties of *Agaricus bisporus* and one variety of *Agaricus subrufescens* have undergone DUS testing according to this guideline. These varieties have only been applied for Community plant variety rights (CPVR) at the level of the European Union, and are DUS tested by the National Food Chain Safety Office in Hungary (NEBIH).

Although, the current Agaricus test guideline works adequately, the Community Plant Variety Office (CPVO – which manages the CPVR system), NEBIH and the various Agaricus mushroom breeders, recommended making a thorough revision to test guideline to make it more precise and pertinent to the needs of stakeholders. It was felt that some characteristics had to be deleted and new ones introduced to make the test guideline more useful and discriminatory for new Agaricus varieties. Furthermore, the breeders of Agaricus mushrooms expressed that the variety descriptions emanating from the current test guideline were not proving to be precise enough for identifying the most recent varieties in the marketplace. Such inadequacies could lead to the protection being afforded by CPVR not being robust enough, with a reduction of minimum distances between varieties and difficulties in enforcing a title of protection.

1.4. The revision process of the UPOV Agaricus guideline: TG/259/2 Proj.

During the 48th session of the TWV in June 2014, the CPVO proposed that a revision to the current UPOV Agaricus test guideline be launched in 2015. The TWV accepted the proposal, and nominated the CPVO as the Leading Expert, with assistance from Interested Experts from Hungary (NEBIH), the Netherlands (Naktuinbouw), France, South Korea, Japan, and breeders representatives. The Agaricus breeders who would be actively contributing to the revision process were: Limgroup, Sylvan Somycel S.A., Amycel SARL, Wageningen University & Research Centre, INRA Bordeaux, and Lambert Spawn.

The CPVO first utilised the electronic Test Guideline template developed by UPOV for the creation or revision of any test guideline. The aforesaid template proposes standard text and additional text which has to be used by the Leading Expert when devising a test guideline, to ensure a common format for each UPOV test guideline. Once some of the standard text normally applied to plants had been adjusted specifically for fungi, the CPVO distributed a document to the other interested experts; this included the 21 characteristics found in the

current Agaricus test guideline, to see which ones needed to be retained in the future TG/259/2.

During the ensuing period between March and April 2015, the interested experts made several proposals for improvements to the Agaricus test guideline, by adding a further 14 characteristics and deleting 4 of the current characteristics. Proposals for improvements to several of the current characteristics in the test guideline were also made. By the time the formal Agaricus sub-group meeting met at the 49th session of the TWV on 16 June 2015, the firsts draft of the revised UPOV Agaricus guideline was almost 50% different to the version presently in force.

In the TWV Agaricus sub-group meeting itself, lively discussions were held between the Leading Expert, the Interested Experts and the UPOV Office on basic principle issues as well as different interpretations on numerous characteristics. Due to a lack of time, the sub-group only managed to work through one third of the table of characteristics. Greater harmonisation was still required amongst the experts, since there were noticeable differences of opinion on which characteristics should be retained and how they should be observed.

A separate day-long working group meeting was consequently organised on 11 December 2015 to make substantial progress on the document in advance of the 50th TWV session at the end of June 2016, when the second draft of TG/259/2 would be formally discussed. The main agreement resulting from this meeting was that the scope of the future TG/259/2 should be reduced from the whole Agaricus genus, to cover only varieties of *Agaricus bisporus*. The working group expressed that most of the current and future Agarics mushroom varieties would be limited to that species, so the test guideline would be more precise and scientifically robust if characteristics were more discriminatory with respect to varieties within a single species, rather than trying to cover the whole genus. By reducing the scope of the test guideline, the number of grouping characteristics could also be reduced to just three: cap colour, colour of the gills, and presence of spores, which meant simplifying the design of the DUS trial and reducing the number of varieties of common knowledge varieties which had to be taken into account.

The positive effect which the proposed reduction of scope of TG/259/2 to just *Agaricus bisporus,* is substantial on the table of characteristics. Most of the characteristics proposed in the first draft of the revised test guideline have been retained, with an improved methodology on how they should be observed. The practical demonstrations with actual fruit bodies during the meeting of 11 December 2015 meant that there is now common agreement between breeders and examination authorities on how characteristics should be interpreted in the DUS test.

CONCLUSIONS

The Leading Expert will now circulate the second draft of TG/259/2 amongst the Interested Experts between March-May 2016 for comments, before its formal presentation to the Agaricus sub-group at the 50th session of the TWV. If there is acceptance at the TWV of the latest draft of TG/259/2, then the document could be proposed for adoption at the UPOV Technical Committee in April 2017; otherwise, discussions would have to be prolonged for a further year.

The eventual adoption of a revised UPOV test guideline TG/259/2 for *Agaricus bisporus* will provide authorities, breeders and users of new varieties of this species with a clear document on how DUS tests are to be undertaken and on the results emanating thereof. This will result on a more robust level of IPR for the protected varieties, which will ensure these are not copied or propagated illegally. In turn this should spur the development of more and improved *Agaricus bisporus* varieties for the marketplace.

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The typical life cycle of the button mushroom *Agaricus bisporus* var. *bisporus*: Implications for breeding and protection of new cultivars

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ABSTRACT

The button mushroom (*Agaricus bisporus*)is one of the world's most cultivated mushroom species. In spite of its economic importance, new cultivars by outbreeding have been hardly produced in the last 40 years. One of the reasons for this lack of breeding effort is the difficulty to introduce new traits. This is caused by the atypical meiosis in button mushrooms. Recombination between homologous chromosomes is restricted to chromosome ends resulting in the inheritance of nearly parental type of chromosomes. In addition, instead of four spores, most basidia produce two spores in which non-sister nuclei are paired. As a result, these spores are fertile and each produce mushrooms that are genetically and phenotypically very similar to the parental line. Genetic analysis has shown that most present-day white commercial cultivars are derived in this way from the first hybrids released in 1980. These were generated by outcrossing and required a considerable investment. The selection of fertile single spore cultures from protected varieties and the use of these to generate new cultivars should be considered as the generation of essentially derived varieties (EDV) similar to how derived varieties in plant cultivars are defined. A working group of 4 spawn/breeding companies and two research groups has been formed to come to a consensus what should be considered as essentially derived varieties in cultivars of edible fungi. The initiative of this group is supported by the international organization of plant protection (UPOV), the European agency that manage the system of plant variety rights (CPVO) and the ISMS. Next generation sequencing technologies can support the identification of copied, derived and genially new cultivars.
Keywords: Plant variety rights, essentially derived varieties, EDV, Agaricus bisporus.

INTRODUCTION

The generation of new cultivars by breeding can play an important role to improve profits for primary producers and to increase the market share of food products. Good examples are the wide range of new cultivars generated in tomatoes and sweet peppers and there are no reasons to

believe that this cannot be done for edible mushrooms. The button mushroom (*Agaricus bisporus*) is one of the most cultivated mushrooms in the world. It is the predominant mushroom cultivated in the western countries and the cultivation area is extending fast in China (Zhang et al, 2014). Cultivars resistant to diseases, improved shelf life and an increased efficiency of substrate utilization can contribute enormously to the profitability of a crop for growers. The generation of cultivars with an improved taste, better nutritional values and health beneficial components can increase the consumption of mushrooms and thus increase the market share. Previous research has shown that there is variation for a number of these traits in collections and thus a source for breeding new cultivars (Gao et al., 2015). It is thus surprising that hardly any breeding is done since the last 40 years for this crop with a considerable economic value. The main reason lies in the typical life cycle of this fungus since the introduction of new traits is often accompanied by dragging in unwanted inferior traits. We discuss here briefly what genetic analysis has been done to unravel the life cycle of *A. bisporus* var. *bisporus* in detail thus explaining the bottleneck in breeding. We will also discuss the relatively easy way to generate look-a-likes from existing cultivars by selecting fertile heterokaryons of existing cultivars and how this hampers investment in breeding. Finally, we will do some suggestions what genetic analysis might be useful to discriminate cultivars generated by outcrossing and the generation of look-a-likes by screening fertile offspring of existing cultivars. This can help to define what should be considered as a new cultivar according to the definition of the UPOV or what should be considered as a cultivar that is essentially derived from protected cultivar and thus being subject to negotiation with the first breeder for a license to produce.

1. Result & Discussion

In *A. bisporus* var. *bisporus* most basidia produce 2 spores each receiving non-sister nuclei after meiosis (Callac et al., 1993). Only a few percentage of the basidia have 3-4 spores which will germinate into homokaryons which can be used for outbreeding. All commercial and most wild collected strains have this so called secondary homothallic life cycle (Xu et al., 1998).

Crossover landscape in *A. bisporus* var. *bisporus* and the hurdles for introduction of new traits

Previous research has shown that in the var. *bisporus* recombination between homologous chromosomes in meiosis I is restricted to chromosome ends (Sonnenberg et al. 2016, submitted). We have used for this analysis one of the first commercial hybrid generated in 1980 (Fritsche 1981) which was made by crossing a homokaryotic single spore isolate (SSI) of a traditional white cultivar and a SSI from a traditional off-white cultivar. The homokaryons of this hybrid (Horst U1) have been *de novo* sequenced (Morin et al, 2012; Sonnenberg et al, 2016 submitted) and were used in our study as high quality reference genomes. We have subsequently resequenced the whole genome of 2 homokaryotic SSI and the constituent nuclei of 2 heterokaryotic SSI of the cultivar Horst U1. This allowed the precise mapping of recombination in these 8 nuclei on each of the 13 chromosomes and revealed 52 crossovers (CO) of which more

than 90% are located within 100 kb from chromosome ends. As a result, the major part of each chromosome has remained unchanged after meiosis and are thus nearly parental types. The introduction of only a relevant part of chromosomes of a donor strain bearing the desirable alleles into cultivars is thus very difficult and this linkage drag is one of the major problem in restoring quality in cultivars when using outcrossing in breeding (Gao et al. 2015). The combination of non-sister nuclei bearing nearly parental type chromosomes leads also to heterokaryotic offspring that has a genetic make-up that is very similar to the parental cultivar. The recombination regions account for less than 8% of the genome and can lead to loss of heterozygosity in case of non-reciprocal recombinations. Next to this, the main difference between the heterokaryotic offspring and its parent is the reshuffling of parental chromosomes over the nuclei due to the independent segregation of chromosomes in meiosis I. Using a limited set of SNP markers, the constituent nuclei of sixteen heterokaryotic SSI derived from Horst U1 were recovered by protoplasting and genotyped. This confirmed the first findings, i.e. all chromosomes are nearly parental type with a different distribution over the nuclei. Similar outcomes have been found with unrelated *A. bisporus* var. *bisporus* strains (Gao et al., 2015).

The origin of present-day white hybrids

We have resequenced the constituent nuclei of two white commercial cultivars that were marketed after the release of Horst U1. The constituent nuclei of first one, Somycel 608, contain chromosomes with genotypes nearly identical to those found in Horst U1. The differences seen are located at chromosome ends and can be explained by recombinations in the Horst U1 genome and except for one, all CO are located within 100 kb from chromosome ends. The chromosomes are also differently distributed over the nuclei compared to Horst U1. For the second white commercial cultivar, Sylvan A15, a similar data were found. The only difference is that Sylvan A15 has twice as many CO than Somycel 608 and a number of chromosome ends show double recombinations within a distance on average of 27 kb. Since nearby CO are seldom due to CO interference, it is very likely that each member of the pairs are generated in different, i.e. subsequent meioses. In both cultivars no new alleles are found, only loss of alleles due to non-reciprocal recombinations. As is discussed in the previous paragraph, the only way to arrive to such genotypes is to select fertile heterokaryons from the first cultivar Horst U1. Somycel 608 is can be considered as a first generation SSI and Sylvan A15 as a second generation SSI. The genotyping with a limited number of SNP markers of constituent nuclei of 3 additional white hybrids marketed after the release of Horst U1 gave similar genotypes. This underlines that the present-day white hybrids are generated by selecting fertile SSI directly or indirectly derived from the first white hybrids that were produced by outcrossing. The 8% of the genome involved in recombination can lead to loss of alleles in heterokaryotic offspring. This might well explain the few phenotypic differences seen between the commercial cultivars. A selection and screening of heterokaryotic SSI can thus be used to generate small differences in phenotype and can thus be a useful tool in breeding. It is very likely that the traditional cultivars that were used before 1980 are generated in a similar way. At that time three types of cultivars were used, i.e. white, off-

white and brown cultivars. We have genotypes 66 of these traditional hybrids with 115 SNP markers. A clear clustering was found based on genetic similarity into white, off-white and brown cultivars with a very low genetic variation within each group, similar to the genetic variation within the present-day white hybrids. A similar genetic analysis of 131 wild collected strain of var. *bisporus* showed a considerable larger genetic variation (not shown). This indicates that the "variation" within each group of the traditional cultivars has been generated also by selecting fertile single spore cultures or multispore cultures and subsequent selection.

Discrimination between copies, derived and new varieties

As for plants, mushroom cultivars can be protected by Plant Variety Right in most countries and several titles of protection have already been granted for mushroom strains (see for example http://www.cpvo.europa.eu/main/nl/). A variety can only be protected if it complies with the DUS criteria: Distinct from protected varieties, Uniform and Stable as defined by UPOV (http://www.upov.int/). Breeding of a new cultivar in button mushrooms using outcrossing is a considerable job requiring much time and a large investment. Once generated, a very similar cultivar can be made in a short time using fertile SSI derived from this first cultivar. These can be marketed under different names and out-compete the original cultivar and thus minimizing the return of investment of the first breeder. Cultivars generated in this way should be considered as essentially derive varieties (EDV) as defined by UPOV (http://www.upov.int/): A cultivar is considered as an EDV if it is generated by the use of mainly the original variety, is clearly distinct from the original variety but has otherwise a strong resemblance to the original variety. Fertile SSI cultures of button mushrooms clearly comply to this definition. For marketing an EDV, a breeder must negotiate with the first breeder for a license to produce. A working group has started a broad discussion on the definition of EDV for button mushrooms. The group consists of the spawn/breeding companies Sylvan, Amycel, Lambert and the Limgroup and representatives of research group of Plant Breeding Wageningen UR (NL) and INRA Bordeaux (Fr). The group has reached a first consensus stating that fertile SSI derived from a protected cultivar should be considered as an EDV. A position paper has been published on websites of all members of the working group. In addition, this initiative is supported by the UPOV and the Community Plant Variety Office (CPVO) of the European Union and supportive letters have been obtained from different companies within the European mushroom industry. The definitions of DUS and EDV are based on distinctness of phenotypes of varieties. Assessment of phenotypes is often difficult requiring replicate tests in different environments. Genotyping methods are, therefore, used as an additional tool. Mushroom cultivars have relatively small genomes and often haplotypes (constituent nuclei) can be recovered as homokaryons. That offers the opportunity to use next generation sequencing technologies (NGS) to thoroughly genotype cultivars and discriminate new from derived or copied cultivars. If whole genome sequences of new varieties are deposited, resequencing of new cultivars as a heterokaryon using HiSeq Illumina Technology can clearly identify the percentage of allele differences with existing varieties. Identical alleles are clearly an indication of a copy. No new alleles and the loss of a few

percentages of heterozygosity compared to an existing variety are indication of an EDV obtained via a fertile SSI. The resequencing of one or both constituent nuclei can also proof if such a variety is derived directly (first generation) or indirectly (second or more generations) from a protected variety since the number of recombinations at chromosome ends indicate in how many meiotic cycles a variety is obtained from the original variety. Resequencing can also adequately detect the percentage of new alleles in a variety thus detect if outcrossing has been done. NGS offers thus an adequate tool to assess genetic similarity between new and existing varieties and can estimate breeding effort. This will help in discussions to set a broader definition of EDV in button mushrooms and in edible mushrooms in general.

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Redefinition of straw mushroom *Volvariella volvacea* as a bipolar heterothallic fungus❖

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ABSTRACT

The straw mushroom *Volvariella volvacea* is an important crop in East and Southeast Asia. Determination of its mating system is particularly difficult because of multinucleate hyphae and lack of clamp connections. About forty years ago, the mating system of this mushroom has been defined as homothalism, yet this has been debated.

In this studies, the basidiospore-forming patterns of 276 basidia were observed with a scanning electron microscope, showing that 193 basidia were tetrasporic, 58 basidia were trisporic and 25 basidia were bisporic (figure 1). If the four nuclei derived from meiosis were segregated randomly into basidiospore, a results could be ratiocinated that 7.23% of spores were heterokaryon, 92.77% were homokaryon.

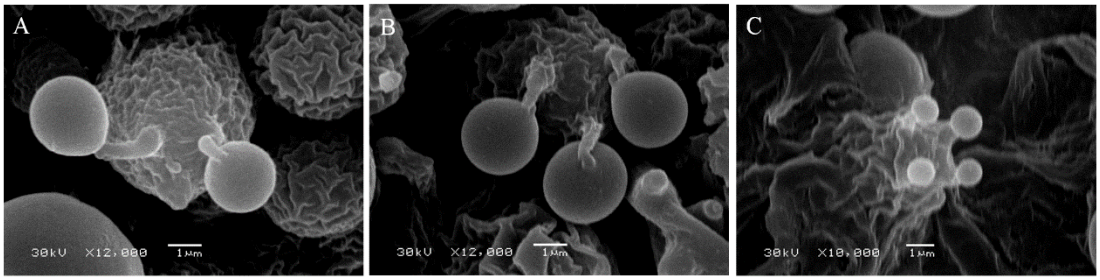


Figure 1. SEM images representing the three different spore patterns encountered on basidia of *V. volvacea* strain PY-1, (**A**) bisporic, (**B**) trisporic, (**C**), tetrasporic. Magnifications are indicated at the bottom, scale bars represent 1 μm.

The nuclei of 2859 basidiospores were examined by fluorescence microscopy (figure 2), showing that 282 spores contained two nuclei and 2577 spores were mononuclear, it also be ratiocinated that 6.58% of spores could be heterokaryotic and 93.42% were monokaryotic.

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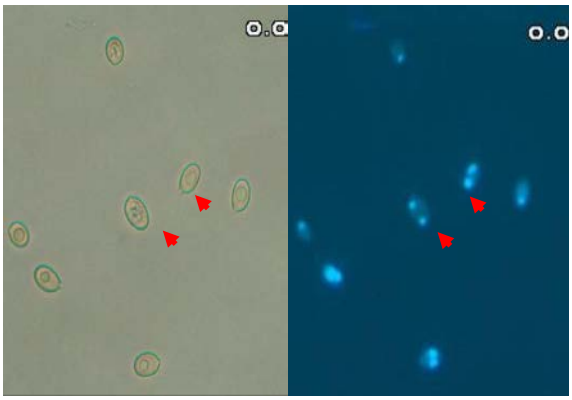


Figure 2. Basidiospores under microscopy. The left was under Ordinary optics microscope, the right was under fluorescent microscope.

A single spore isolate (Pyd21) derived from Py1, a commercial strain, was sequenced (Solexa, *de novo* sequencing) and assembled as the reference genome sequence for *V. volvacea*. Another single spore isolate (Pyd15), also derived from Py1 and mating compatible with Pyd21, was re-sequenced. The genomes of these two strains, together with the available genome of strain V23-1 (downloaded from NCBI) were used to analyze the *MAT* loci. Alignment of 50 Kb genomic regions that surrounded the *MAT-A* loci in PYd15, PYd21 and V23-1 revealed very high sequence conservation except *MAT-A* loci. High sequence

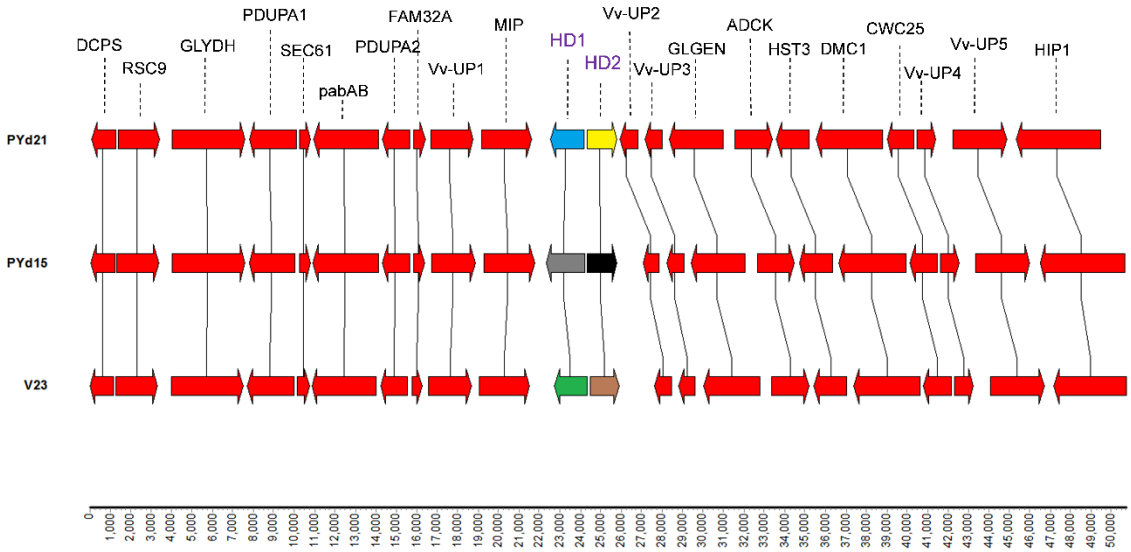


Figure 3. The comparison of genomic regions containing the *MAT-A* locus from *V. volvacea* strains PYd21, PYd15 and V23-1, showing the *MAT-A* locus (homeodomain genes in colored arrows, and purple text) and conserved *MAT-A* locus flanking genes including the *mitochondrial intermediate peptidase* gene (MIP), the Sec61 complex subunit gene (SEC61), the *glycine dehydrogenase* gene (GLYDH), and the glycogenin-2ß gene (GLGEN). Black lines connect homologous genes (depicted as red arrows). Relative gene positions (scale in bp) are indicated at the bottom.

polymorphism of HD1 and HD2 protein encoding genes and their mating type specific combinations indicated that *MAT-A* was involved in mating type discrimination. Synteny mapping showed that the high conservation of these regions over different strains with different mating types indicated that the presented organization is common for *V. volvacea* (Figure 3).

MAT-B loci are typically composed of pheromone receptor and pheromone precursor genes. Homology searches using *C. cinerea* okayama7#130 pheromone receptors indicated four pheromone receptor genes (*VvSTE3.1*, *VvSTE3.2*, *VvSTE3.3* and *VvSTE3.4*) in *V. volvacea* PYd15 and PYd21 as well as in the genome of strain V23-1. In addition, a pheromone precursor gene (*Vvphb1*) was found 719bp upstream in the flanking region of pheromone receptor *VvSTE3.1* in strain PYd21 (Figure 4). Identical sequences for *Vvphb1* were detected in the genomes of PYd15 and V23-1 at the same relative position, demonstrating that *V. volvacea* in general has a complete *MAT-B* locus. Gene *Vvphb1* was predicted to encode a 47 amino acid (AA) with a typical conserved Glutamic Acid (Glu, "E") positioned ~10 AA upstream of the C-terminal CAAX box, and can be expected to mature properly. Alignment of the *VvSTE3.1* (1424 bp, 378 AA),*VvSTE3.2* (1567 bp, 447AA), *VvSTE3.3* (1115 bp, 333 AA) and *VvSTE3.4* (2155

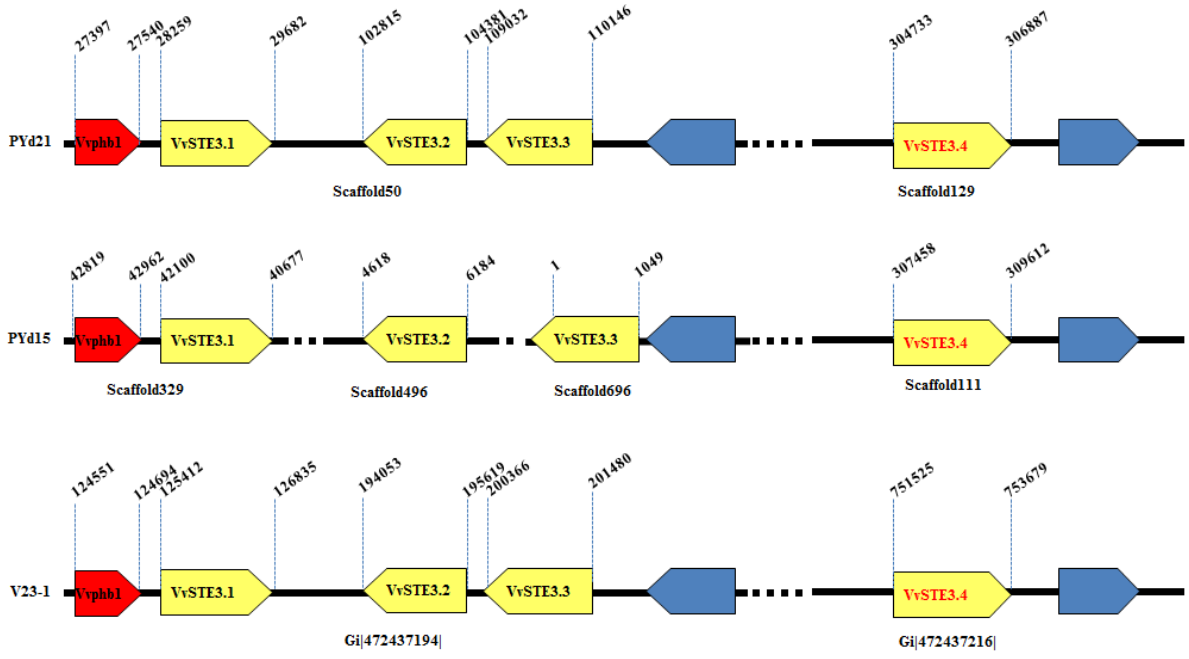


Figure 4. Overview of the MAT-B genes and pheromone receptor like genes of *V. volvacea* PYd21, PYd15 and V23-1. Pheromone precursor genes are indicated in red, pheromone receptor (like) genes in yellow (the new and fourth STE3 gene has red text) and other genes in blue. Relative positions of the genes on their corresponding genome scaffolds are indicated by black numbers showing the start and stop position of each gene. Corresponding scaffold numbers are indicated under the genes for each respective genome.

bp, 601 AA) pheromone receptor genes revealed 100% sequence identity between the respective homologues of PYd15, PYd21 and V23-1, except for one single nucleotide polymorphism (SNP) in *VvSTE3.4*. Thus, no allelic variation was found in the *MAT-B* loci of the three *V. volvacea* genomes.

In addition, the A and B *MAT* loci of 14 more single spore isolates that were derived from 7 wild and commercial strains were amplified and sequenced. The sequences of all *B* mating-type genes showed very high identity that indicated they do not participate in mating-type discrimination. Meanwhile, a total of 8 different *A* mating-type loci, each consisting of a unique HD1-HD2 pair, were demonstrated to control mating as heterokaryotization only occurred between strains that contained different *A* loci.

All our data together indicates that more than ninety percent of *V. volvacea* basidiospores were monokaryon, they needed mating with another compatible monokaryotic strain, and the compatibility was controlled by *MAT-A* only because of *MAT-B* were the same either wild strains or cultivation one. We can conclude that *V. volvacea* has a bipolar heterothallic mating system instead of homothalism .

Keywords: *Volvariella volvacea*, Mating-type loci, Bipolar heterothallic, spore-forming pattern, fruiting.

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Next Generation Sequencing and *Agaricus bisporus* breeding

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ABSTRACT

The advent of Next Generation Sequencing (NGS) technology has allowed cost-effective mass sequencing of genomes and transcriptomes in many organisms. Consequently, Plant Breeders are now routinely using NGS data to discover new genes and to find new markers for economically important traits. The purpose of this paper is to present several emerging NGS technologies which have potential to be tools for commercial mushroom breeding. We conclude with a description of how NGS data was used to define powerful markers for a US Patent on a brown mushroom strain.
Keywords: Mushroom Breeding, Next Generation Sequencing, SNPs

INTRODUCTION

Over the last eight years, progress in reducing the associated costs of Whole Genome Sequencing (WGS) has been both rapid and extraordinary. Costs fell to $100,000 in 2008, to $60,000 later on in the same year, to the 2014 price of $1000 for a whole Human Genome offered by Illumina's HiSeq X Ten platform (Perez-de-Castro et al., 2012; Illumina website). The end result is that NGS technologies, including Whole Genome Sequencing, are now affordable for commercial mushroom breeding programs.

Figure 1: Cost of Whole Genome Sequencing over time (NHGRI website)

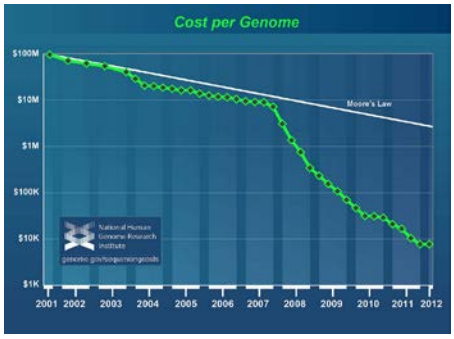


Figure 1 clearly shows the reduction in sequencing costs over time. Cost reductions are driven by technological advances. Cheaper sequencing has resulted in a rush to sequence the major plant and animal genomes. In a breeding context, once the data is collected the hard work of extracting value within the genome can begin.

1. NGS technologies: New Horizons for mushroom breeding

A number of new technologies have emerged over the last five years, and are being used extensively in Animal and Plant Breeding. We envisage a number of these technologies being adapted for Mushroom Breeding.

1.1. Single Nucleotide Polymorphisms (SNPs)

With the accumulation of WGS data from many organisms over the last decade, a new class of genetic markers has become dominant; SNPs. Each SNP represents variation at a single position within a DNA sequence. SNPs are high throughput sequence based markers and as such are highly amenable to computer analysis. Individual SNPs can be located to chromosomal regions and can also enable the identification of nearby coding sequences involved in the inheritance of important traits, including Quantitative Trait Loci (QTLs).

Figure 2: SNP differences between five brown strains of *Agaricus bisporus*

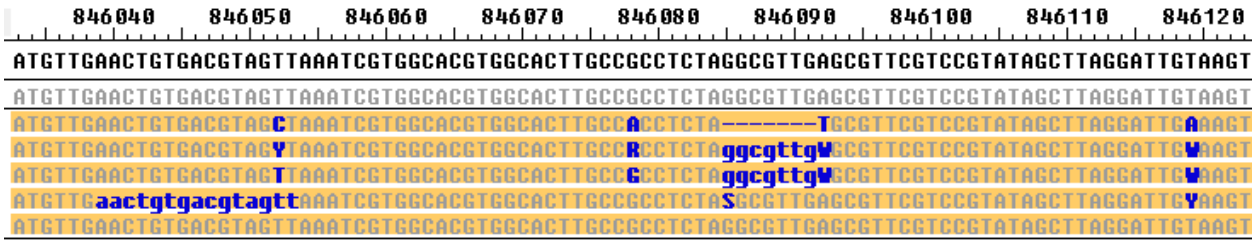


Figure 2 is a demonstration of aligned WGS to show SNP differences. We aligned five different mushroom strains against reference genome H97. Bases in bold show differences with the consensus sequence at the top of the figure. It is a straightforward process to generate tables showing base pair position and sequence variation.

1.2. SNP chips

SNP chips are a fusion of Silicon chip technology with DNA hybridization. Oligonucleotides are bound to a microarray and DNA is hybridized *in silico*. A detection system (for example fluorescence) marks the places where hybridization has occurred. A number of manufacturers sell this technology, for example Affymetrix.

1.3. Genomic Selection

Genomic Selection is a form of marker-assisted selection in which genetic markers covering the whole genome are used so that all QTLs are in linkage disequilibrium with at least one marker. There are significant efforts in Genomic Selection in all of the major Plant Breeding Systems (Goddard & Hayes, 2007). In the well-developed systems, SNPs at precise locations are used along the lengths of each chromosome.

1.4. RNA-seq

Many researchers are using RNA-seq (Wang *et al*., 2009) to get data on which RNAs are produced at various stages in the growth cycle. This technique uses the same platforms used to

generate WGS data. Basically, RNA is purified, cDNA is made and the fragments are modified for Illumina sequencing. Software can align the sequences and databases can be checked for matches. This technology is also known as Transcriptomics.

1.5. TILLING

TILLING (Targeting Induced Local Lesions in Genomes) is a method that allows directed identification of mutants in a specific gene (Comai *et al*., 2004). The TILLING method relies on the formation of DNA heteroduplexes that may occur when multiple alleles are created by PCR. TILLING is now used heavily in most major Plant Breeding systems.

2. Using WGS to develop IP for a brown mushroom hybrid

Sylvan recently published patent number US 9,017,988 (Kerrigan *et al*., 2015) on a new brown cultivar. In contrast to Plant Breeding, there are relatively few measureable points of phenotypic difference in mushroom strains. We focused on measuring differences between mushroom morphology, mushroom density, yield and timing.

For US 9,017,988, we placed heavy emphasis on DNA markers to demonstrate the uniqueness of our invention. We used two marker types; Sequence Characterized Amplified Regions (SCARs; Paran & Michelmore, 1993) and SNPs. SCARs represent known amplified regions in the genome, and alleles are easily separated by restriction digestion or sequencing. In our Patent example, six SCARs were used on the B14528 brown hybrid, and were designated as follows: p1n150, ITS, MFPC-1-ELF, AN, AS and FF.

For SNP analysis the first step was to isolate pure, high molecular length DNA for Whole Genome Sequencing. DNA was sent to a private sequencing company, where the Illumina MiSeq platform was used. The reference sequence was the H97 V2.0 reference genome sequence published by the U.S. Department of Energy Joint Genome Institute (Morin *et al*., 2012). Our service provider made paired-end libraries of approximately 250bp in size. Average genome coverage was 20 to 50 times.

Using SeqMan Pro (DNA Star software) we then aligned three whole genome sequences: reference homokaryon H97, B12998-s39 (one of the parents of B14528), and the F1 hybrid B14528. We began the search for SNPs with a goal of covering all Scaffolds (linkage groups) at distances of approximately 100kb intervals. The SNP marker segments in this project were all 9-mers. Finally, we generated a table comprising 19 scaffolds and 138 markers. The genotypes became Table V in US 9,017,988. Within Table V, we defined precise locations and the sequence of each SNP.

The SNP data were just a snapshot of the distinguishing markers that were discovered. We managed to cover somewhere between 93 and 95% of the genome and software analysis of the

sequence revealed approximately 750,000 genetic marker differences between the strains that were compared.

The marker data we presented provides for the unambiguous identification of Sylvan's invention: the new hybrid strain B14528, and the strains derived or descended from B14528, even though we used only a fraction of the distinguishing markers present. Since these markers are defined by the actual DNA sequence, they are indelible, and can be easily determined by anyone using similar methods.

A strain described in US Patent 9,017,988 was released commercially as Sylvan's "Tuscan Brown" in 2014.

3. Summary

Next Generation Sequencing techniques represent a new suite of techniques which have been embraced by the Animal and Plant Breeding Communities. *Agaricus bisporus* is now beginning to benefit from the adoption of NGS methodologies, in particular SNP markers. Commercial Mushroom Breeding operations should see a consequent improvement in the speed and efficiency in the development of new mushroom varieties.

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Development and yield evaluation of non-browning hybrids in button mushroom (*Agaricus bisporus*)

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ABSTRACT

Browning in white button mushroom after harvest is a major limiting factor affecting the quality and marketability of mushrooms. In the present study, 361 hybrids were developed using non-fertile isolates from 11 strains and evaluated for their bruise resistance by applying mechanical injury using a fork. In majority of the cases, the browning started immediately after the injury whereas a few remained unchanged even after two hours. After preliminary yield evaluation, five of these were selected and each hybrid was evaluated for yield on 1000 kg compost in a commercial unit. Finally, two of them (NBS-1 and NBS-5) were selected on the basis of better quality and higher yield. Final trial of the two strains was done on 4500 bags of 10 kg compost each in three commercial units. The yield of the two strains ranged between 21-25 kg per 100 kg of compost in a total harvesting period of 15 days. These two strains out yielded the strains used by those commercial units.
Keywords: *Agaricus bisporus*, non-browning hybrids, yield evaluation

INTRODUCTION

The quality of *Agaricus bisporus* button mushrooms is determined by various factors like colour, texture, flavor, etc. Of these, colour is the most important factor likely to affect the consumer behaviour and hence commercial value (Burton, 2004). Browning or mushroom discoloration is a post-harvest stress disorder caused by senescence processes and by mechanical damage (bruising during picking, storage and transport) as a consequence of the enzyme-catalyzed oxidation of phenols into quinones. By comparing 2-day post-harvest mushrooms with freshly harvested mushrooms, 20 genes with increased expression levels have been identified (Eastwood *et al*., 2001), showing a probable genetic determinism of post-harvest stress disorders in addition to effects of culture and storage conditions (Burton, 2004). The browning sensitivity of different mushroom strains differs (Weijn *et al*., 2011) and this may be due to loss of water, membrane damage and enzyme activation that result in higher or lower browning sensitivity. It has been shown that enzymatic browning of mushrooms is caused by polyphenol oxidases (PPOs: tyrosinases and laccases) and peroxidases through an enzyme-catalysed oxidation of phenolic substrates into quinines (Jolivet *et al*., 1998). These products undergo subsequent reactions leading to the formation of the dark pigment melanin.

The present study reports the development of hybrids, which are resistant to browning even after the mechanical bruising and also remain white even after storage for few days.

1. Results and Discussion

During the present study, a total of 1000 single spores were isolated from eleven strains, namely, S-11, U-3, A-4, A-6, A-16, A-2, A-15, A-94, S-465, S-130, and a wild strain WI-1. All the strains used in the study were obtained from culture collection of Directorate of Mushroom Research, Solan and Indian Agriculture Research Institute, New Delhi. A total of 39 spores were identified as self sterile. 361 putative hybrids were developed by inter-mating of non-fertile spores. The status of hybrids was confirmed using fruiting trials on 100 kg compost per hybrid. A total of 161 true hybrids were confirmed through fruiting trials. All the confirmed hybrids were evaluated for their browning reaction by applying double streak mechanical injury using a fork. A total of 41 hybrids showed different degrees of browning. Out of the 41 hybrids showing browning resistance, five were selected on the basis of high yield and no browning even after 2 h of mechanical injury (Fig 1 & 2).

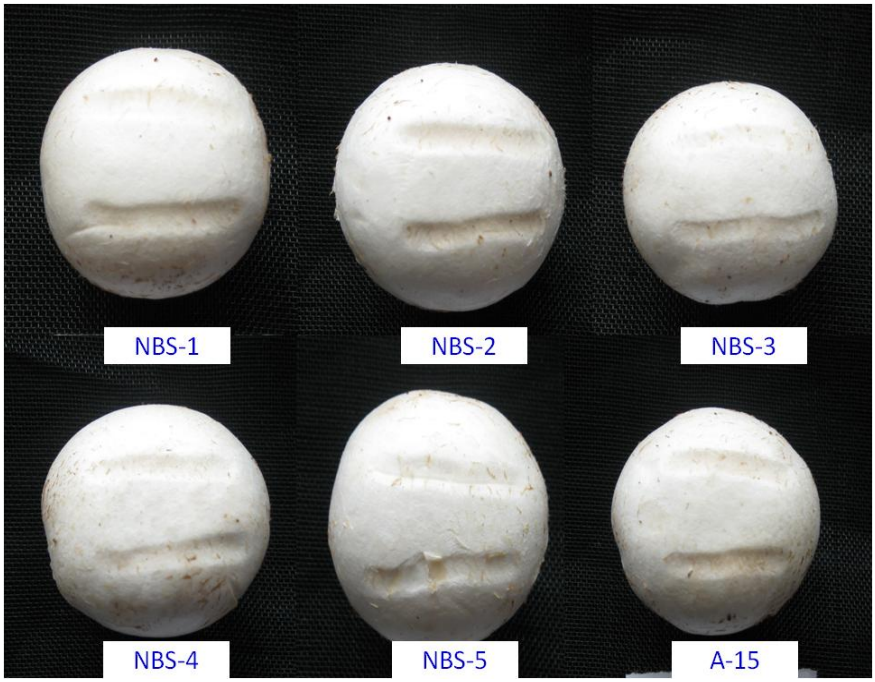


Fig 1. Browning reaction after two hours using double streak mechanical injury



Fig 2. No browning even after 2 h of cutting: upper=NBS-1; lower=NBS-5

These five selected hybrids were named as NBS-1 to NBS-5 and tested for three consecutive years at ICAR-Directorate of Mushroom Research, Solan and simultaneously, they were also tested at five different centres of All India Coordinated Research Project on Mushrooms

(AICRP-M). The data indicated that NBS-1 and NBS-5 performed well over the control strain (U-3). On the basis of trials at the DMR, Solan and AICRP-M, the two strains were given for commercial evaluation at three different large button mushroom farms of 5000 TPA capacities at different locations in India. The Farms were Tirupati Balaji Agro-Products, Baramati, Pune (Maharashtra), Flex Foods, Dehradun (Uttarakhand) and Weikfield's Mushroom farm, Pune (Maharashtra). The varieties were tested at a large scale at these mushroom farms and in some cases resulted in 10-15 per cent higher yield that the imported strains used by these units (Fig 3). The fruit bodies of all the five non-browning hybrids were evaluated for their enzymatic activity and it was observed that laccase, tyrosinase and Poly phenol oxidase activity in the hybrids were very low and almost 50% reduction in the enzymatic activity than control was recorded.



Fig 3. Crops of NBS-1 (Left) and NBS-5 (right) at Balaji Agro-products, Pune (Maharashtra)

Browning after harvest is a common phenomenon in mushroom crops, which decreases the commercial value of the products. Loss of whiteness upon storage is particularly important in the mushroom industry (Burton, 1988). Factors like rough handling, fruiting body senescence and bacterial infection, especially with *Pseudomonas tolaasii* (Olivier *et al*., 1978; Rainey *et al*., 1992; Moquet *et al*., 1996) initiate discolouration reactions. Enzymatic browning is a consequence of the enzyme catalysed oxidation of phenolic substrates into quinones; these slightly coloured products undergo further reactions (oxidations and polymerizations) leading to high molecular mass, dark pigments called melanins. Although hydroxylation of monophenols into diphenols and their subsequent oxidation into quinones can involve various enzymes (reviewed by Robb, 1984), the enzymatic discolouration of mushrooms and other crops is largely mediated by copper oxygenases called polyphenol oxidases (PPOs: laccases and tyrosinases) and peroxidases. In *Agaricus bisporus* (Lange) Imbach sporophore, the signifcance of laccases is very limited because of their low levels, whereas tyrosinase plays the most important role (Lindeberg, 1950; Turner, 1974). As far as the location of mushroom tyrosinase is concerned, Yamaguchi *et al*. (1970) found that tyrosinase activity occurred both in the cap (flesh, skin and gills) and in the stipe. Later, Burton (1986) found that the cap skin from strains U3 and D649 of *A. bisporus* harvested at stage 3 contained three-fold more catechol oxidase specific activity than the cap flesh. Flurkey & Ingebrigtsen (1989) extracted and measured active and total (i.e.

active-latent) l-DOPA oxidase activities in the cap skin, cap flesh, gills and stipe from mature *A. bisporus* and found that cap flesh and stipe tissues contained greater amounts of tyrosinase than skin or gills. These results indicate that the browning activity also takes place in the flesh of *A. bisporus* fruit body. During the present study, we have also visually evaluated the browning in the flesh and stipe of the fruit body by cutting the fruit body longitudinally. The cut fruit body did not show any browning on cutting up to 2 hours.

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Association Analysis of Seven Agronomic Traits with SSR Markers in Chinese Cultivation Germplasm of *Lentinula edodes*

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ABSTRACT

Xianggu (Shiitake, Lentinula edodes) is the second largest commercial mushroom species in terms of total world production. China has been the largest producer, consumer and exporter of Xianggu in the world. Xianggu cultivation in China is gradually transformed from small-scale production pattern to large-scale professional production and requires accurate, high-quality cultivars. At this moment, most commercial strains are nor well defined, i.e. strains might have different names but are genetically identical or the other way around. Thirty-nine commercial Xianggu cultivars used recently in China, two wild strains isolated respectively from Sichuan and Shaanxi province in China, and two commercial cultivars from Japan were genotyped using 92 SSR markers, distributed equally on the whole genome. Seven agronomic traits including fruiting temperature (FT), time from inoculating to fruiting (vegetative growth time, VGT), single fruit body weight (SW), cap color (CC), cap diameter (CD), step length (SL) and total yield (TY) were selected for association analysis with the genotype. A total of 445 bands were detected of which 433 bands (97.6%) were polymorphic between two or more strains. The Polymorphism Information Content (PIC) values varied from 0.19 to 0.75, and the mean value was 0.47. The genetic similarity values varied from 0.56 to 1.00, and averaged to 0.76. Sixteen strains can be distinguished from all tested strains, i.e. have a unique genotype. The rest clustered in six groups containing 2 to 9 strains with no differences within each group. Agronomic traits and SSR loci were analysed by a GLM (general linear model) program in TASSEL software. The results showed that the differences between the corresponding agronomic traits of the materials were highly significant (P<=0.01). The present study suggested that the genetic diversity and linkage disequilibrium of the tested Xianggu germplasm were suitable for association analysis. The marker-trait association SSR primers developed in present study provides references for the construction of germplasm for Xianggu and can be used for molecular marker assisted breeding.

INTRODUCTION

Xianggu is the second most cultivated mushroom of the total mushroom production in the world and is the most important mushroom in east and south Asia. China has the most abundant natural

germplasm and developed many cultivars successively in the last 30 years. Different cultivars are used in different territories of China and apply to different seasons or climate. So these cultivars have obvious differences in some important traits, such as fruiting temperature, vegetative growth time, fruit body appearance and so on. Most of these agronomic and economic traits are controlled by multiple genes and are difficult to improve through traditional breeding. Now most of the Xianggu breeding work in China is derived from these cultivars by crossing with each other or with wild strains, which have some excellent phenotype. Molecular markers used in selection of Xianggu greatly facilitate the breeding work. QTL is the most common strategy in which the molecular markers are used, but still have some problems to be solved right now. Association analysis, which is based on the linkage disequilibrium is an alternative way to link the molecular markers to phenotypes. In this study, we used 92 SSR markers derived from the whole genomic sequencing to scan almost all the commercial cultivars used in China. Genetic diversity of these cultivars can be evaluated and based on the linkage disequilibrium an association analysis can be processed with 7 important agronomic traits.

1. Materials and methods

1.1. UPGMA cluster analysis

An UPGMA hierarchical clustering method was carried out by Mega 7.0 and calculated the similarities of all the strains.

1.2. Population structure analysis

STRUCTURE 2.3 software was used to analyze the population structure. Set the 'Length of Burnin Period' as 10000 and 'Number of MCMC'as 100000, then set K from 1 to 12 and calculate for 20 times. After calculation based on the second order rate of change of the likelihood (ΔK), the ΔK shows a clear peak at the true value of K.

1.3. Linkage disequilibrium analysis

Linkage disequilibrium was analyzed by Tassel 5.0 software for each pair of SSR (markers/bands or primers?).

1.4. Association analysis with agronomic traits

Seven agronomic traits were associated with genotypes by Tassel 5.0 software. Covariate values derived from STRUCTURE analysis were used to combine with genotype and trait data. Fruiting temperature (FT), time from inoculating to fruiting (vegetative growth time, VGT), single fruit body weight (SW), cap color (CC), cap diameter (CD), stipe length (SL) and total yield (TY) of each strain was associated respectively with all the SSR sites.

2. Results

2.1. UPGMA cluster analysis

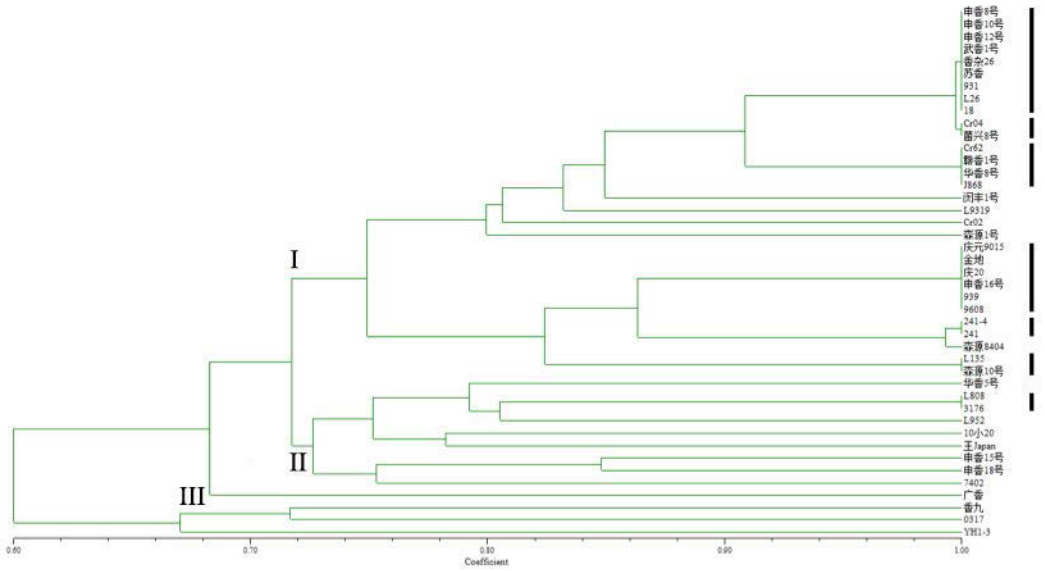


Figure 1. UPGMA cluster analysis of all the cultivar used in China. The phenogram show a clear clustering in three groups (I, II and III), with group representing two commercial lines and wild isolates.

According to the genotyping analysis in 42 strains, a total of 445 bands were detected of which 433 bands (97.6%) were polymorphic between two or more strains. The PIC values varied from 0.19 to 0.75, and the mean value was 0.47. The genetic similarity values varied from 0.56 to 1.00, and averaged to 0.76. Sixteen strains can be distinguished among all strains tested. The rest clustered into six groups containing 2 to 9 strains that could not be differentiated.

2.2. Population structure analysis

The population structure of all strains was estimated using STRUCTURE v2.2 based on all markers. The number of populations (K) was identified based on maximum likelihood and ΔK values, showing 3 groups (a clear peak appearing at K=3). Population 1 contains the log cultivation strain L135, and all strains share genetic markers with L135. Population 2 contains natural germplasm, because strains in this part have some part of wild strain as a parent or domesticate from a wild strain. And population 3 contain strains that are either identical or genetically related to Japanese strains. The STRUCURE analysis has a very similar outcome as the UPGMA analysis with only minor differences.

2.3. Linkage disequilibrium

The linkage disequilibrium result showed that D' values of 30.7% of the detected combinations were lower than 0.5 and 93.7% of the R^2 were lower than 0.3. This result shows that the SSR markers in the cultivar populations show a linkage disequilibrium of the tested Xianggu cultivation germplasm and that these SSR markers were suitable for association analysis.

2.4. Association analysis with agronomic traits

Table 1 provides and overview of the association of SSR markers with agronomic traits.

Table 1. Association analysis of 7 traits with SSR markers (part of the results).

Trait	Marker	marker_p	Marker R^2
Cap diameter (CD)	P4-849-1[c]	3,60E-05	0,381610978
	101-1-2[a]	7,84E-05	0,35535144
Stipe length (SL)	166-1-1	3,86E-05	0,379288739
Single fruit body weight (SW)	P4-849-1[c]	1,33E-04	0,337131623
	101-1-2[a]	2,24E-04	0,318476142
Cap color (CC)	P2-404-3	1,10E-03	0,364241002
	83-3	8,94E-03	0,252186805
Fruiting temperature (FT)	135-88-5	1,58E-04	0,383486239
	181-1-3	2,84E-04	0,360001338
Vegetative growth time, VGT	P2-741-2	3,75E-08	0,666757919
	43-3	2,91E-06	0,548161001
	137-1-1	3,24E-06	0,544760716

P<0.001

CD, SL and SW of all the cultivars had an association with 9, 16 and 8 SSR markers respectively (at P<0.001). This is an obvious association since cap diameter (CD) and stipe length (SL) relates to the size and thus the weight of mushrooms. Three markers were shared for all the three agronomic traits (Table 1). TY, CC, FT and VGT of all the cultivars when P<0.001 had associated with 18, 3, 3 and 32 SSR markers respectively. VGT associated with the most number of SSR sites.

3. Discussion

The values of PIC indicated that the cultivars used in China displayed a high genetic diversity. The population structure and UPGMA cluster results divided the whole cultivar population into 3 main groups. There is a high level of agreement among these two analytic methods. A population with remarkable phenotypic differentiation and a high genetic diversity and multiple genetic constitutions are good conditions to process association analysis. The linkage disequilibrium values also showed that the SSR loci used in this study had low linkage disequilibrium and thus suitable for association analysis. Seven agronomic traits were detected that show a significant association with a total of 56 SSR markers at the P<0.001. Three markers associate with three

agronomic traits: CD, SL and SW and indicate that there is a relation between these traits. Some cultivars have big single weight fruit bodies, attributed to the big cap diameter and long stipe length. Traits TY and VGT had the most SSR sites (18 and 32, respectively) significantly associated with them, and were affected by many factors and regulated by the highest number of genes of all the 7 traits. This kind of agronomic traits is hard to detect with the regular breeding method and are also variable in cultivation. Traits CC and FT had less genomic markers associated with them, which suggests that these two traits are controlled by only a few genes and were relatively stable phenotypes. The results of this study provide additional information for marked assisted selection and might be complementary to QTL analysis. Association analysis can find the most excellent alleles in a fixed population and could be quickly adopted use in breeding with this population. Compared to QTL, association analysis is timesaving and can investigate many traits at the same time. SSR markers distributed over the whole genome were used in this study on the important quantitative traits of Xianggu and these results should be confirmed by later application.

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Regulation of fruiting body development in *Coprinopsis cinerea*

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ABSTRACT

The edible mushroom *Coprinopsis cinerea* is an excellent model to study the genetics of fruiting body development in the Agaricomycetes. Fruiting follows a conserved scheme defined by day and night phases, with well predictable distinct stages over the time. Fruiting starts with primary hyphal knot formation in the dark, followed by aggregation into compact round secondary hyphal knots in which stipe and cap tissues differentiate. Primordia development (stages P1 to P5) takes five days to culminate on day 6 of development in karyogamy and meiosis within the basidia and subsequent basidiospore production which parallels fruiting body maturation (stipe elongation and cap expansion) during the last day of development. This scheme of development is followed up by normal dikaryons as well as by a self-compatible homokaryon which has mutations in both mating type loci. The *matA* genes control fruiting body initiation at the stages of primary and secondary hyphal knot formation and the decision to enter the alternate pathway of sclerotia formation. *matA* induced steps in fruiting end at the P4 state. Activation of the *matB* genes is required for karyogamy and fruiting body maturation. Further, *matB* activation enhances the frequency of *matA*-induced fruiting. *matB* functions as a mediator between light and nutritional signals and *matA* control of development. We have a large collection of about 1500 mutants in fruiting body development. Mutations do not evenly distribute over the developmental pathway of fruiting body development. High numbers of mutants are available from the developmental stages of primary and secondary hyphal knot formation up to stage P1. Comparably few mutations resulted in defects in the developmental progress from P1 to P4 but high numbers of mutants were found also in the later developmental processes occurring normally on day 6 for fruiting body maturation and sporulation. In our studies, we focus on genes that act at the first stages in fruiting. Mutants with defects in primary and secondary hyphal knot formation helped before by complementation to clone genes *cfs1* and a gene of the *NWD2* family. We study the functions of these and further genes with predicted functions in regulation and light control of early fruiting body development.

Keywords: mating types, light, nutrients, signaling, regulation

INTRODUCTION

Coprinopsis cinerea with a very short life cycle grows very fast on artificial medium and completes its whole life cycle with fruiting body and basidiospore production in Petri dishes in less than two weeks. Fruiting bodies in the wild arise on fertile dikaryons with two types of haploid nuclei which formed by mating of monokaryons germinated from basidiospores of different mating types (Kües, 2000). Monokaryons with only one type of haploid nuclei in their cells are unable to produce fruiting bodies. However, mutations in the two mating type loci can render unmated homokaryons self-compatible, allowing them to produce fruiting bodies with meiotic basidiospores. Fruiting body development of homokaryon AmutBmut (*A43mut*, *B43mut*) is well studied. After mycelial growth on YMG/T medium for 6 days at 37°C and transfer into a standard fruiting regime (25°C room temperature, 12 h dark/12 h light regime at high humidity of 85-90 %; Granado et al., 1997), the complete fruiting pathway takes place in seven days. Distinct stages in primordia development have been defined, for every day one, using as reference point the moment at which the 12-h dark phase (night phase) switches to the 12-h light phase (day phase). First small loose hyphal aggregates (primary hyphal knots, PK) are formed in the dark by local intense sub-branching of length-restricted hyphae. A light signal given at the right time induces production of secondary hyphal knots (SK) which are more compact hyphal ball-like plectenchymatic structures. In these, cap and stipe differentiation for primordia formation initiates. The shape of a mushroom becomes visible at the next day (stage P1). During the next four day-night phases, the primordium grows in size while the cap and stipe further differentiate. Primary and secondary gills are established with a hymenium on their surfaces in which basidia arise (stages P2 to P4). An essential light signal given by the day 5 light phase induces karyogamy in the basidia (accomplished at stage P5), directly followed in the day 6 light phase by meiosis and subsequent basidiospore production. In parallel, the stipe elongates and the cap opens. The fruiting body (FB) is fully matured during the next night phase. At the morning of the seventh day of incubation under standard fruiting conditions, the cap autolyzes to release the basidiospores in liquid droplets (Kües and Navarro-Gonzaléz, 2015; Fig. 1C). Here, we present the fruiting pathway in form of a catalog for a dikaryon with defined hallmarks under the same environmental scheme as applied before for homokaryon AmutBmut, underlining that the mutant strain can be used instead of a dikaryon to define regulatory mechanisms of fruiting and, most importantly, to collect dominant as well as recessive mutants in fruiting body development by mutagenesis of haploid mitotic spores (oidia).

1. Results & Discussion

1.1. Cultural conditions for fruiting body formation

Dikaryon PS1x2 was produced by mating of the AmutBmut co-isogenic monokaryons PS001-1 (*A42*, *B42*) and PS002-1 (*A3*, *B1*) on YMG/T (Granado et al., 1997) agar plates. For fruiting, we inoculate strains with a small agar piece (0.2 x 0.2 mm) from a freshly grown YMG/T pre-culture in the middle of freshly made (!) YMG/T agar plates. For good supply of nutrients and water and for good aeration, the agar layer should be ca 5-6 mm thick leaving above 7-8 mm air space in unsealed Petri dishes (9 cm ø) with cams in the lids; i.e., ca 30 ml

of sterile medium should be poured into the bottom half (8.5 cm in ø) of a dish. For gelling, only 1% agar (Serva 11396) should be used to ensure a low agar water potential. Plates (agar surface top) are incubated at the favored growth temperature at 37°C in lightproof ventilated boxes with a layer of wetted paper tissues at the bottom of the box for maintaining good humidity during the incubation. PS1x2 grows with similar speed on YMG/T at 37°C (7.7 ± 1.0 mm/d during logarithmic growth) as AmutBmut (7.9 ± 0.4 mm/d). By standard, plates are inoculated in afternoon hours (between 2 to 6 pm) for growth at 37°C. Plates are checked for their growth front at similar hours 6 days later. For best results in subsequent fruiting body formation, the front hyphal tips of growing colonies should be close to the edges of the Petri dishes (with 0.5-1 mm of still mycelium-free agar) at the time of transfer into standard fruiting conditions but they should not have yet entered the plastic surfaces of the Petri dishes. By the transfer in the afternoon hours into fruiting conditions (with switching on the 12 h light phase at 9 am), a few hours of illumination are provided to the cultures at the same day at 25°C. Illumination (provided by Osram L40/25 Universal-Weiβ tubes, emission spectrum 295-780 nm; at 30 cm distance above shelfs to provide ca 30-90 µE m^{-2} s^{-1} light to plates which increases the temperature at the plate surfaces to 28°C) serves to induce and synchronize development.

Light-induced initiation of fruiting body formation occurs at the mycelial growth front and likely employs fresh hyphal tips. If a culture is transferred too early from 37°C into fruiting conditions, the vegetative hyphal growth will continue over the still free agar surface but at reduced speed and with a change in mycelial morphology from loose fluffy aerial mycelium at 37°C to a less fluffy mycelium produced at 25°C (Fig. 1A). Due to delayed growth with general culture aging, the mycelium may be less active in induction of secondary hyphal knots and these may be more spread over the outer range of the mycelium produced at 25°C due to a less defined time of light induction of fruiting. When the mycelium in contrast already grew onto the plastic edges of the Petri dish, it likely fails to initiate fruiting but may produce on the plastic a dense mass of mycelium, possibly because of suboptimal water supply for the receptive hyphal tips (Fig. 1B). Several hundred secondary hyphal knots may arise under optimal fruiting conditions along the outer edge of a Petri dish. Once the fruiting process is initiated, every day parts of them will give up development in favor of finally a few that complete the fruiting process (Fig. 1C, Fig. 2).

1.2. The developmental pathway of fruiting in a dikaryon as compared to homokaryon AmutBmut

Under standard fruiting conditions, dikaryon PS1x2 will undergo the same developmental pathway as the co-isogenic homokaryon AmutBmut. Light determines the induction of secondary hyphal knots. If missing, primary hyphal knots will transform into sclerotia as in AmutBmut. Light is further needed for proper primordia development from secondary hyphal knots. If lacking, slender, stipe-like structures with underdeveloped caps (“dark stipes”) grow out of secondary hyphal knots. However, with further day-night phases, cap and stipe tissue development instead proceeds to end with stage P5 in the morning of day 6. During this day, the stipe elongates and the cap opens by cell expansion in parallel to basidiospore production.

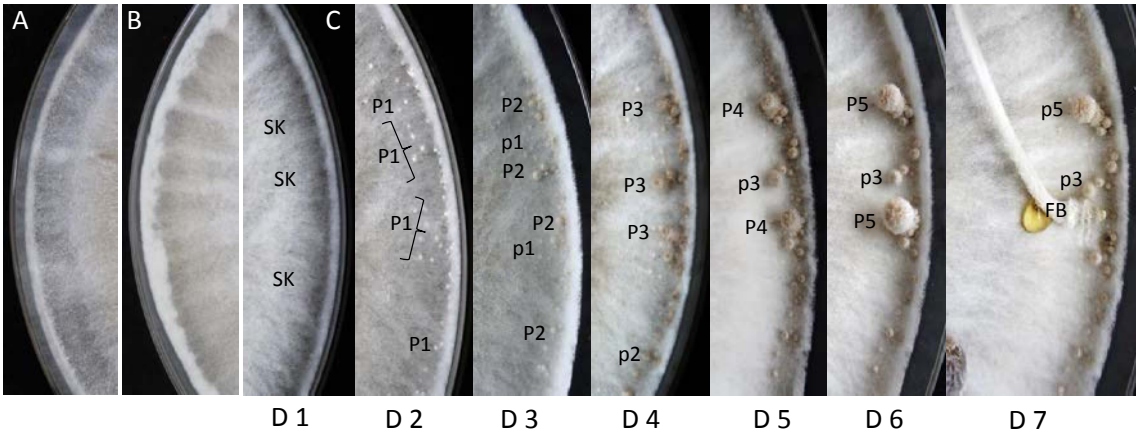


Fig. 1 A. Colony edge of an AmutBmut culture transferred too early and B. of a PS1x2 colony transferred too late into standard fruiting conditions. C. The progress of development (still growing structures: SK, P1 to P5, FB; aborted: p1 to p5) in an AmutBmut culture at the start (when light switches on) of days (D1 to D7) at standard fruiting conditions.

The mushroom is fully matured in the middle of the next night phase (4-5 h in dark). At day 7, the mushroom cap then autolyzes (Fig. 2).

1.3. Regulation of fruiting body development

Previous transformation work showed that the *matA* and the *matB* genes together with light regulate the fruiting pathway. *matA* for homeodomain proteins is most important for induction of fruiting at the secondary hyphal knot stage. Light is needed but too much light is contra-productive. *matB* for pheromones and pheromone receptors can support the *matA* action in induction of fruiting by counteracting negative light effects. *matA*-induced fruiting ends at the P4 state unless *matB* induces further development at the stage of karyogamy (Kües, 2015). By mutations in *matA* and *matB*, AmutBmut performs the light-induced process of fruiting. However, other than dikaryons, the strain produces high numbers of oidia (10^9 versus 10^6 to 10^7 per plate) when cultured in light. This feature of AmutBmut is made use of in mutagenesis of single cells with a single haploid nucleus (Granado et al., 1997). Developmental defects of a large collection of mutants of AmutBmut have so been obtained and assigned to the fruiting pathway shown in Fig. 2. Many mutants are blocked in the formation of either primary or secondary hyphal knots or P1 primordia. Many others are affected in processes of fruiting body maturation at P5 and later whereas few mutants are defective in the developmental progress from P1 to P4. Abundant production of oidia by the mutants in light eases efficient transformation (Granado et al., 1997). Gene *cfs1* for a cyclopropane fatty acid synthase and a gene of the *NWD2* family for a potential regulator in signalling have thus been cloned by restoring defects in hyphal knot formation in AmutBmut mutants (Liu et al., 2006; Yu et al., 2013), showing the value of characterized mutants for studies of regulation of fruiting body development.

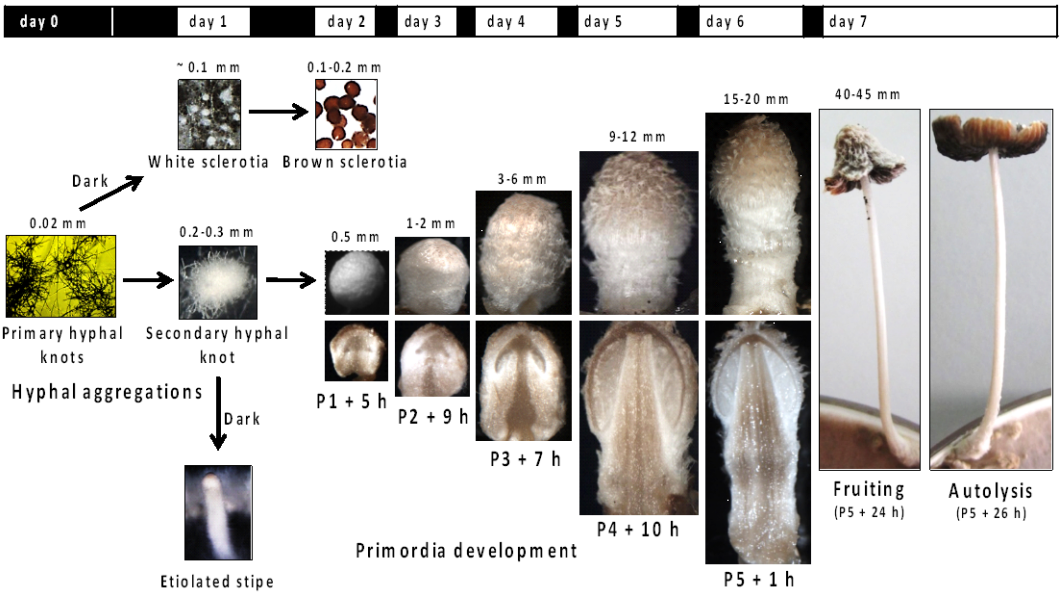


Fig. 2 Fruiting body development in dikaryon PS1x2. Black and white boxes mark dark and night phases (not to scale).

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Establishment of a crossbreeding system for the ectomycorrhizal mushroom *Rhizopogon roseolus*

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ABSTRACT

Rhizopogon roseolus, known as "shoro" in Japanese, is an edible ectomycorrhizal mushroom. Research aiming at elucidating the steps in the life cycle of mushrooms, such as basidiospore germination, subsequent hyphal elongation, and heterokaryosis between compatible mycelia, is fundamental in developing a crossbreeding system. Variability in the germination ability of the basidiospores was observed among the fruiting bodies, implying that selection of the fruiting body is an important step for efficient isolation of germlings. The highest germination ratio was observed in the spore suspension in which spores showed three types of wall, viz., two-layer spore wall, three-layer spore wall and four-layer spore wall. No significant difference in colony morphology was evident between the basidiospore isolates and the hybrid strains obtained from crosses between the isolates. Variability in nuclear number was observed in not only the basidiospore isolates but also in the hybrid strains. Binucleate cells were frequently observed in both, the basidiospore isolates and the hybrid strains, indicating that nuclear number is not a key characteristic that can be used to distinguish between primary mycelia and secondary mycelia. Clamp connections were occasionally observed in the hybrid strains. When the hybrid strains were cultured on a specific medium, clamp connections were consistently observed in the hybrid strains, but never in the basidiospore isolates, indicating that the specific medium is useful to distinguish between primary mycelia and secondary mycelia in *R. roseolus*. These results will contribute to the establishment of a crossbreeding system for shoro mushrooms.
Keywords: clamp connection, crossbreeding, *Rhizopogon roseolus*, spore germination

INTRODUCTION

Rhizopogon roseolus (Corda) Th. M. Fr. (=*R. rubescens* Tul. & C. Tul.) is a basidiomycete that is an important ectomycorrhizal symbiont of the Pinaceae (Molina and Trappe 1994). The fruiting bodies of this mushroom are found in sandy soils of *Pinus thunbergii* Parl. forests in seashore habitats and are prized as an edible mushroom called "shoro" in Japanese (Kawai et al. 2008). For the cultivation of this mushroom, pine seedlings are aseptically inoculated with mycelia or spores; then, these mycorrhizal seedlings are planted in a natural environment, and the mushroom mycelia are encouraged to grow (Yamada et al. 2001). However, cultivation techniques have not been fully refined because of the low efficiency with which mycorrhizal trees are obtained, the low levels of fruiting body production, and the long incubation requirement before fruiting. To overcome these issues, we recently selected

superior isolates that rapidly colonized and produced a lot of ectomycorrhizas within the host root, and developed a new inoculation method using mycelial slurries containing surfactant (Shimomura et al. 2012a). Furthermore, we showed that the inoculation method led to the production of not only abundant mycorrhization of host roots but also rapid fruiting body formation within 1 year after seeding of the host *P. thunbergii* under non-aseptic greenhouse conditions.

Research to elucidate the steps in the life cycle of ectomycorrhizal mushrooms, such as basidiospore germination, subsequent hyphal elongation, recognition of the host root, colonization, ectomycorrhizal formation, and heterokaryosis between compatible mycelia, is fundamental in developing a crossbreeding system. Recently, some parts of the life cycle of *R. roseolus* have been revealed. Distinct karyological characteristics during basidiosporogenesis of *R. roseolus* were studied by light and transmission electron microscope (TEM) (Shimomura et al. 2012b). Furthermore, Shimomura et al. (2008) showed that the basidiospores are discharged by sterigma collapse, and then are processed with accompanying changes in spore wall architecture. Kawai et al. (2008) demonstrated, by mating experiments using basidiospore isolates, that this mushroom exhibits a bipolar incompatibility system. However, little else is known about the life cycle of this edible mushroom.

In this study, we found that selection of the fruiting body is an important step for efficient isolation of basidiospore isolates and then examined mycelial organization of basidiospore isolate and hybrid strains. Furthermore, we developed useful medium to detect clamp connection for distinguish between primary mycelium and secondary mycelium. Here, we propose crossbreeding system of this mushroom.

1. Results & Discussion

1.1. Variability of spore germination and spore wall structure

Fruiting bodies, collected from a pine forest, were classified into three developmental stages based on gleba color, and the germination capacity was evaluated at each stage. Variability in the spore germination rate was observed between individual fruiting bodies. The peak germination rate was recorded for the spores from the fruiting bodies in the beige glebal stage, and the rate decreased for the beige-to-brown glebal stage. When spore wall structures were studied using TEM, the spore wall was found to be multi-layered upon maturation of the fruiting body. The spores of the beige glebal stage showed three types of spore walls, namely two-layered, three-layered, and four-layered spore walls. On the other hand, the spores of the brown glebal stage showed predominantly four-layered spore walls. These results suggest that wall structure of basidiospore may be associated with germination of spore.

Shimomura et al. (2008) reported that multilayered spore wall observed in brown stage may play a role for physical tolerance against harsh environmental conditions that might exist in sandy soil, which could lead to severe dehydration and overheating. Therefore, there is possibility that multilayered spore wall inhibits water permeability that trigger spore germination. On the other hand, spore germination of this mushroom was stimulated by host root exudates, showing that the spore recognize signal molecules secreted by host roots (Kawai et al., 2008). There is a possibility that host root exudates stimulate germination of

multilayered spore. However, we did not have a certain evidence to explain this possibility. Future cytological research will be carried out using multilayered spores exposed to host root exudates.

When the basidiospore isolate was incubated close to another basidiospore isolate, occurrence of hybrid strain will be expected. However, no significant difference in colony morphology was evident between basidiospore isolates and their hybrid strain. To investigate nuclear number per cell, hyphae of cultured mycelia in thin agar plate were stained with DAPI and Calcocfluor White, and then examined under a fluorescence microscope. Variability of nuclear number per cell was recognized not only within basidiospore isolates but also in hybrid strains. Binucleate cells were the most frequently found in both the basidiospore isolates and hybrid strains. Although the most frequent number of nuclei per primary hyphal cell is two, various numbers of nuclei per cell were observed, implying random septum formation and partial anastomosis among hyphae. This instability in nuclear number was also observed in hybrid strains obtained from crosses between two compatible basidiospore isolates, indicating that nuclear number is not reliable for distinguishing between the primary mycelium and secondary mycelium.

When the mycelia of hybrid strains were cultured on agar medium, clamp connections were occasionally observed at 3 to 7 days after incubation at 25 ^{0}C. However, clamp connections were not detected in the colony periphery cultured for 14 days. Variability of clamp formation was observed among culture conditions. Table 1 shows that effect of medium component on clamp formation. Increase in rate of clamp formation was found as decrease concentration of malt extract. Table 2 shows that effect of pH on clamp formation. The most frequent clamp formation was evident at pH 7. From these results, we have developed a inducing medium (TM7) for the clamp formation in *R. roselus*. The medium contained 0.2% (w/v) Tween 80, 0.1% (w/v) malt extract, and 2% (w/v) agar, with pH adjusted to 7.0 with 1 M NaOH. When hybrid strains and basidiospore isolates were cultured on TM7 plates whose medium sides were kept downward, clamp formation was consistently observed in the hybrid

Table 1. Effect of concentration of malt extract on clamp formation in *R. rosolus*

Concentration of malt extract (%)	Clamp formation (%)
0.1	30.8 ± 5.3
0.5	25.2 ± 6.2
1.0	18.8 ± 3.6
1.5	15.0 ± 4.1
2.0	9.2 ± 2.6
2.5	6.6 ± 4.1

Table 2. Effect of pH of medium on clamp formation in *R.roseolus*

pH of medium	Clamp formation (%)
4.0	3.3 ± 1.4
5.0	8.0 ± 3.4
6.0	14.3 ± 1.7
7.0	21.9 ± 5.0
8.0	17.9 ± 2.3
9.5	0.0 ± 0.0

Clamp formation (%)=(no. of hypha bearing clamp/no. of hypha observed) ×100

strains at 5 days after inoculation, but never in the basidiospore isolates. These results suggest that TM7 medium developed in this study is useful to distinguish between primary mycelium and secondary mycelium in *R. roseolus*.

Clamp connections were also reported on specific medium containing a lot of components (Kawai et al. 2008). TM7 medium developed in study is a more rapid and simple method than the specific medium developed by Kawai et al. (2008). In this study, we did not reveal a key factor that control clamp formation in this mushroom. Effect of culture condition on clamp formation is very interesting and important to promote mushroom breeding and to better understanding the biology of the mushroom. Therefore, future research will be required to reveal the mechanism leading to clamp formation.

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Effect of Temperature Treatment on Myceliel Growth Rate Distribution Patterns of 164 Self-bred Dikaryons from *Lentinula edodes* L808

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ABSTRACT

A total of 164 self-bred dikaryotic progenies derived from *Lentinula edodes* strain L808 were selected from 207 isolates based on the formation of myclial clamp connections and, together with the parent stain, were inoculated in glass tubes filled with a substrate consisting of 80% sawdust and 20% wheat bran (moisture content 50%, pH 6.5). After the mycelia had colonized the surface of the substrate, the tubes were subjected to a series of six subsequent periods of 5 days each at different temperatures: 25℃, 30℃, 32℃, 34℃, 25℃(recovery) and 25℃(growth), with each incubation period lasting five days. Mycelial growth rates were recorded in each case. Our data revealed that MGR of the isolates varied considerably during exposure to the poikilothermic regimes and no isolate exhibited the same MGR throughout the entire experimental period, indicating a complex system of gene regulation. Consequently, the MGR under constant temperature and the MGR under poikilothermic regimes must both be taken into account in strain evaluation, especially when the mushroom cultivation period is long and the ambient temperatures during growth are highly variable.
Keywords: *Lentinula edodes*; mycelial growth rate; differentiation; poikilothermic treatment

INTRODUCTION

Mycelial growth rate is often widely used as an important index in the characteristic evaluation and breeding screening of edible fungi. However ,the temperature range and the optimum temperature of the mycelial growth is usually obtained under constant temperatures. Whether the fastest mycelial growth rate at constant temperature represents the mycelial growth capacity, has yet to be studied. Our previous studies showed that *Lentinula edodes* and *Auricularia auricula* which had the same growth rate under the constant temperature exhibit different degrees of differentiation during exposure to the poikilothermic regimes.

Lentinula edodes, a low-temperature, temperature change firmness fungus, has a long production cycle during non-industrialized cultivation (substitute cultivation 4 to 6 months, the wood planting 2 to 3 years). It will inevitably go through the seasons and temperature changes during the entire process of cultivation, so that the hyphal characteristics and mycelial growth rate under different temperature conditions is particularly important. Mycelium has strong cold hardiness characteristics in *Lentinula edodes*, but is sensitive to high temperature. Substrate rot and contamination occurs during production, often due to high

temperatures. In this paper, self-bred progenies were established by multi-spore selfing methods , derived from *Lentinula edodes* strain L808 as a parent. Inbred progeny hyphae were cultured in 6 c temperature regimes hoping to understand the changes of mycelium growth in the status of optimum temperature, high temperature, the optimum temperature again.

1. Results & Discussion

A total of 164 self-bred dikaryotic progenies derived from *Lentinula edodes* strain L808 were selected from 207 isolates based on the formation of myclial clamp connections. The mycelial growth rate distribution of 165 strains together with the parent strain L808 at the different temperatures during the poikilothermic regime is shown in Fig. 1. For each period of 5 days mycelial growth rate was measured. A high peak negative skewness distribution composed at 25℃ incubation period, but it was larger different when the mycelia were incubated at 30℃. The average growth rate of progeny strains significantly decreased at 32℃ incubation period. None of the isolates showed any growth during the 34℃ incubation period. When transferred back to 25℃ for first five days (the recovery period), the isolates reverted back to growth, but at a low mycelial growth rate. Hence, the distribution pattern changed markedly. Mycelial growth rate of all the isolates induced differentiation during second five days incubation period at 25℃, showing the normal population.

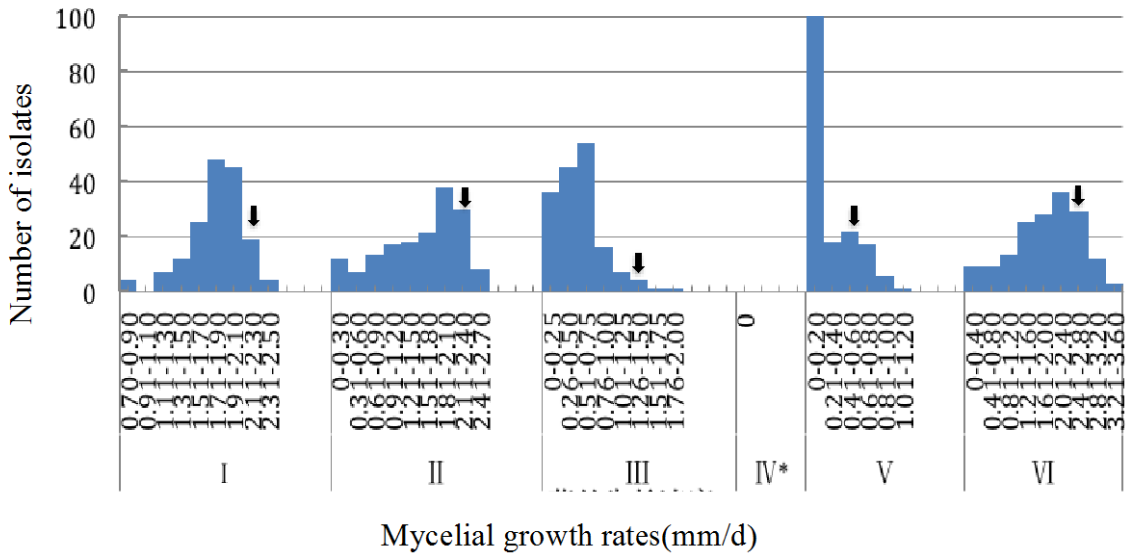


Figure. 1. Distribution of Mycelial Growth Rates (MGR) among 164 self-bred isolates derived from *Lentinula edodes* parent L808 during exposure to different temperature regimes I - VI represent MGR from first to sixth period of temperature treatment (i.e. I=**25**℃**, II=30**℃**, III=32**℃**, IV=34**℃**, V=25**℃**(recovery) and VI=25**℃**(growth))**.
*None of the isolates showed any growth during the fourth period. Arrows indicated the location of the parent strain

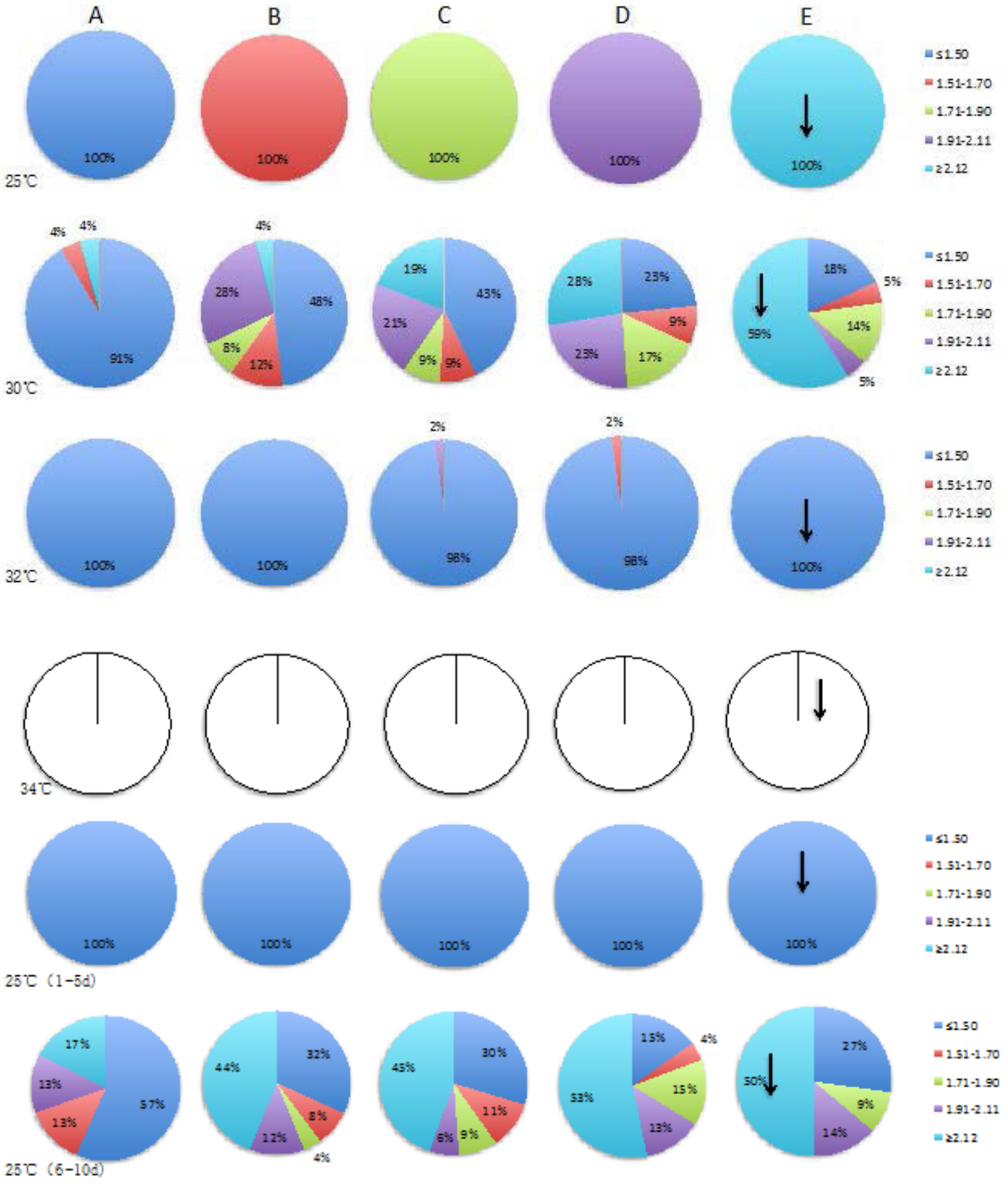


Figure 2. Distribution of MGR among self-bred progenies during exposure to different temperature regimes（Arrows indicated the location of the parent strain）

Lentinula edodes L808 parent strain and 164 self-bred isolates were divided into five groups (A-E) （Fig 2）） according their MGR at 25 ℃ (top row of differently-colored circles). Group A contained 22 isolates (MGR≤1.50 mm/d), Group B contained 22 isolates (MGR 1.51～1.70 mm/d), Group C contained 46 isolates (MGR 1.71～1.90 mm/d), Group D contained 48 isolates (MGR 1.91～2.11 mm/d), Group E contained 27 isolates (MGR≥2.12 mm/d).

Data in subsequent rows show the distribution of the isolates in each group based on the MGR under different temperature regimes.

For example, the MGR of all the isolates in Group A (first column) was less than 1.5 mm/d when incubated at 25℃. However, when transferred to 30℃, most isolates still exhibited a slow rate of growth but 4% showed a slightly higher MGR (1.51～1.70 mm/d) and the MGR of another 4% was greater than 2.12 mm/d. When transferred to 32℃, all the isolates exhibited the lower MGR (≤1.50 mm/d), but no growth was recorded after transfer to 34℃. When transferred back to 25℃ for five days, all the isolates reverted back to the lower MGR (≤1.50 mm/d). However, during a subsequent five day incubation period at 25℃, the distribution pattern changed markedly. Fifty-seven percent of the isolates exhibited a MGR of ≤1.50 mm/d, 13% a MGR of 1.51～1.70 mm/d, 13% a MGR of 1.91～2.10 mm/d, and 17% a MGR of ≥2.12 mm/d.

Nineteen isolates in the green circle (denoted by the isolate number) exhibited MGR higher than the parent stain during the first incubation period (25 ℃); 58 isolates in the pink circle exhibited MGR higher than the parent stain during the fifth incubation period (initial five-day period following transfer from 34 ℃ back to 25 ℃);15 isolates in the blue circle exhibited MGR higher than the parent stain during the sixth period (second five-day period following transfer from 34 ℃ back to 25 ℃). Strains 12 and 20 exhibited the highest MGR compared with the parent strain under the three temperature regimes shown.

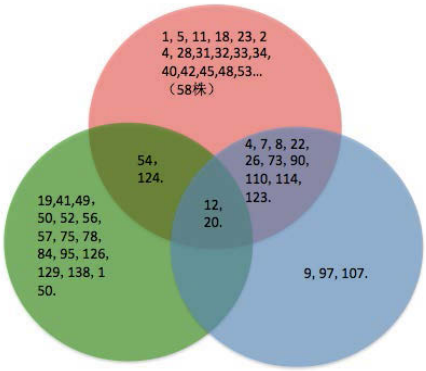


Figure 3. Venn diagram identifying isolates with MGR higher than the parent strain when exposed to different temperature regimes

Consequently, the MGR under constant temperature and the MGR under poikilothermic regimes must both be taken into account in strain evaluation, especially when the mushroom cultivation period is long and the ambient temperatures during growth are highly variable.

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Molecular characterization of browning resistant hybrids of button mushroom using retro-element based markers

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ABSTRACT

Post-harvest browning in white button mushroom is a major problem affecting the quality and marketability of this mushroom. It has been shown that enzymatic browning of mushrooms is caused by polyphenol oxidases (PPOs: tyrosinases and laccases) and peroxidases through an enzyme-catalysed oxidation of phenolic substrates into quinones. These products undergo subsequent reactions leading to the formation of the dark pigment melanin. In the present study, 361 hybrids were produced using non-fertile isolates from 11 strains and evaluated for their browning reaction by applying double streak mechanical injury using a fork. Forty-one hybrids were selected on the basis of varying browning resistance (after two hours of mechanical injury) and yield. Five of these with high browning resistance have been finally selected for large scale evaluation. Further, the five high browning resistant hybrids were characterized using ISSRs, SSRs, and retro-element based markers. In the study, to amplify around pairs of insertion sites through IRAPs technique, we have used outward facing primers of the two consecutive retro-elements. For REMAP studies, we have used successful IRAP primers along with microsatellite primers. These retro-element based markers have shown very high resolution between the parents and hybrids. It can be inferred from the study that these can be effectively used in confirmation of hybridization in *Agaricus* breeding programme. We have also got a number of fertility linked markers in *Agaricus*, strain specific markers in different strains of *Agaricus* and also genus and species specific markers in different mushrooms. Retro-element based markers showed high diversity in *Agaricus bisporus*, where diversity is always revealed to be very low with most of the other marker systems.

Keywords: white button mushroom, *Agaricus bisporus*, browning resistance, molecular characterization, retro-element markers

INTRODUCTION

Though commercial cultivation of mushroom is over three centuries old, yet very little has been achieved in strain improvement. Even the white strain in *A. bisporus* was a spontaneous mutation in a population of cream fruiting bodies. The life cycle and the sexuality pattern of only a few species of basidiomycetes are known, yet there seem to be the predominance of

heterothallic sexual pattern in majority. Development of new strains by mating of single spore isolates (SSI) is generally evident by clamp connections in dikaryotic mycelium but unfortunately, conventional breeding in *Agaricus bisporus* is time consuming and complicated proposition due to secondary homothallic nature of the species (where majority of the basidia produce two spores each containing two nuclei of opposite mating-type and only a few basidia are tri- or tetrasporic yielding homokaryotic spores), absence of clamp connections in mono and dikaryons, lack of well-defined morphological differences in fertile and non-fertile isolates *in vitro*. The multinuclear mycelial features and the absence of asexual spores in *A. bisporus*, make it further difficult to generate mutants. Most commonly used technique for breeding button mushroom is to isolate and evaluate single spore isolates through fruiting trial for identification of non-fertile isolates and intermating these to produce hybrids. Together, these features made mushroom breeding a relatively unfavourable area of research among scientists. Biotechnological interventions are a beautiful support system to handle some of the difficult problems in mushroom diversity analysis and breeding. It is practically impossible to screen all the single spore isolate for their fertility. It is thus important to look for some molecular markers, which can efficiently differentiate between fertile and non-fertile single spore isolates.

Because of their mobility and activity, transposons have proved to be valuable markers for genetic diversity and variability. The TEs have strains/species specific signatures, which can be used for identification of non-fertile isolates in *Agaricus bisporus* while it can also be used for speedy species or strain identification for strain protection.

Post-harvest browning in white button mushroom is a major problem affecting the quality and marketability of mushroom. It has been shown that enzymatic browning of mushrooms is caused by polyphenol oxidases (PPOs: tyrosinases and laccases) and peroxidases through an enzyme-catalysed oxidation of phenolic substrates into quinones. These products undergo subsequent reactions leading to the formation of the dark pigment melanin, which is seen as the browning of the mushroom fruit body.

The study is aimed to charecterize the browning resistant hybrids using retro-element based markers and aslo develop some DNA based markers useful in identification of hybrids so that cumbersome fruiting trials can be ommitted for confirmation of hybrid stauts.

1. Results & Discussion

During the present study, 361 putative hybrids were developed by intermating of 39 non-fertile spores from 11 genetically distant parents including wild collections. The status of hybrids were confirmed using fruiting trials on 100 kg compost per hybrid. A total of 161 true hybrids were confirmed through fruiting trials. All the confirmed hybrids were evaluated for their browning reaction by applying double streak mechanical injury using a fork. A total of 41 hybrids showed different degrees of browning resistance. Out of the 41 hybrids showing browning resistance, five were selected on the basis of no browning (Fig 1) even after two hours of mechanial injury and higher yield potentials. All the five browning resistant hybrids (named as NBS-1 to NBS-5)

were assayed for phenols and browning resitant enzymes i.e. Laccase, Tyrosinase and Polyphenol oxidase. The results are presented as enzyme activity / µg protein (Table 1). The results indicated that enzyme activity varied in different hybrids but invariably it was significantly lower in the hybrids. The lower enzyme activity may have resulted in browning resistance in these hybrids.

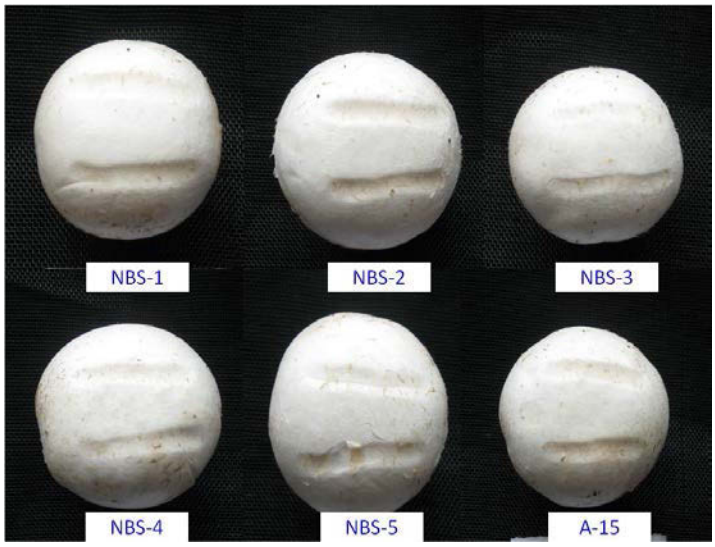


Fig 1. Browning reaction after two hours using double streak mechanical injury

Table 1. Phenol content and enzyme activity of browning resistant hybrids of button mushroom

Hybrids	Phenol (µg/g fresh mushroom)	Laccase*	Tyrosinase*	Polyphenol oxidase**
NBS-1	166.36	0.053	9.66	0.97
NBS-2	230.10	0.046	15.99	1.23
NBS-3	220.69	0.025	16.76	1.40
NBS-4	171.44	0.033	13.26	1.44
NBS-5	168.36	0.035	9.51	1.43
A-15	220.16	0.137	18.28	2.17
Critical Difference (5%)	19.00	0.027	3.46	0.24
Standard Error	8.53	0.012	1.55	0.11
Coefficient of variance (%)	5.32	27.470	13.69	9.24

* = δA_{470} by 0.001/min/µg protein; ** = δA_{495} by 0.001/min/µg protein

Enzymatic browning is a consequence of the enzyme catalysed oxidation of phenolic substrates into quinones; these slightly coloured products undergo further reactions (oxidations and polymerizations) leading to high molecular mass, dark pigments called melanins. Although hydroxylation of monophenols into diphenols and their subsequent oxidation into quinones can involve various enzymes (reviewed by Robb, 1984), the enzymatic discolouration of mushrooms and other crops is largely mediated by copper oxygenases called polyphenol oxidases (PPOs: laccases and tyrosinases) and peroxidases. In *Agaricus bisporus* (Lange) Imbach sporophore, the signicance of laccases is very limited because of their low levels, whereas tyrosinase plays the most important role (Lindeberg, 1950; Turner, 1974). Two non-exclusive explanations have been

proposed for this complex process : (i) a decompartmentation between substrates and enzymes following breakdown of intracellular membranes; (ii) the activation of latent PPOs (Mayer & Harel, 1979; Robb, 1984; Mayer, 1987; Nicolas *et al*., 1994).

Further, the hybrids were molecularly characterized alongwith their parents for developing markers for confirmation of hybrid using retro-element based markers i.e. Inter Retroelement Amplified Polymorphism (IRAPs) and Retroelement Microsatellite Amplified Polymorphism (REMAPs). A total of 32 outward facing IRAP and 14 REMAP primers were used in the study singly and in pairs. The results indicated that the the retroelement based primers were very much effective and showing clear resolution in identification of hybrids in *Agaricus bisporus* and can be successfully used for developing markers for the same (Fig 2). The fragments from the single spore isolates and the hybrids were isolated and cloned for the sequencing purpose. The clones have been sent for sequencing. The sequences obtained will be used for developement of codominant markers and will be tested on large populations.

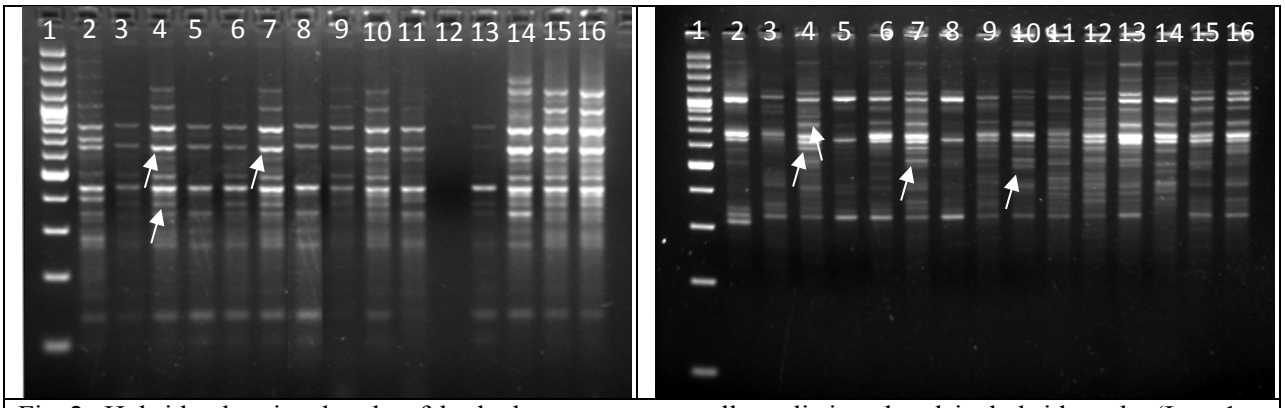


Fig 2. Hybrids showing bands of both the parents as well as distinct band in hybrids only (Lane1 = marker; Lane 2 & 3= parents; Lane 4= hybrid; Lane 5&6= parents; Lane 7= hybrids; Lane 8&9= parents; Lane 10= hybrids; Lane11&12= parents; Lane 13= hybrid; Lane 14&15= parents; Lane 16= hybrid)

Because of their mobility and activity, transposons have proved to be valuable markers for genetic diversity and variability. Retroelements have been recognized in a wide range of eukaryotes and, in some cases, used as genetic markers to investigate phylogeny, to generate mutants and recombinants, and to identify mutated genes (Bennetzen, 2000; Bingham and Zachar, 1989; Boeke, 1989; Flavell and Smith, 1992; Hirochika, 1997; Lucas *et al*., 1995; Peterson et al. 2000). In particular, use of outward facing primers to amplify around pairs of insertion sites, the IRAP technique, (or between retroelements and simple sequence repeats, the ISSR method) first developed by Schulman *et al*., (2004) has been useful to provide multi-locus anonymous markers. The SSAP, IRAP and REMAP methods are multiplex and are used to generate several anonymous marker bands. REMAP markers are also applicable in several phylogenetic and biodiversity studies. Retrotransposon-based insertional polymorphisms (RBIP) have been developed, with the advantages of being co-dominant markers (Flavell *et al*., 1998; Schulman *et al*., 2004; Kalendar and Schulman, 2006; Schulman *et al*., 2012). These markers are

now used in several studies to observe biodiversity and phylogenetic relations of the species and to investigate the retrotransposons.

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Interruption of a MSH4 Homolog in *Pleurotus ostreatus* is Responsible for the Absence of Spore Production

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ABSTRACT

The sporeless oyster mushroom (*Pleurotus ostreatus*) variety SPOPPO, introduced to the market in 2006, has been generated by using an existing sporeless mutant (ATCC58937) as the donor and the commercial variety HK35 as acceptor strain. The sporeless phenotype appeared to be recessive and was mapped in both constituent nuclei on the same locus, suggesting the involvement of one gene. The present research was directed to identify the gene(s) involved, thus facilitating breeding additional sporeless varieties and see if this gene can be a target for breeding sporeless varieties in other edible fungi. The locus containing the sporeless trait was fine mapped to 78.9 kb containing 27 predicted genes. The equivalent region of the sequenced sporulating strain N001 PC15 was subcloned as individual genes or pairs of genes and used to transform the non-sporulating mutant. One gene was able to restore the sporulation completely. The commercial sporeless variety shows, next to the absence of spores, also the loss of orientation of fruiting body in each bunch indicating a disturbance of negative gravitropism. With restoration of sporulation, also the negative gravitropism of the fruiting bodies was restored in the transformants, indicating the involvement of the gene also in this phenotype. The 3,864 bp gene encoding an 852 amino acid protein was identified as a MutS protein homolog 4 (MSH4) homolog. MSH4 is known to be meiosis specific and required for reciprocal recombination and proper segregation of homologous chromosomes at meiosis I. The cDNA sequence of the wild type of MSH4 revealed 26 introns ranging in size between 8 and 300 bp. Comparing the coding regions of the wild type and the mutant MSH4 homologs, the latter constructed from the Illumina reads, showed two amino acid shifts in the mutant that do not seem to be located in positions crucial for its function. A *de novo* assembly of one of the homokaryons of the non-sporulating strain indicates that the gene is interrupted, possibly by a 5 kb sequence. Due to the poor quality of the *de novo* assembly, only part of the sequence of insertion is known. A BLAST search on the whole genome indicates that it represents a repetitive sequence, possibly a LTR-transposon.
Keywords: Pleurotus ostreatus, sporulation, MSH4

INTRODUCTION

Pleurotus ostreatus (oyster mushroom) is amongst the most cultivated edible mushrooms worldwide. It can produce enormous amounts of spores (Sonnenberg et al. 1996) which cause severe problems for people harvesting and handling the crop. Repeated exposure to high spore numbers causes extrinsic allergic alveolitis (EAA), an inflammation of the alveoli in the lung

provoked by inhalation of spores (Cox et al. 1988). In addition, spores can clog filters in the climate-control system and spread viral disease (Grogan at al. 2003, Kim at al. 2008). A sporeless commercial strain would solve all these problems.

Mutagenesis by chemical treatment and UV irradiation have been used previously to generate sporeless mutants in *Agrocybe cylindracea, Pleurotus ostreatus* (Joh et al. 2004, Pandey et al. 2010)*, Pleurotus pulmonarius, Pleurotus eryngii* (Obatake et al. 2003) and *Pleurotus sajor-caju* (Pandey et al. 2010). It appeared, however, difficult to restore yield and quality in these mutants to an acceptable level by breeding. In a breeding program for a commercial sporeless *P. ostreatus* strain (Baars et al. 2004) a spontaneous sporeless *P. ostreatus* mutant, ATCC58937 (F42 x 11; Eger et al. 1976), and a commercial variety HK35 were used. In this breeding program, Baars et al. (2000) studied the inheritance of the sporeless phenotype and concluded that it is recessive and mapped in both haploid genomes of the sporeless mutant on the same chromosome at the same locus, suggesting that only one gene is involved. This sporeless phenotype mapped on the same chromosome as the *A* mating-type. The breeding program yielded a commercially acceptable sporeless strain although fruiting bodies somehow lost their normal orientation in the bunches. The strain was introduced on the market in 2006 as SPOPPO. The intention of the present research was to identify and characterize the gene(s) responsible for the sporeless phenotype. This will facilitate breeding for additional sporeless varieties since it will allow a more accurate selection and reduce linkage drag. If this gene is conserved in edible fungi it will allow development of sporeless varieties in other species too.

1. Results and discussion

Identification of candidate genes

Two *Pleurotus ostreatus* strains were used: a normal sporulating dikaryotic strain N001 (Larraya et al. 1999) with its two constituent monokaryons PC9 (Sp^+hap1) and PC15 (Sp^+hap2) and a non-sporulating dikaryotic mutant, ATCC58937 (F42 x 11; Eger et al. 1976), with its two constituent monokaryons EP25 (Sp^-hap1) and EP57 (Sp^-hap2). A mapping population was generated by collecting 188 monokaryotic offspring of a cross between Sp^+hap2 and Sp^-hap2. Sp^+hap2 was selected as parent for this cross since, the whole genome sequence was available (http://genome.jgi.doe.gov/PleosPC15_1/PleosPC15_1.home.html) and therefore could be used as a reference genome. Sp^-hap2 was resequenced by Illumina Genome Analyzer II technology (ServiceXS) and sequencing reads were mapped against the Sp^+hap2 reference for SNP analysis. A *de novo* genome assembly resulted in 2717 contigs. A selection of 384 SNP's was made of which 98 were evenly distributed over scaffold 3 harbouring the sporeless locus.

A genetic map was made based on 358 SNP-markers, 3 CAPS-markers and 4 phenotypes; *A* mating-type, *B* mating-type,

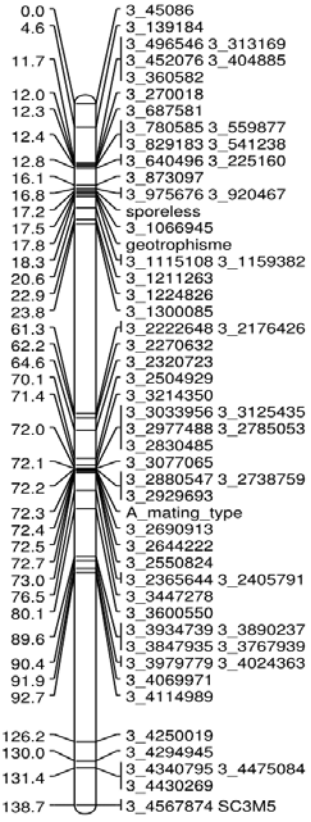


Figure 1: Linkage map for scaffold 3 of *P. ostreatus*, based on 94 markers on 153 homokaryotic progeny. Marker positions (cM Haldane) on the left and marker names on the right.

sporulation and disturbed gravitropism using JoinMap 4 (Kyazma). The resulting map consisted of 12 linkage groups covering 1050 cM, on average 32.7 kb/cM. The sporeless phenotype, *A* mating-type and the disturbed gravitropism are located on linkage group 3 (Figure 1) which has a length of 138.7 cM. The genomic region containing the sporeless trait had a length of 78.9 kb. Using the Sp^{+}hap2 genome annotation (http://genome.jgi.doe.gov/PleosPC15_1/PleosPC15_1.home.html), 27 predicted genes were identified in this region of which 4 genes are predicted to play a role in transcription, replication, recombination and DNA repair.

Transformation of P. ostreatus

The 27 candidate genes were amplified and cloned from the Sp^{+}hap2 genome including a 1 kb region upstream as a promoter region and 500 bp downstream as a terminator region. Protoplasts of the Sp^{-} dikaryon were transformed with each of the constructs in combination with the carboxin resistance bearing vector pTM1 (Honda et al. 2000). Carboxin resistant colonies were screened for the presence of the Sp^{+}hap genes by PCR and for the presence of clamp connections. For each construct, 5 transformants were cultured to produce fruiting bodies. Gills were microscopically examined for spore production. Only one of the constructs did restore sporulation in all transformants of the non-sporulating host (Figure 2). With restoration of sporulation, also the negative gravitropism was restored. The sporeless phenotype and disturbed gravitropism mapped at nearly the same position indicating that the mutation affects both phenotypes.



Figure 2: Morphology of *P. ostreatus* transformants; the sporulating strain N001 (A), the wild type non-sporulating mutant ATCC58937 (B), a non-sporulating transformant (C) and a transformant with restored sporulation (D).

Sequence and structure analysis of the MSH4 homolog.

The gene restoring sporulation in the Sp^{-} dikaryon, encodes protein ID 1101251 (http://genome.jgi.doe.gov/cgi-bin/dispGeneModel?db=PleosPC15_2&id=1101251). This protein was identified as a MutS protein homolog 4 (MSH4) homolog. MSH4 is meiosis specific and, in a heterodimer with MSH5, required for reciprocal recombination and proper segregation of homologous chromosomes at meiosis I. The genomic DNA sequence of the Sp^{+}hap2 MSH4 homolog is 3,864 bp in length. Total RNA was extracted from gill tissue of the Sp^{+} dikaryon, reverse transcribed into cDNA, cloned and sequenced. This resulted in a total length of 2,556 bp containing 26 exons and encoding a protein of 852 amino acids. The Sp^{-}hap2 sequencing reads were mapped against the annotated Sp^{+}hap2 genomic DNA sequence of the MSH4 homolog and based on this, a Sp^{-}hap2 RNA sequence was predicted. Comparing the RNA sequence of the Sp^{+} dikaryon with this predicted RNA sequence of Sp^{-} hap2 revealed two positions where a difference in RNA sequence resulted in a change in the amino acid composition of the encoded protein; an arginine to cysteine shift at position 638 and an asparagine to serine shift at position 789. Studying a predicted 3D protein structure

and the conserved domains revealed that these two amino acid shifts are not located at positions crucial for the proteins function.

Since the differences between the active and inactive MSH4 homolog could not be explained by their (predicted) RNA sequences, the Sp$^-$hap2 Illumina reads mapped against the Sp$^+$hap2 MSH4 homolog genomic DNA sequence were studied in more detail. The entire region was mainly covered with paired reads, except for the region between 1800 and 2100 bp, where a high number of orphan reads were mapped. This indicates the presence of an insertion in the Sp$^-$hap2 MSH4 homolog. Amplification of the relevant region with MSH4 specific primers revealed that the insertion is ~5 kb long. Although cloning and sequencing of the insert has to be done, the few nucleotides known at both ends suggests that the Sp$^-$hap2 MSH4 is interrupted by a LTR-transposon.

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Studies on *Tricholoma giganteum* and *Agaricus bisporus* Protoplast Fusion Breeding

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ABSTRACT

Tricholoma giganteum is one of the nutritious rare edible fungi. However，its mycelia grows slowly. In order to improve the speed of its growth, the protoplast fusion was conducted using *T. giganteum* and *Agaricus bisporus* in this paper.

The condition of protoplast fusion of *T. giganteum* and *A. bisporus* was optimized by single factors and uniform design method. The results showed that the optimum protoplast fusion condition was: PEG 26.17%, fusion time 24.4 min, fusion temperature 30.8 ℃, Tris 0.016 mol/L, CaCl2 0.014 mol/L, pH 8.5. The highest fusion rate was 3.87×10^{-4} at this condition.

One zygote (H49) having good character and the genetic different with their parents was obtained. It was identified from its colony character，morphological state，growth rate and ISSR. The colony character，morphological state of the zygote was different from its parents. Its growth rate was faster than *T. giganteum* and slower than *A. bisporus*. Its ISSR genetic correlation coefficients and cluster analysis was analyzed. The result showed that the genetic correlation coefficient between H49 and *T. giganteum* was 0.65 and between H49 and *A. bisporus* was 0.47. This indicated that the fusion between *T. giganteum* and *A. bisporus* was preliminary successful.

The mycelia growth medium of H49 was optimized. The optimum medium of H49 was: sucrose 1%, peptone 1%, yeast extract 0.11%, VB1 10 mg/L, light calcium carbonate 0.11%, magnesium sulfate 0.29%, potassium dihydrogen phosphate 0.11%; The mycelium of H49 grew fastest (0.98 cm/d) in this medium at its suitable pH 6.

The cultivation property of the fusion strain H49 was studied. It indicated that the mycelium of H49 grew fastest in corn kernels medium .The mycelium was thick and germination time was short. While the cultivation medium of H49 was baggasse 60%, cotton seed hull 30%, wheat bran 9%, yeast extract 0.5%, KH_2PO_4 (fertilizer grade) 0.3%, $MgSO_4$0.1%, light calcium carbonate 0.2%. Its fruiting time was shorter than the parent strain *T. giganteum*.

Key words: *Tricholoma giganteum Agaricus bisporus* Protoplast fusion

INTRODUCTION

Tricholoma giganteum which has a great potential as a producing breed is one of the nutritious rare fungus for edible and officinal use (Liu et al., 2000; Mau et al., 2002; Lee et al., 2004; Wang et al., 2005). It was showed that the fruit bodies of *T. giganteum* could be stored at 8 ~ 12 ℃ for 30 days without browning and decay, and had long shelf-life according our previous results (Yang et al., 2010; Mo et al., 2011). However，its mycelia growth was slowly and long fruiting time, the industrial and large-scale development was restricted. *Agaricus bisporus* is a basidiomycete mushroom belonging to the *Agaricaceae* family which is cultivated in over 70 countries (Ma et al., 2014 and Saravanan et al., 2013), and has for decades been the most widely grown of all mushrooms (Foulongne-Oriol et al., 2014 and Tautorus and Townsley, 1984). Protoplast fusion is potentially important technique to develop mushroom hybrids, especially when conventional methods cannot achieve this result. Protoplast fusion can be performed intraspecifically, interspecifically, intergenerically and interhetero generically. Protoplast fusion from different species may dramatically influence the course of biological processes compared to their occurrence in the parental strains (Dhitaphichit et al., 2005). The protoplast fusion between *T. giganteum* and *A. bisporus* was studied in order to obtain a strain of *T. giganteum* with fruiting in a short time in this paper.

1. Results & Discussion

1.1. Optimization of the protoplast fusion condition

The condition of protoplast fusion of *T. giganteum* and *A. bisporus* was optimized by single factors and uniform design method. The results showed that the optimum protoplast fusion condition was: PEG 26.17%, fusion time 24.4 min, fusion temperature 30.8 ℃, Tris 0.016 mol/L, $CaCl_2$ 0.014 mol/L, pH 8.5. The highest fusion rate was 3.87×10^{-4} at this condition. The protoplast regeneration and fusion rate of the optimal results were significantly higher than those of the best treatment results in the uniform design.

1.2. Fusant ISSR identification

One zygote having good character and the genetic different with their parents was obtained. Its ISSR genetic correlation coefficients and cluster analysis was analyzed. The result showed that the genetic correlation coefficient between H49 and *T. giganteum* was 0.65 and between H49 and *A. bisporus* was 0.47 (Table 1). This indicated that the fusion between *T. giganteum* and *A. bisporus* was preliminary successful.

Table 1. The genetic similarity coefficient matrix of fusant H49 and its parents

Strain	*Tricholoma giganteum*	*Agaricus bisporus*	H49
Tricholoma giganteum	1		
Agaricus bisporus	0.47	1	
H49	0.65	0.47	1

1.3. Optimization of the mycelia growth medium

The mycelia growth medium of H49 was optimized. The optimum medium of H49 was: sucrose 1%, peptone 1%, yeast extract 0.11%, VB1 10 mg/L, light calcium carbonate 0.11%, magnesium sulfate 0.29%, potassium dihydrogen phosphate 0.11%; the mycelium of H49 grew fastest (0.98 cm/d) in this medium at its suitable pH 6.

1.4. The cultivation property of the fusion strain H49

The cultivation property of the fusion strain H49 was studied. It indicated that the mycelium of H49 grew fastest in corn kernels medium (Table 1) .The mycelium was thick and germination time was short. While the cultivation medium of H49 was baggasse 60%, cotton seed hull 30%, wheat bran 9%, yeast extract 0.5%, KH_2PO_4 (fertilizer grade) 0.3%, $MgSO_4$0.1%, light calcium carbonate 0.2%. Its fruiting time was shorter than the parent strain *T. giganteum* (Figure 1).

Table 2. Growth rate of fusant H49 and its parent

Strain	Colony diameter after being cultured 7 days（mm）
H49	35.5667±0.5207 [b]
Agaricus bisporus	71.533±0.4333 [a]
Tricholoma giganteum	29.500±0.3215 [c]

All experiments were performed in triplicate and the value is the average, the different letter means the significant difference in Duncan's new multiple range test ($P < 0.05$).

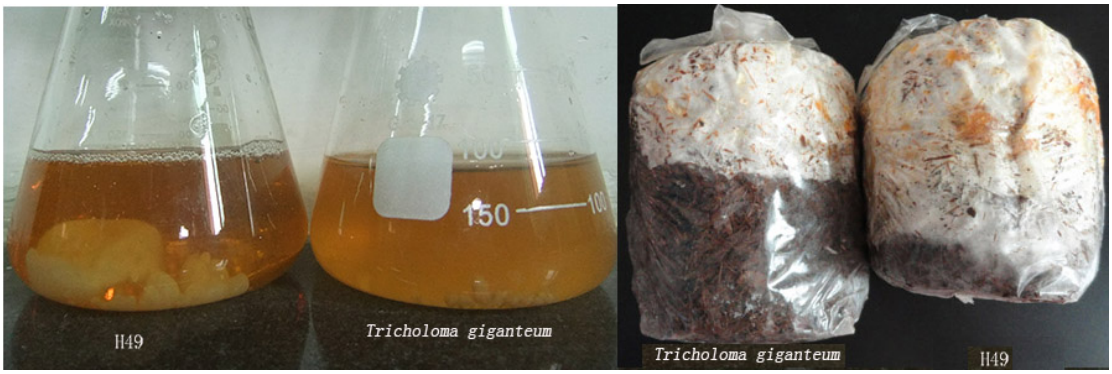


Figure 1. Comparison of the growth rate of fusion strain H49 and the parent strain *Tricholoma giganteum*

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VIII Molecular biology & Biochemistry

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Increasing carbohydrate degradation in compost during commercial mushroom production of *Agaricus bisporus*?

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ABSTRACT

After 2-3 flushes of *Agaricus bisporus* mushroom production, champost still contains about 50% of the carbohydrates and 45% of the lignin originally present in compost. Increased uptake of the unused pool of polysaccharides may increase mushroom yield. Lignin is removed during the vegetative growth phase of *A. bisporus* (phase III) but less efficiently during mushroom formation (phase IV). Here, we overexpressed the manganese peroxidase gene *mnp1* in *A. bisporus* by placing it under control of the actin promoter to improve the removal of lignin, thereby promoting accessibility of hemicellulose and cellulose. Transformants produced MnP activity in liquid malt extract while the wild type strain did not. MnP activity was 3-4 fold increased in wheat bran medium. During a semi-commercial production cycle, MnP activity per gram wet compost was increased significantly at the end of phase III (30%) while the activity was similar to the wild type strain at the initiation of mushroom formation in phase IV. This indicates that other factors than mRNA accumulation may be limiting in MnP1 production at this stage. After the 1st and 2nd flush, MnP activity was increased 3-4 fold. There was no difference in mushroom yield or biomass formation in compost as measured by chitin release. In addition, carbohydrates released after enzymatic treatment of milled compost and carbohydrate content was not affected. Finally, lignin was not affected differently by the *mnp1* overexpressor as determined by pyrolysis. Efficient MnP activity in compost may have been impaired by cofactor limitation.
Keywords: *Agaricus bisporus*, manganese peroxidase, overexpression, carbohydrates, lignin

INTRODUCTION

Mushrooms of *A. bisporus* are produced throughout the world on compost. In the Netherlands, the substrate of *A. bisporus* contains wheat straw and horse manure as C-source. Compost is produced in two phases of composting that results in a humic-rich substrate highly preferred by *A. bisporus*. After phase II (PII), *A. bisporus* is introduced by mixing spawn through the compost. The compost is colonized by *A. bisporus* in large tunnels, this is referred to as phase III (PIII). In phase IV (PIV), the colonized compost is transported to mushroom growers where it is topped with casing layer on which mushrooms are formed. *A. bisporus* preferentially removes carbohydrates in PIV (Patyshakuliyeva et al 2015). Conversely, lignin degradation occurs mainly in phase III (Jurak et al 2015a, Jurak et al 2015b). After 2-3 flushes of mushroom production, champost still contains about 40% of the

carbohydrates and 45% of the lignin originally present in compost. Lignin can interfere with cellulases and hemicellulases by shielding their substrates and enzyme adsorption. Therefore, increasing lignin degradation may increase carbohydrate availability and by this increased mushroom production.
The degradation of lignin correlates with the activity and expression of laccase (Lcc) and manganese peroxidase (Wood and Goodenough 1977, Bonnen et al 1994, Patyshakuliyeva et al 2015). The genome of *A. bisporus* contains two manganese peroxidases (MnPs) that are likely involved in lignin degradation (Kirk and Farrell 1987, Hofrichter 2002, Morin et al. 2012). MnP uses H_2O_2 to oxidize Mn^{2+} to Mn^{3+} which is able to oxidize phenolic substrates. Additional mediators like unsaturated lipids are required for the oxidation of non-phenolic substrates (Hofrichter 2002, Hammel and Cullen 2008). Therefore, MnP is an interesting target for overexpression to increase ligninolysis. Here, we overexpressed *mnp1* (ID 221245) in *A. bisporus* with the aim to increase lignin degradation, improve carbohydrate accessibility and thereby increase mushroom yield.

Results and Discussion

A. bisporus gills were transformed with *mnp1* controlled by *actin* regulatory sequences using the *Agrobacterium tumefaciens* transformation system (Chen et al 2000). Transformants were identified on malt extract (ME) supplemented with hygromycin. To identify transformants overexpressing *mnp1*, MnP activity of commercial type strain A15 and transformants were assessed in liquid malt extract medium (ME) during 38 days. Transformants MnP1-1 and MnP1-2 produced MnP activity in liquid ME, while A15 wild type did not. On wheat bran medium, MnP activity relative to Lcc activity (MnP/Lcc) was increased 3-4 fold. MnP activity was corrected for Lcc activity to correct for fungal biomass. MnP/Lcc was significantly increased by ~40% ($p < 0.05$ after Bonferroni correction) in compost colonized with *A. bisporus* for 13 days.
Next, the effect of MnP overexpression on lignin, carbohydrate availability and mushroom yield was assessed in a semi-commercial cultivation. To this end, 18 boxes of strain A15 and MnP1-1 were inoculated and used for 2 flushes of mushroom production. Samples were taken at casing (applying of casing layer), venting (induction of mushroom formation) and after the 2nd flush (15, 30, and 56 days after inoculation, respectively). For each time point 4 boxes were sacrificed for each strain. After the 1st flush, compost samples were taken from one box containing A15 and two boxes containing MnP1-1, boxes were not sacrificed. There was no difference in Lcc activity per gram wet compost between strain A15 and the *mnp1* overexpressor. In contrast, MnP activity per gram wet compost was significantly higher in strain MnP1-1 at casing and after the 2nd flush (~30% and ~250%, respectively ,$p < 0.05$ after Bonferroni correction). No difference was observed at venting, indicating that other factors than mRNA accumulation are limiting for MnP1 activity at this stage. There was no difference in fungal biomass based on chitin content of the compost. In addition, no effect was found on carbohydrates released after enzymatic treatment of milled compost and carbohydrate content at casing or after the 2nd flush. Finally, no difference in mushroom yield between strain A15 and MnP1-1 was found. Therefore, carbohydrate consumption, accessibility and mushroom yield were not affected by increased MnP activity. Analytical pyrolysis was used to assess lignin degradation and modification by strain A15 and MnP1-1. No significant differences in relative abundance of lignin derived pyrolysis residues were

identified. Therefore, increasing MnP activity did not affect ligninolytic activity of *A. bisporus*. Possibly, MnP activity was not efficient in the compost conditions. This may be due to limiting amounts of co-factors like H_2O_2, Mn^{2+}, organic acids or unsaturated lipids. Preliminary data indeed show that H_2O_2 production in compost extract is greatly reduced in PIV as compared to PIII providing a new target for increasing lignin degradation by *A. bisporus* in compost.

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Fate of Lignin and Substituted Xylan during Commercial Cultivation of *Agaricus bisporus*

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ABSTRACT

Wheat straw based compost is the substrate for commercial growth of *Agaricus bisporus* mushrooms, but it is unknown to which extent the carbohydrate-lignin matrix changes and how much is metabolized during commercial cultivation. In this paper we report yields and remaining structures of the major compost carbohydrates and lignin. Hereto, a mass balance was conducted in a tunnel-experiment at industrial scale, and total dry matter, cellulose, hemicellulosic xylan and lignin were quantified (Jurak *et al*., 2015a). Remaining (substituted) xylan structures were extracted and analysed in detail by enzymatic fingerprinting with use of HPAEC and MALDI-TOF MS. Remaining lignin structures were subjected to analytical pyrolysis GC/MS and typical lignin units were determined. In addition, to determine why certain fractions remain unused in the compost, activities of water extracted carbohydrate degrading enzymes were analysed for their ability to degrade a range of polysaccharides (Jurak *et al*., 2015b,c). During growth of *A. bisporus*, carbohydrates were only slightly consumed and xylan was partially degraded. At the same time, lignin was metabolized for 45% based on pyrolysis GC/MS. Remaining lignin was modified, as observed by an increase in the ratio of syringyl (S) to guaiacyl (G) units from 0.5 to 0.7 during growth, while fewer decorations on the phenolic skeleton of both S and G units remained (Jurak *et al*., 2015a). During mushroom formation, mainly endo-xylanase, endo-glucanase, β-xylosidase and β-glucosidase activities were determined in the compost extracts. Arabinofuranosidase activity able to remove arabinosyl residues from doubly substituted xylosyl residues nor α-glucuronidase activity were detected (Jurak *et al*., 2015b). The latter correlated with the observed accumulation of xylan fragments substituted with arabinosyl and glucuronic acid substituents in the compost towards the end of the cultivation (Jurak *et al*., 2015c). Hence, it was concluded that compost grown *A. bisporus* lacks the ability to degrade and consume highly substituted xylan fragments.
Keywords: *Agaricus bisporus*, compost, carbohydrates, xylan, enzymes

INTRODUCTION

The white button mushroom, *Agaricus bisporus* is cultivated worldwide for its edible, nutritious fruiting bodies. For commercial purposes, mainly the *A. bisporus* heterokaryon strain A15 is cultivated. In the last years, in the Netherlands, 250 million kg of this mushroom were produced yearly (personal communication CNC-C4C). Commercially, *A. bisporus* is grown on a compost based on wheat straw, straw bedded horse manure, chicken manure and gypsum (Gerrits, 1988).

When it comes to obtaining economically feasible yields of good quality *A. bisporus* mushrooms, the compost recipe and composting process are well established. Some flexibility in composting conditions, such as changing the duration of a phases or adding additives (personal communication, CNC-C4C, Milsbeek, The Netherlands) is common practice to maintain compost quality with the existing variations in substrates used. Finding the optimal composting conditions is, so far, mainly based on empirical experience. It is unclear how the composting conditions influence changes on a molecular level and on the final yield. Therefore, up to now conceptualized processing with the aim to improve the mushroom yield is not possible. Hence, our research aimed to obtain detailed information on the content and composition of structural remaining components in the compost throughout each of the composting phases and *A. bisporus* growth, as well as on the ability of *A. bisporus* to degrade carbohydrate structures in the compost. In addition, it was aimed at elucidating the structures hindering the complete saccharification of carbohydrates, analyzing the lignin structures and identifying the enzyme activities present or lacking in the compost. This knowledge is expected to lead to possibilities for improving the composting process and utilization of carbohydrates.

1. Results & Discussion

1.1. Vegetative mycelium growth- changes in plant cell wall structures of compost

A main finding was that in compost both xylan and lignin structures were altered to the largest extent during 16 days of mycelium growth in the so-called Phase III (PIII).

At the end of Phase III (PIII-16), about 20% of the total remaining xylan in the compost was water soluble, compared to 3% in compost at the start of Phase III and PIII-16 alkali extracted xylan showed a decreased molecular weight (Jurak et al., 2014). Albeit xylan was found to be degraded, the carbohydrate composition, and the degree of substitution of the xylans in compost at the beginning and end of PIII was rather similar (Jurak et al., 2014). Also, less than 6% of xylan was metabolized during PIII. In contrast, 40% of lignin was metabolized. The remaining lignin showed a different fingerprint in PIII-16 compost compared to earlier stage composts, deducted from the Py-GC/MS pyrograms. In PIII-16, the ratio of syringyl-like and guaiacyl-like units (S:G ratio) increased from 0.5 to 0.7 and the ratios of Py-GC/MS analysed vinyl-substituted guaiacol to guaiacol and vinyl-syringol to syringol decreased (Jurak *et al*., 2015a).

Previously, studies on lignin degrading fungi (white rot fungi) have shown that the extracellular lignin system involved in lignin degradation is induced by starvation (Ten Have and Teunissen, 2001). Therefore, it can be speculated that due to the metabolization of 50% of carbohydrates in the composting stages prior to PIII, only more difficult to degrade carbohydrates remain in the compost at the start of PIII. As a result, the lignin degrading machinery of *A. bisporus* is induced in Phase III, resulting in the lignin degradation observed. In grasses (wheat straw), the presence of lignin in the plant cell wall is often negatively related to the digestibility of the cell wall carbohydrates (Grabber *et al*., 2004). Therefore, it is proposed that the observed lignin changes during mycelium growth have an important impact in improving the digestibility of carbohydrates in the later stages of mushroom growth.

1.2. Fruiting body formation of A. bisporus - Changes in plant cell wall structures (Filling, Pinning, 1st flush and 2nd flush)

During the fruiting body formation phases of *A. bisporus* (Filling, Pinning, 1st flush, and 2nd flush) the main finding was the accumulation of arabinosyl- and (4-*O*-methyl-)glucuronic acid xylan substituents in the compost (Jurak *et al*., 2015b,c).

Next to the accumulation of xylan substituents, genome-expression analysis showed that *A. bisporus* did not have the ability to degrade this substituted xylan in the compost during the mushroom growth conditions applied. Specifically, *A. bisporus* genes encoding the enzyme activity required to degrade the accumulated structures, namely to remove (4-*O*-methyl-)glucuronic acid or arabinose from xylosyl residues having two arabinosyl residues, were not present in the genome or not expressed. This was confirmed by the lack of the above named enzymatic activities in water extract from compost during fruiting body formation (Jurak *et al*., 2015b,c; Patyshakuliyeva *et al*., 2013). The compositional changes in xylan structure do not reflect changes in absolute amounts of carbohydrates in the compost during fruiting body formation. Therefore, the decrease in dry matter amounts of compost (http://edepot.wur.nl/299892) for the same composting process was used in order to quantify the degradation of carbohydrates. To that end, by using the carbohydrate content values (% w/w) obtained (Jurak *et al*., 2015b), the mass balance was calculated of total carbohydrates, xylan and cellulose as shown in Table 1. A consumption of 40% of total carbohydrates from the compost was calculated based from end of PIII to 2nd flush (reported by Baars and Sonnenberg, 2014). In more detail, xylan was found to be consumed to a larger extent (50%) compared to glucan (35%) (Table 1). Since glucan can be present in multiple forms, although mainly as cellulose and as glucan in mycelium cell walls, it could not be distinguished how much cellulose was consumed and how much glucan was built in mycelial cell walls.

Table 1. Dry matter, total carbohydrates, xylan and glucan content (% w/w based on dry matter (DM)) and mass balance of compost from end of PIII to after 2nd flush.

	Content (w/w% DM)		Mass balance (kg/m^2)			Loss
	End PIII[a]	2nd flush[a]	End PIII[b]	2nd flush[b]	Loss	Relative to end PIII
Dry matter	37	39	30.9	25.9	5	16%
Total carbohydrates	21.9	15.7	6.8	4.1	2.7	40%
Glucan	10.4	8.1	3.2	2.1	1.1	35%
Xylan	9.3	5.5	2.9	1.4	1.5	50%

[a]values based on Jurak *et al*. (2015b).

[b]based on report, Baars and Sonnenberg, 2014 (http://edepot.wur.nl/299892).

1.3. Proposed model of conversion of wheat straw during composting and mushroom growth

A schematic overview of changes in wheat straw cell walls during composting and growth of *A. bisporus* is proposed in Fig. 1. In wheat straw (Fig. 1A) primary cell walls are mainly built from cellulose fibers (20-30% w/w) and xylan (20-40% w/w). The secondary cell walls make up to 50% (w/w) of stem cell walls and consist of cellulose (35-45% w/w), xylan (40-50% w/w/) and lignin (20% w/w). In the phase prior to PIII 50% of the compost carbohydrates are metabolized, mainly by bacteria and some fungi (Jurak *et al*., 2015a). Also, separation along middle lamella has been

observed, which is expected to additionally reduce the rigidity of the connected cells (Atkey and Wood, 1983) and shown in Fig. 1B. After the mycelium was fully grown through the compost (PIII-16) xylan was found to become more water soluble and partially degraded next to metabolization of 40% of lignin and modifications of remaining lignin structures. Less bacteria were found to be present on the wheat straw cell wall surface compared to the prior phases and mycelium was thinly spread over the surface of the straw (Atkey and Wood, 1983) (Fig. 1C).

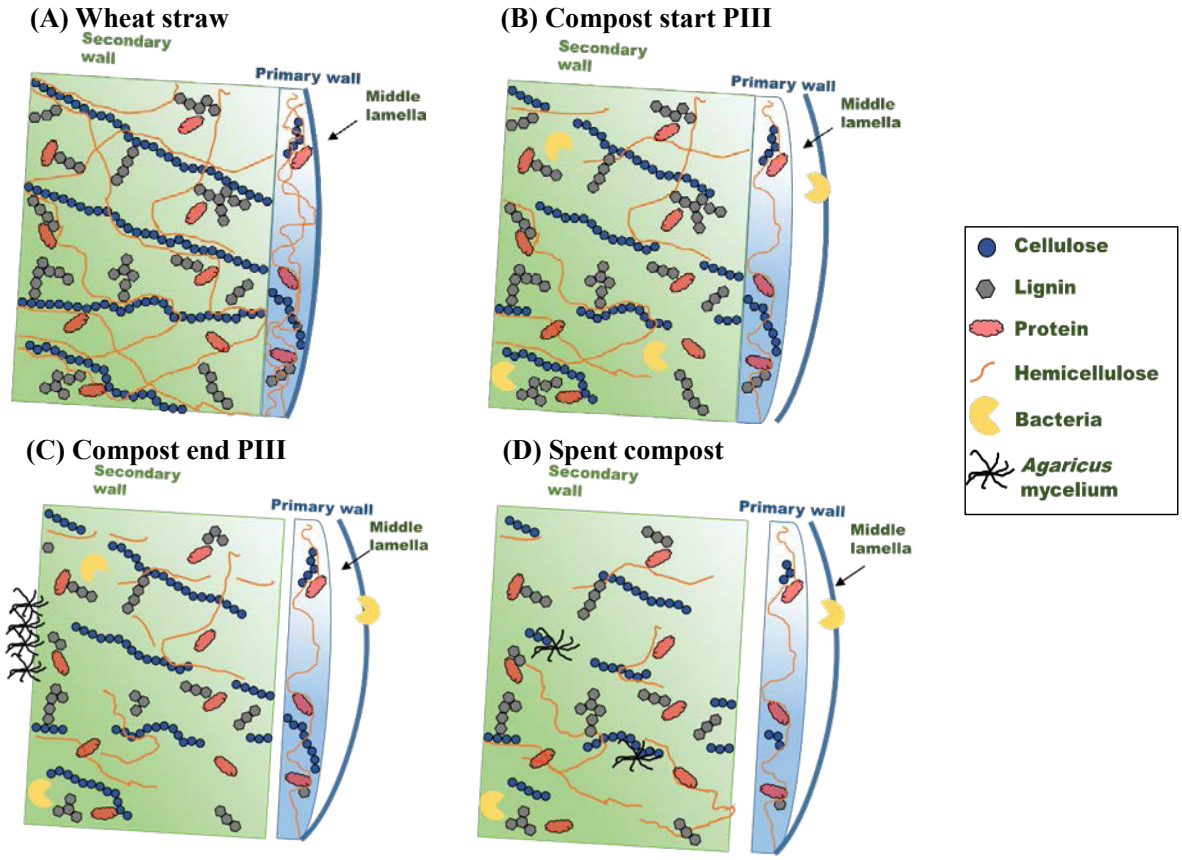


Figure 1. Proposed cell wall model of wheat straw (adapted from Achyuthan *et al*. (2010)) and modifications in PIII-start, PIII-16 and 2nd flush spent compost.

After the 2nd flush of mushrooms was collected, compost was considered spent and remaining cell wall structures are expected to be severely disrupted (Fig. 1D). Overall 40% of carbohydrates are expected to be degraded in spent compost compared to compost at PIII-16 (see Table 1). Remaining xylan in the spent compost was found to have a higher degree of substitution compared to PIII-16 compost indicating that mainly linear xylan was degraded during fruiting body formation (chapter 4). Mycelium hyphae were found to penetrate into the cellulose fibrils (Atkey and Wood, 1983).

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How (post-)genomic insights can provide new leads for improvements of mushroom cultivation

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ABSTRACT

The economically important edible basidiomycete mushroom *Agaricus bisporus* thrives on decaying plant material in forests and grasslands of North America and Europe. It degrades forest litter and contributes to global carbon recycling, depolymerizing (hemi-)cellulose and lignin in plant biomass. Relatively little is known about how *A. bisporus* degrades compost in the controlled environment in commercial production facilities and utilizes this substrate to produce mushrooms. Using transcriptomics and proteomics linked to analysis of the compositional changes in the compost, we showed that changes in plant biomass degradation by *A. bisporus* occur throughout its life cycle. Lignin degradation appears to only occur during the spawning stage. In contrast, (hemi-)cellulolytic genes were highly expressed at the first flush, whereas low expression was observed at the second flush. The essential role for many highly expressed plant biomass degrading genes was supported by exo-proteome analysis, demonstrating the presence of these enzymes during specific stages. The out-of-phase enzyme production in the second flush may explain the reduction in the number of mushrooms during the second flush.

Combined with other data generated in our lab we have now generated the first detailed model of the genes and enzymes involved in compost degradation and utilization by *A. bisporus*, which has provided many leads for a better understanding and improvement of the commercial mushroom cultivation.

Keywords: *Agaricus bisporus*, compost, gene expression, enzyme activity, commercial cultivation

INTRODUCTION

Agaricus bisporus is the most commonly cultivated commercial mushroom in the world and represents a multi-billion euro industry. Its cultivation occurs on compost, a mixture of wheat straw, horse manure and gypsum (Gerrits 1988). Typically two to three harvests (flushes) of mushrooms are obtained for each cultivation, with the second and third having progressively lower productivity.

The reason for this is not clear as there appears to still be sufficient substrate left after the second and third flush to support mushroom formation (unpublished data).
Despite a long history of cultivation and detailed knowledge about the technical aspects that result in high mushroom production, up to recently very little was known about the biological and molecular processes that occur during commercial mushroom cultivation. Strain breeding has resulted in strains with better productivity, shelf-live and other properties, but also for these aspects little is known about the underlying biological mechanisms. With the availability of the genome sequence of *A. bisporus* (Morin et al. 2012) and subsequent post-genomic studies (Jurak et al. 2015c; Jurak et al. 2015a; Jurak et al. 2015b; Patyshakuliyeva et al. 2013; Patyshakuliyeva et al. 2015), more insight has been gained into the molecular mechanisms that contribute to its life in compost. These insights will be summarized here, focusing in particular on substrate utilization.

1. Results & Discussion

1.1. Insights from the genome sequence of A. bisporus

Two haploid strains of *A. bisporus* were used for genome sequencing, *A. bisporus* var. *bisporus* H97 and *A. bisporus* var. *burnetti* JB137-S8, resulting in 10348 and 11289 protein-coding genes, respectively (Morin et al. 2012). The genome revealed a generalist life style with respect to plant polysaccharide utilization, although growth profiling revealed a preference for xylan over cellulose. Most genes involved in plant biomass utilization are classified in the Carbohydrate Enzyme database (CAZy, www.cazy.org) (Lombard et al. 2014), and comparison of the CAZy content of the *A. bisporus* genome to other basidiomycetes, showed that it was overall similar to that of white-rot and to a lesser extent brown-rot fungi (Morin et al. 2012; Rytioja et al. 2014). However, some notable differences are the low number of GH7 (1, acting on cellulose) and GH10 (2, acting on xylan), and the high number of GH25 (4, acting on mannan) genes. *A. bisporus* only has a limited ability to degrade lignin, based on the number of genes present in the genome (Morin et al. 2012) and in this resembles more brown-rot fungi. However, it has a strongly enlarged set of HTP genes, including chloroperoxidases and aromatic peroxygenases, which are likely related to the high content of humic compounds in compost. This may reflect a different method of lignin decomposition and its accessibility in compost compared to woody substrates. Moreover, the high number of HTPs could compensate for the almost absence of heme-including peroxidases in *A. bisporus*.
Transcriptome profiling revealed that the CAZy genes encoding plant biomass active enzymes were mainly expressed in compost grown mycelium, and hardly in the casing layer or fruiting bodies (Fig. 1A) (Morin et al. 2012; Patyshakuliyeva et al. 2013). In contrast, expression of genes encoding fungal cell wall related CAZy genes was detected in all samples (Fig. 1A), but the gene sets differed, with only 36% of the genes expressed in all three samples.
Analysis of the expression of genes involved in carbon metabolism revealed that the vegetative mycelium growing in compost consumes a variety of monosaccharides, matching the compounds present in the substrate (Fig. 1B). In contrast, mainly hexose metabolism was observed, which suggests, combined with the absence of pentose accumulation in fruiting bodies, that hexoses are selectively transported from the mycelium to the fruiting body (Patyshakuliyeva et al. 2013). The other sugars could be used as an energy source for the mycelium or metabolic converted in the mycelium before transported to the fruiting body.

1.2. Detailed analysis of substrate utilization during commercial cultivation.

To obtain insight into how *A. bisporus* obtains carbon from compost during commercial cultivation, we harvested compost samples from different stages of the cultivation, starting from the spawning stage until just after the second flush was harvested (Patyshakuliyeva et al. 2015). Interesting, based on gene expression lignin modification only occurred during the spawning stage, while at later stages no significant expression of ligninolytic genes was observed (Fig. 2). In contrast, expression of genes encoding polysaccharide degrading enzymes was mainly observed after the spawning stage, with peaks in expression during the first flush and after the second flush. This is likely due to the higher requirement of carbon source during mushroom formation, resulting in a higher need to degrade the polysaccharides. The same genes were expressed in these two peaks, suggesting a cyclic expression pattern of gene expression. However, gene expression appears to be a bit out of phase for the second flush, possibly explaining why the mushroom production is so much lower compared to the first flush. The carbohydrate related genes included genes involved in both xylan and cellulose degradation, indicating that both polysaccharides are degraded simultaneously by *A. bisporus*. The proteomics data largely confirmed the transcriptome results, although the reduction observed for the transcriptome between the flushes was less obvious for the proteome. This is likely due to stability of the proteins, enabling them to persist after gene expression is turned off.

The absence of expression of genes encoding α-glucuronidases correlates well with the accumulation of a xylan fraction that is rich in glucuronic acid side groups (Jurak et al. 2015c; Jurak et al. 2015a). Why *A. bisporus* does not express these genes when their enzymes would clearly be beneficial to the substrate degradation process is unclear, but may be related to commercial compost not being a fully natural substrate, and that the commercial strain (A15) has not yet evolved the expression of these genes accordingly.

1.3. Outlook on how molecular insights can help to improve mushroom production

With more knowledge on what actually happens at the molecular level during growth of *A. bisporus* in compost it has become clear that many questions remain to be answered to fully understand the physiology of the fungus and how this translates to mushroom productivity. The accumulation of xylan with high glucuronic acid content and doubly-substituted arabinose demonstrates that part of the hemicellulose is not available to the fungus and explains why some of the substrate is left unused (Jurak et al. 2015c). This accumulation of non-usable substrate may also explain why second and third flushed result in lower mushroom productivity than the first flush. Extensive screening for strains that do express the α-glucuronidase encoding genes may provide the basis for strain improvement strategies that will provide strains that can utilize a larger part of the compost substrate. The absence of genes in the *A. bisporus* genome encoding enzymes that can remove the doubly-substituted arabinose, questions whether this problem can be solved by classical strain improvement. While there may be strains that do contain this activity, they will likely be very distantly related to the current commercial strain and crosses between them will probably introduce many unwanted other traits. Introducing a gene encoding this activity from another fungus into the commercial strain is not a feasible approach either, as acceptance for genetically modified food is still low in many parts of the world.

The data obtained in the last years has provided a starting point for our understanding of the physiology of *A. bisporus* during commercial cultivation. While many thinks remains unanswered, such as why no lignin modification occurs after spawning, it has provided a baseline of the process and a much deeper insight in the genes and enzymes involved. Extending this data set to cultivations with different productivity and cultivations using other types of compost of cultivation regimes may provide answers to why productivity can vary so strongly between cultivations.

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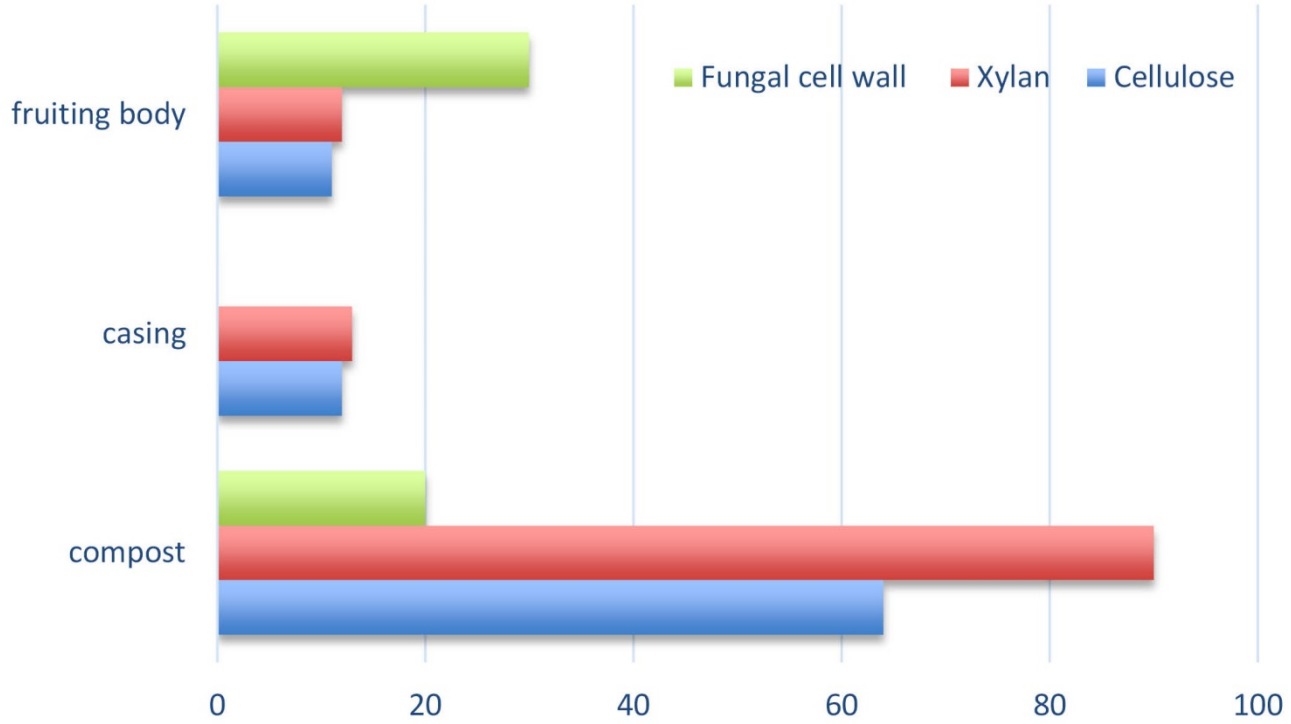


Figure 1. Percentage of genes acting on fungal cell walls, xylan or cellulose that are upregulated in compost, casing layer or fruitiung bodies (Patyshakuliyeva et al. 2013).

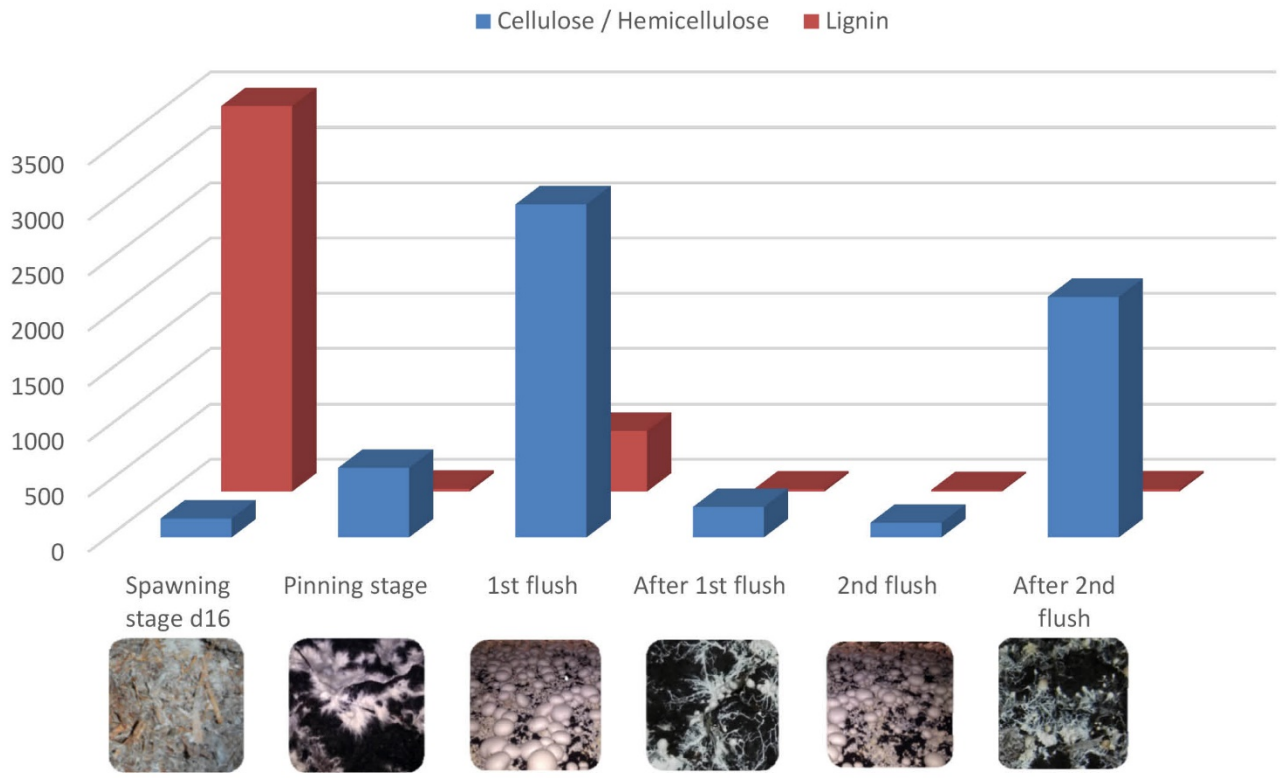


Figure 2. Summarised expression (in RPKM values) of highly expressed ligninolytic (red) and (hemi-)cellulolytic (blue) genes during the different stages of commercial mushroom production (Patyshakuliyeva et al. 2015).

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Gene expression patterns of *Pleurotus ostreatus* under heat stress revealed by genomic and transcriptomic data

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ABSTRACT

We report the genome sequence of a monokaryotic *Pleurotus ostreatus* strain, CCEF00389_1, which is widely cultivated in China. A 34.9-Mb genome assembly was obtained from approximately 81 million Illumina 100 bp reads, giving rise to over 13,000 predicted genes. RNA-Seq data was obtained from four time points under heat stress conditions, and these data were also used to improve genome assembly and gene prediction. By k-means clustering for expression profiles of 450 selected genes related to heat stress, four different expression patterns were detected. Some protein families show coincident expression trends, suggesting that there are different response for different functions to the high temperature. For example, the expression of chitin synthetases and most transcription factors decreased with time. The expression of heat shock proteins and proteins related to trehalose increased at the beginning of heat stress and peaked at 0.5 hour after stress. There were different expression patterns for different sub-families of glucoside hydrolase.

Keywords: *Pleurotus ostreatus,* sequencing, heat stress, RNA-seq.

INTRODUCTION

Pleurotus ostreatus (Jacq.: Fr.) Kumm. (Dikaryia, Basidiomycota, Agaricomycotina, Agaricales), is an edible fungus, and is the most popular mushroom in Northern China. It is widely cultivated worldwide, and represents an important complementary foodstuff [1, 4]. This fungus can grow easily in various temperature ranges on a variety of organic substrates, including agricultural wastes [1, 2]. It has been used as a model for the study of mechanisms involved in lignin biodegradation [3]. In addition to its delicious taste and nutritional value, this mushroom also has health-promoting effects [5, 6].

A higher temperature is one of the most important environmental factors that can negatively affect the growth and development of *P. ostreatus*[7]. In this study, we investigated the pathways involved in heat resistance and heat stress responses in *P. ostreatus*. The genomic data combined

with the RNA-seq data under heat stress conditions can improve our understanding of the fundamental and specific cellular processes of cultivation under high temperatures.
Here we report the genome sequence of the monokaryotic *P. ostreatus* strain, CCEF00389_1, along with predicted genes and select annotations. RNA was extracted from the mycelia which were subjected to heat stress at 37 ºC for different time periods (0, 0.5, 1, 1.5 hours). Among these data, 450 genes reported to be associated with heat stress were selected for further analysis. The differential expression analysis and k-means clustering method were used to detect the expression patterns of different protein families.

1. Results and discussion

1.1. The genome information of CCEF00389_1

A 34.9-Mb genome sequence was obtained by assembling approximately 81 million Illumina reads (~300x coverage) (Table 1), using the tools PLATANUS [8] and L_RNA_SCAFFOLDER [9] with assembly guided by the assembled transcriptome. Gene prediction from the assembled genome and RNA-seq data generated 13438 gene models. The genome size, number of predicted genes and the basic information of predicted genes are very similar as those of related edible Agaricales, such as *Volvariella volvacea* [10], *Agaricus bisporus* [11] and *Flammulina velutipes* [12]. GO annotations were found for 6566 proteins (48.9%) with homologous sequences in the NR database. In addition, 9931 (73.9%) of all predicted genes could be annotated by their protein domains with InterproScan.

Table 1: general features of the *P. ostreatus* CCEF00389_1 genome

Number of all scaffolds	2535
Length of all scaffolds (Mb)	35.8
GC content (%)	51
Scaffold N50 value (bp)	394,787
Number of large scaffolds (>1000)	799
Length of large scaffolds (Mb)	34.9
Number of protein-coding genes	13438

1.2. Detecting the gene expression patterns for different protein families associated with heat stress

There were 10047 genes which were expressed during at least one time point. In order to detect the expression patterns associated with heat stress, only genes responding to heat were selected for further analysis. The function of these genes included cell wall integrity, cell wall sensor, chitin synthase, glucan synthase, heat shock proteins, catalase, peroxidase, oxidoreductase, superoxide dismutase, monooxygenase, nitric oxidase, trehalose, cell cycle, some transcription factors and so on. Among these, 450 genes representing the above functions were selected, and the differential expression analysis was conducted for this gene set. There were 142 genes with

significant differential expression (FDR<0.05) between at least two time points. The k-means clustering method was applied to the expression profiles of these genes to distinguish different expression patterns. Four patterns (clusters) were detected for the differential expressed gene set (Figure 1). Genes with each expression pattern had a different expression peak during four time points. The expression peak of genes with the first expression pattern (46 genes) was at 0 hour, and the expression decreased continuously with continued heat stress. The second gene expression pattern (25 genes) peaked half an hour after onset of heat stress, while gene expression with the third pattern (41 genes) peaked at 1 hour after heat stress. The expression of genes with the last pattern (30 genes) increased sequentially and did not show a peak up to 1.5 hour of heat stress.

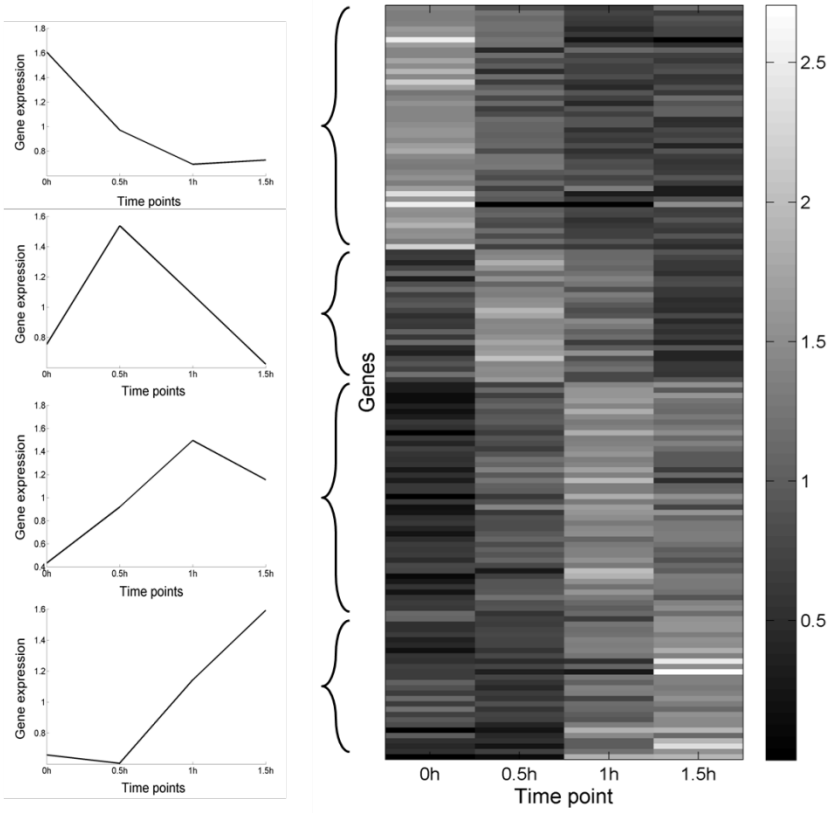


Figure 1: Four expression patterns of 142 differential expressed genes (grey-scale heat-map on the right). The expression profiles are shown on the left.

The expression of all three genes encoding chitin synthase, and the expression of most transcriptional factors (9 out of 14) belonged to the first pattern. This suggests that the chitin synthesis and activity of transcriptional factors are suppressed after heat stress. There were 44 genes encoding heat shock proteins in our gene set, and the expression of 26 of them (59%) belonged to the second pattern. So most heat shock proteins had the highest activity at half an hour after heat stress. The expressions of all three genes related to trehalose synthetis also

belonged to the second pattern, suggesting that the trehalose was produced in response to the heat stress in the first half an hour. Some subfamilies of glucoside hydrolase showed different expression patterns after heat stress. The expression of genes encoding glucoside hydrolase subfamilies 1 and 35 belonged to the first pattern, while the expressions of genes encoding glucoside hydrolase subfamily 3 belonged to the last patterns. It seems that some subfamilies of glucoside hydrolase play different roles in heat stress condition.

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IX Germplasm & Taxonomy

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Towards standardizing taxonomic ranks using divergence times – a case study for reconstruction of the *Agaricus* taxonomic system

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ABSTRACT

The recognition of taxonomic ranks in the Linnean classification system is largely arbitrary. Some authors have proposed the use of divergence time as a universally standardized criterion. *Agaricus* (Agaricaceae, Agaricales) is a mushroom genus that contains many species of high commercial value. Recent studies using ITS sequence data discovered eleven new phylogenetic lineages within the genus, however their taxonomic ranks were uncertain due to the lack of criteria to define them within traditional taxonomy. In this study, we analyzed ITS sequence data from 745 collections (nearly 600 being newly generated) including 86 from type specimens of previously recognized subgenera and sections. Many monophyletic groups were recognized, but most basal relationships were unresolved. One hundred and fourteen representatives of the identified ITS clades were selected in order to produce a multi-gene phylogeny based on combined LSU, *tef-1α*, and *rpb2* sequence data. Divergence times within the multi-gene phylogeny were estimated using BEAST v1.8. Based on phylogenetic relationships and with respect to morphology, we propose a revised taxonomic system for *Agaricus* that considers divergence time as a standardized criterion for establishing taxonomic ranks. We propose to segregate *Agaricus* into five subgenera and 20 sections. Subgenus *Pseudochitonia* is substantially emended; circumscription of the subgenera *Agaricus* and *Flavoagaricus* is restricted to taxa of sections *Agaricus* and *Arvenses*, respectively; and two new subgenera (*Minores* and *Spissicaules*) are introduced. Within *Pseudochitonia*, sections *Bivelares*, *Brunneopicti*, *Chitonioides*, *Nigrobrunnescentes*, *Sanguinolenti* and *Xanthodermatei* are maintained, but the latter two are reduced because we raise subsection *Bohusia* section *Sanguinolenti* and a clade within section *Xanthodermatei* to sectional rank as sections *Bohusia* and *Hondenses* respectively; and sections *Rubricosi,*

Crassispori, Flocculenti, and *Amoeni* are introduced. Section *Laeticolores* is placed in the subgenus *Minores* and sections *Rarolentes* and *Subrutilescentes* are placed in the subgenus *Spissicaules*. Twenty-two new species belonging to various sections are described. This work exemplifies that ITS data, while useful at lower taxonomic levels (i.e., detection of species and species groups), are of limited value for inferring deeper phylogenetic relationships. Finally, we suggest that the establishment of a standardized taxonomic system based on divergence times could result in a more objective, and biologically more meaningful, taxonomic ranking of fungi.
Keywords: Agaricaceae, divergence times, multi-gene phylogenetics, systematics, taxonomy

INTRODUCTIONS

Current systematic and taxonomic research focuses on organizing and classifying species, genera, and higher ranks using a combination of characters, including molecular, morphological, ecological, and biogeographical data. However, as several biologists have pointed out, the Linnean system lacks universal standardized criteria that can comprehensively recognize taxonomic ranks. Fifty years ago, W. Hennig suggested that a taxonomic rank should reflect its geological age, and that time divergence could be used as a universally standardized criterion in the systematics of all known organisms (Hennig 1966). However, it was not until the advent of DNA sequence data analyses and molecular clocks that this idea was further considered. Advances in divergence time estimation based on DNA sequence data provide a way to convert molecular change into evolutionary time (Drummond et al. 2012). Fossil isotopic ages aid estimation of molecular divergence time analyses by providing points of calibration (Berbee and Taylor 2010). Two fungal fossil specimens from Basidiomycotina (Berbee and Taylor 2010; Smith et al. 2004) provide a means for divergence time calibrations in the basidiomycetes.

The saprotrophic genus *Agaricus* is the type genus of the family *Agaricaceae* (*Agaricales*), and has a worldwide distribution. *Agaricus* includes many species that have high nutritional and medicinal value. Historically, the bulk of morphotaxonomic and systematic foundations in *Agaricus* has stemmed from mycological work in Europe, North America, tropical Asia, and Africa (such as Heinemann 1978; Kerrigan 1986). In recent years, *Agaricus* has been well-studied in Europe in a combination of molecular (ITS or ITS and LSU) and morphological analyses (Parra 2008; 2013). Some sections have been shown to be monophyletic, while others were not (Kerrigan 2016 in press; Parra 2008; 2013). In a more global analysis based on the ITS sequence data eleven unnamed clades from tropical areas were revealed (Zhao et al. 2011).

Results & Discussion

First, preliminary NJ tree using the 745 ITS sequences served as a map for sampling strategies, both for sequencing other genes and for the divergence time analysis. Then we selected 114 taxa for dating analyses using ITS, LSU, *tef-1α*, and *rpb2* sequences and BEAST v1.8 (Drummond et al. 2012). Divergence times of the genus *Agaricus* and those nodes within this genus were estimated (Fig.1).

We used the following criteria to recognize subgenera and sections: (i) they must be monophyletic and statistically well-supported in the multi-gene analyses; (ii) their respective stem ages should be roughly equivalent, and subgenera stem ages must be older than section stem ages; and (iii) they should be identifiable phenotypically, whenever possible. With regard to the second criterion, estimated stem ages for subgenera and sections were estimated to be ca. 30 Ma and subgenera ca. 20 Ma, respectively.

Based on the criteria as the above, the genus *Agaricus* is divided into 5 subgenera and 20 sections. Two new subgenera are introduced (*Minores* and *Spissicaules*), one of which is emended (subgenus *Pseudochitonia),* and the subgenera *Agaricus* and *Flavoagaricus* are amended with a restricted circumscription. The subgenus *Lanagaricus* (Heinemann, 1956) becomes a heterotypic synonym of the subgenus *Pseudochitonia*. Five new sections are introduced (*Rubricosi*, *Crassispori*, *Flocculenti*, *Hondenses*, and *Amoeni*), and two remain with a much more restricted circumscription (*Xanthodermatei* and *Sanguinolenti)*. Subsection *Bohusia* is elevated to the rank of section. Finally, 22 new species are introduced with illustrated accounts (Zhao et al. 2016).

A comparison of phylogenetic classifications in *Agaricus* that only used ITS (Zhao et al., 2011), with that of multi-gene analysis (Fig. 1), show that in general, only the younger clades in the ITS tree that were well-supported, are consistently retrieved in the multi-gene phylogeny, whereas deeper, older nodes were generally not or weakly supported. Zhao et al. (2011) were tempted to recognize TRV and TRVI clades as new sections, however, this study indicates that they are 13 and 14 Ma old and embedded in a larger clade with many members of section *Minores*. In this study, we only recognize sections that are approximately the same age clades (ca. 20 Ma), and thus we conclude that TRV and TRVI should remain in section *Minores*.

Taxonomic ranking by using divergence times. Recognition of taxonomic groups has, to date, mainly been based on phenotype and phylogenetic reconstruction with different ranks being applied in a subjective manner. Comprehensive studies on species concepts are moving towards understanding speciation. However, standard definitions for the higher ranks lack universal, standardized criteria. This results in constant changes to the taxonomic system, especially in complex groups, which is not helpful to the end user. Our study is an example towards providing a more objective taxonomic ranking by using divergence times; if such standardized rankings are adopted it could help to stabilize systematics. It has been suggested that divergence times could be used as universal criteria in taxonomic ranking (Hennig 1966; Avise & John 1999). In this paper we explore criteria that could be used as principles in taxonomic classification above the species level. It is feasible that the systematics of all organisms could be arranged using the criteria of divergence times, so that the systematics of all organisms could be represented as a whole, even though they have completely different morphologies and distant phylogenetic relationships.

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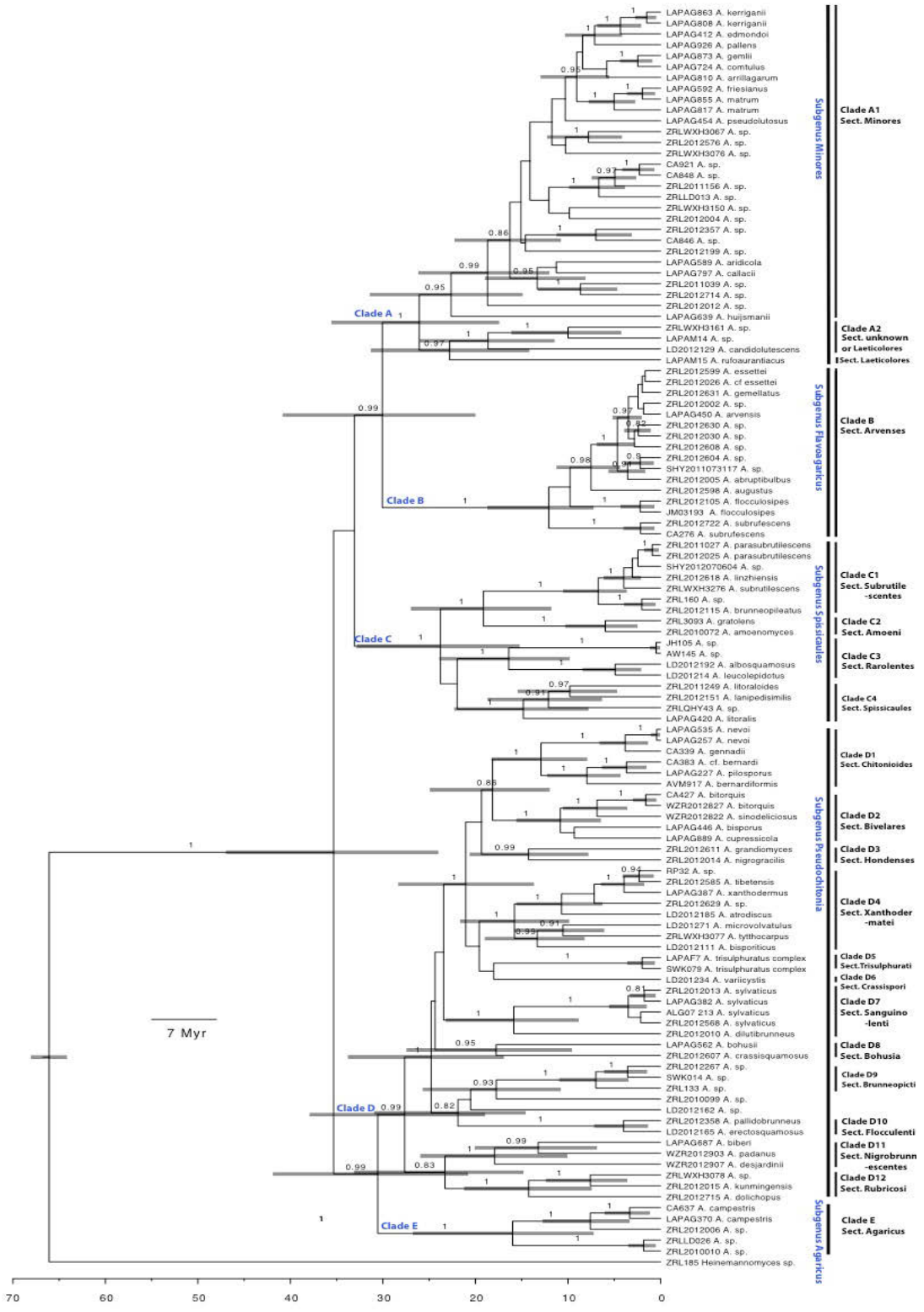


Fig. 1. Maximium Clade Credibility tree of *Agaricus* based on ITS, LSU, *tef-1α*, and *rpb2* genes sequences with the outgroup *Heinemannomyces* sp.. Posterior probability which are equal and above 80% are annotated at the internodes. The 95% highest posterior density of divergence time estimation are marked by horizontal bars. (Zhao et al. 2016)

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African mycodiversity, a huge potential for mushroom trade and industry

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ABSTRACT

A comprehensive study was conducted in tropical Africa and allowed us to update a list of 138 wild edible mushrooms available on the continent. Findings on this huge mycodiversity rely on traditional knowledge recorded from the local population whereas taxonomic data are supported by reference specimens collected in the field. Restoration of endangered miombo woodlands is encouraged to boost the local production and promote the exportation of ectomycorrhizal fungi. Valorization of wild saprotrophic strains through successful cultivation tests reveals the huge potential of the African mycoflora.
Keywords: Africa, Non-Timber Forest Products (NTFPs), wild edible mushrooms, market potential.

INTRODUCTION

In tropical Africa, household food insecurity still constitutes a major challenge with over two thirds of the population relying on non-timber forest products, namely wild edible mushrooms. According to former literature surveys, more than 300 mushroom species are putatively edible in Africa (Boa 2004, Rammeloo & Walleyn 1993), many were anecdotally mentioned in very ancient papers without further confirming or attesting of their food use. Field expeditions and surveys on the whole continent were conducted by the authors to enhance this inventory. Significant advances from this study are that: i) data on edibility rely on traditional knowledge, their truthfulness being ensured by crossing information recorded from the local populations in Central, East and West-Africa; ii) taxonomic information is supported by collecting of reference herbarium specimens thus available for further

validation; iii) cultivation tests were conducted using strains from reference specimens collected in the field throughout Africa.

1. Results & Discussion

A species list was established confirming the edibility and local consumption of 138 fungal taxa in Africa. Their habitat and ecological features were also noticed. One reference specimen is mentioned per region within tropical Africa (Table 1).

Table 1. Edible mushrooms from tropical Africa. [1] – Df = dense evergreen forest (incl. gallery), Mf = montane forest, Mw = miombo woodland, Ra = ruderal area, Sv = savanna; [2] – EM = ectomycorrhizal, S = saprotrophic, P = parasitic, T = termitophilic; [3] – C = central Africa, E = Eastern Africa (incl. Albertine Rift and Zambezian region), W = Western Africa; [4] – collections ADK = A. De Kesel, HEN = H. Eyi Ndong, JD = J. Degreef, SDM = S. Dibaluku Mpulusu (all from BR herbarium).

	Habitat[1]	Ecology[2]	Distribution[3]	Reference specimens[4]
Afroboletus luteolus (Heinem.) Pegler & T.W.K. Young	Mw	EM	E, W	ADK5028, ADK2006
Agaricus arvensis Schaeff.	Ra	S	E	JD1334
Agaricus croceolutescens Heinem. & Gooss.-Font.	Ra	S	E	ADK5517
Agaricus goossensiae Heinem.	Ra	S	W	ADK2751
Agaricus subsaharianus L.A. Parra, Hama & De Kesel	Ra	S	E	ADK4732
Agaricus silvicola (Vitt.) Lév.	Mf	S	E	JD1205
Agaricus volvatulus Heinem. & Gooss.-Font.	Ra	S	W	ADK2566
Amanita crassiconus Bas	Mw	EM	W	ADK2212
Amanita loosii Beeli	Mw	EM	E, W	JD861, ADK4378
Amanita mafingensis Härk. & Saarim.	Mw	EM	E	ADK6196
Amanita masasiensis Härk. & Saarim.	Mw	EM	E, W	ADK6237, ADK1945
Amanita pudica (Beeli) Walleyn	Mw	EM	E	ADK6229
Amanita rubescens Pers. s.l.	Df, Mf, Mw	EM	C, E, W	JD607, ADK6218, ADK2170
Amanita strobilaceovolvata Beeli	Mw	EM	W	ADK1971
Amanita subviscosa Beeli	Mw	EM	W	ADK2149
Armillaria borealis Marxmüller & Korhonen	Mf	S, P	E	JD1312
Armillaria cepistipes Velen.	Mf	S, P	E	JD1314
Armillaria heimii Pegler	Df, Mf, Mw	S, P	C, E	JD443, JD1191
Armillaria lutea Gillet	Mf	S, P	E	JD1284
Armillaria ostoyae (Romagn.) Herink	Mf	S, P	E	JD1372
Armillaria tabescens (Scop.) Emel	Mf	S, P	E	JD1369
Auricularia auricula-judae (Bull.) Quél.	Mf	S	E	JD1258
Auricularia cornea Ehrenb.	Mf, Mw	S	E, W	JD1265, ADK2888
Auricularia delicata (Mont. ex Fr.) Henn.	Mf	S	E	JD1210
Boletus loosii Heinem.	Mw	EM	E, W	ADK5040, ADK2678
Boletus spectabilissimus Watling	Mw	EM	E	ADK5297
Calvatia subtomentosa Dissing & M. Lange	Sv	S	W	ADK2204
Cantharellus addaiensis Henn.	Mw	EM	E, W	ADK5377, ADK1942
Cantharellus afrocibarius Buyck & V. Hofst.	Mw	EM	E	ADK5418

Table 1 (continued). Edible mushrooms from tropical Africa.

	Habitat[1]	Ecology[2]	Distribution[3]	Reference specimens[4]
Cantharellus congolensis Beeli s.l.	Df, Mw	EM	C, E, W	JD454, ADK5471, ADK3179
Cantharellus defibulatus (Heinem.) Eyssart. & Buyck	Mw	EM	E	ADK5511
Cantharellus densifolius Heinem. s.l.	Mw	EM	E	JD854
Cantharellus floridulus Heinem.	Df	EM	C	JD341
Cantharellus isabellinus Heinem.	Mw	EM	E	ADK5414
Cantharellus luteopunctatus (Beeli) Heinem.	Df	EM	C	ADK5836
Cantharellus microcibarius Heinem.	Mw	EM	E	JD980
Cantharellus miniatescens Heinem.	Df	EM	C	ADK5216
Cantharellus miomboensis Buyck & V. Hofst.	Mw	EM	E	ADK6006
Cantharellus platyphyllus Heinem.	Mw	EM	E, W	ADK5510, ADK2028
Cantharellus pseudocibarius Henn.	Mw	EM	E	ADK5373
Cantharellus ruber Heinem.	Mw	EM	E	ADK6032
Cantharellus rufopunctatus var. *rufopunctatus* (Beeli) Heinem. s.l.	Df	EM	C	ADK5949
Cantharellus splendens Buyck	Mw	EM	E	JD896
Cantharellus subcyanoxanthus Buyck, Randrianj. & Eyssart.	Mw	EM	E	JD961
Cantharellus subincarnatus Eyssart. & Buyck	Df	EM	C	JD400
Cantharellus sublaevis Buyck & Eyssart.	Mw	EM	E	ADK6057
Cantharellus symoensii Heinem.	Mw	EM	E	JD912
Clavulina albiramea (Corner) Buyck & Duhem	Mw	S	E	ADK5390
Collybia aurea (Beeli) Pegler	Mf, Mw	S	E	JD1412
Cookeina speciosa (Fr.) Dennis	Df, Mw	S	C, W	JD466, ADK2994
Cotylidia aurantiaca (Pat.) A.L. Welden	Mf	S	E	JD1315
Craterellus aureus Berk. & M.A. Curtis	Df	EM	C	JD646
Cystodermella elegans (Beeli) Harmaja	Mf	S	E	JD1320
Dacryopinax spathularia (Schwein.) G.W. Martin	Mf	S	E	JD1224
Gerronema hungo (Henn.) Degreef & Eyi	Df	S	C	JD370
Gymnopilus zenkeri (Henn.) Singer	Df, Mw	S	C, E	JD447, ADK5758
Hypholoma subviride (Berk. & M.A. Curtis) Dennis	Mf	S	E	JD1182
Lactarius acutus Heim	Df	EM	C	ADK5946
Lactarius kabansus Pegler & Piearce	Mw	EM	E	ADK2430
Lactarius saponaceus Verbeken	Mw	EM	W	ADK2281
Lactarius tenellus Verbeken & Walleyn	Mw	EM	E, W	ADK5329, ADK3598
Lactifluus densifolius Verbeken & Karhula	Mw	EM	E, W	ADK5331, ADK4285
Lactifluus edulis (Verbeken & Buyck) Buyck	Mw	EM	E, W	ADK2385, ADK2063
Lactifluus flammans (Verbeken) Verbeken	Mw	EM	W	ADK3392
Lactifluus gymnocarpoides (Verbeken) Verbeken	Mw	EM	E, W	ADK5410, ADK4302
Lactifluus gymnocarpus Heim ex Singer	Df, Mw	EM	C, E, W	JD308, ADK6236, ADK4442
Lactifluus heimii (Verbeken) Verbeken	Mw	EM	E, W	ADK2451, ADK4327
Lactifluus inversus (Gooss.-Font. & Heim) Verbeken	Mw	EM	W	ADK4316
Lactifluus laevigatus (Verbeken) Verbeken	Mw	EM	E	ADK5013
Lactifluus latifolius (Gooss.-Font. & Heim) Verbeken	Df	EM	C	SDM37
Lactifluus longisporus (Verbeken) Verbeken	Mw	EM	E	ADK2966
Lactifluus luteopus Verbeken	Mw	EM	E, W	ADK5328, ADK3396

Table 1 (continued). Edible mushrooms from tropical Africa.

	Habitat[1]	Ecology[2]	Distribution[3]	Reference specimens[4]
Lactifluus pelliculatus (Beeli) Buyck	Df, Mw	EM	C, E, W	SDM28, ADK5009, ADK3301
Lactifluus medusae (Verbeken) Verbeken	Mw	EM	E	ADK5424
Lactifluus pumilus (Verbeken) Verbeken	Mw	EM	E, W	ADK5427, ADK1987
Lactifluus rubroviolascens (R. Heim) Verbeken	Mw	EM	E, W	ADK5475, ADK3169
Lactifluus sesemotani (Beeli) Buyck	Mw	EM	E	ADK2409
Lactifluus velutissimus (Verbeken) Verbeken	Mw	EM	E	JD886
Lactifluus volemoides (Karhula) Verbeken	Mw	EM	E, W	ADK2395, ADK2019
Lactifluus xerampelinus (Karhula & Verbeken) Verbeken	Mw	EM	E	ADK2394
Lentinus brunneofloccosus Pegler	Df	S	C	HEN74
Lentinus cladopus Lév.	Mf	S	E	JD1219
Lentinus retinervis Pegler	Mf	S	E	JD1269
Lentinus sajor-caju (Fr.) Fr.	Mf	S	E	JD1274
Lentinus squarrosulus Mont.	Df, Mw	S	C, E, W	ADK5278, ADK6223, ADK3632
Lepista sordida (Schumach.) Singer	Mf	S	E	JD1381
Leucocoprinus cretatus Locq. ex Lanzoni	Sv	S	W	ADK3384
Leucopaxillus brasiliensis (Rick) Singer & A.H. Sm.	Mw	S	W	ADK2674
Macrocybe lobayensis (R. Heim) Pegler & Lodge	Ra	S	W	ADK2174
Macrolepiota africana (R. Heim) Heinem.	Mf, Mw	S	E	JD1181
Macrolepiota dolichaula (Berk. & Broome) Pegler & R.W. Rayner	Mf, Mw, Sv	S	E, W	JD1260, ADK2900
Marasmiellus inoderma (Berk.) Singer	Df, Mf, Sv	S	C, E, W	ADK5737, JD1223, ADK3273
Marasmius arborescens (Henn.) Beeli	Df, Mf, Mw	S	C, E, W	ADK5799, JD1220, ADK4199
Marasmius bekolacongoli Beeli	Df, Mf, Mw	S	C, E, W	ADK5779, JD1341, ADK2671
Marasmius buzungolo Singer	Df, Sv	S	C	JD436
Mycoamaranthus congolensis (Dissing & M. Lange) Castellano & Walleyn	Mw	EM	E	ADK5446
Neonothopanus hygrophanus (Mont.) De Kesel & Degreef	Df, Mw, Sv	S	C, E, W	ADK5911, ADK5472, ADK1976
Octaviania ivoryana Castellano, Verbeken & Thoen	Mw	EM	W	ADK2197
Paxillus brunneotomentosus Heinem. & Rammeloo	Df, Mf	S	C, E	ADK5655, JD1232
Phlebopus sudanicus (Har. & Pat.) Heinem.	Mw	EM	E, W	ADK5285, ADK2862
Pleurotus cystidiosus O.K. Mill.	Df, Mf, Mw	S	C, E, W	ADK5272, JD1283, ADK2052
Pleurotus djamor (Rumph. ex Fr.) Boedijn	Mf, Mw	S	E	JD1344
Pleurotus flabellatus Sacc.	Df, Mf, Mw	S	C, E	ADK5771, JD1203
Pleurotus tuber-regium (Fr.) Singer	Df, Mw, Sv	S	C, E, W	ADK5183, ADK5287, ADK2531
Polyporus tenuiculus (P. Beauv.) Fr.	Df, Mf, Mw	S	C, E, W	ADK5613, JD1375, ADK2000
Psathyrella atroumbonata Pegler	Mf	S	E	JD1335
Psathyrella tuberculata (Path.) A.H. Sm.	Mf, Mw	S	E, W	JD1393, ADK2300
Royoporus spatulatus (Rungh.) A.B. De	Df, Mw	S	C, E	ADK5652, ADK5435
Rubinoboletus balloui (Peck) Heinem. & Rammeloo	Mw	EM	E, W	ADK5464, ADK2054
Russula afronigricans Buyck	Mw	EM	E	ADK6240

Table 1 (continued). Edible mushrooms from tropical Africa.

	Habitat[1]	Ecology[2]	Distribution[3]	Reference specimens[4]
Russula congoana Pat.	Mw	EM	E, W	ADK5282, ADK2153
Russula cellulata Buyck	Mw	EM	E, W	ADK5342, ADK2827
Russula ciliata Buyck	Mw	EM	E	ADK6239
Russula compressa Buyck	Mw	EM	E, W	ADK6050, ADK2187
Russula roseoviolacea Buyck	Mw	EM	E	ADK5313
Schizophyllum commune Fr.	Df, Mf, Mw, Sv	S	C, E, W	JD478, ADK5289, ADK2350
Termitomyces aurantiacus (R. Heim) R. Heim	Mw	T	E, W	ADK5283, ADK2830
Termitomyces clypeatus Heim	Df, Mw, Sv	T	C, E, W	JD335, ADK6075, ADK2193
Termitomyces fuliginosus Heim	Df	T	C, W	JD469, ADK4377
Termitomyces letestui (Pat.) R. Heim	Mw, Sv	T	E, W	JD1014, ADK2053
Termitomyces mammiformis R. Heim	Df, Mw, Sv	T	C, E	HEN20, JD1162
Termitomyces medius R. Heim & Grassé	Mw	T	E, W	JD1036, ADK2496
Termitomyces microcarpus (Berk. & Broome) R. Heim	Df, Mw, Sv	T	C, E, W	HEN86, JD1042, ADK2242
Termitomyces reticulatus Van der Westh. & Eicker	Mw	T	E	ADK5461
Termitomyces robustus (Beeli) R. Heim	Df, Mf, Mw, Sv	T	C, E, W	ADK5910, JD1396, ADK1968
Termitomyces schimperi (Pat.) R. Heim	Sv	T	W	ADK2366
Termitomyces singidensis Saarim. & Härk.	Mw	T	E, W	ADK5286, ADK4835
Termitomyces striatus (Beeli) R. Heim	Df, Mw, Sv	T	C, E	ADK5759, ADK5090
Termitomyces titanicus Pegler & Piearce	Sv	T	E	JD1015
Termitomyces tylerianus Otieno	Mw	T	E	ADK5087
Trogia infundibuliformis Berk. & Br.	Mw	S	E	JD1044
Tubosaeta brunneosetosa (Singer) E. Horak	Mw	EM	E, W	ADK5034, ADK4537
Volvariella earlei (Murrill) Shaffer	Mw	S	E, W	ADK5081, ADK1948
Volvariella volvacea (Bull.) Singer s.l.	Mw	S	W	ADK2205
Xerocomus subspinulosus Heinem.	Mw	EM	E, W	ADK5466, ADK2239

Termitomyces representatives are unanimously seen as a delicacy by the African populations. Some species are amply but only locally sold throughout the continent because they deteriorate soon making them difficult to market.

Ectomycorrhizal species are mainly found in the remaining miombo woodlands of West- and East-Africa associated with indigenous trees (Dipterocarpaceae, Euphorbiaceae, and Fabaceae). Tremendous yields (10 to 50 kg fresh weight/ha for the most commonly sold chanterelles) recorded in these natural ecosystems during the 5-months duration of a rainy season foretell a high trade potential. Production plots with artificially-inoculated local trees combined with efficient harvesting plans are worth to be developed in the miombo ecozone for promoting the export of e.g. African chanterelles and milk-caps to the international market.

Although less palatable, saprotrophic fungi have nevertheless a significant food potential in dense and montane forests of Central, East- and West-Africa. Living strains were isolated for some of the collected species in order to test their potential for cultivation. Results obtained locally with low-cost technology reveal that some wild African strains of *Auricularia cornea*,

Lentinus brunneofloccosus, *L. cladopus*, *L. squarrosulus*, *Marasmiellus inoderma*, *Marasmius buzungolo*, *Pleurotus cystidiosus*, *P. flabellatus*, and *Schizophyllum commune*, all popular edible, are definitely good candidates for domestication.

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X Cultivation techniques

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Spawn cryopreservation of *Pleurotus pulmonarius* and *Lentinula edodes* strains

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ABSTRACT

One of the main problems for conservation of germplasm of edible mushrooms is that the traditional method of subculturing facilitates aging and contamination of strains. The conservation at ultra-low temperature in liquid nitrogen (-196 °C) is a widely used method, however, it becomes expensive in collections with large numbers of strains and requires highly specialized personnel. Cryopreservation at -80 °C of mycelium on cereal grains (spawn) has also been shown to be an efficient and low cost method for *Agaricus* species. It could be used for other edible mushrooms. The aim of this study was to evaluate the viability of spawn of *Pleurotus pulmonarius* and *Lentinula edodes* strains frozen at -20 or -80°C.
Two strains of each species were studied. The spawn was prepared in sorghum seeds and incubated for 3 weeks at 25 ºC to allow the grains were completely covered by mycelium. Fully-incubated sorghum grains were placed in polycarbonate vials containing 1.5 ml of sterile cryoprotectant solution (10% glycerol v/v) or in vials without solution. The grains remained in contact with the cryoprotectant solution for 1 h at room temperature and then vials of both treatments were transferred directly into freezers at -20 or -80 ºC. After 3, 6 and 12 months the samples were thawed for 10 min in a bath water at 30 °C and the viability of the spawn grains was evaluated. Mycelial growth rate was measured on potato dextrose agar (PDA) at 25 °C for 7 days. The percentage of sample recovery was recorded from the number of spawn grains giving rise to a mycelial colony. Recovered mycelia were further used in small scale cultivation experiments to check their fruiting ability.
In the samples frozen at -20 °C the recovery was 24 - 100 % and 4 - 94 % for *Pleurotus* and *Lentinula* strains respectively. However, the samples frozen at -80 °C with or without cryoprotectant showed a recovery of 100% from 1 to 5 days after thawing. The recovered samples had a normal mycelial growth in PDA. In all cases the recovered samples produced normal basidiomata when cultivated on substrate prepared with pasteurized barley straw. The proposed method may facilitate handling and reduce maintenance for edible mushroom strains.
Keywords: Oyster mushroom, shiitake, edible mushroom, germplasm conservation

INTRODUCTION

Most of Basidiomycetes used to produce edible mushrooms are usually preserved in

laboratories and collections by successive mycelial sub culturing but this method occasionally causes genetic degenerations, loss of commercial characteristics and contamination by other microorganisms (mainly moulds and bacteria). Furthermore this method becomes expensive when maintaining collections with many strains and requires highly specialized staff for managing them. On the other hand, cryopreservation with liquid nitrogen at -196 ºC has been successfully used for preservation of fungal species [1]. Other methods have been also tested for mycelial preservation [2], however one of the cheapest method is to use spawn of the strains as an alternative support for preservation [3 - 7]. With the objective to find options to spawn preservation, the aim of this work was to evaluate two different temperatures (-20 and -80 ºC) to preserve spawn of *Pleurotus pulmonarius* and *Lentinula edodes.*

1. Material & methods

1.1. Studied strains and spawn preparation

Two strains of *Pleurotus pulmonarius* (IE 115, IE 136) and two strains of *Lentinula edodes* (IE 40, IE 245) were studied. They were cultured for 7 days in Petri dishes with medium of potato dextrose agar (PDA). Spawn was prepared according to the method of Guzman *et al.* [8] on sorghum seeds (65% hydrated and sterilized at 121 °C for 1 h). The seeds, placed in Petri dishes were inoculated with mycelium precultures (in PDA) of each strain and incubated in the dark for 3 weeks at 25 °C.

1.2. Freezing (-20 and -80 ºC)

For freezing samples, the method proposed by Mata and Perez Merlo [4] was used. Fully-incubated sorghum seeds were placed in sterile polycarbonate (Nalgene) vials (25 seeds per vial) each vial containing 1.5 ml of sterile cryoprotectant solution (10% glycerol v/v). The seeds remained in contact with the cryoprotective solution for 1 h (G) and then samples were placed in polycarbonate boxes and transferred directly into the freezer at -20 or -80 ºC. Moreover vials containing 25 seeds without glycerol were also prepared (WG) and immediately placed into the freezers. The samples were kept frozen at -20 or -80 for 3, 6 and 12 months (3M, 6M, 12M). After that time they were thawed in water at 30 °C for 10 min. Once thawed, the vials were cleaned for 1 min in an alcohol solution (70% v / v) and then seeds were removed from vials and placed in Petri dishes with PDA and incubated at 25 °C.

1.3. Viability and vitality of the samples

After treatments, the percentage of sample recovery was evaluated through daily observations of the seeds. A sample was considered recovered when mycelial growth was noted by observing the seeds with a stereoscopic microscope. The delay for recovering was also recorded. For each treatment (G, WG) and time of freezing (3M, 6M, 12M) recovery was evaluated with 50 spawn seeds. Mycelia recovered from freezing treatments were used to prepare new spawn in order to evaluate mycelial growth in PDA. Mycelial diameter was recorded by placing a spawn seed in PDA and taken from 2 perpendicular axes after 7 days of incubation at 25 ºC [3]. Ten samples were prepared per treatment and strain. These data were analyzed using ANOVA followed by Tukey's multiple-range test (p = 95%).

1.4. Basidiomata production test

To asses basidiomata formation, samples of 2 kg of steam pasteurized (65 ºC/6h) barley straw were inoculated with spawn (5 %) prepared as above from each strain recovered after freezing at – 80 ºC. After incubation at 25 ºC (three weeks for *Pleurotus* strains and 6 weeks for *Lentinula* strains), samples were placed in a room with relative humidity of 90 – 95 %, ventilation for 10 min 4 times a day, 12 h light and 12 h dark at 22 ºC for *Pleurotus* strains and at 18 ºC for *Lentinula* strains. Formation of basidiomata was registered.

2. Results & discussion

The freezing temperature affected the percentage of recovery in the strains (Table 1). At -20 °C, recovery rates ranged from 2 to 100%, but the viability decreased in function of time and no strain was recovered at 12M. On the other hand the strains showed a full recovery from 1 to 5 days after thawing when they had been frozen at -80 °C, both with and without cryoprotectant (G, WG), whatever the freezing times tested (3M, 6M, 12M). The delay for total recovery was slightly longer in strains of *L. edodes.* Once again there were no differences between G or WG treatments. In all strains and conditions tested at -80 ºC, the recovered mycelia showed normal appearance in color and texture, with no apparent alteration.

Table 1. Percentage and time of recovery in samples of *Pleurotus pulmonarius* (IE 115, IE 136) and *Lentinula edodes* (IE 40, IE 245) strains frozen at two different temperatures

	-20 ºC					-80 ºC					
Strain and	3M		6M		12M	3M		6M		12M	
treatment	R	D	R	D	R	R	D	R	D	R	D
IE 115 WG	100	4	100	6	0	100	1	100	1	100	1
IE 115 G	88	10	24	9	0	100	1	100	1	100	1
IE 136 WG	100	6	100	5	0	100	1	100	1	100	1
IE 136 G	100	7	52	8	0	100	1	100	1	100	1
IE 40 WG	94	10	42	12	0	100	2	100	4	100	4
IE 40 G	4	14	2	10	0	100	3	100	5	100	3
IE 245 WG	80	14	4	10	0	100	2	100	5	100	3
IE 245 G	8	14	0		0	100	2	100	5	100	5

G = Treatment with glycerol. WG = Treatment without glycerol. 3M = Treatment with 3 months of freezing. 6M = Treatment with 6 months of freezing. 12M = Treatment with 12 months of freezing. R= Recovery percentage. D = Days of total recovery.

The average mycelial growth of the samples recovered from the WG treatment at -80°C was 47.1 mm while in treatment G was 47.6 mm (Figure 1). These results confirm those obtained in other studies on the recovery of mycelia frozen without cryoprotectant using seeds as vectors [4, 6, 9]. The average mycelial growth in all samples recovered from

the 3M (58.0 mm) and 12M (53.5 mm) showed no significant differences, suggesting that there is not a negative effect due to freezing increasing with the time. Mycelial growth of *L. edodes* strains showed no differences with respect to the values of its own control. However, the strain IE 115 of *P. pulmonarius* presented slight differences with respect to its control. The lower mycelial growth rate in samples recovered after 6 months observed in all the samples might be an artifact due to problems in the quality of the culture medium and incubation temperature.

Spawn prepared from mycelia recovered after freezing at -80 ° C showed a normal growth in barley straw. The basidiomata obtained in all cases showed normal morphological (color, size, shape) and organoleptic (odor) characteristics, even though yield was not assessed there was no apparent differences with controls. The present data on *L. edodes* and *P. pulmonarius* are in agreement with previous ones on *A. bisporus* and *A. subrufescens* [4, 6]. Cryopreservation at -80°C of mycelia of edible mushrooms pre-cultivated on sorghum seed is a promising alternative to the conventional conservation in liquid nitrogen using cryoprotectants. It should be adopted by spawn makers as well as academic laboratories, after controls for longer storage times.

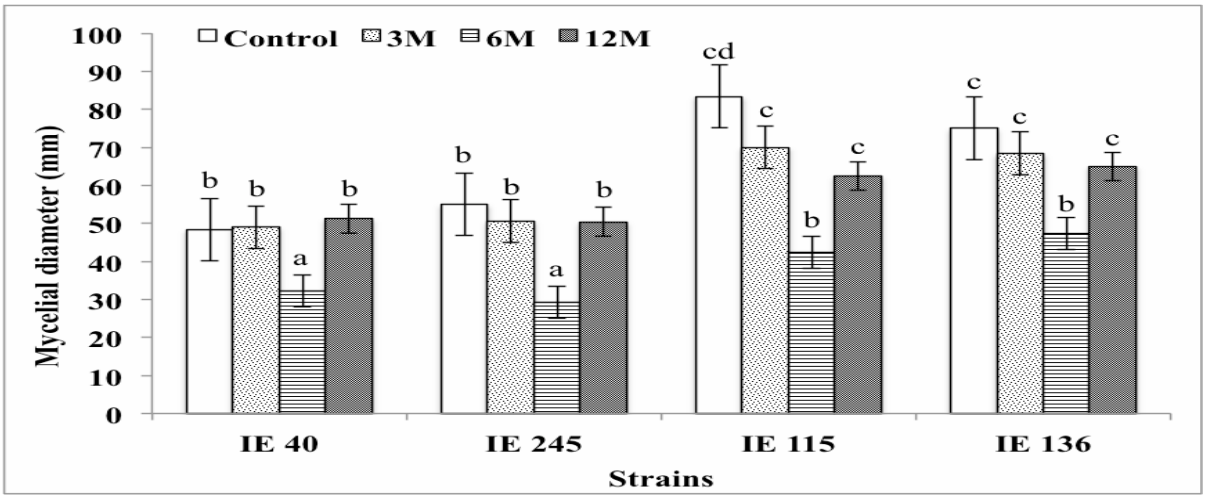


Figure 1. Mycelial diameter (mm) of *Lentinula edodes* (IE 40, IE 245) and *Pleurotus pulmonarius* (IE 115, IE 136) strains after freezing during 3, 6 and 12 months (3M, 6M, 12M) at -80 ºC. Different letters indicate significant differences (p = 95%).

ACKNOWLEDGEMENTS

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Domestication of Wild Edible Mushrooms in Eastern Africa: A Review of Research Advances and Future Prospects

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ABSTRACT

Mushroom farming is an emerging industry in Africa with great potential to provide a stable income to small-scale farmers. However, the mushroom farming is still dominated with a few exotic varieties (*Agaricus, Pleurotus, Lentinula, Ganoderma*) cultured from imported mother cultures whose lineages have poor regional adaptability, low yields and increased pests and diseases susceptibility. Although there has been increased effort to exploit and domesticate native strains of mushroom species with desirable characteristics for commercial cultivation, the information concerning their domestication status, challenges and future prospects is still scattered and not clear. The purpose of this paper was to review and detail the diversity of potential and already domesticated wild edible (medicinal) mushrooms species in Eastern Africa, their domestication status, nutritional value in comparison to introduced exotic species and availability of their germplasm (mother cultures). To achieve this, a detailed review of published and un-published research articles, books and thesis from Eastern Africa was conducted. Data was collected only from articles focusing on diversity of wild edible (medicinal) mushrooms, their nutritional composition and domestication methods and status.
From the review, 258 wild edible mushrooms species are shown to have desirable characteristics for utilization as food and medicine, with Tanzania documenting the highest number of wild edible species (118) followed by Malawi (47). Among this, 82 species were edible ectomycorrhiza species with great potential in supporting mushroom industry if sustainably harvested and managed. The rest were saprophytic fungi species. Only 9 species *(Pleurotus flabellatus, Coprinus cinereus*, *Volvariella volvocea, Pleurotus citrinopileatus, Auricularia auricula, Pleurotus djamor*, *Pleurotus HK-37, Pleurotus sp* and *Oudemansiella tanzanica)* among saprophytic group have been tissue cultured, tested for spawn production and cultivation. 40 species have been analyzed for nutritional composition. However, none of these have been commercially introduced in cultivation and availability of their mother cultures (germplasm) for research and propagation purpose is uncertain. The result from this study clearly shows research on domestication of wild edible mushrooms in Africa is still in its infancy stages. Establishment of a national and regional Fungi Gene Bank and Spawn Production Centers in Africa may enhance storage of potential fungi germplasm for further research and propagation purposes.
Key words: Wild edible mushrooms, Domestication, Nutritional composition, Diversity, Germplasm

INTRODUCTION

Mushroom farming is an emerging industry in Africa with great potential to provide a stable income to small-scale farmers. However, mushroom sector in Africa still remain underexploited as a source of income in Africa, and is dominated with a few exotic temperate varieties (*Agaricus, Pleurotus, Lentinula, Ganoderma*) cultured from imported mother cultures whose lineages have poor regional adaptability, low yields and increased pests and diseases susceptibility. Over the past years, there has been increased interest to exploit and domesticate wild edible and medicinal native strains of mushroom species with desirable characteristics (good regional adaptability and low susceptibility to pests and diseases) for commercial mushroom cultivation in Eastern Africa (EA). But their diversity and potential of in commercial mushroom industry still remain less explored, their exact number of remains unknown and information concerning their domestication status, challenges and future prospects is still scattered and not clear. The purpose of this paper is to review and detail the diversity of WEMs in with special reference to EA, its potential in mushroom industry, domestication status, nutritional value in comparison to introduced exotic species and availability of their germ-plasm (mother cultures).

Results & Discussions

Diversity of edible (medicinal) mushrooms and their nutrition status

This review has revealed diverse community of wild edible mushroom (WEMs) species that could be brought to cultivation in EA. A total of 258 species of wild mushroom distributed in 35 families and 43 genuses were documented from 7 East African countries (Kenya, Tanzania, Malawi, Somalia, Ethiopia, Uganda and Burundi). The WEMs diversity comprised of saprophytic fungi (45.74%), ectomycorrhiza (43.02%) and *Termitomyces* species (11.24%). Tanzania and Malawi had the highest number while Somalia had the least (Figure 1). Miombo woodland dominated by *Brachystegia*, *Julbernardia* and *Isoberlinia* tree species known to symbiotically associate ectomycorrhizal mushrooms species, makes 40% of Tanzania land and 20% of land Malawi, explaining the high number of ectomycorrhiza mushroom species found in these two countries[1] *Termitomyces* mushrooms live in a mutualistic obligate relationship with termites, and are important wild edible species also found in Miombo woodland, although are widely distributed in other areas [1]. Saprobic mushrooms colonize rotting wood and organic matter found in soil found in forested ecosystems, woodlands, grasslands and in less intensive agricultural systems. Their diversity in EA was high (118 species), and comprised of 32 cultivatable species, with good representation of *Agaricus* (18) and *Pleurotus* (17*)* species whose economic significance in mushroom industry is widely recognized.

Nutritional status of some these WEMs species was comparable to cultivated exotic species with protein levels between 17-39.2 g/100g among the 72 species analyzed for the nutritional composition. This is within the range of protein levels of exotic cultivated species like *Pleurotus ostreatus* (35.6 g/100g), indicating highly nutritious resources for future mushroom industry in EA [2].

Domestication status of edible and medicinal Mushrooms in East Africa

Domestication of WEMs in EA provides an enormous opportunity to expand mushroom industry in this region since most of WEMs have regional adaptability, grow rapidly at higher temperature (>25^0c) and their growth conditions can easily be replicated using locally available agricultural and industrial waste. Domestication of WEMs in EA is however still in its early stages with only 11 *(Pleurotus flabellatus, Coprinus cinereus*, *Volvariella volvocea, Pleurotus citrinopileatus, Auricularia auricula, Pleurotus djamor*, *Pleurotus HK-37, Pleurotus sp* and *Oudemansiella tanzanica)* species under experimental trials (Table 1). Nine saprophytic species have been tissue cultured, tested for spawn production and their production tested under different growth condition (Table 1). But none of the 9 species have been commercially introduced into cultivation and availability of their mother cultures (germplasm) for research and propagation purpose is uncertain. Two of the species (saprophytic and *Termitomyces* species) are still in tissue culture stage. However, the growth rate of tested WEMs species was amazing, producing comparable results with exotic species (*Pleurotus and Agaricus)* from tissue culture, spawn production and cultivation under different substrate (Sisal waste, Rice straw, Saw dust, Sugarcane bagasse, Wheat straw, Grass straw, Maize cobs, Banana leaves, Ground nut residue, Soya bean residues) (Table 1). *Auricularia auricula* did not fruit despite successful spawn run and pinning. Although the protein levels of cultivated species was quite low compared to that of exotic species (*Pleurotus ostreatus;* 22.1 vs. 35.5 (g/100g), their yield was 10 times higher under saw dust and 2 times higher in other substrates. Supplementation of substrates with other nutrient rich substrate may be necessary to improve the protein levels of these mushrooms.

Our result clearly shows research on domestication of wild edible mushrooms in Africa is still in its infancy stages, although WEMs has great potential in supporting mushroom industry.

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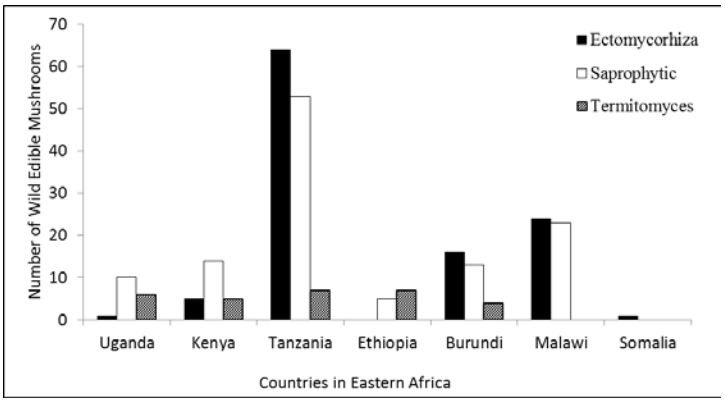


Figure 1: Distribution of wild edible (medicinal) mushrooms in Eastern Africa

Table 1: Domestication status of edible and medicinal Mushrooms in Eastern Africa

Mushroom species	**Tissue culture**		**Spawn production**		**Mushroom cultivation**					
	Media	Incubation Period	Substrate	Incubation period	Substrate	Spawn run	Pinning	Fruiting	Yield	
		-----days-----		**-----days---**		**----------days----------**			--g/kg--	**Reference**
Coprinus cinereus	PDA	4	a	10	d	7 (12)	9 (15)	11 (17)	238	3,4
Volvariella volvacea	PDA	5	a	12	d	9	11	13	114	3
Pleurotus flabellatus	PDA	6	a	13	d	12	14	17	371	3
Oudemansiella tanzanica	MALT	7	b	21	f, e, d	18 (19)	20 (21)	19 (22)	–	5
Auricularia auricula	MALT	5 (7)	a, b	12	g, h, i, j	8 (14)	13 (23)	–	–	6
Pleurotus citrinopileatus	PDA	7 (10)	c	–	e, f, h, j, k, l, m, n, o	8 (21)	13 (28)	17 (33)	36 (397.7)	7
Pleurotus sp.1	PDA	7	b	14	a, k, l, m, n, p	8 (23)	39	45	177 (500.6)	8
Pleurotus sp.2 (HK-37)	MALT	5 (7)	a		d	12 (14)	18 (43)	20 (46)	119.2	4
Pleurotus djamor	PDA	7	a, b	14	m, n, f ,n, p, o	8	19	25	158.3	8
Macrolepiota	MALT	10	–	–	–	–	–	–	–	
Termitomyces	PDA	10	–	–	–	–	–	–	–	

Spawn substrate included (a) Sorghum grains, (b) Millet grains and (c) Wheat grains. Cultivation substrate included (d) Sisal waste (e) Rice straw, (f) =Saw dust, (g) Sugarcane bagasse (h) =Wheat straw, (i)=Grass straw, (j)= Maize cobs, (k)=Banana leaves, (l) Ground nut, (m) Soya bean, (n) Coffee husk, (o) cotton waste and (p) Bean straw. The values in parenthesis are maximum number of days taken or yield produced. The yield data was compared to yield of *Pleurotus ostreatus* under rice straw (85.5)m Wheat straw (83), Maize cob (27.2), bean straw (105) and saw dust (3.2) [9]

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Adapting the Commercial Cultivation Technique of *Lepista Nuda* to Obtain a Constant Production and Non-stop Supply of Volumes

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ABSTRACT

To successfully increase the economic value of mushrooms on the market, a constant supply of production volumes, when there is a demand, is a very important issue. This is also the case for the niche market of *Lepista nuda*, the wood blewit or blue stalk mushroom. In order to have a better view on this topic a practical test to explore the possibilities in adapting the commercial cultivation technique was set up. The effect of using two different batch numbers of commercially available *Lepista nuda* spawn in combination with adding casing soil at two separate time points was evaluated. These adaptations in cultivation technique resulted in a twofold harvest period of eight weeks, showing a regular production level over this period. This study provides further information on the implementation of a viable, commercial cultivation technique for *Lepista nuda*.
Keywords: *Lepista nuda;* blewit*;* cultivation techniques; production spread; marketing pied bleu

INTRODUCTION

Although the market for *Lepista* in Western Europe is a seasonal one, a constant supply of production volumes is an important issue and the more for a niche market product. Irregular production can easily destroy the success of a new product and being constantly available on the market is a must.

Upon request by the auction REO (Roeselare, Belgium), a trial was set up to spread the production and market supply and minimize fixed production costs. Transport, filling costs and energy costs are as good as the same for a whole growing room than for a partially filled growing room. To minimize these fixed costs a complete growing room was filled with traditionally in tunnels prepared phase II compost. At spawning, two batch numbers of commercially available *Lepista nuda* spawn were used and the casing activity was done on two different moments; both strategies were evaluated to obtain a better spread in the production of *Lepista nuda*.

Results and Discussion

Phase I compost was delivered by the company Sterckx (Roeselare, Belgium) and pasteurized in the tunnels of the test farm of the Belgian mushroom research center Inagro. A total of 95kg phase II compost/ m² was filled in 1,5 m² aluminum containers wherein plastic was placed on the bottom. Before filling the containers, the compost was spawned with Sylvan nr 4102 at spawn rate 2,5 % wet weight spawn/wet weight compost. One half of the compost was spawned with batch number 821496 and the other half with 821497. Production was set up in the same controlled-climate growing room (GICOM).
After 15 days of spawn run, half of the number of containers of each batch was cased with a layer of 5 cm traditional casing soil [1] type 'wet', based on black peat mixed with sugar beet lime (Sterckx, Roeselare, Belgium; code *-15d). The remaining containers of each batch were cased 2 weeks later (code *-29d). The cac-ing technique was not applied. In the trial each combination batch/casing moment was performed in 10 parallels. Physical conditions like homogeneity, humidity, structure and acidification of the casing soil were scored and no difference was observed between the two deliveries. Consequently, possible influence of the casing soil on later production was not expected.
After each casing moment, the casing layer was watered with 5,5l water/m² spread over the first 5 days, and then covered with perforated plastic [2]. After the first flush casing layer was watered with 3l/m² and another 2l was giving after the second and third flush [3].
During spawn run in compost and in casing layer, room temperature was controlled between 20 and 23°C to obtain a compost temperature of 26 to 30°C. Table 1 shows the overview of the trial set up for each object.

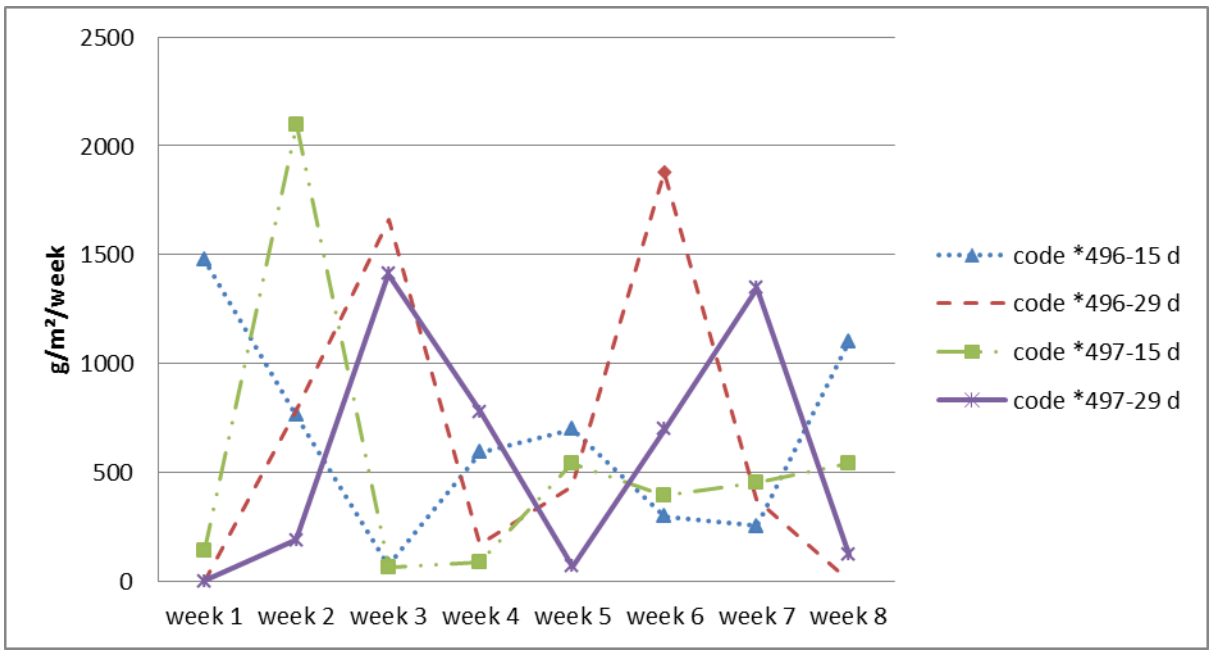
Table1: Overview of trial set up in number of days from spawning to start harvest

Object	Casing day	Cooling day	1st picking day
1 code 496-15d	15	36	72
2 code 496-29d	29	36	74
3 code 497-15d	15	36	79
4 code 497-29d	29	36	83

Start of cooling was set at day 36 for each object. At that time, evaluation of height of mycelium was scored. The mycelium at cooling was significantly higher for the 496-15d plots, what was expected. For the plots with code 497 no significant difference of the height of the mycelium at cooling could be noticed.
In total we registered harvest for 4 flushes covering 15 weeks. The production of the first 8 picking weeks was 5,3 kg/m² (55,3 kg/ton phase II compost) for object code 496-15d and 5,3 kg/m² for object 496-29d (55,8 kg/ton phase II compost). Object 497-15d produced 4,3 kg/m² (45,3 kg/ton phase II compost) and object 497-29d 4,6 kg/m² (48,4 kg/ton phase II compost).
Yield of the 29 day spawn run plots was higher than that of the 15 day spawn run plots, irrespective of used spawn batch. Results of weekly yield are shown in graph 1.

Results showed that the first flush from objects cased at day 15 started approximately two weeks earlier than the objects cased at day 29. The same trend was observed for the second flush.

Graph 1: Yield spread during the first 8 picking weeks

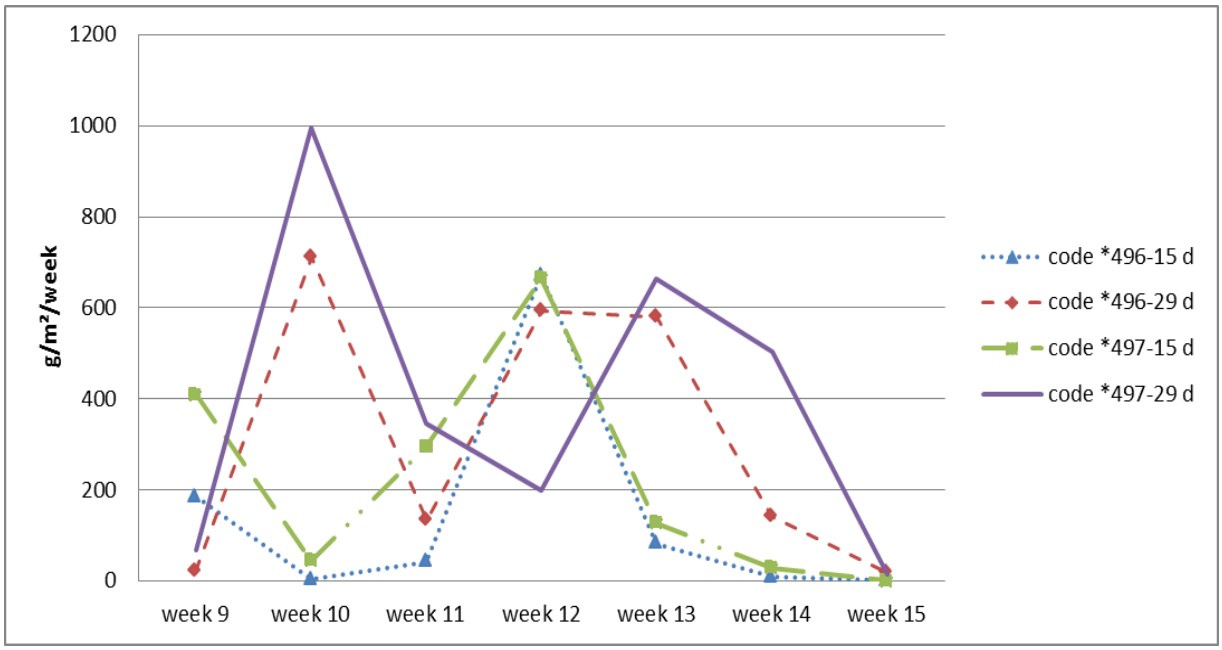


The followed strategy using 2 different batch numbers had limited effect on production spread. This production spread was about one week depending of the number of the flush.
Weekly yield for the first 8 weeks showed a constant production level but with considerable fluctuations. In fact, the relative yield for the whole room was respectively 8%, 19,7%, 16,5%, 8,3%, 8,9%, 16,8%, 12,4% and 9% for week 1 to 8.

The production of the last 7 weeks was respectively 1 kg/m², 2,2 kg/m², 1,6 kg/m² and 2,8 kg/m² for objects 1 to 4. The overall results for the total cropping cycle for 15 weeks were 6,2 kg/m² for the code 496-15d, 7,5 kg/m² for the code 496-29d, 5,9 kg/m² for the code 497-15d and 7,4 kg/m² for the code 497-29d. Again, yield of the 29 day spawn run plots was higher than that of the 15 day spawn run plots, irrespective of used spawn batch.
Evaluation of yield data of third and fourth flush indicated that production spread was present between the objects cased at different moments (see graph 2). Similarly as in the first 8 weeks, spread between the two batches was less apparent.

Variation in relative yield of the complete room was considerable. The relative yield was respectively 9%, 23,2%, 10,8%, 28,1%, 19,2%, 9% and 0,5% for week 9 to 15.

Graph 2: Yield spread from week 9 to week 15



We can conclude that when adding casing soil at two separate time points, in combination with using 2 different spawn batches, it was possible to spread the harvest and ensure a more constant supply of the market. In addition we can state that the object cased on day 29 produced better than the object cased after 15 days, irrespective of used spawn batch.

ACKNOWLEDGEMENTS

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Successful commercial cultivation of morels in China

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ABSTRACT

Morchella (*morel*) is one of the most prized edible and medical mushrooms in the world. Despite artificial cultivation of morels has been studied for over 100 years, commercial cultivation was still a difficult task. In recent years, the successful commercial cultivation of morels was rapidly developed in China, especially in Sichuan Province. Here we report the commercial cultivation of *Morchella importuna* and *M. sextelata* in Sichuan Province. Identification of the cultivated morels were based on morphological characters and molecular evidence of ITS and RPB2 sequences. Both *M. importuna* and *M. sextelata* follow the same cultivation procedures and conditions. Firstly, the mycelia were incubated in PDA for 7days at 20°C. Then, mycelia were inoculated on wheat substrate supplemented with wood shavings and soil; and then incubated for about 20 days at 20°C until mycelia fully colonized and sclerotium formed on the surface of substrate. Sclerotium usually appeared after 7-10 days of inoculation. After sclerotium formation, the spawn was then transferred to the field and inoculated in the soil at 10-18°C and 60-80% humidity. Watering was performed when necessary. The primordia were formed about 80-100days after incubation in the field, and mature fruiting bodies were observed about 5-7days after primordial formation. Mature fruiting bodies of *M. importuna* reached 7-15 cm in length, and 6-16cm for *M. sextelata*. *M. importuna* has conical cap that is olive brown to brown, and its stipe is grayish white, covered with granules; the cap of *M. sextelata* is conical, reddish brown with brown ridges, and its smooth stipe is pure white. Average yield of *M. importuna* and *M. sextelata* was 1200 kg and 1500 kg per acre, respectively. The maximum yield of *M. importuna* reached to 3048 kg per acre, and 3120 kg for *M. sextelata*. *M. sextelata* was less sensitive to temperature rise than *M. importuna*.

Keywords: Morels; *Morchella importuna*; *Morchella sextelata*; Commercial cultivation; Southwest China

INTRODUCTION

Morchella (morel) is one of the most prized edible and medical mushrooms in the world. It is widely distributed throughout the world and well known for its delicious taste. Members in this genus are economically important and of high commercial value at national and international

level. In nature, morels mainly occur in spring and could be found in the markets in production areas for a few weeks. In recent years, the market demand and price of morels has been increasing steadily, which has caused unrestrained collection that strongly destroyed the ecological environment and threatened seriously the wild resources of morels. This aroused through attention from both home and abroad, and resulted in the need to develop an effective biotechnological process to cultivate morels.

Morels are widespread in China, and morel cultivation has been studied more than 60 years in this country. In recent years, researchers in Soil and Fertilizer Institute (SFI), Sichuan Academy of Agricultural Sciences (SAAS) conquered technical difficulty of morel commercial cultivation, and morels cultivation was rapidly developed in China, especially in Sichuan Province. Additionally, we found that the cultivated morels might represent two different species other than the long hypothesized *M. conical*. Here we report the germplasm resources, commercial cultivation, breeding and new variety protection of morels in Sichuan Province, and identification of the cultivated morels based on morphology and molecular evidence.

1. Results &Discussion

1.1 Germplasm resource collection and identification

More than 200 *Morchella* strains were collected from four provinces in southwest China during recent ten years, and 105 strains were selected for artificial cultivation test. Forty-five strains were fruited under the controlled condition, among which M8 and M9-1 demonstrated better agronomic traits. Morphological characters of the two strains are corresponding to those of *M. importuna* and *M. sextelata*, respectively (Kuo et al. 2012). Additionally, ITS and RPB2 sequences ofM8 were 99-100% similar to those of *M. importuna*, and sequences of M9-1 were almost identical to those of *M. sextelata*.

1.2 Breeding and variety protection

Three varieties of morels have been selected and bred in our study. The first cultivation variety in China, “Sichuan Morel No.1”, was authorized by Sichuan Provincial variety Examination and Approval committee in 2014. Subsequently, another two varieties, “Sichuan Morel No.3” and “Sichuan Morel No.4”, were also approved by the Sichuan Provincial variety Examination and Approval committee in 2016. These three varieties belong to *M. importuna*. Variety “Sichuan Morel No.1” has been commercially cultivated in large area for three years (2013-2015) with stable yields. Recently, a new variety of *M. sextelata* with good commercial characters was selected and bred.

For variety protection, a “Guidelines for the conduct of tests for distinctness, uniformity and stability (*Morchella importuna* M. Kuo, O’Donnell & T.J. Volk and *Morchella sextelata* M. Kuo)” was drawn up by our research team and has been approved by the Department of Agriculture of China on June 18th, 2015. It means that the artificially cultivable morels “*M. importuna* and *M. sextelata*” are very possibly going to be included in the tenth batch of the

"protection index of the new agricultural plant varieties" in China, which would strengthen variety protection of morels.

1.3 Spawn production

Quality of spawn is directly related to the success or failure of morel cultivation. Spawns were prepared as follows: Firstly, mycelia were incubated in PDA for 7days at 20°C. Secondly, mycelia were inoculated on sterilized substrate and incubated for about 15 days at 20°C until mycelia fully colonized. Finally, mycelia were inoculated on wheat substrate supplemented with wood shavings and soil. Sclerotium usually formed and appeared on the surface of substrate after 7-10 days of inoculation at 20°C. When the sclerotium color turns golden yellow; the spawns were ready for sowing.

1.4. Artificial cultivation and yields

The spawn was sown in the field at 10-18°C and 60-80% humidity. Mushroom sheds are built above the field to control sunshine, moisture and temperature conditions. Watering was performed when necessary. The primordia were formed about 80-100 days after sowing in the field, and mature fruiting bodies were observed about 5-7 days after primordial formation. Mature fruiting bodies of *M. importuna* reached 7 to 15 cm in length, and 6 to 16 cm for *M. sextelata*. *M. importuna* has a conical cap that is olive brown to brown, and its stipe was grayish white, covered with granules; the cap of *M. sextelata* is conical, reddish brown with brown ridges, and its smooth stipe is pure white. Average yield of *M. importuna* and *M. sextelata* was 1200 kg and 1500 kg per acre, respectively. The maximum yield of *M. importuna* reached to 3048 kg per acre, and 3120 kg for *M. sextelata*. Both *M. importuna* and *M. sextelata* follow the same cultivation procedures and conditions, and *M. sextelata* is less sensitive to temperature rise than *M. importuna*.

Our results showed that morels could be cultivated in various terrains including plain-hills zones, plateau zones, mountain zones. Space among fruit trees or horticultural plants in orchard could





Figure 1. Successful cultivation of *Morchella importuna*. Figure 2. Successful cultivation of *M. sextelata*.

also be used for morel cultivation. In our study, cultivation of morels under orange trees and osmanthus trees showed a high yields.

1.5. Discussion

Morel cultivation techniques explored in the present study are effective and quite practical, which makes it widely accepted and popularized in China. Our highly efficient way of morel cultivation has proven to be stable in fruiting, and successful commercial cultivation of morels was rapidly developed in China. It is expected that cultivation areas of morel are over 2500 acres in 2016. In Sichuan province, diverse cultivation patterns together with related techniques and varieties have been developed.

The successful domestication and cultivation of *M. importuna* and *M. sextelata* in Sichuan province suggested the blooming of morels in China, from laboratory research to commercial cultivation and large-scale popularization. We hope this would make a contribution to the international morel industry development and the ecological-friendly solutions of global green-agricultural revolution.

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XI Mycorrhiza’s

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Induction of salt tolerance in *Rhizopogon roseolus* by ethyl methanesulfonate

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ABSTRACT

Rhizopogon roseolus (Corda) Th. M. Fr. (=*R. rubescens* Tul. & Tul.), known as "shoro" in Japanese, is a hypogeous basidiomycete and an important ectomycorrhizal symbiont of Pinaceae. Recent studies have suggested that ectomycorrhizal fungi play an important role in protecting host roots from environmental stresses such as high concentrations of heavy metals and salts. However, little is known about fungal strains that promote host defense mechanism against environmental stresses. Moreover, no mutagenesis studies on the selection of salt-tolerant ectomycorrhizal of *R. roseolus* strains have been performed. Here, to induce mutations, we treated two structures, the basidiospores and the homokaryotic mycelial fragments, of *R. roseolus* with ethyl methanesulfonate (EMS), a widely used chemical mutagen, and subsequently spread the cultures on diluted Modified Melin-Norkrans agar plates. On recovering the strains, we evaluated their salt-tolerance. Following EMS treatment, the number of colonies of isolates derived from the EMS-treated basidiospore suspension and homokaryotic mycelial fragments decreased in a concentration-dependent manner. We recovered *R. roseolus* strains from basidiospores and homokaryotic mycelia growing on both EMS-free media and EMS-containing media. The recovered strains were cultured on agar medium containing 0 mM NaCl and on that containing 300 mM NaCl. Among the isolates recovered from basidiospores grown on EMS-containing media, we identified salt-tolerant and salt-sensitive strains that showed a more vigorous or weaker mycelial growth in medium containing 300 mM NaCl, compared to that observed with the isolates derived from basidiospores grown on EMS-free media. Although salt-tolerant isolates derived from EMS-treated homokaryotic mycelial fragments were observed as well, most of these showed a significantly negative mycelial growth in medium containing 300 mM NaCl, compared to that observed with the parent strain. These results suggest that although the mutagen EMS may expand the salt tolerance of isolates derived from basidiospores, it induces negative mutation in isolates derived from homokaryotic mycelial fragments of *R. roseolus*.

Keywords. Basidiospore, homokarytic mycelium, mutation, ethyl methanesulfonate, *Rhizopogon roseolus*.

INTRODUCTION

Saline soils occupy approximately 7% of the earth's land surface (Dixon et al.,1993). The main mediators of salt stress which inhibit or reduce plant survival and development, include unfavorable pH, altered soil structure, ion toxicity and osmotic stress (Dashtebani et al., 2014). Recent studies have suggested that ectomycorrhizal fungi can help host plants by facilitating more efficient uptake of phosphorus and water, thereby diluting the effects of toxic ions and indirectly increasing plant growth. *Rhizopogon roseolus* (Corda) Th.M. Fr. (*R.rubescens* Tul. & C. Tul.), known as "shoro" in Japanese, is a hypogeous basidiomycete and an important ectomycorrhizal symbiont of plants of the family Pinaceae (Kawai et al., 2008; Shimomura et al., 2008). *R. roseolus* is known to be salt tolerant, since it inhabits seashores (Nakano et al., 2015).

Chemical mutagens and UV irradiation have been widely used to induce a large number of functional variations in several different organisms. Ethyl methanesulfonate (EMS) is the most commonly used chemical mutagen, because of its potency and the ease with which it can be used. However no mutagenesis studies to select *R. roseolus* strains of ectomycorrhizal fungi have been reported to date. Here, we treated two structures of *R. roseolus*, basidiospores and homokaryotic mycelial fragments, with EMS to induce mutations. Subsequently, we evaluated the relative growth of the resulting mutant strains on 300 mM NaCl agar plates, and selected salt-tolerant strains of *R. roseolus*.

1. Materials and Methods

1.1. Preparation of basidiospores suspension

Fresh wild fruiting body of *R. roseolus* MCL2014Rhz41was collected from a *Pinus thunbergii* forest at the Arid Land Research Center, Tottori University on March of 2014. The fruiting body was cut with a scalpel and soaked in sterilized water to obtain basidiospore suspensions. In general, the basidiospores concentrations obtained from *R. roseolus* was 10^6-10^7 spores / ml. Suspensions were stored in a refrigerator at 4 °C until used.

1.2. Treatment of basidiospores with EMS mutagen

EMS was added to microtubes containing basidiospore suspensions at different concentrations (5-50 mg / ml). An untreated basidiospore suspension was used as a negative control. After 5 h, each microtube was centrifuged and the basidiospores rinsed with sterilized distilled water. Subsequently, EMS-treated basidiospore suspensions were spread on 1/5 diluted Modified Melin-Norkrans (MMN; Marx, 1969) agar plates. Following culture for 20 days, the numbers of colonies derived from each

basidiospore suspension was counted. Five replicates of each experiment were performed.

1.3. Presentation of mycelial fragments of mutant homokaryotic

Liquid mycelial slurries were prepared, following the method of Shimomura et al. (2012), form *R. roseolus* MCL2014Rhz41·Sp2, selected from non-mutated salt-tolerant single spore isolates. EMS (20 mg / ml) was added to the resulting mycelial slurries and the samples incubated for approximately 5 h. Subsequent processing was the same as that for mutated basidiospores, mycelial slurries were collected by centrifugation and washed. Homokaryotic mycelial fragments were obtained by filtering with a cell strainer (100 μm), and spread on 1/5 MMNA medium containing 0, 150, 300 and 450 mM NaCl. Following culture for 30 days, the numbers of colonies derived from the recovered homokaryotic mycelial fragments were recorded.

1.4. Salt tolerance selection of recovery isolates

We isolated colonies formed by germinated basidiospores and recovered homokaryotic mycelial fragments on 1/5 MMNA plates. Circular agar blocks (diameter 6.0 mm) of recovered homokaryotic isolates were cut and inoculated on 1/5 MMNA medium containing 0 and 300 mM NaCl. After culturing for 25 days, we measured the diameter of the colonies in different NaCl and calculated the relative growth rate of each isolate in 300 mM NaCl to select salt-tolerant and sensitive strains. Three replicate experimenths were performed for each strain.

2. Results and discussion

Following EMS treatment, the number of colonies derived from isolates from the EMS-treated basidiospore suspensions of MCL2014Rhz41 fruiting body decreased in a concentration-dependent manner. When EMS concentrations exceeded 40 mg / ml, no colonies were formed. Both salt and EMS treatment resulted in a decrease in the number of colonies from isolates derived from mycelial fragments of the salt-tolerant homokaryotic strain MCL2014Rhz41·Sp2, in a concentration-dependent manner. Due to bacterial infection, we did not observe colonies of isolates derived from EMS treated mycelial fragments on 150 or 450 mM NaCl medium, however the average numbers of colonies growing on medium containing 300 mM NaCl was 1.52 / plate.

In the absence of salt stress, we did not observed a significant difference in mycelia growth between EMS-treated and untreated isolates recovered from basidiospores. In conditions of 300 mM salt stress, mycelial growth of all isolates recovered from basidiospores increased with EMS concentration, peaked at 20 mg / ml EMS treatment. The mycelia growth of each isolate varied significantly. Among isolates recovered from basidiospores grown on EMS-containing media, we identified salt-tolerant and salt-sensitive strains that showed more vigorous or weaker mycelial

growth in medium containing 300 mM NaCl, compared to that of isolates derived from basidiospores grown on EMS-free media.

One hundred and twenty three mutant strains were isolated from 20 mg / ml EMS treated mycelial fragments of the salt-tolerant homokaryotic strain MCL2014Rhz41·Sp2, grown on 300 mM salt and salt-free medium. Although salt-tolerant isolates derived from EMS-treated homokaryotic mycelial fragments were observed, the majority demonstrated significantly impaired mycelial growth in medium containing 300 mM NaCl, compared to that of the parent strain. These results suggest that although the mutagen EMS may increase the salt tolerance of isolates derived from basidiospores, it induces negative mutations in isolates derived from salt-tolerant homokaryotic mycelial fragments of *R. roseolus.*

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Development of suitable irrigation regime for a summer truffle *(Tuber aestivum)* orchard in Israel

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ABSTRACT

The weather conditions of Israel were thought to be unsuitable for the cultivation of truffles. Nevertheless, part of northern Israel, namely the "Upper Galilee" is mountainous with heights of 700-1000 meters, and at this region weather and calcareous soil are suitable for growing crops like apples and cherries. Therefore we decided to make an attempt to introduce truffle cultivation to this region too. In order to achieve this goal, truffles fruit bodies were imported from Italy and used to inoculate seedlings of local and introduced oaks and hazelnuts and an experimental plot was planted, in kibbutz Bar'am, at the Upper Galilee. Summer truffles *(Tuber. aestivum)* were collected in this experimental plot, since the spring of 2009 and yields were calculated. The Upper Galilee region, unlike the known truffle cultivation regions in Europe, lacks summer rains. Therefore low soil humidity during the summer, was thought to be the main limitation for appropriate fruit bodies development. Irrigation is used in truffle cultivation regions. Sprinkler irrigation is usually applied if it does not rain for 3 consecutive weeks. An irrigation experiment in Bar'am plot with 4 different watering regimes has proven the need for irrigation. The regimes differed by the daily additional water amounts (1-2mm) and irrigation intervals. A dramatic, eight fold yield increase, was recorded during the first irrigation season, as compared to former years yields. During 2015, 50 kilograms, of about 1600 truffle ascocarps were collected from the1.0 hectare plot. The best irrigation regime was with 2mm daily water addition in 5 days watering interval, until June and 1mm daily water addition, in 13 days watering interval, later until the summer end. Highest monthly truffle yields were collected during the period of May to July. During this period the biggest fruit bodies were collected, highest daily collection score for a working hour and also highest daily yields were recorded. The collected truffles were tested for spore maturation .It was found that from the early spring in to the summer there was an increase in spore maturation. A correlation was found between spores maturation and improvement of the truffles taste, according to organoleptic tests. These results are an encouraging sign that truffles cultivation could be possible in this region.

Keywords: Summer truffles, *Tuber aestivum,* irrigation.

INTRODUCTION

The "Upper Galilee" is a mountainous region in northern Israel with heights of 700-1000 meters. The weather and calcareous soil of this region are suitable for growing crops like

apples and cherries. Therefore an attempt was made to introduce truffle cultivation to this region too. Several different tree species were initially introduced for this purpose. These included both European and local oak species, as well as hazelnuts. The different tree species were randomly planted within the 1.0 hectare grove[1]. The plantation setting was necessary to facilitate our identification of the most suitable host species for producing the best yields under Israeli conditions. It was later found that local oak species produced higher yields as compared to introduced oak species, chosen for their reputation of being good Tuber symbionts. Truffles fruit bodies were imported from Italy and used to inoculate the seedlings. Summer truffles *(Tuber aestivum)* were collected in this experimental plot, since the spring of 2009[2]. The Upper Galilee region, unlike the known truffle cultivation regions in Europe, lacks summer rains. Therefore low soil humidity during the summer, was thought to be the main limitation for appropriate fruit bodies development[3]. The available data on truffle plantations irrigation is limited, since the traditional truffle plantations are located in summer rain regions. Therefore the objective of the present study was to develop the proper irrigation system and regime for truffle cultivation in mountainous Israeli regions.

1. Results & Discussion

An irrigation experiment in the Bar'am experimental plot with 4 different watering regimes was installed. The regimes that differed by the daily additional water amounts (1-2mm) and irrigation intervals are presented in Table 1.

Table 1. The different irrigation regimes at Bar'am experimental truffle plot

Treatment	until 10/6/15		after 10/6/15	
	Interval (days)	Daily addition (mm)	Interval (days)	Daily addition (mm)
1	5	1.5	5	1
2	10	1.5	10	1
3	13	1.5	20	1
4	5	2	13	1

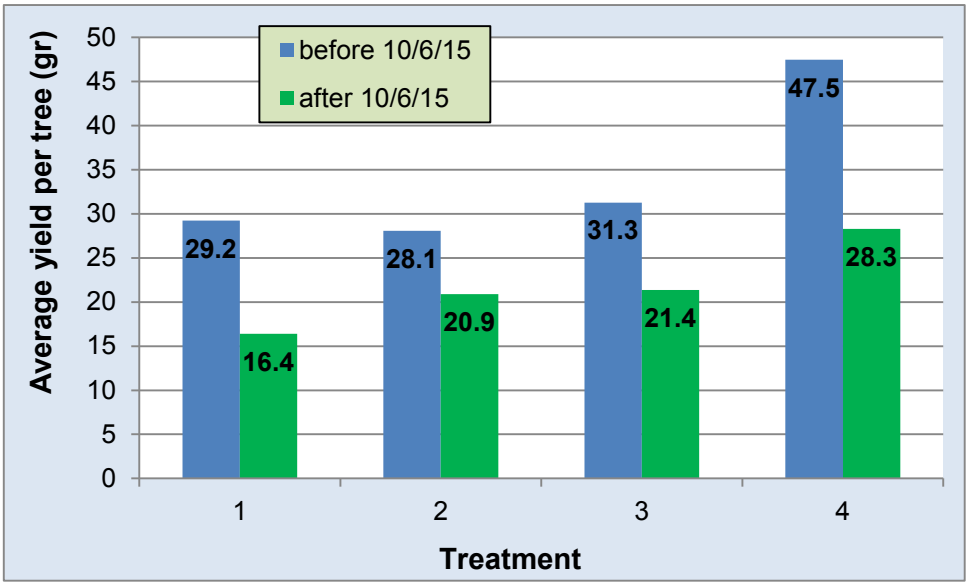


Fig 1 The effect of irrigation regime on the average single tree truffle yield

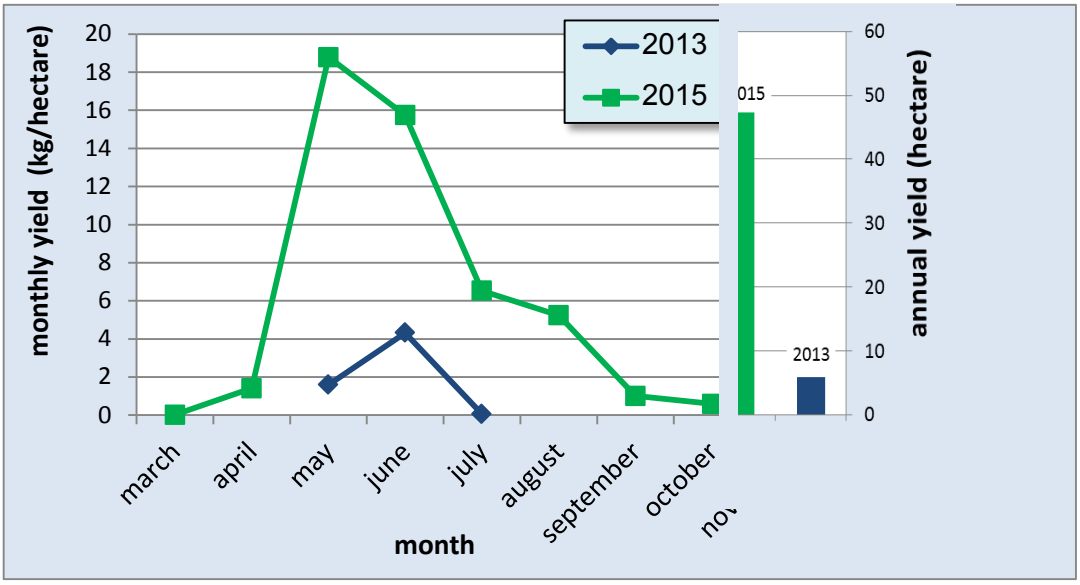


Fig 2. The effect of irrigation on the distribution and annual truffles yields.

More than eight fold yield increase, was recorded during 2015 irrigation season, as compared to 2013 truffle yields (Fig. 2.) During 2015, 50 kilograms, of about 1600 truffle ascocarps were collected from the1.0 hectare plot. The best irrigation regime (treatment 4) was with 2mm daily water addition in 5 days watering interval, until June and 1mm daily water addition, in 13 days watering interval, later until the summer end (fig 1.). Highest monthly truffle yields were collected during the period of May to July (fig 2.). During this period the biggest fruit bodies were collected, highest daily collection score for a working hour and also highest daily yields were recorded. The collected truffles were tested for spore maturation .It was found that from the early spring in to the summer there was an increase in spore maturation. A correlation was found between spores maturation and improvement of the

truffles taste, according to organoleptic tests (un presented data). The development of an optimal truffle plantation's irrigation regime is locally dependent on several parameters. These include: weather, soil characteristics, tree species, distances between the trees, plantation orientation etc. There is limited published data on truffle plantation irrigation. In southern European (mainly in Spain) truffle plantations, irrigation is applied only toward the beginning of ascocarps initiation, in order to create the proper conditions for fruit bodies development (personal data). Earlier irrigation can cause too fast and deep roots development. Irrigation quantities are limited (compared fruits orchards), in order to avoid too fast and massive trees development and truffle rot. Nevertheless our initial results are promising that there is an actual potential for truffles plantations cultivation in these regions.

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Re-domesticating Termitomyces fungi for human consumption

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ABSTRACT

Roughly 30 million years ago an intriguing, obligate symbiosis arose between termites and basidiomycete fungi of the genus *Termitomyces* (Aanen et al. 2002; Aanen and Eggleton 2005). This symbiosis has most often been described as a farming system in which the farming termites cultivate their domesticated fungus for food (Mueller et al. 2005). We want to investigate how we could re-domesticate *Termitomyces* independently of its partners in order to cultivate the fungus for its nutritionally valuable, protein-rich and delicious mushrooms. By doing so, we will gain insight into the life history characteristics of this fungus and thereby deepen our understanding of its symbiotic lifestyle.
The first goal of our research is to investigate the fructification process of a single *Termitomyces* species that is associated with the termite species *Macrotermes natalensis*. As most *Termitomyces* species, this symbiont employs a horizontal mode of transmission. Within a nest the fungus is propagated by asexual spores found in nodules (spore balls eaten and subsequently secreted by termites). New nests are inoculated with sexual spores produced by fruiting bodies. Until now, it has been presumed that the nodules, which are essential for asexual propagation, are also the primordia of the fruiting bodies for sexual reproduction (Aanen 2006; Heim 1942). Our aim is to test whether nodules are indeed primordia of *Termitomyces.*

INTRODUCTION

The symbiosis between macrotermitinae termites and their *Termitomyces* fungus has a single evolutionary origin in an African rain forest. Being a white-rot fungus, *Termitomyces* is adapted to the warm, humid conditions of the rain forest. Yet, the cooperation with termites allowed *Termitomyces* to appear and thrive in the more variable and unfavourable conditions of the savannas (Aanen and Eggleton 2005). The termites and their fungi are now major decomposers of these areas and have been found to be responsible for roughly 20 % of all C-mineralisation in the dry savannas (Wood and Sands 1978). The symbiosis is so successful that no reversals to a free-living state have ever been found, not for the termites and neither for their fungi. Consequently we currently know roughly 330 species of fungus-growing termites, placed in 11 genera. Of the symbiotic fungus about 40 extant species have been described, all placed in the genus *Termitomyces* (Aanen et al. 2002).
The fungus-farming termites are highly advanced eusocial insects. Each mound is founded by a king and queen, who are the only reproductives within a nest. Together they produce all of the other castes of termites that constitute the colony and maintain the nest. The termite mound is a large, intricate structure with tunnels and chambers in which the conditions are

tightly regulated (Korb 2003). In these chambers the termites keep their crop, the *Termitomyces* fungus, on airy structures called fungus combs. Termite workers forage for dead plant material and bring it back to the nest. There the plant material is consumed together with fungal nodules, i.e. asexual spore balls, that grow on the comb. The faeces that are produced after this first gut passage, mainly consist of practically undigested plant material and the asexual fungal spores that remain from the nodules. These faeces are secreted to form new parts of fungus comb; they are essentially pre-digested substrate, perfectly inoculated with fungal spores (Badertscher et al. 1983; Gerber et al. 1988).
In mature colonies, each year the king and queen produce winged, sexually mature termites called alates. The alates of a given species usually swarm synchronously from their nests during fairly specific seasons, often in response to rainfall (Darlington 1994; Wood and Sands 1978). After this nuptial flight and the finding of a mating partner, alates lose their wings and bury themselves in the ground. In this primary royal chamber copulation commences to produce the first workers of the new colony. Most species of fungus-growing termites then need to acquire their symbiont via horizontal transmission, because the colony will not survive unless it is inoculated with *Termitomyces* (Johnson et al. 1981). Intriguingly, the formation of *Termitomyces* fruiting bodies, and the accompanying production of asexual spores, coincides with the appearance of the first workers of the given species with whom they live in symbiosis (Kone et al. 2011).
The *Termitomyces* fruiting bodies are important to ensure propagation of horizontal-symbiont-acquisition-dependent fungus-growing termites and their Termitomyces fungi. However, they are also of considerable importance to many African and Asian people. First of all, *Termitomyces* basidiocarps are edible and considered to be a delicacy (Oso 1975). The mushrooms have been shown to have high nutritive value as they contain high amounts of protein (Ogundana and Fagade 1982), crude fibre, minerals and polyunsaturated fatty acids (Kansci et al. 2003; Malek et al. 2012). Moreover, much of the mycelial protein content has been shown to consist of essential amino acids especially in the species *T. umkowaani* and *T. sagittaeformis* (Botha and Eicker 1992). In addition, several *Termitomyces* species have been implied to have medicinal value, e.g. *Termitomyces eurhizus* in the accelerated healing of gastric ulcers in mice (Chatterjee et al. 2013)and *Termitomyces miscrocarpus* in the lowering of blood cholesterol levels in rats (Nabubuya et al. 2010). As a consequence, the mushrooms gathered in the wild during the fruiting season create a considerable income for the people who have access to them (e.g. Kone et al. 2011). Logically, several attempts have been made to cultivate *Termitomyces* for its mushroom, but so far nobody has recently succeeded (e.g. De 1983; Olila et al. 2007). We believe that by learning more about the natural fructification processes of *Termitomyces* we will eventually be able to develop an evidence based method for *Termitomyces* mushroom cultivation.

The relation between nodules and mushrooms

Asexual structures, like *Termitomyces* nodules, are rare in basidiomycete fungi, and their role within the symbiosis has been much debated (Aanen 2006; Nobre et al. 2011). As all known *Termitomyces* species form nodules it is likely that they either have evolved as a consequence of the symbiotic lifestyle or perhaps that they were already present in the *Termitomyces*

ancestor, which was therefore pre-adapted. At least it is reasonable to assume that there is an important role for the nodules in the stability of the symbiosis. Indeed it has been found that the nodules help the establishment of a monoculture in each nest, because the propagation of the fungus by the termites is positively frequency dependent (Aanen et al. 2009). Also, the nodules were shown to be a protein-rich food source for the termites (Aanen 2006; Leuthold et al. 1989) and it has been hypothesized that enzymes in the nodules survive the first gut passage and enable the fungus to quickly colonise new comb (Nobre and Aanen 2012).
Most researchers who have speculated about the origin of nodules proposed that nodules are in fact primordia of the sexual fruiting bodies which are suppressed in development by grazing termites (Aanen 2006; Bathellier 1927; de Fine Licht et al. 2005; Heim 1942). This is supported by an observation made by de Fine Licht, Anderson et al. (2005). When they incubated pieces of fungus comb in glass containers in the absence of termites, there was one piece of fungus comb that was not overgrown by the ascomycete *Xylaria*, which usually happens. Instead, on this piece of comb sexual fruiting bodies seemed to develop from nodules. This observation is in line with the theory that it is not in the direct interest of the termites to allow their partner to fruit, because it is a waste of resources, and therefore the termites actively supress fruiting of the fungus (Aanen 2006; Korb and Aanen 2003). According to this hypothesis, a colony normally has enough capacity to graze away all nodules before they can continue their development. However, when alates are produced up to 40 % of a colony's biomass may disperse (Wood and Sands 1978) and therefore not enough termites remain to prevent the fungus from fruiting. This hypothesis is supported by the observation that only termite colonies that produced alates were observed to produce *Termitomyces* fruiting bodies (Kone et al. 2011)

Research outline

We want to test the hypothesis that nodules indeed are the very early stages of mushroom formation. To investigate this hypothesis we have incubated many excavated garden fragments without termites from the *Macrotermes natalensis* species, as this is the species in which fruiting bodies were found on incubated fragments once before (de Fine Licht et al. 2005). From these incubated fragments, we have isolated nodules, mushrooms and several intermediate stages to make sections for light microscopy to study the successive stages in development. Also we have isolated total RNA for RNAseq of the different stages to investigate differences and similarities between the expression profiles of nodules and possibly later stages of development. We hope to present these data at the ISMS conference. Eventually, we will use the RNAseq data to compare expression profiles between different stages of mushroom formation and investigate whether the expression profiles can be linked to transcription factors or other genes involved in fruiting body formation of better studied fungi e.g. *Schizophyllum commune* and *Agaricus bisporus* (Amongst others very well described by Moore 1998). Also for this latter part of our research it is convenient to use the symbiont of *Macrotermes natalensis*, since its whole genome has recently been sequenced (Poulsen et al. 2014).

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APPENDIX

POSTER SESSIONS

Session Research and innovations in the industry

COMPUTER CONTROL IN **MUSHROOM GROWING** IN **2020**

Inventory of applications

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Fancom BV

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www.fancommushroom.com

PROGRAM

- Climate control
- Logistical control
- Automatic Watering
- Weighing
- Labour registration
- Automatic harvesting
- Farm Management System
- Culture reports

CLIMATE CONTROLS

Compost preparation:

- Phase I
- Phase II
- Phase III

Growing Rooms

- Central Airhandling
- Growing room control
  - Phase I
  - Phase II
  - Phase III
  - Phase IV



FIRST COMPUTER 80's



CLIMATE COMPUTER 2016

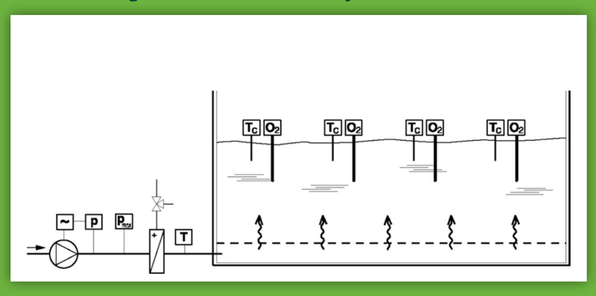
COMPOST PREPARATION

PHASE I CLIMATE CONTROL


Naturally aerated compost rows

- Naturally aerated rows
- Mechanically aerated row/floors
  - pre-wetting
  - pre-fermentation
- Open bunkers
- Closed top bunkers
- Closed fermentation tunnels

Diagram Individual airsystem bunkers



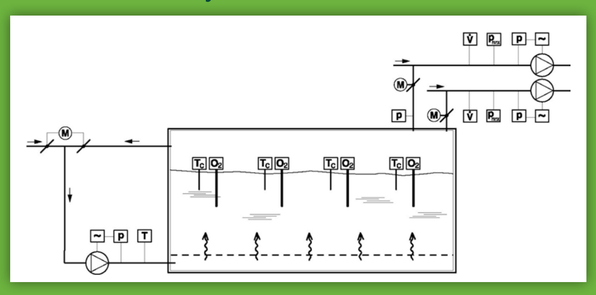
Airsystem with mixed air


Open / Closed-top bunkers


Air system bunkers


Air system open bunkers

Control system fermentation - Phase I in open air



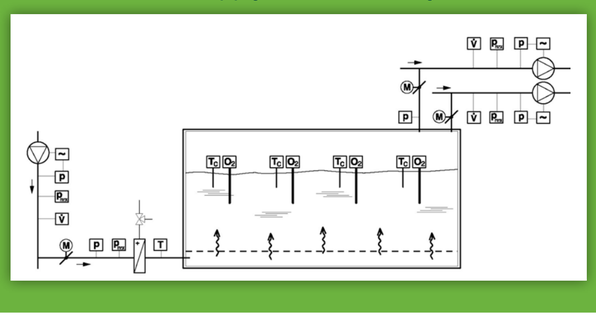
Central supply and exhaust system







Control system - Phase I in Tunnels/Bunkers



Central supply and exhaust system

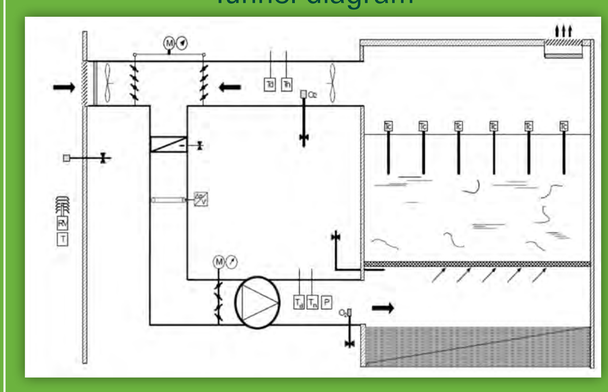






PHASE II & III TUNNELS

Tunnel diagram



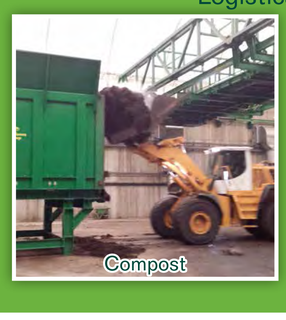
Ventilation system



Control system



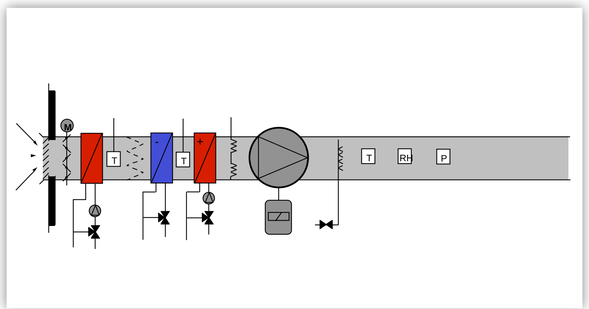
Logistical controls


Compost

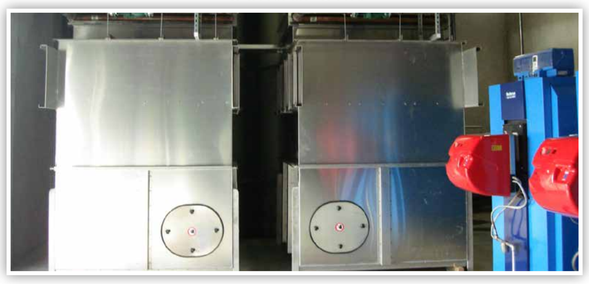

Mushroom

GROWING ROOMS

Central Unit Control



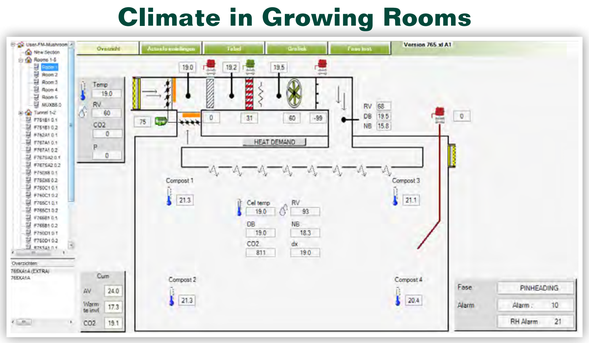
Central Unit



Watering and climate control



Climate in Growing Rooms



Airsystem Growing Room



Climate Control System Growing Room



Automatic Watering Control



Automatic harvesting



Logistical Controls



Central Farm Management



Conclusion status automation 2016

- Developed countries:
  - phase II: 100 % adopted
  - phase III: 100% adopted
  - phase I : 90% adopted
- Less developed countries:
  - increasing demand

2020

- Automatic harvesting still illusion?
- Automatic watering on moisture level?
- Culture reports?

Photography: Christiaens Group,
Van den Top Machinebouw & GTL Europe

Effect of Pulsed Electric Fields on Proximate Composition and Bioactive Compounds in Mushrooms Cultivated in La Rioja, Spain

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19th ISMS Congress International Society for Mushroom Science
Amsterdam 2016
29th May - 2nd June

INTRODUCTION

Mushrooms are a good source of health-related compounds, such as β-glucans, dietary fibre and antioxidants, without-providing calories to the human diet. On the other hand, pulsed electric field (PEF) is a nonthermal technology that could activate the secondary metabolism of cell tissues leading to an increase of these bioactive compounds.

Objective: The objective of this study was to evaluate the effect of PEF technology on the proximate composition, β-glucan and dietary fibre contents as well as the antioxidant activity in the fruiting bodies of five mushroom species cultivated in La Rioja (Spain): *Agaricus bisporus, Pleurotus ostreatus, Pleurotus eryngii, Lentinula edodes* and *Ganoderma lucidum*.

METHODS

Cultivation at CTICH facilities

Agaricus bisporus AB
Pleurotus ostreatus PO
Pleurotus eryngii PE
Lentinula edodes LE
Ganoderma lucidum GL



PEF application
- Intensity: 1.2 and 2kV/cm
- Number of pulses: 5, 18 and 30

Lyophilisation and powdering



1. Proximate composition analysis
- Moisture
- Ash
- Fat
- Proteins
- Carbohydrates
- Energy

A.O.A.C. (1995)

2. Glucans content
Absorbance at 420 nm

3. Fibre

4. Antioxidant activity and phenols content

Statistical analysis: one-way analysis of the variance (ANOVA) and Duncan's test ($P < 0.05$). Relationship between the different variables was carried out by computing the relevant correlation coefficient (Pearson's linear correlation) at the $p < 0.05$ confidence level.).

RESULTS AND DISCUSSION

β-glucan concentration ranged from 11.47% of *Agaricus bisporus* to 47.45% of *Pleurotus eryngii.* No significant differences were found in treated mushrooms respect untreated ones except in *Pleurotus eryngii* in 2kV/cm-5 pulses and *Ganoderma lucidum.*

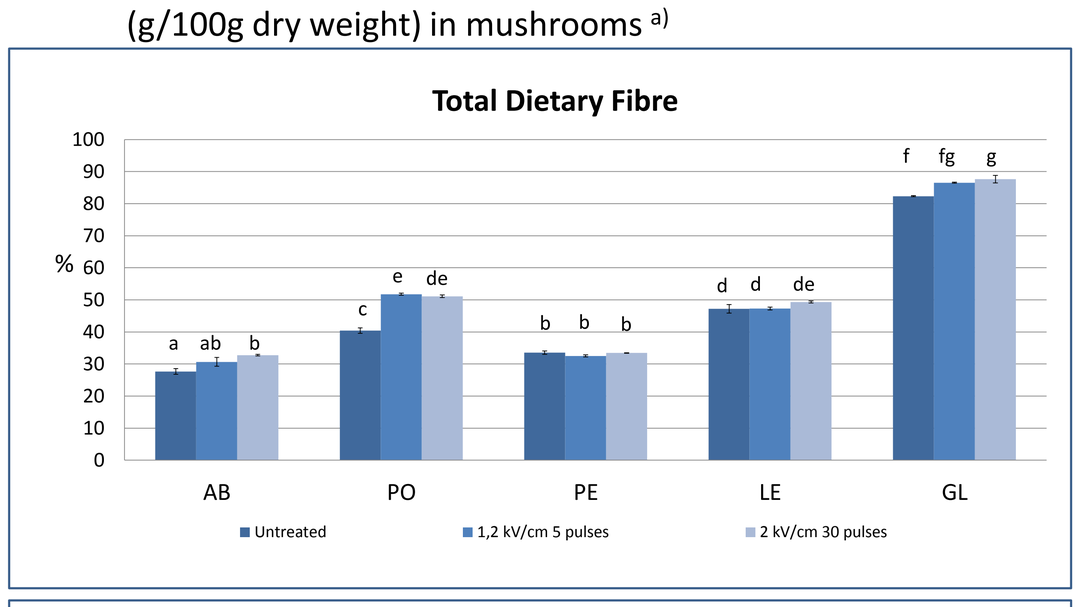
Dietary fibre content varied from 27.66% of *Agaricus bisporus* to 87.64% of *Ganoderma lucidum.* Significant differences were observed only in PO and whit 2kV/cm-30 pulses in AB and GL.

Table 1. Antioxidant activity and phenols content in mushrooms [a)]

	Treatments	Phenols	DPPH	ABTS	FRAP
Mushrooms	kV/cm - pulses	µg EQ galic/100mg	µmol EQ trolox/100mg		
Agaricus bisporus	UNTREATED	182,0 ± 2,3^{bc}	0,75 ± 0,01^{e}	2,33 ± 0,02^{b}	2,36 ± 0,03^{d}
	1.2 - 5	198,8 ± 6,0^{c}	0,62 ± 0,01^{d}	2,40 ± 0,06^{bc}	2,47 ± 0,03^{e}
	1.2 - 18	187,7 ± 6,8^{c}	0,47 ± 0,00^{c}	3,01 ± 0,15^{d}	2,73 ± 0,03^{g}
	1.2 - 30	186,9 ± 3,1^{c}	0,41 ± 0,00^{b}	2,70 ± 0,06^{cd}	2,59 ± 0,01^{f}
	2 - 5	159,2 ± 3,6^{a}	0,45 ± 0,00^{c}	2,45 ± 0,09^{bc}	2,24 ± 0,02^{c}
	2 - 18	147,1 ± 2,4^{a}	0,45 ± 0,01^{c}	3,02 ± 0,07^{d}	1,99 ± 0,03^{b}
	2 - 30	163,8 ± 4,2^{ab}	0,34 ± 0,02^{a}	1,76 ± 0,02^{a}	1,78 ± 0,03^{a}
Pleurotus ostreatus	UNTREATED	82,3 ± 1,2^{d}	0,47 ± 0,01^{a}	0,77 ± 0,01^{c}	0,48 ± 0,01^{a}
	1.2 - 5	74,1 ± 1,9^{bc}	0,57 ± 0,02^{bc}	0,52 ± 0,00^{a}	0,83 ± 0,02^{c}
	1.2 - 18	71,4 ± 1,4^{ab}	0,61 ± 0,01^{c}	1,22 ± 0,03^{d}	1,01 ± 0,01^{e}
	1.2 - 30	76,0 ± 0,8^{bc}	0,57 ± 0,01^{b}	1,30 ± 0,02^{de}	0,85 ± 0,01^{c}
	2 - 5	76,7 ± 1,8^{bcd}	0,61 ± 0,01^{bc}	1,32 ± 0,02^{e}	0,95 ± 0,01^{d}
	2 - 18	78,2 ± 1,1^{cd}	0,65 ± 0,00^{d}	0,67 ± 0,05^{b}	0,99 ± 0,02^{de}
	2 - 30	66,0 ± 1,1^{a}	0,47 ± 0,01^{a}	0,81 ± 0,01^{c}	0,70 ± 0,01^{b}
Pleurotus eryngii	UNTREATED	76,6 ± 0,9^{a}	0,41 ± 0,00^{a}	0,37 ± 0,01^{a}	0,54 ± 0,01^{a}
	1.2 - 5	84,1 ± 1,3^{b}	0,56 ± 0,00^{bc}	0,53 ± 0,00^{b}	0,56 ± 0,01^{a}
	1.2 - 18	88,9 ± 1,8^{bc}	0,64 ± 0,01^{d}	0,98 ± 0,05^{c}	0,66 ± 0,01^{b}
	1.2 - 30	96,1 ± 1,7^{d}	0,67 ± 0,01^{d}	0,92 ± 0,02^{c}	0,67 ± 0,01^{b}
	2 - 5	104,6 ± 1,6^{e}	0,67 ± 0,01^{d}	1,18 ± 0,03^{d}	0,67 ± 0,01^{b}
	2 - 18	95,1 ± 1,9^{cd}	0,58 ± 0,01^{c}	0,93 ± 0,03^{c}	0,64 ± 0,01^{b}
	2 - 30	83,3 ± 1,2^{b}	0,54 ± 0,01^{b}	0,52 ± 0,00^{b}	0,55 ± 0,01^{a}
Lentinula edodes	UNTREATED	104,4 ± 1,1^{a}	1,01 ± 0,02^{a}	0,41 ± 0,01^{b}	1,38 ± 0,02^{a}
	1.2 - 5	117,9 ± 0,4^{bc}	1,38 ± 0,02^{b}	0,38 ± 0,01^{a}	1,52 ± 0,03^{b}
	1.2 - 18	117,4 ± 1,2^{bc}	2,20 ± 0,01^{c}	0,54 ± 0,00^{cd}	1,63 ± 0,02^{c}
	1.2 - 30	125,3 ± 1,4^{cd}	2,16 ± 0,01^{c}	0,53 ± 0,00^{c}	1,69 ± 0,02^{cd}
	2 - 5	116,2 ± 1,1^{b}	2,17 ± 0,01^{c}	0,55 ± 0,01^{d}	1,62 ± 0,02^{bc}
	2 - 18	133,3 ± 1,9^{d}	2,20 ± 0,02^{c}	0,54 ± 0,01^{c}	1,75 ± 0,04^{d}
	2 - 30	121,3 ± 2,5^{bc}	1,41 ± 0,01^{b}	0,48 ± 0,06^{b}	1,52 ± 0,03^{b}
Ganoderma lucidum	UNTREATED	110,1 ± 3,4^{c}	0,59 ± 0,01^{b}	1,17 ± 0,04^{c}	0,96 ± 0,03^{bc}
	1.2 - 5	102,1 ± 1,3^{bc}	0,43 ± 0,02^{a}	1,00 ± 0,03^{b}	0,85 ± 0,01^{ab}
	1.2 - 30	74,2 ± 0,7^{a}	0,80 ± 0,02^{c}	1,13 ± 0,02^{c}	0,86 ± 0,03^{ab}
	2 - 5	83,0 ± 3,6^{a}	0,95 ± 0,03^{d}	1,32 ± 0,04^{d}	0,99 ± 0,04^{c}
	2 - 30	96,5 ± 1,1^{b}	0,39 ± 0,02^{a}	0,87 ± 0,02^{a}	0,81 ± 0,01^{a}

[a)] Values are means ± SEM, n = 3. Different letters within a column indicate significant differences between samples of each mushroom ($P < 0.05$).

Figure 1. Contents of total dietary fibre and β-glucans (g/100g dry weight) in mushrooms [a)]



a) Values are means ± SEM, n = 3. Different letters indicate significant differences samples of each mushroom ($P < 0.05$)

CONCLUSION

Proximate composition was not statistically different between PEF-treated and untreated mushrooms. In β-glucan content, only the treatment effect of pulsed electric fields is appreciated in *Ganoderma lucidum*, but without differences between the intensities or the number of pulses applied.

Although *Agaricus bisporus* showed the lowest values in dietary fibre and β-glucan content, it has the highest total phenolic content among all the mushrooms evaluated, being untreated and those treated at 1.2 kV/cm the mushrooms with the greatest values (approximately 190 µg GAE/100mg). All studied species presented statistically significant differences in antioxidant activity between treated and untreated mushrooms, being higher in those submitted to PEF treatment, especially in *Pleurotus eryngii, Lentinula edodes* and *Ganoderma lucidum*, which showed the highest ABTS, DPPH and FRAP values when treated with 5 pulses at 2 kV/cm. As a result, mushrooms should be taken into account as a non-caloric functional food due to their high content of β-glucan, dietary fibre and antioxidant activity, which can be enhanced by PEF technology.

Acknowledgments: This research was financed by the Department of Industry, Innovation and Employment of La Rioja and the Economic Development Agency of La Rioja (ADER) (2014-I-IDD-00051.

NEW APPLICATOR FOR THE WOOD DESTROYING FUNGI INOCULATION

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Actual research is aimed on utilization of the waste dendromass of the broadleaved trees in various dimensions according to their presence in forestry practice. Wood material with diameter over 7 cm – so called "thick wood" – is the greatest source of the deadwood at the forest stand. It consists mainly of short and long fragments of branches, whole trunks laying and decomposing on the ground, and stumps remaining on the forest stand after tree cutting. Naturally, this dendromass provides the nutrients for the living trees of the forest stands. Its decomposition can be sped up with the assistance of wood decaying fungi, providing fruiting bodies of interest while leaving the nutrients to absorb into the soil.






Fig. 1-4: Dead dendromass being decomposed by naturally occurring fungi

At the beginning of research, the wood blocks, stumps and logs were inoculated by various species of wood destroying fungi over the cut surface, possibly into the snicks at the side of wood block. That system of inoculation was very simple and was not very laborious, however wood overgrowth by inoculated fungus was not very intense and losses of fungal inoculum were significant.







Fig. 5-9: Older methods of inoculation

Nowadays, a new method of the intensive inoculation has been developed; it requires a special applicator. According to this method, fungal inoculum (grain spawn) is pressed by wooden wedge through a thin gap of special metal applicator (in a shape of irregular triangular prism) into the longitudinal, 2-3 cm deep snicks at the side of stumps, logs or wood block, and is subsequently is painted protective painting of wax or ecological covering paint.







Fig. 10-14: Applicator and its using

Inoculation is practically without loss of grain spawn, painting is relatively cheap and application is very easy, without undesired effect on mushroom growing. The mycelium overgrowth and fruiting bodies production is already very intensive in the first and the second year.
Very positive impact on successful inoculation and rapid growing of mycelium is evident by application of protective painting over the snicks. Its positive function is mainly based on inoculum protection against wet loss, ants, snails and insects, as well as against the other wood destroying fungi. Fungal mycelium overgrows the wood during several months, and the mycelium already occupies a great part of the wood block by the time the protective coating falls off.
Intense inoculation is the basis for intensive overgrowth and rapid decomposition of wood. It is very important because of the determined aim of our research – effective and rapid decomposition of the waste, practically unused wood at the forestry conditions.
Applicator for the inoculation of wood by fungus is protected as a utility model in Slovakia.







Fig.15 – 19: Fruiting bodies growing out of the notch (oyster mushroom, shiitake, jelly ear, turkey tail)

ACKNOWLEDGEMENT: This research was supported by grant from the Grant **Agency KEGA No. 007TUZ-4/2015**

Session Pests and diseases

Characterization of genes associated with antifungal activity of a bacterium against mushroom pathogen *Trichoderma viride*

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Introduction

Green mold caused by the fungus *Trichoderma viride* is the main constraint in production of mushroom worldwide. On the mushroom hyphae period, it can infect many different mushrooms, such as *Pleurotus ostreatus*, *Flammulina velutipes*, *Agaricus bisporus*, *Ganoderma lucidum*, and so on. However, the availability of useful fungicides within the mushroom industry is limited not only by the fact that the pathogen and the crop are both fungi but also resistance of pathogen to the fungicide has reduced disease control efficacy.

Therefore, development of biologically-based disease management for mushroom cultivation is in demand. The aim of this study was to identify the genes of bacteria associated with the production of antifungal activity against *T. viride.*



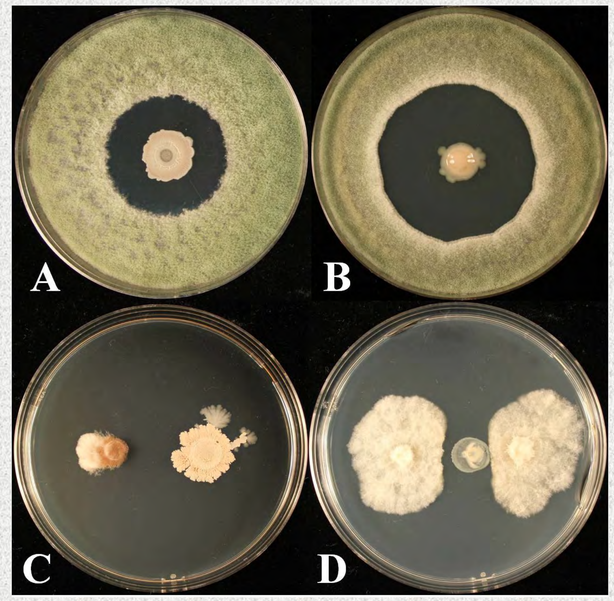


Bacterial Isolates with Antifungal Activities

In order to find the appropriate antifungal strains and methods to inhabit *T. viride*, 12 isolates of biocontrol bacterium inhibiting growth of *T. viride* significantly were selected by inhibition zone method. 12C2-4 and MS82 were two isolates that had distinct antifungal activities against *T. viride*.

Bacterial isolates 12C2-4 (A, C) and MS82 (B, D) were inoculated in the center of the PDA plates and incubated for 3 days at 28℃. Then different pathogenic indicator fungus and mushroom were overspread on the plates, and the clear zones are indication of antifungal activities. The indicator fungus used in the study are A and B: *Trichoderma viride*; C and D: *Agaricus bisporus*. Strain MS82 did not influence the growth of mushroom mycelium.

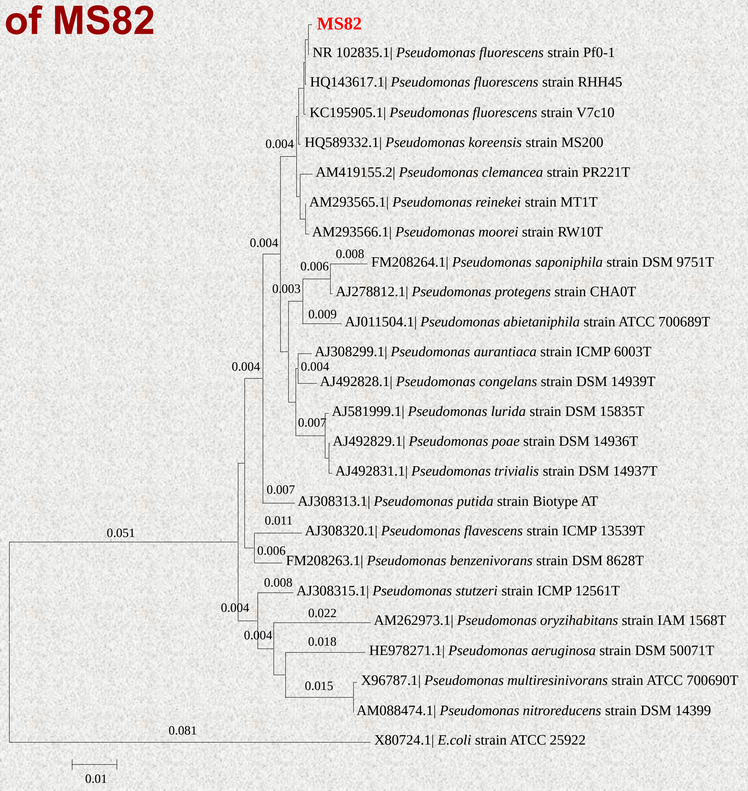
Strains	Radius of inhibition zone (mm)		Strains	Radius of inhibition zone (mm)	
	NBY	PDA		NBY	PDA
12A5-7	11.00e	0e	12C7-5	11.33e	11.33bc
12A7-6a	13.67d	3.33d	12C9-1a	11.67de	11.00bc
12B10-5	19.33a	2.67d	12D13-10a	17.33bc	10.33c
12C1-1	9.67e	11.33bc	MS82	18.67ab	18.33a
12C2-4	11.67de	11.67bc	13B5-11	17.33bc	13.00b
12C3-6	10.00e	11.33bc	13C13-5	16.33c	11.33bc



Identification of MS82

Characteristic*	1	2	3	4	5
Nitrate reduction[a]	+	+	-	-	+
Arginine dihydrolase[a]	+	+	+	+	V
Assimilation of:					
N-acetyl-glucosamine[a]	+	+	-	+	+
Phenylacetic acid[a]	-	-	+	-	-
L-Arabinose[b]	+	+	-	+	-
D-Ribose[b]	+	+	-	+	+
D-Xylose[b]	-	-	-	-	-
D-Galactose[b]	+	+	-	+	-
D-Mannose[b]	+	+	-	+	-
D-Mannitol[b]	+	+	-	+	+
Esculin[b]	-	-	-	-	+
D-Trehalose[b]	-	-	-	-	-
D-Arabitol[b]	+	+	-	+	ND

*Designated characteristics were determined with: a, API20 NE; b, API50 CH. Strains: 1, MS82; 2, *P.fluorenscens*; 3, *P.putida*; 4, *P. koreensis*; 5, *P.aeruginosa*. +, Positive reaction; -, negative reaction; V, doubtful reaction, ND, not determined. Values in parentheses were read after 48h.



Cells of strain MS82 are Gram-negative, aerobic and rodshaped. The results obtained with API20 NE and API50 CH biochemical and physiological tests indicated that strain MS82 most closely resembled the phenotypic characteristics of *P. fluorescens*. The nearly full-length 16S rRNA gene sequence of strain MS82 shared highest similarities (99–98%) with those of Pseudomonas species that were archived in GenBank. The phylogenetic tree based on 16S rRNA gene sequences indicates that strain MS82 belongs to *P. fluorescens*.

Genetic Analysis of Antifungal Activity of Strain MS82

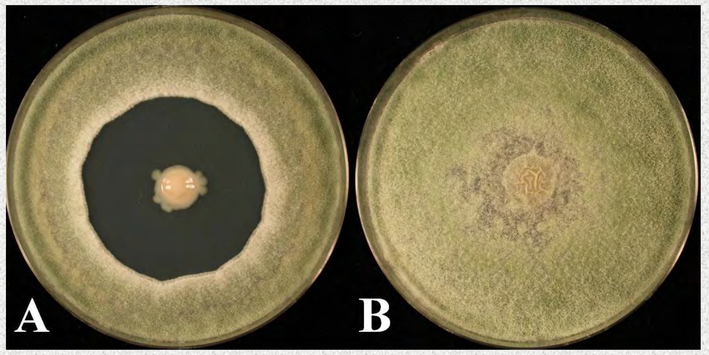


Plate bioassays of the bacterium *P. fluorescens* MS82 (A) and the mutants MS82MT31 (B) through overspraying with the indicator fungus *T. viride*. BLAST analyses revealed that Tn5 transposon was inserted into the *rpoB* gene.

Conclusions

- ✓ *Pseudomonas fluorescens* strain MS82 is potential using in mushroom cultivation and controlling fungal pathogens *Trichoderma viride*.
- ✓ Mutants defective in antifungal activities have been obtained using Tn*5* random mutagenesis.
- ✓ The genes cloned from mutants are associated with secretion and biosynthesis of antifungal compounds.
- ✓ Further genetic analyses and chemical structure elucidation are under way.

CENTRE OF EXPERTISE IN BIOTECHNOLOGY AND ANALYSIS **HAN BIOCENTRE**

Hogeschool van Arnhem en Nijmegen
HAN University of Applied Sciences

High-throughput screening of microorganisms and plant extracts with antifungal activity against *T.aggressivum*

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Introduction

The commercial cultivation of mushrooms is susceptible to the growth of 'weed' fungi which can cause substantial loss of yields. Due to increasing resistance against the currently used antifungals combined with their often harmful nature there is a growing demand for new antifungals. In this research a reporter system to measure fungal stress, previously developed in *A.niger*, was implemented in the fungus *Trichoderma aggressivum* allowing us to screen for antifungal activity against this strain.

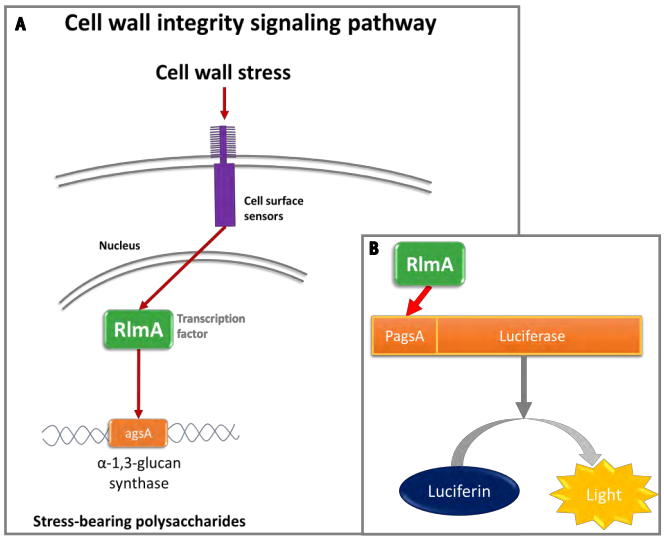


Figure 1. Our system allows us to identify compounds that specifically affect the fungal cell wall (A) Stress activates the fungal Cell Wall Integrity (CWI) pathway which results in the activation of the transcription factor RlmA. **(B)** In our reporter strains RlmA induces luciferase expression, resulting in light emission in response to stress.

Objectives

- Validate our *T.aggressivum* reporter strain
- Screen for antifungal activity of bacteria present in mushroom compost & 20 selected plant extracts

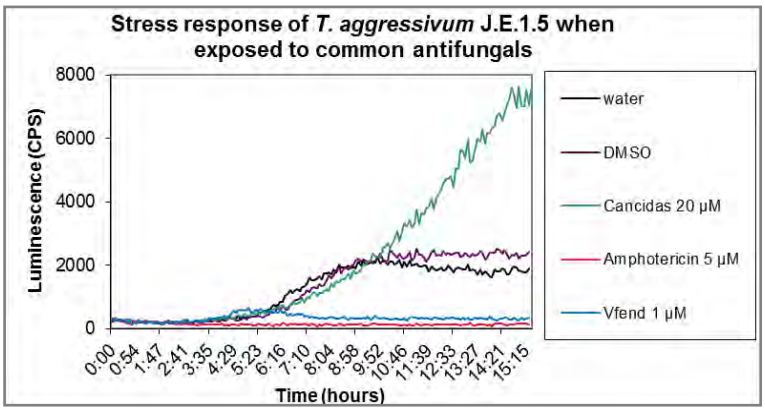


Figure 2. Validation of the *T.aggressivum* reporter strain using common antifungals. Luminescence is a measure of experienced stress, lack of luminescence indicates the fungus was killed. With the three common antifungals either an increase in signal or a lack of signal is detected. This indicates the antifungal effect of all three antifungals could be detected, validating our assay.

Results

- Our reporter strain was validated using common antifungals (figure 2)
- Out of compost 450 bacteria were isolated. 20 of these bacteria exerted antifungal activity against *T.aggressivum* in our assay
- 20 selected plant extracts were tested in our assay, 6 killed *T.aggressivum* and 4 caused a stress response

Conclusions

- Our assay was validated
- 20 bacteria and 10 plant extracts were detected with antifungal activity against *T.aggressivum*, those will be further researched

HAN

About HAN BioCentre

The central theme of HAN BioCentre is Biodiscovery; the discovery, analysis, production and application of biomolecules. We are in search for new bioactive compounds and screenings assays which can be applied in agriculture, food and health. All our projects are bringing together multiple disciplines ranging from microbiology and molecular biology to chemical analysis and bio-informatics.

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Effectiveness of chlorothalonil, metrafenone and prochloraz-Mn on dry bubble disease (*Lecanicillium fungicola*)

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The aim of this study was to test the *in vitro* sensitivity of the *Lecanicillium fungicola* isolates to chlorothalonil, metrafenone and prochloraz-Mn and the effectiveness of these fungicides to control dry bubble disease in mushroom crops artificially infected with *L. fungicola*.

In vitro assessment of fungicide sensitivity

Dose response relationships for the inhibition of mycelial growth by the fungicide were assessed in radial growth experiments on fungicide-amended malt extract agar (*L. fungicola*) and agar-compost (*A. bisporus*).

ED_{50} values and selectivity indices of fungicides for *L. fungicola* and *A. bisporus*

Fungicide	*Lecanicillium fungicola* ED_{50} (µg mL^{-1})			*Agaricus bisporus* ED_{50} (µg mL^{-1})			Selectivity index
	n	Mean	Range	n	Mean	Range	
Chlorothalonil	31	5.8	0.004-53.3	6	1.4	0.74-3.20	4.143
Metrafenone	8	> 200	-	6	2074.5	1490.3-3013.0	-
Prochloraz-Mn	31	3.3	1.01-7.17	12	14.3	3.58-27.91	0.231

Efficacy of fungicides in a mushroom crop infected with *Lecanicillium fungicola*

The *in vivo* effectiveness of these fungicides was evaluated in two mushroom cropping trials infected with *L. fungicola* at a rate of 10^6 conidia m^{-2}. In each trial, there were four different fungicide treatments and two controls. The numbers and the total weight of the fruiting bodies were recorded for each treatment. Disease incidence (DI) was calculated.

Effect of fungicide treatments on yield (kg m^{-2}) and dry bubble incidence (%) in two *A. bisporus* crops artificially infected with *L. fungicola*

Trial	Treatments[a]	1st flush		2nd flush		Total crop	
		Yield	DI[b]	Yield	DI	Yield	DI
1	C	16.6 a[c]	0 a	11.0 c	0.4 a	27.7 b	0.1 a
	IC	18.0 ab	20.0 cd	3.8 a	86.8 c	21.8 a	58.7 d
	ICTL	17.5 ab	0.4 ab	9.84 c	4.3 a	27.3 b	1.8 a
	IM	19.8 b	2.3 b	6.8 b	30.8 b	26.5 b	12.5 b
	IP	17.3 ab	26.6 d	5.0 ab	77.6 c	22.2 a	54.5 cd
	IP2	18.6 ab	13.7 c	3.3 a	78.9 c	21.9 a	43.5 c
2	C	13.5	0.0 a	12.3 c	16.1 a	25.8 b	8.2 a
	IC	14.0	37.0 d	7.4 ab	85.8 c	21.4 a	73.3 c
	ICTL	13.4	2.2 ab	13.1 c	10.5 a	26.5 b	6.3 a
	IM	15.1	10.5 bc	10.4 bc	48.9 b	25.5 b	30.9 b
	IP	14.2	24.5 cd	5.5 a	88.9 c	19.7 a	74.3 c
	IP2	14.5	32.2 d	6.1 a	78.2 c	20.6 a	61.7 c

[a] Treatments: C: control with water; IC: inoculated control; IP: prochloraz-Mn, 0.5 g m^{-2}; IP2: prochloraz-Mn, 1 g m^{-2}; IM: metrafenone, 1 mL m^{-2}; ICTL: chlorothalonil, 2 mL m^{-2}. [b] DI: Disease incidence (%). [c] In each trial, means within a column followed by a common letter do not differ significantly according to Tukey's test at $P = 0.005$.

The most effective fungicide for inhibiting the *in vitro* growth of *L. fungicola* was prochloraz-Mn.
The most effective treatment in both mushroom crops was with chlorothalonil.

Chemical control of mushroom cobweb disease caused by *Cladobotryum mycophilum*

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PROCHOLRAZ-Mn

Is the fungicide recommended to fight cobweb disease (*C. mycophilum*) within button mushroom crops in EU, but...

- ❑ Risk of resistance
- ❑ Yield losses

Effective and selective to the parasite

...the continuous use of this pesticide may imply...

...so pesticides must be...

...alternative fungicides have been evaluated...

Clortalonil

Metrafenone

temporary approved for use on mushrooms in Spain



Cobweb disease

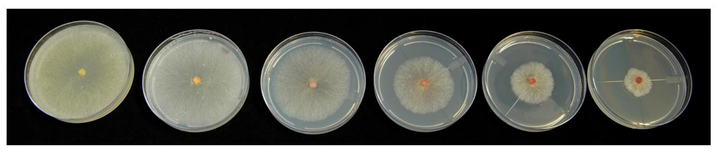
OBJECTIVES:

I) *In vitro* sensitivity was assessed to specify toxicity and selectivity of chemical fungicides.

II) Fungicide control was evaluated when *C. mycophilum* is artificially inoculated in mushroom crops.

In vitro sensitivity assays

ED_{50}, resistance factor and selectivitity index have been calculated. 4 replicates per fungicide / strain in duplicate.



Fungicide	*C. mycophilum* n[1]	*C. mycophilum* ED_{50} (µg mL^{-1})	*C. mycophilum* RF[2]	*A. bisporus* n	*A. bisporus* ED_{50} (µg mL^{-1})	Selectivity index[3]
Chlorothalonil	50	0.542±0.02b*	2.3	6	1.4±0.5a	0.38c
Metrafenone	26	0.025±0.03a	4.5	6	2074.5±460.7c	0.00001a
Prochloraz-Mn	26	0.053±0.03a	11.7	12	14.3±7.4b	0.003b

[1]Number of ED_{50} values considered per fungicide. [2]RF: Resistance factor, expressed as the ratio of the highest ED_{50} to the mean ED_{50}. [3]SI: Selectivity index calculated by dividing the mean ED_{50} value for *C. mycophilum* by the corresponding mean ED_{50} value for *A. bisporus*. *Same letters in columns: no significant difference.

Efficacy of fungicides in mushroom growing crops

2 trials. 32 randomized blocks (10 kg compost; 0.15 m^2 in area) in 20 m^3 experimental growth chambers. 3 fungicides, 6 treatments (*Inoculation: 9 days after casing at 10^6 conidia m^{-2}):

Code	*Inoc.	Treatment (1 L m^{-2})
C	No	Only tap water
IC	Yes	Only tap water
IP1	Yes	Prochloraz-Mn at 0.05% on day 4
IP2	Yes	Prochloraz-Mn at 0.05% on day 4 and 21
IM	Yes	Metrafenone at 1 ml L^{-1} on day 4
ICTL	Yes	Chlorothalonil at 2 ml L-1 on day 4





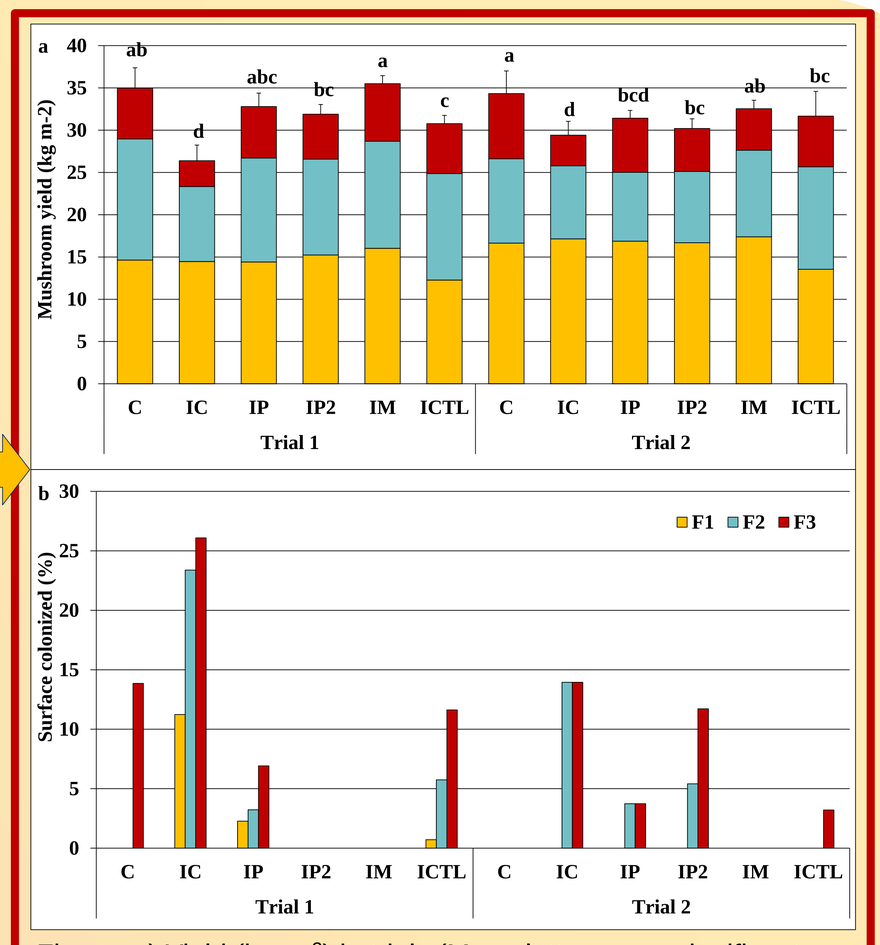


Figure. a) Yield (kg m^{-2}) in trials (Mean letters: non-significant diffrences.). b) Evolution of the crop surface colonized by cobweb (%) during crop cycle. F1: 1st flush; F2: 2nd flush; F3: 3rd flush.

- ❑metrafenone & prochloraz show high toxicity to *C. mycophilum* and adequate selectivity index;
- ❑ chlorothalonil was the least toxic showing reduced selectivity to the pathogen. However, the risk of resistance to this a.s. is reduced.
- ❑ yield losses up to 24.6% were detected due to cobweb disease and a reduction of the crop surface up to 26% (IC);
- ❑metrafenone was the most effective treatment in crop to control cobweb;
- ❑chlorothalonil led to significant differences in production in both trials.

Metrafenone presents a high potential to be used as an alternative to prochloraz-Mn to control cobweb disease in commercial mushroom crops.

19th ISMS Congress International Society for Mushroom Science
Amsterdam 2016
29th May - 2nd June

REFERENCES

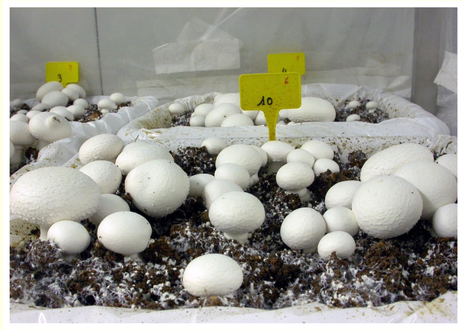
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Toxicity of Chlorothalonil, Metrafenone and Prochloraz-Mn Fungicides towards *Agaricus bisporus*: *In vitro* and *In vivo* Evaluation

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Chemical control of fungal diseases requires highly selective fungicides in order to prevent infection without affecting the growth of *A. bisporus*. The aim of this study was to evaluate the potential toxicity on *A. bisporus* mycelium of some fungicides commonly used in mushroom growing crops, and their effect on yield after fungicide drenches applied to the casing layer.

***In vitro* evaluation:** three commercial *A. bisporus* strains were used for the *in vitro* assays. Dose response relationships for the inhibition of mycelium growth by the fungicide were assessed in radial growth experiments on fungicide-amended compost agar. ED_{50} values (µg ml^{-1} of a.i. inhibiting radial mycelial growth by 50%) were calculated for each isolate and for each fungicide.

Table 1. ED_{50} and range of values (µg ml^{-1}) of selected fungicides against *A. bisporus*.

Fungicide	ED_{50}	Range
Chlorothalonil	1.412	0.796- 3.197
Prochloraz-Mn	14.254	3.576- 27.905
Metrafenone	2074.460	1490.301- 3013.045

✓ **Chlorothalonil exhibited some degree of toxicity against the commercial spawns of *A. bisporus* mycelium evaluated.**

✓ **The strains tested showed low sensitivity towards metrafenone.**

✓ **Prochloraz-Mn showed an intermediate value.**

Mushroom growing crops: the toxicity of these three fungicides was also evaluated *in vivo*. Commercial formulations of chlorothalonil 50% and metrafenone 50% in tap water were added as single drench applications (doses of 3 and 1 ml m^{-2}, respectively) and prochloraz-Mn 46% was added as single (one drench, 0.5 g m^{-2}) and double (two drenches, 1 g m^{-2}).
The mushrooms were harvested daily in three flushes, and the biological efficiency (BE) was calculated for each treatment.

✓ **Prochloraz-Mn produced falls in yield between 0-8% (PCL) and between 0-8.6% (PCL2).**

✓**Chlorothalonil produced falls of around 7%.**

✓**Metrafenone caused yield falls lower than 6%.**

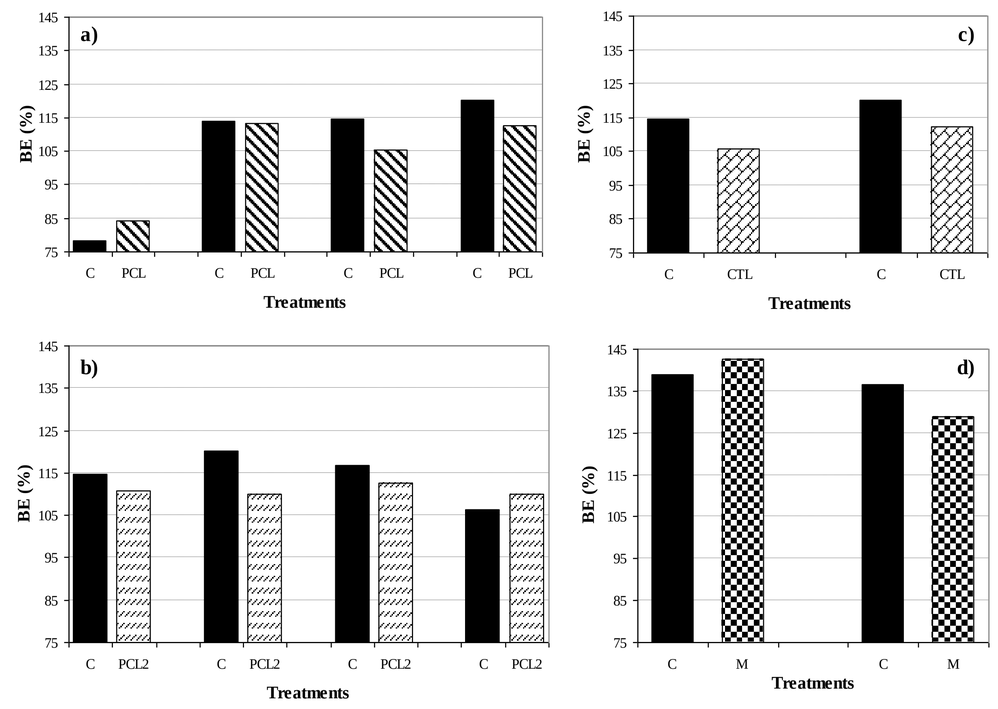


Figure 1. Biological efficiency (kg mushroom harvested on 100 kg dried compost) obtained in control treatment (C), with water, vs different fungicide treatments: a) PCL: prochloraz-Mn, 0.5 g m^{-2}; b) PCL2: prochloraz-Mn, 1 g m^{-2}; c) CTL: chlorothalonil, 2 ml m^{-2}; d) M: metrafenone, 1 ml m^{-2}.



***In vitro* and *in vivo* results suggest that chlorothalonil fungicide is the most detrimental to *Agaricus* mycelium. The *in vitro* differences found between metrafenone and prochloraz-Mn were not supported by *in vivo* results, where the reductions in yield turned out similar.**

Funding for this research was provided by the Ministerio de Ciencia y Competitividad (INIA) from Spain (Project E-RTA2014-00004-C02-01)

19th ISMS Congress International Society for Mushroom Science
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29th May - 2nd June

Diversity of *Bacillus* spp. strains during *Agaricus bisporus* substrate production and their antagonistic activity against four *Trichoderma* species

153.

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INTRODUCTION

The commercial production of *Agaricus bisporus*, one of the most commonly cultivated edible mushrooms worldwide, has been seriously affected by green mould epidemics caused by *Trichoderma* species. The disease is characterized by the occurrence of dense white mycelia on casing soil or compost which changes colour into green after an extensive sporulation. Also, mushroom fruiting bodies might develop brown coloured necrotic lesions whereas during serious outbreaks of the disease, no fruiting bodies are formed. One of the most promising alternatives to synthetic fungicides is the biological control of pathogens, which includes the use of biopesticides based on antagonistic *Bacillus* spp.





MATERIALS AND METHODS

The isolation of bacteria was carried out from straw and manure samples, compost during various stages of composting and casing soil used for growing button mushrooms. The method for isolation of *Bacillus* spp. strains was based on the resistance of their endospores to elevated temperatures (80 C).

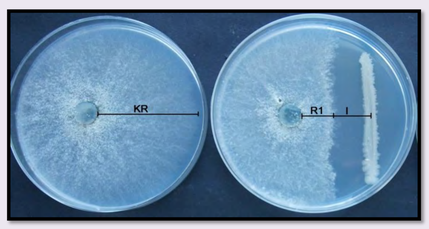
Further identification of *Bacillus* spp. was based on the partial sequence of the 16S rDNA, specifically the hypervariant region.

A preliminary screening of bacterial antagonism against *Trichoderma aggressivum* f. *europaeum*, as well as the screening against all four indicator strains of fungi, was carried out using the dual culture method.

The size of the growth inhibition zone was measured and the percent of the growth inhibition (PGI) was calculated using the formula:

$$PGI(\%)=[(KR-R1)/KR]x100$$

where KR represents the colony diameter of pathogen in the control plate, and R1 represents the colony diameter in the treated plate.



RESULTS AND DISCUSSION

After preliminary tests, the bacterial isolates characterized as Gram-positive and catalase positive were subjected to molecular identification based on the of hypervariant region of 16S rDNA.The results revealed the presence of four *Bacillus* species during the mushroom production process, namely *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus pumilus* (Table 1). *B. subtilis* strains were the most numerous and were isolated from all tested sources. *B. pumilus* strains were predominant in manure, but also present in compost stage I. *B. amyloliquefaciens* strains were found in compost stages I and II, and were the only *Bacillus* species found in casing soil. *B. licheniformis* was isolated only from compost stage III.

Table 1. Preliminary screening of antagonistic activity of bacterial isolates against *Trichoderma aggressivum* f. *europaeum* and characterization of isolates

Isolate	Source	Antagonism	Gram differentiation	Catalase test	Species	Accession number
B-106	Straw	+	+	+	*B. subtilis*	KT692716
B-107	Straw	+	+	+	*B. subtilis*	KT692717
B-108	Straw	+	+	+	*B. subtilis*	KT692718
B-109	Straw	+	+	+	*B. subtilis*	KT692719
B-110	Straw	+	+	+	*B. subtilis*	KT692720
B-111	Straw	+	+	+	*B. subtilis*	KT692721
B-112	Straw	+	+	+	*B. subtilis*	KT692722
B-124	I stage 8. day	+	+	+	*B. subtilis*	KT692723
B-126	I stage 14. day	+	+	+	*B. subtilis*	KT692724
B-128	II stage	+	+	+	*B. subtilis*	KT692725
B-233	I stage 3. day	+	+	+	*B. subtilis*	KT692729
B-253	Manure	+	+	+	*B. subtilis*	KT692731
B-270	III stage	+	+	+	*B. subtilis*	KT692736
B-138	Manure	+	+	+	*B. pumilus*	KT692727
B-217	I stage 14. day	+	+	+	*B. pumilus*	KT692728
B-254	Manure	+	+	+	*B. pumilus*	KT692732
B-256	Manure	+	+	+	*B. pumilus*	KT692733
B-257	Manure	+	+	+	*B. pumilus*	KT692734
B-129	II stage	+	+	+	*B. amyloliquefaciens*	KT692726
B-241	I stage 14. day	+	+	+	*B. amyloliquefaciens*	KT692730
B-268	Casing soil	+	+	+	*B. amyloliquefaciens*	KT692735
B-276	III stage	+	+	+	*B. licheniformis*	KT692737

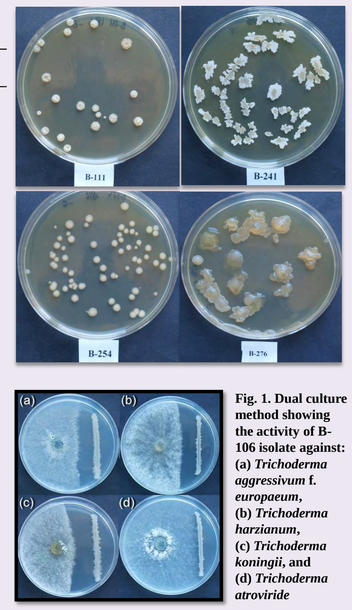


Fig. 1. Dual culture method showing the activity of B-106 isolate against: (a) *Trichoderma aggressivum* f. *europaeum*, (b) *Trichoderma harzianum*, (c) *Trichoderma koningii*, and (d) *Trichoderma atroviride*

In screening for antagonistic activity against *Trichoderma aggressivum* f. *europaeum*, 22 out of 108 tested isolates inhibited the mycelial growth of that mycopathogenic fungus. Further screening revealed that these bacterial isolates inhibited the growth of *Trichoderma harzianum* and *Trichoderma koningii* as well, while only 13 isolates inhibited the growth of *Trichoderma atroviride*. Therefore, *T. atroviride* was the least sensitive fungus while the most sensitive pathogen was *T. aggressivum* f. *europaeum* (Fig. 2).

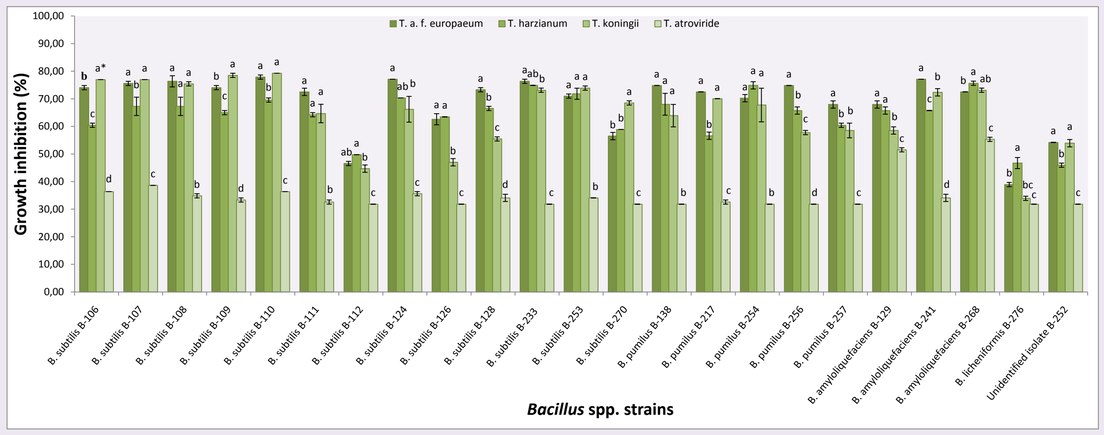


Fig. 2. Antifungal activity of *Bacillus* spp. *in vitro* against *Trichoderma* spp. Mean values of percent of inhibition (n=3) of fungal growth with standard error for each antagonistic strain were shown. *Values followed by the same letter, within columns with all tested fungi for each isolate, are not significantly different ($P<0.05$), according to Tukey HSD test.

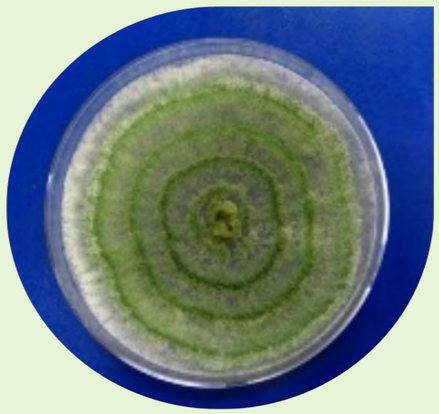
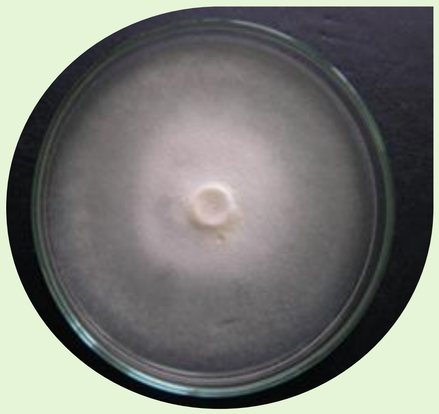
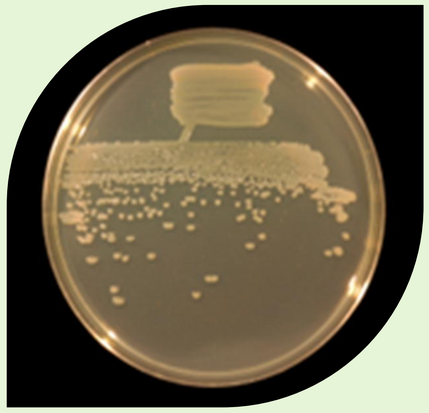
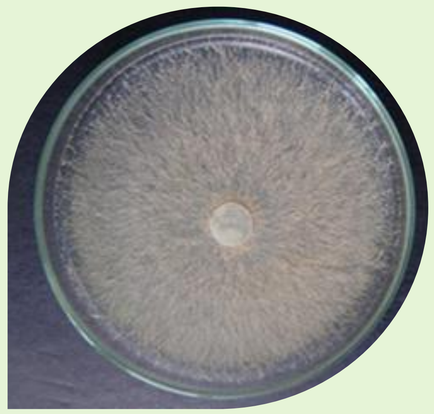
CONCLUSION

Bacillus spp. strains isolated from compost material, compost under various stages of composting and casing soil belonged to four species: *B. subtilis, B. pumilus, B. amyloliquefaciens* and *B. licheniformis.* These 22 strains proved to be, in various extent, efficient in antagonising mycopathogenic *Trichoderma* spp.. The majority of *Bacillus* spp. strains were exceptional in antagonism of *T. aggressivum* f. *europaeum.* Half of the strains showed remarkable inhibition of *T. harzianum* and *T. koningii* mycelial growth while only two *B. amyloliquefaciens* strains lead to a significant inhibition of *T. atroviride* growth.

155. Antimicrobial activity of biochemical substances against pathogens of cultivated mushroom in Serbia

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Introduction

After antibiotic and fungicide resistance among pathogen populations, a major challenge for mushroom growers is disease control with few or no chemicals. Special attention is now being paid to the possibility of using biochemicals, such as extracts or essential oils of different plants, to inhibit pathogen activity.




Biochemical substances in disease control of edible mushrooms

Testing of 22 essential oils from Germany and Albania against *Pseudomonas tolaasii* showed the strongest activity of the oils of wintergreen (*Gaultheria procumbens* L.) and oregano (*Origanum vulgare* L.). Respective MBC values were 0.16 and 0.32 µl ml^{-1}.

Six essential oils extracted from plants originated from Serbia assayed against *Trichoderma agressivum* f. *europaeum* and the strongest activity was shown by the oils of basil (*Ocimum basilicum* L.) and peppermint (*Mentha piperita* L.). Peppermint oil showed MFC=0.64 µl ml^{-1} (Đurović-Pejčev et al., 2015).

Among eight commercial essential oils from Serbia tested against *Mycogone perniciosa*, *Lecanicillium fungicola* and *Cladobotryum* sp., oregano oil possessed the highest antifungal activity with a both MIC and MFC of 0.02 µl ml^{-1} and geranium *(Pelargonium graveolens)* et higher concentrations (Tanović et al., 2009). Out of seven oils, thyme *(Thymums vulgaris)* showed the strongest activity against *Mycogone perniciosa* (Potočnik et al., 2010).

Tea tree oil caused a significant reduction in cobweb disease *(Cladobotryum dendroides)* and green mould levels *(Trichoderma harzianum*) in the *Agaricus bisporus* experimental growing room (Potočnik et al., 2010; Kosanović et al., 2013). The oil was not toxic to *Pseudomonas tolaasii* (Todorović et al., 2012).

Acknowledgement

This study was carried out with financial support from the Ministry of Education, Science and Technological Development of the Republic of Serbia, project TR 31043.

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Monitoring of bacterial diseases of *Agaricus bisporus* in Serbia

159.

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INTRODUCTION

Button mushroom (*Agaricus bisporus* (Lange) Imbach) is one of the most common cultivated edible mushrooms in Serbia. Its production is mostly intended for fresh market but although market demands are increasing, it is still at the level of small-scale growers. Unlike fungal pathogens that were thoroughly studied in Serbian mushroom farms (Potočnik et al. 2015), bacterial pathogens in *A. bisporus* were not studied until 2006 when symptoms resembling brown blotch were observed on several mushroom farms and *Pseudomonas tolaasii* was reported for the first time (Milijašević-Marčić et al., 2012). Since there are several fluorescent pseudomonads with the ability to cause diseases of *A. bisporus* (Munsch et al., 2000; Godfrey et al., 2001; Iacobellis, 2011), after these findings, an extensive survey of button mushroom bacterial diseases both in farms and fresh markets was conducted to estimate the presence and identity of mycopathogenic bacteria and to determine the predominant bacterial pathogen in Serbia.

MATERIAL AND METHODS



Symptoms



Samples of diseased button mushroom were collected from mushroom farms and fresh markets in 18 localities within a ten year period. Bacteria were isolated from the *A. bisporus* fruiting bodies with bacterial blotch symptoms or different degrees of brown discoloration on caps and stalks following the method of Lelliott and Stead (1987).

Pathogenicity tests were carried out using healthy, one-day-old *A. bisporus* whole sporophores and cubes of cap tissue excised with a sterile scalpel. For both trials tissue was placed into a sterile petri dish containing a filter paper dampened with sterile distilled water. Bacterial isolates and reference strains of *P. tolaasii* (CFBP 2068^T) and *P. agarici* (CFBP 2063^T) were cultured on solid KB medium were suspended in sterile distilled water to reach the density of approximately 1×10^8 CFU ml^{-1}. Bacterial isolates were evaluated by determining LOPAT characteristics and the some differential phenotypic properties (Lelliott and Stead 1987).

Molecular identification was conducted to confirm the identification of *P. tolaasii* isolates according to the PCR protocol given by Lee et al. (2002) using primer pair Pt-1A, Pt-1D1 specific for its detection. Reference strain of *P. tolaasii* CFBP 2068^T was used as positive control. Other group of isolates was identified based on analyses of the 16S rDNA sequence. A PCR assay with the universal primers 27F and 1541R was performed for amplification of the bacterial 16S rDNA (Edwards et al., 1989; Lane, 1991). Positive amplicons were sequenced in forward direction with the 27F primer. Sequencing was performed by a commercial service (Macrogen Inc., Korea). The obtained sequences were assembled using Pregap4 from the Staden program package.

RESULTS AND DISCUSSION

A monitoring of bacterial diseases in mushroom farms and fresh markets in Serbia revealed the presence of symptoms of brown blotch or different degrees of brown discoloration on cultivated button mushrooms. Over a hundred bacterial isolates were obtained after isolation from symptomatic samples. Forty-six green fluorescent, Gram negative isolates from different sources and localities were selected for pathogenicity tests. The bacterial isolates showed different levels of discoloration and tissue degradation on the mushroom cubes varying from light to dark brown. Thirty four bacterial isolates caused sunken brown lesions on *A. bisporus* cap tissue blocks after 72 h, consistent with those caused by a reference strain of *P. tolaasii*. The remaining isolates showed light brown superficial discoloration both on tissue blocks and sporophores similar to *P. agarici* reference strain.

Results of monitoring of bacterial diseases

Locality	Strain designation	Year of isolation	Strain identity
1. Novi Slankamen	NSl6B6	2006	*P. tolaasii*
2. Čačak	Ča1B6	2006	*P. tolaasii*
3. Mol	Mol4B6	2006	*P. tolaasii*
4. Padina	Pad1B6	2006	*P. tolaasii*
6. Vinarce, Niš	Vin7B6; Vin31B6	2006	*P. tolaasii*
7. Borča, Beograd	Bo42B6	2006	*P. tolaasii*
8. Novi Sad	NS3B6; NS1B6	2007	*P. tolaasii*
9. Veliko Gradište	Vg1/1;Vg1/2 Vg3/1;Vg3/2	2010	*P. tolaasii*
10. Veliko Gradište (market)	S-54; S-55, S-56; S-57; S-58; S-76; S-77; S-78; S-79; S-91; S-92; S-93; S-94; S-95; S-96, S-97	2014/2015	*P. tolaasii*
11. Jošanica	S-80; S-81; S-82; S-83; S-84; S-85	2015	*P. tolaasii*
12. Padinska Skela	OP-6	2006	*P. agarici*
13. Padina	Pad-4	2006	*P. agarici*
14 . Požarevac	Poz 8/7	2006	*P. agarici*
15. Veliko Gradište	Veg 2	2006	*P. agarici*
16. Vračev Gaj	Vg 1	2006	*P. agarici*
17. Vinarce	Vin 6	2006	*P. agarici*
18. Jakovo	Jak 4	2006	*P. agarici*

Biochemical characterization

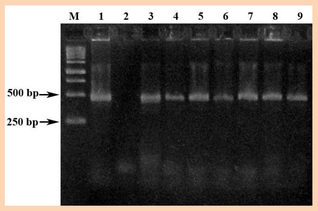
	P. tolaasii strains (36) and CFBP 2068^T	*P. agaricii* strains (7) and CFBP 2063^T
Gram stain	-	-
Glucose (O/F)[1] metabolism	O	O
Levan production	-	-
Oxidase activity	+	+
Potato rot	-	-
Arginine dehydrolase	-	-
Tobacco hypersensitivity	-	+
Fluorescence on KB medium	+	+
Catalase activity	+	nt
Aesculin hydrolysis	-	nt
Tween 80 hydrolysis	+	nt
Casein hydrolyses	+	nt
Gelatine hydrolyses	-	nt
Nitrate reduction	-	-
Utilization of:		
mannitol	+	+
erythritol	+	-
sorbitol	+	-
inositol	+	nt
sucrose	-	-
trechalose	+	-
L-arabinose	-	-
L-arabitol	nt	-
D(-)tartrate	-	-
L-rhamnose	nt	-

+ = Positive reaction; - = Negative reaction; O = Oxidative metabolism of glucose; 1 = Oxidative-fermentative metabolism of glucose, nt-not tested

Pathogenicity tests



PCR identification of *P. tolaasii*



Amplification of 449 bp PCR products with DNA of following *P. tolaasii* strains: 1 - positive control strain CFBP 2068^T; 2 – negative control; 3 – NSl6B6; 4 – Mol4B6; 5 – Pad1B6; 6 – Vin7B6; 7 – Bo42B6; 8 – NS3B6; 9 – Vg1/1; M - molecular weight marker 1000 bp ladder.

Generally, the results of this survey showed that *P. tolaasii* was a predominant bacterium associated with brown discoloration symptoms on *A. bisporus* which is not unexpected considering that *P. tolaasii* has been recognized as the most common button mushroom pathogen. However, recent studies pointed out that brown blotch of *A. bisporus*, caused by *P. tolaasii*, is actually a complex disease since some other pseudomonad species also have the ability to cause blotch symptoms with various levels of discolorations (Godfrey et al., 2001; Iacobellis, 2011). Moreover, the pathogenicity tests in our study also showed differences in the level of discoloration of the inoculated mushroom cap tissue implying that some other fluorescent pseudomonads participated in the expression of the disease symptoms. On the other hand, *P. agarici* was detected and identified in seven different localities, from 2006-2010. Further monitoring in fresh markets did not show repeated isolations of this bacterium. It is also noteworthy that in samples infected with *P. agarici,* typical drippy gill symptoms were not observed. The same observations had been made by Geels et al. (1994) and Lo Cantore and Iacobellis (2004) suggesting pathogen ability to induce different symptoms in various environmental conditions.

160. Evaluation of a native *Bacillus subtilis* strain for biological control of green mould disease in *Agaricus bisporus* cultivation

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Introduction

The main idea of this study was to identify favorable indigenous microorganisms in mushroom substrate, that have antagonistic activity against *Trichoderma aggressivum* f. *europaeum* and *Trichoderma harzianum,* causal agents of green mould, *in vitro* and in mushroom growing rooms.



Material and Methods

Efficacy against *Trichoderma aggressivum* f. *europaeum* T77 and *Trichoderna harzianum* T54 (10^6 conidia/m^2) from Serbia and effects on mushroom yield of the chosen *Bacillus subtilis* strain (B-38) were tested in comparison with both commercial formulation of *Bacillus subtilis* QST 713 and fungicide prochloraz-Mn.

The efficacy evaluation: Abbott's formula: E (%) = [(Ic-It)/Ic] x 100; Ic - disease incidence in inoculated control; It - disease incidence in treated samples.

The effects on mushroom productivity: BE (%) = (fresh total fruiting body yield/dry spawned substrate mass) x 100.

Results and discussion

The most promising strain B-38 induced 48.08% *in vitro* growth inhibition of *Trichoderma harzianum* and 52.25% of *Trichoderma aggressivum* f. *europaeum*.

Mushroom productivity improvement in the inoculated plots was observed after treatment with prochloraz-Mn against *Trichoderma harzianum* T54 and when *Bacillus subtilis* QST 713 was applied facing *Trichoderma aggressivum* f. *europaeum* T77 (Table 1). The strain *Bacillus subtilis* B-38 decreased yield more than *Bacillus subtilis* QST 713 in plots inoculated with both *Trichoderma* species.

The fungicide prochloraz-Mn showed the strongest capacity of preventing symptoms caused by both *Trichoderma* species. *Bacillus subtilis* QST 713 showed higher efficacy in disease control than the B-38 strain (Figure 1).

Our further research will focus on discovering other strains of the *Bacillus* genus that occur in mushroom composting material which could be used as an alternative to commercial fungicides and incorporated in integrated green mould management.

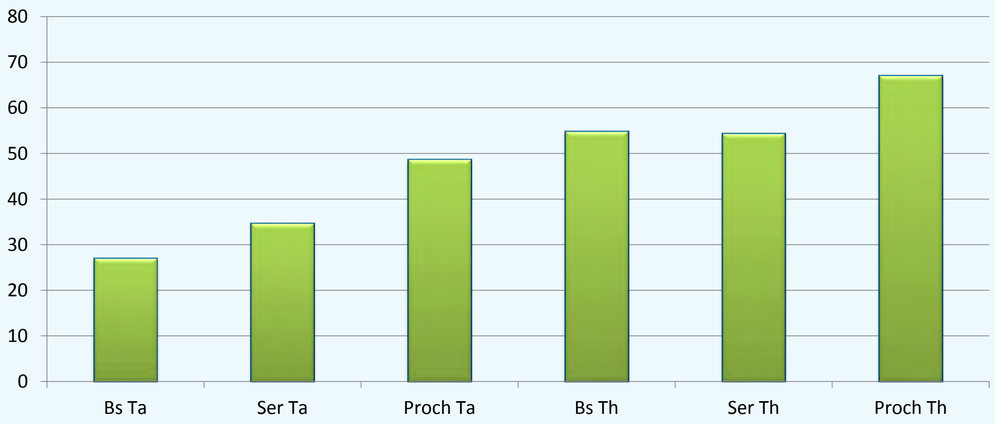
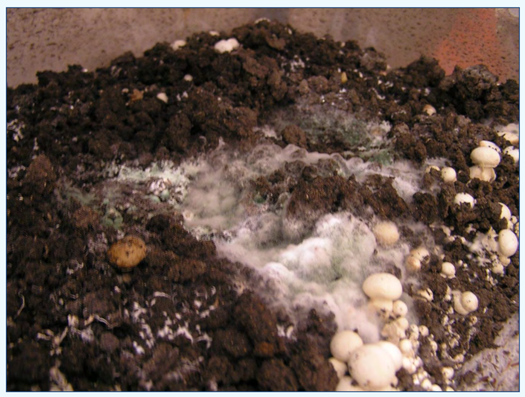


Figure 1. Efficacy of fungicides in the control of *Trichoderma harzianum* T54 (Th) and *Trichoderma aggressivum* f. *europaeum* T77 (Ta) on *Agaricus bisporus*; Bs – *Bacillus subtilis* B-38; Ser – *Bacillus subtilis* QWT 713; Proch – prochloraz Mn.

Table 1. Disease incidence on *Agaricus bisporus* inoculated with *Trichoderma*; data are means of three replicates ± SE, standard error of means

Treatment	*Trichoderma harzianum* T54	*Trichoderma aggressivum* f. *europaeum* T77
Control inoculated	26.16±15.90	23.47±2.22
Bacillus subtilis B-38	14.19±7.50	15.03±2.94
Bacillus subtilis QST 713	12.26±5.06	12.20±2.44
Prochloraz-Mn	11.81±3.50	11.88±3.45



Acknowledgement

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ISMS 2016

Mushroom tumor: A new disease on Flammulina velutipes caused by *Ochrobactrum pseudogrignonense*

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1. Introduction

Mushroom tumor has become the main disease during the off-season cultivation of *F. velutipes,* while the causal organism has remained unknown. Once infected by the pathogen, *F. velutipes* mycelia form nodular lesions which stops the formation of primordia and fruiting bodies. In 2012-2014 in the off-season cultivation areas of northwest Sichuan, 30-40% of the yellow *F. velutipes* was affected during the first harvest which rose to more than 80% during second harvest. In 2013, factory cultivated white *F. velutipes* was also found to be affected in Sichuan, Fujian and Henan.

The present study was aimed at identifying the pathogen confirming its pathogenisity following Koch's Postulates, characterizing it using morphological, physiological, biochemical and molecular features, and studying its current distribution.

2. Materials and methods

Five diseased samples (HY1, HY2, HY3, HY4 and HY5) were collected from Hongyuan, Songpan, and Dujiangyan cities in Sichuan, and the suspected pathogen was isolated from the five diseased samples. The test cultivar of *F. velutipes* was chuanjin No. 3, which is the main cultivar grown in Sichuan. Pathogenicity tests followed Koch's Postulates, and were repeated twice. The pathogen was identified by 16S RNA.

3. Results

During the early stage of infection the *F.velutipes* mycelia turned yellow which quickly spread to the whole bag. After 3–5 days, the surface of the mycelia wrinkled and small white to light yellow tumors formed on the surface of the substrate. After 7 days, the compact and viscoelastic tumors expanded to cover the whole surface of the substrate. Although the *F.velutipes* mycelium was not killed, initiation of primordia and fruiting body formation was completely inhibited. After one month new tumors grew up as old ones died (Fig.1a, b, c, d).

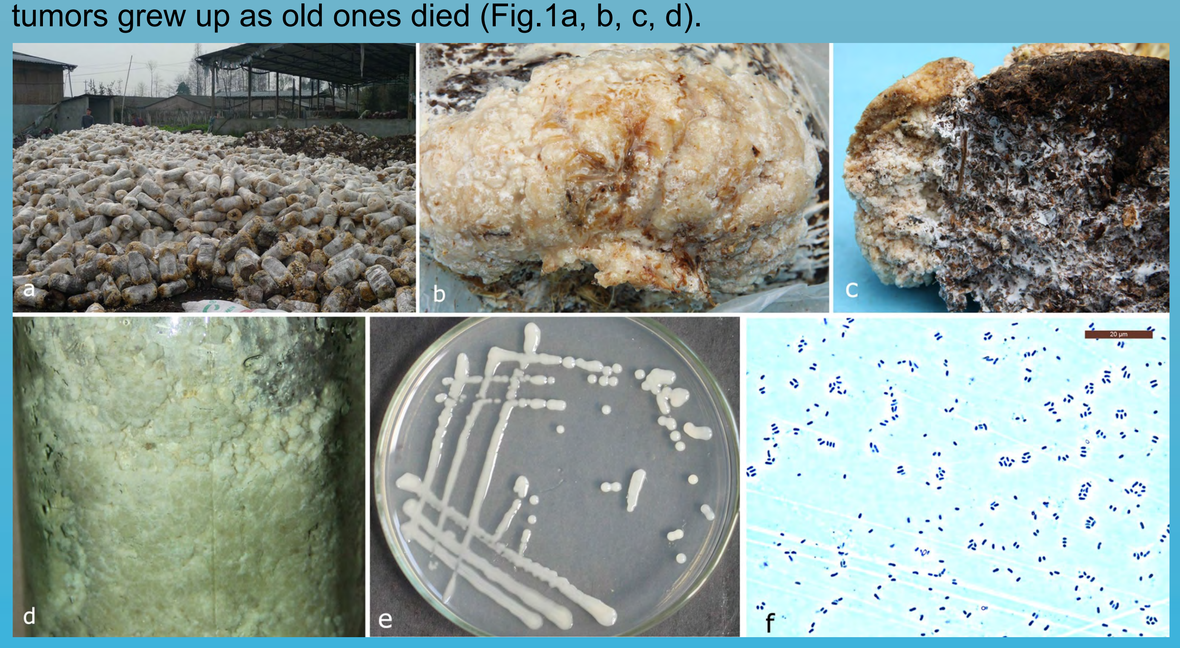


Fig. 1. Symptoms of mushroom tumor in Flammulina velutipes and the pathogenicity test. (a, b) Late stage of mushroom tumor infection showing no fruiting bodies formed. (c) Transverse view of hyphal bodies. (d) Successful infection of HY5-3 strains on F. velutipes (e, f) Morphological characters of Ochrobactrum pseudogrignonense.

A bacterial strain HY5-3 (Fig.1e, f) was recognized as the pathogen of mushroom tumor on *F.velutipes*. HY5-3 was identified as *Ochrobactrum pseudogrignonense* based on 16S rRNA sequence, physiological, biochemical characters (Fig.2; Table 2, 3).

Ochrobactrum pseudogrignonense is a fermentative bacteria and its characteristics are as follows: cells are rod, approximately 3–5 μm (Fig.1e,f). It has the ability of utilizing tartrate and 25 types of carbon sources (Table 2), and it is sensitive to 13 chemical reagents (Table 3).

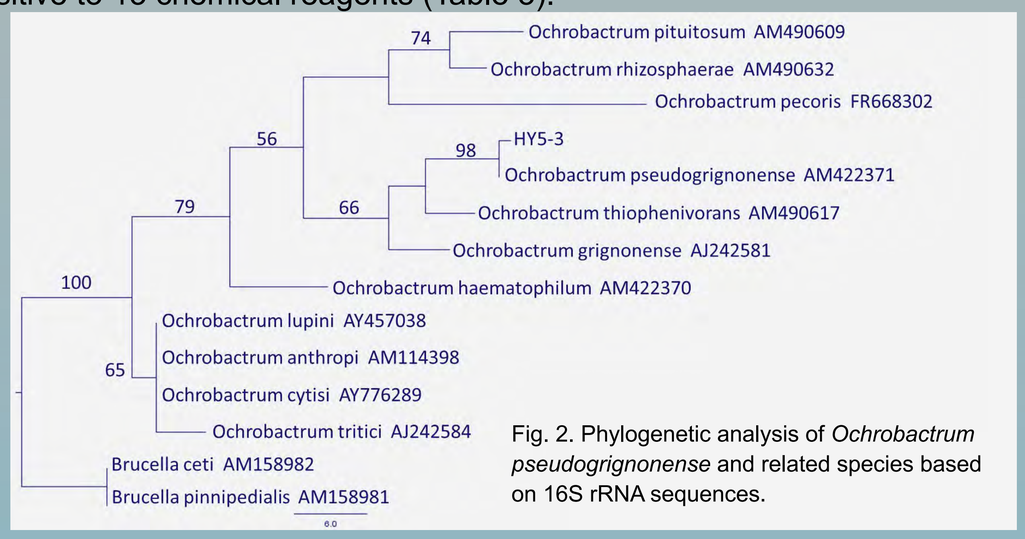


Fig. 2. Phylogenetic analysis of *Ochrobactrum pseudogrignonense* and related species based on 16S rRNA sequences.

Table 2. The carbon source utilization ability of *Ochrobactrum pseudogrignonense*.

Test	Strain (HY5-3)	Test	Strain (HY5-3)
N-Acetyl-D-Galactosamine	+	Glycyl-L-Proline	+
α-D-Glucose	+	L-Alanine	+
D-Mannose	+	L-Aspartic Acid	+
D-Fructose	+	L-Glutamic Acid	+
D-Galactose	+	L-Serine	+
D-Fucose	+	L-Galactonic Acid Lactone	+
L-Fucose	+	L-Lactic Acid	+
L-Rhamnose	+	α-Keto-Glutaric Acid	+
D-Sorbitol	+	D-Malic Acid	+
D-Mannitol	+	L-Malic Acid	+
D-Arabitol	+	Bromo-Succinic Acid	+
myo-Inositol	+	Acetic Acid	+
Glycerol	+		

Note: + Positive

Table 3. Chemical sensitivity test of *Ochrobactrum pseudogrignonense*.

Test	Strain (HY5-3)	Test	Strain (HY5-3)
pH 6	+	Guanidine HCl	+
1% NaCl	+	Vancomycin	+
4% NaCl	+	Tetrazolium Violet	+
1% Sodium Lactate	+	Tetrazolium Blue	+
Troleandomycin	+	Lithium Chloride	+
Rifamycin SV	+	Aztreonam	+
Lincomycin	+		

Note: + Positive

4. Discussion

Fungal or bacterial diseases have been the causes of serious economic losses in the cultivation of *Agaricus bitorquis*, *A. bisporus, Auricularia polytricha, F. velutipes* and *Pleurotus*. This is the first time that *Ochrobactrum* has been shown to be involved in the formation of a disease of a cultivated mushroom.

The identification of tumor disease in *F.velutipes* has provided a theoretical basis for the effective control of this disease, but control methods remain uncertain. In view of the importance of this mushroom in China urgent studies are needed if we are to be able to manage this significant problem in the future.

Bioprotection of the cultivated mushroom *Agaricus bisporus* by *Bacillus subtilis* biofilms

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ISMS Congress, 29 th May - 2nd June 2016 , Amsterdam, The Netherlands

INTRODUCTION

Worldwide, food losses amount to about one third of food for human consumption, equivalent to about 1.3 billion tonnes per year (FAOSTAT). A large fraction of these losses is due to microbiological deterioration. Until recently, chemical pesticides were the most commonly used solution in agriculture because of their radical effectiveness. In France, since 2008, the government aims by 2018 to reduce by 50% the use of such pesticides through for sustainable agriculture (French plan *EcoPhyto 2018*), which led to the intensification of biological control in agriculture. The different modes of action of microbial biocontrol known to date are the stimulation of the host natural defences, toxin or antimicrobial substances production and nutrient competition, but the concept of biofilm which could involve other mechanisms of bioprotection is rarely mentioned. Biocontrol represents at the present time, 5% of the plant protection sector. In the sector of button mushrooms (*Agaricus bisporus*) in France, the biocontrol agent used since 2008 by more than 80% of the sector and compatible with organic agriculture is *Bacillus subtilis* QST713. It shows a clear biofungicidal effect against *Trichoderma aggressivum* (compost flooding) during cultivation of *A. bisporus*. However, the mechanisms involved in the bio-protection phenomenon are poorly documented to date. The study of *Bacillus subtilis* QST713 biofilm formation at different stages of *A. bisporus* cultivation will allow a better understanding of the mechanisms involved as spatial and nutritional competition towards competitors such as *T. aggressivum* or *Pseudomonas tolaasii*.

RESULTS

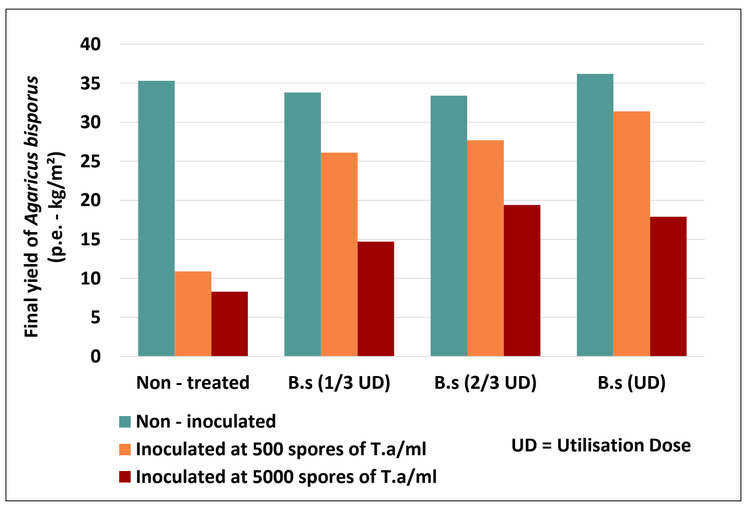
Inoculated compost at spawning by *T. aggressivum*



- Symptoms at the end of incubation and impact at the first harvest.
- Treatment by *Bacillus subtilis* QST713 protects *Agaricus bisporus* from *Trichoderma aggressivum*.

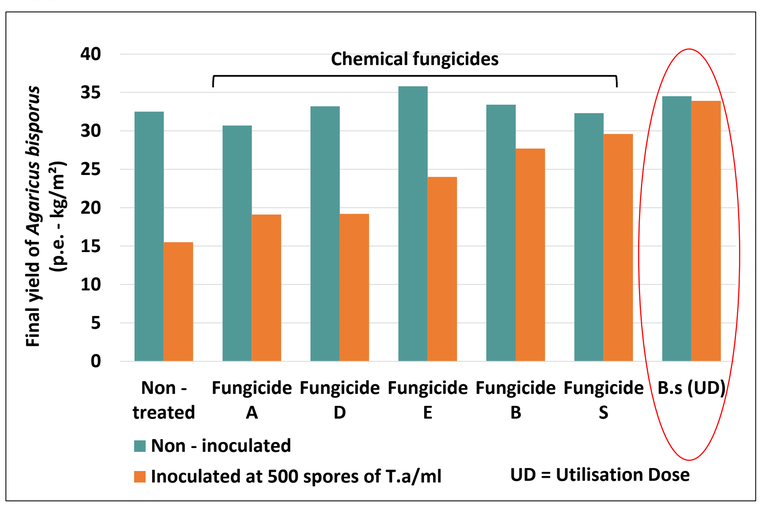
Yield efficiency of *Agaricus bisporus* with treatment by *B. subtilis* against *T. aggressivum*

Efficiency of 3 doses of *Bacillus subtilis* QST713 (B.s) on 2 levels of inoculation with *T. aggressivum* (T.a)



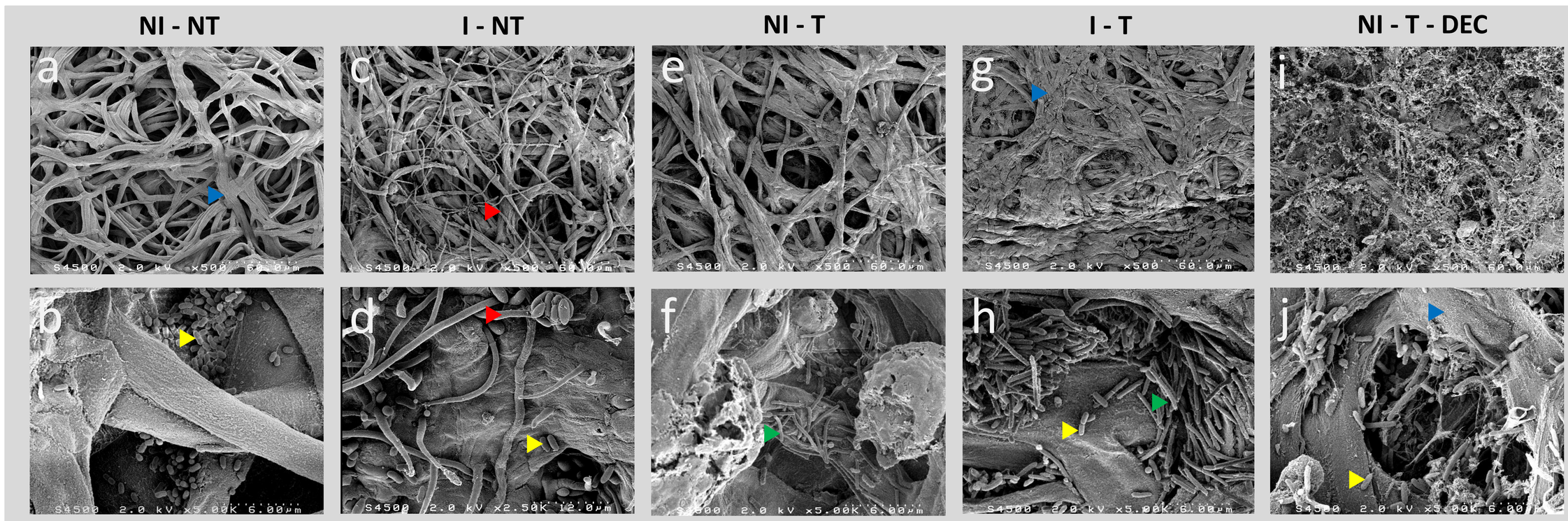
- Final yield is higher than the others doses with the utilisation dose of product with *Bacillus subtilis* QST713 compared to untreated and Fungicide S with 500 spores/ml of *Trichoderma aggressivum* inoculated.

Highlighting of the effectiveness of *Bacillus subtilis* QST713 (B.s) compared to 5 fungicides on *T. aggressivum* (T.a)



- Compared to the chemical fungicides used in this experiment, the biofungicide product with *Bacillus subtilis* QST713 restores at 98,2 % the final yield of *Agaricus bisporus* crops.

Scanning Electron Microscopy assay of *Agaricus bisporus* end crop, inoculated or not by *T. aggressivum* and treated or not by *Bacillus subtilis* QST713 at spawning



NI - NT : Non - inoculated, Non - treated
I - NT : inoculated (*T. aggressivum*), Non - treated
NI - T : Non - inoculated, treated
I - T : inoculated, treated
NI - T - DEC : Non - inoculated , treated (*B. subtilis* QST713), in decomposition (2 months)

▶ *Agaricus bisporus* ; ▶ *Trichoderma aggressivum* ; ▶ *Bacillus subtilis* ; ▶ *Pseudomonas*-like

- *Agaricus bisporus* microflora is composed largely by bacterial biofilms with a dominant *Pseudomonas*-like morphotype (a ; b ; i ; j). Most bacteria are lodged in the cavities of the mesh mycelial.
- Without treatment with *Bacillus subtilis* QST713, encroachment of the *Agaricus bisporus* carpophore by biofilms of *Pseudomonas*-like bacteria (a ; b ; c ; d) and *Trichoderma aggressivum* (c ; d).
- With treatment, spatial competition between biofilms of *Pseudomonas*-like bacteria with *B. subtilis* on the carpophore (e ; f ; g ; h ; i ; j). Only a few *T.aggressivum* spores were found on the sample Inoculated - Treated, no mycelial filaments were observed (g ; h).

CONCLUSION

- *Bacillus subtilis* QST713 has a protective efficacy against *Trichoderma aggressivum* at compost level during the growth of *Agaricus bisporus*, it has also a potential role on the protection of *A. bisporus* fruiting body against *Pseudomonas*-like bacteria and *Trichoderma aggressivum*.
- *T.aggressivum, Pseudomonas*-like bacteria and *B. subtilis* formed spatially-organized biostructures on *A. bisporus* fruiting body that resemble plurimicrobial biofilms described in other fields. It has been described that the biocontrol agent *Bacillus subtilis* QST713 partly acts by nutrient competition and by the action of specific lipopeptides that target cells membranes of the pathogen, but specific biofilm-associated mechanisms should also be considered.

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Occurrence and characterisation of strains *Trichoderma pleuroti* from the Czech Republic

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The study is supported by the The Technology Agency of the Czech Republic (project No. TA03020356



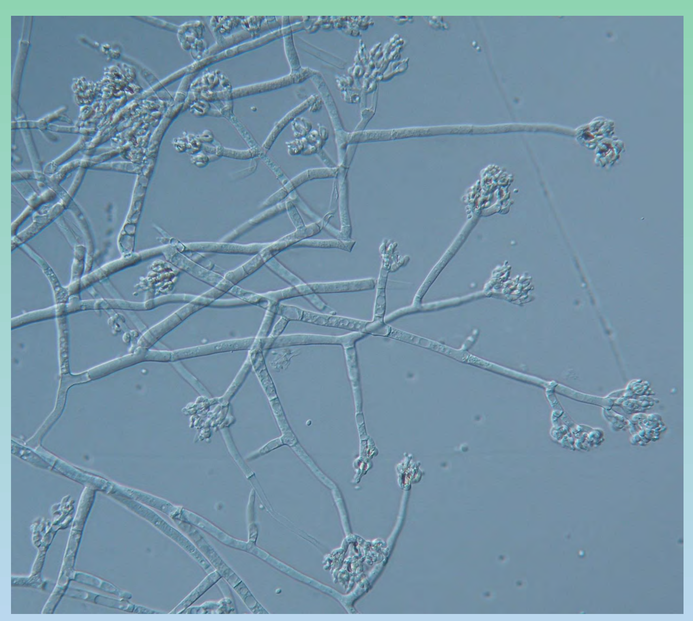
Introduction

Oyster mushroom (*Pleurotus ostreatus*) is the second the most important mushroom in the Czech Republic. *Trichoderma* species caused large losses at oyster mushroom cultivation during last seven years. *Trichoderma pleuroti* is the most important harmful fungal species in oyster mushroom farms in the Czech Republic in present time.

Materials and methods

The samples of oyster mushroom substrate with symptom of infection of Trichoderma species were pick up in oyster mushroom farms in the Czech Republic and then incubated on malt extract agar medium in Petri dishes. The recorded strains of Trichoderma species were inspected by stereomicroscope and microscope and isolated in pure culture. The identification on species level was based on macro-, micromorphological and molecular genetics characters. The influence of temperature and pH on growth of *T. pleurotum* on agar media and influence temperature on surviving *T. pleurotum* on wheat straw were investigated.







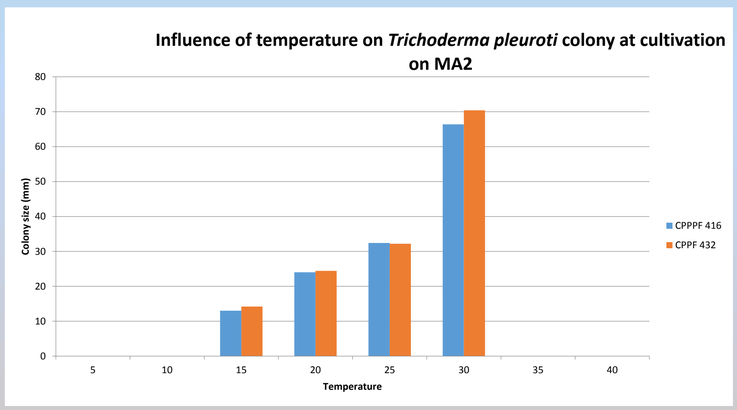
Results

There were eight oyster mushrooms farms visited and *Trichoderma* species were recorded in all of them. More than 30 *Trichoderma* strains were obtained. *T. pleurotum* was found out in seven (Blatnička, Biskupice, Bravantice, Jaroměř, Kojátky, Smržovka, Vrbno nad Lesy) of sites only. It was not find out in locality Soběslav.

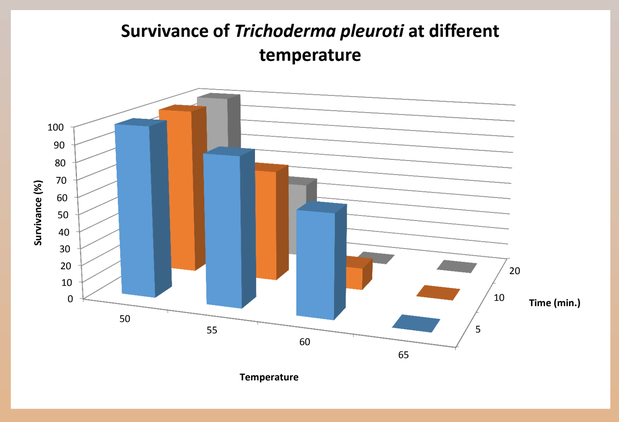




None of the evaluated strains survived of exposure to any time interval of temperature 65°C. The differences among the strains were observed at lower temperature values. Three strains were viable after exposure to 65°C for 5 minutes or and one strain was viable after exposure to 65°C for 10. None strain was viable after exposure to 65°C for 20 minutes.

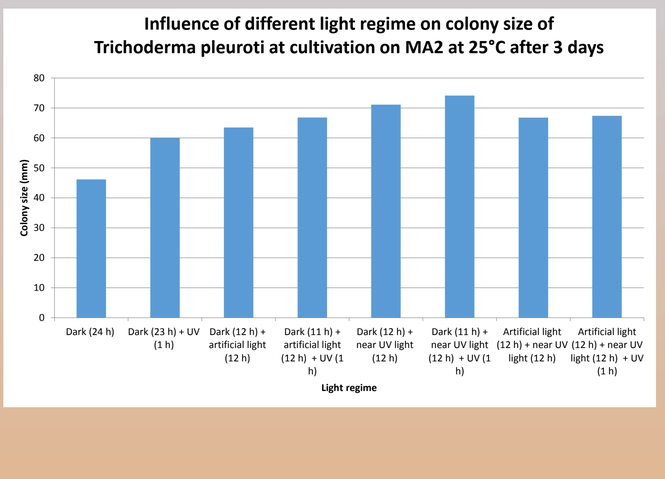


The investigated strains of T. *pleurotum* grew at temperature 15-30°C. The quickest growth was recorded at temperature 30°C. Small differences among the investigated strains were observed only.





The slowest growth of colonies of the investigated *T. pleuroti* strains was found out at cultivation at dark. The quickest growth was recorded at cultivation in regime (dark - 11 hours, near UV light (12 hours) and UV (1 hour). Artificial light and near UV light induced faster growth of mycelium of *T. pleuroti*.

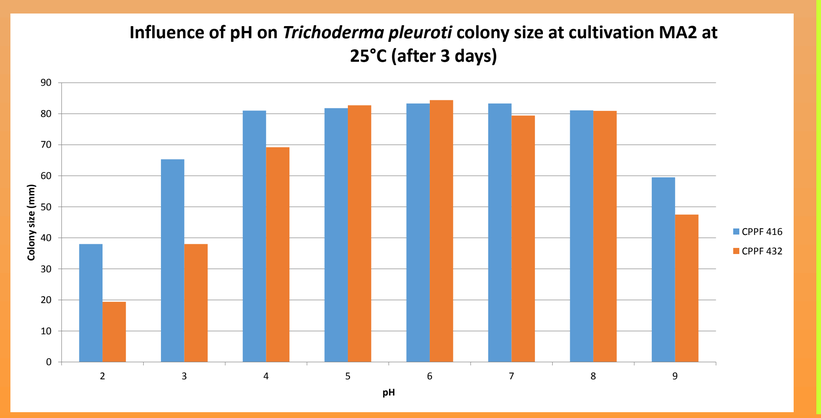


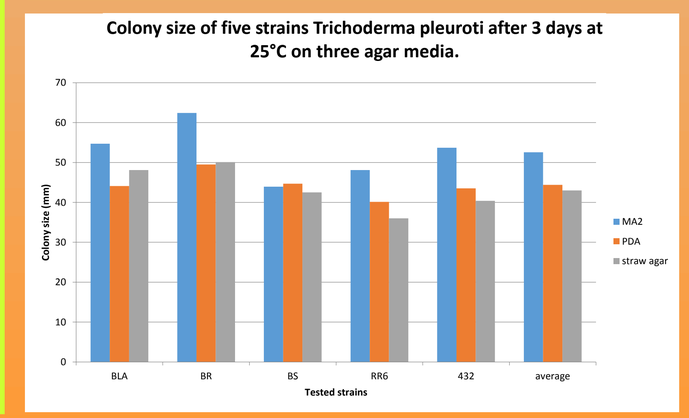
Sequencing of ITS region was used for identification on species level and until now has not be completely finished. The studied strains belong to two *Trichoderma* clade. Majority of the strains belong to to clade Harzianum (*T. pleuroti* and *T. pleuroticola* are ordered to this clade). None strain from this clade was identified in locality Soběslav. Minority of the strains belong to clade Viride. Strains from this clade were recorded in sites Soběslav, Bravantice and Jaroměř.

The strains grew most quickly on agar medium with pH 5-8. Considerably slow grow rate was found out on agar medium with pH 2, 3 and 9, but at none studied strains of pH value the growth rate of was zero. The visible differences among the strains were observed.

Morphological studies of *T. pleuroti* were carried out on potato dextrose agar and malt extract agar at 25°C . The colonies were initially white or greenish white, later turn top green. Colony reverse on 2% malt extract agar colourless. The average mycelial growth rate was 25.7 mm per day at 25. Conidiophores were hyaline, unbranched near the base, branches near the apex of conidiophore, singly, irregularly or in whorls (*Gliocladium* type). Phialides were lageniform, hyaline 6 to 8 × 2.5 to 3.5 µm. The conidia were one-celled, hyaline, ellipsoid, 2 to 3 × 3 to 4 µm, and the majority of conidia were held in heads. Chlamydospores were not observed in culture.

The quickest growth of colonies of the investigated *T. pleuroti* strains was observed on 2% malt extract agar (MA2). Growth speed on potato dextrose agar and growth speed on straw agar were similar. There were recorded differences among the strains.





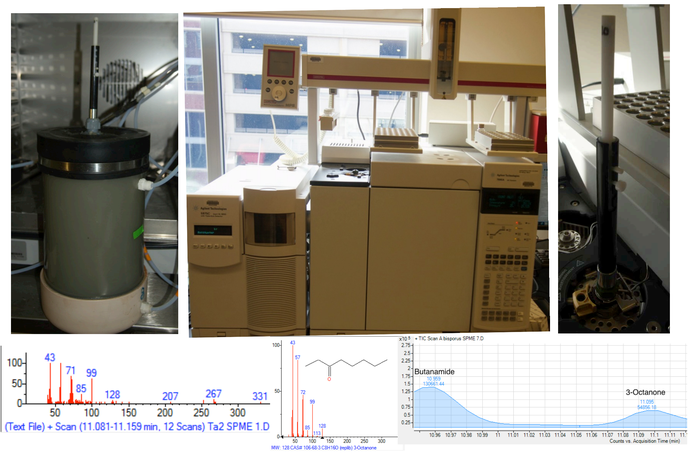
Identifying *Trichoderma aggressivum* Volatile Compounds During a Phase III Spawn Run

PENNSTATE College of Agricultural Sciences

[1]Department of Plant Pathology and Environmental Microbiology
[2]Department of Food Science

Garrett Morrison[1], David Beyer[1], Ryan Elias[2], John Pecchia[1]

Introduction



- *Trichoderma aggressivum aggressivum, (Ta2),* the aggressive "green mold" of *Agaricus*, persists despite advanced composting technologies
- Genetic approaches to detect *Ta2* in bulk substrate are inadequate and tunnel systems limit regular sampling
- Monitoring volatile compounds (VC) of phase III (P3) compost may provide an early detection method of *Ta2*

Objective

- **Identify VCs associated with *Ta2* infestation and green mold disease during phase III**
- **Calibrate a Portable-MS to target marker VCs associated with *Ta2***

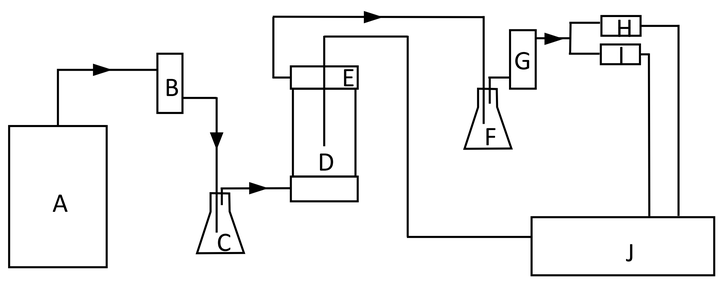
Methods

Compost and Inoculum
- Horse bedding based, finished phase II compost acquired from industry
- 'White' *A. bisporus* inoculum acquired from North American spawn supplier
- Spores from Penn State culture collection*Ta2* strain #342

Phase III Bioreactor
- Airflow maintained at rate equivalent to a phase III tunnel system
- Compost temperature maintained at 25-27°C
- Spawn was applied at 18g/kg wet compost
- Compost inoculated with 2mL of *Ta2* spore suspension (5x10^5 spores/mL)

Sampling & Analysis
- Headspace solid-phase microexactraction (SPME) fibers for VC sampling
- Sampling occurred at three-day intervals beginning day after spawning
- Volatiles were analyzed using Agilent Technologies GC-MS
- GC-MS method set to "scavenge" mode with a slow ramp speed over 36 minutes to detect identifier VCs

Phase III System



A. Air Compressor
B. Flow Restrictor
C. Humidification Flask
D. Bioreactor Vessel
E. Temperature Probe
F. Condenser Flask
G. Flow Meter
H. Oxygen Sensor
I. Carbon Dioxide Sensor
J. Data Logger

Results

Figure 1 A. B. *Agaricus bisporus* finished phase III after 14 days showing healthy surface growth and fully colonized substrate. **C**. *Ta2* infected phase III compost after 14 days. Spore maturity and greening occurred in only 1/5 replicates. Note the lack of mycelium and dark coloration. **D.** Bioreactor parameters of treatment's mean compost temperature, oxygen and carbon dioxide at day 6-7 after spawning. Air temperature and airflow within vessels maintained near P3 tunnel parameters.

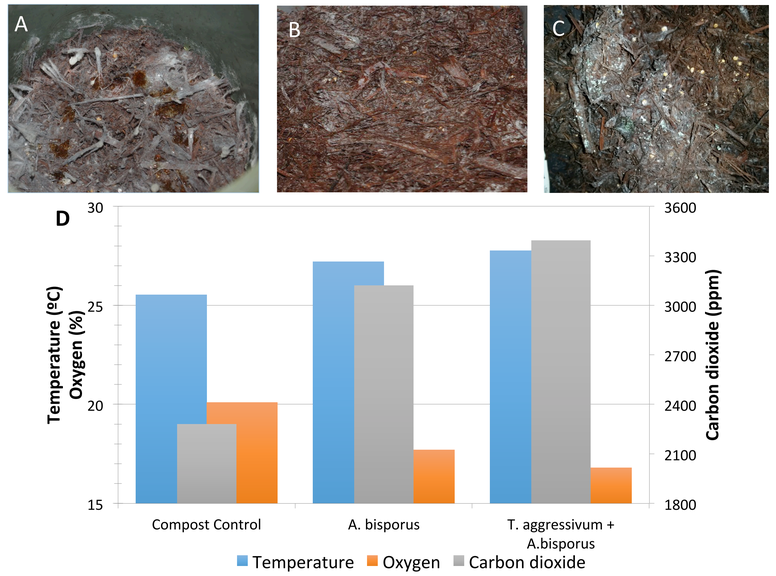
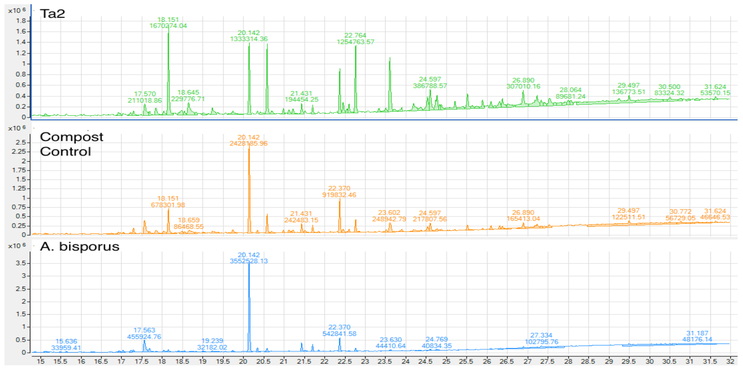


Table 1. Subset of GC-MS results acquired from NIST library showing unique and shared VCs across treatments. Compounds below were sampled on spawn run day 6. Area % based on total VC abundance.

Treatment	RT (minutes)	% Area	NIST Library Compound Name	% Match
Compost	14.5405	0.5729	Decanal	91
A.bisporus	14.5405	0.4418	Decanal	91
Ta2	14.5405	0.4548	Decanal	90
Compost	11.5177	0.1435	D-Limonene	95
A.bisporus	11.5177	0.3805	D-Limonene	98
Ta2	11.5176	0.2584	D-Limonene	98
Compost	19.0029	0.3519	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	94
A.bisporus	19.01	0.6842	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	93
Ta2	19.0173	0.3437	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	92
Compost	13.0363	2.9048	Nonanal	91
A.bisporus	13.036	4.6515	Nonanal	91
Ta2	13.0433	2.2458	Nonanal	91
A.bisporus	9.9631	0.2741	Bicyclo[4.2.0]octa-1,3,5-triene	93
Ta2	9.9704	0.2478	Bicyclo[4.2.0]octa-1,3,5-triene	87
A.bisporus	16.7465	0.1957	.alpha.-Copaene	78
Ta2	16.7466	0.1269	Copaene	93
A.bisporus	11.0951	0.7077	3-Octanone	91
Ta2	11.1022	0.8044	3-Octanone	91
Ta2	29.4968	1.256	1,10b(2H)-Dihydropyrano[3,4,5-jk]fluorene	90
Ta2	18.0361	0.6084	1,3,5,7-Cyclooctatetraene	76
Ta2	16.0734	0.1603	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	98
Ta2	24.1891	0.9807	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	93
Ta2	9.9777	0.1424	Phthalic acid, di(2-propylpentyl) ester	93
Ta2	9.6409	0.3204	n-Tridecan-1-ol	97

Discussion

Figure 2. Agilent MassHunter Qualitative Analysis chromatographic results from samples taken on spawn run day 6. Horizontal axis represents retention time (minutes), vertical axis represents abundance while x-axis represents abundance.



- Similarities between treatments require targeting of 150-300g/mol compounds using selective-ion monitoring (SIM)
- Eliminating manure, a 100% synthetic N based compost may reduce interference for higher precision on targeted compounds
- Differentiation between *A. bisporus* and *Ta2* VCs detected as early as spawn run day 6.

Future

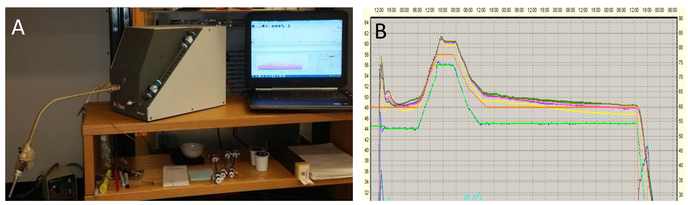


Figure 3. Incorporating VC analysis may provide insight to activities within a tunnel **A.**Portable-MS requires less training and inexpensive than GC-MS, able to perform constant monitoring from multiple sources. **B.** Phase II tunnel temperature profile.

Optimize a dedicated VC monitoring system, a Portable-MS, to identify *T. aggressivum* in a phase III tunnel system

Monitor phase II activity to identify and attempt to correlate VCs to inadequate pasteurization and poor conditioning

Are volatiles produced by *T. aggressivum* inhibitory to *A. bisporus*?

What is the impact of repurposing phase III *T. aggressivum* infested compost for use in phase I and phase II?

Acknowledgements

This research project is supported by the Specialty Crop Research Initiative of USDA's National Institute of Food and Agriculture
Special thanks to Penn State's Dept. of Plant Pathology and Environmental Microbiology, Food Sciences, and everyone at the Mushroom Research Center

USDA NIFA

Insecticidal Activity of Two Plant Extracts against *Lycoriella* sp.(Diptera: Sciaridae) in Button Mushroom Cultivation

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The sciarid mushroom fly *Lycoriella* sp.(Diptera: Sciaridae) is one of the most common and serious pests of commercial production of button mushroom[*Agaricus bisporus* (Lange) Imbach] in Korea. The present study investigated insecticidal activity of two plant extracts against *Lycoriella* sp. larvae. The extract parts of two plants tested were fruit of *Ginko biloba* and root of *Phytolacca americana*. The extracts of two plants was found to have an high insecticidal effect on the larvae of *Lycoriella* sp. and no inhibitory effect on the mycelial growth of *A. bisporus* at 1% concentration. They were applied at spawn run, after casing soil, and pinning with 2.5L/m^2 each time and compared with diflubenzuron and water-treated control. Insecticidal effect of *Ginko biloba* fruit and *Phytolacca americana* root on adult mushroom flies were 77.2 and 78.3%, respectively. Fruiting body damages of *A. bisporus* by mushroom fly larvae in the treatments of *Ginko biloba* fruit and *Phytolacca americana* root were 13.5 and 12.7%, respectively compared to water-treated control. Treatments with *Ginko biloba* fruit and *Phytolacca americana* root resulted in 13~14% significantly higher fruiting body yield of *A. bisporus* compared to to water-treated control. The results suggest that both plant extracts tested may have a potential as natural insecticides for the control of *Lycoriella* sp. mushroom flies in environment friendly cultivation.

▣ Materials and Methods

◈ Preparation of plant extracts

Ethanol extracts of *Ginkgo biloba* fruits(seedcoat) and *Phytolacca americana* root was tested for insecticidal activities against sciarid mushroom fly *Lycoriella* sp. The seeds of *Ginkgo biloba* fruits were removed and *Phytolacca americana* roots were cut into small segments and then they were dried at 60℃ for 3 hours in a dry oven. For ethanol extraction 100g of each sample were placed in the 500ml of 70% ethanol at room temperature for 1 month. The solution was filtered with Whatman filter paper No. 2 and the extracts were stored at 4℃ until used for the experiments.

◈ Mushroom fly culture

Adult *Lycoriella* flies were collected by use of aspirators from commercial button mushroom growing rooms in Buyeo district, Korea and then they were transferred and reared on mycelial culture in Petri dishes according to Lee et al.(2015). Hatched larvae were reared on *A. bisporus* mycelia grown on PDA medium until emergence of second and third instar larvae of *Lycoriella* sp. and then they were treated with four different concentrations of ethanol extracts by topical application. The number of dead larvae were checked for 3 days. .

◈ Application of plant extracts

Mycelial cultures of *A. bisporus* 'Seolgang' were transferred to 450g of sterile cooked wheat grain, buffered with 5% $CaCO_3$. Colonized grains (40g) were used to inoculate plastic container (57×41×18cm) containing a complex biologically modified, straw-based substrate. Pasteurized soil was used as the casing material. The plant extracts at 1% concentration were applied at spawn run, after casing soil, and pinning with 2.5L/m^2 each time and compared with diflubenzuron and water-treated control. In positive control, diflubenzuron were tested at 0.16% concentration of 2.5L/m^2 after casing soil treatment.

◈ Data analysis

Insecticidal effect for the each treatment was calculated by using the formula as follows. Insecticidal effect(%)= [(A-B)/A]×100, where, A= total number of emerging adults in the negative control and B= total number of emerging adults in the each treatments. The percentage of damaged fruiting body was determined by using the formula as follows. Fruiting body damage(%) = (no. of fruiting body damaged/ total no. of fruiting body) x 100. The means were separated and analyzed statistically by Duncan's multiple range test at P≤0.05 level.

▣ Results

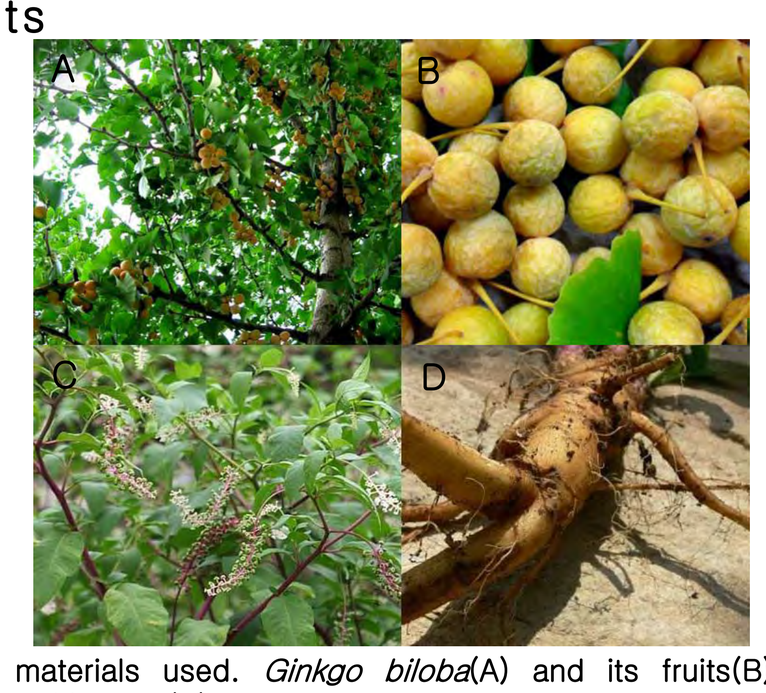


Fig 1. Plant materials used. *Ginkgo biloba*(A) and its fruits(B), *Phytolacca americana*(C) and its root(B)

Table 1. Insecticidal effect of plant extracts in the mortality of mushroom fly, *Lycoriella* sp. larvae at different concentrations

Concentration (%)	*Ginkgo biloba* fruit (%)	*Phytolacca americana* root (%)
0.01	8.2	7.1
0.1	21.1	19.4
1	66.5	69.4
10	87.3	94.8

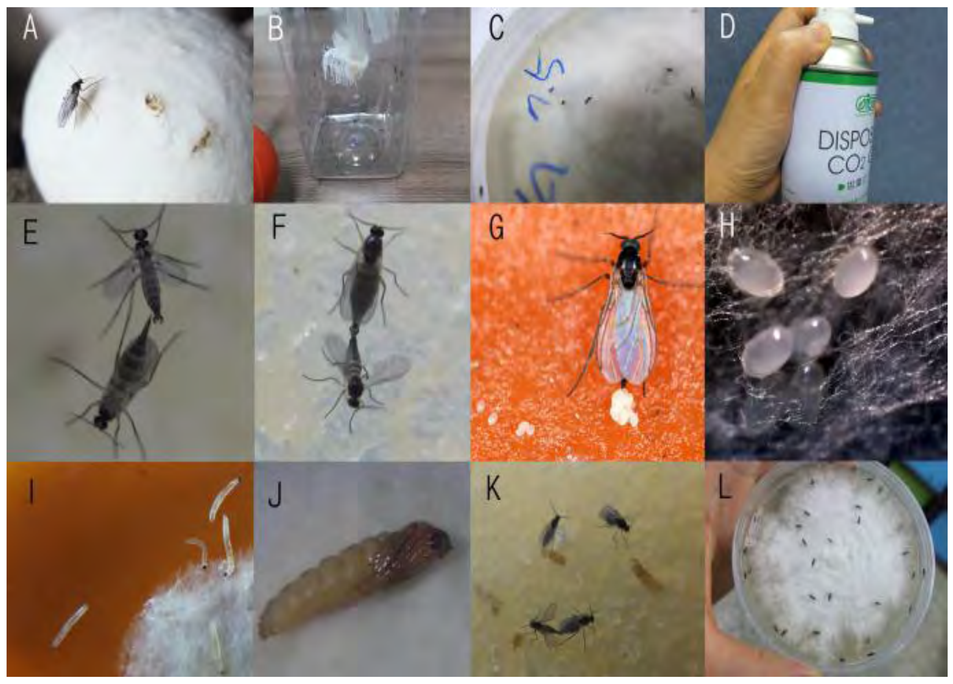


Fig. 2. Rearing and the life cycle of mushroom fly, *Lycoriella* sp. A; adult flies on *A. bisporus* fruiting body. B; captured adult flies with aspirator, C; transferred adult flies to Petri dish, D; immobilization with carbon dioxide, E, male and female adult flies trying to mate, F; mating, G; ovipositoin, H; eggs, I; larvae; J; pupa, K; exuviae and adult flies , L; 2nd generation adult flies..

Table 2. Mycelial growth of *A. bisporus* at different concentrations of plant extracts

Concentration (%)	*Ginkgo biloba* fruit (mm/day)	*Phytolacca americana* root (mm/day)
0.01	3.4 a	3.5 a
0.1	3.3 a	3.4 a
1	3.2 a	3.1 ab
10	2.5 b	2.7 b

[a]Means within a column followed by the same letter are not significantly different (DMRT, P≤0.05)

Table 3. Insecticidal effect in the reduction of adult mushroom fly, *Lycoriella* sp. and fruiting body damages of *A. bisporus* in the treatments of plant extracts

Treatments	Insecticidal effect (%)	Fruiting body damage (%)
Ginkgo biloba fruit	77.2	13.5
Phytolacca americana root	78.3	12.7
Diflubenzuron	72.7	14.2
Tap water	0.0	27.4

Table 4. Fruiting body yield of *A. bisporus* in the treatments of plant extracts

Treatments	Total yield (kg/container)	Yield index (%)
Ginkgo biloba fruit	2.14 a	114
Phytolacca americana root	2.11 a	113
Diflubenzuron	2.03 a	109
Tap water	1.87 b	100

[a]Means within a column followed by the same letter are not significantly different (DMRT, P≤0.05)

▣ References

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Session Substrate and composting

THE EFFECT OF DIFFERENT SUBSTRATES and DISINFECTION TREATMENTS ON YIELD IN OYSTER MUSHROOM (*Pleurotus ostreatus*) CULTIVATION

Hatice KURTCEPHE **Ersin POLAT**

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Introduce

The aim of this study was to investigate the effect of different substrates and disinfection treatments on yield and quality in oyster mushroom (*Pleurotus ostreatus*) cultivation.

Material and method

The study carried out in a controlled-mushroom production room, which is located at Akdeniz University. The spawns(strain: HK35) of mushroom were inoculated on **W**heat **S**traw + wheat **B**ran (2:1), wheat straw + wheat bran + **C**arob **P**ulp (2:1:0.5) and wheat straw + wheat bran +carob pulp (2:1:1) as substrates. These substrates were treatmented by %1, %2, %3 of sodium hypochlorite (SH) solution, and the control was sterilized by autoclave. Mycelium growing duration, total yield, biological efficiencies and some physical charecteristics of oyster mushroom were determined.





The composition of the Carob pulp	Value
Dry matter (%)	84.5
Raw protein (%)	4.4
Raw cellulose (%)	7.3
Raw lipid (%)	0.4
Ash (%)	3.0
Starch (%)	0.6
Total sugar (%)	39.8
Calcium (g/kg)	4.3
Phosphorus (g/kg)	0.9
Magnesium (g/kg)	0.5



Results

The fastest mycelium growing durations (35 days) and the highest total yield (154.23 g/kg bag) and also the best biological efficiencies (%69.70) were obtained from wheat straw + wheat barn (2:1) with autoclaved treatment. The highest cap size and stem length of oyster mushroom were obtained from control substrate (7.42 cm and 2.66 cm) respectively, However when carob pulp added into the subsrate, cap size increased and also stem of the mushroom become thick. The ratio of mycelium development in substrate treatmented by sodium hypochlorite was about %50, but mushroom yield wasn't obtained from sodium hypochlorite treatment, except %1 of SH concentration.

pH and humidity values of substrates after treatments

SUBSTRATES	Disinfection	pH	Humidity(%)
2WS + B (Control)	Autoclave	6.5	75.86
2WS + B + ½CP		6.2	72.00
2WS + B + CP		6.0	71.50
2WS + B (Control)	%1 SH	7.0	79.60
2WS + B + ½CP		5.1	78.03
2WS + B + CP		4.9	73.94
2WS + B (Control)	%2 SH	6.7	77.60
2WS + B + ½CP		5.7	76.58
2WS + B + CP		5.4	70.77
2WS + B (Control)	%3 SH	6.6	76.22
2WS + B + ½CP		6.0	72.97
2WS + B + CP		4.8	72.63

Durations of mycelium growing (day)

SUBSTRATES	Disinfection treatments			
	Autoclave	%1 SH	%2 SH	%3 SH
2WS + B (Control)	35	55	61	66
2WS + B + ½CP	42	59	63	75
2WS + B + CP	48	64	74	82

Effects of treatments on yield and BE

SUBSTRATES	Total yield (g/kg bag)				Biological efficiency(BE) (%)			
	Autoclave	%1 SH	%2 SH	%3 SH	Otoklav	%1 SH	%2 SH	%3 SH
2WS + B (Control)	154.24 a	–	–	–	69.71 a	–	–	–
2WS + B + ½CP	134.06 ab	–	–	–	48.80 b	–	–	–
2WS + B + CP	119.54 b	–	–	–	35.77 c	–	–	–

Effects of substrates on yield of mushroom

SUBSTRATES	Yield (g/kg bag)			
	15 days	30 days	45 days	Total yield
2WS + B (Control)	64.31	61.27 a	25.86	154.24 a
2WS + B + ½CP	63.74	39.96 b	29.56	134.06 ab
2WS + B + CP	58.55	36.48 b	24.51	119.54 b

Effects of of treatments on some physical propertites of mushroom

SUBSTRATES	Cap size(cm)	Diameter of stem (cm)	Length of stem (cm)
2WS + B (Control)	7.42	0.96 a	2.66
2WS + B + ½CP	5.89	0.62 b	2.08
2WS + B + CP	7.33	1.02 a	2.27





Wheat Straw + wheat Bran (2:1)


Wheat Straw+Wheat Bran+Carob Pulp (2:1:0.5)


Wheat Straw + wheat Bran +Carob Pulp (2:1:1)



ISMS Congress in Amsterdam, The Netherlands, 29th May - 2nd June 2016

Cellulases production by a White Rot Fungus using grape pulp as substrate

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[2] Laboratoire de Microbiologie Industrielle, Université de Reims, France

INTRODUCTION

The solid state fermentation (SSF) process is one of the most applied in industry with low investment and operation costs. This has made it an attractive alternative for the production of cellulases, the enzymes that break down cellulose, which are widely used in the textile industry, in detergents, pulp and paper industry, improving digestibility of animal feeds, in food industry, and the enzymes account for a significant share of the world enzyme market. However, the most promising use in the current economic climate seems to be the production of alternative fuels. Production of cellulases using agricultural residues or wastes is one of the most efficient biological ways by which these residues can be recycled using a value added process for conversion of these materials into renewable chemicals and fuels.

OBJECTIVE

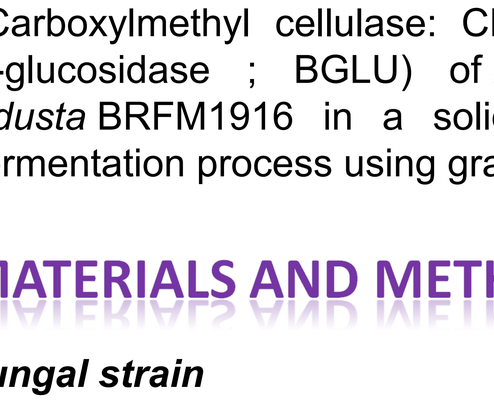
The purpose of this work is to evaluate the growth and cellulases production (Carboxylmethyl cellulase: CMCase and β-glucosidase ; BGLU) of *Bjerkandera adusta* BRFM1916 in a solid substrate fermentation process using grape pulp.

MATERIALS AND METHODS

Fungal strain

- Isolation : Yakourene forest (Tizi-Ouzou, Algeria)
- Authentified and Deposited in CIRM- Marseille, France as

Bjerkandera adusta **BRFM1916**



Subculture
(PDA medium 28°C/
10 days)

Production of Cellulases by Solid Fermentation

SUBSTRATE PREPARATION

(45 g moistened with distilled water)
(121 °C /20 min)



INOCULUM

20 agar discs with mycelia
Nutritive medium
(Elisashvili et *al*.,2008)
28°C / 5 days



SUBSTRATE INOCULATION
(12 ml inoculum
28°C / 20 days)



FERMENTED SUBSTRATE



ENZYME EXTRACTION

Mixing of 50 ml of distilled water and 10 g of the fermented substrate

↓

Centrifugation (10,000 rpm/ 30 min/4 °C)

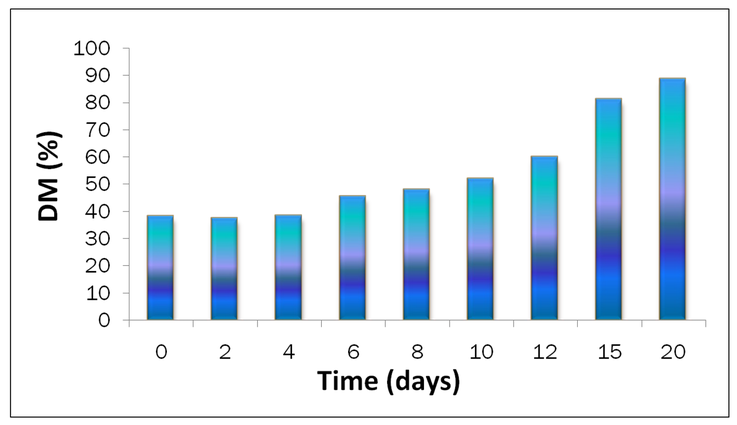
The supernatant = crude enzyme extract

Determination of enzyme activities

CMCase (Ghosse,1984)

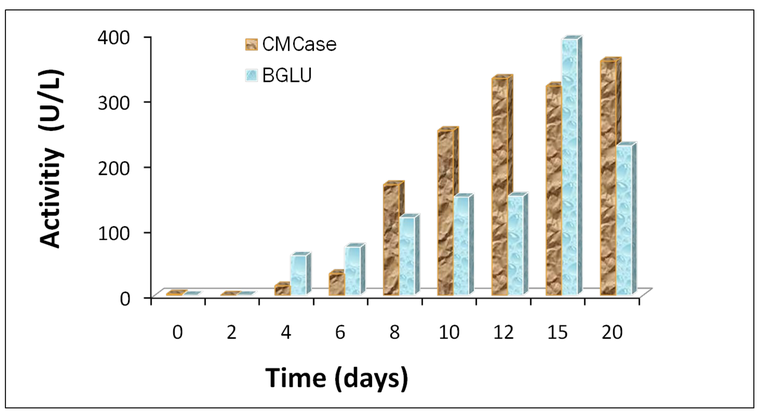
BGLU (Norkrans, 1957)

RESULTS



Dry Matter evolution

Dry matter was increased gradually during the fermentation from 38 % at 88.39 % at the end of SSF .



Enzyme activities

During the growth of the strain the endoglucanase secretion was started after the 4th day of fermentation and the highest activity was found on the 20 th day (358 U/L).The production of β-glucosidase was observed after the 2d day of culture for reaching a maximal activity of 390 U / L in 15 days.

CONCLUSION

Based on the obtained results, it is concluded that grape pulp can be used and valorized for the cellulases production by *Bjerkandera adusta* under SSF.

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The circulation agricultural technology of utilizing by *Agaricus blezei*

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Division of Agriculture Environment Research, Gyeongsangbuk-do Agricultural Research and Extention Services, Daegu, Republic of Korea

Abstract

In korea, *Cucumis melo* var. *makuwa* (=Oriental water melon) belongs to the major fresh fruit produced from February, and 2015 year's total output was $600 million. Growing season of oriental water melon are usually from December to July. After oriental water melon cultivation , many farmers generally choose a sesame or pepper. After second crop cultivation, farmer have to supply of fertilizers for next year's the melons growing. *Agaricus blezei* are thermophilic mushroom than other well known mushroom. Our research team try to grow *Agaricus blezei* as second crop which is more sustainable and environmentally cultivation system than other vegetables. *Agaricus blezei*'s cultivation facilities are simple greenhouse which was 8M width, 50M length, and plastic vinyl coated on steel frame for growing oriental water melon. For grow a mushroom in that green house, we added only 2 facilities. First, Light-blocking film was installed to create an environment scattered light and to let down temperature. Second, Irrigation system was installed at the upper of greenhouse to make a lower temperature. As a result, it was possible to adjust that the illumination is from 30 to 350 lux and the temperature is under 30 degree except august. After preparing the these facilities, Mix of bagasse and chicken manure were fermentation at outdoor during 30 days. Compost fermentation was processed during 10 days. After fermentation, a medium was transferred to greenhouse, and spawn inoculation, spawn running, casing, watering were processed from May 20. First mushroom were harvested from 29 June. 23kg/3.3m2 of mushrooms could be harvested during four months. In particular, Because of the high temperature of August, mushroom's harvest amount was reduced. Morphological characteristics of *Agaricus blezei* was so different from indoor cultivation product, but mushrooms quality was generally excellent. Spent mushroom compost's physicochemical characteristics was analyzed for cultivation of oriental water melons. In pre-harvest, total nitrogen content of compost was 2.1%. After harvest's total nitrogen content of compost was 1.5%. In nutritional terms of *Agaricus blezei*'s SMC will be a good material for oriental water melon cultivation. Using SMC on oriental water melon was currently in progress, and we knew some problems that compost's amount is too much for oriental water melon's fertilizer. Our research team will solve these problem and find a better compost material for these two crop combination.

* keyword : *Agaricus blezei*, Oriental water melon, *Cucumis melo* var. *makuwa*, crop rotation

Methods & Results

Crop rotation system and additional facilities at green house

A. Crop rotation system of *Cucumis melo* var. *makuwa* and *Agaricus blezei* in green house

Crops	Cultivation of *Cucumis melo* var. *makuwa*							Cultivation of *Agaricus blezei*					
Month	Nov	Dec	Jan	Feb	Mar	Apr	May	May	Jun	Jul	Aug	Sep	Oct
Period	7 Months							6 Months					
Stage	Seeding	Seedling	Fruiting	Fruit growth	Harvest			Spawning Incutationg	Casing Pinning	Harvest			
Photo													

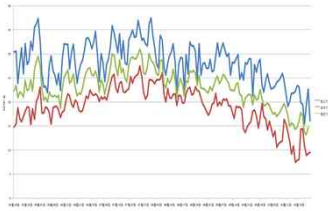
B. Crop rotation system of *Cucumis melo* var. *makuwa* and *Agaricus blezei* in green house



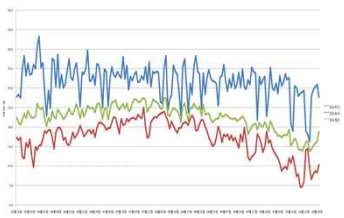


Analysis on cultivation environment of *Agaricus blezei*

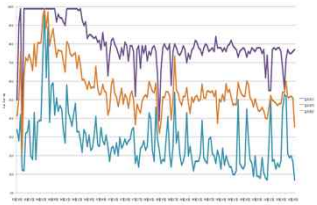
A. Temperatures in out-side of green house (Blue – Highest, Green – Avr, Red – Lowest)



B. Temperature in green house (Blue – Highest, Green – Avr, Red – Lowest)



C. Humidity in green house (Purple – Highest, Orange – Avr, Blue – Lowest)



- Most of environment were variable and different from general mushroom far, but *Agaricus blezei* grow well except early of August because of high temperature.
- Cucumis melo var. makuwa and *Agaricus blezei* were powerful crop rotation combinations that give to farmer an economic income and a biofertilizer.

D. Lux in case of different weather conditions

Weather condition	Out-side	In-side
Cloudy	43,000 lux	30～100 lux
Cleany	105,000 lux	300～350 lux

E. Physicochemical properties of mushroom compost for *Agaricus blezei*

T-N (%)	T-C (%)	T-S (%)	P2O5 (%)	K2O (%)	CaO (%)	MgO (%)	Water (%)	pH (1:5)
2.15	35.98	0.97	0.91	1.95	4.63	0.71	72.22	7.4

Summary of green house cultivation processing of *Agaricus blezei*

A. Photo of *Agaricus blezei* cultivation processing in green house

Phase1 (20 Apr ~ 10 May)	Phase2 (11 May ~17 May)	Spawn running (18 May~7 Jul)
		
Casing (7 Jul)	**Pin heading (15 Jul~22 Jul)**	**Harvest (29 Jul ~ 26 Oct)**
		

B. Analysis of harvest data during 4 months

Harvest Period	1st Period (kg)	2nd Period (kg)	3rdPeriod (kg)	4th Period (kg)	5th Period (kg)	6th Period (kg)	Total (kg)
Harvest Days	29 Jun ~5 Jul	12 Jul ~17 Jul	28 Jul ~1 Aug	18 Aug ~22 Aug	1. Sep ~10 Sep	16 Sep ~23 Sep	
Yield (kg)	180	150	50	100	80	60	620 /87.3m²
Variation (%)	0	-17%	-67%	200%	-20%	-25%	
Yield/ 3.3㎡(kg)	6.8	5.6	1.9	3.7	3.0	2.2	23.4

C. Analysis of economic report between two crops.

Crops	*Cucumis melo* var. *makuwa* (A)	*Agaricus blezei* (B)
Total income (C)	$8,556/10a	$36,756/10a
	Data Source	Output statement
	From 2013 RDA in korea Information of agricultural income	• Harvest amount : 3,030kg/10a - 6.06kg/㎡ x 500㎡=3,030kg • Total income : $36,756/10a - $12.1 x 3,030kg=$36,756
Farm costs (D)	$3,170/10a	$14,070/10a
	Data Source	Output statement
	From 2013 RDA in korea Information of agricultural income	• Compost : $7,278 ($14.5/㎡ x 500㎡) • Casing : $2,426 ($4.85/㎡ x 500㎡) • Spawn : $606 ($1.21/㎡ x 500㎡) • Light blocking film : $323 • Sprinklers : $606 ($60.6/10m x 100m) • Employment and labor : $2,426 (300hour) • Electricity bill : $404
Income (C-D)	$5,391/10a	$22,685/10a
Rate (%)	100	421

☆ Question & Answer : ilovegeoje@korea.kr

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ISMS Congress 19th International Society for Mushroom Science
Amsterdam 2016 29th May - 2nd June

MICONET s.r.l.
ACADEMIC SPIN-OFF
Pavia University

Different approaches for *Hericium erinaceus* spawn in the perspective of gluten free products

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Background - Gluten-related disorders have becoming a problem for many people which can suffer of Celiac Disease (CD), Wheat Allergy (WA) or Non-Celiac Gluten Sensitivity (NCGS). The last one is still an undefined syndrome with several unsettled issues despite the increasing awareness of its existence. These patients have to avoid to consume wheat, barley and rye grains (Elli et al., 2015).

Dietary supplements based on medicinal mushrooms are commonly prepared also using grain spawn, mainly with wheat or rye grain.


Picture by E. Savino

Hericium erinaceus (Bull.) Pers. in Asia has a long history of use to treat various human diseases. It has been adduced for a variety of physiological effects, including anti-aging, anti-cancer, anti-gastritis, anti-inflammatory and anti-metabolic disease properties (Ma et al., 2010; Khan et al., 2013). Nowadays the main interest regards its role as adaptogen for brain and nerves. Hence, it is an attractive target resource for developing functional foods or dietary supplements. As it **could be used also by people with gluten-related disorders, it is becoming of great importance to have gluten free products too** (Alvarez-Jubete et al., 2010; Amboni et al., 2013)**.**

THE EFFICACY OF *H. ERINACEUS* GROWTH ON SPAWN PREPARED WITH GLUTEN FREE GRAINS WAS TESTED.

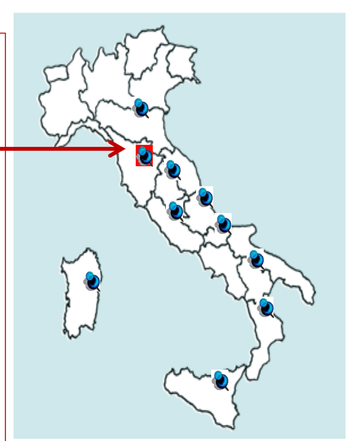
1) **ISOLATION OF *H. ERINACEUS* STRAIN.** *H.e.* basidioma was collected in Centre of Italy (Siena province) on a living tree of *Ilex aquifolia.*

From field to laboratory:





H. erinaceus STRAIN
(pure culture on 2% MEA)



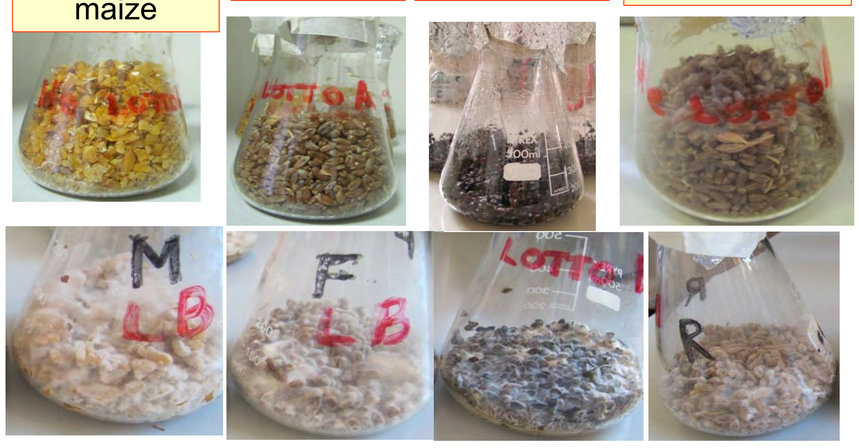
2) The *H. erinaceus* strain (1.2.H.e.1) has been included into the Culture Collection of Pavia University. (MicUNIPV) and it is owned by Miconet S.r.l. It was maintained on 2% Malt Extract Agar (MEA) slant at 4° C.

To our knowledge, IT SHOULD BE THE FIRST ITALIAN STRAIN.

The spawn bags were prepared according to the most common procedures and **the following grains were tested: wheat (gluten +); brown rice and maize (gluten-free); buckwheat (*Fagopyrum esculentum*, fam. *Polygonaceae*, gluten-free).**
Yet well-known because its grain-like seeds, in Italy buckwheat is cultivated in Valtellina (Central Alps) only.
Buckwheat is regarded as functional food and it is also proved to contain medicinal compounds (Zhou et al., 2015)

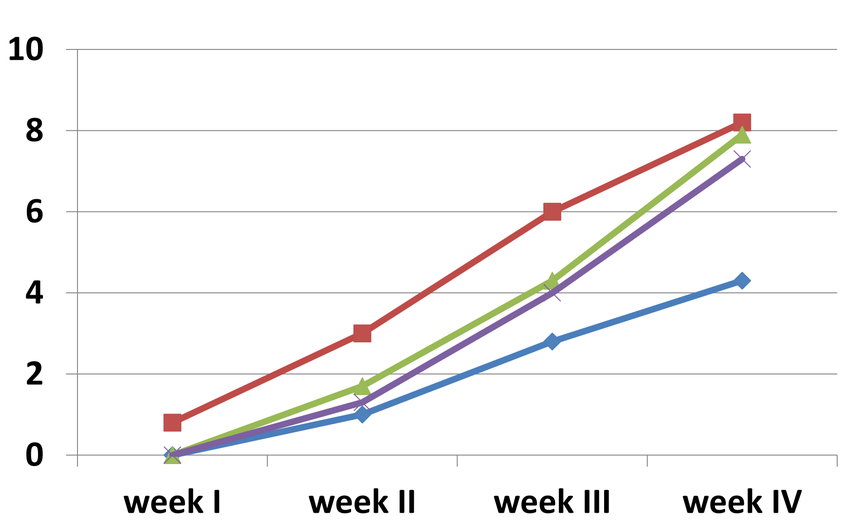


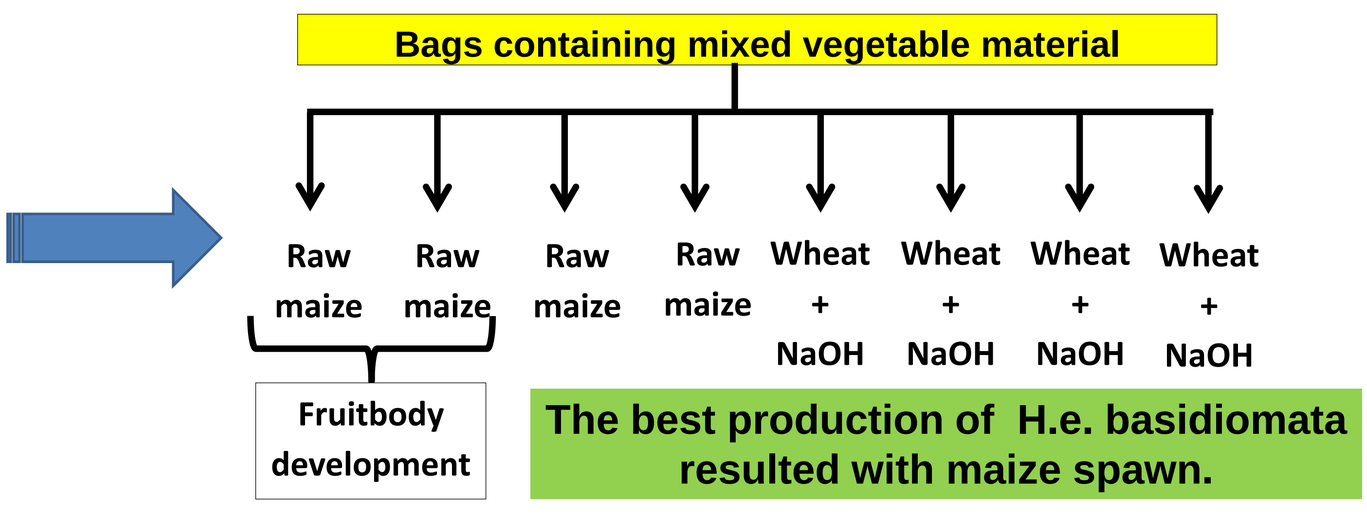

chopped maize | wheat | buckwheat | rice



The **growth of *H. erinaceus*** was obtained **in all the different spawn bags**, however a **faster** result was highlighted with **chopped maize and wheat grains**.
Therefore, these two spawns were selected to cultivate *H. erinaceus*;

The best performing spawns were used as inoculum for the cultivation of *H. erinaceus* basidiomata.
Beside, half spawn (chopped maize and wheat grains) were treated with NaOH 0,1 M 24 h instead of pure water.





The best production of H.e. basidiomata resulted with maize spawn.

Conclusions - *H. erinaceus* dietary supplements prepared using **maize grain spawn** can be an effective alternative, useful to let a wider consumption of this medicinal mushroom. Buckwheat could also be used to increase functional properties.

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OPTIMIZATION OF *GANODERMA LUCIDUM* CULTIVATION AND COMPARISION OF BIOACTIVE COMPOUNDS IN FRUITING BODIES, SPORES AND MYCELIUM

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INTRODUCTION: *Ganoderma lucidum* has been traditionally used in Chinese medicine for the prevention and treatment of various diseases. The major bioactive components extracted from their fruiting body, mycelium or spores include polysaccharides and triterpenoids. These compounds are of great interest for their pharmacological effects, such as immunomodulation, anti-cancer or anti-diabetic activity. Traditionally, bioactive components have been extracted from fruiting bodies. Therefore, to obtain a satisfactory yield of mushrooms, it is essential to optimize growing conditions and substrate formulation. Due to the long time required for fruiting body growing, research with new alternatives such as using liquid medium culture for the production of mycelia biomass and bioactive compounds is been conducted.

OBJECTIVE: The objective was to evaluate the optimum combination between three strains with different substrates formulations in terms of cultivate yields and content of glucans and dietary fibre. Also, comparison between bioactive compounds production obtained from mycelium, spores and fruiting body cultivation was established.

METHODS

The cultivation experiments were carried out in the facilities of CTICH in La Rioja. Three strains of Ganoderma lucidum (S1, S2 and S3) had been cultivated on three different substrates mixtures (G1, G2 and G3) varying percentages of raw materials. Once the mushrooms achieved the mature state they were harvested, and production yield and biological efficiency were calculated. The mycelium of Ganoderma lucidum was grown in liquid medium in a shaking incubator at 30˚C and 160rpm. Then, fruiting bodies, spores and mycelium were freeze-dried, powdered and analyse for glucan content and dietary fibre in triplicate. Glucans content was determined spectrophotometrically using a β-glucan kit (Megazyme, Ireland). Total, insoluble and soluble dietary fibre were measured according to AOAC methodology (991.43).

3 Strains of *G. lucidum*

3 Substrate mixtures			
	S1G1	S2G1	S3G1
	S1G2	S2G2	S3G2
	S1G3	S2G3	S3G3

→ **FRUIT BODIES SPORES MYCELIUM** →

- **PRODUCTION YIELD**
- **BIOLOGICAL EFFICIENCY**
- **GLUCANS CONTENT**
- **DIETARY FIBRE**

RESULTS AND DISCUSSION

The cultivation experiments were carried out in the facilities of CTICH in La Rioja. Three strains of *G.lucidum* (S1, S2 and S3), showed in Figure 1, were cultivated on three different substrates mixtures (G1, G2 and G3) varying their percentages of raw materials: oak sawdust, wheat straw, wheat bran, crushed corn and oat grain, calcium carbonate and oil seed supplement. Once the mushrooms achieved the mature state they were harvested, and production yield and biological efficiency were calculated.

Significant differences were found in yield production between the combinations of the three substrates and strains. A wide range in the results was observed (from 0.3 to 4.3%). It is possible to increase 150% total yield depending on the strain and substrate used. S3 cultivated on substrate G2 and G3 achieved the highest yields. It is essential to find an optimal substrate-strain combination to obtain the highest *G.lucidum* production.

Fruiting bodies were harvested from the combination of three strains (S1, S2 and S3) and three substrates (G1, G2 and G3) and spores were collected from them. Mycelium of *G. lucidum* was grown in liquid medium in a shaking incubator at 30˚C and 160rpm.

Table 1. Composition of glucans and dietary fibre in three strains of *Ganoderma lucidum* (S1, S2 and S3) cultivated on three different substrates mixtures (G1, G2 and G3), spore and mycelium (% dry matter). [a)]

	IDF	SDF	TDF	α-glucans	β-glucans	Total Glucans
S1G1	80.78 ± 0.38^{a*}	2.25 ± 0.10^{a*}	83.01 ± 0.42^{a*}	0.37 ± 0.01^{a*}	19.69 ± 0.24^{a}	20.06 ± 0.24^{a*}
S1G2	68.61 ± 0.26^{b*}	3.50 ± 0.11^{b}	72.11 ± 0.30^{b*}	3.03 ± 0.16^{b*}	16.20 ± 0.71^{a*}	19.23 ± 0.59^{a*}
S1G3	65.63 ± 0.24^{c*}	2.77 ± 0.41^{ab}	68.39 ± 0.20^{c*}	5.01 ± 0.03^{c*}	18.13 ± 0.31^{b*}	23.14 ± 0.32^{b*}
S2G1	67.69 ± 0.14^{a†}	4.31 ± 0.33^{a†}	72.00 ± 0.46^{a†}	0.66 ± 0.02^{a†}	18.28 ± 0.04a	18.92 ± 0.02^{a†}
S2G2	89.56 ± 0.17^{b†}	3.79 ± 0.15^{a}	93.35 ± 0.31^{b†}	1.35 ± 0.01^{b†}	22.42 ± 0.35^{b†}	23.77 ± 0.34^{b†}
S2G3	80.81 ± 0.28^{c†}	1.72 ± 0.57^{b}	82.53 ± 0.86^{c†}	1.91 ± 0.01^{c†}	22.29 ± 0.36 ^{b†}	24.20 ± 0.36^{b*†}
S3G1	77.91 ± 0.20$^{a\delta}$	2.67 ± 0.28^{a*}	80.58 ± 0.32$^{a\delta}$	0.43 ± 0.02^{a*}	19.37 ± 0.09^{a}	19.79 ± 0.11^{a}*
S3G2	83.51 ± 0.40$^{b\delta}$	3.68 ± 0.22^{b}	87.19 ± 0.27$^{b\delta}$	2.36 ± 0.02$^{b\delta}$	23.76 ± 0.59^{b†}	26.11 ± 0.57$^{b\delta}$
S3G3	78.40 ± 0.73$^{a\delta}$	2.92 ± 0.16^{a}	81.33 ± 0.60^{a†}	1.71 ± 0.06$^{c\delta}$	22.81 ± 0.47^{b†}	24.52 ± 0.41^{b†}
SPORE	74.68 ± 1.15	0.22 ± 0.08	74.90 ± 1.22	0.84 ± 0.01	10.44 ± 0.19	11.27 ± 0.19
MYCEL.	15.4 ± 0.59	6.27 ± 0.14	21.67 ± 0.45	18.12 ± 0.28	7.76 ± 0.07	25.88 ± 0.34

a) Different letters within a column indicate significant differences between substrates with the same strain. Different symbols indicate significant differences between strains with the same substrate ($P < 0.05$). IDF: Insoluble Dietary Fibre; SDF: Soluble Dietary Fibre; TDF: Total Dietary Fibre

As indicated in the Figure 2, fruiting bodies obtained in the combination of the three strains and the three substrates had higher β-glucans content than in mycelium obtained in liquid culture. The results of the study indicated that fruiting bodies of *Ganoderma lucidum* are an important source of dietary fibre and β-glucans.



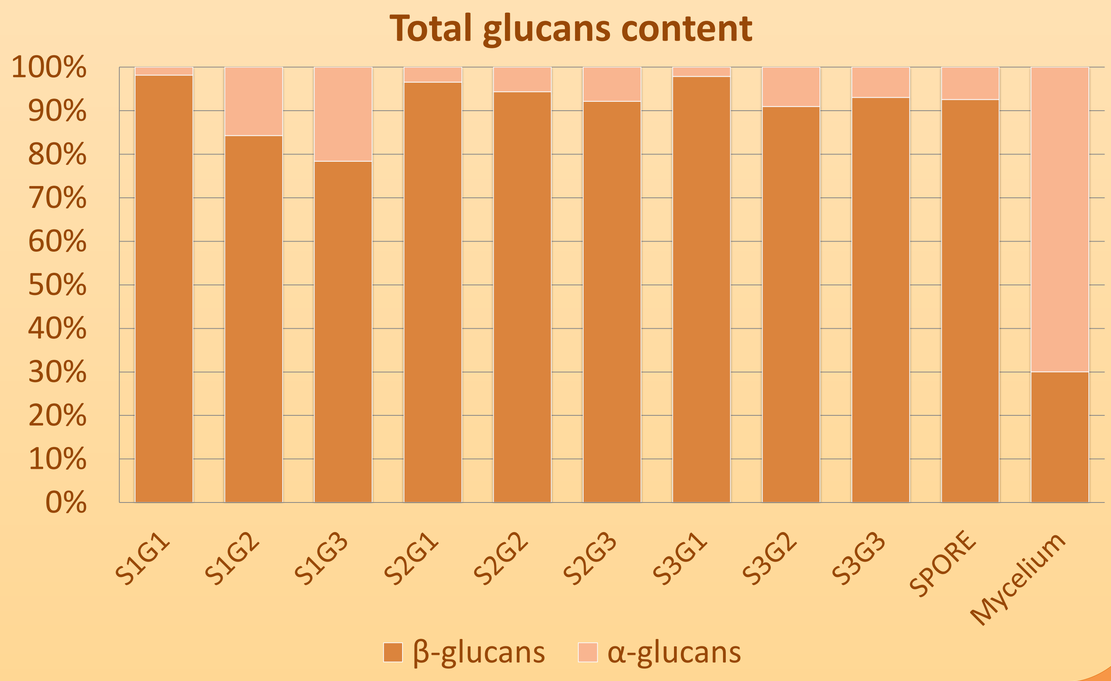
Figure 1. Images of *Ganoderma lucidum* fruiting bodies of S1, S2 and S3 strains.

Fruiting bodies, spores and mycelium were freeze-dried, powdered and analyse for dietary fibre and glucans content in triplicate. Glucans content was determined spectrophotometrically using a β-glucan kit (Megazyme, Ireland). Total, insoluble and soluble dietary fibre were measured according to AOAC methodology (991.43).

Table 1 showed data collected from the dietary fibre and glucans content. Fruiting bodies from S2-G2 combination had the highest values of dietary fibre content (93.35%) and mycelium had the lowest content (21.67%).

Spores presented similar dietary fibre content but less quantity of glucans than fruiting bodies. Stain S3 in combination with G2 and G3 produced the highest total glucans content. Differences between α-glucans (0.37-5.01%) and β-glucans (16.20-23.76%) content were found in fruiting bodies. Although mycelium and fruiting bodies showed similar amount of total glucan content, fruiting bodies presented higher value of β-glucans, which are renowned as bioactive molecules (Figure 2).

Figure 2. Total glucans content (divided in α -glucans and β-glucans content) in *Ganoderma lucidum* (fruiting bodies, spore and mycelium).



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Efficacy of spent blewit mushroom (*Clitocybe nuda*) compost for controlling vegetable crop diseases

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- The culture filtrates of *Clitocybe nuda* (wood blewit) were found to completely suppress spore germination of *Alternaria brassicicola*, *Colletotrichum higginsianum* and *Phytophthora capsici* and also inhibited growth of *Xanthomonas axonopodis* pv. *vesicatoria* and *X. campestris* pv. *campestris*.
- The purposes of the study were to develop a container medium from spent blewit mushroom compost for the control of Pythium damping-off of cucumber seedlings and clubroot disease of crucifers.
- We have formulated a new container medium composed of 50% (v/v) SBMC, 50% (v/v) peat moss, 0.3% (w/v) lime and *Bacillus aryabhattai* for controlling vegetable crop diseases.

Introduction

Pythium damping-off of cucumber seedlings caused by *Pythium aphanidermatum* and clubroot disease of crucifers caused by *Plasmodiophora brassicae* both are the soil-borne Pathogens. They affect a wide range of hosts and causes economic losses in several important vegetable crops. Therefore, the purposes of this study are to (i) develop a formulated container medium from spent blewit mushroom compost (SBMC) for the control of Pythium damping-off of cucumber and clubroot diseases in cruciferous vegetables, (ii) isolate the beneficial micro-organisms from SBMC and evaluate the applicability for vegetable disease control, (iii) develop a bio-formulation for cultivation of cruciferous vegetables.

Results

Culture filtrates of 27 fungal strains belonging to 14 species of edible mushrooms were screened for antimicrobial activity against four fungal and six bacterial pathogens. Culture filtrates of *Clitocybe nuda* also inhibited growth of *Xanthomonas axonopodis* pv. *vesicatoria* and *X. campestris* pv. *campestris*. Also, culture filtrates of *C. nuda* were found to completely suppress spore germination of *Alternaria brassicicola, Colletotrichum higginsianum* and *Phytophthora capsici*. Since *C. nuda* is an edible mushroom, its culture filtrate is considered safe for use in the disease control by organic farmers or commercial nurseries.

Table1. Effects of culture filtrates of 27 mushroom species on growth inhibition of six plant bacterial pathogens

Mushroom cultural filtrate	Inhibition zone (mm)[1]					
	Acidovorax avenae subsp. *citrulli*	*Erwinia chrysanthemi*	*Raistonia solanacearum*	*Xanthomonas campestris* pv. *oryzae*	*Xathomonas campestris* pv. *campestris*	*Xathomonas axonopodis* pv. *vesicatoria*
Ganoderma lucidum	20.5±0.6	0.0	0.0	0.0	18.6±0.5	0.0
Grifola frondosa	22.0±0.4	0.0	15.3±0.3	0.0	18.1±0.5	9.8±0.3
Clitocybe nuda	0.0	0.0	0.0	0.0	20.5±0.5	10.3±0.7
CK (PDB)	0.0	0.0	0.0	0.0	0.0	0.0

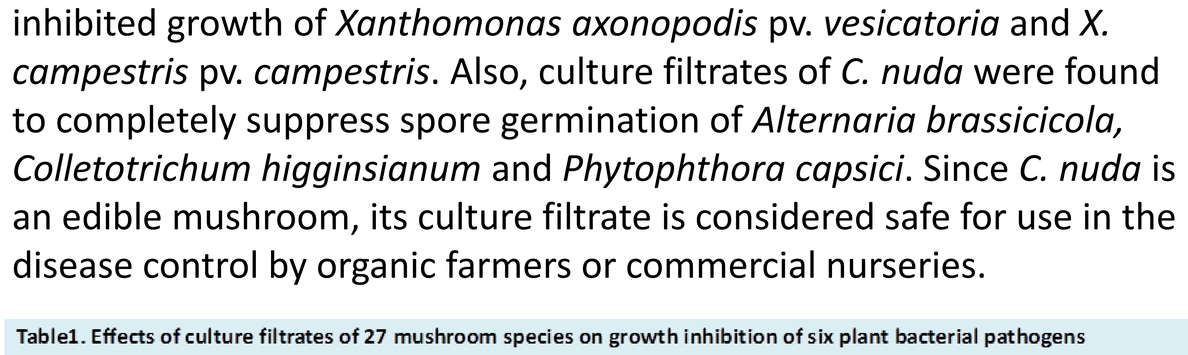


Figure 1. Inhibition of bacterial growth (left) and spore germination of *Phytophthora capsici* (right) by culture filtrates of *Clitocybe nuda.*

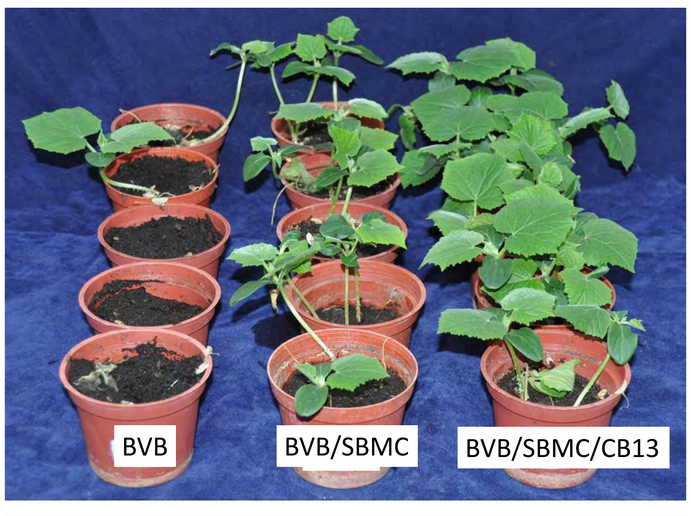
Table2. Effects of culture filtrates of 27 edible mushrooms on spore germination of fungal plant pathogens

Mushroom cultural filtrate	Spore germination (%)			
	Alternaria brassicicola	*Colletorichum higginsianum*	*Fusarium oxysporum* f. sp. *lactucae*	*Phytophthora capsici*
Ganoderma lucidum	0.0±0	76.0±3.8	100.0	97.2±2.0
Lentinus edodes	0.0±0	0.0±0	98.0±2.1	0.0±0
Tremella aurantialba	89.8±2.8	85.4±1.5	100.0	0.0±0
Clitocybe nuda	0.0±0	0.0±0	57.6±2.3	0.0±0
Control (PDB)	98.4±0	87.2±1.9	99.8±0.5	98.8±1.3

Twenty microorganisms were isolated from SBMC compost and screened for their ability in the growth inhibition of *P. aphanidermatum*. In particular, *Bacillus aryabhattai* CB13 was able to significantly restore the suppression of *P. aphanidermatum* in the steamed SBPC medium. Thus, the bioformulation of SBPC medium with a beneficial microbe *B. aryabhattai* CB13 was conducted. The disease incidence of Pythium damping-off was reduced to 4% in the bioformulated container medium. For control of clubroot disease of cabbage, the seedlings were planted on 50% (v/v) SBMC and 50% (v/v) infested soil for one month. The disease incidence of clubroot disease was less than 10%.

Table 3. Effect of seven culture media on growth or Pythium damping-off of cucumber seedlings caused by *Pythium aphanidermatum* for 14 days in the greenhouse

Medium	Plant height (cm)	Fresh weight (g/5 seedlings)	Disease incidence (%)
Carbonized rice hull (CRH)	10.4 d	5.1 d	100.0 a
Field soil (FS)	14.6 b	6.0 b	100.0 a
Raw growth medium (RGM)	13.3 c	5.3 c	71.0 b
Rice hull (RH)	13.4 c	5.4 c	100.0 a
Rice straw (RS)	13.6 c	5.5 c	67.0 b
Spent blewit mushroom compost (SBMC)	13.2 c	4.9 d	46.0 c
BVB No. 4	15.1 a	6.8 a	99.0 a





Clitocybe nuda

Figure 2. Effect of *Bacillus aryabhattai* CB13 on spent blewit mushroom compost (SBMC) for controlling *P. aphanidermatum.*



Figure 3. Efficacy of spent blewit mushroom (SBMC) compost for controlling clubroot disease of crucifers.

Conclusion

Pythium damping-off of cucumber seedlings and clubroot disease of crucifers are the most important soil-borne diseases. In order to reduce soil-borne diseases, we have formulated a new container medium composed of 50% (v/v) SBMC, 50% (v/v) peat moss, 0.3% (w/v) lime for culturing vegetable seedlings. *Bacillus aryabhattai* CB13 isolated from SBMC was capable of suppressing *P. aphanidermatum* growth. Thus, a novel bioformulation was based on spent blewit compost inoculated with a potential biocontrol agent *B. aryabhattai*. Further studies on the mechanisms of this new bio-formulation are needed.

Goody water in compost production – a valuable microbial inoculum or a source of disease?

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Introduction

- Production of high-yielding button mushroom compost requires addition of water up to 70-80% of compost mass. The process water used is often recycled by commercial composting operations for economic and environmental reasons ("goody water"; usually combined runoff water from all parts of the operation (Fig. 1)).

- Goody water is thought to inoculate the raw ingredients with microbes beneficial for the composting process. However, concerns about spreading undesirable microbes in this way have also been raised by the industry. In addition, anaerobic conditions arising from poor management of goody water may cause odour problems due to generation of volatile sulfur compounds by bacteria.

To investigate the dynamics of microbial communities in goody water, we studied changes in bacterial community composition and anion concentrations over a period of four weeks under one compost yard's standard operating procedures.

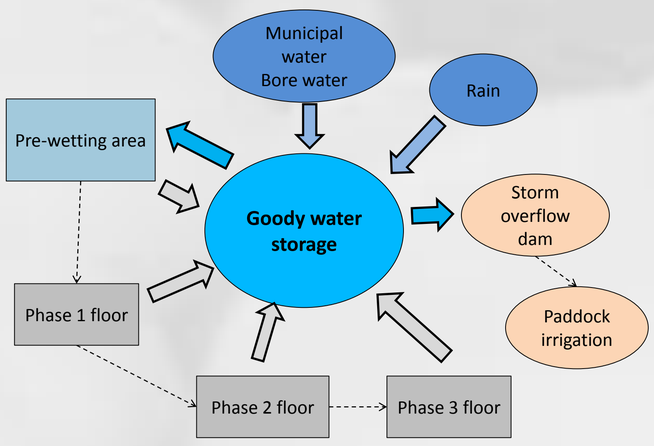


Figure 1. Goody water use and recycling in mushroom compost production.

Process day	Week starting					
	31 Mar	7 Apr	14 Apr	21 Apr	28 May	5 May
0			8	12	16	20
2		3	9			
3		4		13	17	
4		5	10	14	18	
5	2	6	11	15	19	
6		7				

Figure 2. Study sampling schedule and sample coding. Samples sequenced with Illumina MiSeq are shown in red.

Microbial populations in goody water are dynamic and related to phosphate concentration and pH

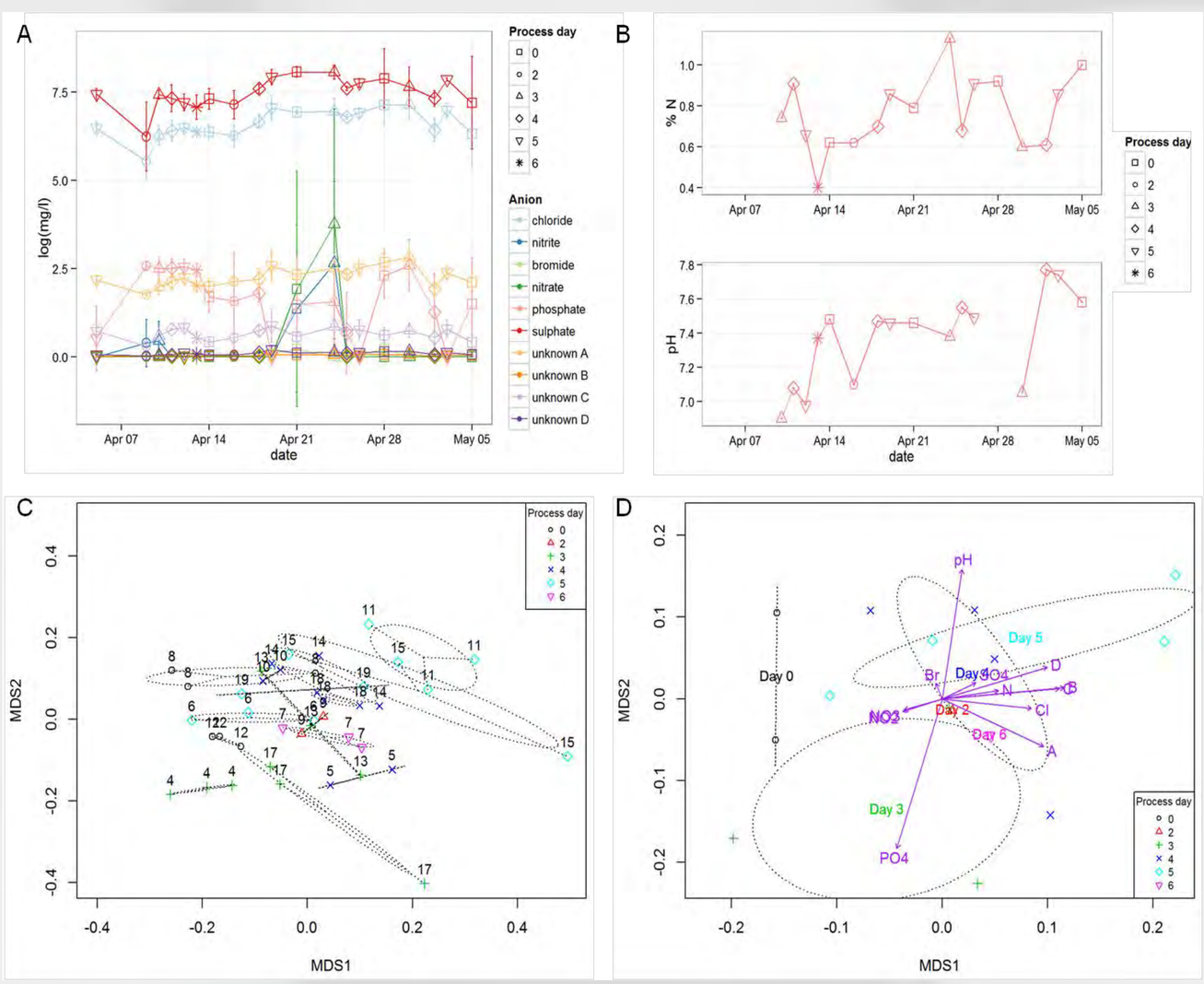


Figure 3. Fluctuations of anion concentrations (A), pH and nitrogen content (B) and bacterial population fingerprints (C, D) in goody water during one month of compost yard's standard operations. Numbers refer to sample codes in day-to-day variability analysis (C); correlations between microbial community fingerprints and ion concentrations are shown as arrows in process-related variability analysis (D).

Methods

Goody water samples were collected from main tank into a 10 l bucket 3-4 times a week after major operation events (Fig. 2) and subsampled in triplicate for DNA analysis and for anion determination.

- Direct MiSeq Illumina sequencing analysis using bacterial 16S primers (515F-806R, Earth Microbiome Project) was carried out at Research and Testing Laboratory (Lubbock, Texas) on total DNA extracts of four goody water samples (Fig. 2).

- pH was measured with portable pH meter at the time of sampling. Anion analysis was used to measure concentration of chloride, sulphate, phosphate, bromide, nitrate and nitrite in goody water (Jackson), and was done by HPLC on a Dionex system with IonPac AS14/AG14 anion-exchange column and ED50 electrochemical detector, coupled to an ASRS300 micromembrane suppressor. The ions were eluted in 3.5 mM Na_2CO_3/ 1 mM $NaHCO_3$ at 25°C with a flow rate of 1.2 ml/min.

- Changes in bacterial communities were investigated using terminal restriction fragment length polymorphism (T-RFLP) (Liu *at al.* 1997) using 338F-1050R primer pair targeting the bacterial 16S rRNA gene (Winsley *et al.* 2012) and *HhaI* enzyme (NEB, Ipswich, MA). DNA fragments were separated using capillary electrophoresis in a commercial laboratory (Macrogen, Seoul, South Korea).

- Data analysis was carried out using R v3.1.2 and RStudio v.0.98.1091 with markdown support and "knitR" package (R_Core_team, 2014; Racine, 2012; Xie, 2013) with packages "vegan" (Oksanen *et al.* 2007), "RawGeno"(Arrigo *et al.* 2000), and "random forest" (Liaw & Wiener, 2002). Multivariate analysis (non-metric multidimensional scaling (NMDS)) was used to visualize differences between samples.

Conclusions

Goody water is a source of microbes beneficial for composting, such as *Thermus thermophilus* and *Bacillus* sp.

However, goody water's microbial population is highly responsive to external events and therefore not stable.

Regular monitoring may be required to optimize manipulation of the microbial population in goody water for compost management purposes.

Results

- Sulphate and chloride were the most abundant anions in goody water and their concentrations fluctuated differently from that of phosphate anion (Fig. 3 A). Nitrogen content and pH did not follow any pattern (Fig. 3 B).
- The goody water bacterial community was highly dynamic and related to the variation in phosphate anion concentration and pH (Fig. 3 C and D).
- Some periodicity was observed in the results (Fig. 3 D), despite marked within-sample and day-to day variability (Fig. 3 C), suggesting causal links between compost yard management events and changes in the bacterial community.
- Over 300 species were detected in four goody water samples (Table 1). The most abundant bacterial species was *Thermus thermophilus*, but *Bacillus* and related bacteria, associated with active phase 1, and anaerobes from phylum *Bacteroidetes*, associated with the pre-wetting stage, were also detected.
- Sulfate-reducing bacteria (*Desulfovibrio*) were present only in small numbers.
- Several bacterial species related to known poultry pathogens were detected in low numbers, and are thought to derive from the poultry manure used in composting process.

Area drained/event	Sample			
	4	9	13	16
Prewetting windrows	✓	✓	✓	
Phase 1 bunkers	✓		✓	✓
Ammonium nitrate added	✓		✓	

Table 1. Bacteria present in goody water on four sampling dates within a one month period. Only microbes making up an average >0.5% of the population are listed.

Bacterial phylum	Bacterial species	% of goody water bacteria				Associated with Prewet leachate or Phase 1 (P1) leachate?	Found in compost?	Comments
Deinococcus-Thermus	*Thermus thermophilus*	56.8	15.1	44.9	41.6	P1	P1	Thermophile, important for organic degradation in Phase 1
Bacteroidetes	*Bacteroides* sp.	5.7	11.6	2.1	4.3	Prewet	Prewet	Anaerobes, common human commensals
Firmicutes	*Bacillus* sp.	3.2	2.7	6.1	7.1	P1	Both	Aerobic, common in the composting process
Bacteroidetes	*Prevotella albensis*	3.1	6.7	2.8	1.1	Prewet	Prewet only	Also called *Bacteroides ruminicola*, rumen bacterium
Firmicutes	*Clostridium* sp.	1.2	3.6	3.1	3.7	Both	n.d.	Anaerobe, can be sulfate reducer
Bacteroidetes	*Petrimonas* sp.	0.5	8.3	0.9	1.6	Prewet	n.d.	Anaerobic fermentor, can be nitrate reducer
Bacteroidetes	*Prevotella* sp.	5.3	2.9	1.2	1.3	Prewet	Prewet only	Common oral bacteria
Firmicutes	*Ureibacillus suwonensis*	1.4	1.1	2.6	3.3	P1	Both, mainly P1	Aerobic spore forming bacteria
Proteobacteria	*Alcaligenes* sp.	0.8	3.4	1.0	2.0	Prewet	Both	Aerobic, can be nitrate-reducing bacteria
Firmicutes	*Caloramator* sp.	1.4	0.4	2.0	2.4	P1	Prewet only	Thermophilic anaerobe
Proteobacteria	*Lampropedia hyalina*	0.0	4.5	0.0	1.2	Prewet	n.d.	Aerobe
Proteobacteria	*Comamonas denitrificans*	0.5	0.7	1.0	1.6	P1	Both	Efficient nitrate-reducing bacteria
Proteobacteria	*Arcobacter skirrowii*	0.1	0.1	1.4	1.9	P1	Prewet only	Associated with chickens, related to *Campylobacter*
Firmicutes	*Enterococcus cecorum*	1.1	0.7	1.2	0.4	Both	Prewet only	Associated with chickens
Firmicutes	*Clostridium stercorarium*	0.4	0.4	0.7	1.1	P1	n.d.	Anaerobic cellulose-degrading thermophile
Firmicutes	*Kurthia gibsonii*	0.6	0.6	1.3	0.1	Both	n.d.	Heterotrophic aerobe, associated with chickens
Firmicutes	*Streptococcus* sp.	1.1	0.8	0.6	0.2	Both	Prewet only	Common oral bacteria

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A Genomic Approach to Understanding and Improving Mushroom Compost Utilisation.

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Abstract

Agaricus bisporus (common button mushroom) has an annual global value of over $4.7 billion (Eastwood et al, 2015) with a domestic farmgate value of €120 million, making it Ireland's largest horticultural sector. However, significant profits are lost due to *A. bisprous* failure to fully utilise the compost it is grown on. The overall objective of this research is to identify potential means of improving mushroom yields, particularly addressing the issues at the third flush through improved compost utilisation and strain selection.

Introduction

It is standard practice that commercially grown *A. bisporus* mushrooms are picked from mostly the first and second flush. This is due to the third flush of *A. bisporus* resulting in reduced yields (Royse and Sanchez, 2008), which are also often more prone to disease. This occurs despite significant nutrients and nitrogen being available in the compost for *A. bisporus* to utilise. To further understand why this is occurring microarray analysis will be carried out on compost samples throughout a full commercial growth cycle with the aim of identifying genes of interest. Promoter analysis will then be carried out on several of the most interesting genes.



Fig. 1: First flush of *A. bisporus*.

Project Overview

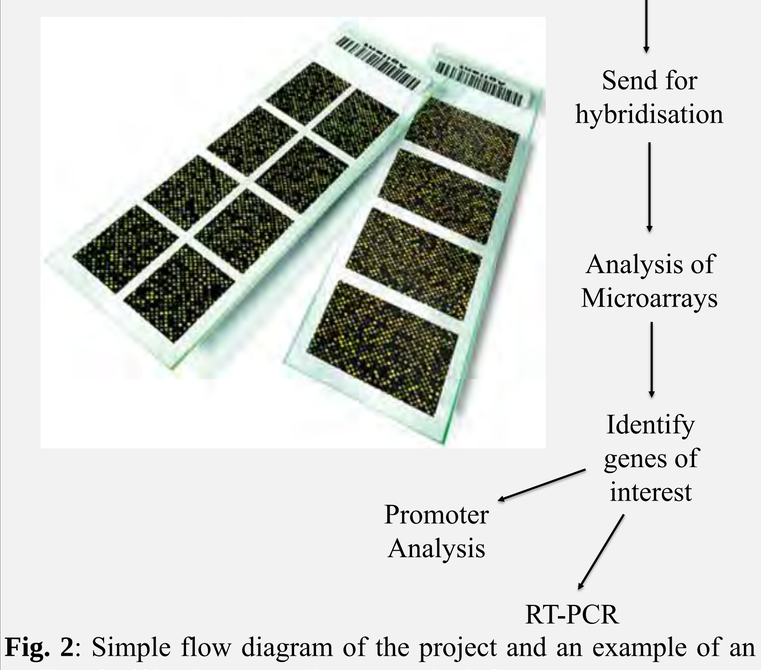


Fig. 2: Simple flow diagram of the project and an example of an Agilent 8x60k microarray (image on the left).

Promoter Analysis

- Promoter analysis will be carried out on several genes of interest identified during the microarray analysis.
- The promoters will be inserted into the vector and transformed into *Coprinopsis cinerea* a model basidiomycete, in order to evaluate gene expression levels.
- To aid the rapid construction of an expression plasmid the plasmid pGFPi004 (Fig. 3; Heneghan et al., 2007) will be modified by removing the *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase (gpdII) promoter and replacing it with a multiple cloning site (MCS).
- The new MCS will be constructed by designing a forward primer complementary to the 5' end of the intron in pGFPi004 and a reverse primer complementary to the 3‘ end of eGFP. The forward primer will include an additional sequence for a MCS (Fig. 4 A).
- PCR will be performed using these primers and pGFPi004 as a template. The resulting product will be subcloned into TOPO vector (Fig. 4B).
- The MCS-Intron-eGFP fragment will be digested out of TOPO and ligated into the backbone as a *EcoR* I-*BamH* I fragment overhangs (Fig. 4C).

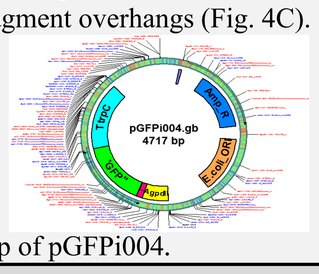


Fig. 3: Plasmid map of pGFPi004.

Expression Plasmid Design

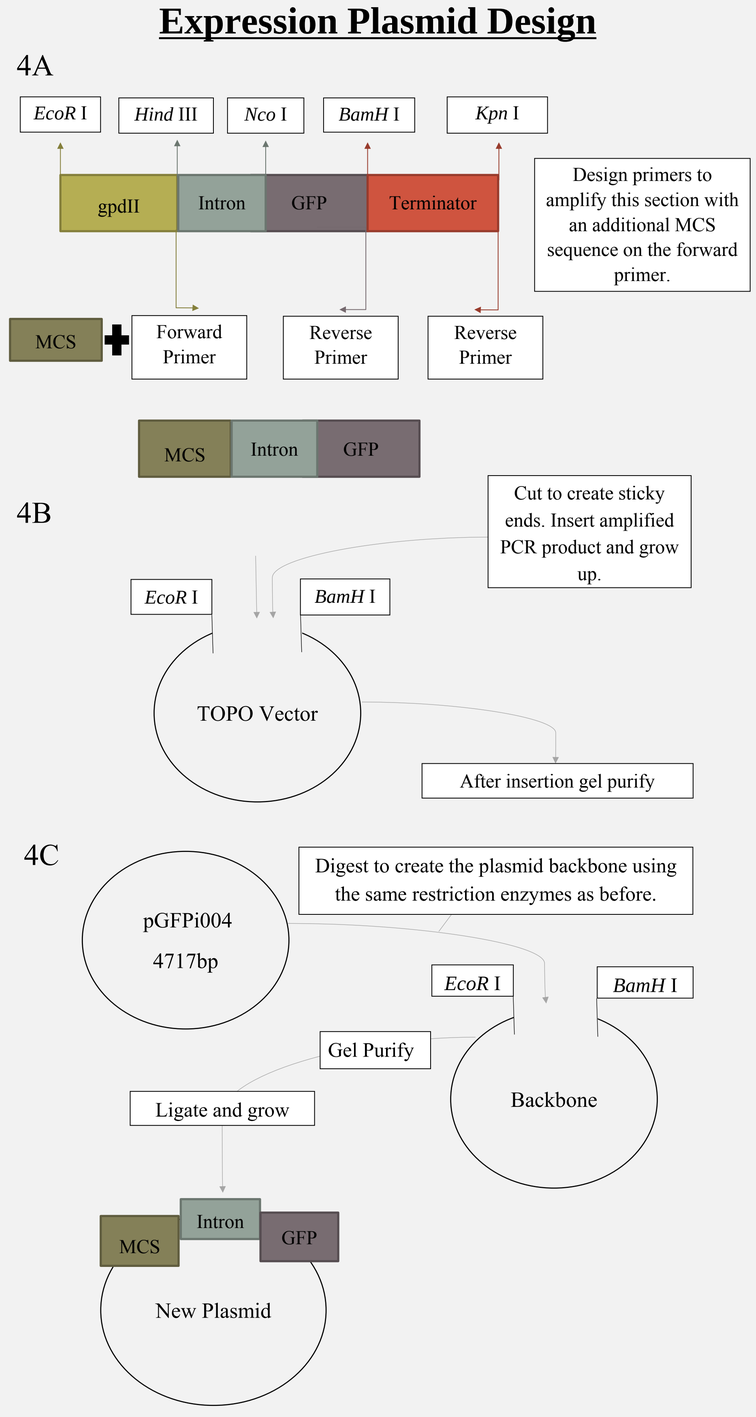


Fig. 4: Schematic of the construction of plasmid pMCSIeGFP004.

Results

- Sampling for the microarrays was carried out every 48 hours on strain A15 from pre-1st flush up until the end of the 3rd flush. At each sampling point mushroom, casing and compost samples were collected in four replicates across the entire mushroom shelf.

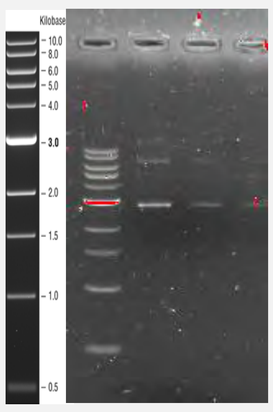


Fig. 5: A miniprep was performed on *E. coli* DH5α cells transformed with pGFPi004.

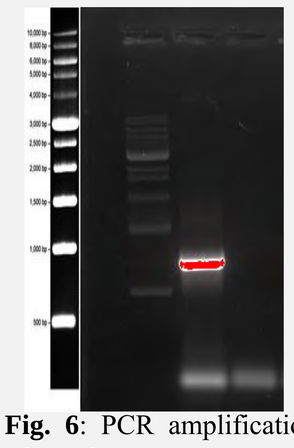


Fig. 6: PCR amplification of eGFP present in pGFPi004 at approximately 700 bp.

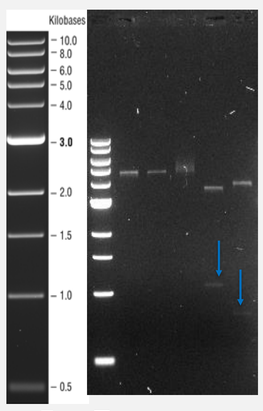


Fig. 7: To prepare the backbone of pMCSIeGFP004, pGFPi004 was digested with *BamH* I and *EcoR* I.

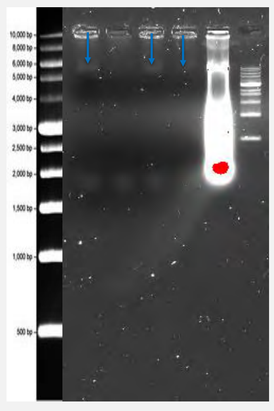


Fig. 8: Genomic DNA was extracted from *C. cinerea* grown in liquid cultures.

Discussion

Previous work has demonstrated that an *A. bisporus* promoter linked to eGFP can be used to generate an expression profile for that gene throughout the *C. cinerea* lifecycle (Fig. 9) (Heneghan et al 2009).

To facilitate rapid construction of a promoter expression plasmid for *C. cinerea*, a new vector was designed to include a MCS upstream of eGFP. This vector pMCSIeGFP004, is based on the previously published pGFPi004 (Heneghan et al 2010).

Preliminary work on the construction of this plasmid is underway. This includes the preparation of the plasmid backbone by removing the gpdII promoter-Intron-eGFP fragment and the design of primers engineered to contain a MCS preceding the Intron. The final MCS will contain several widely used restriction enzymes (*EcoR* I, *Bcl* I, *Nde* I, *Not* I, *Sac I*, *Sac* II, *Sal* I, *Swa* I, and *XHO* I) to facilitate prompt digestion.

Future work will focus on the amplification of the Intron-eGFP fragment using these primers and the cloning of this fragment on an *EcoR* I- *BamH* I digestion into the plasmid backbone.

Genomic DNA will be isolated from *A. bisporus,* following the same method used to extract DNA from *C. cinerea (*Fig. 8), in order to isolate promoters. The promoters of interest will be identified through microarray analysis.

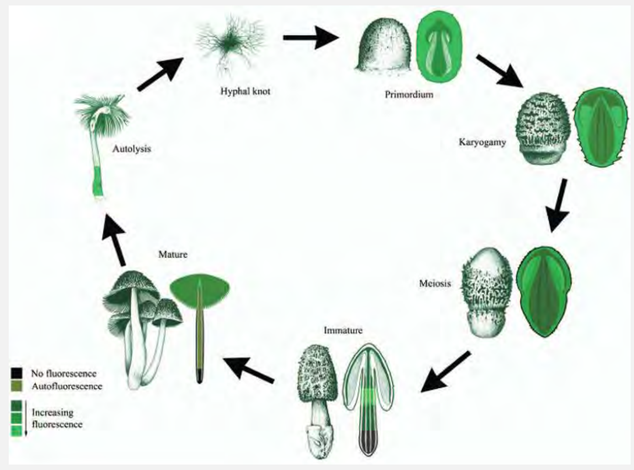


Fig. 9: Illustration of the *A. bisporus* SPR1 expression profile throughout the *C. cinereus* lifecycle (Heneghan et al 2009).

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Agriculture and Food Development Authority

IT Sligo An Institiúid Teicneolaíochta, Sligeach

CERIS Centre for Environmental Research Innovation and Sustainability

Department of Agriculture, Food and the Marine

An Roinn Talmhaíochta, Bia agus Mara

A Deep Exploration Into the Microbial Community in an Experimental Composting Condition for *Agaricus bisporus* Cultivation

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INTRODUCTION

Substrate preparation for Agaricus cultivation is the most critical point of a mushroom production and as such has been the impetus for many studies. Among many factors involved in the composting process, the microbial ecology of the system is the underlying drive of composting, which can be influenced by composting management techniques. We evaluated three common pasteurization temperature regimes used during Phase II compost preparation for *A. bisporus*.

METHODOLOGY

Advances in molecular biology in the past 10 years, mainly the metagenomic approach, has revealed a new frontier to explorer the microbial ecology of different environments (soil, sea and human body). Utilizing this approach, we investigated the microbial ecology of Phase II composting for Agaricus with a focus on the pasteurization stage. Pasteurization temperatures were simulated in bioreactors following 3 different pasteurization conditions (57°C/6h, 60°C/2h and 68°C/2h), simulating conditions adopted by many producers. Compost samples were submitted to high throughput sequencing using the *MiSeq Illumina* Platform with bacterial and fungal primers.

Phase I compost - 6 days in bunker
- Straw-bedded horse manure
- Distiller's Grain
- Poultry manure
- Gypsum N = 1.40%

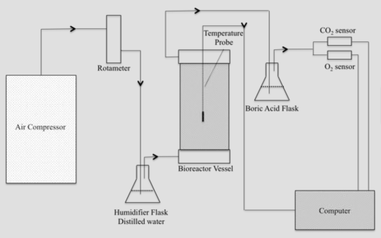


Pasteurization
P57°C
P60°C
P68°C

Conditioning
C57°C
C60°C = 47°C
C68°C

Phase II compost – bioreactors
- Pasteurization – P57°C/6h
- Pasteurization – P60°C/2h
- Pasteurization – P68°C/2h
- Conditioning – 47°C/5 days

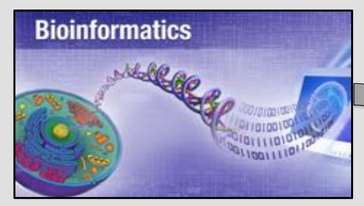




- Extraction metagenomic DNA
- Amplification of 16S and ITS1 targets
- Sequencing via *MiSeq* Illumina



- Bioinformatics analysis
- Chemical analysis
- Looking forward to analyze the data
- Mothur Software



RESULTS

Bacterial communities showed higher diversity compared with fungal populations. Among nearly 15 bacterial phyla detected, Firmicutes and Proteobacteria were the most abundant across the composting process (Figure 1A). The abundance of Firmicutes increased and Proteobacteria decreased when submitted to high temperature pasteurization (68°C/2h). Fungal phyla consisted mainly of Ascomycota (Figure 1B). The number of bacterial and fungal genera identified was higher than documented in previous studies (~350 and 80, respectively, data not showed). Fungal sequences showed the presence of two important thermophile genera, *Thermomyces* and *Mycothermus,* even when exposed to high temperature pasteurization (68°C/2h) (Figure1D). The composition of bacterial genera varied utilizing different pasteurization conditions (Figure 1C).

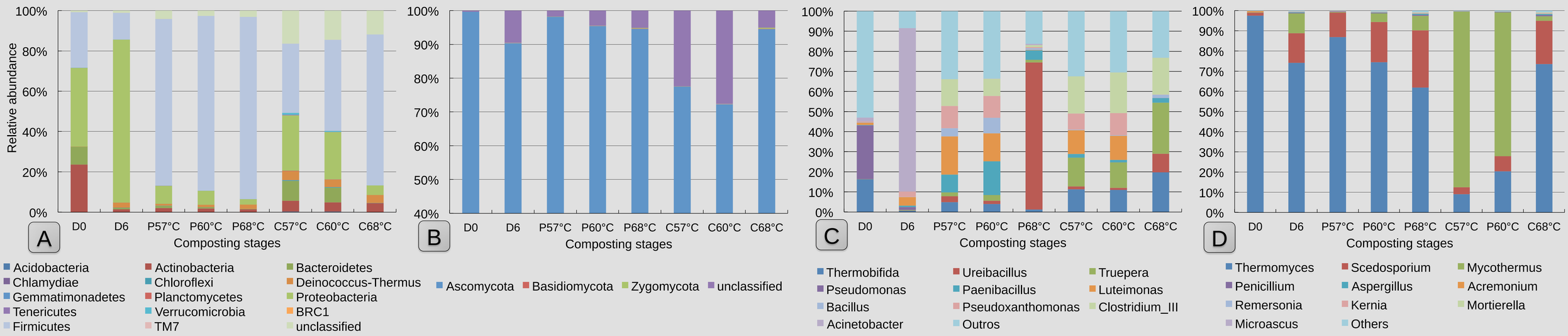


Figure 1. Distribution of phyla across the composting process (Phase I and II). **A.** Bacterial phyla; **B.** Fungal phyla; **C.** Bacterial genera; **D.** Fungal genera; D0 – beginning Phase I; D6 – end Phase I (6 days); end pasteurization: P57°C – pasteurization at 57°C/6h; P60°C – pasteurization at 60°C/2h; P68°C pasteurization at 68°C/2h; end conditioning: C57°C – conditioning at 47°C/5 days following 57°C pasteurization; C60°C – conditioning at 47°C/5 days following 60°C pasteurization; C68°C - conditioning at 47°C/5 days following 68°C pasteurization.

DISCUSSION

High temperature pasteurization (68°C) decreased the number of bacteria genera (data not showed) and increased the nitrogen loss (ammonia), mainly during the conditioning stage (Figure 2C). On the other hand, carbon loss was more pronounced during pasteurization at 60°C (Figure 2A). In this experimental condition a lower organic matter content was obtained through pasteurization at 60°C and solids showed more stability during Phase II (Figure 2B). From the biological point of view, the pasteurization at 57°C showed higher diversity of bacteria and predominance of important fungal thermophilic genera as *Mycothermus*.

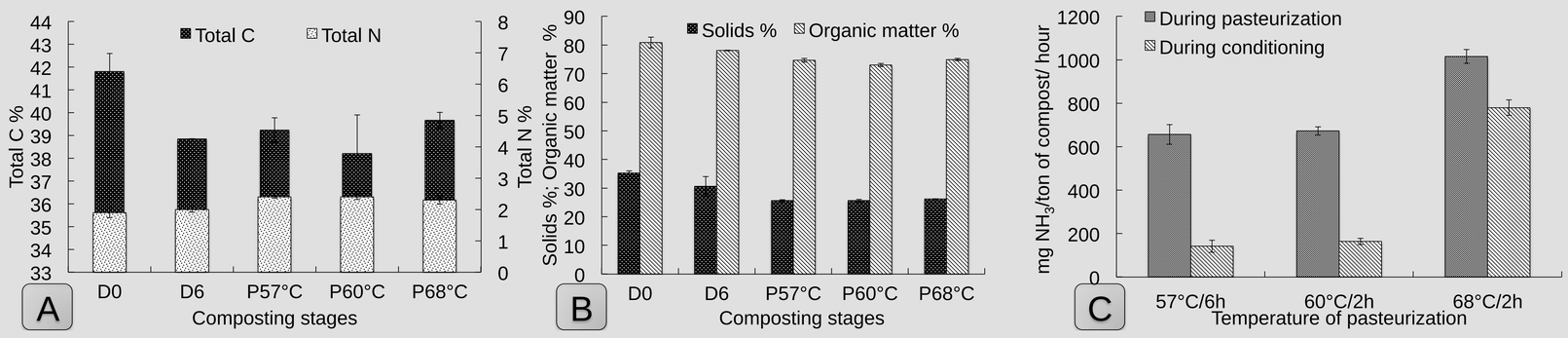


Figure 2. Chemical changes during the composting process (Phase I and II). **A.** Total C (Carbon); **B.** Solids (%) and organic matter (%); **C.** NH_3; D0 – beginning Phase I; D6 – end Phase I (6 days); P57°C – pasteurization at 57°C/6h; P60°C – pasteurization at 60°C/2h; P68°C pasteurization at 68°C/h; **A** and **B** represent the final compost after conditioning; **C** ammonia emissions during pasteurization and conditioning, measured after each step and expressed in mg of NH_3 per ton of compost per hour.

CONSIDERATIONS

Looking forward, we expect to complete the full report about ecological studies of Phase II composting with this new approach and encourage future investigations to look into the black box of composting aiming to develop best practices to manage the composting process for Agaricus cultivation.

ACKNOWLEDGEMENTS
- CAPES Foundation, Ministry of Education of Brazil – N° 005707/2014-05
- Penn State University Mushroom Industry Endowment

NIR hyperspectral imaging to characterize spent mushroom substrate package without destructive preparation

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Introduction

Commercial mushroom growth on substrate material, produces a heterogeneous waste that can be used for bioenergy purposes. The utilization of this resource is largely based on its chemical composition. Thus, NIR hyperspectral imaging is considered as an efficient methods for a characterization of spent mushroom substrate (SMS) .

Material and method

The substrate material was initially composed of 75-80% sawdust and in cylinders' form (Fig. 1), from a *Pleurotus ostreatus* cultivation. A line-scanning camera from Specim (Umbio AB, Umeå Sweden) was used for imaging and measuring samples on a belt. The images are of 320 pixels wide in 256 wavelengths (900 to 2500 nm). Multivariate image analysis was applied to analyze the images [1] .

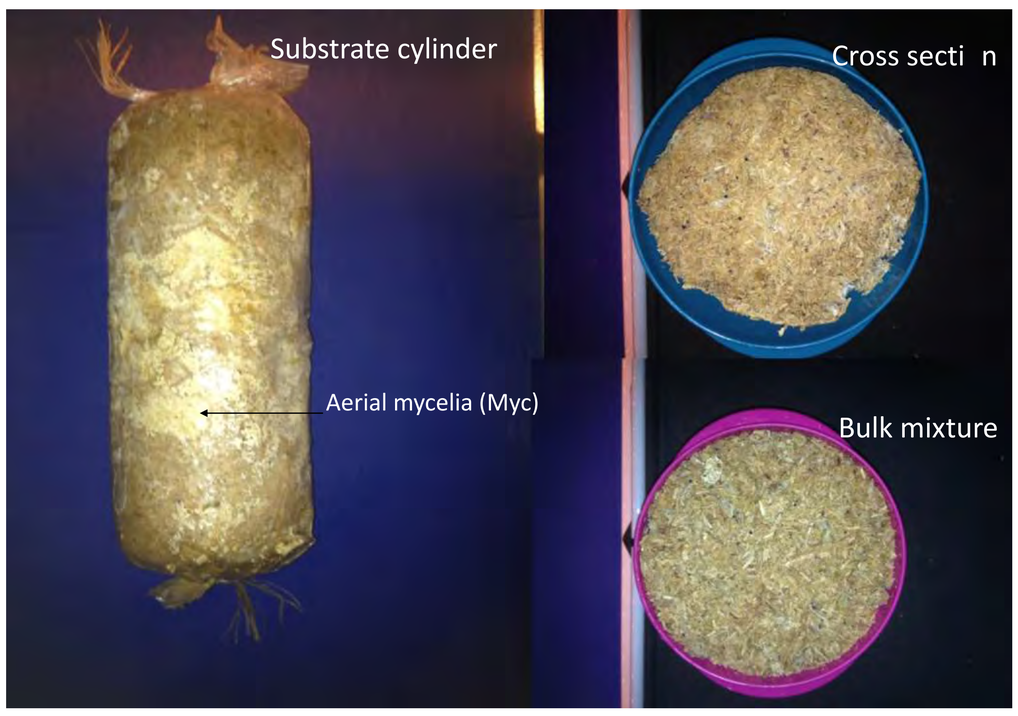


Fig. 1 Digital images of the materials. The average diameter of the cylinder is 100 mm.

Results

PLS-DA model suggested that plastic cover caused little influence on the light radiation penetration (Fig. 2) and a side face hyperspectral image could represent the information for the whole SMS cylinder when the Myc was removed (Fig. 3). The best results were obtained for dried cylinders, but it was shown that almost equally good results could be obtained for wet material and for wet material covered by plastic (Fig. 4).

Conclusion

NHI has great potential to make calibration models after remove the Myc by taking images on the side surface for predicting the water, carbohydrates, lignin and protein contents of the wet and plastic cover SMS cylinder.

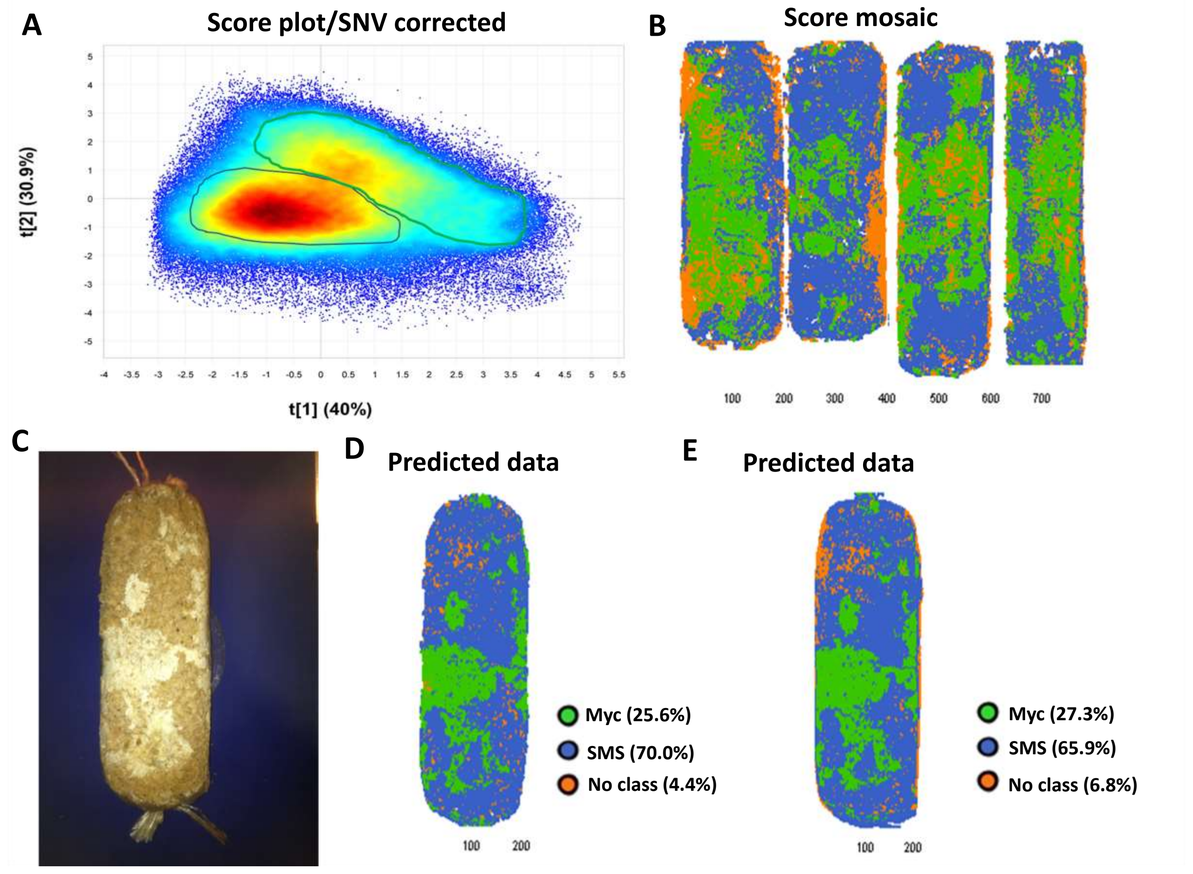
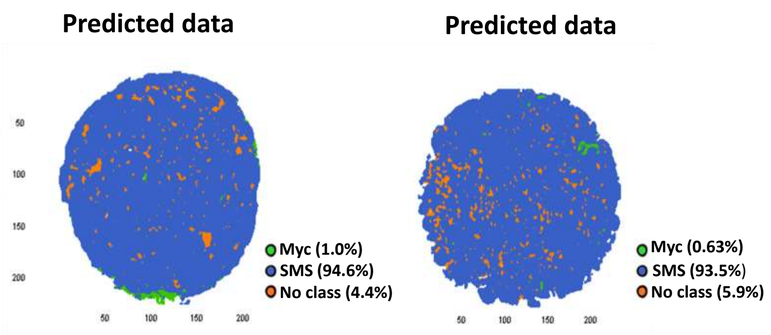


Fig. 2 The PLS-DA modeling for mycelia (Myc) and spent mushroom substrate (SMS) classification based on the wet and covered cylinders. A: the pixel clusters of Myc and SMS. B: the selected classes for Myc (green) and SMS (blue) in the mosaic. C: The digital image of no covered surface. D and E: the predicted results of the same surface (C) but without and with plastic covered. The number within the bracket is the proportion of the right predicted pixels.

Fig. 3 The cross section (left) and the bulk mixture (right) SMS were predicted correctly by the PLS-DA model. The number within the bracket is the proportion of the right predicted pixels.



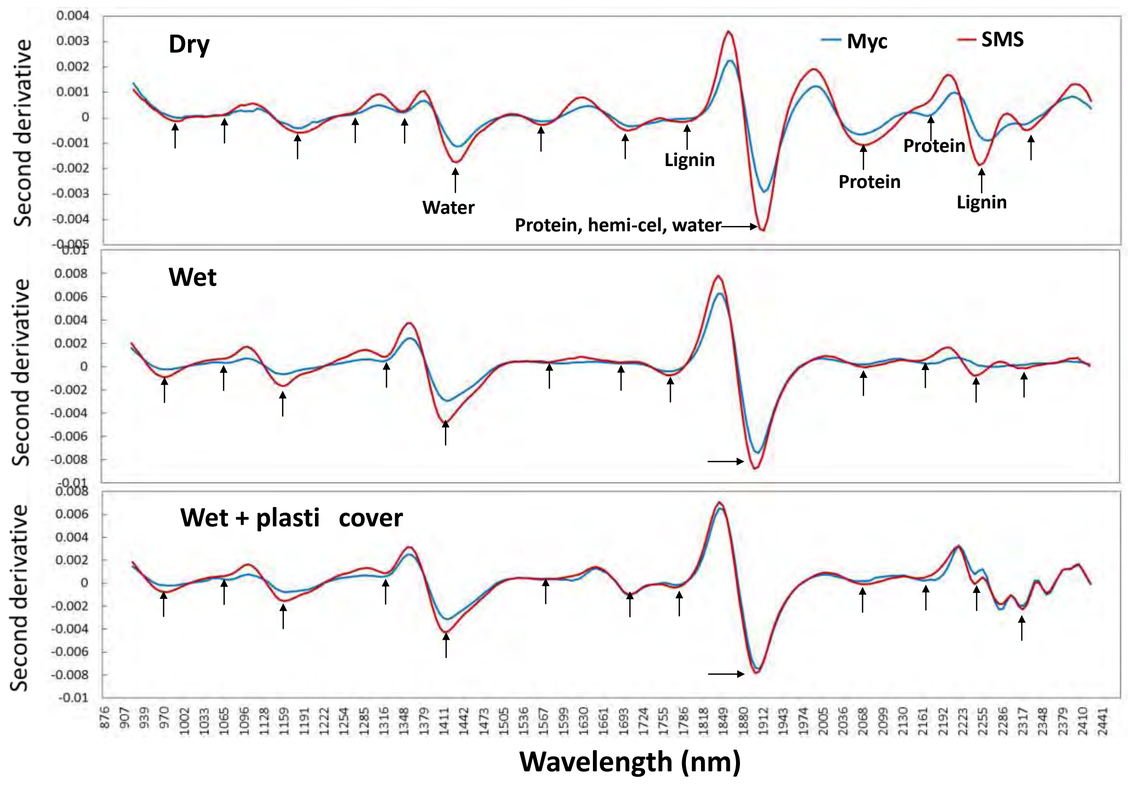


Fig. 4 The chemical differences between mycelia (Myc) and spent mushroom substrate (SMS) based on the mean spectra of pixel clusters of dry, wet, and wet and covered materials. The chemical assignments of the wavelengths are from previous studies [2, 3].

Acknowledgements

This research was financed by the Swedish State Department of Innovation (VINNOVA 2013-02015) and Swedish State Energy Agency (Project 37816-1).

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Session Nutrition, Bioactive Compounds, Medicinal Aspects

Unleashing the potential of mushroom value addition in India

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Introduction

Mushrooms are assuming increasing importance as a source of food because of its pleasing flavor exotic preference and awareness about nutritional and medicinal effects. Majority of Indians are vegetarians with cereal based diet but deficient in intake of good quality protein. FAO has recommended the inclusion of mushrooms in the daily diet of Indians to compensate the protein deficiency in countries where rice is the staple food. Changing food habits, increasing health consciousness and awareness of nutritional benefits have led to an increase in the demand of fresh and processed mushrooms among urban and rural consumers.


Pleurotus


Calocybe


Auricularia


Tricholoma


Lentinus


Agaricus


Volveriella

PLEUROTUS RECIPES


SOUP


SAMOSA


BAJI

CALOCYBE RECIPES


MASALA


MUSHROOM 65


WINE

AURICULARIA RECIPES



MUSHROOM KOFTA


PICKLE


DOUGH NUT

TRICHOLOMA RECIPES


OMLETTE


PAYASAM


PUFFS

LENTINUS RECIPES


BISCUIT


CUTLET


SCRAMBLE

AGARICUS RECIPES


STEW


SWEET BALLS


KURUMA

VOLVARIELLA RECIPES


PAPPAD


PIRALAN


CHUTNEY POWDER

Results

- Lip smacking soups and glorified snacks can be prepared using fresh oyster mushrooms grown throughout the year
- For preparation of wine Calocybe the Indian beauty among mushrooms is the most suited.
- Petite and juicy Auricularia produces delicious fritters and dried ones good for pickles.
- Egg savouries like mint omlette, egg rolls are quite common in South India and Tricholoma excels in tasty dishes. Payasam, a sweet desert is prepared with Tricholoma
- Lentinula known for its superb flavor is used for making cutlets ,scramble and in powder form as supplement for biscuit making
- Agaricus through dominates the International market is less popular in south India because of the expensive cultivation technology. Kuruma, stew and even sweet balls can be prepared out of this
- Pappads, honey coated doughnuts and biscuits can be prepared with dried mushroom powder.

Value added products have always been a gourmet's delight. Mushrooms not only produces delicious dishes, but also a very balanced cholesterol free and almost fat free items. Thus there remains ample scope for popularizing mushroom cultivation and development of mushroom based recipes.

Fungal Immunomodulatory Protein from Tiger Milk Mushroom, *Lignosus rhinocerotis*



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INTRODUCTION

Fungal immunomodulatory proteins (FIPs) are a family of novel proteins purified from edible medicinal mushrooms that possess extensive biological functions including anti-tumor, anti-allergy and immunomodulatory responses. To date FIP from *Lignosus rhinocerotis* (Tiger Milk Mushroom, TMM) has not yet been reported. A new FIP from TMM (FIP-Lrh) was isolated in a previous study and the protein sequence was provided. An *in silico* study was carried out to analyse the protein and compare it structurally to other FIP proteins. *L. rhinocerotis* is an important medicinal mushroom found in Malaysia and it has also been used as a food source. The sclerotium has the most medicinal value and is widely used indigenously as treatment for breast cancer, fever, cough, asthma and food poisoning among others. Biocomputational studies offer powerful new methods to predict molecular structures and understand the complex behavior of living organisms, as well as to analyze vast amount of molecular data to make predictions that guide experimental work. Homology modeling is an important technique to obtain three dimensional (3D) structure of the proteins that have not yet been identified. The goals of molecular docking are the identification of a ligand that binds to a specific receptor binding site and of its preferred, energetically most favorable, binding pose.

OBJECTIVE

•To predict the three dimensional structure of fungal immunomodulatory protein from *L. rhinocerotis* (FIP-Lrh)

•To determine the active site and important residues of the FIP-Lrh by performing docking studies on the binding site using various test ligands

METHODOLOGY

BLAST analysis of FIP-Lrh protein sequence

↓

The 3D structure of protein with nearest match (1OSY.pdb, FIP-fve template) was downloaded from PDB

↓

Modeller 9v12 was used to model the sequence

↓

Target sequence written as PIR format

".ali" file used as input for construction of 3D model	Structure with lowest objective function was selected and superimposed on template

↓

The target protein was validated using PROCHECK

↓

14 different glycans downloaded from PubChem

↓

AUTODOCK 4.2 and AUTODOCK Vina used for docking followed by analysis of glycans to both target and template protein

RESULTS

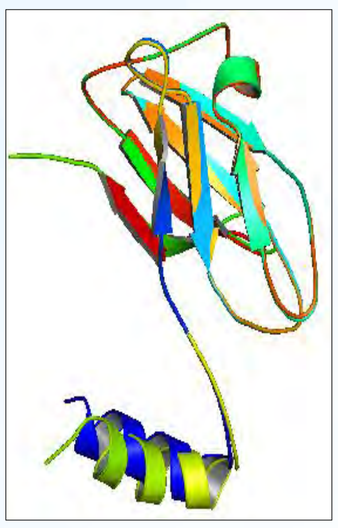


Figure 1. FIP-Lrh superimposed on the FIP-fve template protein

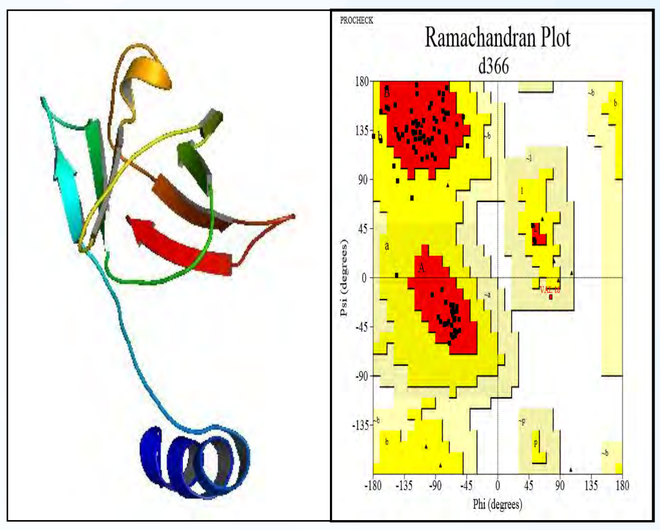


Figure 2. The Model of FIP-Lrh and its Ramachandran Plot

- The FIP-Lrh is structurally similar to FIP-fve (Fig.1).
- A total of 91.8% of the amino acids were in the core region
- Quality of the predicted structure was good (Fig. 2).

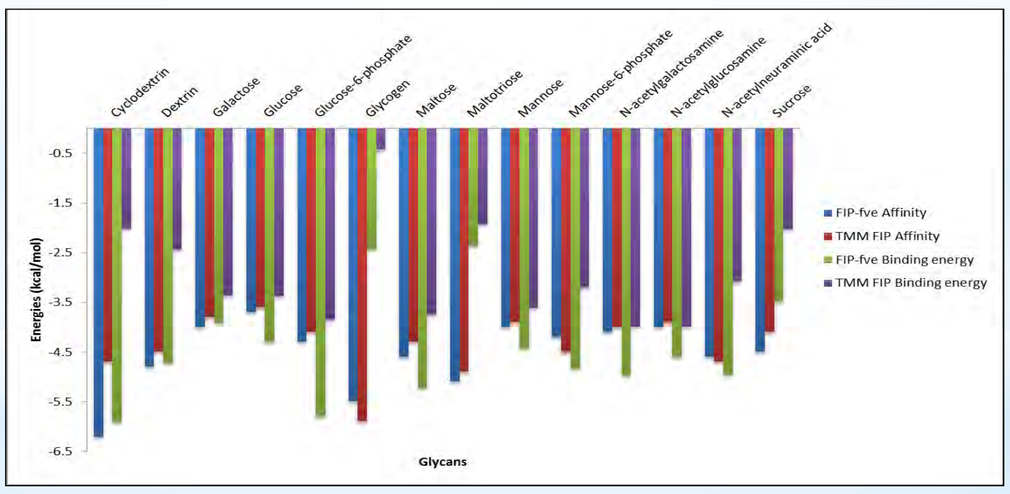


Figure 3. Chart showing the binding affinities and binding energies of the ligands

- Of a total of 14 glycans tested, glycogen had the highest binding affinity (-5.9 kcal/mol) and N-acetylgalactosamine had the highest binding energy (-3.98 kcal/mol) for the predicted protein.
- N-acetylgalactosamine docking into the binding pocket of FIP from TMM is as shown in Fig. 4.

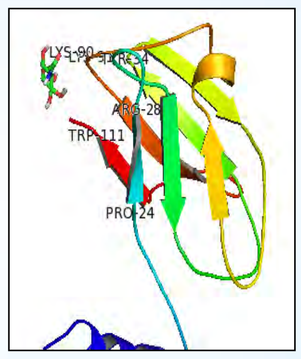


Figure 4. N-acetyl galactosamine bound to the predicted protein

CONCLUSION

- The FIP isolated form *L. rhinocerotis* was successfully modeled using FIP-fve as a template.
- Its protein sequence is similar to other FIPs and is structurally similar to FIP-fve.
- Based on the docking studies, FIP-Lrh could bind to sugars with high affinity to glycogens and N-acetylgalactosamine.
- Therefore it is predicted to properties similar to other FIPs which are lectin mitogens such as:
  - a dose dependant mitogenic effect *in vitro* for human peripheral blood lymphocytes and mouse splenocytes
  - haemagglutination
- Wet laboratory research will help to verify the results of the predictions reported in this study.

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The Cultivation of *Ganoderma leucocontextum* in Tibet

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Abstract

***Ganoderma leucocontextum*, a new species in the *G. lucidum* complex, was recently described following molecular characterisation. The fruiting bodies were subsequently found to contain 16 new lanostane triterpenes, ganoleucoins, together with 10 known tripterpenes. *G. leucocontextum* is now a commercially important species. In this paper we describe a method for its cost effective cultivation in relatively low cost polythene-covered greenhouses in Lhasa, Tibet.**

Keywords: *Ganoderma leucocontextum*, edible mushroom, cultivation, bioactive compounds, lanostane triterpenes, ganoleucoins, Tibet, China

Background

***Ganoderma* along with the allied taxa *Inonotus* and *Phellinus* are significant pathogens of woody trees, or saprobes that grow on dead wood. More than a hundred species of *Ganoderma* have been characterized using biological and ecological traits but a lack of useful morphological features has led to chaos in the taxonomy of the group (Martin et al. 2015; Richter et al. 2015). However, the application of molecular tools has begun to resolve many of the species complexes and misidentifications (e.g. Coetzee et al. 2015). For example, the widely cultivated species ling-zhi has long been ascribed the name *G. lucidum*, a species described from an English specimen, whereas the correct name is *Ganoderma sichuanense* (Wang et al. 2012).**

The medicinal effects of *G. sichuanense* have been recognised for more than 2000 years and were given considerable attention in the Eastern Han Dynasty (25–220) and Ming (1368–1644) Chinese pharmacopoeias (神农本草经, Shen Nong Ben Cao Jing 2009; 本草纲目, Ben Cao Gang Mu 2016). The common name for *G. sichuanense* ling-zhi first appears during the Eastern Han Dynasty (Yeo 2013). So important were its medicinal effects that images of ling-zhi appear in Chinese paintings, carvings, furniture, figurines and other objets d'art (Yeo 2013; Figures 1 and 2). *G. sichuanense* is now known to contain a number of bioactive compounds including triterpenoids and polysaccharides (Li et al. 2015) and is widely cultivated in China.



Figure 1 (left). Ling-zhi fungus and rocks - an Illustration from the Ten bamboo studio manual of calligraphy and painting (Shizhuzhai shuhua pu). Date painted: after 1633 - before 1703. Harvard Art Museums, USA.



Figure 2 (right). Coral figure holding ling-zhi. 17th Century. Worcester Art Museum, MA, USA.

Because *Ganoderma* had never been cultivated in Lhasa, I decided to collect a number of specimens from Lin Zhi, in southeast Tibet, and isolate cultures. Close examination of the fruiting bodies showed that they were somewhat different from *G. sichuanense* so specimens were sent to Professor Li Tai Hui at the Guangdong Institute of Microbiology. He determined that they were identical to *Ganoderma leucocontextum*, a new species in the *G. lucidum* complex that had just been described (Li et al. 2015). At the same time specimens were analysed by Professor Liu Hong Wei, at the Institute of Microbiology, Chinese Academy of Sciences, Beijing, where it was found to contain 16 new lanostane triterpenes, ganoleucoins, together with 10 known triterpenes (Wang et al. 2015).

Our first attempt to cultivate *G. leucocontextum* in a greenhouse was a complete failure which required a complete rethink. We eventually devised a cost effective method for growing this mushroom in relatively low cost polythene covered greenhouses in Lhasa, which we briefly describe below.

Cultivation

In August and September cultures of *G. leucocontextum* maintained on standard PDA at 25° C, were used to inoculate autoclaved wheat seeds in 250 mL bottles which were then incubated at 25° C for 20-25 days. These were then used immediately or stored in a refrigerator at 4° C until needed. Between September and November a mixture of cotton waste, *Quercus semecarpifolia* (高山栎, gao shan li) sawdust, wheat husk, corn starch and fine calcium carbonate (ratio 40 : 45 : 10 : 5 : 2 v/v) was used to fill 17 cm x 33 cm autoclavable bags which were then closed with rings and stoppers. A hole 1 cm diameter and about 18 to 20 cm deep was bored into the centre of the bag through the ring, a dowel inserted to prevent the hole collapsing and the bags autoclaved. When the temperature of the bags fell below 30° C after autoclaving, under aseptic conditions the dowels were removed, 30 to 40 mL of the wheat inoculum inserted, and the stoppers replaced. The bags were then incubated at 20° C to 25° C for 50 to 60 days.

In late March the bags, rings and stoppers were then removed from the bags and the bags placed either vertically or horizontally with their upper surfaces just under the soil surface in black polythene tunnels where the air temperature ranged from 5° C at night during March to 28° C during summer, and the soil temperatures ranged from 3° C to 20° C. Temperature control was maintained by lifting the covers on the sides to allow air to circulate. The tunnels had previously been used to grow a variety of crops including garlic and celery. Bags were placed in tunnel houses during late March, began fruiting in early July and continued for about one month. Fresh yields ranged from c.0.09 kg to 0.5 kg per half kilogram bag with a mean of 0.13 kg.

Commercialisation

After preliminary discussions a company, Tibet Ganoderma Biotechnology Limited, was established in Lhasa and licensed the technology for the cultivation of *G. leucocontextum*. There has since been keen interest in growing this species elsewhere in Tibet with wide ranging climates. So far the most successful location is in a greenhouse at 4000 m in Jiangzi. *G. leucocontextum* is now a commercially important species.

Acknowledgements

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Figure 3. .



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Screening study of ß-glucan content in commercial and wild mushroom species

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Introduction

Mushrooms have a long history in culinary and medicinal usage in Asia. For years they have become the focus of international medicinal research. Besides a wide variety of health beneficial compounds like phenolic substances, sterols, alkaloids, lactones, terpenes or ceramides, bioactive polysaccharides and polysaccharide-protein complexes are the most studied groups of functional compounds in medicinal mushrooms. These glucans show bioactive properties such as immunomodulating, antitumor, antiviral effects. Beta-glucans and their protein -complexes show positive results in cancer treatment. Natural immune responses are activated and can be used as an adjuvant therapy in combination with chemotherapy and radiotherapy. Many studies proved the positive effect of mushroom products and the increasing numbers of cancer incidences will ask for new treatments in the future[1-5] The main objective of this study was the screening of ß-glucan content in cap and stalk in six commercially cultured mushrooms like *Agaricus bisporus* or *Lentinula edodes* and several wild mushroom species to maintain valuable information about content and distribution of the polysaccharide in mushroom species.

Methods

Fruiting bodies of six common culinary mushrooms were purchased in local supermarkets in Mönchengladbach, NRW, Germany. Several wild mushroom species were collected in a local area of 1 km² in the western part of NRW, Germany between 24.9.2015 and 08.11.2015. The temperature varied between -1,2 °C and 20 °C with rainfall between 0,05 and 42 l/m² per day. Samples were divided into cap and stalk, if possible, dried at 60°C for 16 h and were analysed with an enzymatic assay ß-glucan-kit (Megazyme Ltd, Ireland): Glucans were extensively hydrolysed and incubated with purified enzymes. All over glucan content and α-glucan content were measured photometrically (coloration of glucose molecules with GOPOD-reaction, images 1 and 2) and ß-glucan content was calculated. The method was described before and seems to deliver reliable results for beta-glucan content determination.[6-9] Also a complete statistical validation of the assay kit shows reliability. All wild mushroom samples were also identified with DNA -analysis.



Image 1 and 2: GOPOD reaction with glucose

Results and Discussion

The screening results of several culinary and wild mushrooms show interesting results. The ß-glucan content of culinary mushrooms like *Lentinula edodes* (Shiitake), *Pleurotus eryngii* (King oyster mushroom) *and Pleurotus ostreatus* (Oyster mushroom) show high ß-glucan content of 20-35 % in cap and 25 to over 50 % in stalk. Other species like *Agaricus bisporus* (white and brown Mushroom) show lower content (about 8 to 12 %). (table 1)

Table 1: six common culinary mushrooms and their ß-glucan content in cap and stalk in g per 100 g dry matter ±sd

Name[10]	ß-glucan content in g/100 g dry matter
Agaricus bisporus white cap	8,61±2,37
Agaricus bisporus white stalk	12,30±4,08
Agaricus bisporus brown cap	8,84±3,05
Agaricus bisporus brown stalk	10,08±2,23
Lentinula edodes cap (Shiitake)	19,79±6,24
Lentinula edodes stalk (Shiitake)	25,31±4,38
Pleurotus ostreatus cap (Oyster mushroom)	35,04±6,84
Pleurotus ostreatus stalk (Oyster mushroom)	53,95±10,50
Pleurotus eryngii cap (King oyster mushroom)	28,58±2,15
Pleurotus eryngii stalk (King oyster mushroom)	49,78±5,01
Cantharellus cibarius cap (Chanterelle)	23,59±1,66
Cantharellus cibarius stalk (Chanterelle)	26,93±2,46

The ß-glucan content of 15 wild species varies greatly. For example *Armilliaria mellea* (Honey fungus) showes high ß-glucan content with over 30 % in cap and stalk. Also *Kuehneromyces mutabilis* (Sheated woodtuft) holds high ß-glucan content in its stalk (more than 40%), but a lot less in the cap (18,7%). The study could show that different species of the same genus also show differences: *Xerocomus chrysenteron* (Red cracking bolete) seems to own low ß-glucan values (cap 7,8% and stalk 13,1%), but *Xerocomus baduis* (Bay bolete) owns higher ß-glucan content (20,2% and 31%) . Also *Coprinus comatus* (shaggy ink cap, lawyers wig) does not present very high ß-glucan content in cap (15%) or stalk (17,5%). (table 2)

Table 2: 15 wild mushroom species and their ß-glucan content in cap and stalk (if possible) in g per 100 g dry matter ± sd

Name[10]	ß-glucan content in g/100 g dry matter
Armilliaria mellea cap (Honey fungus)	33,53±1,68
Armilliaria mellea stalk (Honey fungus)	38,77± 5,10
Boletus edulis cap (Penny bun)	16,89±3,03
Boletus edulis stalk (Penny bun)	57,90±9,91
Coprinus comatus cap(shaggy ink cap)	14,98±0,62
Coprinus comatus stalk (Shaggy ink cap)	17,48±1,66
Fomes fomentarius (Hoof fungus)	22,50±2,33
Grifola frondosa (Maitake)	25,99±3,63
Kuehneromyces mutabilis cap (Sheathed Woodtuft)	18,70±2,36
Kuehneromyces mutabilis stalk (Sheathed Woodtuft)	40,60±3,71
Laccaria amethystina cap (Amethyst Deceiver)	26,25±3,47
Laccaria amethystina stalk (Amethyst Deceiver)	37,38±2,92
Laetiporus sulphureus (Chicken of the woods)	47,01±2,52
Lepista nuda cap (Wood blewit)	14,64±3,30
Lepista nuda stalk (Wood blewit)	26,84±0,42
Phallus impudicus cap (Common stinkhorn)	15,92±0,74
Phallus impudicus stalk (Common stinkhorn)	17,27±1,55
Phlebia tremellosa (Jelly rot)	53,56±2,45
Suillus grevillei cap (Larch bolete)	12,93 1,93
Suillus grevillei stalk(Larch bolete)	26,75±2,44
Trametes versicolor (Turkey tail)	60,79±1,80
Xerocomus badius cap (*Imleria badia*, bay bolete)	20,20±1,36
Xerocomus badius stalk (*Imleria badia*, bay bolete)	31,04±2,48
Xerocomus chrysenteron cap (Red cracking bolete)	7,80±0,62
Xerocomus chrysenteron stalk (Red cracking bolete)	13,10±0,95

Especially bracket fungi seem to own very high ß-glucan content. *Grifola frondosa* (Maitake) might be the most famous species and is well known for being rich in ß-glucans and owning pharmacological effectiveness. Our study shows that also other species of bracket fungi show very high content: *Trametes versicolor* (Turkey tail) shows extremely high ß-glucan content with 60% ß-glucan in its dry mass, also *Laetiporus sulphureus* (chicken of the woods, 47%) and *Phlebia tremellosa* (Jelly rot, 54%) own high ß-glucan content. For morphological reason the bracket fungi species were not devided into stalk or cap material.



Image 3-5: *Coprinus comatus, Trametes versicolor, Laccaria amethystina*

Glucan content in stalk is commonly higher than in cap, based on structural composition of the fruiting body. This fact may give rise to new approaches for extraction of ß-glucans from stalkcuts, which are cut off in mushroom production. All over this comparative study provides interesting data about rare mushroom species and their ß-glucan content in comparison with well-known studied species. The results can give new impulses for ß-glucan research and studies.

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Lignosus rhinocerotis on Airway Muscle Relaxation

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Background & Aim

- *Lignosus rhinocerotis* (Tiger Milk Mushroom) has been used for 400 years by the communities in South East Asia for treating asthma and cough. It is from the family of Polyporaceae (Basidiomycota) (***Fig. 1***). The sclerotium is particularly of medicinal value[1] .
- Patients with asthma and chronic cough are known to have airway smooth muscle contraction and hence airway narrowing, leading to breathing difficulty. Asthmatic attack consists of early phase (airway contraction) and late phase (airway inflammation). Current mainstay of treatment of asthma is bronchodilators for early phase and corticosteroid for late phase[2,3].
- No pharmacological studies published to date to support its most popular claims for treating cough and asthma. This study aims to investigate the direct relaxant effects of the *L. rhinocerotis* cold water extract on isolated rat airways.



Fig. 1. L. rhinocerotis comprises pileus (cap), stipe and sclerotium.

Methods

Cultivated sclerotia of *L. rhinocerotis* (TM02) were provided by Ligno Biotech Sdn. Bhd. (Selangor, Malaysia). Cold water extract (see ref. 4 for extraction method) was subjected to organ bath experiment to study the direct effect on airway smooth muscle contractility. Male Sprague-Dawley rats (260-360g; 2-3 months) were sacrificed and dissected. Trachea and bronchus were isolated and hooked in the bath[5]. Carbachol (muscarinic receptor agonist) $1x10^{-6}$M was added to induce tissue contraction.

Then, cold water extract was added cumulatively to the bath until maximal response reached. All the data were expressed as mean ± standard deviation (SD) of *n* number of animals. E_{max} represents maximum relaxation response.

Results

Cold water extract of the mushroom exhibited marked relaxation on the rat tracheal ***(Fig. 2a)*** and bronchial rings ***(Fig. 2b)*** when compared to vehicle control. The extract fully relaxed the tracheal rings (E_{max}: 106.10±8.11%) at 3.75mg/ml and bronchus (E_{max}: 106.45±19.02%) at 2.5mg/ml.

In subsequent experiments ***(Fig. 3)***, tissues were exposed to the extract at varying duration between each concentration to investigate any impact of incubation time. The concentration required to fully relax trachea decreased with longer incubation time intervals, from 4.75mg/ml (5-minute; 49.30 ± 13.57%) to 2.5mg/ml (30-minute; 118.19 ± 17.13%) ***(Fig. 3a)***. Similarly, lower concentrations were required to fully relax bronchus with longer incubation time intervals, from 5.5mg/ml (5-minute; 96.68 ± 8.56%) to 1.75mg/ml (30 -minute; 116.25 ± 19.56%) ***(Fig. 3b)***.

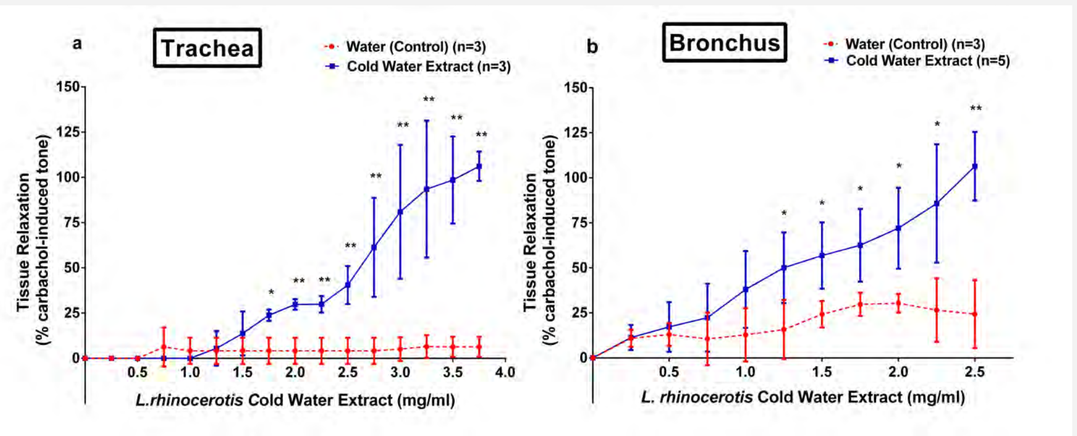


Fig. 2 **The relaxation effect of *L. rhinocerotis* cold water extract on carbachol-induced contraction in isolated rat (a) trachea and (b) bronchus. Incubation time between the additions of each concentration was 15 minutes. The mushroom extract caused significant relaxation in tracheal and bronchial segments when compared to vehicle control (Unpaired T-test; *p< 0.05 and **p<0.01).**

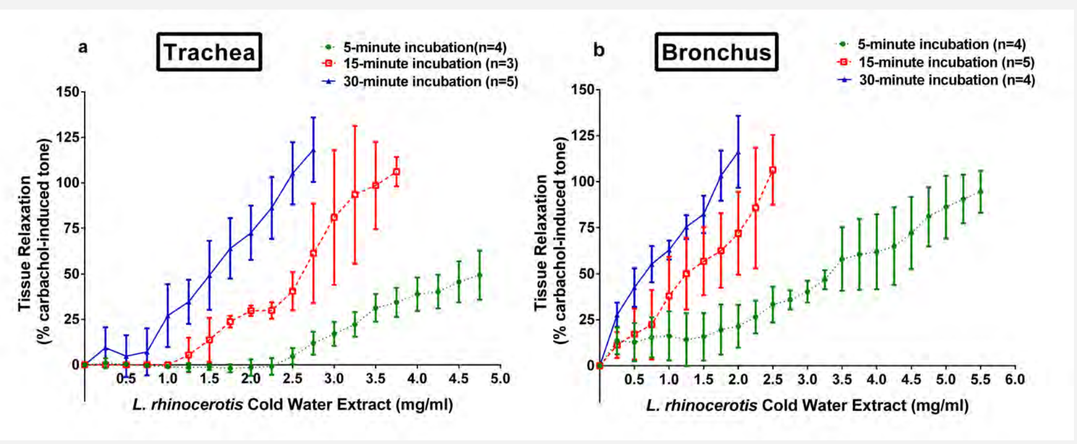


Fig. 3 **Effect of different incubation time of *L. rhinocerotis* cold water extract in isolated rat (a) trachea and (b) bronchus. Overall, a longer incubation time resulted in a greater relaxation response for each concentration tested.**

Overall, the findings suggest that the longer the tissues were exposed to the extract, a greater relaxation response was observed for each concentration tested.

L. rhinocerotis cold water extract was reported to contain mainly carbohydrates and proteins. *β*-glucan is the dominant form of glucan composition[6]. We postulate that the bioactive component in the cold water extract is large polysaccharide or polysaccharide-protein complex, which are less likely to penetrate cells directly. Therefore, a longer response time may be linked to the requirement of these large molecules to adsorb and bind to these surface proteins/ receptors .

Conclusion

- Overall, our finding revealed a **direct relaxant effect of *L. rhinocerotis* cold water extract on pre-contracted rat airways**, in support for its most popular claims to treat cough and asthma.
- The airway relaxant effect is **time– and concentration-dependent**.

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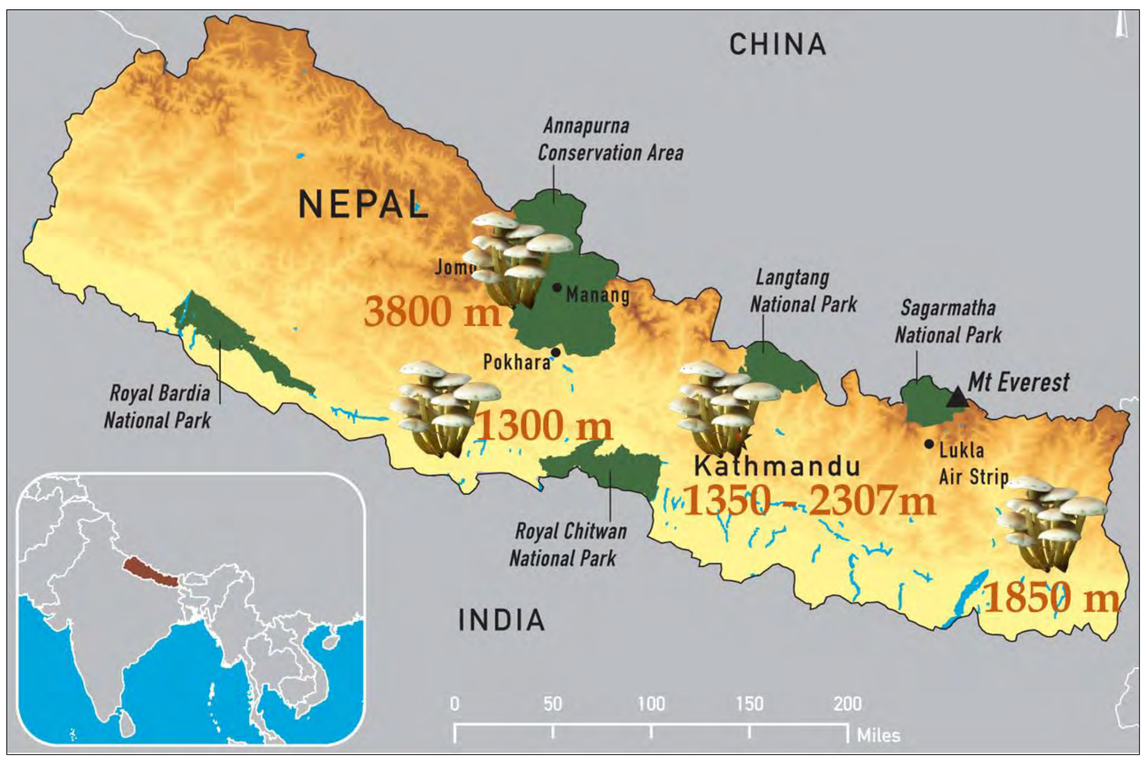
Nepalese Wild Mushrooms: A Treasure Trove of Various Biological Activities

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Background

- Nepal is a country rich in biodiversity, due to its unique geographic location and climatic conditions.
- It is home to rare natural medicinal resources, including various kinds of mushrooms.
- Mushrooms are being recognized for its nutritional as well as medicinal benefits.
- The therapeutic potential of Nepalese wild mushrooms remains largely unstudied[1].
- The aim of this study is to fill this gap by exploring various biological activities of the **ethanol extracts** of the wild mushrooms collected from different parts of Nepal.



Sample collection sites in Nepal

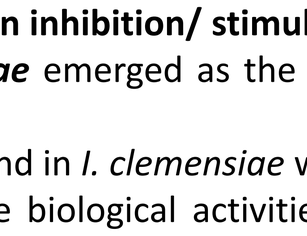
Major Findings

- This study suggests that Nepalese wild mushrooms are a reservoir of various bioactive properties, such as **antioxidant, antibacterial, anti-allergy, and melanin inhibition/ stimulation** effects.
- ***Inonotus clemensiae*** emerged as the strongest candidate among the **91 samples** tested.
- The major compound in *I. clemensiae* was found to be **hispidin**.
- Investigation of the biological activities and bioactive compounds of these mushrooms shall pave way for its commercial use as functional foods.

Methods

Antioxidant Activities
- Free radical scavenging
  ORAC, DPPH, and ABTS assays
- Reducing Capacity
  Total phenol content (TPC),
  Reducing power (RP)

Antibacterial activities
- Inhibition % for *Staphylococcus aureus* and *Propionibacterium acnes*
- Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) for samples with high inhibition%



Ethanol extraction of 91 mushroom samples

Anti-allergy activity
- Inhibition of β-hexosaminidase release % from RBL-2H3 cells
- Cell viability checked by MTT assay

Melanin synthesis activity
- Inhibition or stimulation of melanin content in B16 melanoma cells
- Cell viability checked by MTT assay

Reference: [1]Hai Bang Tran, Hiroto Suhara, Katsumi Doi, Hiroya Ishikawa, Katsuya Fukami, Gopal Prasad Parajuli, and others, 'Wild Mushrooms in Nepal: Some Potential Candidates as Antioxidant and ACE-Inhibition Sources', *Evidence-Based Complementary and Alternative Medicine*, 2014 (2014)

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Contact details

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Results

Table 1: **Antioxidant activities** of the samples with highest TPC values

Sample name	TPC (mg GAE/g)	ORAC (μM TE/g)	DPPH Inhibition %	ABTS Inhibition %	RP 700 nm
Inonotus clemensiae	643.2±21.32	31966.9±198.6	83.2±0.06	92.2±0.05	1.73±0.03
Cyclomyces setiporus	423.7±19.65	11017.9±401.3	86.1±0.11	83.3±0.79	1.64±0.03
Inonotus andersonii	310.8 ± 2.7	21015.4 ± 121.3	36.4 ± 1.0	72.9 ± 2.1	NI
Phellinus gilvus	258.7 ± 4.3	9564.0 ± 281.5	38.1 ± 0.4	55.2 ± 3.2	NI
Cyclomyces setiporus	245.0±2.73	6570.9±169.7	85.3±0.17	67.3±1.14	1.77±0.11
Cyclomyces setiporus	204.5±0.10	2952.3±71.6	88.0±0.07	70.9±0.58	2.01±0.10
Inonotus sp.	155.7±14.55	6015.9±90.2	89.9±0.11	18.7±0.21	1.00±0.01
Cyclomyces setiporus	155.3±8.73	9988.7±664.5	85.1±0.09	75.2±0.65	2.20±0.06
Cyclomyces setiporus	145.0±17.07	7548.79±261.1	86.1±0.10	75.5±0.87	1.97±0.14
Pseudomerulius curtisii	131.6±0.48	11204.9±414.9	89.0±0.29	53.0±0.34	1.10±0.07

GAE is gallic acid equivalent; TE is trolox equivalent; NI is not investigated; values are expressed as mean ±standard deviation; final concentrations of 80, 20, and 110 μg/mL were used for DPPH, ABTS, and RP assays respectively

Table 2: The MIC and MBC values of samples with highest **antibacterial activity**

	S. aureus		*P. acnes*	
Scientific name	MIC	MBC	MIC	MBC
Inonotus clemensiae	100	100	100	200
Postia stipica	-	-	100	200
Pseudomerulius curtisii	100	200	200	400
Cantherellus ferruginascens	100	400	-	-
Inonotus andersonii	200	200	200	200
Polyporus sp.	200	200	-	-
Gymnopus dryophilus	200	ND	-	-
Nigroporus sp.	200	ND	-	-
Oudemansiella sp.	250	400	-	-

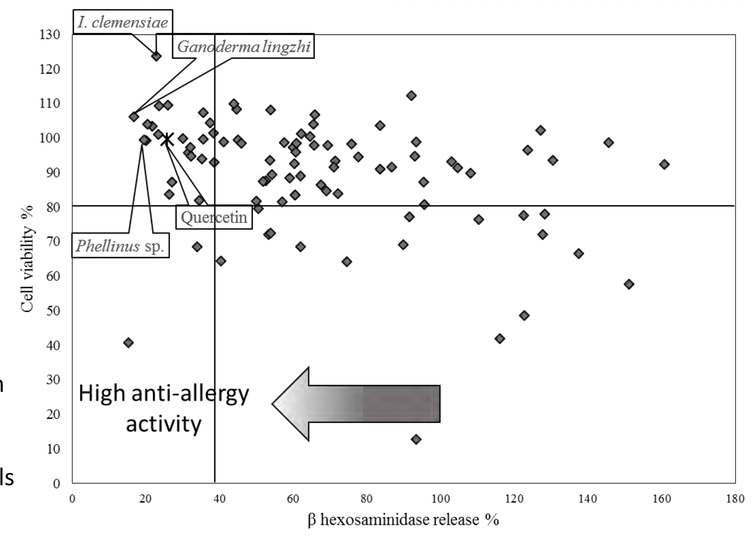
The values are expressed as μg/mL; - means not detected



Anti-allergy



Fig 1. Effect of the mushroom extracts (100 to 400 μg/mL) on β-hexosaminidase release & cell viability in RBL-2H3 cells



Melanin synthesis

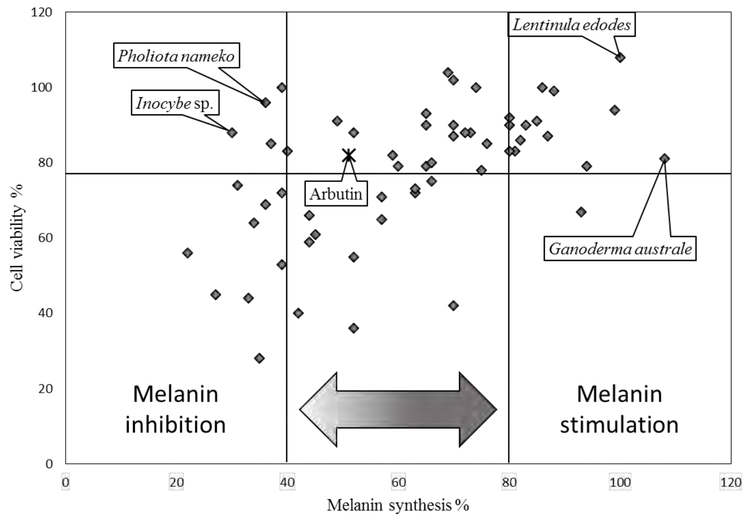
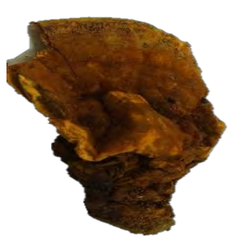


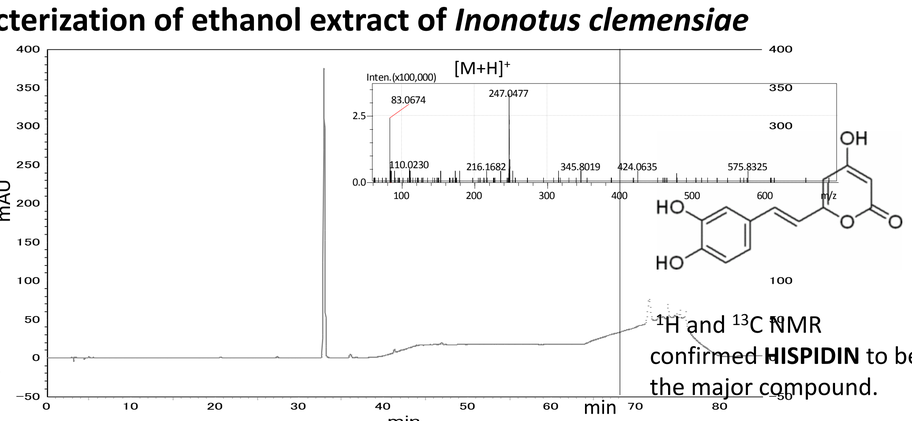


Fig2. Effect of the mushroom extracts (40 μg/mL) on melanin synthesis and cell viability in B16 Melanoma cells

Chemical characterization of ethanol extract of *Inonotus clemensiae*



Inonotus clemensiae



^{1}H and ^{13}C NMR confirmed **HISPIDIN** to be the major compound.

Fig3. HPLC chromatogram and IT-TOF-MS analysis of ethanol extract of *I. clemensiae*

EVALUATION OF 5α-REDUCTASE INHIBITORY ACTIVITY OF CERTAIN HERBS USEFUL AS ANTIANDROGENS

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19th ISMS Congress International Society for Mushroom Science

Amsterdam 2016
29th May - 2nd June

OBJECTIVE OF THE STUDY

With a view to find out some potential 5α-Reductase (5αR) inhibitors from plant origin, we screened eleven herbs reported to have antiandrogenic properties for their 5αR inhibition potential.

HERBS USED IN THE STUDY



Ganoderma lucidum (Curtis) P. Karst
Ganodermataceae GL



Urtica dioica Linn.
Urticaceae UD



Caesalpinia bonducella Fleming
Caesalpiniaceae CB



Tribulus terrestris Linn.
Zygophyllaceae TT



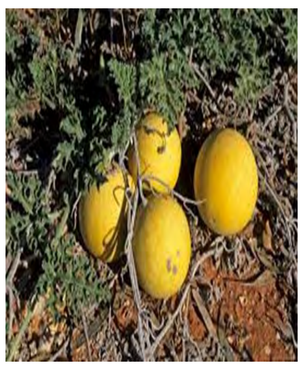
Pedalium murex Linn.
Pedaliaceae PM



Sphaeranthus indicus Linn.
Compositae SI



Cuscuta reflexa Roxb.
Cuscutaceae CR



Citrullus colocynthis Schrad.
Cucurbtaceae CC



Benincasa hispida Cogn.
Cucurbtaceae BH



Phyllanthus niruri Linn.
Euphorbiaceae PN



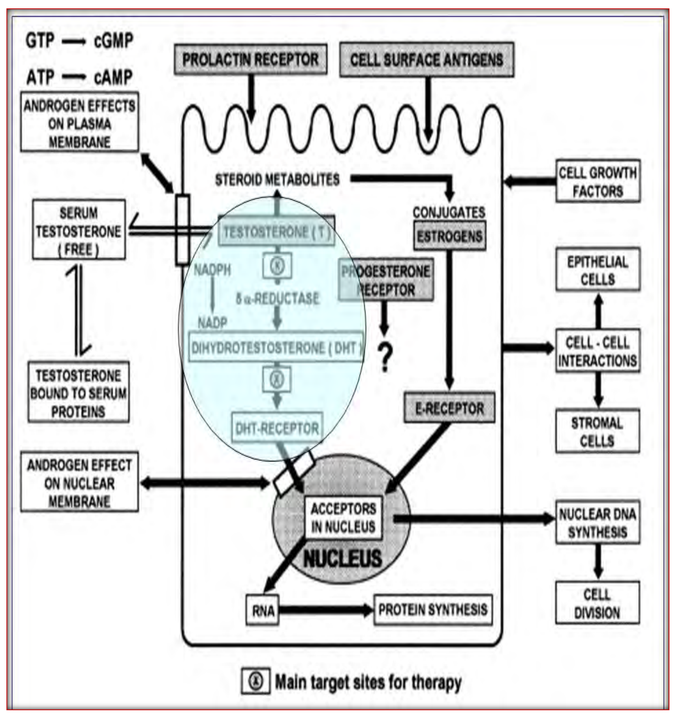
Echinops echinatus Roxb.
Compositae EE

Ganoderma lucidum powder was obtained as a gift from Gano Excel Industries Pvt. Ltd, Malaysia. *Urtica dioica* roots were obtained from Nainital, India. CB, TT, PM, SI, CR, CC, BH, PN and EE were collected from the forest areas of Sagar India and were identified at the Department of Botany, Dr. Hari Singh Gour University, Sagar. All the herbs were coarsely powdered and extracted successively with petroleum ether, ethanol and water using Soxhlet apparatus to obtain the extracts.

ENZYME INHIBITION ASSAY

Preparation of 5αR enzyme solution

Human prostate (about 200 mg) supplied from the local hospital of Sagar was minced in small pieces and homogenized in 10 ml of medium (20 mM sodium phosphate, pH 6.5, containing 0.32 m sucrose and 1 mM EDTA). The homogenate was centrifuged at 4000 rpm (716 g) for 15 min. The supernatant was used as a source of enzyme (Nahata & Dixit, 2012a,b). The concentration of enzyme in the supernatant was determined by Bradford method of protein estimation.



Preparation of standard curve of NADPH

Standard curve of NADPH was prepared in methanol at 340 nm spectrophotometrically using concentrations from 2 to 20 µg/ml. Equation of straight line was y = 0.0555x + 0.0242, and the correlation coefficient (r^2) was = 0.9939 indicating good linearity between absorbance and concentration.

Preparation of test solutions

NADPH solution (22 µM) prepared in methanol; extract solution (1 mg/ml) in parent solvent; Finasteride solution (200 nM) in methanol; testosterone solution (75 µM) in methanol; Tris–HCl buffer (0.5 M) in distilled water.

Preparation of reaction mixtures

Following reaction mixtures were prepared for the experiment:

1 Blank control: It consisted of 3 ml of NADPH (22 µM), 1 ml of Enzyme and 4 ml of Tris–HCl buffer (0.5 M).

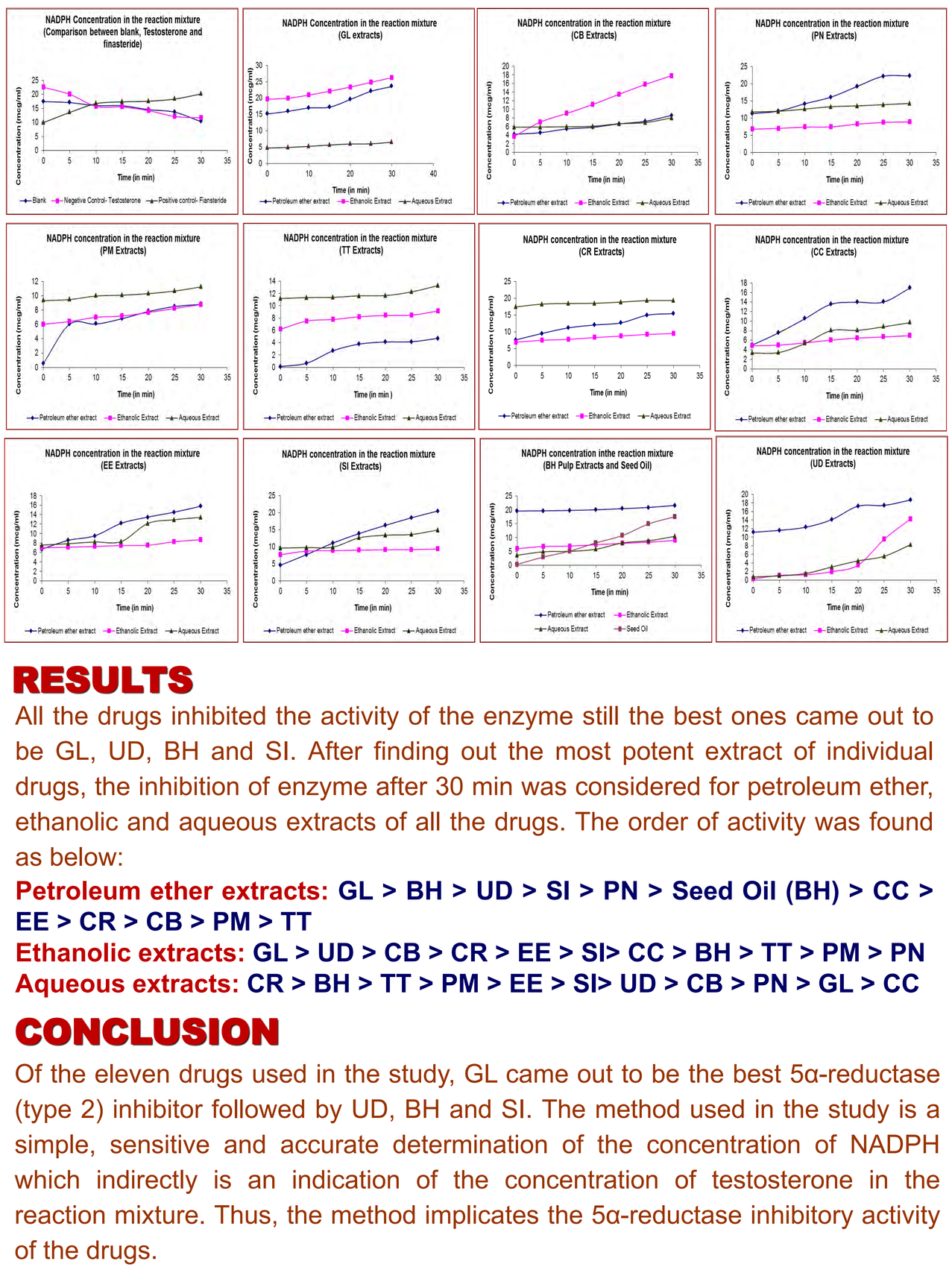
2 Negative control: It consisted of 3 ml of NADPH (22 µM), 1 ml of enzyme, 4 ml of Tris–HCl buffer (0.5 M) and 2 ml of testosterone (75 µM).

3 Positive control: It consisted of 3 ml of NADPH (22 µM), 1 ml of enzyme, 4 ml of Tris–HCl buffer (0.5 M), 2 ml of testosterone (75 µM) and 2 ml of finasteride (200 nM).

4 Test sample: It consisted of 3 ml of NADPH (22 µM), 1 ml of enzyme, 4 ml of Tris–HCl buffer (0.5 M) and 2 ml of testosterone (75 µM) and 2 ml of test extract solution (1 mg/ml in parent solvent).

All the reaction mixtures were scanned spectrophotometrically at 340 nm at the time interval of 5 min for a period of 30 min. The absorbance was noted, and the corresponding concentration of NADPH was calculated from the standard curve. This concentration was used to find out the residual amount of testosterone in the reaction mixture.

Comparative NADPH scavenging activity of the extracts in presence of 5α reductase enzyme in the reaction mixture



RESULTS

All the drugs inhibited the activity of the enzyme still the best ones came out to be GL, UD, BH and SI. After finding out the most potent extract of individual drugs, the inhibition of enzyme after 30 min was considered for petroleum ether, ethanolic and aqueous extracts of all the drugs. The order of activity was found as below:

Petroleum ether extracts: GL > BH > UD > SI > PN > Seed Oil (BH) > CC > EE > CR > CB > PM > TT

Ethanolic extracts: GL > UD > CB > CR > EE > SI> CC > BH > TT > PM > PN

Aqueous extracts: CR > BH > TT > PM > EE > SI> UD > CB > PN > GL > CC

CONCLUSION

Of the eleven drugs used in the study, GL came out to be the best 5α-reductase (type 2) inhibitor followed by UD, BH and SI. The method used in the study is a simple, sensitive and accurate determination of the concentration of NADPH which indirectly is an indication of the concentration of testosterone in the reaction mixture. Thus, the method implicates the 5α-reductase inhibitory activity of the drugs.

Acknowledgements



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29th May - 2nd June

SUITABILITY OF PINK OYSTER MUSHROOM FOR RAINY SEASON CULTIVATION IN THE BELOW MEAN SEA LEVEL TRACTS OF KUTTANAD

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INTRODUCTION

Kuttanad - Rice Bowl of Kerala

Area : 35500 Hectrares
69 Panchayaths :
Districts Covered Alappuzha, Kottayam and Pathanamthitta
Features : 0.5 – 3m <MSL
Climate : Tropical Humid Monsooon
Annual Mean Temperature : 27.6 C
Annual Rainfall : 2746 mm
Humidity : >85%
Total Rice production : 1.6 - 1.75 Lakh tonnes
Total Straw production : 2.25 - 2.5 Lakh tonnes







Straw Utilisation

Left in the field for soil incorporation
Burning of straw
Viable alternative - Mushroom Cultivation
Conversion of Agro waste – Protein rich food

MATERIALS AND METHODS

Varieties used



Pleurotus florida (white oyster)



P.eous (pink oyster)



P.sajor-caju (grey oyster)

- Methodology- Participatory technology development
- Design - CRD- 3 treatments - 6 replications
- Weight of bed-1 Kg dry straw (chemically sterilized)
- Multilayered spawn technique
- Season- Rainy (June- July, 2012)
- Field trial- 10 locations
- Spawn used- first generation

Nutrient analysis of harvested mushroom- Cashew Export Promotion Council, Kollam , Kerala

PTD TRIALS



Mr. Ouseppachen C.K



Mr. Nazar Mustaffa



Mrs. Jolly Joseph



Mrs. Sreelatha Nair

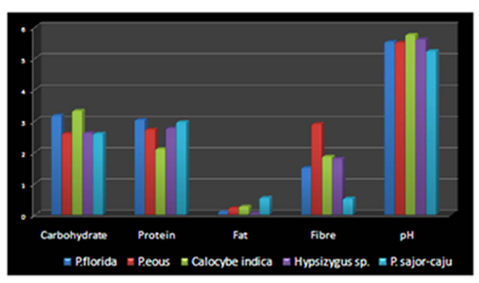
WEATHER FACTORS

Mean max. temperature - 30.75 C Total rainfall - 238.15 mm
Mean relative humidity - 82.45% Total number of rainy days - 34

RESULTS

Table 1 - Yield And Biological Efficiency Of Mushroom

Sl. No	*Mushroom*	*Yield (gm)*	*Biological efficiency (%)*
1	*Pleurotus florida*	*786*	*78.6*
2	*Pleurotus eous*	***875.50***	***87.5***
3	*Pleurotus sajor-caju*	*671.33*	*67.1*
	CD value	79.01	7.9



Y Axis - g/100gm

Figure 1 - Relative Concentration Of Nutritional Factors Of Mushroom Types Cultivated In Kuttanad

Table 2 - Other Observations Of PTD Trial

Sl. No	Varieties	Days for				
		Spawn running	1st harvest	Interval 1st and 2nd harvest	Interval 2nd and 3rd harvest	Cropping period
1	*Pleurotus florida*	16	19	8	6	47
2	*Pleurotus eous*	**10**	**12**	**6**	7	*46*
3.	*Pleurotus sajor-caju*	20	25	10	8	43

Table 3 - Relative Concentration Of Minerals In Mushroom Types Cultivated In Kuttanad

Sl.No	*Parameter*	*Content(mg.kg-1)*				
		P. florida	*P.eous*	*Calocybe indica*	*Hypsizygus*	*P. sajor-caju*
1	Sodium	**100**	40	**100**	50	**100**
2	Potassium	1840	2800	3200	2500	**3300**
3	Calcium	9.03	1.67	4.30	1.31	**118**
4	Magnesium	141.46	65.62	60.14	59.43	**191.1**
5	Iron	10.19	10.91	4.73	12.95	**75.26**
6	Molybdenum	BDL	BDL	BDL	BDL	BDL
7	Selenium	BDL	BDL	BDL	BDL	**0.871**
8	Zinc	5.85	9.2	0.91	5.05	**29.27**

• Chemically sterilized paddy straw

Table 4 - Relative Content Of Nutrient Factors In Fruit Bodies Of Different Mushroom Types And Vegetables

Nutrient factor	Content (g/100g)						*Vegetable		
	P.fl	P. eous	P. sajor caju	Hypsizygus	Milky	Range	Cow pea	Brinjal	Cucumber
Carbohydrate	3.17	1.42	2.59	2.61	3.32	**1.4-3.3**	2.5	4.0	8
Protein	3.03	2.72	2.95	2.75	2.10	**2.1-3.0**	3.8	1.4	0.4
Fat	0.09	0.21	0.54	0.04	0.26	**0.04-0.5**	0.7	0.3	0.3
Fibre	1.5	2.9	0.52	1.79	1.86	***0.5-3.1***	2	1.3	0.45
pH	5.58	5.23	5.23	5.6	5.75				

Table 5 - Relative Content Of Minerals In Fruit Bodies Of Different Mushroom Types And Vegetables

Sl.No	*Parameter*	*Content (mg.kg-1)*							
		P. florida	*P.eous*	*Milky*	*Hypsizygus*	*P. sajor-caju*	Cow pea	Brinjal	Cucumber
1	Sodium	**100**	40	**100**	50	**100**	**232**	30	102
2	Potassium	1840	2800	3200	2500	**3300**	1130	**2000**	500
3	Calcium	9.03	1.67	4.30	1.31	**118**	**720**	180	100
4	Magnesium	141.46	65.62	60.14	59.43	**191.1**	**2100**	153	145
5	Iron	10.19	10.91	4.73	12.95	**75.26**	**25**	3.8	6
6	Molybdenum	BDL	BDL	BDL	BDL	BDL	**18.9**	0	7
7	Selenium	BDL	BDL	BDL	BDL	**0.871**	0	0	0
8	Zinc	5.85	9.2	0.91	5.05	**29.27**	46	2150	**2330**

Table 6 - Relative Concentration Of Nutrient Factors Fruit Bodies Of *P. florida* and *P.eous* Upon Cultivation On Variable Substrates

Sl. No	Mineral	Content (g/100g)						
		P. florida			*P.eous*		*Hypsizygus sp.*	
		Paddy straw	Saw dust	Banana waste	Paddy straw	Saw dust	Paddy straw	Saw dust
1.	Carbohydrate	3.17	**7.59**	1.83	2.59	2.59	2.61	1.87
2.	Protein	3.03	**5.46**	4.65	2.72	2.8	2.76	2.47
3.	Fat	0.09	**0.46**	0.19	0.21	**0.99**	0.04	**0.13**
4.	Fibre	1.5	**1.69**	0.54	2.9	**3.3**	1.79	0.72
5.	pH	5.52	**5.43**	5.32	5.5	5.18	5.6	5.28

Table 7 - Relative Concentration Of Minerals In Fruit Bodies Of *P. florida* And *P.eous* Upon Cultivation On Variable Substrates

Sl. No	Mineral	Content (mg.kg-1)						
		P. florida			*P.eous*		*Hypsizygus sp.*	
		Paddy straw	Saw dust	Banana waste	Paddy straw	Saw dust	Paddy straw	Saw dust
1.	Sodium	100	**250**	100	40	**70**	50	60
2.	Potassium	1800	3050	**5800**	2800	800	2500	2200
3.	Calcium	9.03	**67.07**	*0.01	1.08	0.005	1.31	**233.8**
4.	Magnesium	141.46	196.45	**259.3**	65.62	**203.2**	59.47	**272**
5.	Iron	10.19	**41.89**	37.24	10.23	146.3	12.95	**70.39**
6.	Molybdenum	BDL	BDL	**2.02**	BDL	BDL	BDL	BDL
7.	Selenium	BDL	7.07	BDL	BDL	**0.28**	BDL	**9.84**
8.	Zinc	5.85	**15.39**	10.44	11.95	**59.95**	5.05	**31.95**

Table 8 - Relative Concentration Of Nutritient Factors Of Mushroom Types Under Different Modes Of Sterilization

Sl no	Parameter	Content (g/100g)					
		Pleurotus florida		*Pleurotus eous*		*Pleurotus sajorcaju*	
		Chemical	Steam	Chemical	Steam	Chemical	Steam
1.	Carbohydrate	3.17	**10.1**	2.61	**15**	2.59	3.4
2.	Protein	3.03	2.6	2.76	2.3	2.96	3.1
3.	Fat	0.09	0.1	0.04	0.1	0.54	0.2
4.	Fibre	1.5	**2.2**	2.9	**3.4**	0.52	**1.2**
	pH	5.52	**6.6**	5.5	**6.1**	5.23	**7.0**

Table 9 - Relative Concentration Of Minerals Factors Of Mushroom Types Under Different Modes Of Sterilization

Sl no	Parameter	Content (mg.kg-1)					
		Pleurotus florida		*Pleurotus eous*		*Pleurotus sajor-caju*	
		Chemical	Steam	Chemical	Steam	Chemical	Steam
1.	Sodium	100	34.6	40	21.4	1 0 0	62.3
2.	Potassium	1840	**3821**	2800	2754	3 3 0 0	**3382**
3.	Calcium	9.03	12	1.67	4.6	118	1.9
4.	Magnesium	141.46	138	65.62	**174**	191.1	185
5.	Iron	10.19	13.8	10.91	8.0	75.26	8.5
6.	Molybdenum	BDL	BDL	BDL	BDL	BDL	BDL
7.	Selenium	BDL	BDL	BDL	BDL	**0.871**	BDL
8.	Zinc	5.85	6.9	9.2	16.9	29.27	5.4

CONCLUSION

- Identified ***Pleurotus eous*** (pink mushroom) as the most suited mushroom variety for rainy season cultivation in Kuttanadu region of Kerala state - average yield 875.5g/ bed. Minimum period for completion of spawn running - 10 days
- Nutrient status of mushrooms harvested from the same substrate (paddy straw) varied with mushroom types - Highest concentration of Carbohydrate in Milky mushroom, Protein in ***P.florida*** and Fibre in ***P.eous***
- Mushroom can be substituted for common vegetables like cowpea, brinjal and cucumber
- Nutrient status of mushrooms varied with different Substrates - Molybdenum only in P.florida. from banana waste.
- Rubber wood sawdust - Recommended for cultivation of ***P.florida*** and ***P.eous*** with higher quantity of Proximates - presence of Selenium (antioxidant) Molybdenum only in P.florida.
- Mushrooms from steam sterilized paddy straw – Increased carbohydrate and fibre – pH above 6.0.

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FREE-RADICAL SCAVENGING AND METABOLIC ACTIVITIES OF CULTURED MYCELIUM OF *PAECILOMYCES HEPIALI* EXTRACTS

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ABSTRACT: **In this paper, current research is focused on testing of the antioxidant activity of the mycelium of *Paecilomyces hepiali* Chen cultivated on various plant substrates and metabolic activity assays of this mycelial biomass with a qualitative interesting substances. The antioxidant activity of the mycelial biomass was performed by DPPH˙ radical scavenging method for different ethanolic extracts base of a millet substrate (No. 1) and chickpeas substrate (No. 2). The extract No. 1 corresponds to the value of IC_{50}^{DPPH} = 1.73 mg/ml and the ethanol extract of No. 2 value was almost 4.5 times higher, that is IC_{50}^{DPPH} = 7.92 mg/ml. Extracts No.1 and No. 2 were separated by column chromatography to the fractions and determine the chemical structure of substances which formed the most effective fraction of the sample No. 1. The chemical structure of all compounds of most active fraction of sample No.1 was analyzed by ^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC spectra. Antiproliferative effects of extracts from mycelium of *Peacilomyces hepiali* Chen (strain of fungus Cs-4, maintained by company Mykoforest Velčice under catalogue number MFTCCB022/022013), cultivated on selected substrates of cereals, were tested on three cancer cell lines, namely HeLa, MCF-7 and Jurkat by MTT assays. We observed differences in antiproliferative effects of extracts among tested cell lines, as well as differences related to dilution of tested extracts, usually the most pronounced at highest tested concentration of 1mg/ml.**

The mycelial powder of *Paecilomyces hepiali* has been intensively studied and developed into functional food in China for many years. Polysaccharides, adenosine and cordycepin in the mycelial powder of *Paecilomyces hepiali* are considered as the major functional compositions for the health effects.[2] Studies have shown that *Paecilomyces hepiali* can inhibit tumor proliferation, invasion, metastasis, and neovascularisation, induce apoptosis, reverse drug resistance, enhance immunity, and protect hepatic function.[1]

Within the framework of our project, **fungal biomass has been produced using solid-state fermentation** under precisely defined conditions without free water. The use of a solid substrate is closer to the growth conditions in the natural environment, and also allows the composition of the substrate to be modified in order to change the content of biologically active compounds in a final product and eliminate loss of extracellular metabolites into the surroundings medium.

We now report the ethanolic extraction and chromatographic separation of biologically interesting organic compounds from cultured mycelium of *Paecilomyces hepiali*, the DPPH radical-scavenging activity of total and fractionated extracts, and the elucidation of the chemical structure of isolated compounds by 1D and 2D NMR spectroscopy.

Cervical cancer is the second most common cancer in women worldwide and is a major cause of morbidity and mortality.[2,3] The first human cell line was **HeLa** – named after Henrietta Lacks, the lady from the cells was derived, who had cervical carcinoma. **MCF-7 human breast adenocarcinoma** established in 1973 at the Michigan Cancer Foudation. The popularity of MCF-7 is largely due to its exquisite hormone sensitivity through expression of oestrogen receptor (ER), making it ideal model to study hormone response.[4] **Jurkat** is a **human T cells lymphocyte leukemia**. The Jurkat cell line was established in the late 1970s from the peripheral blood of a 14 year old boy with T cell leukemia.

Antiproliferative effects of extracts from mycelium of *Paecilomyces hepiali*, cultivated on millet and chickpea substrates of cereals, were tested on three cancer cell lines, namely HeLa, MCF-7 and Jurkat.

Extract of sample No.1 (cultured on millet) caused concentration dependent decrease of metabolic activity in HeLa and Jurkat, while antiproliferative effect in MCF-7 was not strong and we did not observe significant differences at any from tested concentration.

Extract of sample No. 2 (cultured on chickpea) caused concentration dependent decrease of metabolic activity in Jurkat, highest tested concentration (1 mg/ml) was lethal. In case of MCF-7 and HeLa we observed significant decrease of metabolic activity in these cell lines, however, dilution factor was not strong enough to be significant.

The structures of the components of the most effective Fraction **IV** of sample No. 1 were determined by NMR spectroscopy (1D - ^{1}H and ^{13}C; 2D - COSY, HSQC, HMBC). Figure 1 shows the proton spectrum of Fraction **IV** of sample No. 1.

TABLE 1 ^{1}H NMR signals of compounds 1-5

Compounds 1-5	Chemical shifts (ppm)	The integrated intensity	Proton	Multiplicity	Coupling constants (Hz)
Glycerol 1	3.43	1	H-2	qi	$^{3}J_{H2\text{-}H1A} = {}^{3}J_{H2\text{-}H1B} = {}^{3}J_{H2\text{-}H3A} = {}^{3}J_{H2\text{-}H3B} = 3.6$
	3.36	2	$H\text{-}1_A,3_A$	dd	$^{2}J_{H1A\text{-}H1B} = 7.2$, $^{3}J_{H1A\text{-}H2} = 3.6$, $^{2}J_{H3A\text{-}H3B} = 7.2$, $^{3}J_{H3A\text{-}H2} = 3.6$
	3.29	2	$H\text{-}1_B,3_B$	dd	$^{2}J_{H1B\text{-}H1A} = 7.2$, $^{3}J_{H1B\text{-}H2} = 3.6$, $^{2}J_{H3B\text{-}H3A} = 7.2$, $^{3}J_{H3B\text{-}H2} = 3.6$
***cis*-oleic acid 2a**	5.32	1	H-10	m	-
	5.31	1	H-9	m	-
	2.17	2	H-2	t	$^{3}J_{H2\text{-}H3} = 4.8$
	2.00-1.98	4	H-8,11	m	-
	1.46	2	H-3	qi	$^{3}J_{H3\text{-}H2} = {}^{3}J_{H3\text{-}H4} = 4.8$
	1.33-1.24	20	H-4,5,6,7,12,13,14,15,16,17	m	-
	0.85	3	H-18	t	$^{3}J_{H18\text{-}H17} = 2.8$
1-monoacylglycerol 3	4.03	1	$H\text{-}1_A$	dd	$^{2}J_{H1A\text{-}H1B} = 7.2$, $^{3}J_{H1A\text{-}H2} = 2.4$
	3.88	1	$H\text{-}1_B$	dd	$^{2}J_{H1B\text{-}H1A} = 7.2$, $^{3}J_{H1B\text{-}H2} = 4.4$
	3.64	1	H-2	m	-
	3.42	1	$H\text{-}3_A$	dd	$^{2}J_{H3A\text{-}H3B} = 7.2$, $^{3}J_{H3A\text{-}H2} = 3.6$
	3.36	1	$H\text{-}3_B$	dd	$^{2}J_{H3B\text{-}H3A} = 7.2$, $^{3}J_{H3B\text{-}H2} = 3.6$
	2.02	3	CH_3	s	-
Succinic acid 4	2.30	4	H-2,3	s	-
Acetic acid 5	1.90	3	H-2	s	-

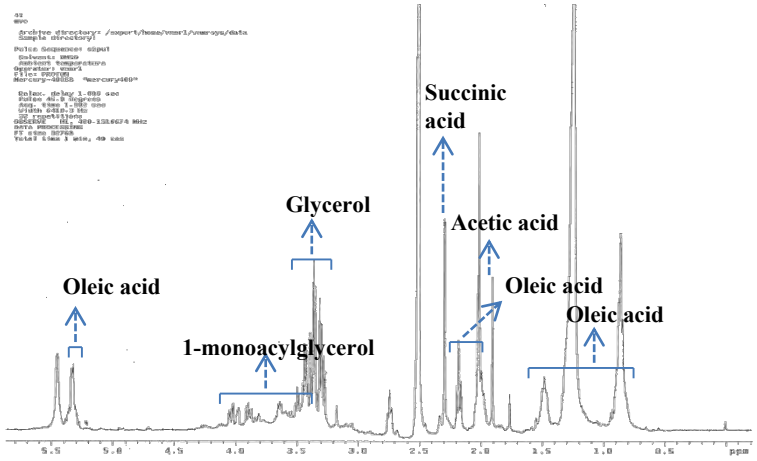


FIGURE 1 ^{1}H NMR spectrum (400 MHz) of the ethanolic extract of *P.hepiali* fraction IV from sample No. 1 measured in DMSO-d_6

Below, we selected 2D NMR spectrum of a mixture of compounds **1-5** and principles for assessing the chemical structure of oleic acid **2**. After careful analysis of HMBC (Fig. 2), based on the transfer of magnetization from the protons on the carbon atoms through three bonds, we managed to confirm the structure of oleic acid **2a** and to determine the quaternary carbon C=O of the carboxyl group. We can see a significant transfer of protons H-2 and H-3 to the carbon of a carbonyl group in a carboxylic acid **2a** (Fig.2). Interesting transfer of magnetization through three bonds were also observed from the olefinic protons H-9 and H-10 to the C-7 and C-12, and from the proton H-8 and H-11 to the sp^2 carbon C-9 and C-10.

The fact this is oleic acid **2a** and not the *trans*-isomer, e.g. elaidic acid **2b** could not be determined based on the size of the coupling constants of H-9,10 due to the difficult digestion of these protons.

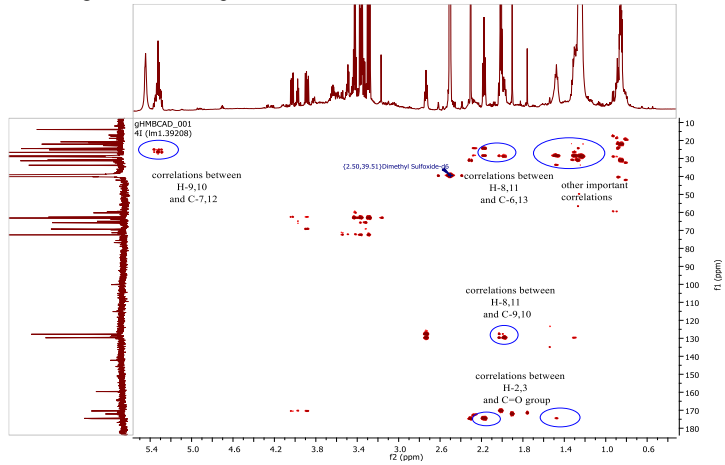


FIGURE 2 HMBC spectrum of oleic acid 2a. Selected cross peaks (in the ring) confirming the structure.

The geometry of the multiple bond can be determined on the basis of the chemical shift of carbon atoms present in *α*-position relative to the double bond, and which are extremely sensitive to steric effects (Fig. 3).

Since the chemical shifts of the carbons C-8, C-11 obtained from the HSQC spectra of oleic acid **2a**, were in 26.3 ppm (elaidic acid **2b** has spectrum in 32.0 ppm, this value was obtained from the database NMR) therefore, we can say that this is a *cis*- isomer of oleic acid **2a**.

The same analysis of other signals in the 1D and 2D NMR spectra were used for determination of the chemical structure of the four other compounds **1**, **3**, **4** and **5**.



cis- **2a** *trans*- **2b**

FIGURE 3 Use of chemical shifts of carbon signals in determining the stereochemical configuration *Z*-, respectively. *E*-.

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ACKNOWLEDGMENTS

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Effect of Different Cooking Methods on Nutritional Value and Antioxidant Activity in Cultivated Mushrooms

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INTRODUCTION

The most mushrooms are commonly cooked before being consumed. Thermal processing may lead to change the nutritional value of the final product, it is well-known that most of the bioactive compounds are relatively unstable to heating. On the other hand, new substances can be formed with interesting biological properties such as antioxidant activity. Thus, chemical and nutritional characteristics of mushrooms are not only strictly dependent on the species but also on the cooking method applied.

Objective: This study aimed to evaluate the effect of different cooking methods on proximate composition, β-glucans content and antioxidant activity of two cultivated mushrooms species.

METHODS

Cultivation at CTICH facilites



Agaricus bisporus



Lentinula edodes



Culinary treatments

- Boiling
- Microwaving
- Grilling
- Deep frying





Lyophilisation and powdering

1. Proximate composition analysis



A.O.A.C. (1995)

- Moisture
- Ash
- Fat
- Proteins
- Carbohydrates
- Energy

2. Glucans content

3. Absorbance at 420 nm



4. Antioxidant activity determination



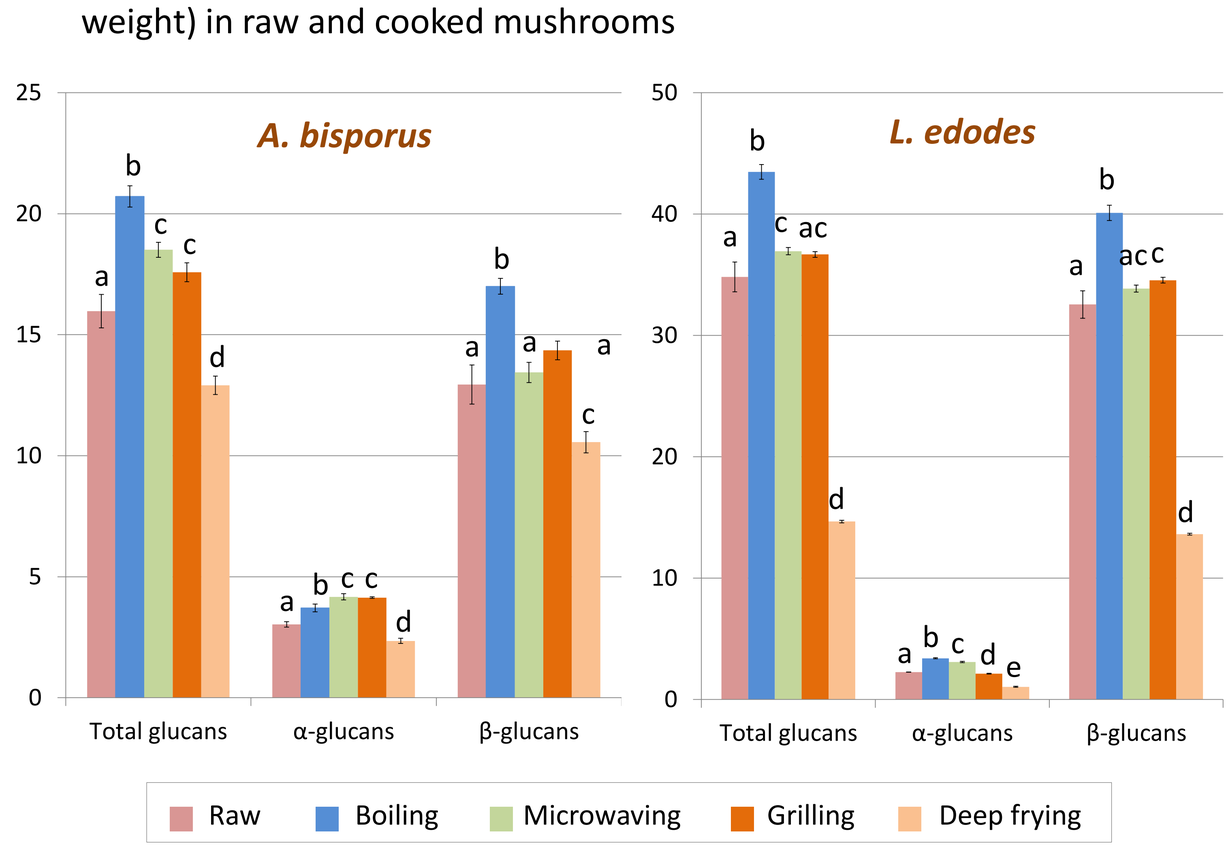
RESULTS AND DISCUSSION

Table 1. Proximate composition (g/100g dry weight) of raw and cooked mushrooms [a)]

	Cooking method	Moisture	Ash	Protein[b]	Fat	Carbohy-drates	Energy
		%					
A. bisporus	Raw	89.71 ± 0.25[a]	9.40 ± 0.06[a]	24.64 ± 0.17[a]	2.34 ± 0.03[a]	63.66 ± 0.24[a]	374.2 ± 0.5[a]
	Boiling	85.87 ± 0.24[b]	5.46 ± 0.09[b]	26.56 ± 0.37[b]	1.19 ± 0.02[b]	66.55 ± 0.37[b]	384.4 ± 0.2[b]
	Microw.	82.14 ± 0.72[c]	8.21 ± 0.04[c]	23.87 ± 0.14[c]	1.42 ± 0.01[b]	66.62 ± 0.09[b]	374.4 ± 0.2[a]
	Grilling	79.62 ± 1.09[d]	8.81 ± 0.10[d]	23.67 ± 0.18[c]	3.27 ± 0.08[c]	64.06 ± 0.04[a]	381.0 ± 1.0[c]
	Frying	56.24 ± 1.01[e]	5.01 ± 0.05[e]	15.27 ± 0.14[d]	45.08 ± 0.19[d]	34.81 ± 0.10[c]	605.3 ± 0.6[d]
L. edodes	Raw	87.83 ± 1.08[a]	7.36 ± 0.01[a]	16.82 ± 0.36[a]	2.06 ± 0.01[ab]	73.43 ± 0.21[a]	380.9 ± 0.1[a]
	Boiling	89.14 ± 0.24[a]	4.49 ± 0.10[b]	16.88 ± 0.08[a]	1.57 ± 0.03[a]	77.02 ± 0.14[b]	389.5 ± 0.3[c]
	Microw.	81.11 ± 0.75[b]	6.73 ± 0.07[c]	16.75 ± 0.10[a]	1.98 ± 0.03[ab]	74.40 ± 0.14[a]	382.9 ± 0.2[ab]
	Grilling	78.31 ± 0.54[c]	6.89 ± 0.07[c]	16.50 ± 0.04[a]	3.00 ± 0.04[b]	73.62 ± 0.01[a]	387.5 ± 0.7[bc]
	Frying	39.44 ± 0.26[d]	2.66 ± 0.02[d]	5.90 ± 0.08[b]	62.27 ± 0.75[c]	29.20 ± 0.88[c]	700.6 ± 3.7[d]

[a)] Values are means ± SE, n = 3. Different letters within a column indicate significant differences between raw and cooked samples of each mushroom ($P < 0.05$).

Figure 1. Contents of total glucans, α-glucans and β-glucans (g/100g dry weight) in raw and cooked mushrooms



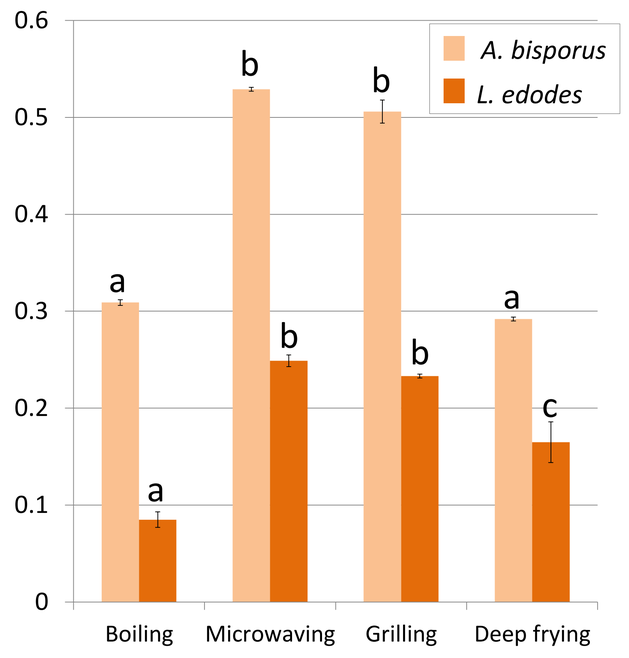
Different letters indicate significant differences between raw and cooked samples of each mushroom ($P < 0.05$)

Table 2. Antioxidant activity and total polyphenols content in raw and cooked mushrooms [a)]

Sample	Cooking method	Polyphenols	ABTS	DPPH	FRAP
		μg GAE/100 mg	μmol TE/100 mg		
A. bisporus	Raw	181.99 ± 2.29[a]	2.333 ± 0.018[a]	0.752 ± 0.005[a]	2.358 ± 0.030[a]
	Boiling	96.31 ± 1.21[b]	1.120 ± 0.025[b]	1.268 ± 0.015[b]	1.243 ± 0.008[b]
	Microwav.	170.79 ± 1.97[c]	1.388 ± 0.033[c]	1.880 ± 0.020[c]	1.927 ± 0.017[c]
	Grilling	166.87 ± 2.68[c]	1.581 ± 0.021[d]	1.808 ± 0.039[d]	2.198 ± 0.032[d]
	Frying	130.87 ± 2.35[d]	1.372 ± 0.012[c]	1.426 ± 0.018[e]	1.811 ± 0.013[e]
L. edodes	Raw	104.35 ± 1.13[a]	0.413 ± 0.007[a]	1.012 ± 0.024[a]	1.376 ± 0.017[a]
	Boiling	54.64 ± 0.66[b]	0.383 ± 0.004[b]	0.350 ± 0.006[b]	0.439 ± 0.006[b]
	Microwav.	147.03 ± 1.91[c]	0.405 ± 0.003[a]	2.310 ± 0.015[c]	2.100 ± 0.014[c]
	Grilling	151.67 ± 1.62[d]	0.480 ± 0.004[c]	2.221 ± 0.027[d]	1.985 ± 0.014[d]
	Frying	44.01 ± 0.49[e]	0.329 ± 0.004[d]	0.334 ± 0.009[b]	0.393 ± 0.006[e]

[a)] Values are means ± SE, n = 3. Different letters within a column indicate significant differences between raw and cooked samples of each mushroom ($P < 0.05$).

Figure 2. Mean absorbance values at 420 nm (unspecific index of Maillard reaction development).



Different letters indicate significant differences between samples of each mushroom ($P < 0.05$)

CONCLUSION

Frying produced more severe losses in protein, ash and carbohydrates content but increased the fat and energy. Boiling improved the β-glucans content. Grilled and microwaved mushrooms shown higher antioxidant activity, especially in *L. edodes*. Probably, the antioxidant activity increases due to Maillard reaction development as supported by the highest absorbance values at 420 nm. Microwaving and grilling were the more adequate culinary treatments to preserve the nutritional profile of mushrooms.

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Method with High-Throughput Screening Potential Using *E. coli* Biosensor Enables Searching for Bioactive Compounds from Fungi

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Introduction

Endophyte fungus strains were isolated and pure cultured from Scots pine root tips grown in organic soil in western Finland. The 130 fungus strains were sequenced (ITS1/ITS4) and they belonged to ten different fungus species. Water extracts of the fungal mycelium were tested for their antioxidant properties with H_2O_2 scavenging test [1]. Also a new microplate technique was used for the rapid real-time screening of antioxidant and antimicrobial properties from extracts and fractions using a recombinant *Escherichia coli* DPD2511 and DPD2794 biosensors [2,3].

Materials and methods

The microbial biosensors used here are manipulated to produce luminescent light in the presence of oxidative or DNA damaging agents and can be used to measure either both oxidative and antioxidant activity or DNA damage induced to the bacteria. Three endophytic fungus species *Acephala applanata* (A), *Phialocephala fortinii* (R) and *Humicolopsis cephalosporioides* (S16) showed activity with biosensor methods used and were selected for further testing. The fungal extracts were fractionated with preparative HPLC (Shimadzu Prominence) (Fig. 1a) and the bioactive fractions were further analysed with LC-MS analysis (Agilent 1200 LC- system with Bruker maXis HR-QTOF mass spectrometer coupled to the LC system).

Results and discussion

The results obtained in this study show that the developed biosensor method with using recombinant *E. coli* harboring *katG'::lux* fusion is suitable to screen for the antioxidative and antimicrobial properties of sample extracts and fractions in a simple, rapid and reliable manner, and it embodies real HTS potential also for the screening of very minor concentrations of sample material. The results found with LC-MS are shown in the Table 1 and the unknown compounds found are highlighted in blue. Because water extracts were used, they also contain levels of water soluble primary metabolites such as arginine, mannitol and adenosine. These metabolites are, however, common in all fungi including those not showing bioactivity and are also produced by the *E. coli* and do not themselves show activity in the biotests used. It is likely that the peaks of the unknown components with potential pharmacological interest are overlapping with the large peaks of known metabolites and can therefore not be detected with the method used. Further analysis is in progress.

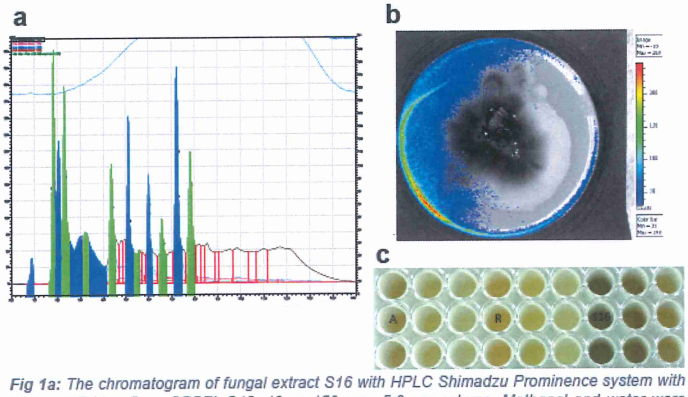


Fig 1a: The chromatogram of fungal extract S16 with HPLC Shimadzu Prominence system with Waters XBridge Prep OBD™ C18, 19 × 150 mm, 5.0 µm column. Methanol and water were used as the eluents. Extracts were fractioned and the activities of the fractions were tested separately for their bioactivity.

Fig 1b: S16 fungus on Hagem agar plate with bacterial strain producing constitutive luminescent light signal. Fungal mycelium is producing antimicrobial metabolites, which disturbs the light signal by inducing death of bacterial cells.

Fig 1c: Fungal extract dilution triplicates in microplate wells before further dilution for the measurements with biosensors.

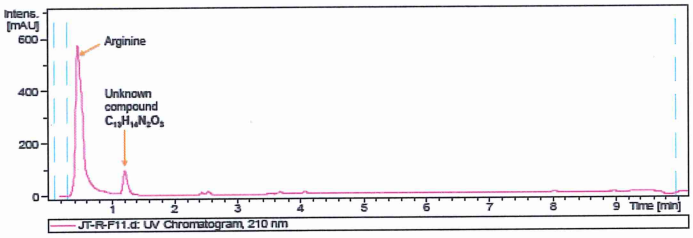


Fig 2: The UV Chromatogram of the P. fortinii fraction 11 shows peaks of arginine and an unknown compound. LC-MS eluent A consisted of 10% acetonitrile and 90% water with 0.01% trifluoroacetic acid and 1.3 mM ammonium formate, while eluent B was 90% acetonitrile and 10% water with 0.01% trifluoroacetic acid and 1.3 mM ammonium formate. In most of the fractions the peaks occurred immediately after the start of the run and are most likely overlapping.

Table 1: The identified fractions and their activities in the biosensor and hydrogen peroxide scavenging tests. O = E. coli DPD2511 Oxidative stress; D = E. coli DPD2794 DNA damage; A = E. coli DPD2511 Antioxidative activity and S = Hydrogen peroxide scavenging test.

A. Applanata	Activity	*P. fortinii*	Activity	*H. cephalosporioides*	Activity
F2 – Arginine, Mannitol	O,D,S	F2 – Arginine	O,D,S	F1 - Arginine	A
F3 – Arginine	O,D,A, S	F3 – Arginine	O,D,A	F2 – Arginine, $C_{10}H_{15}N_3O_5$	O,D
F5 – Arginine and $C_{14}H_{26}O_{10}$	A,S	F4 – Arginine, Rhamnose	D,A,S	F3 – Arginine, $C_{10}H_{15}N_3O_5$	O,A
F13 – Adenosine, $C_{10}H_{22}O_8$, Tryptophan, $C_{11}H_{20}N_2O_3$	A	F7 – $C_{16}H_{30}O_{11}$, $C_8H_{15}NO_3$	O,S	F7 – Arginine, $C_{14}H_{16}O_{10}$	O
		F10 – Arginine, $C_{14}H_{28}O_3$	D,A,S	F13 – Adenosine	D,A,S
		F11 – Arginine, $C_{13}H_{14}N_2O_3$	O	F14 – Arginine, Adenosine, Tryptophan	O,S
		F13 – Arginine	A		
		F14 – Arginine, Adenosine	D,A,S		
		F15 – Arginine, Tryptophan	A,S		

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luke.fi

Proximate Composition, β-Glucan Content, Dietary Fibre and Antioxidant Activity in Mushrooms Cultivated in La Rioja, Spain

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INTRODUCTION

Several mushroom species have been pointed out as sources of antioxidant compounds, besides other bioactive compounds such as health-related polysaccharides. Moreover, mushrooms are a potential source of dietary fibre and interesting bioactive components like β-glucans which are proved to be useful for the treatment of cancer and various infectious diseases. Also mushrooms contain phenolic compounds that have been found to be excellent antioxidants.

Objective: The objective of this study was to determine proximate composition, dietary fibre and β-glucan contents as well as antioxidant activity in seven edible mushrooms cultivated in La Rioja, Spain

METHODS

Cultivation at CTICH facilities

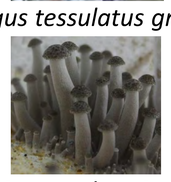
Agaricus brunnescens Portobello ABp



Hericium erinaceus HE



Hypsizygus tessulatus grey HTg



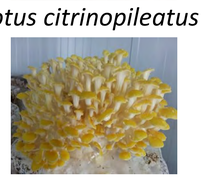
Hypsizygus tessulatus white HTw



Hypsizygus ulmarius HU



Pleurotus citrinopileatus PC



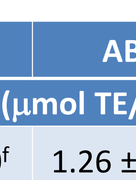
Pholiota nameko PN





Lyophilisation and powdering

1. Proximate composition analysis



A.O.A.C. (1995)

- Moisture
- Ash
- Fat
- Proteins
- Carbohydrates
- Energy

2. Glucans content Absorbance at 420 nm



3. Fibre

4. Antioxidant activity and phenols content



Statistical analysis: one-way analysis of the variance (ANOVA) and Duncan's test ($P < 0.05$). Relationship between the different variables was carried out by computing the relevant correlation coefficient (Pearson's linear correlation) at the $p < 0.05$ confidence level).

RESULTS AND DISCUSSION

Table 1. Proximate composition (g/100g dry weight) of mushrooms [a)]

Mush-rooms	% Humidity	% Ash	% Fat	% Protein	% Carbohy-drates	Energy (kcal)
ABp	88,7±1,26^{bc}	10,4±0,01^{d}	1,7±0,11^{a}	27,8±0,28^{d}	60,1^{b}	367^{a}
HE	87,1±0,13^{a}	7,8±0,14^{b}	3,6±0,43^{abc}	15,3±0,02^{a}	73,2^{de}	386^{bcd}
HTg	90,3±0,05^{d}	7,5±0,24^{b}	4,2±0,67^{bcd}	19,3±0,13^{b}	69,1^{cde}	390^{cd}
HTw	89,7±0,08^{cd}	9.0±0,19^{c}	2,8±0,28^{abc}	22,3±0,05^{c}	66.0^{bcd}	378^{b}
HU	89,1±0,09^{bc}	10,2±0,09^{d}	5,1±0,38^{cd}	36,7±0,10^{e}	48,3^{a}	385^{bc}
PC	88,1±0,33^{ab}	8,5±0,02^{c}	6,1±0,03^{d}	26,6±0,14^{d}	58,8^{bc}	396^{d}
PN	91,3±0,14^{e}	6,4±0,12^{a}	2,0±0,67^{ab}	15,6±0,15^{a}	75,6±^{e}	384^{bc}

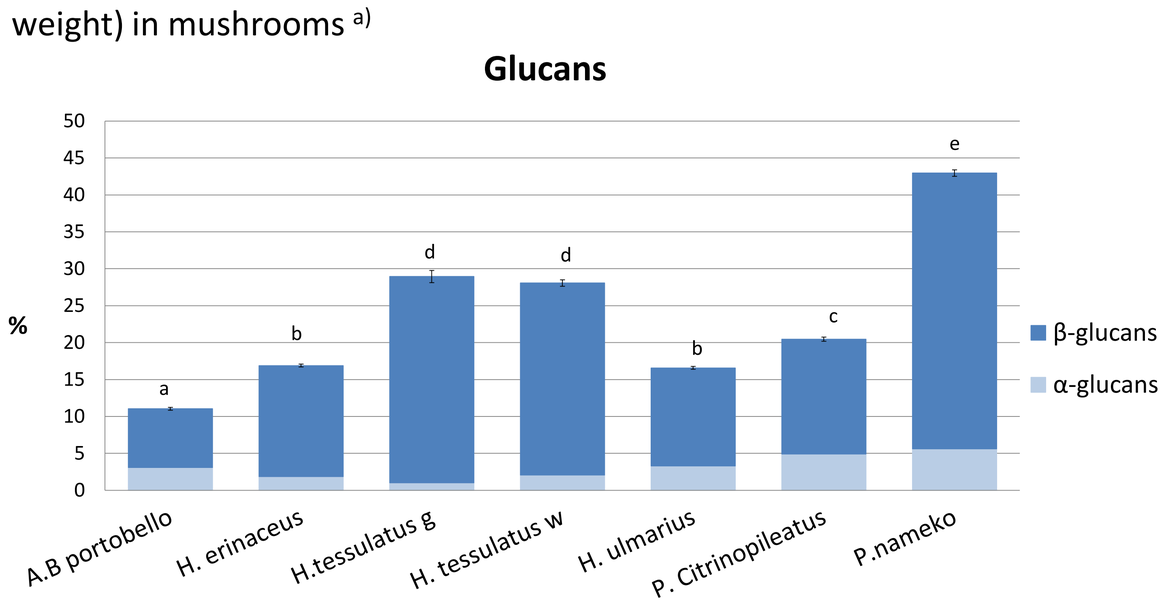
[a)] Values are means ± SEM, n = 3. Different letters within a column indicate significant differences between samples of each mushroom ($P < 0.05$).

Table 2. Antioxidant activity and total phenols content in mushrooms [a)]

Mushrooms	Total Phenolic content (μg GAE/100 mg)	DPPH	ABTS	FRAP
		(μmol TE/100 mg)		
Abp	252.5 ± 3.66^{d}	2.38 ± 0.00^{f}	1.26 ± 0.04^{d}	2.38 ± 0.03^{c}
HE	169.6 ± 5.18^{c}	1.77 ± 0.02^{d}	2.46 ± 0.05^{e}	2.45 ± 0.06^{c}
HTg	86.6 ± 2.11^{b}	0.53 ± 0.00^{b}	0.38 ± 0.01^{a}	0.49 ± 0.01^{a}
HTw	63.6 ± 1.18^{a}	0.28 ± 0.01^{a}	0.38 ± 0.00^{a}	0.52 ± 0.02^{a}
HU	286.9 ± 3.97^{e}	1.83 ± 0.01^{e}	0.52 ± 0.02^{bc}	0.97 ± 0.01^{b}
PC	274.7 ± 5.18^{e}	0.70 ± 0.01^{c}	0.54 ± 0.00^{c}	2.41 ± 0.03^{c}
PN	98.3± 1.49^{b}	0.66 ± 0.01^{c}	0.41 ± 0.02^{ab}	0.58 ± 0.01^{a}

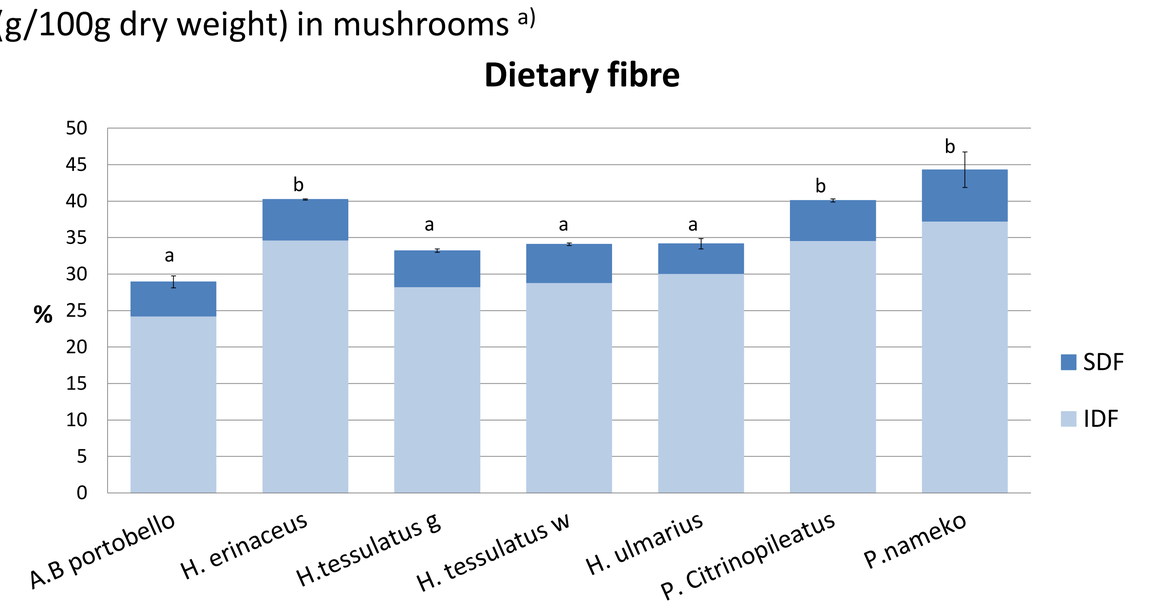
[a)] Values are means ± SEM, n = 3. Different letters indicate significant differences between samples of each mushroom ($P < 0.05$)

Figure 1. Contents of total glucans, α-glucans and β-glucans (g/100g dry weight) in mushrooms [a)]



[a)] Values are means ± SE, n = 3. Different letters within a column indicate significant differences between samples of each mushroom ($P < 0.05$).

Figure 2. Contents of total dietary fibre, Insoluble and Soluble dietary fiber (g/100g dry weight) in mushrooms [a)]



[a)] Values are means ± SEM, n = 3. Different letters indicate significant differences between samples of each mushroom ($P < 0.05$)

CONCLUSION

Significant differences in proximate composition among the seven mushrooms were found, being remarkable the low fat content in ABp and the high content of protein in *H. ulmarius.*

The β-glucan content ranged from 7.96 % to 37.35 % *for A. brunnescens* and *P. nameko* respectively and from 28.93 % to 44.30 % in the TDF content in both species. There were significant differences among the species, but it may be noted that *H. tessulatus* did not have differences in both grey and white colour strains.

The antioxidant activity showed that mushrooms are a good source of antioxidants, being *A. brunnescens, P.citrinopileatus* and *Hericium erinaceus* those with highest antioxidant activity, while both *Hypsizygus tessulatus*, white and grey, showed the lowest concentration.

Acknowledgments: This research was financed by the Department of Industry, Innovation and Employment of La Rioja and the Economic Development Agency of La Rioja (ADER) (2014-I-IDD-00051).

ANTIOXIDANT ACTIVITY OF SUBMERGED MYCELIUM, CULTURE FILTRATE AND FRUITING BODY OF CLAVARIADELPHUS TRUNCATUS

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To improve defence system capacity of the body, a lot of different synthetic antioxidants have been used. But, natural antioxidants are preferred because of toxic and mutagenic effects of synthetic ones. Edible mushrooms are good candidates for natural antioxidants sources. Because they includes compounds have antioxidant activity such as phenolics, polysaccharides, proteins, organic acids, vitamins, carotenoids, and alkaloids.

Clavariadelphus truncatus is a wild edible mushroom species. This species is known as producer of clavaric acid, a compound to reduce the rate of tumor development. But, there is a gap about antioxidant activity of the species. Life form of the mushroom species is one of the major factors affect chemical composition and antioxidant potential of them. Therefore, the main goal of the present work was to compare the antioxidant potential of *Clavariadelphus truncatus* culture filtrate and mycelia produced by in vitro culture, and fruiting bodies. As a preliminary study, DPPH radical scavenging activity of culture filtrate and mycelia produced by *in vitro* culture and fruiting body of *Clavariadelphus truncatus* were determined as 89.65 ± 0.08, 17.88 ± 0.15, and 42.91 ± 0.06, respectively.

AIMS



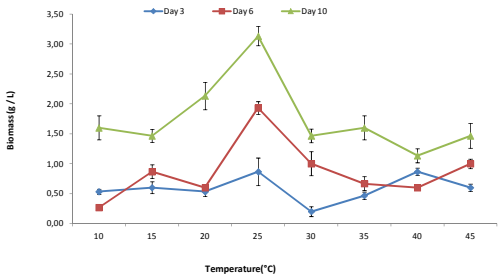
To optimize mycelial growth

To determine life form demonstrated higher antioxidant activity

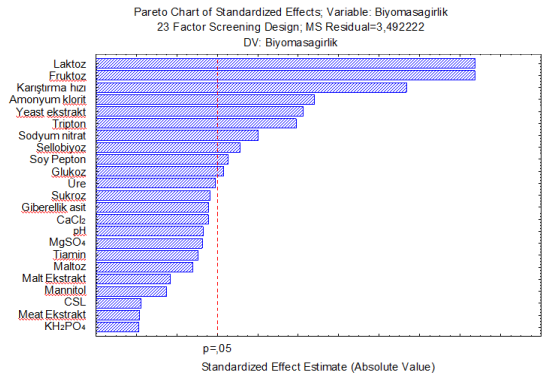
To determine the best incubation time for the harvest of the culture.

OPTIMIZATION OF MYCELIAL GROWTH

Incubation Temperature



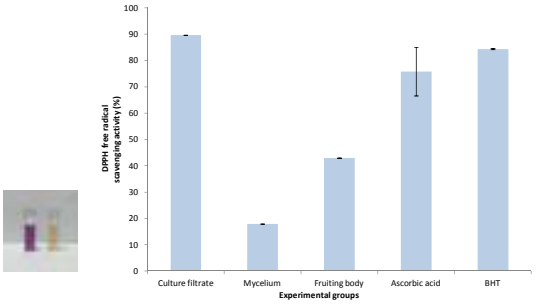
Plackett-Burman Design



Parameters		Value
Carbon source (g/L)	Lactose or Fructose	10
Nitrogen source (g/L)	Ammonium chloride or Yeast extract	5
Mineral source(g/L)	$MgSO_4$ and $CaCl_2$	2
Incubation temperature (°C)		25
Medium pH		5

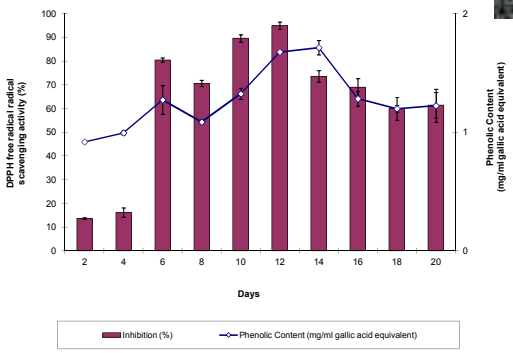
SELECTION OF LIFE FORM

DPPH Free Radical Scavenging Activity





TIME DEPENDENT ANTIOXIDANT ACTIVITY



CONCLUSIONS

The best;

Medium : Lactose or Fructose (20 g), Ammonium chloride or yeast extract (5 g), $MgSO_4$ (2 g) , $CaCl_2$ (2 g), Distilled water (1000 ml), pH: 5.

Incubation temperature : 25 °C

DPPH activity : Culture filtrate

Incubation time : 12 days

Acknowledgement

The study was supported financially by Eskisehir Osmangazi University Research Foundation (Grant number: 2015-675)

Cultivation of Chaga in Low-productive Peatland Forests – Comparison of Antioxidative Properties of Conks and Inoculants

Eira-Maija Savonen, Niko Silvan, Tytti Sarjala

Introduction

Chaga mushroom (*Inonotus obliquus*) is a wood-rotting fungus of deciduous trees. The sterile sclerotial conk on the trunk of living tree has been used as folk medicine for centuries. Later comprehensive studies have revealed chaga mushroom to contain a large variety of biologically active substances with antioxidant, antitumoral, antiviral and immune response effects. Phenolic compounds, melanins, and triterpenoids among others have been identified. Several chaga-containing products for wellbeing have been developed and are available on the market. In order to ensure constant supply of raw material for entrepreneurs, and to get additional incomes from nutrient poor, low-productive drained peatland forests a non-sterile cultivation protocol for chaga mushroom was developed.

Materials and methods

We inoculated 90 birch trees altogether by planting a small piece of mycelium containing agar into hollows drilled into the trunk. We used three different chaga strains in order to study the differences between the strains in the success of infection, growth and antioxidative properties.

Inonotus obliquus mycelium pure cultures were performed by cutting small pieces from the conk and laying them on Hagem agar plates after a short bath in H_2O_2. Identification of the isolated fungus strain was confirmed by sequencing the amplified ITS-region of the fungal DNA by using ITS1and ITS4 primers.

Antioxidative properties of the mycelium and conks of the different *Inonotus obliquus* strains were studied with 96-microplate techniques. The fungus mycelium was extracted either with ethanol or hot water and the antioxidative properties were tested with ORAC (Oxygen radical absorbance capacity) (Prior et al 2003 [1]) and SCAV (H_2O_2 scavenging) (Hazra et al 2008 [2]) tests.

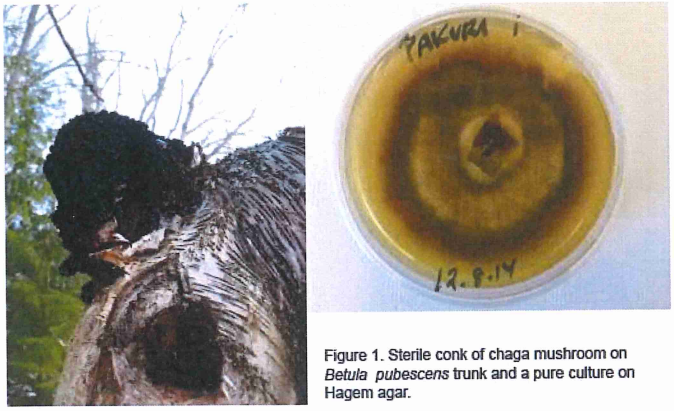


Figure 1. Sterile conk of chaga mushroom on *Betula pubescens* trunk and a pure culture on Hagem agar.

Results

Hot water extracts of all the conks but only one mycelium extract showed antioxidative effect in SCAV test. In ORAC test hot water extracts of both the mycelium and the conk showed antioxidative strength. Ethanol extracts of the conks were also tested. They did not show H_2O_2 scavenging effect in SCAV test whereas in ORAC test they were active. Our first results show that bioactivity of the mycelium does not correlate with the results obtained from the conks. The differences between the strains of *Inonotus obliquus*, however, emphasize the importance of selection of the inoculants for the cultivation before establishment of large scale field studies.



Figure 2. A) Three holes/tree were drilled, B) filled with a lump of mycelial agar and C) closed with a wooden tap. D) An inoculated tree in June, seven months after inoculation, showed faint signs of infection.

Conclusions

- The inoculation method used was easy and functional
- The first faint indications of infection were visible after 7 months from the inoculation
- Conks showing bioactivity do not necessarily produce mycelium with the same bioactivity properties on pure culture

References cited:

[1] Prior, R. L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B., Jacob, R.(2003) Assays for Hydrophilic and Lipophilic Antioxidant Capacity (oxygen radical absorbance capacity (ORAC)) of Plasma and Other Biological and Food Samples, J. Agric. Food Chem. 51(11):3273-3279

[2] Hazra B, Biswas S, Mandal N (2008) BMC Complementary and Alternative Medicine 8:63.

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Influence of *Grifola frondosa* polysaccharide on preventive effects of avian influenza inactivated vaccine

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Introduction

Maitake (*Grifola frondosa*) is a kind of rare mushroom. Modern medical research showed that, as the main active ingredient, *Grifola frondosa* polysaccharide (GFP) showed significant anti-cancer, liver protection, immunity improvment and many other pharmacological activities. As a serious threat to the poultry industry around the world, bird flu (Avian Influenza, AI), especially the H5 and H7 subtypes, which caused highly pathogenic avian influenza, had leaded to tremendous economic losses in the poultry industry. Furthermore, highly pathogenic avian influenza virus (e.g.. H5N1 subtype) could directly infect humans. In order to provide reference for the development and application of new animal feed additives, the co-effects of H5N1 subtype inactivated vaccine and GFP as feed additives of Beijing fatty chicken were investigated.

Table 1 The contents of AIV-H5 Ab

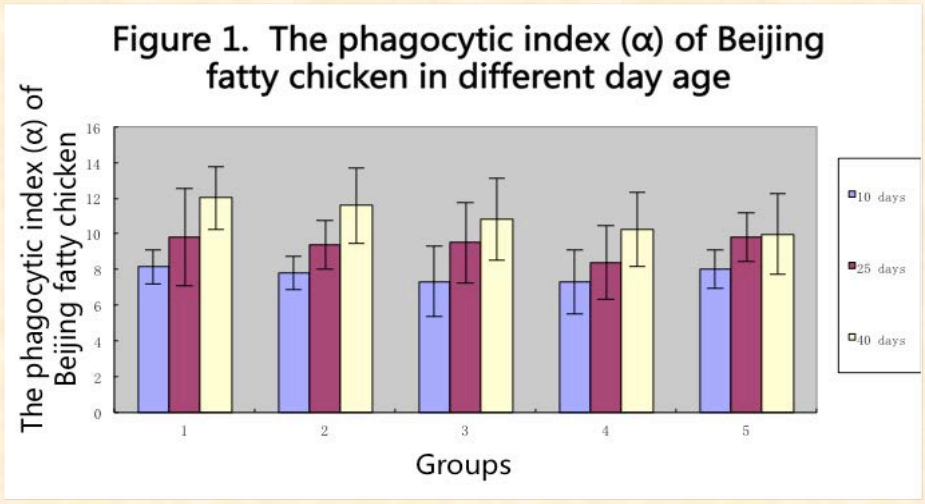
Groups	First immunization	Second immunization	Third immunization
1%GFP	220.81±7.91 ^{a}	315.23±7.25^{a}	420.74±17.83 ^{a}
0.5%GFP	225.39±7.19 ^{a}	291.56±10.68^{ab}	410.11±14.07 ^{a}
0.25%GFP	236.56±3.91 ^{a}	279.72±7.28^{b}	397.15±21.36 ^{ab}
0%GFP	223.66±4.46 ^{a}	276.82±9.73^{b}	385.46±18.68^{b}
ck	226.82±8.66 ^{a}	274.61±8.91^{b}	305.05±15.95^{b}

Note: Standard letters represented that the difference between values in each column was significant (P<0.05); The contents of H5 subtype antibody were regular when ranged in 23.5-141.2 ng/L.

Table 2 The T cell subpopulations (CD3+/CD4+/CD8+) contents of Beijing fatty chicken in different day age (pg/mL)

Groups	10 days	25 days	40 days
1%GFP	189.982±14.452 ^{ab}	386.673±31.721^{a}	421.546±36.527 ^{ab}
0.5%GFP	180.603±22.066^{a}	377.676±28.667^{a}	418.748±31.622 ^{a}
0.25%GFP	179.213±18.570^{a}	342.358±31.721^{a}	390.691±39.301^{a}
0%GFP	166.372±13.551^{a}	375.726±37.766^{a}	396.565±40.018 ^{ab}
ck	201.554±12.744 ^{ab}	394.281±35.338^{a}	457.345±33.323 ^{b}

Note: Standard letters represented that the difference between values in each column was significant (P<0.05).



Results

Beijing fatty chickens were inoculated by recombinant avian influenza virus inactivated bivalent vaccine of H5 subtype, and the contents of H5 subtype antibody (AIV-H5 Ab) in the immune serum samples for different times were investigated. As shown in Table 1, after the second immunization, there were significant differences in the 1%GFP and 0.5%GFP groups compared with other groups (P<0.05). After the third immunization, higher levels of antibody contents were showed in the GFP groups than other groups, and the differences were significant (P<0.05). So the contents of serum antibodies of H5 subtype were promoted by adding GFP.

Compared with the positive control group, the phagocytic indexes of 25-day and 40-day-old groups with GFP were 13.35%-16.90% and 5.45 %-17.06% higher respectively, and the differences were significant (P<0.05) (Figure 1). There were also positive correlations between the amounts of phagocytic index and the contents of GFP. Thereby, the phagocytic index and the immune system were improved by the promoter GFP.

Table 2 showed that the contents of T lymphocyte subsets in the same group were gradually increased, and compared to the positive control group, the contents in groups added with 0.5% and 1% GFP were higher, but the difference was not significant (P>0.05). It is inferred that addition of GFP into feed played a certain role in promoting lymphocyte transformation, thus, the cellular immune functions of chickens were enhanced.

Conclusion

By adding certain amounts of GFP into feed, phagocytic activity of macrophages of Beijing fatty chicken could be enhanced, and the proliferation and differentiation of lymphocytes were stimulated. Meanwhile, the phagocytic ability of neutrophils was further strengthened. Furthermore, the contents of T lymphocyte subsets were positive correlation with the contents of GFP added. In conclusion, *Grifola frondosa* polysaccharide plays an important role in promoting immune system of Beijing fatty chicken.

Enhancement of the ergothioneine in shiitake (*Lentinula edodes*) fruiting bodies through sawdust media with different nitrogen sources

국립산림과학원
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Abstract

Ergothioneine(ERG) is a naturally occurring amino acid analogue, which has been found in bacteria, plants and animals, cannot be made in human cells. ERG has antioxidant, protectant against radiation and anti-inflammatory properties. The objective was to enhance the ergothioneine content in shiitake(*Lentinula edodes*) fruiting bodies through the culture of sawdust media with different nitrogen sources. To assess the effect of different nitrogen sources on ergothioneine contents, the fungus was grown in sawdust media with four different nitrogen sources. Nitrogen sources including ammonium nitrate, sodium nitrate, ammonium sulfate and histidine at the concentrations of 0, 0.1, 0.2, 0.3% were used. The contents of ergothioneine in shiitake fruiting bodies were quantified by high performance liquid chromatography (HPLC). As a result, ergothioneine was detected in all samples. The fruit bodies cultivated with 0.2% ammonium sulfate showed the highest ergothioneine content, which is 1.7-fold higher than the control. Our results demonstrate that the cultivation of shiitake mushroom in sawdust medium with suitable ammonium sulfate concentration was effective to enhance ergothioneine contents. There was no significant effect on the enhancement of ergothioneine content in sawdust medium with sodium nitrate and histidine group.

Materials & Methods

- **Preparation of onion peel water extract**
  - **Onion peel was washed three times in tap water and were dried in a drying oven at 60℃ for 15h and then were ground using high speed mixer.**
  - **Onion peel powder was mixed with distilled water at a concentration of 20mg/mL and extraction was carried out at boiling temperature.**
  - **The extract was filtered through filter paper and the solid residue was extracted one more time.**
- **Concentrations of onion peel water extracts: 0, 10, 20, 30%**
- **Three shiitake strains used in this study: KFRI 169, 547, 623 • Medium were incubated for 2 months at 25℃**

Results

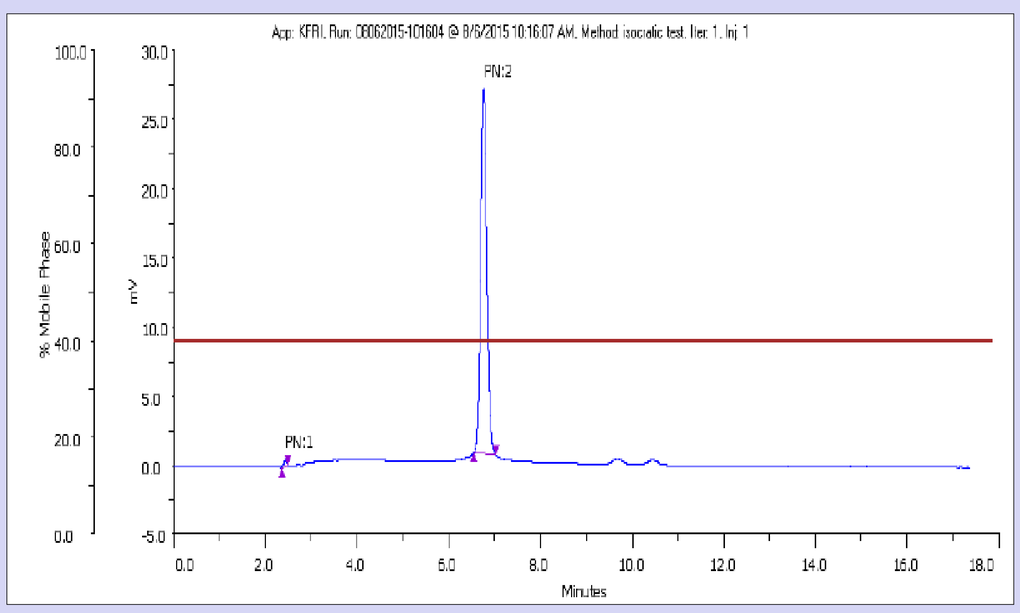


Figure 1. HPLC chromatogram of standard quercetin.

A, Solid control; B, Solid OE10%; C, Solid OE20%; D, Solid OE30%; E, Liquid control; F, Liquid OE10%; G, Liquid OE20%; H, Liquid OE30%. OE: onion peel extract.

Figure 2. HPLC anlaysis of quercetin in the mycelium of *Lentinula edodes* strain KFRI 169 on solid and liquid media with onion peel extracts.

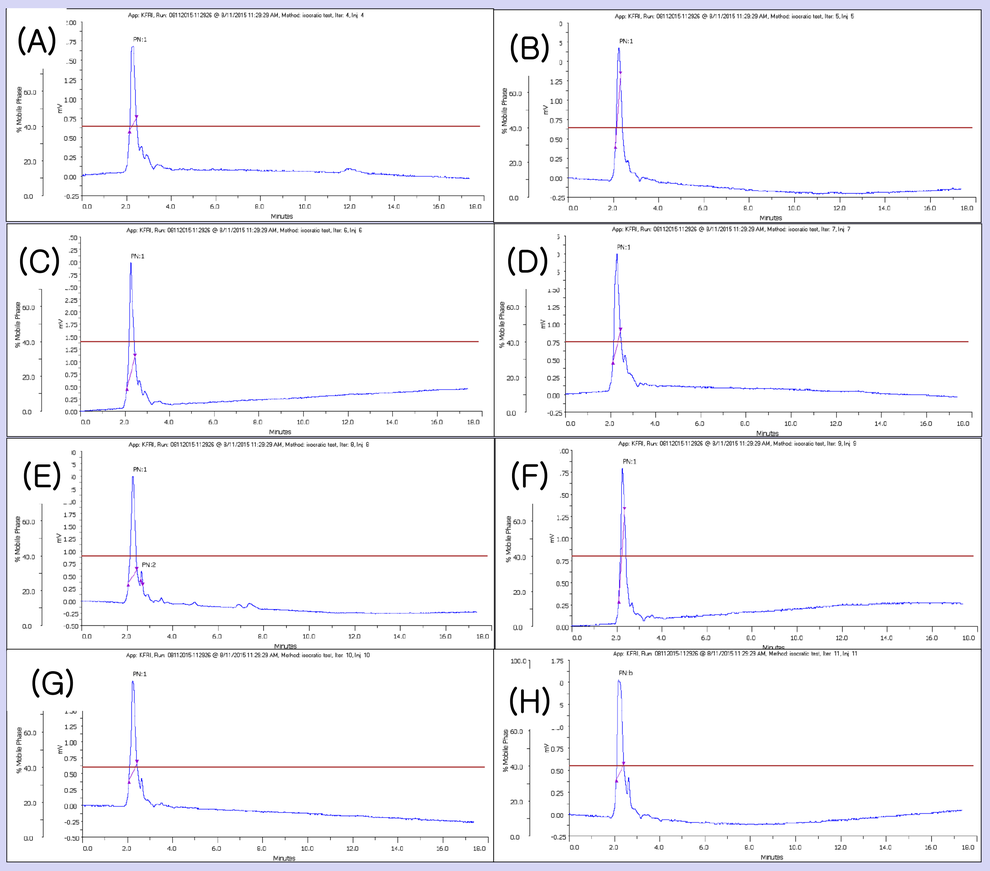


Figure 3. HPLC anlaysis of quercetin in the mycelium of *Lentinula edodes* strain KFRI 547 on solid and liquid media with onion peel extracts.

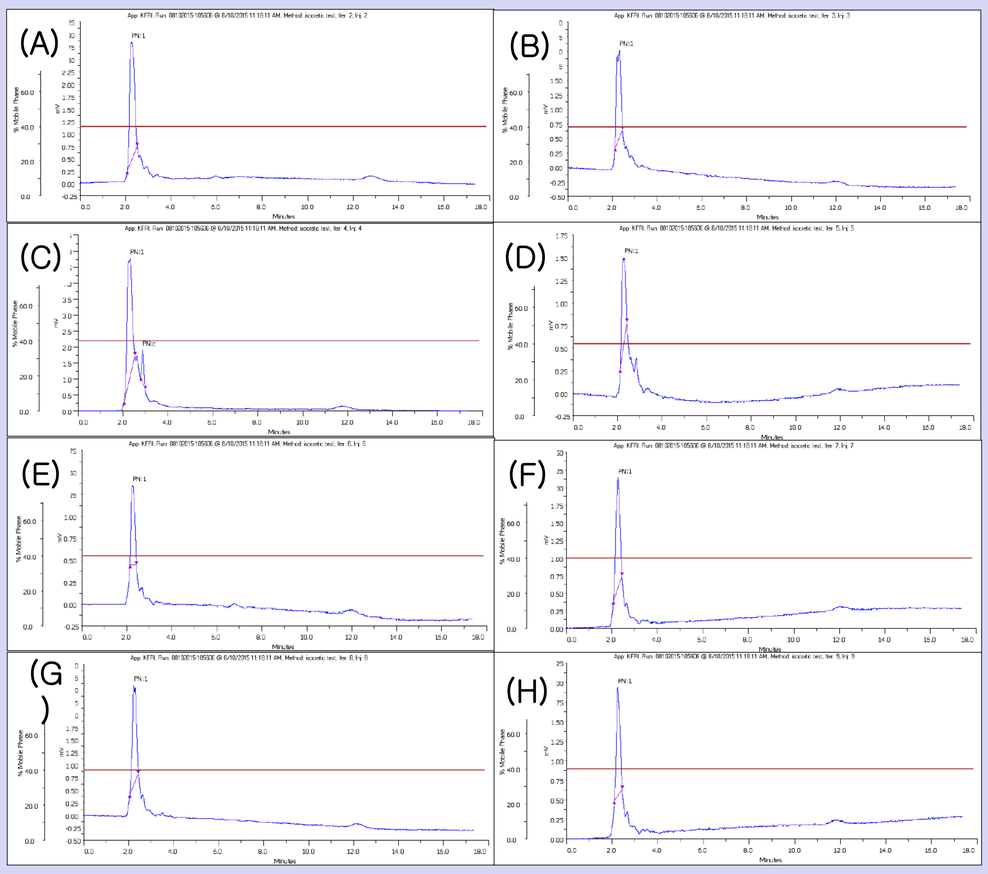


Figure 4. HPLC anlaysis of quercetin in the mycelium of *Lentinula edodes* strain KFRI 623 on solid and liquid media with onion peel extracts.

IN VITRO PREBIOTIC ACTIVITY OF *PLEUROTUS OSTREATUS* POLYSACCHARIDES

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Prebiotics are nondigestible food components or ingredients mainly including oligosaccharides such as fructo-oligosaccharides, gluco-oligosaccharides, and mannan-oligosaccharides which positively influence the host by their selective growth or activation of certain number of bacterial species present in intestines.

Mushrooms have prebiotic activity that are rich in nondigestible dietary fibres belonging to β-Glucan, chitin, mannan, xylan, galactose and hemicellulose etc. They may prevent viral infection by enchaning the growth of probiotic bacteria in the large intestine. β-Glucans, homo-glucans, and hetero- glucans with β(1→3), β(1→4), and β(1→6) glucosidic linkages were thought to be responsible for their bioactivity.

Material and Methods

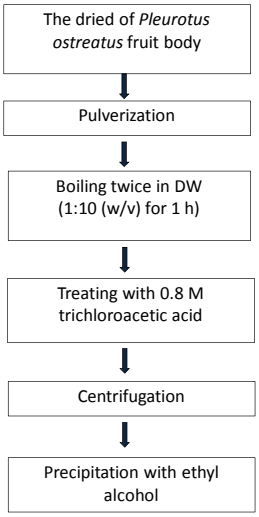


Figure 1. Extraction of polysaccharides of *P. ostreatus* (POP)

The obtained polysaccharides add into MRS broth for growth of Lactobacillus acidophilus and Lactobacillus plantarum in different concentrations (20, 10, 5 and 2.5 mg/ml) and incubated at 37°C, 48 h. The growth of the bacteria was monitored hourly with the using of nephelometer as RNU (relative nephelometric unit).

The same concentrations of glucose and inulin were used as control groups. The obtained results were evaluated by analysis of variance.



Figure 2. Nephalometer

Table.1 Nephelostar settings used throughout validation studies

Number of cycles	49
Measurement time/well (s)	1.0
Positioning delay (s)	0.5
Gain	0
Laser beam focus (mm)	2
Shake time (s)	10
Orbital shake width (mm)	3
Laser intensity (%)	25

Results

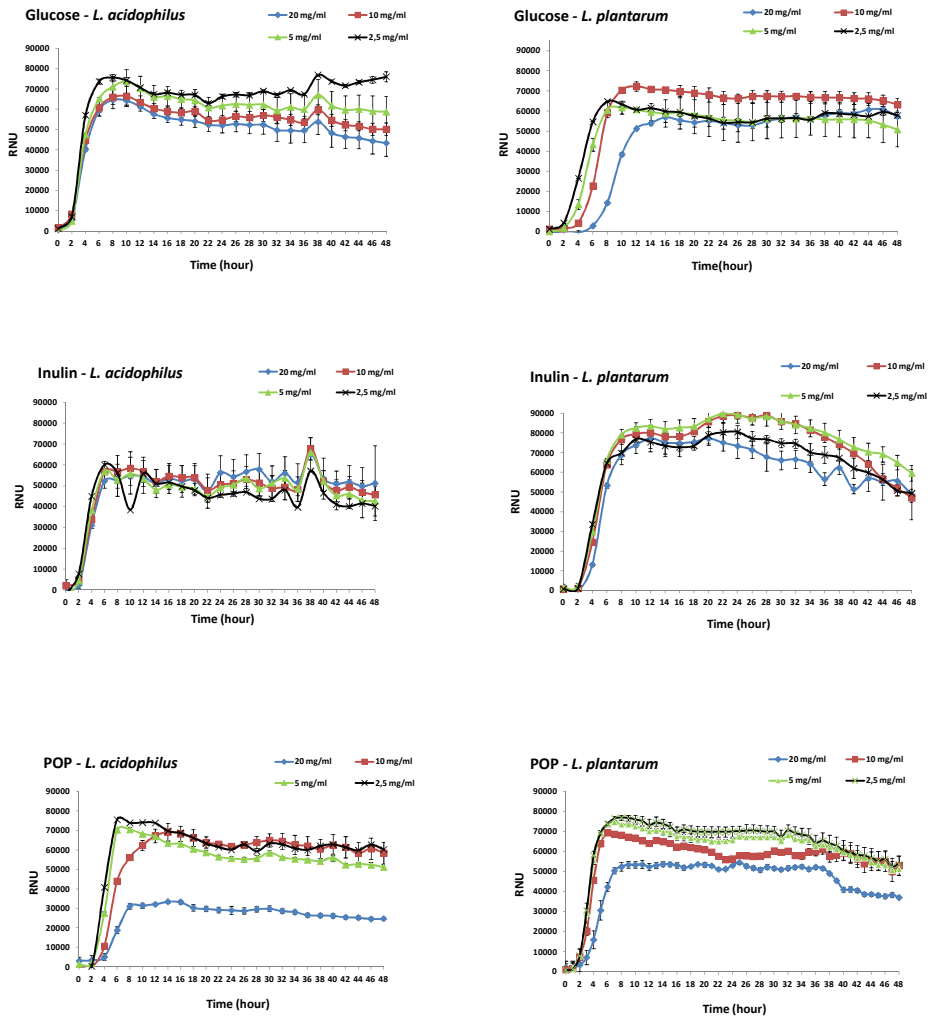


Figure 3. Nephalometric data



Conclusions

- 5 and 2.5 mg/ml POP showed higher prebiotic activity. In these concentrations, the prebiotic activity of polysaccharides can be described in the order of L. plantarum in MRS with inulin > L. plantarum in MRS with POP > L. acidophilus in MRS with POP > L. acidophilus in MRS with inulin.
- L. plantarum showed higher growth in all concentration (20, 10, 5 and 2.5 mg/ml) than L. acidophilus in the experimantel groups (MRS with POP).
- POPcan be used to increase the number of selected bacteria.

The Diet Composed of One Portion of Meat, Vegetable and Mushroom Respectively Contributes to a Healthy and Stable Food Structure for Human Being

Han Shenghua
China
Hangzhou Huadan Agri-product Co.,Ltd

Introduction

The humankind has been living mainly on animal and plant foods since its birth over 3 million years ago. Animal food contains rich proteins, fat, carbohydrates, minerals and vitamins essential for human bodies and provides sufficient energy to maintain their internal and external activities, adequate proteins for growth, development, tissue repair and regeneration and maintenance of normal physiological functions. Vitamins, celluloses and inorganic salts contained in plant food participate in building-up the tissues and adjusting the physiological functions to keep the normal body development and increase its resistance. An adequate amount of food fibres are used to maintain the normal excretion, prevent some kind of intestinal diseases and provide ample water for the normal physiological processes.

All human beings long for strengthening their vitality and extending their life span. The overall life span and living quality has witnessed a great improvement since the global attention to mushroom development in the 1980s. In human practices in the past more than four decades, mushroom production and resultant food, healthy products and medicines made the humankind realize its dream for longevity. Scientists have realized that as a new protein source other than animal and plant, mushroom has made an important contribution to the human being.

I proposed the dietary concept composed of one portion of meat, vegetable and mushroom respectively in 1995. Since then, this concept has been promoted under the name of FAO, UNSCO and WHO, though in fact it was not initiated by them. It was presented in various media and was widely spread among ordinary people, which shows that the actual functions of edible mushrooms are significant and the dietary structure composed of meat, vegetable and mushroom is reasonable, and thus has been widely recognized worldwide.

The Nutrients and Structure of Edible Mushroom

The nutrients and structure of edible mushroom meet the nutritional requirement of people nowadays, which is the theoretical basis of this concept. The protein content of edible mushroom usually accounts for 4% of its fresh weight, or 30-45% of its dry weight. It contains very low fat content, mainly unsaturated fatty acid, and 8 essential amido acids needed by human bodies. Vitamin contents in mushroom are higher than those in ordinary vegetables. The bioactive substances of mushroom have healthy functions.

Proteins: protein contents in Agaricus bisporous, Lentinula edodes, Pleurotus ostreatus and Volvarellae volaceae, which are widely commercialized, account for 1.75-3.63% of the fresh weight of the fruitbody, with an average of 3.5%. Calculated at dry weight, mushroom generally contains 19-35% proteins. Mushroom grows fast with high biological efficiency. Industrial cultivation of A. bisporous all year round, 1 hectare of growing rooms can produce 22 tons of proteins a year, while 1 hectare of most crops can only produce 1-2 tons of proteins a year. Therefore, mushroom industry is one of the effective ways to solve worldwide food shortage, especially severe shortage of proteins.

Amino acids: proteins consist of 20 amino acids. There are 8 essential amino acids which the human body can't synthesize and can only be obtained from food. They must exist simultaneously and have the right percentages in amount when synthesizing into proteins. The utilization of all other amino acids in cells will be reduced at an appropriate percentage if one or more of them are insufficiently supplied. Edible mushrooms contain the 8 essential amino acids needed by human body.



Fat: crude fat content in common edible mushrooms accounts for 1.1-8.0% of its dry weight, with an average of 4%. At least 74% of fatty acid contained in edible mushroom is unsaturated, which is mainly linoleic acid. In daily diet, unsaturated fatty acid is an essential nutrient. The high percentage of unsaturated fatty acid contained in mushroom is one factor that it is regarded as healthy food.

Cholesterol: cholesterol is a member of lipid, steroid compound and an important structural component of Eukaryotic membrane, but a high content of cholesterol in serum will increase the risk of cardiovascular diseases. In human food, animal food contains high content of proteins and cholesterol. The consumption of animal food increases with the improvement of people's living standards, which cause the increase of cholesterol intake and the risk of cardiovascular diseases. Mushroom contains high content of proteins, but low content of cholesterol. More consumption of mushroom can balance the cholesterol content.

Vitamins: mushroom contains various vitamins such as thiamine (VB1), riboflavin (VB2), biotin (VH) and ascorbic acid (VC) etc.

Minerals: mushroom is a good source of minerals. Potassium ranks No. 1 in minerals contained in mushrooms, followed by phosphorous, sulphur, sodium, calcium and megnasium, which are the main mineral elements or called bulky mineral elements, accounting for 56-70% of the total ash content. Mushroom contains trace elements like copper, iron, manganese and molybdenum.

Nucleic acid: Nucleic acid content in mushroom is 2.7-4.1%, 2.66% in white button mushroom, 2.93% in Abalone mushroom, 4.06% in Phoenix mushroom and 3.88% in paddy straw mushroom.

The maximum safe intake of nucleic acid for an adult is 4 grams daily. This is because the human body can't oxidate uric acid as it lacks in urea oxidase. Uric acid is a metabolic substance of guanine and adenine, which is difficult to solve. When high in serum, uric acid can cause the sedimentation of uric salt in tissues and joints, thus leading to stone formation in kidneys and bladder.

Nucleic acid content of Phoenix mushroom is the highest among the common edible mushrooms, accounting for 4.06% of its dry weight, equivalent to 0.51% of its fresh weight. Daily consumption of 392 g fresh Phoenic mushroom is safe. It is not necessary to restrict the consumption of mushrooms as a vegetable.

Mushroom can supplement the nutrients of plant food for the vegetarians, especailly the amino acids like lysine, methionine and tryptophan lacked in plant food.



Edible Mushroom can Strengthen and Regulate Human Bodies' Immunity

Most human diseases are caused by damaged body's inability to recover normal functions. The body's resistance is gradually decreasing after middle ages, the balance of the internal micro-flora is broken and disease occurrence increases, which is related to immunity. Now medical community has reached common understanding about cancers: cancer cells in the body are produced everyday, but will be eradicated or restrained in a co-existence state with the body if the immune function is normal. Cancer cells and stem cells are seeds and internal body environment is soil, where immunity is the most important factor. An ideal method can not only increase both the activity of antigen presenting cells (APC, the intelligence system of human body) and the number of T-lymphocyte, but also regulate the ratio of Th to Ts and Th1 to Th2. This is a high level balance. Edible mushroom is conducive to completing this high level balance. It is possible to cure chronic and old-age diseases and resist senility. To regulate the balance of immune system is a good approach to improve sub-health conditions.



Edible mushroom is closely related to human life, health and longevity. Edible mushroom is both a nutritional food and a functional food. It can not only provide the human body with complete and balanced nutrients, but also has good efficacy for preventing and treating chronic diseases and restraining tumor growth. More and more research and the wide applications of mushroom food show that dietary mushroom food contains comprehensive nutrients and has significant healthy function. Polysaccharides contained in mushroom can obviously regulate human bodies' immunity. The anti-oxidation function of mushroom can accelerate metabolism of cells, delay aging and reduce the incidence of senile dementia. Long-term consumption of mushroom food can reduce blood fatty level including cholesterol and triglyceride and blood viscosity, slow down the occurrence of arteriosclerosis, decrease the incidence and risk of cardiac and cerebral vascular diseases and cancers.

Frequent consumption of mushroom food is more effective than long-term use of medicines for those who are in sub-healthy conditions or have chronic diseases.

Edible mushroom may perhaps make an epoch-making contribution to human health again. Most deadly diseases today are internal diseases, caused by the abnormal immune systems and aging tissues and organs. It is necessary to adjust dietary structure to regulate the abnormal immune system and slow down the aging of tissues and organs. The dietary structure composed of edible mushroom, animal and plant food builds up a new food base for regulating immunity and slowing down tissue and organ aging process. The effective and low-toxicity medicine, which can regulate the abnormal immune system and slow down the tissue and organ aging process, will probably be discovered from fungi again, as many on-going researches have revealed the twilight now. The frequent, sufficient and long-term consumption of edible mushroom food can possibly delay senility and extend life span. The purpose of a scientific diet is to balance nutrition and to live long healthily, which is a permanent topic for continuous research.



In vitro assessment of the nematocidal activity of *Pleurotus djamor* hydroalcoholic extract against the sheep parasitic nematode *Haemonchus contortus* (L_3)

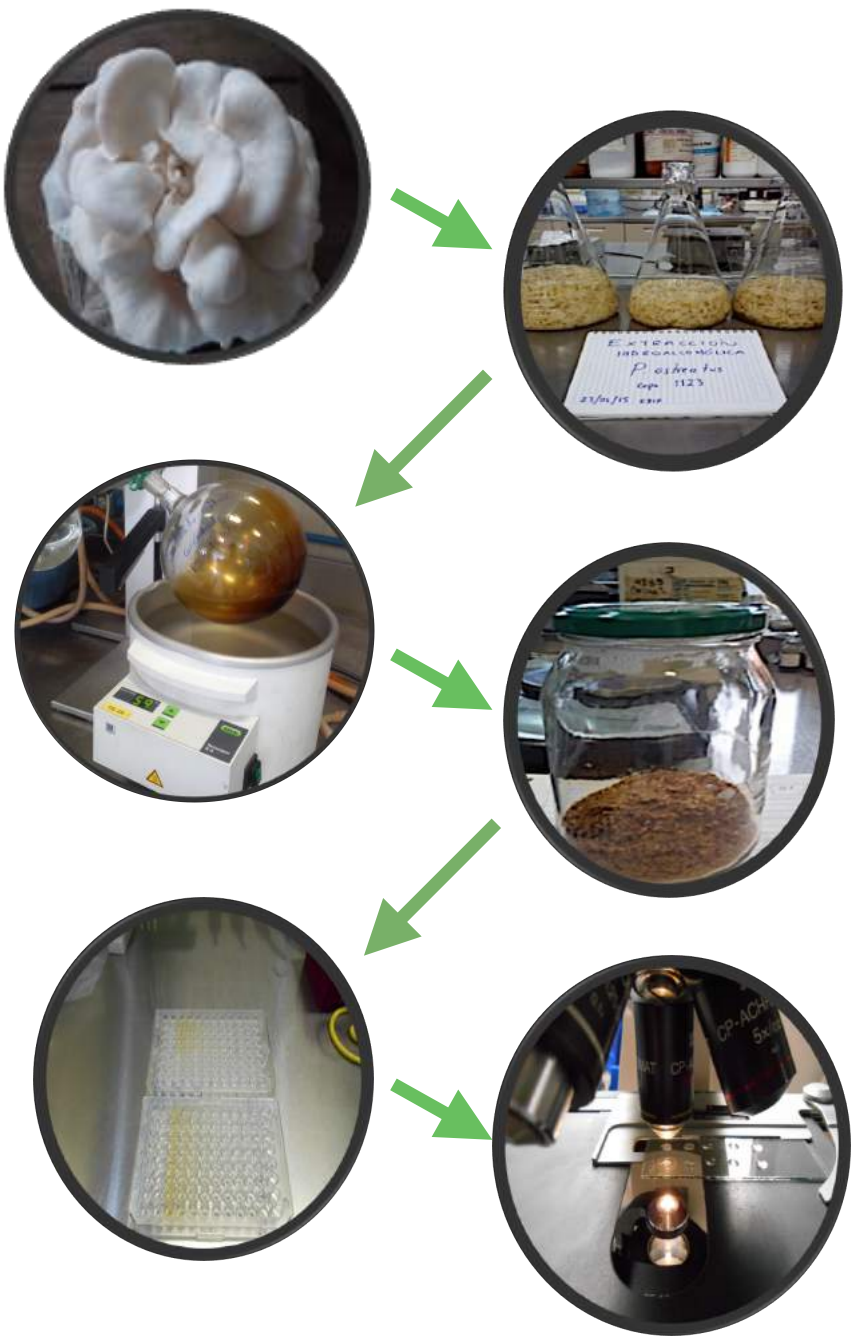
Jesús Antonio Pineda Alegría[2], José E. Sánchez[1], Pedro Mendoza de Gives[3], María Eugenia López Arellano[3], Manases González Cortázar[4], Alejandro Zamilpa Álvarez[4], Edgar Josué Cuevas Padilla[3], Liliana Aguilar Marcelino[3].

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Introduction.

Haemonchus contortus (Hc) is an important parasite affecting sheep industry worldwide. The use of anthelmintic drugs is limited by a number of disadvantages in their use; ie, anthelmintic resistance, the public health risk and environmental pollution. Mushrooms possess a number of important nutritional, medicinal and nematocidal properties. The *in vitro* nematocidal activity of a hydroalcoholic extract (HE) from *P. djamor* against infective larvae of Hc (L_3) was assessed.

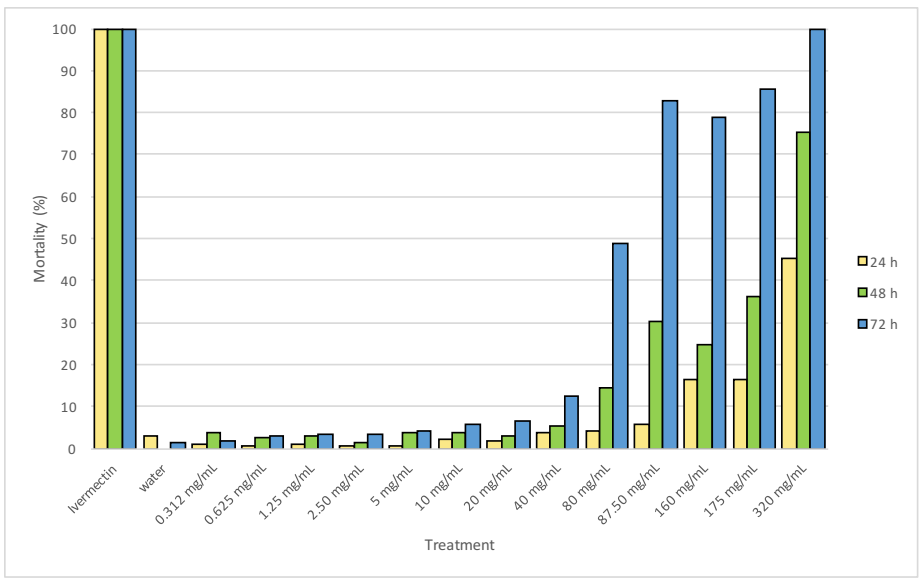
Materials and Methods



Results and Discussion.

The highest mortalities were 82.8%, 79.9%, 85.6% and 100% at 87.5 mg/mL, 160 mg/mL, 175 mg/mL and 320 mg/mL concentrations, respectively, at 24, 48 and 72 hours (Figure 1).

Figure 1. Mortality of infective larvae of *Haemonchus contortus* to 24, 48 and 72 hours post-confrontation against a hydroalcoholic extract from *P. djamor* ECS 0123.



Conclusion

The *P. djamor* HE showed an important nematocidal activity against *H. contortus* (L3). The highest activity (100% of mortality) was obtained with the highest concentration of the extract (320 mg/mL). The purification of the extracts could probably increase the nematocidal activity.

Acknowledgments

This research was supported by resources of the INIFAP (Project No.834432984).

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Zinc, Selenium and Manganese Accumulation Experiments with Cultivated King Oyster Mushroom (*Pleurotus eryngii*)

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Introduction

There are many mushrooms capable of accumulating metals in their fruitbodies. In case of cultivated species, under controlled conditions, it is possible to enhance the level of certain elements that are essential for the human body by adding them to the substrate. Our goal was to examine the zinc, manganese and selenium accumulation capability of a *P. eryngii* strain.

Material and method

Variety: 'PES' strain isolated from Hungarian habitat and selected for large-scale cultivation. The spawn was prepared at the Biokékes Mushroom Spawn Plant and Strain Research Laboratory.

Substrate composition and preparation: 37 % oak wood chip, 49 % wheat straw, 11 % cooked rye, 3 % gypsum (dry weight). The elements were mixed into the substrate and filled into 15 liter filtered bags (SACO2, Belgium). Each bag contained 4 kg substrate (wet weight) and sterilized at 126 C° for 2 hours.

Tested chemicals and dosage: ZnCl2, MnCl2, Na2SeO3 in 50, 150, 300 and 600 ppm (in dry weight).

Cultivation: the bags were inoculated with rye based spawn (10%) and colonized on 25 C° for 8 days. After shaping the bags, they were placed on 20 C° for 10 days. For fructification the bags were incubated on 10 C° for 5 days, then on 2 C°. The bags were placed into a growing room for fruitbody development. No casing was applied.

Elements determination: 400 mg dry, homogenized mushroom fruitbody powder was fractured by nitric acid using a microwave assisted drilling machine. The solution was analyzed by Perkin Elmer Elan DRC II. (Dynamic Reaction Cell) ICP-MS instrument in Argon gas flow. Following analysis, two parameters were calculated:

- EMF = element content in treated mushroom/element content in control mushroom
- EMF* = element content in treated mushroom/element content of previous treatment

Results

No significant levels of zinc and manganese elements accumulation was detected in the fruit bodies, however 600 ppm zinc caused an intense yield decrease. The 600 ppm manganese had no effect on yield (Table 1). Based on the result, the selenium enriched substrate increased the amount of selenium in the mushroom. 300 ppm selenium concentration had no negative effect on the yield, but 600 ppm lowered it. In case of zinc the highest degree of accumulation was measured in the 300 ppm treatment (untreated control: 25 mg/kg; 300 ppm: 40.03 mg/kg/d.w.). The 300 ppm manganese supplementation gave the best result as well: control: 4.13 mg/kg/d.w.; 300 ppm: 7.3 mg/kg/d.w. While the fruitbodies did not accumulate significant amounts of zinc and manganese, in case of selenium high levels were detected in the treated samples: control: 0.15 mg/kg; 600 ppm: 520.03 mg/kg/d.w.

Table 1. Zinc, Selenium and Manganese accumulation in *Pl. eryngii* fruit bodies

Treatment	Concentration (mg/kg d.w.)	EMF	EMF*	Yield (%)
Kontroll	25,00	-	-	27,8
Zn 50	30,50	1,22	-	26,3
Zn 150	34,60	1,38	1,13	27,3
Zn 300	40,03	1,60	1,16	32,6
Zn 600	29,50	1,18	0,74	18
Treatment	**Concentration (mg/kg d.w.)**	**EMF**	**EMF***	**Yield (%)**
Kontroll	4,13	-	-	27,8
Mn 50	5,30	1,28	-	29,6
Mn 150	7,07	1,71	1,33	25,5
Mn 300	7,30	1,77	1,03	24,3
Mn 600	6,70	1,62	0,92	25,3
Treatment	**Concentration (mg/kg d.w.)**	**EMF**	**EMF***	**Yield (%)**
Kontroll	0,15	-	-	27,8
Se 50	76,03	506,89	-	26,5
Se 150	99,30	662	1,30	28
Se 300	230,20	1534,67	2,32	28,8
Se 600	520,03	3466,89	2,26	20,5

The degree of selenium accumulation was evaluated both from the cultivation and from the dietary point of view. Although, based on the yield results, a maximum level of 300 ppm is recommended, but taking the RDA (Recommended Dietary Allowances) into consideration, only a 1-50 ppm supplementation should be applied. A higher dose of selenium present in the substrate could lead to an accumulation degree, which can be toxic to the human body.

Based on our results, selenium enriched *P. eryngii* could serve as an excellent source of this element. This mushroom product could be used as a functional food for selenium supplementation to the deficient populations.

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Session Genetics & Breeding

Mushroom: Biodiversity of Indigenous species in the Niger Delta Region of Nigeria



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INTRODUCTION

Mushrooms are non-green vegetables. They are cultivated worldwide for their taste, nutritional attributes and potential application in industries. They are known to grow on a wide variety of substrates and habitat (Adesina *et al*.2011). Advocacy on consumption and cultivation of mushroom is increasing by the day (Adedokun and Akuma 2013). This calls for researching indigenous mushrooms, their morphology and genotype.

OBJECTIVES

- To identify the edible mushroom species from Nigeria

MATERIALS AND METHODS

- Fruiting bodies of wild mushrooms were collected from different locations in the Niger Delta region of Nigeria.

- Genomic DNA was extracted from mushroom fruiting bodies using Zymo Fungal/Bacteria DNA extraction kit. Molecular characterization was done using internal transcribed spacer (ITS) DNA barcoding of the fungal ribosomal RNAs for species identification. Using ITS barcoding, the mushrooms were adequately identified.

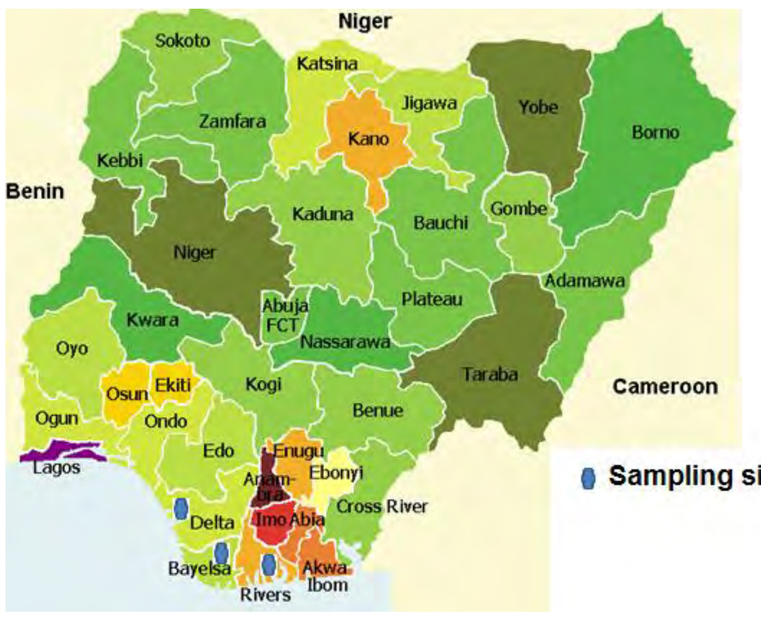
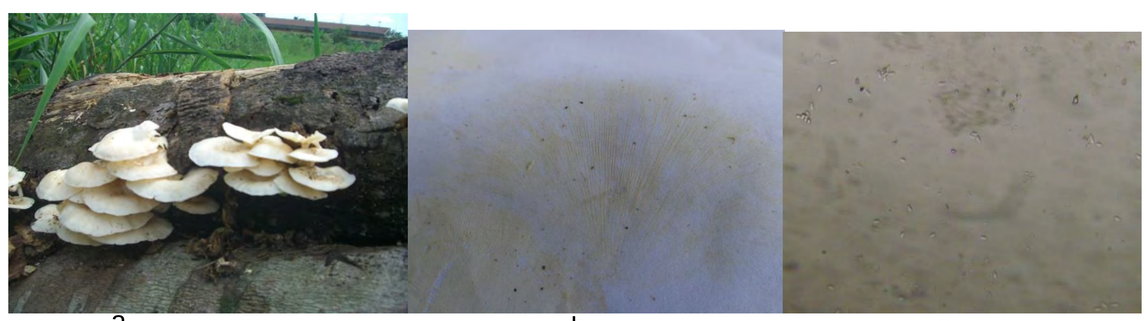


Figure 1. Nigerian map showing the sampling sites

RESULTS



a b c

Figure 2. *Lentinus squarrosulus,* a-Fruiting body; b-Spores print; c-Spores



d e f

Figure 3. *Agaricus argyropotamicus,* d-Fruiting body; e-Spores print; f-Spores



g h i

Figure 4. *Pleurotus djamour,* g-Fruiting body; h-Spores print; i-Spores

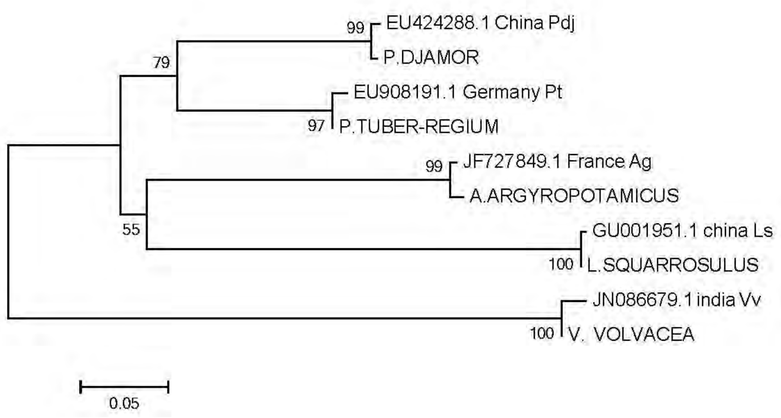
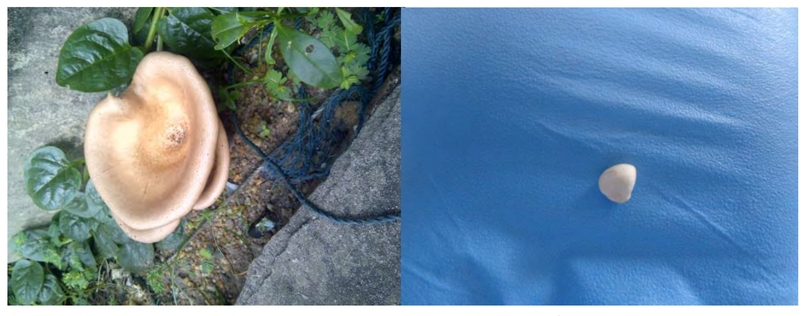


Figure 7. Phylogenic tree of the identified edible mushroom



j k

Figure 5. *Pleurotus tuberregium*, j-Fruiting body; k- sclerotium



l m

Figure 6. *Volvariella volvacea*, l-Fruiting body; m-Spores

Scientific name	Common name	Local name (language)	habitat	Edibility	Uses
Lentinus squarrosulus	Lentinus mushroom	Atakata ero (Igbo) Olu ayunre (Yoruba)	Rubber trees	Edible	Food, antioxidant properties, Bioremediation
Agaricus argyropotamicus	Button mushroom	Olu (Yoruba) Ero (Igbo)	Newly cleared land	Edible	Food
Pleurotus djamour	Oyster mushroom	Olu resu-resu (Yoruba) Ero (Igbo)	Mango trees/sawdust	Edible	Food, antimicrobial properties
Pleurotus tuberregium	Oyster mushroom	Olumoyi (Yoruba)	Rotten treestrees/cocoa pods/wheat/rice straw	Edible	Food, antimicrobial activities, Bioremediation, Nutraceutical properties
Volvariella volvacea	Straw mushroom	Ogiri agbe (Yoruba) Ero (Igbo)	Mostly on rice/wheat straws, cotton wastes	Edible	Food, antimicrobial/anti-tumour properties

Conclusion and future work

There is a huge diversity of edible mushroom in Nigeria. These mushroom are used as food. Therefore, there is need to understand their nutritional profile.

References

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Genome sequence of the edible mushroom *Lentinula edodes* (shiitake) reveals insights into Lignocellulose degradation

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ABSTRACT

Lentinula edodes, one of the most popular edible mushroom species with high nutrition and special flavor, is widely cultivated in many Asian countries, especially in China, Japan and Korea. As a white rot fungus with strong wood degradation ability, *L. edodes* has the potential application in bioenergy production. However, genes, pathways and mechanisms regarding this function are still unknown. Here, we report its 41.8-Mb genome, encoding 14,889 predicted genes, obtained via next-generation sequencing technology. *L. edodes*' phylogenies and wood-degrading machinery were investigated by a comparative analysis with the model species of fungi. A total of 93 genes predicted to encode transcription factors and 41 genes related to lignocellulose degradation are up-regulated when *L. edodes* mycelia were cultured in sawdust medium versus CYM medium. These differentially expressed genes may play important roles in lignocellulose degradation, and the research of these genes may be beneficial to bioenergy industry, environmental improvement and mushroom production. The overall results will facilitate a better understanding of the molecular mechanism of lignocellulose degradation and provide the basis for partial replacement of wood sawdust with agricultural wastes in *L. edodes* cultivation.

INTRODUCTION

Lentinula edodes, also known as Xianggu or shiitake, belonging to the Agaricales of Agaricomycetes in Basidiomycota, is one of the white-rot fungi that grow on the dead tree or sawdust by degrading cellulose, hemicellulose and lignin. Meanwhile, *L. edodes* is the second most widely cultivated mushroom species all over the world, only second to *Agaricus bisporus*.

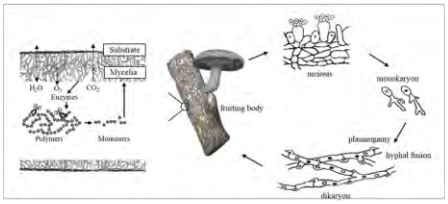


Fig1. Life cycle and the wood degrading ability of *L. eddoes*.

RESULTS AND DISCUSSION

Genome sequencing and general features

Table 1. Genome assembly and annotation of *L. edodes* W1-26

Number of Scaffolds	340
Length of the genome assembly (Mb)	41.8
Scaffold L50 (kb)	300.7
Contig L50 (kb)	106.4
GC content (%)	46.1
Sequencing Coverage	230x
Number of protein-coding genes	14,889
With GO terms	7,713 (51.8%)
With InterPro Terms	8373 (56.2%)
With CDD Terms	9.984 (67.1%)
With KAAS annotation	3,252 (21.8%)

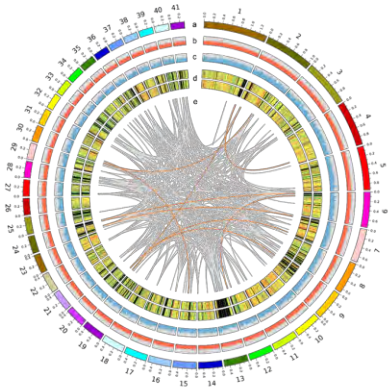


Fig 2. The ideogram showing the genomic features of *L. edodes*.

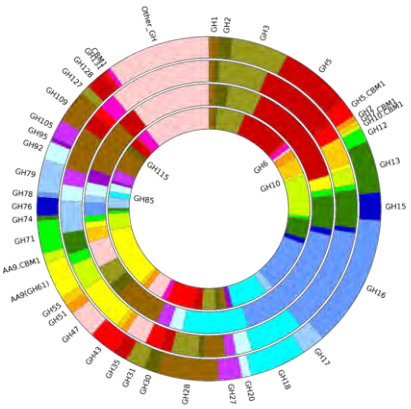


Fig 5. Distribution of various glycoside hydrolases and carbohydrate binding module 1 in *L. edodes* (out ring), *Phanerochaete chrysosporium* (second ring counted from out), *Postia placenta* (third ring) and *Volvariella volvacea* (inner ring).

Comparisons with other fungal genomes

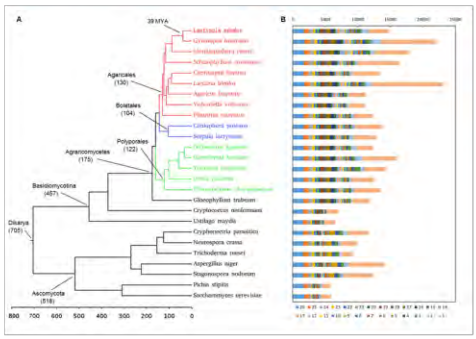


Fig 3. Orthologous gene number statistics and phylogenetic tree of *Lentinula edodes* with other 25 fungal species.

matA and matB gene locic

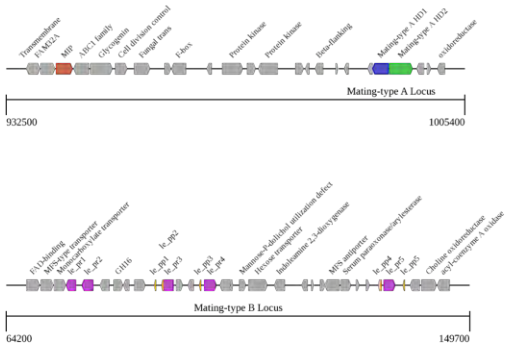


Fig 4. Distribution of matA and matB genes

Genes involved in Lignocellulose decomposition

Table 2. The gene distribution of lignocellulose-degrading genes in *Lentinula edodes* and other edible fungi or model fungi

Class	Enzyme	EC	CAZY	*L. edo*	*S. com*	*G. luc*	*P. chr*	*P. ost*	*P. pla*	*S. lac*	*C. cin*	*V. vol*	*L. bic*	*A. bis*
				White rot					Brown rot		Straw rot			
Cellulase	Endo-beta-1,4-glucanase	EC:3.2.1.4	GH5, GH7, GH9, GH12, GH44, GH45, AA9	21	24	21	19	34	7	12	36	26	9	15
	1,4-β-cellobiosidase	EC:3.2.1.91	GH6, GH7	5	3	4	8	16	0	1	10	16	0	2
	β-glucosidase	EC:3.2.1.21	GH1, GH3	12	12	10	10	11	6	11	9	12	2	6
	Total	-	-	38	39	35	37	61	13	24	55	54	11	23
Hemicellulase	Endo-1,4-beta-xylanase	EC:3.2.1.8	GH10, GH11	5	6	7	7	5	3	1	12	16	0	4
	β-xylosidase	EC:3.2.1.37	GH3, GH39,GH43	2	4	4	1	5	1	1	1	3	0	2
	α-glucuronidase	EC:3.2.1.131	GH67	0	0	0	0	0	0	0	0	0	0	0
	acetylxylan esterase	EC:3.1.1.72	CE1, CE5	1	4	2	4	1	0	0	3	2	1	3
	feruloyl esterase	EC:3.1.1.73	CE1	0	6	0	0	1	0	0	2	2	0	0
	α-L-arabinofuranosidases	EC:3.2.1.55	GH51, GH54, GH62	1	1	1	1	1	0	0	0	0	0	0
	Total	-	-	9	21	14	13	13	4	2	18	23	1	9
Pectinase	pectin lyase	EC:4.2.2.10	PL1	0	0	0	0	0	0	0	0	0	0	0
	pectate lyase	EC:4.2.2.2	PL1, PL3, PL9	3	10	0	0	12	0	0	3	14	0	3
	pectinesterase	EC:3.1.1.11	CE8	2	2	3	2	2	2	2	0	3	4	2
	polygalacturonase	EC:3.2.1.15	GH28	2	0	1	1	1	2	1	0	0	0	1
	Total	-	-	7	12	4	3	15	4	3	3	17	4	6
Lignin Oxidase	multicopper oxidase	EC:1.10.3.2	AA1	14	3	15	3	12	4	6	16	11	12	13
	Lignin peroxidase	EC:1.11.1.14	AA2	0	0	2	10	0	0	0	0	0	0	0
	Manganese peroxidase	EC:1.11.1.13	AA2	2	0	2	5	0	0	0	0	2	0	0
	Versatile peroxidase	EC:1.11.1.16	AA2	1	0	5	1	9	0	0	0	5	1	2
	Other peroxidase	-	AA2	6	2	1	1	1	2	1	4	3	2	3
	Cellobiose dehydrogenase	EC:1.1.99.18	AA3_1	1	1	1	1	1	0	2	1	1	0	1
	Total	-	-	24	6	26	21	23	6	9	21	22	15	19
Lignin Degrading Auxiliary enzyme	aryl-alcohol oxidase	EC:1.1.3.7	AA3_2a	8	0	7	0	0	0	0	27	0	4	22
	glucose oxidase	EC:1.1.3.4	AA3_2b	2	7	3	7	2	3	3	1	1	1	0
	alcohol oxidase	EC:1.1.3.13	AA3_3	4	4	4	3	4	5	5	2	5	2	5
	Pyranose oxidase	EC:1.1.3.10	AA3_4	1	1	0	1	0	0	0	0	0	0	0
	vanillyl-alcohol oxidase	EC:1.1.3.38	AA4	2	3	2	2	2	2	3	0	2	1	1
	Glyoxal oxidase	EC:1.1.3.-	AA5_1	3	0	8	6	8	1	2	5	2	5	9
	Galactose oxidase	EC:1.1.3.9	AA5_2	2	2	1	1	7	1	1	1	2	5	0
	Benzoquinone reductase	EC:1.6.5.6	AA6	2	4	1	4	2	1	2	3	2	2	4
	Total	-	-	24	21	26	24	25	13	16	39	14	20	41
Total	-	-	-	102	99	105	98	137	40	54	136	130	51	98

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China Agriculture Research System

Comparison between mycelial growth and morphogenesis of fruiting of *Agaricus bisporus* in high temperature

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Abstracts

The white button mushroom, *Agaricus bisporus* is commercially the fifth important edible mushroom, accounting for a Korean production of 11,493 tons in Korea, 2014. The button mushroom mostly cultivated at mushroom farm controlled 16-19℃ temperature during fruiting period in all seasons. For this reason, the production costs including the cost of energy, is very expensive to keep optimal cultivation temperature. In this study, strains collected from various countries were investigated on the mycelial growth at different incubation temperature and cultivated at different growth temperature conditions to use baseline data to develop high temperature-tolerant varieties. Mycelial growths of the strains were measured after incubation in CDA (Compost Dextrose Agar) during two weeks. 12 strains among total 197 strains were divided into 3 groups. Strains incubated well in low temperature(15, 20℃) as A group, high temperature(25, 30℃) as B group and all incubation temperature(15, 20, 25, 30 ℃) as C group. These strains cultivated in 16℃ and 25℃ temperature to confirm relationships between mycelial growth and morphogenesis of fruiting bodies in different temperature. As this cultivation results, strains including A group was 6 days faster than those including C group at fruiting formation in high growth temperature ($p<0.05$). We will analyze genes related incubation, growth temperature and fruiting formation with mycelial and fruiting bodies.

Method and Results



Fig. 1. Phylogenic relationships of 197 collected *A. bisporus* strains by RAPD analysis. All stains were used in this study.

• Red box, strains with tercile-based mycelial growth at high temperature(25, 30℃) ; Blue box, strains with tercile-based mycelial growth at low temperature(10, 15℃); Green box, strains with tercile-based mycelial growth at all temperature(10, 15, 25, 30℃)

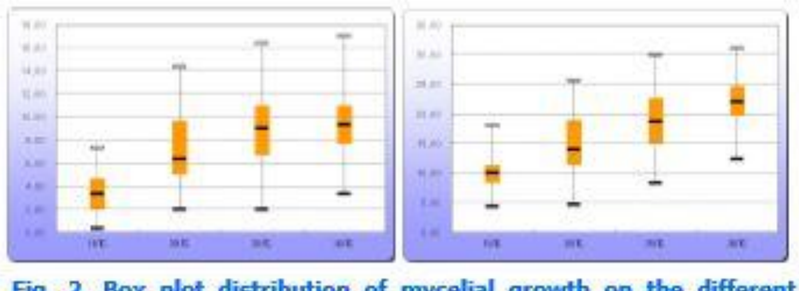


Fig. 2. Box plot distribution of mycelial growth on the different incubation temperature after cultivation for 7 days(left) and 14 days(right).

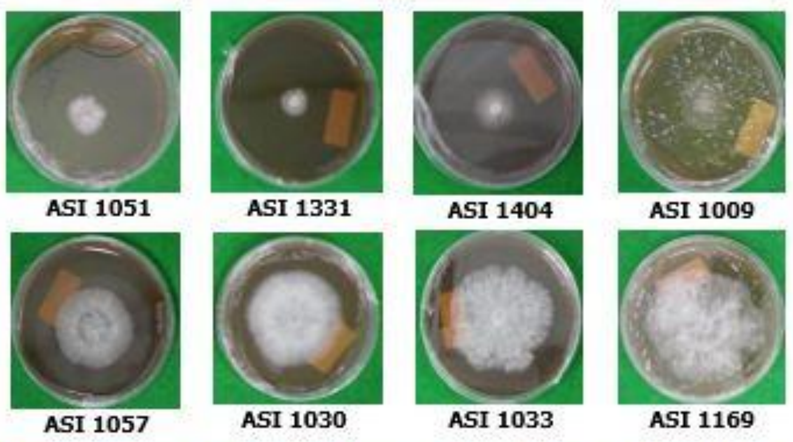


Fig 3. Comparison strains of minimal mycelial growth(above) with those of maximal growth(below) on the different incubation temperature(from left: 15, 20, 25, 30℃)

Table. 1. Selected strains with faster mycelial growth at low incubation temperature, 15, 20℃ (A group), in both low and high incubation temperature 25, 30 ℃(B group) and high incubation temperature(C group) of 197 collected *A. bisporus* strains.

NO	Group	Strains	Species	Coloor of pileus	Origin
1	A	1011	*Agaricus bisporus*	white	Korea(Daegu)
2		1018	*Agaricus bisporus*	white	Korea(Gwangju)
3		1021	*Agaricus bisporus*	white	Korea(Busan)
4		1057	*Agaricus bisporus*	white	Korea(Namwon)
5	B	1015	*Agaricus bisporus*	white	Korea(Iksan)
6		1019	*Agaricus bisporus*	white	Korea
7		1042	*Agaricus bisporus*	white	UK
8		1114	*Agaricus bisporus*	white	America
9	C	1030	*Agaricus bisporus*	white	America
10		1062	*Agaricus bisporus*	white	Japan
11		1087	*Agaricus bisporus*	white	Taiwan
12		1169	*Agaricus bisporus*	white	Korea
13	Control	Saea	*Agaricus bisporus*	white	Korea
14		Saejoong	*Agaricus bisporus*	white	Korea
15		Seureum	*Agaricus bisporus*	white	Korea
16		Seado	*Agaricus bisporus*	white	Korea



Incubation condition
: 25 ℃, 80~85%
Cultivation condition
1. Control : 16±2℃, 87~90%
2. FHT: 25±2℃, 92~95%

Fig. 4. Cultivation condition of *A. bisporus* to confirm the ability of fruiting in high temperature(FHT).

Table. 2. The yield and morphological traits on the different cultivation temperature(asterisks indicates the significance of the t-test ($p<0.05$)).

Group	NF[a]	YE[b]	MW[c]	DP[d]	TP[e]	TS[f]	LS[g]	HP[h]	HS[i]	LP[j]	E[k]
16℃	29±13	928±323	33±7	45±2	24±2	18±3	34±7	4±1	4±1	92±3	24±5
25℃	10±9	321±223	36±13	47±3	24±2	18±3	34±7	5±1	5±1	93±2	33±6

[a]Number of fruiting bodies, [b]Yield, [c]Mushroom Weight, [d]Diameter of Pileus, [e]Thickness of Pileus, [f]Thick of Stipe, [g]Length of Stipe, [h]Hardness of Pileus, [i]Hardness of Stipe, [j]Lightness of Pileus, [k]Earliness

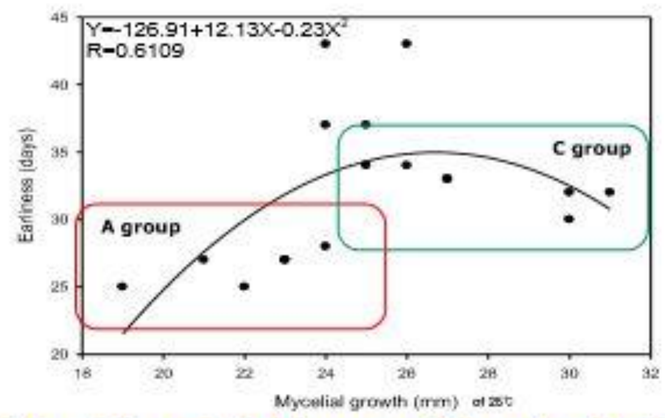


Fig. 5. Correlation analysis between mycelial growth and earliness in high temperature.

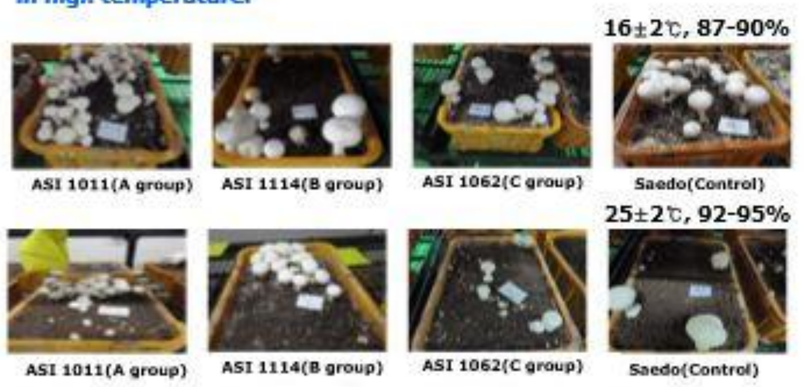


Fig. 6. The FHT trait of each group(A: faster mycelial growth in low temperature, 15, 20℃, B: faster mycelial growth in low and high temperature, 25. 30℃, C: faster mycelial growth high temperature)

Conclusion

- The morphological traits, yield and earliness of *A. bisporus* are different in low and high temperature.
- The strains with faster mycelial growth in low temperature were earlier generated fruiting bodies than strains with thermotolerance during mycelial growth in high temperature.

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Table. 3. The yield and morphological traits of three groups(A: faster mycelial growth in low temperature, 15, 20℃, B: faster mycelial growth in low and high temperature, 25, 30℃, C: faster mycelial growth high temperature) on the different cultivation temperature(asterisks indicates the significance of the t-test ($p<0.05$)).

Control condition : 16±2℃, 87~90%

Group	NF	YE	MW	DP	TP	SS	LS	HP	HS	LP	E
A	30±15	847±379	30±8	45±1	25±3^{bc}	22±19	37±5	4±1^{ab}	4±1^{ab}	93±2	23±5
B	30±9	992±219	34±5	46±2	23±1^{a}	19±2	32±4	5±1^{b}	5±1^{b}	92±3	24±5
C	25±10	865±359	35±6	45±2	24±2^{ab}	20±4	33±6	4±1^{ab}	4±1^{ab}	92±3	25±6
Control	32±17	993±348	34±7	46±3	26±2^{c}	19±2	35±10	4±1^{a}	3±1^{a}	92±3	24±3

FHT condition: 25±2℃, 92~95%

Group	NF	YE	MW	DP	TP	SS	LS	HP	HS	LP	E
A	13±14	353±291	34±10^{a}	49±3	24±1	18±1^{a}	35±3^{a}	5±2	4±1^{a}	93±1	**29±3^{a}**
B	13±8	379±213	36±15^{a}	46±5	24±1	17±2^{a}	39±7^{b}	5±1	5±1^{ab}	93±1	30±6^{ab}
C	10±6	341±199	37±9^{a}	47±2	25±2	18±2^{a}	34±4^{a}	6±1	5±1^{ab}	93±1	**35±6^{bc}**
Control	32±17	993±349	34±7^{b}	46±3	26±2	19±2^{b}	35±10^{c}	4±1	3±1^{b}	92±3	38±6^{c}
Control B	8±5	291±140	38±10^{ab}	47±2	24±2	27±4^{c}	23±5^{d}	5±1	5±1^{ab}	5±1	28±7^{a}

* NF, Number of fruiting bodies; YE, Yield; MW, Mushroom Weight; DP, Diameter of Pileus, TP, Thickness of Pileus, TS, Thick of Stipe, LS, Length of Stipe, HP, Hardness of Pileus, HS, Hardness of Stipe, LP, Lightness of Pileus, E: Earliness

On the usefulness of cytometric tools to select homokaryons in *Agaricus bisporus*

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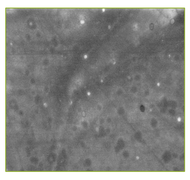
Agaricus bisporus is one of the most commonly cultivated mushrooms worldwide. The current commercial cultivars are derived from a very narrow genetic basis. Despite its economic relevance, breeding efforts in this crop species are clearly hampered by its unfavorable life cycle equivalent to a pseudoclonal reproductive system. Most of the strains are bisporic with basides bearing a majority of heterokaryotic spores and a very small number of homokaryotic ones. A major bottleneck in the development of a breeding program for *A. bisporus* lies in the difficulty of isolating homokaryons (n) from heterokarons (n+n) among single spore isolates (SSIs). Several methods based on growth rate, fruiting ability or molecular markers are practiced, with, for each, their own drawbacks and limitations. Based on the difference in spore size between bisporic and tetrasporic *A. bisporus* strains (Callac et al. 2003), the aim of the study was to evaluate the usefulness of cytometric tools as a new method of isolating homokaryotic spores from SSI's. While the feasibility of such an approach has been already demonstrated to characterize mushroom spores (Allman, 1992, Kullman et al. 2005, Veselska et al, 2014), to our knowledge, our work is one of the first attempt on *A. bisporus*.

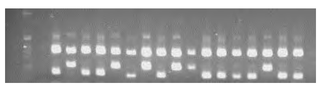
Material & Methods.



- 22 *A. bisporus* strains among which 16 and 6 produce bisporic and tetrasporic basidia respectively
- Collection of spore print after fruiting trials
- Counting of spores and calculation of average spore number per basidia (ASN) (Imbernon et al. 1996)
- Measures of spore size parameters : length (L), width (l), volume (V) (Callac et al. 2003)
- Measures of FSC (Forward Scatter ~ cell size) and SSC (Side Scatter ~ granularity) parameters in PBS buffer using FACS Aria BD Biosciences flow cytometer sorter

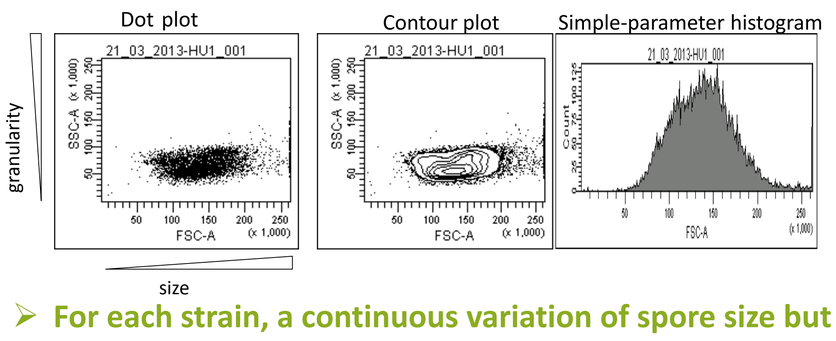
- Staining DNA nuclei of living spores with DAPI+DMSO
- Detection and quantification of fluorescence with cytometer
- Sorting of living spores according to their level of fluorescence
- Single spore isolates (SSI) and DNA extraction
- Confrontation with level of ploidy using 2 independent molecular markers (Kerrigan 1992) : PR006 linked to the *MAT* locus on Chrom I + PR066/*Rsa*I on Chrom III (Foulongne-Oriol et al. 2010)





Results.

- Example of cytometer output (HU1 strain)

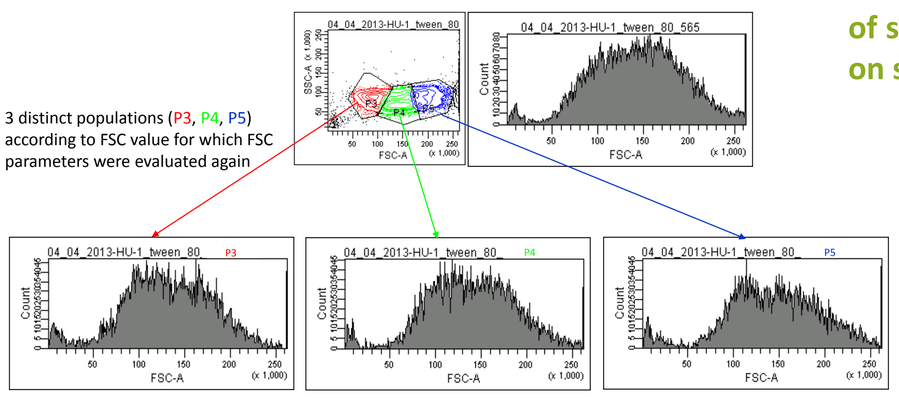


➢ **For each strain, a continuous variation of spore size but …**



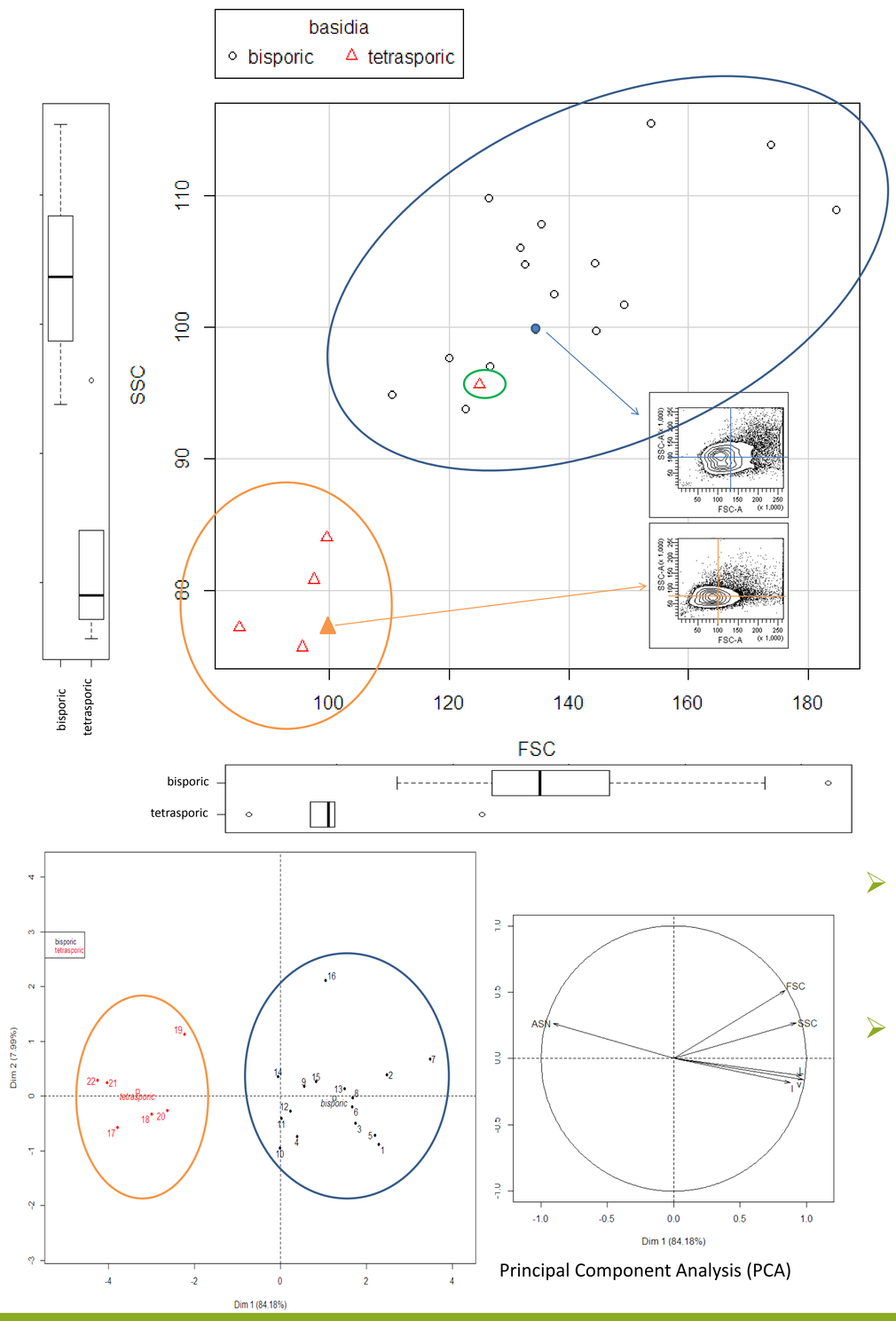
- Sorting on the basis of FSC parameter

… without any differentiation of spore sub-populations based on size parameter.



Ovoid shape of the spore ? Low representativeness of homokaryotic spores vs. heterokaryotic ones ?

- Characterization of the 22 strains



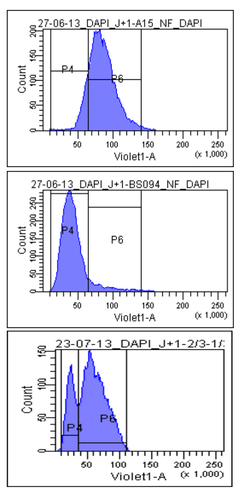
➢ **A clear discrimination between bisporic and tetrasporic strains was possible based on flow cytometer parameters.**

➢ **A phenotypic variability within each group could also be highlighted.**

➢ **The ASN trait structured our sample of strains.**

➢ **FSC and SSC were found highly correlated with other size parameters.**

- Measure of fluorescence after staining



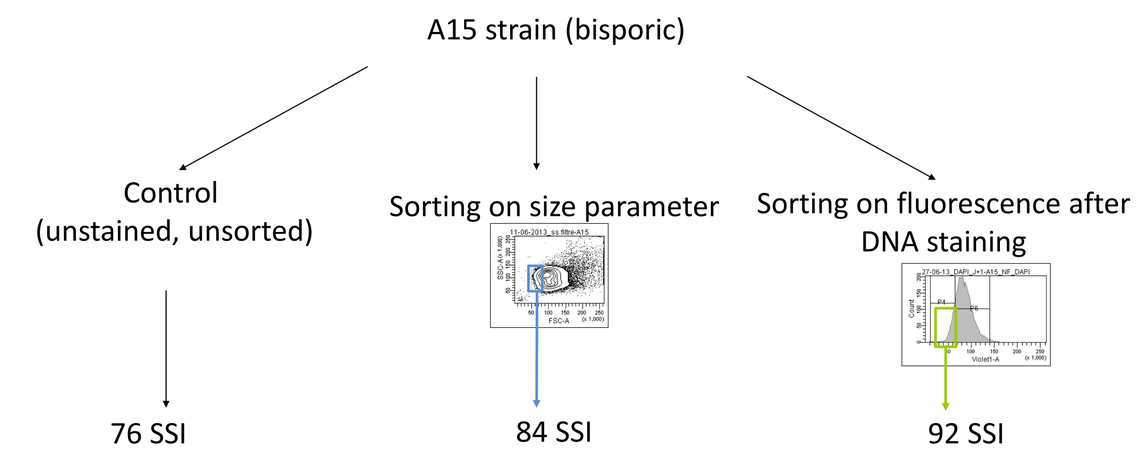
Bisporic basidia strain (mainly heterokaryotic spores)
(*A. bisporus* var. *bisporus*)

Tetraporic basidia strain (mainly homokaryotic spores)
(*A. bisporus* var. *burnettii*)

Mix 2/3 bisporic/tetrasporic

➢ **The level of DAPI fluorescence reflects the level of ploidy of the spores mainly produced by a strain.**

- Feasibility of sorting on the fluorescence level to select homokaryotic spores



➢ **Sorting on size parameter did not allow an enrichment in homokaryons.**

➢ **Sorting on fluorescence increased the efficiency of homokaryon recovery by 5.5 fold.**

Conclusions.

- The flow cytometry tool has been used to characterize *A. bisporus* strains. While it was possible to distinguish bisporic strains from tetrasporic ones, it did not allow to individualize spore sub-populations that coexist in each group on the basis of size parameters only.
- Sorting on the level of ploidy with a non destructive protocol is an alternative. The DAPI was found efficient to stain DNA in living spores, however its use raises the question of its remanence in the mycelium.
- This work is a proof of principle that the flow cytometry associated with DNA staining and subsequent sorting on fluorescence is a promising way to select quickly and efficiently homokaryotic spores.

Allman 1992, Myc. Res. 96:1016-1018 - Callac et al. 2003, Mycologia 95:222-231 - Foulongne-Oriol et al. FGB, 47:226-236 - Imbernon et al. 1996, Mycologia 88:749-761 - Kerrigan, 1992, Mycologia, 84:575-579
Kullman et al. 2005, in Proc. XVI Symp. of Mycologists and Lychenologists of Baltic State. 119–123 - Veselska et al. 2014, Cytometry 85A:854861

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Genetic transformation of the model basidiomycete *Coprinopsis cinerea*

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Coprinopsis cinerea was amongst the first fungi for which a functional transformation system had been established. Transformation made use of a *trp1.1,1.6* auxotrophy that can be complimented by the cloned *trp1*$^+$ wild type gene which randomly integrates into the host genome (Binninger et al., 1987). Transformation in *C. cinerea* is especially efficient since the fungus produces unicellular haploid oidia in its aerial mycelium which are easily transformed into protoplasts and also easily regenerated (Dörnte and Kües 2012). With time, also the *trp3*$^+$ and the *pab1*$^+$ genes have been appointed for complementation of auxotrophies in *C. cinerea* (Bhattiprolu et al. 1993; Granado et al. 1997). In addition, the bacterial hygromycin and phleomycin resistance genes *hph* and *ble* and fungal carboxin-resistance *sdi1*^{R} genes are available for selection of transformants (Kilaru et al. 2009). Despite of using DNA-mediated transformation in numerous molecular analyses for nearly 30 years, there are still factors limiting its applicability. More selection markers of homologous and heterologous origin are desirable, for example to allow sequential transformations of a strain or as markers in knocking out host genes without any interference at markers´ homologous sites. Therefore, the focus of this study is on the further improvement of the oidia transformation technique of *C. cinerea*, better vector design, development of new selection markers and optimizing screening techniques for further characterization of transformants. We summarize important factors in generating highly competent protoplasts, present new homologous and heterologous *trp1*$^+$-based vectors, introduce partly inactivated bifunctional enzymatic markers, visual screening for successful expression of co-transformed genes and a fast microwave-PCR transformant screening method.

Host cells for DNA uptake and established marker

Haploid oidia of *C. cinerea* are easily protoplasted by a lytic enzyme mix of cellulase Onozuka R-10 from *Trichoderma viride* (Serva 16419) and chitinase from *Streptomyces griseus* (Sigma C6137) and afterwards also easily regenerated. For high transformation rates, a level of 50-70% protoplasting should be achieved, a stage which is recognized by clumping of protoplasts and partially digested spores (spheroplasts). Several hundreds of transformants can be obtained per µg plasmid DNA from 0.5-1x10^8 of such protoplasts (Binninger et al. 1987, EMBO J 6:835–840). For potential pitfalls and further explanations in protoplasting, transformation and regeneration see the trouble-shooting guide by Dörnte & Kües 2012, CTPB 6:340-355.

Protoplasting and transformation protocol

Oidia

from fresh fully grown YMG/T Petri dishes washed with MM buffer
(2:1:1 mix of 1 M mannitol, 0.2 M Na maleate, pH 5.5 and bidest. H_20)

↓

Protoplasting

at 37°C for 4-6 h with 80 mg cellulase Onozuka R-10 + 1 mg chitinase in 2 ml MM
reaction stop by addition of 10 ml MMC (2.5% 1 M $CaCl_2$ in MM)
2x washing in MMC, uptake of protoplasts in 500 µl MMC

↓

Transformation

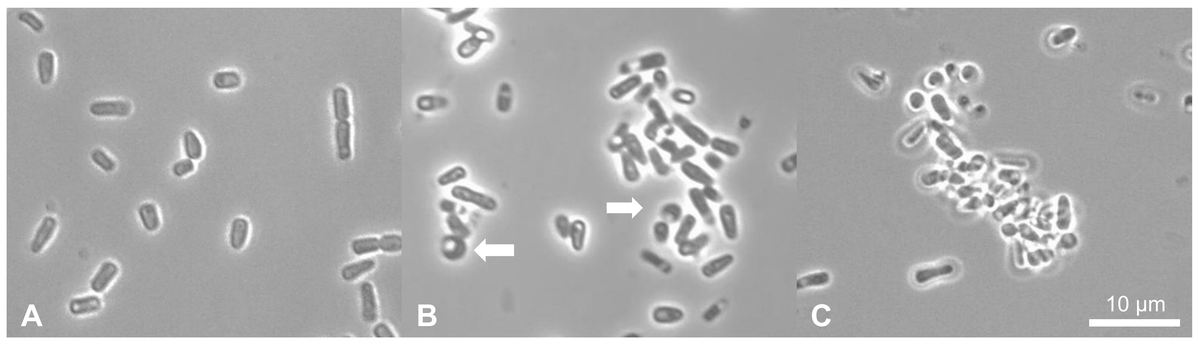
20 min incubation on ice of 50 µl protoplasts plus 1 DNA + 12.5 µl 5% PEG 4000
addition of 500 µl 5% PEG 4000 and incubation for heat shock at RT for 5 min
dilution of protoplasts by addition of 1 ml STC buffer
(1 M sorbitol, 25 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5)

↓

Plating and regeneration

plating of 300 µl aliquots of protoplasts on fresh soft regeneration agar (RM + 1% agar)
incubation at 37°C in humid conditions
evaporation of surplus liquid on agar surface 2 to 3 d later
picking of small colonies as soon as they appear

Protoplasting of oidia



A. Rod-shaped oidia of strain FA2222 prior to enzymatic digestion. **B.** and **C.** Clumping of oidia, partially digested spores and protoplasts (see arrows) after 3 h and 5 h incubation with the enzyme mix.

Table 1: Marker genes available for complementation of auxotrophic mutations or conferring resistance to antibiotics in *C. cinerea*

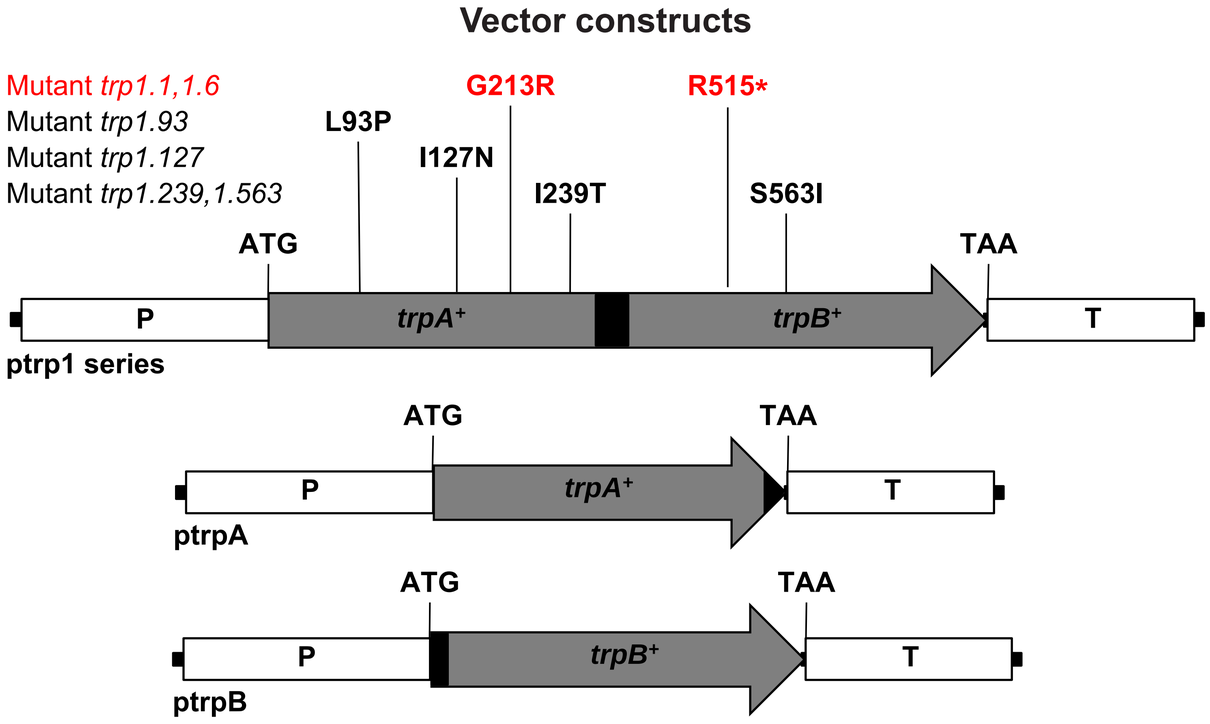
Marker	Protein	Selection	Reference
ade8$^+$	*C. cinerea* aminoimidazole ribonucleotide synthetase	Complementation of *ade8*$^-$	Casselton et al. (1989) Proc. EMBO-Alko workshop 6:139-148
pab1$^+$	*C. cinerea para*-aminobenzoic acid synthase	Complementation of *pab1*$^-$ Sulfonamid resistance	James et al. (2002) J Basic Microbiol 42:91-103
trp3$^+$	*C. cinerea* anthranilate synthase	Complementation of *trp3*$^-$	Burrows (1991) PhD-thesis, Bhattiprolu et al. (1993) Myc Res 97:1281-1286
trp2$^+$	*C. cinerea* trifunctional enzyme with glutamine amidotransferase, phosphoribosyl-anthranilate-isomerase and indole-glycerol-phosphate synthase activities	Complementation of *trp2*$^-$	Casselton et al. (1989) Curr Genet 16:35-40
trp1$^+$	*Schizophyllum commune* trifunctional enzyme with glutamine amidotransferase, phosphoribosyl-anthranilate-isomerase and indole-glycerol-phosphate synthase activities		
trpC$^+$	*Phanerochaete chrysosporium* trifunctional enzyme with glutamine amidotransferase, phosphoribosyl-anthranilate-isomerase and indole-glycerol-phosphate synthase activities		
trp1$^+$	*C. cinerea* tryptophan synthase	Complementation of *trp1*$^-$	Binninger et al. (1987) EMBO J 6:835–840
trpB$^+$	*S. commune* tryptophan synthase		Dörnte & Kües unpublished
sdi1	*C. cinerea* mutated succinate dehydrogenase	Flutolanil & carboxin resistance	Ito et al. (2004) Mol Gen Genomics 272:328-335
hph	*Escherichia coli* hygromycin B phosphotransferase	Hygromycin resistance	Cummings et al. (1999) Curr Genet 36:371-382
ble	*Streptoalloteichus hindustanus* phleomycin binding protein	Phleomycin resistance	Kilaru et al. (2009) Curr Genet 55:543-550

New selection markers for sequential transformation

C. cinerea tryptophan synthase Trp1 is a bifunctional enzyme that catalyzes the final two reactions in the tryptophan biosynthesis. The N-terminal A-domain (encoded by the 5´ *trpA*$^+$ half of *trp1*$^+$) is responsible for the conversion of indole-3-glycerol-phosphate (IGP) into indole, while the C-terminal B-domain (encoded by the 3´ *trpB*$^+$ half of *trp1*$^+$) catalyzes the subsequent production of tryptophan from serine and indole. The *trp1.1,1.6* mutant allele used in *C. cinerea* hosts in transformation carries a mutation in each gene half, both of which prevent the strain from completing tryptophan biosynthesis. Here, we wanted to exploit the situation of the two independent mutations in *trp1*$^-$ for sequential transformation of a same nucleus. Since it is possible to add indole to the medium, it is the *trpB* mutation (*trp1.6)* that must be complemented in the first round of transformation.

By erroneous PCR, we amplified mutated versions of *trp1*$^+$ (see below). We first tested mutant *trpA* mutant L93P in transformation under indole selection, but complementation of the *trp1.6* mutation was not achieved. Next we separated the two gene half's of *trp1*$^+$ to create vectors containing either only a functional *trpA*$^+$ sequence or only a functional *trpB*$^+$ under regulatory control of the original *trp1*$^+$ promotor and terminator sequences. The *trpB*$^+$ construct has successfully been used for selection of transformants on regeneration agar with added indole and the *trpA*$^+$ construct in subsequent transformation using simple regeneration agar for selection of indole production.

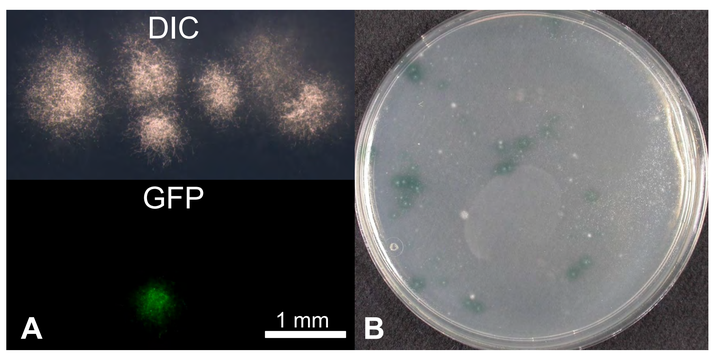
Vector constructs



Easy screening techniques for co-transformants

In transformation *C. cinerea* protoplasts take up multiple DNA molecules, a feature which allows efficient cotransformations with different plasmids. Detection of cotransformants directly on regeneration agar can be achieved by using visual markers like the product of the enhanced *gfp* gene (fluorescence emission) or enzymatic reactions such as mediated by the laccase gene *lcc1*$^+$ of *C. cinerea* (ABTS color assay) .

Fast microwave-based DNA extraction (Dörnte & Kües 2012, CTPB 7:825-836) in connection with PCR is another quick option for further characterization of transformants to detect specific transformed DNA fragments. An example for molecular detection of integration of the *trp1*$^+$ gene at its homologous site is shown below.



A. Emerging *trp1* + *gfp* cotransformants on RM plates after 4 days of incubation at 37°C, examined by differential interference contrast (DIC) and fluorescence (GFP) microscopy. **B.** *trp1* + *lcc* cotransformants growing on ABTS-supplemented RM plates after 5 days of incubation. Green halos indicate laccase expression by positive clones.

Molecular screening of transformants

Material

collect a tiny piece of mycelium with a needle
transfer into 100 µl sterile H_20 in E-cup

↓

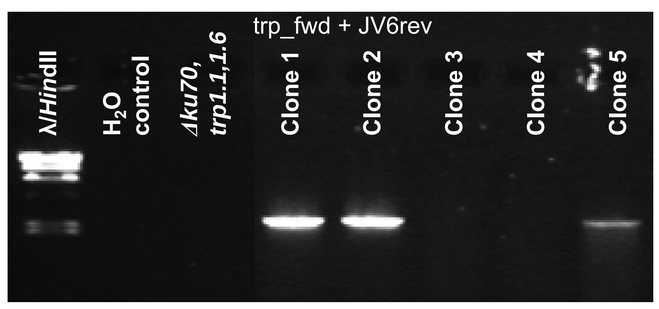
DNA extraction

1 min microwaving at 600 W
30 sec vortexing at RT
1 min microwaving at 600 W
10 min at -20°C
5 min centrifugation at 9,300 x *g*

↓

Standard PCR

use 1 µl in PCR



PCR amplification of a 2.6 kb fragment with *trp1*$^+$-specific sequences from microwave-isolated DNA to confirm homologous integration in a *Δku70*, *trp1.1,1.6 C. cinerea strain*. *trp1*$^+$ inserted at the natural site of the gene but not the mutant allele at the site is amplified by the allele-specific *trp1*$^+$ primer JV6_rev combined with an outside located primer trp_fwd.

Asexual spores as means for de-dikaryotization of mycelia

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Mitospores have been observed on mycelia of various Agaricomycetes but usually have found only limited interest in the past. A general believe was that mitospores are more often produced on monokaryons than on dikaryons [1,2]. A recent literature account on multiple species of Agaricomycetes comes to a different conclusion, i.e. that mitospore production on dikaryons might be much more widespread on dikaryons than so far thought [3]. Mitotic spores can be broadly distinguished into arthroconidia (oidia), blastoconidia and chlamydospores. The latter are thick-walled and produced within aging hyphae and serve for dormancy. Arthroconidia and blastoconidia in contrast might have functions in fungal distribution and in mycelial spermatisation. Such spores when formed on monokaryons can naturally only germinate into monokaryons. Mitospores on dikaryons might either be homokaryotic or heterokaryotic although the nuclear status of the spores is often still unknown. Here we will give a summary of species where the nuclear status of spores formed on dikaryons is known (Table 1). Species differ in whether only homokaryotic spores are formed on dikaryons, or only heterokaryotic spores or percentages of both. Homokaryotic spores with only one type of haploid nuclei can be of impacts in genetic structures of populations when they mate with new compatible partners [4]. *Coprinopsis cinerea* is a species that forms abundant uninucleate oidia on monokaryotic mycelia and lower numbers of uninucleate oidia upon light induction on the dikaryon. Oidia are also formed on dikaryons of other *Coprinopsis* species and of *Coprinellus* species [3,5]. We now analyze the nuclear status of these spores formed on dikaryons, their numbers of production and their germination abilities.

Introduction

Table 1 present data on asexual spore production on dikaryotic (heterokaryotic) mycelia of different Agaricomycetes as collected from the literature (3). For dedikaryotization, i.e. formation of either hyphal cells or spores with only one nuclear type, nuclei of different mating types have to become separated from each other. This may happen by outgrowth of monokaryotic hyphae from dikaryotic hyphal cells, or more specifically by outgrowth from (unfused) clamp cells at dikaryotic hyphae, or by nuclear migration of individual nuclei into sporophores or spores, respectively. In any case, the mating type communication between distinct nuclei has to become interrupted for dedikaryotization. Spores of preferentially one distinct mating type might be produced by a dedikaryotization process.

Table 1. Asexual spores formed on dikaryons of selected Agaricomycetes and their nuclear status

Species	Spores types and their nuclear status	Further observations
Agaricus arvensis	Binucleate oidia	-
Bjerkandera fumosa	Uninucleate arthrospores	Outgrowth of clampless monokaryotic hyphae from dikaryotic hyphae prior to sporulation
Coprinopsis cinerea	Uninucleate oidia	Formation on clamped cells Production is light induced Formation is biased towards on mating type There is an *A* mating type allele hierarchy in preference of nuclei present in oidia
Coprinus xerophilus	Binucleate oidia	-
Flammulina velutipes	Uninucleate oidia	Formation on clampless hyphal branches Formation is biased towards on mating type There is an *A* mating type allele hierarchy in preference of nuclei present in oidia
Galzinia incrustans	80% binucleate blastoconidia, >10% uninucleate, rest trinucleate	-
Heterobasidion annosum s.l.	Binucleate heterothallic and binucleate or uninucleate homothallic blastoconidia	Formed on vesicles of conidiophores Strain dependent, 25% of spores might be of one mating type, 20% of the other and 50% of spores heterokaryotic, or in the other extreme, there might be spores of preferentially only one mating type
Hydnum raduloides	Uninucleate blastoconidia	Formed on uninucleate conidiophores produced by dikaryotic cells
Hyphoderma mutatum	Uninucleate blastoconidia	Formed on vesicles of conidiophores
Moniliophthora perniciosa	Binucleate oidia	-
Pholiota aurivella	Binucleate arthrospores	-
Pholiota gummosa	Binucleate oidia	-
Pholiota microspora	70% uninucleate , 15-25 % binucleate oidia, remaining polynucleate	Outgrowth of short monokaryotic hyphae 90% of all oidia germinate into monokaryons Many of the binucleate and polynucleate spores are homokaryotic There is a mating type allele hierarchy in preference of nuclei present in oidia
Polyporus adustus	Uninucleate arthrospores	Outgrowth of clampless monokaryotic hyphae prior to sporulation
Polyporus varius	90% uninucleate, 10% dikaryotic oidia	On short hypha with clamped and non-clamped septa
Simocybe sumptosa	Binucleate conidia	-
Termitomyces	Binucleate heterokaryotic	-
Trametes hirsuta	Uni- and binucleate oidia	-

Asexual spore production on some *Coprinellus* and *Coprinopsis* species

We are more specifically interested in asexual sporulation of the Coprini and reported before oidia production on dikaryons of *Coprinopis cinerea* and different *Coprinellus* species (5; Fig. 1). Here we further investigate mitospore production of four different *Coprinellus* species, using in total selected 7 strains, and compare this with sporulation of a dikaryon *C. cinerea* (Table 2). Dikaryons in *C. cinerea* are easily recognized by formation of true clamps on hyphal septa (Fig. 1A). *Coprinellus* strains in contrast have no clamps or only pseudoclamps on some hyphal septa. However, all *Coprinellus* strains produce fruiting bodies, identifying them as dikaryons (5). *Coprinellus* strains as *C. cinerea* produce asexual spores on oidiophores (Fig. 1). We discovered that all *Coprinellus* strains produce more spores in light than in dark as *C. cinerea* dikaryons (Table 2).

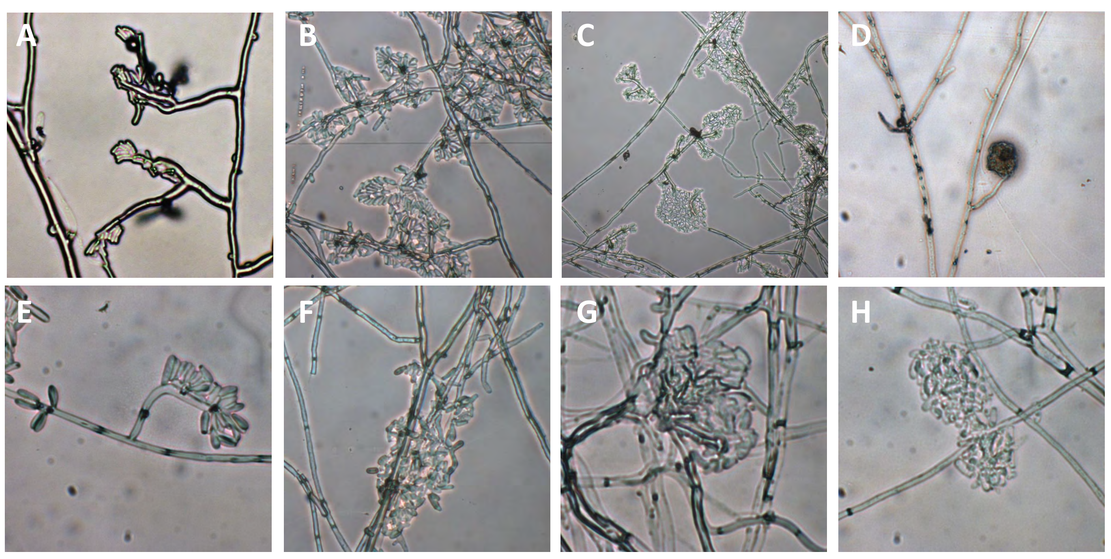


Figure 1. Asexual sporulation of *Coprinellus* species and *Coprinopsis cinerea* cultivated on microscope slides and observed under the microscope
(A) *Coprinopsis cinerea*, (B) *Coprinellus domesticus* C72, (C) *Coprinellus ellisii* C140,
(D) *Coprinellus radians* C22, (E) *Coprinellus* aff. *radians* C35, (F) *Coprinellus xanthothrix* C482,
(G) *C. xanthothrix* 398, (H) *C. xanthothrix* 144

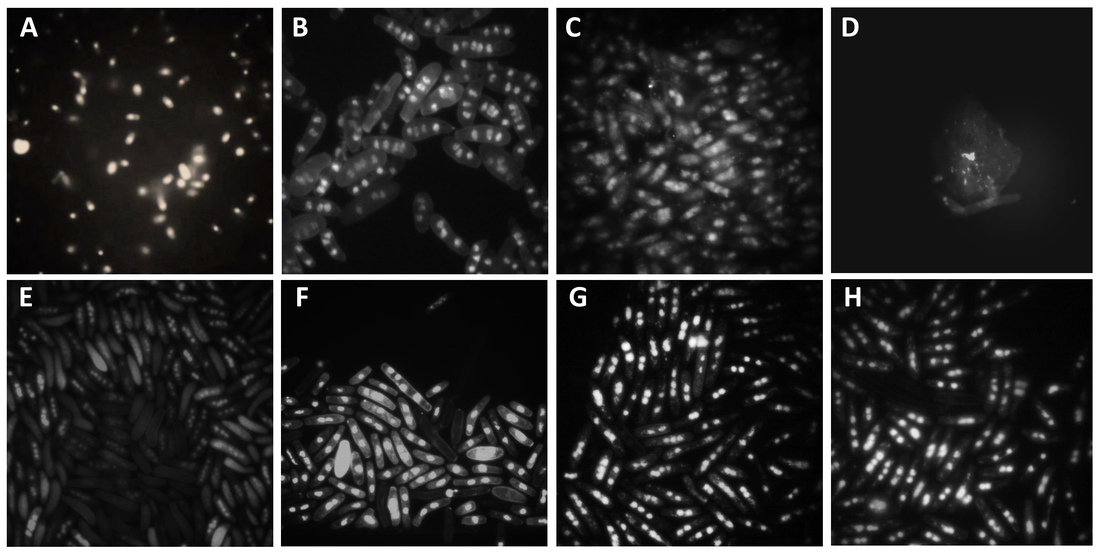


Figure 2. DAPI staining of asexual spores of *Coprinellus* species and *Coprinopsis cinerea*
(A) *Coprinopsis cinerea*, (B) *Coprinellus domesticus* C72, (C) *Coprinellus ellisii* C140,
(D) *Coprinellus radians* C22, (E) *Coprinellus* aff. *radians* C35, (F) *Coprinellus xanthothrix* C482,
(G) *C. xanthothrix* 398, (H) *C. xanthothrix* 144

Nuclei were stained of spores of all strains. *C. cinerea* oidia contained always only a single nucleus per spore for germination into monokaryons, unlike the *Coprinellus* strains that differed in numbers of nuclei per spore in ranges from usually 2 up to 6. Only strain *C. xanthothrix* C482 had also spores with only a single nucleus (Table 2). Spores of all strains were plated for germination on YMG/T medium. Germination rates for most strains were low (<10%), with exception of *C. cinerea* PS1x2 and *C. xanthrotrix* C398 which were about 40% (Table 2). Different colony morphologies (mycelial densities, degree of feathery growth, growth speed) were noted for germinated spores from strain *C. xanthrotrix* C482 (Fig. 3). Spores of one first mycelium (Oid7) with more feathery look as compared to the parental strain were analyzed. As the parental strain, spores had either one (29%), two (43%), three (21%) or four nuclei (7%), respectively. Fruiting tests may give insight of whether strains are still heterokaryotic and whether any homokaryotic strain arose from the procedure of spore germination.

Table 2. Oidia of dikaryotic strains of *Coprinellus* species in comparison to *Coprinopsis cinerea*

Strain	Clamp formation	Number of oidia production per YMG/T plate			No. of nuclei per spore (% of total)	% Germination
		Cultivation in dark	Cultivation in light	Increase in light		
Coprinopsis cinerea PS1x2	True clamps	$9.2x10^4 \pm 2.7x10^4$	$9.3x10^5 \pm 3.1x10^5$	10x	1 (100%)	1.1
Coprinellus domesticus C72	No	$5.3x10^6 \pm 1.7x10^6$	$3.6x10^8 \pm 5x10^7$	68x	2 (45%) 3 (30%) 4 (18%) 5 (3%) 6 (3%)	42.9
Coprinellus ellisii C140	No	$7.5x10^6 \pm 1.5x10^6$	$1.7x10^8 \pm 2.6x10^8$	23x	2 (32%) 3 (45%) 4 (23%)	0.5
Coprinellus radians C22	Some pseudoclamps	$3.5x10^2 \pm 1.8x10^2$	$6.1x10^3 \pm 2.9x10^3$	203x	nd	5.3
Coprinellus aff. *radians* C35	Pseudoclamps	$2.6x10^7 \pm 3.5x10^6$	$7.8x10^8 \pm 2.7x10^8$	30x	2 (19%) 3 (35%) 4 (32%) 5 (12%) 6 (2%)	5.7
Coprinellus xanthothrix C482	Pseudoclamps	$3.0x10^6 \pm 9x10^5$	$1.2x10^7 \pm 2.3x10^6$	4x	1 (46%) 2 (45%) 3 (7%) 4 (1%)	8.5
Coprinellus xanthothrix C398	Pseudoclamps	$2.0x10^4 \pm 1.1x10^3$	$4.1x10^7 \pm 8.0x10^6$	2050x	2 (75%) 3 (25%)	41.3
Coprinellus xanthothrix C144	Pseudoclamps	$7.5x10^6 \pm 1.8x10^4$	$1.9x10^8 \pm 2.3x10^5$	25x	2 (73%) 3 (27%)	14.8

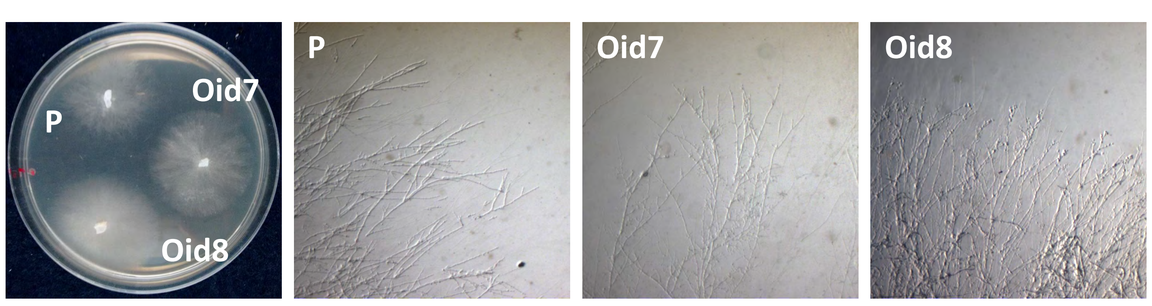


Figure 3. Mycelial morphology of *Coprinellus xanthothrix* C482 (P) and two of its progenies (Oid7, Oid8) obtained from germination of oidia

References
[1] Brodie HJ (1936) The occurrence and function of oidia in the Hymenomycetes. Am J Bot 23:309-327.
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[5] Badalyan SM, Navarro-González M, U Kües (2011). Taxonomic significance of anamorphic characteristics in the life cycle of coprinoid mushrooms. In: Proceed. VII Int. Conf. Mushroom Biology and Mushroom Products, Arcachon, France, Vol. 1, pp. 140-154.

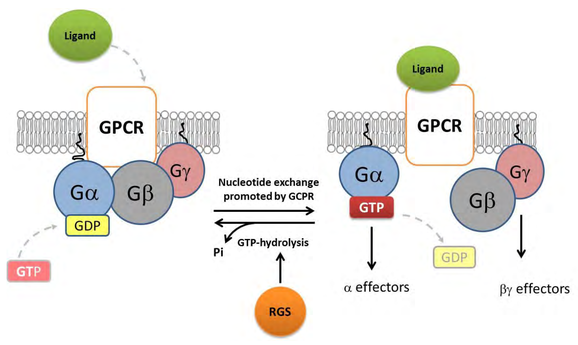
Characterization of some developmental regulators in the mushroom *Coprinopsis cinerea*

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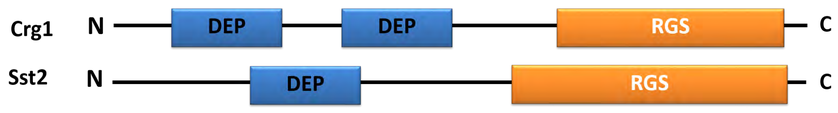
Three putative genes involved in developmental processes in *Coprinopsis cinerea*, *crg1* and two *flu1-II* copies, are being investigated. The homologous genes in the ascomycete *Aspergillus nidulans flbA* and *fluG* work mutually to activate the process of conidiation by initiation of *brlA* expression. Inactivation of these two genes in *A. nidulans* results in fluffy colony growth [1]. FluG contains an N-terminal amidohydrolase domain and a C-terminal glutamine synthetase I (GSI)-like domain and activates conidiation as a specific developmental pathway. For function as a regulator, the GSI-like domain is essential but it does not confer glutamine synthetase activity [2]. The two fluG-homologs in *C. cinerea* contain the C-terminal GSI-like domain but not a amidohydrolase domain. Crg1 as FlbA homolog possesses two DEP (Dishevelled, Egl-10, and Pleckstrin) domains which function in subcellular targeting and as a C-terminal RGS (regulator of G protein signaling) domain. Crg1 homologs in other fungi take part in regulation of processes such as vegetative growth, asexual sporulation, mating, mycotoxin and pigment production and pathogenicity [3]. Genes *crg1* and *crg2* in the basidiomycetous yeast *Cryptococcus neoformans* and the homologous gene *thn1* in the filamentous *Schizophyllum commune* are functionally coupled to G-protein signaling in the pheromone- and the cAMP-response pathways [4,5]. The *C. cinerea nwd2* gene encodes a signal transduction protein with NACHT-NTPases and has been found to suppress a defect in primary hyphal knot formation (*pkn1*) of *C. cinerea* mutant Proto159. In *C. cinerea*, we are investigating the functions of *crg1* and *fluG* in vegetative growth of mono- and dikaryons, in asexual sporulation (oidiation), in mating and in fruiting body formation. To examine the functions of *crg1* and *flu1-II* genes, we approach overexpression of the proteins as well as knocking out the genes in suitable strains with a *ku70* mutation [6], with and without activated mating type pathways.

Function of regulators of G protein signaling (RGS) such as Crg1



[After: Siderovski and Willard 2<005]

RGSs modulate the level of heterotrimeric G protein signaling by acting as GTPase-accelerating proteins (GAPs) for Gβγ-unbound Gα, elevating the rate of GTP hydrolysis, resulting in assembly of Gαβγ and termination of signaling.



Structures of *Coprinopsis* Crg1 and *Saccharomyces* Sst2, two types of DEP domain containing RGS proteins in fungi: Crg1 contains N-terminal two DEP (Dishevelled, Egl-10, and Pleckstrin) domains which play a role in subcellular targeting [5] and a C-terminal RGS domain.

Structure of Flu1-II homolog

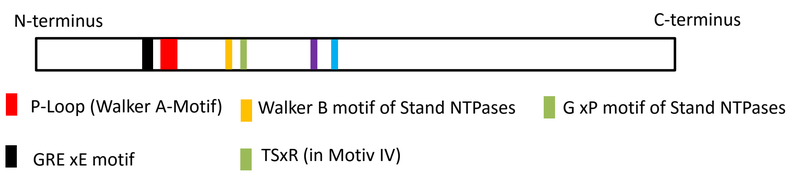


Flu1-II contains an N-terminal amidohydrolase and a C-terminal glutamine synthase I (GSI)-like domain. In filamentous Ascomycota, most the species have at least one copy of a complete *fluG* gene [2]. In addition, many species have one or more copies for proteins containing only a GSI-like superfamily domain. In contrast, a few species (*Cercospora zeae-maydis*, *Ilyonectria* sp., *Mycosphaerella fijiensis*, *Penicillium chrysogenum*) have genes for a protein with just a amidohydrolase domain (own observations).

Of the Basidiomycetes, only two species (*Dacryopinax* sp. and *Sphaerobolus stellatus*) with complete FluG proteins were detected. In most basidiomycetes, the two domains are separated into two independent proteins. Some of the Agaricomycotina have multiple copies of genes for proteins with the GSI-like domain. Some of the Agaricomycota including *C. cinerea* and some species of the Pucciniomycota lack genes for a protein with the amidohydrolase domain (own observations.

Structure of NWD2

NWD2 encodes for a NACHT-NTPase (signal transduction protein with a NACHT domain which is found in animal, fungal and bacterial proteins and named after four different types of P-loop NTPases NAIP, CIITA, HET-E and TP1). As typical for NACHT-NTPases, the NWD2 protein contains an ATP/GTPase specific P-loop and an Mg^{2+}-binding site (Walker A and B motifs, respectively) and other specific motifs such as GRExE and GxP [7].



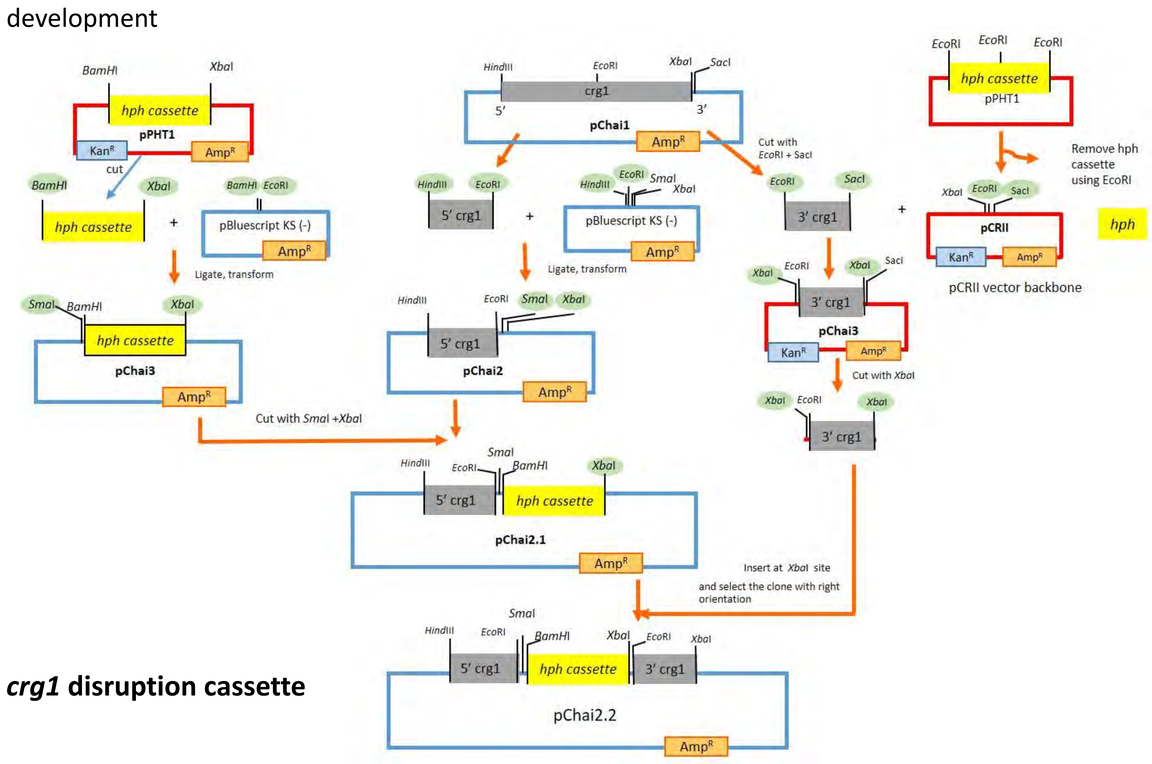
The *C. cinerea nwd2* gene has been found to suppress a defect in primary hyphal knot formation (*pkn1*) of *C. cinerea* mutant Proto159 [8]. This mutant strain was isolated from protoplasting and regenerating oidia of the selfcompatible homokaryon strain AmutBmut.

References

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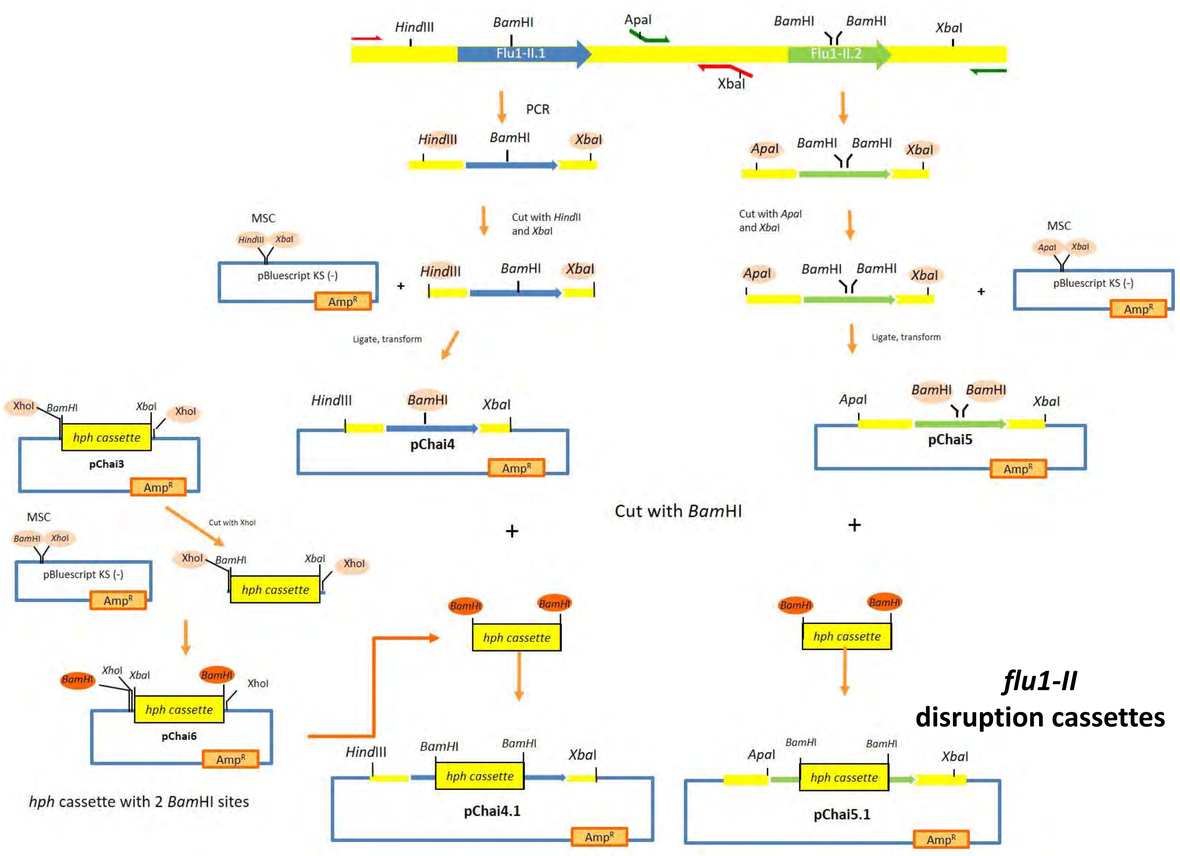
Construction of disruption cassettes

Disruption cassettes will be used to delete *crg1* and *flu1-II* in wildtype monokaryons and in dikaryotic and mutant homokaryotic strains able to undergo fruiting. Knock-out *crg1* strains in *C. cinerea* are expected to behave like the *thin* mutants in *S. commune* that have defects in vegetative mycelial growth (thin aerial mycelium, curly hpyhal growth) and in the *B* mating type control of sexual development



***crg1* disruption cassette**

The *C. cinerea crg1* fragment comprising promoter, coding sequence and terminator was amplified from a Δ*ku70* knockout strain and cloned into MSC site of pBluescript KS(-). The fragment contains in the middle of the gene an *Eco*RI site used for insertion of the hygromycin B resistant cassette (*hph*) giving around 2000 bp flanking homology sequences for gene targeting.



***flu1-II* disruption cassettes**

Two *flu1-II* genes locate adjacently in the *C. cinerea* genome. Two pair of primers with restriction site were used to amplified each fragment individually to clone them into pBluescript KS(-). Both fragment of *flu1-II* contain *Bam*HI restriction sites into which hygromycin resistant cassettes were inserted, giving the disruptions cassette for both *flu1-*II with the 1200-1300 bp homology sequences for homologous gene targeting.

Strategy for overexpression of *crg1*, *flu1-II and NWD2*



An expression cassette consisting of either *crg1* , *flu1-II* or *nwd2* placed under the *gpdII* promoter from *Agaricus bisporus* will be used for transformation of *C. cinerea* monokaryons and *AmutBmut* strains for studying the effects of overexpressions. Inhibition of fruiting body formation and increase in asexual spore production might be expected.

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Institute of Agricultural Resources and Regional Planning of CAAS

Genetic linkage mapping for *Pleurotus tuoliensis* by genome-wide genotyping, 2b-RAD approach

Wei Gao, Jinxia Zhang, Chenyang Huang, Jibin Qu, Qiang Chen, Xiangli Wu, Zhiheng Qiu

Introduction

Pleurotus tuoliensis, Chinese Bailinggu is a rare mushroom species and discovered wildly in Xinjiang of China. The fruitbody is pure white with a delicate texture. It is delicious, nutritious and medicinally valuable. It has been produced commercially and industrially in China. The gross product has increased gradually during the last 10 years, that of 2014 is 0.3 million tones. However, the profit of Bailinggu industry is relatively low compared to that of other mushrooms (e.g. *Pleurotus eryngii* and *Flammulina velutipes*) because of its high production cost, resulted from a long growing period. Breeding for varieties of a short growing period is the urgent need for lowering the production cost of Bailinggu industry. Genetic linkage mapping of this study is the preliminary step of marker-assisted breeding. A prerequisite for constructing a linkage map of high-resolution is the availability of a large number of genetic markers, preferably, SNPs. A high-throughput 2b-RAD method was used to discover and genotype SNPs across the whole genome in this study.





Results

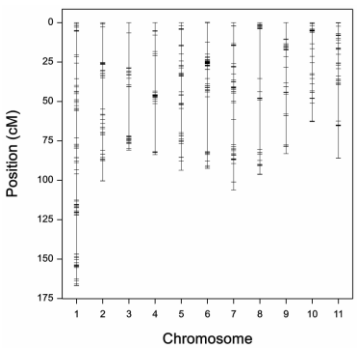
Map characteristics

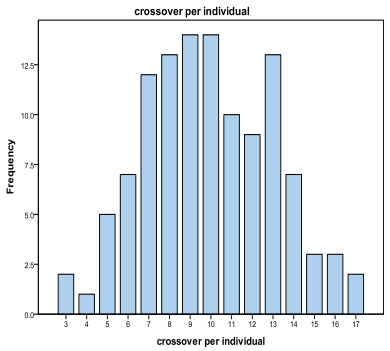
A population of 115 monokaryotic offspring of the hybrid strain (H6) was isolated and genotyped by 2b-RAD method. The two monokaryotic parental lines (H6PA and H6PB) were recovered through protoplasting. In total, 1888 SNP markers were identified. Doubtful markers with errors were omitted in subsequent analysis. A total of 1397 SNP markers were included in the linkage analysis, of which 23 (1.6%) markers showed more than 10% missing value. The analysis of segregation ratio showed that 120 markers (8.6%) deviate from the expected 1:1 ratio (P<0.05). At the LOD threshold of 7.0, 1381 markers were assigned to 12 linkage groups. The map covers a total length of 1077 cM, with an average distance between adjacent markers of 3.9 cM. The size of linkage groups ranged from 25 cM to 167 cM, with an average of 90 cM (see the figure of map chart). The number of markers per group was variable ranging from 26 to 229, with an average of 115 markers per group. Co-segregating markers represented 59% of all the markers. The largest marker interval per group ranged from 14.2 cM to 34.9 cM.

linkage group	map length (cM)	No. of markers	No.of co-segregating markers	Average marker spacing (cM)	largest interval (cM)	No. of crossovers						Mean crossover frequency
						a	b	1	2	3	4	
LG1	167	229	141	0.7	24.6	9	8	55	31	9	3	1.36
LG2	106	130	73	0.8	15.9	21	13	46	19	7	9	1.23
LG3	93.6	166	111	0.6	15.3	16	25	57	16	1	0	0.8
LG4	100.4	116	63	0.9	22.5	19	24	51	17	4	0	0.84
LG5	92.5	131	67	0.7	34.9	26	19	50	5	9	6	0.97
LG6	96.3	126	82	0.8	31.5	19	25	54	12	0	5	0.85
LG7	83.7	100	57	0.8	30.5	22	30	46	10	4	3	0.78
LG8	86	107	65	0.8	23.1	17	28	60	7	3	0	0.72
LG9	83.1	80	39	1.0	18.6	30	14	57	4	8	2	0.84
LG10	62.8	75	36	0.8	14.4	27	30	51	0	7	0	0.63
LG11	80.9	95	60	0.9	31.2	28	23	50	5	6	3	0.78
LG12	24.7	26	17	1.0	14.2	54	39	20	2	0	0	0.21
Total	1077	1381	811	9.8	276.7	234	239	577	126	58	31	10
Average	97.9	125.5	73.7	0.9	25.2	21.3	22	52	11	5	3	0.83

Recombination and Crossover events

Most of the monokaryotic offspring inherited half of the genetic information from each parental lines. Among all the individuals, the minimal proportions of parental genotype were 26% and 18% from H6PA and H6PB, respectively. The average number of crossovers per individual was 10. The distribution of crossovers in the progeny was plotted in the histogram below. For every linkage group (LG), many individuals inherited the intact linkage group (data presented in the Table). The crossover frequency per linkage group per individual varied from 0.21 for LG12 to 1.36 for LG1, and the average of that was 0.83. The A and B mating type factor was mapped to LG2 and LG6.





Discussions

This study presented the first genetic linkage map of *Pleurotus tuoliensis* for future genetic and breeding studies. The map information has assisted the assembly of the whole genome sequencing to assign scaffolds to chromosomes. SNP markers on the map showed an even distribution over the scaffolds (data not shown). The large ratio of co-segregating markers may indicate that crossovers were suppressed within some regions over the genome. The map of *Pleurotus tuoliensis* (1077 cM) was longer than that of *Pleurotus eryngii* (837 cM) as a closed related species (Okuda, 2012), with a chromosome number of 12 (Lee, 2009). If the number of linkage groups correspond to the chromosome number of *Pleurotus tuoliensis* still needs to be further investigated.

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Characteristics of Korean Variety 'Sae-Ah', *Agaricus bisporus*, developed by Genetic Pholymorphism Analysis

Kab-Yeul Jang*, Youn-Lee Oh, Eun-Sun Kim, Minji Oh, Pyung-Gyun Shin and Won-Sik Kong

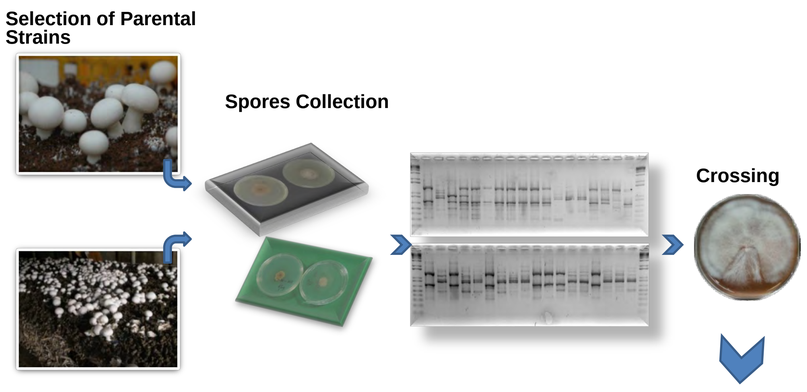
Mushroom Research Division, National Institute of Horticultural & Herbal Science, RDA, Chungbuk Eumseong 27709, Republic of Korea.

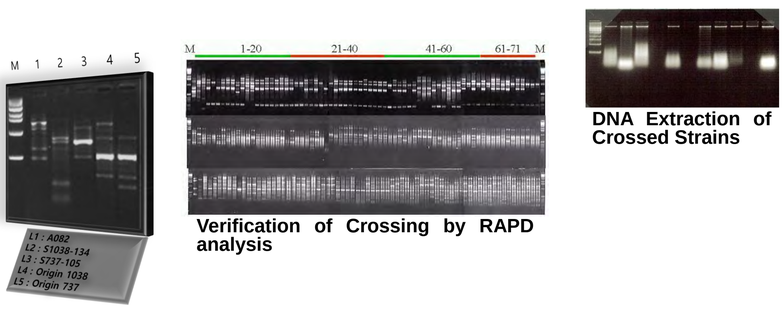
RDA 농촌진흥청 국립원예특작과학원 National Institute of Horticultural & Herbal Science

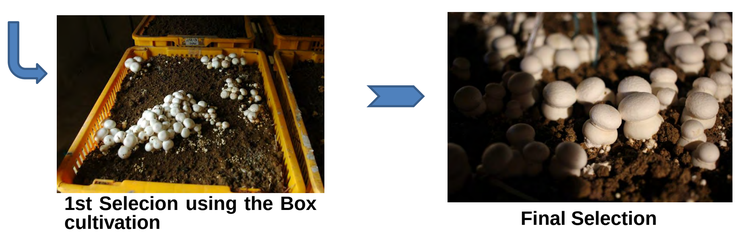
Abstracts

The production of mushroom cultivation in Korea is approximately 600 billion won, which is 1.6% of the total Korean agricultural output. Annually, ca. 200,000 tons of mushrooms are harvested in Korea. *Agaricus bisporus* production in 2014 was 11,000 tons. *A. bisporus* is occupied the fourth largest production following *Lentinula edodes, Pleurotus ostreatus, Flamulina velutipes* in Korea. In addition, *A. bisporus* production value in 2014 was 79 billion won. Production scale is small but turnout is high value-added mushrooms. More than 60% of the mushroom varieties currently being cultivated are the foreign varieties in Korea. Moreover variety development has been suspended since 1980. This study is intended to replace the foreign varieties imported to develop and disseminate indigenous variety. It was also carried out to increase the income of mushroom farms. Most of commercial mushrooms including oyster mushroom are heterotallic and have clamp connection. So It is easy to make a variety by using a single spore crossing method. We were trying to develop hybrid varieties of *A. bisporus* for improve the efficiency of breeding. First, Parental strains, ASI 1038 and 737, were selected from collected strains. And single spores of 1,000 were isolated from ASI 1038 and 737 individually. A new variety, 'Sae-Ah', was made by crossing ASI1038-297 and S737-26, homokaryons selected by genetic analysis. Variety 'Sae-Ah' of button mushroom, *A. bisporus*, has been developed by domestic first crossed line. By using the nucleic acid analysis it has been developed a technique to determine the crossing among homokaryons of *A.bisporus*. Breeding period in the development of this technology has been reduced to three years in five years. After breeding, this variety was confirmed productivity and stability through cultivation in mushroom farm. Mycelium of 'Sae-Ah' on CDA, Compost Dextrose Agar, grew well at 25℃ when it was compared with that of '505'. This variety can be started fruiting at 13~15℃ and grew well at 13~20℃. The diameter of mature cap and length of stipe was longer than control, '505'. The color of fruiting body was typical white. The mycelial running periods on compost were twenty nine days and the days required from casing until harvest of the first fruiting bodies was fourteen days. Varieties of mushrooms 'Sae-Ah' have been growing in 215 of 602 farmers in the shortest period as 35.7% penetration rate and completed contract of ordinary license.

Material & Method







Results



Fig. Parental Strains used in the study

* ASI 1038 has very pure white color, 737 was collected from mushroom farm

#OPA11 / S737 No.1 ~ No.120

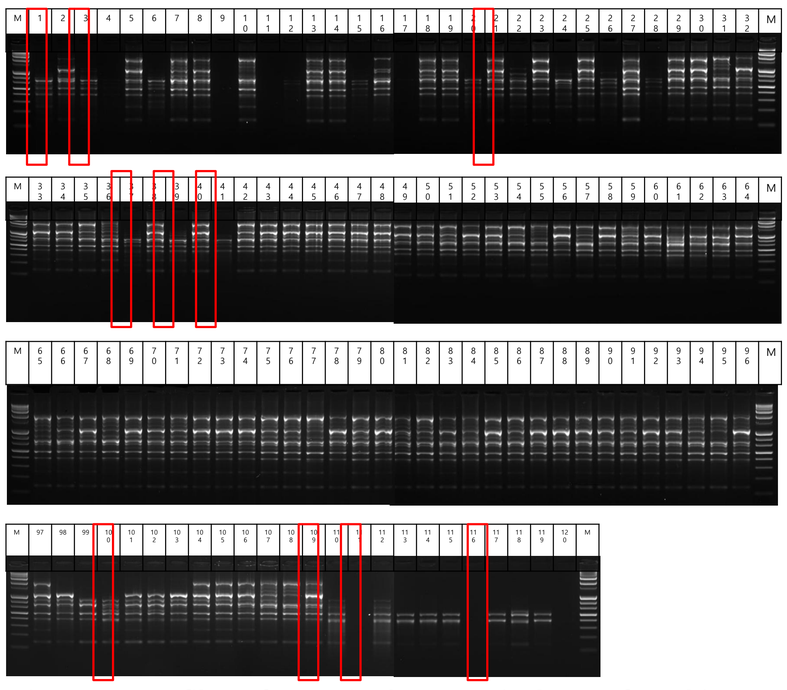


Fig. DNA profiles of 737 single spore isolates of *A. bisporus* by RAPD-PCR

*M : 1kb plus ladder marker, Primer ; OPA11

Parental Strains	Spores isolated	1st selection presumably homokaryotic strain by mycelial growth	2nd Selection Homokaryon by RAPD analysis
ASI 1038	314	250	26
SU 413	131	131	13
A15	1,000	120	4
737	1,000	120	19

Fig. Selected homokaryons to breed a new variety in *A. bisporus*

Table. Crossing among selected homokaryons from ASI 1038, 737 strains

Crossed line	Crossing with homokayrons			Confirm of DNA extraction	Gentic variation with parental strain
A001	S1038-72	*	S737-118	O	X
A002	S1038-72	*	S737-26	O	X
A003	S1038-72	*	S737-28	O	X
A004	S1038-46	*	S737-26	O	X
A005	S1038-211	*	S737-115	O	X
A006	S1038-211	*	S737-109	O	O
A007	S1038-211	*	S737-105	O	X
A008	S1038-211	*	S737-41	O	O
A009	S1038-54	*	S737-116	O	O
A010	S1038-46	*	S737-22	O	O
.	.	.	.	.	.
A304	S1038-297	.	S737-26	O	O
.	.	.	.	.	.
A341	S1038-226	*	S737-3	O	O
A342	S1038-226	*	S737-115	O	O
A343	S1038-235	*	S737-116	O	O
A344	S1038-235	*	S737-3	O	O
A345	S1038-206	*	S737-120	O	O
A346	S1038-206	*	S737-28	O	O
A347	S1038-212	*	S737-105	O	O
A348	S1038-212	*	S737-109	O	O
A349	S1038-212	*	S737-115	O	O
A350	S1038-235	*	S737-20	O	O
A351	S1038-37	*	S737-37	O	O
A352	S1038-37	*	S737-119	O	O
A353	S1038-37	*	S737-20	O	O
A354	S1038-65	*	S737-15	O	O
A355	S1038-65	*	S737-37	O	O
A356	S1038-69	*	S737-20	O	O
A357	S1038-72	*	S737-4	O	O
A358	S1038-131	*	S737-17	O	O
A359	S1038-131	*	S737-105	O	O
A360	S1038-131	*	S737-120	O	O

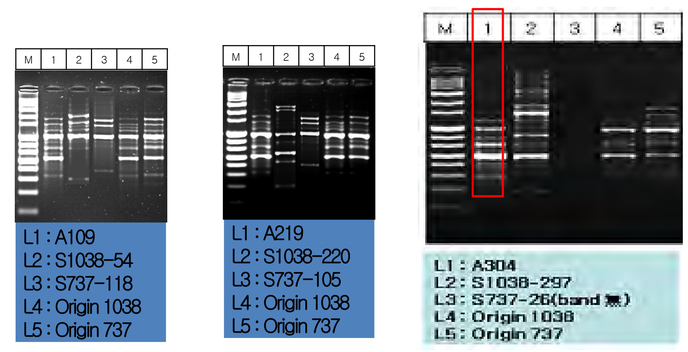


Fig. Crossed lines were identified by RAPD analysis

Table.. Comparison of morphological traits and yield between Sae-Ah and 505

Name of vairety	Morphological trait (mm)				MW[e] (g)	H[h] (g/mm)
	DP[a]	TP[b]	LS[c]	TS[d]		
Sae-Ah	42±5	2.4±0.3	26±6	17±3	12	176
505	39±4	2.3±0.3	28±4	15±3	11	178

* [a] Diameter of Pileus, [b] Thickness of Pileus, [c] Length of Stipe, [d] Thick of Stipe, [e] Mushroom Weight, [h] Hardness of Pileus



Fig. The appearance of *A. bisporus* variety 'Sae-Ah' in mushroom farm

Conclusion

1. **Sae-Ah is the first variety crossed line among homokaryons of *A. bisporus* in Korea.**
2. **Sae-Ah is the variety with high yield as 2.7kg/box and good appearance in mushroom farm.**
3. **35.7% farmers had cultivated in the shortest period after breeding in 2010.**
4. **RAPD analysis can be saved the time to breed in *A. bisprous*.**

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Expression analysis of polysaccharide hydrolyzing enzyme genes from *Tricholoma matsutake*

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INTRODUCTION

Tricholoma matsutake (S. Ito et Imai) Singer, the most famous edible mushroom in Japan that is an ectomycorrhizal fungus. However, artificial cultivation of the mushroom has not been established in a stable condition. This problem is mycelial growth is very slow. Also, sugars available for growth of *T. matsutake* are limited (e.g. Glucose, Fructose, Maltose, Trehalose, Cellobiose,Starch). As well as considered the weak of cellulase and hemicellulase that necessary degraded cellulose and hemicellulose.

Kusuda et al. reported that productivity of the fungus shows very strong activity of β-glucosidase and the enzyme was purified and characterized (Kusuda et al, 2006). But productivity of cellulases (Endglucanase and Cellobiohydrolase) and glucoamylase of the fungus is very weak. Therefore, the cultivation method similar to other saprophytic mushrooms are to be difficult to artificial cultivation. In the artificial cultivation of *T.matsutake*, it analysis whether *T.matsutake* are expressing any carbohydrate degradation system, it is important to search for effective sugars for growth. In this study, it is intended to clarify the carbohydrate degrading enzyme genes inducible and subjected to expression analysis by Real-time PCR.

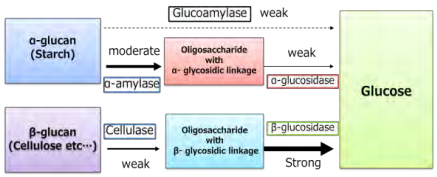


Fig.1 Carbohydrate utilization profiles of *T. matsutake*

【*Material and method*】

Organism : *Tricholoma matsutake* NBRC 30605

Agar medium

Glucose-Yeast extract agar medium (Glucose 2%, and Yeast Extract 0.5%, pH 5.1) prepared with tap water and then incubated at 24℃ for 30 days.



Culture medium

Yeast-saccharide liquid medium (various saccharides 1%(Table 1), and Yeast Extract 0.5%, pH 5.1) prepared with deionized water and were then incubated at 24℃ for 30 days.



Each cDNA synthesized by RT-PCR

- Real Time PCR
- Amylase activity (Glucoamylase, α-Glucosidase)
- Cellulase activity (Endoglucanase, Cellobiohydrolase)

Extracted the sequence information of the carbohydrate degrading enzyme-like genes by DOE Joint Genome Institute (JGI) *Tricholoma matsutake* genome database (Table 2). Each of gene expression using cDNA reverse transcribed from the supplemented culture of each saccharide was subjected to relative quantification by CYBR Green method using the LightCycler® Nano (Roche).

Table 1 Saccharide substrate were used in this study.

Saccharide substrate	Major linkage type (monomer)
Glucose (Control)	-
Mannose	-
Galactose	-
Xylose	-
Arabinose	-
Maltose	α-1,4 (Glc)
Cellobiose	β-1,4 (Glc)
Lactose	β-1,4 (Gal/Glc)
Amylose A (M.W. 2,900)	α-1,4 (Glc)
Amylose B (M.W. 16,000)	α-1,4 (Glc)
Amylopectin	α-1,6 (Glc)
Soluble starch	α-1,4/1,6 (Glc)
Starch from corn	α-1,4/1,6 (Glc)
Avicel	β-1,4 (Glc)
Cellulose powder	β-1,4 (Glc)
Curdlan	β-1,3 (Glc)
Laminaran from Eisenia Bicyclis	β-1,3/1,6 (Glc)
Xylan from Beechwood	β-1,4 (Xyl)
Arabinogaractan from Larch Wood	β-1,4 1,6 (Gal) ; α-1,5 (Gal/Ara)
Glucomannan	β-1,4 (Glc/Man)
Pectin from Citrus	α-1,4 (GalA)

Glc: Glucose, Gal: Galactose, Xyl: Xylose, Man: Mannose, GalA: Galacturonic acid.

Table 2 List of putative polysaccharide hydrolyzing genes analyzed in this study.

Gene	Description	Protein ID
TmCBH	Putative Cellobiohydrolase (GH 7)	1246307
TmEG1	Putative endoglucanase (GH 9)	1320754
TmEG2	Putative endoglucanase(GH 5)	1465229
TmEG3	Putative cellulase (GH 5)	1250585
TmEG4	Putative cellulase (GH 5)	1306486
TmEG5	Putative cellulase (GH 5)	1297329
TmEG6	Putative endoglucanase (GH 5)	1306486
*TmGlu 1**	Glucoamylase (GH 15)	1426725
TmAGL	Putative α-glucosidase (GH 31)	1261725
Actin	Housekeeping gene	1237462

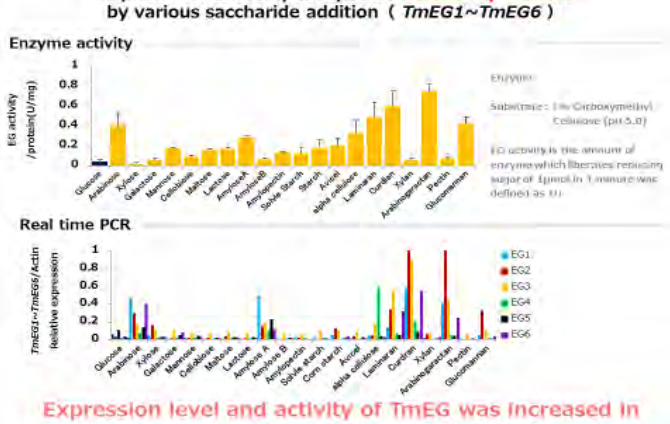
*Wan et al : Mycoscience 53, 194-202 (2012)

Expression variability analysis of CBH activity · mRNA by various saccharide addition (*TmCBH*)



Expression level of *TmCBH* and CBH activity in the maltose addition group was increased

Expression variability analysis of EG activity · mRNA by various saccharide addition (*TmEG1~TmEG6*)



Expression level and activity of TmEG was increased in arabinogalactan and β-1,3,1,6 -glucan addition group

Expression variability analysis of GLA activity · mRNA by various saccharide addition (*TmGlu1*)



Expression level of *TmGlu1* and the GLA activity in starch and hemicellulose addition group was increased

CONCRUSION

	Enzyme activity and gene expression level was increased
TmEG expression, EG activity	Arabinose, Laminaran, Curdlan, Arabinogaractan, Glucoamannan
TmCBH expression, CBH activity	Maltose
TmGLA expression, GLA activity	Amylose A, Xylan, Arabinogaractan, Glucomannan
TmAGL expression, AGL activity	–

▼In the present study, used as a growth substrate of *T. matsutake*, among hemicellulose and β-1,3 / 1,6-glucan such as the polysaccharide, it was suggested that endoglucanase or glucoamylase gene is induced-expression.

And improvement of the enzyme productivity , the construction of medium conditions aimed to artificial cultivation can be expected.



Schizophyllum commune

Fruiting body development of *Schizophyllum commune* on twigs and branches

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Dikaryons of *Schizophyllum commune* easily form fruiting bodies on artificial medium. The fruiting process can be divided into first loose hyphal aggregate (knot) formation, stalk formation by upwards organized growth of the aggregate, apical pit formation (stage III primordium), peripheral expansion of the cup-like pileus (advanced apical pit stage), and split forked pseudolamellae formation into the young stage IV mushroom that continues to grow at the periphery. Basidiospores are continuously produced by the mushroom. In nature, fruiting bodies are observed on twigs and branches of various tree species. Over 260 host species are known for the fungus. Among other living hosts, we have observed fruiting bodies on branches and stems of walnut, cherry, and lilac. Fruiting bodies appear on their hosts on sunny south to west sides at cracks within the bark. More regularly, the fungus was detected on fresh deadwood of multiple hardwood species. Similarly, fruiting bodies are seen to emerge in cracks of bark on the sunny sides on dead twigs, branches or also felled stems. We are interested in the process of fruiting body development. Here we present a system in the lab to initiate fruiting body formation on infested twigs of different tree species collected from nature. We use this system to follow up the early steps in fruiting body development on natural substrate.

Schizophyllum commune in nature



Fig. 1: *Schizophyllum commune fruiting bodies* on living hardwoods (A. *Gleditsia trecanthos* f. *inermis* , B. *Prunus incisa,* C. *Juglans ailantifolia*, D. *Syringa tigerstedtii*) and deadwood (branches of E. *Fagus sylvatica* and F. *Acer pseudoplatanus*, G. cut *Quercus robur* stem, H. burned wood of unknown species)

S. commune was often observed on the grounds of the North Campus of Göttingen University, on living hardwoods, as early colonizers on recently fallen and still barked deadwood and once also on burned wood. Fruiting bodies usually appeared on the sides of wooden substrates best exposed to the sun, most often in S or W directions. When formed on other sides of the substrates, shadows of buildings, other trees or other stems of a shrub blocked sunshine from S and/or W (Fig. 1; Table 1). Where living trees and shrubs were attacked as seen by fruiting body production, this correlated quite often with either replanting or with previous road and ground digging works in close neighborhood with likely root stress for the plants (root damage, disturbances in water supply). Stretches of the branches with fruiting bodies increased often with time while the better shadowed opposite sides of branches were still fully functional. Fruiting bodies were born in cracks of the bark or in lenticells as natural bark openings. Mushrooms of strong wood-decaying fungal species were often also found on the same or on directly connected branches and stems, below and above the fruiting bodies of *S. commune* (Fig. 1; Table 1). *S. commune* itself is only a weak degrader of wood. The fungus may live from the easily accessible nutrients from the parenchym in the sapwood, the cambium and the bast but may also profit from the presence of strong white-rotting species which more easily degrade the wood.

Table. 1. *Schizophyllum commune* and other fungal infestations on living shrubs and trees

Plant Name	Branch condition	Other fruiting bodies on same substrate	Direction*
Acer caudatum	Living stem	*Flammulina velutipes* + *Radulomyces molaris* (below)	W (N,S)
Acer ginnala (multi-stem free standing)	Living stem	*Auricularia auricula-judae* (above) *Trametes gibbosa* + *F. velutipes* (below)	E, W (S)
Alnus glutinosa	Living stem	*Ganoderma lucidum* (same level, NE)	SW
Corylus tibetica	Dead branch	*Exidia glandulosa* + *Trametes pubescens* (connected dead branch)	W, S (N)
Cotoneaster moupinensis	Living branch	-	E
Crataegus monogyna	Living stem	-	W
Euonymus sanguineus	Many living branches and twigs	*Trametes versicolor* (2 branches, above)	E, S, W
Gleditsia triacanthos f. *inermis*	Living stem	-	W
Juglans ailantifolia (free standing)	Living branches	*Antrodiella fragrans* (above) *Trametes* sp. (above) *Coniophora arida* (below)	S, SW
Juglans mandschurica	Living branch	*F. velutipes* (below) *Trametes multicolor* (connected branch)	W
Lonicera korolkowii	Living branch	-	W, S
Lonicera maximowiczii	Living branch	*Skeletocutis nivea* (below)	W, NW
Malus angustifolia	Living stem	-	W
Malus spec. cf. *coronaria*	Living stem	*Bjerkandera adusta* (above)	W
Malus x *zumi*	Living stem	-	SE
Prunus incisa	Living stem	*Hypholoma fasciculare* + *Coprinellus disseminatus* + *Mycena* sp. (below at stem base)	W
Salix discolor	Living branch	-	SW (N)
Syringa tigerstedtii	Dead branch	*E. glandulosa* + *Byssomerulius corium* (connected branch)	W, E
Syringa wolfii	Living branch	-	E, NW
Viburnum burejaeticum	Living branch	*Skeletocutis subincarnata* (below)	NE, SE

* Main geographic directions of mushroom formation on the substrate are given without brackets. Further directions with only a few mushrooms are shown in brackets

Schizophyllum commune in the lab

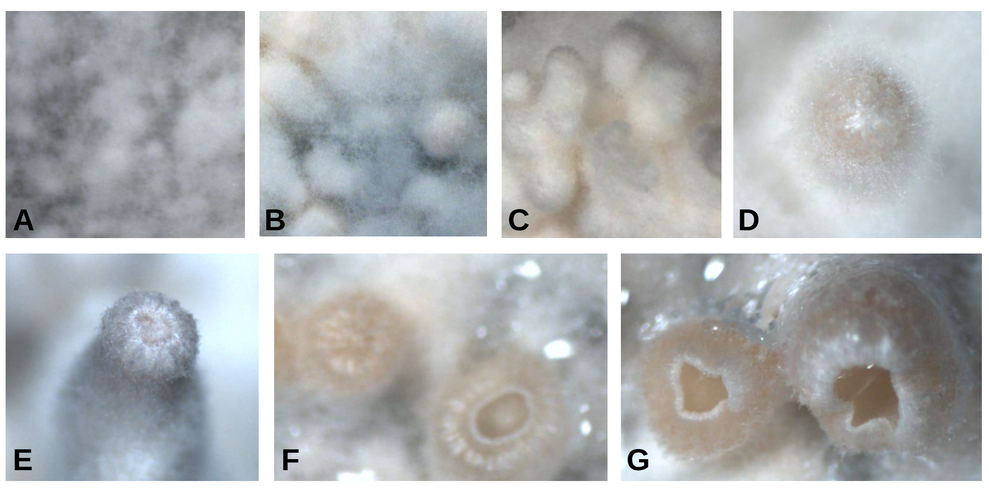


Fig. 2: *Schizophyllum commune* fruiting body development on agar plates with artificial medium. A, B: Hyphal aggregate. **C:** Stalk formation. **D, E:** Apical pit formation (stage III primordium). **F:** Advanced apical pit stage. **G:** First pseudolamellae formation.

Fruiting body development with dikaryotic strains on *S. commune* minimal medium is easily achieved when plates are incubated at 25°C under constant light supplied by white fluorescent light tubes (Fig. 2). Here, we show in addition a simple technique to observe *S. commune* fruiting body development on wood in the lab (Fig. 3).
Infested twigs and branches were laid into aquaria with wet paper beneath for creating a constant humid atmosphere. The aquaria were covered by glass plates which were slightly shifted to leave vents for aeration and were then incubated at room temperature in front of a laboratory window. Water was added daily to need in order to keep the paper sufficiently wet. Spacers were required between paper and wood in order to avoid water logging of the samples. Shadowing the aquaria towards the sun by hanging wet paper over their side glass surfaces (Fig. 3D) or by partially closing venetian blends delayed and reduced frequencies of fruiting. Strong direct sunshine during the morning hours (Fig. 3C) resulted in effective fruiting body development on the surfaces of wood and bark. When positioned at South windows, blends needed to be closed during the noon hours to avoid overheating the system. All stages in fruiting body development from hyphal aggregate over stalk and apical pit formation, peripheral cap expansion, split gill (pseudolamellae) formation and fully developed mushrooms were observed (Fig. 4).

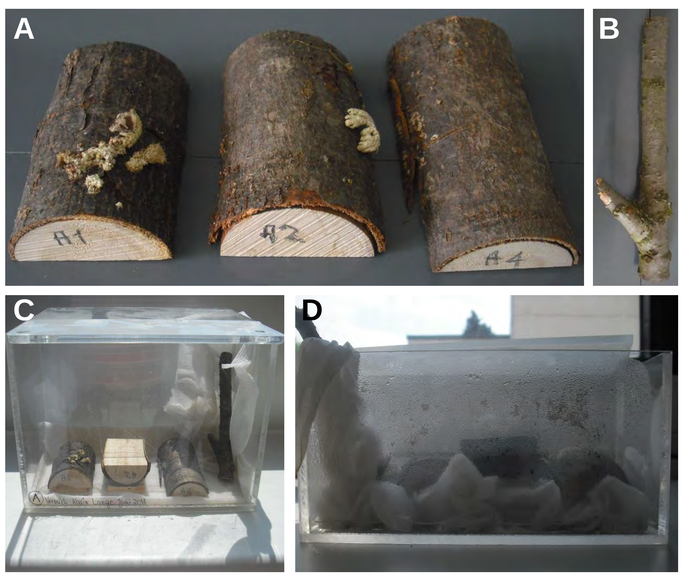


Fig. 3. Cultivation of *Schizophyllum commune* for fruiting body production on infested wood in the lab.
A. *Prunus incisa* branches and **B.** a *Syringa tigerstedtii* twig infested with *Schizophyllum commune*. Aquaria with infested wood inside placed onto a bench at a window, exposed throughout the morning to **C.** strong sunshine, **D.** less sunshine by partial shading through wet paper tissues covering the aquarium walls and partial closing venetian blinds.



Fig. 4: Stages of *Schizophyllum commune* fruiting body development on infested wood.

Characteristics of new cultivar ‘Pyogo(*Lentinula edodes*)’ growing on natural logs or in eco-friendly sawdust bags

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Abstract

Lentinula edodes **called ‘Pyogo’ mushroom in Korea has been known as nutritious and healthy diet without ages, culture or region in the worldwide. Globally, ‘Pyogo’ mushroom industry has been grown rapidly. Korea government, politically, takes more interest of new cultivar nurturer for ‘Pyogo’. National Institute of Forest Science has performed ‘Golden Seed Project’ to breed the new cultivar of ‘Pyogo’ by the policy. Among 15 national new cultivars in Korea, ‘Cheon-Jang 3ho’ for natural logs and ‘San-Beak-Hyang’ for eco-friendly sawdust introduce in the study. ‘Cheon-Jang 3ho’ is grown up a lot of fruitbodies on low temperature(8-15℃) on logs of Oak tree(*Quercus* spp.). ‘San-Beak-Hyang’ is formed abundant fruitbodies through 100 days incubation. These mushroom products have managed according to Good Agricultural Practice (GAP) from Korean government and controlled into food safety and eco-friendly deity without food poisoning bacteria, chemical pesticides, heavy metals, viruses, mycotoxins, and parasites ect.**

Results

New cultivar for sawdust cultivation

San-Baek-Hyang lots of mushroom formation after the short incubation period(100days)

Pileus size	Pileus thickness	Incubating period	Temperature for fruitbody formation	Sawdust media shape
67 mm	16 mm	81~100 days	15~20℃	Cubic shape



Incubating period



Primary harvest : 38g (individual weight)



Secondary harvest : 35g (individual weight)

New cultivar for logs cultivation

Cheon-Jang 3ho high quality mushroom formation on law temperature (7-16℃)

Pileus size	Pileus thickness	Stipe length	Temperature for fruitbody formation	Logs of oak tree
62 mm	14 mm	38 mm	7~16℃	*Quercus acutissima* *Quercus mongolica*



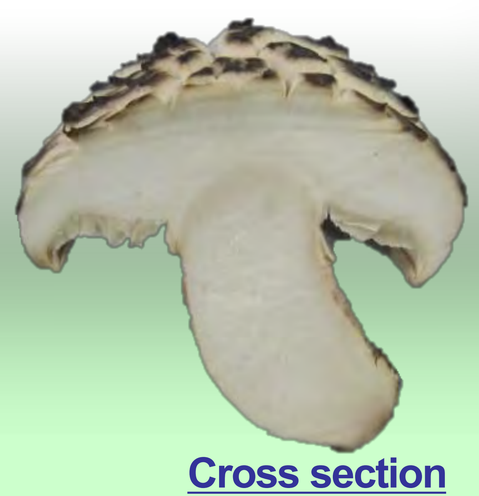
Oak tree



Pileus



Lamellae



Cross section

Briefly introduction of Crossbreeding



① Collection



② Quality property survey



③ Monospore isolation



④ Crossbreeding culture

Individualization of nucleus from mycelium of edible mushrooms using fluorescence dyeing

Se Hun Kim, Rhim Ryoo*, Kang-Hyeon Ka and Myung Kil Kim

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Abstract

***Tricholoma matsutake*, *Sarcodon aspratus* and *Polyozellus multiplex* are commercially important edible mushrooms in Korea. However, there are few monokaryons of those fungi since it is not easy to isolate new strains from the basidiospores of them. However, we could get the monokaryons and dikaryons from the badiospores of those species by using PDA (Potato dextrose agar) medium. We could identify one nucleus or two nuclei within each cell by double staining of nuclei and septa. We expect that the monokaryons could be used as a good source for studying cultural characteristics, mating system, analysis of functional genes, and so on.**

Materials and Methods

Cultural studies

1) Basidiospores Print on Petri dishes with PDA (potato dextrose agar)

2) Streaking plate, Incubation period 40 days, incubating temperature 23ºC

3) Subcultured culture mat on MMN liquid media;
1 % Glucose, 0.3% Malt extract, 0.025% $(NH_4)_2HPO_4$,
0.015% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.1% KH_2PO_4,
0.0001% $FeCl_3$, 0.0025% NaCl

Fluorescence microscopy

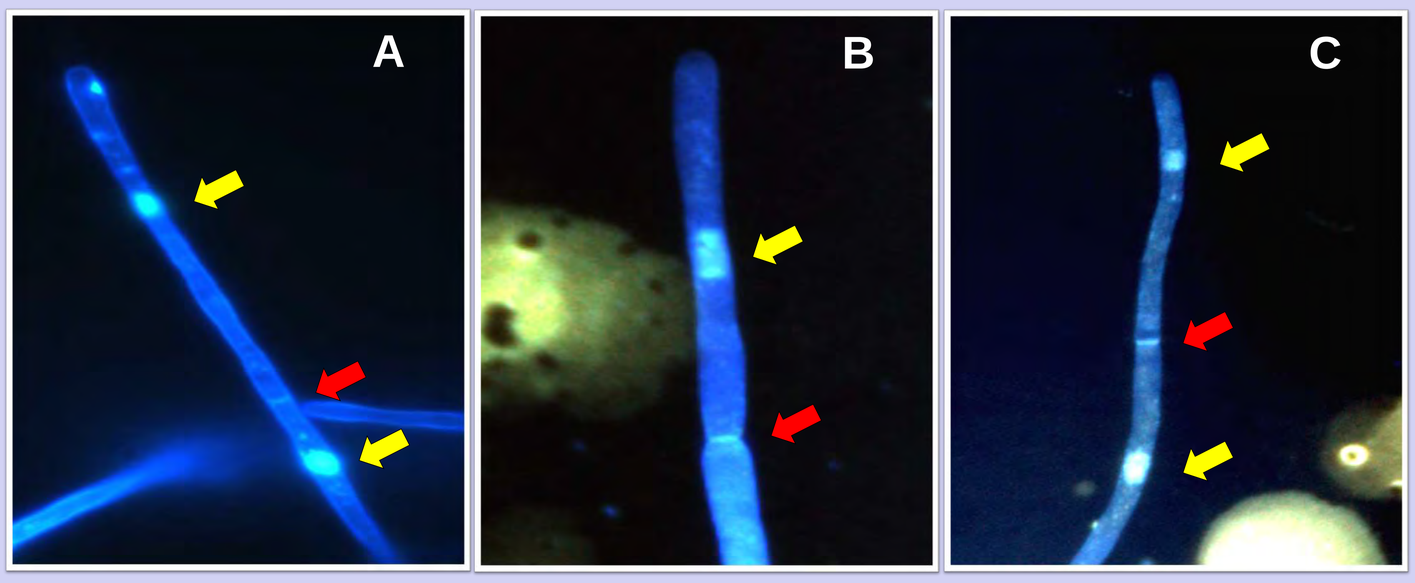
1) Nuclear stain
DAPI staining; 0.25 µg of 4',6-diamidino-2-phenylindole per 1 ml of 0.05 M Phosphate buffer

2) Septa stain
Calcofluor white staining; 200 µg Calcofluor white M2R per 1 ml of distilled water

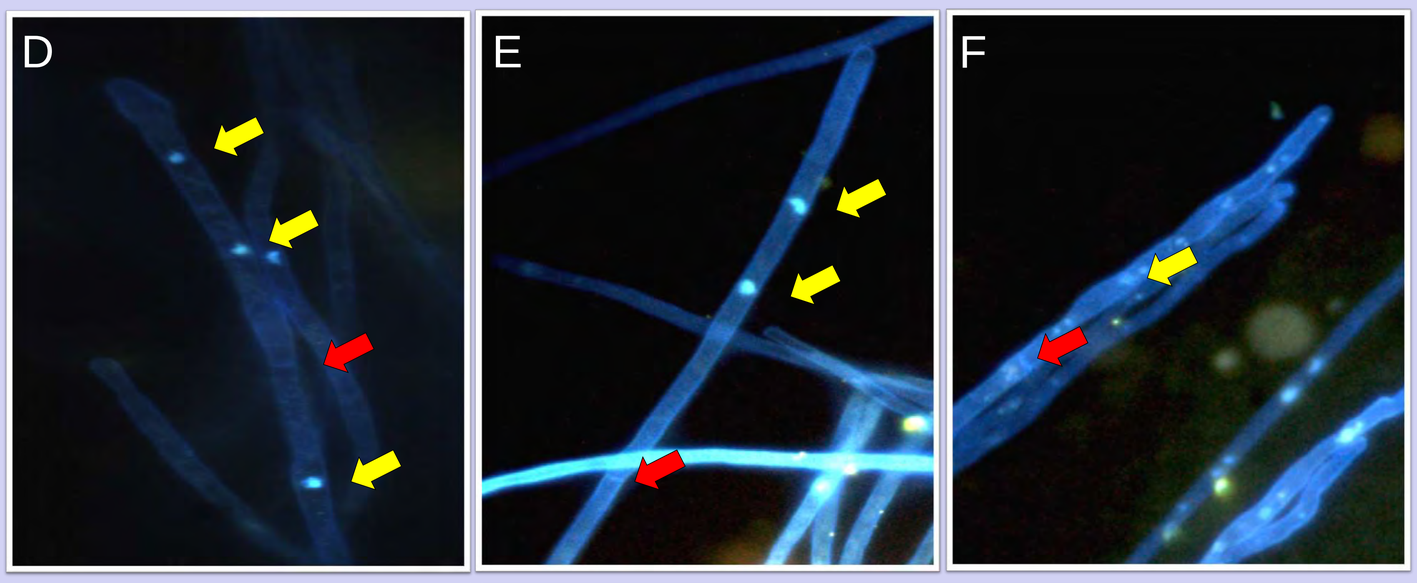
Illumination photo using Fluorescence microscopy

Nuclear

Septa

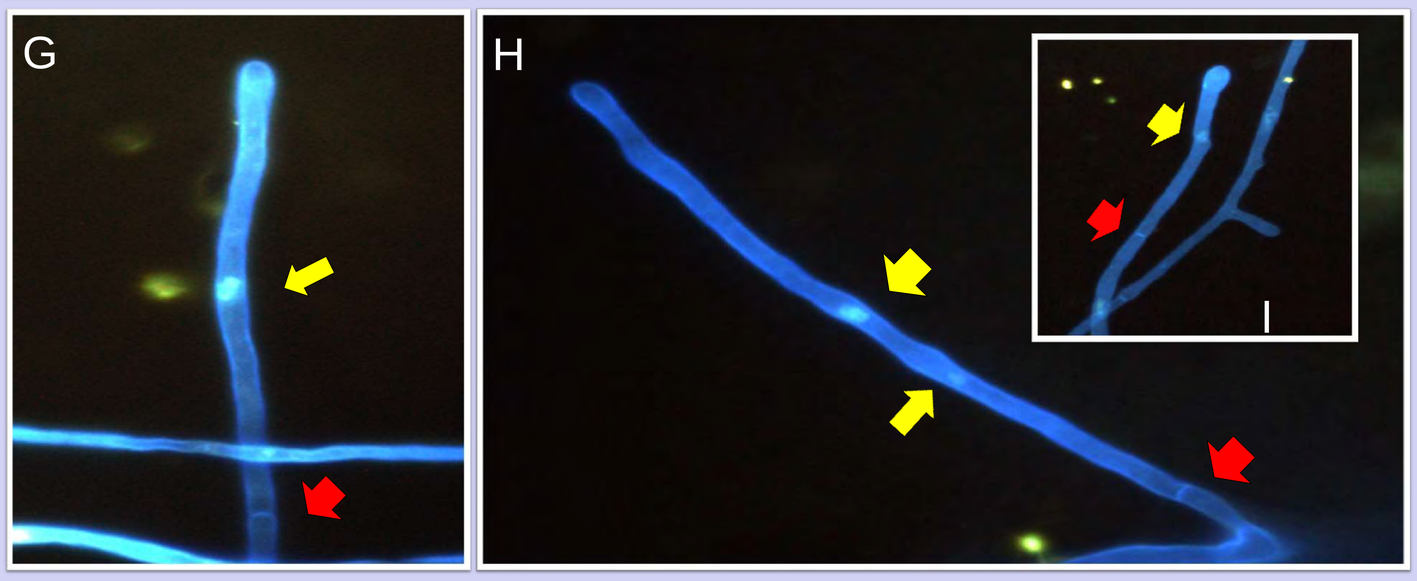


A-C; Monokaryon of *Tricholoma matsutake*



D-E; Dikaryon of *Tricholoma matsutake*

F; Monokaryon of *Polyozellus multiplex*



G, I; Monokaryon of *Sarcodon aspratus*

H; Dikaryon of *Sarcodon aspratus*

Comparative cultivation experiments with some traditional oyster mushroom (*Pleurotus ostreatus*) strains and hybrids

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Introduction

The successful production of oyster mushroom strongly depends on the cultivation character of the selected strains and hybrids. There are some old, almost forgotten hybrids and some new ones; the latter ones are offered by the spawn producers as results of novel breeding activities. In the frame of our work we performed comparative cultivation experiments with some traditional P. ostreatus strains were stored in stock cultures and were used formerly. The aim of the work was to get a comprehensive view about the cultivation character of some new strains and hybrids, and those old strains that are still preserved in cultures.

Material and method

In present experiment 1 different Pleurotus ostreatus and Pleurotus x hybrida varieties were tested: BL, G24, H1, H7, LSZ, Po462, 1-0-30, 357-213, 480, BAK, DSN, HD-35, VL80, ZN, 357-145 and P80 as control. The rye based spawn were produced in the Korona Mushroom Spawn Plant and Strain Research Laboratory. Pastuerized wheat-straw based commercial oyster substrate was used as a growing media from Pilze-Nagy Ltd. 20 kg substrate with 3 w/w % of spawn were mixed and filled into polyethylene plastic bags. After pressing the bags were closed and placed for spawn-running. 5 replicates were prepared. The following parameters were investigated and compared: productivity (P%), biological efficiency (BE%) and other cultivation related features (e.g. spawn running time, ripening time, appearance of flushes, inter-flush time etc.). Photo documentation and a detailed description were made for each strain. The most important chemical parameters of the substrate (pH, water content, dry matter, ash, total C, total N and protein ratio) were measured at spawning and at the end of the cultivation.

Results

A short spawn-running time was shown by the G24 (10th day) and H7 (11th day) strains, while the longest spawn running time was observed at the H1 strain (17th day). Relative short ripening time was found at the G24; 1-0-30; P80 and BAK strains, while the longest ones were shown by the LSZ; BL; DSN and H1 strains (primordia were found on the 20th-21st day after spawning). The first harvest of the 1st flush was performed on the G24 hybrid (25th day). At the same time, this hybrid produced the highest average yield with 37.56 kg on 100 kg Phase II substrate. The worst results were given by the H1 hybrid in every respect. The most widely accepted fruiting bodies were given by the P80, 357-213 and 480 hybrids. Furthermore, some additional strains (HD-35; 357-145; VL80; ZN; BAK; 1-0-30) produced fruiting bodies in such a good quality that is required by the European customers (Table 1). The chemical changes during the cultivation are visible in Table 2.

Table 1: Yield and biological efficiency of the tested *Pleurotus* cultivars

Strain	Fresh yield (kg/100 kg)	Biological Efficiency (%)	Productivity (%)
G 24	37,6	137,7	37,6
H 7	35,8	131,3	35,8
1-0-30	32,0	117,4	32,0
480	28,5	104,7	28,5
DSN	26,1	95,6	26,1
P 80	36,1	132,3	36,1
357-213	25,2	92,3	25,2
357-145	30,1	110,4	30,1
HD 35	30,7	112,5	30,7
BAK	31,5	115,6	31,5
VL-80	34,7	127,1	34,7
ZN	35,4	129,6	35,4
Po462	29,3	107,6	29,3
BL	20,8	76,3	20,8
LSz	25,9	95,1	25,9
H 1	8,4	30,6	8,4

Table 2: Chemical changes in the subsrates

Strain	pH	Water content (%)	Dry matter (%)	Ash	Total C	Total N	C/N	Protein
At spawning	7,8	72,7	27,3	5,1	47,5	1,0	46,5	6,4
Measured in spent mushroom substrate (after 3rd flush)								
G 24	5,3	59,0	41,1	16,7	41,6	0,7	57,8	4,5
H 7	5,4	68,8	31,3	21,1	39,4	1,3	31,6	7,8
1-0-30	5,2	64,0	36,0	22,7	38,7	1,3	30,7	7,9
480	5,4	62,0	38,0	22,1	39,0	1,0	38,6	6,3
DSN	5,4	68,5	31,5	20,5	39,8	1,1	36,5	6,8
P 80	5,3	55,9	44,1	16,6	41,7	0,9	46,4	5,6
357-213	5,5	64,5	35,5	24,5	37,8	1,1	34,0	6,9
357-145	5,5	65,1	34,9	18,6	40,7	1,0	39,5	6,4
HD 35	5,2	67,5	32,4	20,2	39,9	1,2	34,4	7,3
BAK	5,3	65,1	35,0	20,0	40,0	1,1	35,7	7,0
VL-80	5,4	65,9	34,1	20,2	39,9	1,1	38,7	6,9
ZN	5,3	64,5	35,5	19,2	40,4	1,0	40,8	6,2
Po462	5,2	70,0	30,0	19,8	40,1	1,0	42,2	5,9
BL	5,2	67,7	32,3	19,7	40,1	0,9	44,6	5,6
LSz	5,3	72,7	27,4	20,5	39,7	1,0	41,0	6,1
H1	5,8	75,9	24,1	20,4	39,8	1,4	28,6	8,7

As a result of the work, we have a description about the qualitative and quantitative traits of the fruiting bodies of the tested strains and hybrids. Summarizing the results it can concluded, there are several hybrids amongst the 'old' oyster mushroom strains that are suitable for the recent cultivation purposes.

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De novo assembly of the *Pleurotus ostreatus* CCEF89 hyphea transcriptome and identification of candidate unigenes and enzymes related to decomposing process

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Introduction

Lignins are the most abundant high-molecular-mass aromatic compounds, which are complex phenolic polymers that reinforce the walls of certain cells in the vascular tissues of higher plants. Using the genome sequence as a reference, a transcriptome approach has been used to examine the lignocellulolytic enzymes produced by fungi when cultured in a complex biomass. In recent decades, Pleurotus ostreatus (Jacq.) P. Kumm. has attracted particular interest because of its edibility and efficiency in producing lignocellulolytic enzymes. P. ostreatus can be easily cultivated using various agronomic waste products as a substrate, such as wheat, rye and rice straw, sugarcane bagasse, corn cobs, and sawdust. Over the life cycle, the degradation and decomposition of lignocellulose has a significant influence on a fungi's ability to take advantage of the necessary nutriments. Sequencing has demonstrated that P. ostreatus possesses a large repertoire of genes encoding lignocellulolytic enzymes. We examined the transcriptomes of P. ostreatus grown in these substrates treated with deionized water, a certain concentrate of $CuCl_2$ and guaiacol. We also attempted to integrate the transcriptome data. Since the molecular functions of many of the genes that were differentially regulated under our culture conditions are unknown or are poorly characterized, we focused our analysis on degradation enzymes.

Materials and methods

The strain was cultivated in PDA medium and inoculated on the surfaces of the mixtures with two treatments. RNA was extracted using trizol reagent, then the transcript sequences and data were analyzed by using the RNA-Seq method of the Illumina HiSeq 2500/MiSeq at NovaGene.

Results and Discussion

Annotation and Classification of Assembled Unigenes

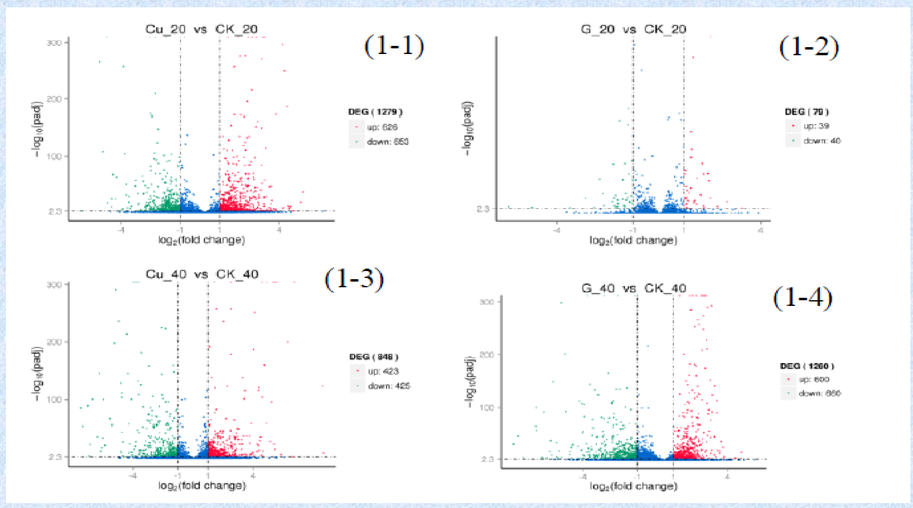
➢Among these fungi, *Trametes velutina* Dai 10149 and *Trametes pubescens* Cui 7571 were most stable and with fast growth. *Antrodia wangii* Cui 7568 was selected to further study because of its high yield and higher activity of cellulase (value = 1.53) To obtain a comprehensive transcriptome, six cDNA libraries denoted "CK_20", "CK_40", "Cu_20", "Cu_40", "G_20" and "G_40" prepared from pure hyphae which thrived on the deionized water-treated, CuCl2-treated and guaiacol-treated substrates were subjected to paired-end read sequencing using the Illumina platform. Using this method for de novo assembly, all high-quality clean reads were assembled into 39,578 contigs with a median length of 1663 bp. GO annotation can provide a standardized vocabulary for assigning the functions of uncharacterized sequences. Based on sequence homology, 8,581 unigenes were categorized into 55 GO terms. The most frequently identified unigenes classified in these categories were "metabolic process" (5,186) and "catalytic activity" (4,470). A total of 4,803 unigenes were assigned to the KOG classification. There were 25 KOG classes. Among them, "secondary metabolites biosynthesis transport and catabolism" involved in 330 unigenes(6.83%). The results indicated that decomposition and degradation did attach much importance.

Manganese peroxidase and Cytochrome P450

➢ 66.23% of unigenes were annotated as NR (NCBI non-redundant protein sequences). Among them, 61 unigenes belonged to manganese family, like short manganese peroxidase, manganese peroxidase 5, manganese peroxidase 1, manganese peroxidase 3 manganese peroxidase 4, manganese superoxide dismutase, manganese peroxidase 2, CDF manganese transporter, chain A (crystal structure of manganese peroxidase 4) and manganese peroxidase.Cellulose content in substrates varied from 34.72% to50.72%, while hemicellulose content did from 22.48% to 25.02%

➢Cytochrome P450 was essential for primary metabolism based on which Pleurotus ostreatus CCEF89 got to preserve cell wall integrity and to form the spore outer wall. Cytochrome P450 played a more important role in secondary metabolism, which enabled fungi to catalyze reactions in the pathways of mycotoxins, hormone production, xenobiotic detoxification, n-alkane assimilation, fatty acid hydroxylation and nitrite reduction (Črešnar and Petrič 2011). According to transcriptomeanalysis of Pleurotus ostreatus CCEF89, 520 unigenes blasted in NR were correlated with cytochrome P450, like cytochrome P450 monooxygenase pc-3, cytochrome P450, PAH-inducible cytochrome P450 monooxygenase PC-PAH4, high nitrogen upregulated cytochrome P450 monooxygenase 2, cytochrome P450 monooxygenase 51, bifunctional P450-NADPH-P450 reductase, cytochrome P450 oxidoreductase OrdA-like protein, cytochrome P450 monooxygenase pc-1, cytochrome P450 monooxygenase 32, cytochrome P450 monooxygenase 110, cytochrome P450 oxidoreductase (putative) and benzoate 4-monooxygenase cytochrome P450.

Identification of DEGs



➢We used the FPKM method to calculate the expression levels of unigenes in CuCl2-treated, guaiacol -treated, and deionized water -treated samples. 1279 and 848 differentially expressed unigenes (Fig. 1-1, 1-3) were obtained when it came to contrast between CuCl2-treated and control-treated (deionized water) samples which were got on the 20th and 40th day. Among those genes, 626 and 423 were up regulated and 653 and 425 were down regulated. Similarly, 79 and 1260 unigenes (Fig. 1-2, 1-4) were discovered between guaiacol- and control-treated samples (20 and 40 d). Among those genes, 39 and 600 were up regulated and 40 and 660 were down regulated. These differential unigenes would explain which genes impact or involve in the decomposition process.

Aromatic compound enzyme

Table 1 Unigenes related to aromatic compound enzymes

Unigenes	Gene length	Description	Biological process	EC
c4207_g1 c3964_g1 c6807_g1	1947 2008 1191	Catalytic LigB subunit of aromatic ring-opening dioxygenase	Cellular aromatic metabolic process (ferrous iron binding)	
c11041_g1	1435	Catechol 1,2-dioxygenase	Catalytic activity (ferric iron binding)	1.13.11.1
c14182_g1 c14513_g1 c10814_g1	4086 4101 2397	Dioxygenase	Catalytic activity/cellular aromatic compound metabolic process (ferric iron binding)	
c20605_g1	204	9-cis-epoxycarotenoid dioxygenase	Catalyzing epoxy (ferrous iron binding)	1.13.11.51
c14594_g1	4280	2 iron, 2 sulfur cluster binding	Aromatic compound catabolic process	

➢the most important aromatic compound enzymes were catalytic LigB subunit of aromatic ring-opening dioxygenase with ferrous iron binding and dioxygenase with ferric iron binding involved in catalytic activity. Meanwhile, catechol 1,2-dioxygenase (EC 1.13.11.1) with ferric iron binding and 9-cis-epoxycarotenoid dioxygenase (EC 1.13.11.51) with ferrous iron binding acted on catalyzing the catechol (some broken patterns of lignin complex) intermediates through aromatic ring-cleavage catabolic pathways. Besides, 9-cis-epoxycarotenoid dioxygenase catalyses (3S,5R,6S)-5,6-epoxy-3-hydroxy-5,6-dihydro-12'-apo-beta-caroten-12'-al, which was considered as a indispensable intermediate products of lignin. Therefore, 9-cis-epoxycarotenoid dioxygenase got to influence lignin decomposition in large extent.

Laccase

Table 2 Laccase activities from *Pleurotus ostreatus* CCEF89 and lignin contents of different substrates

Substrates	Time	AIL (%)	ASL (%)	Lignin (%)	Laccase activities (U/L)
Picea jezoensis	5 d	20.74	4.09	24.83	24.7
	10 d	21.81	3.86	25.67	123.1
	15 d	20.43	4.05	24.48	22.1
	20 d	15.66	4.32	19.98	21.6
	25 d	13.52	4.53	18.05	22.5
Populus tomentosa	5 d	16.03	7.74	23.77	27.4
	10 d	16.50	6.75	23.25	135.2
	15 d	15.92	7.35	23.27	26.9
	20 d	15.55	8.73	24.28	25.8
	25 d	14.27	8.94	23.21	26.1
Corn stalks	5 d	15.52	6.22	21.74	23.5
	10 d	15.30	6.31	21.61	120.6
	15 d	15.58	6.20	21.78	25.2
	20 d	15.73	6.14	21.87	24.5
	25 d	15.94	5.93	21.87	24.7

AIL, acid insoluble lignin; ASL, acid soluble lignin; Lignin= AIL + ASL.

➢In terms of laccases from Pleurotus ostreatus CCEF89,there were no correlation between laccases and lignin contents (Table. 2). Therefore, key lignin-degrading enzymes excluded laccases.

Conclusions

➢We first presented three comprehensive transcriptome datasets of P. ostreatus CCEF89 hyphea. 19,486 unigenes were identified in total. FMN, FAD and NAD(P)H were important cofactors in degrading process. Also MnP, GLOX and CPY P450 played a significant role in decomposing system. Besides, several aromatic compound enzymes were first attached much importance. A putative reaction process was first put forward. These information will facilitate future studies of edible mushrooms' biology and molecular breeding.

Acknowledgments

➢The study was supported by the state key basic research development program of China (No. 2014CB138301).

Purification and Characterization Xylan Degrading Enzyme from Micelium of *Tricholoma matsutake*

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Background

The ectomycorrhizal fungus, *Tricholoma matsutake,* is difficult to cultivate artificially, because the fangus has low ability to decompose polysaccharides as growth substrate, and grows slowly on artificial medium (20 mm/month). There are only a few reports for carbohydrate degrading enzymes from *T. matsutake.* Therefore, we have been studied carbohydrase productions from *T. matsutake* on a modified artificial liquid medium.

Polysaccharide degrading profiles of *T. matsutake*

Reaction substrates	Growth substrates					
	glucose	cellobiose	maltose	laminaran	starch	dextrin
Locust beangum	+	±	++	+	++	++
Arabinogalactan	±	±	++	++	++	++
Xylan	+	+	++	++	++	++
Laminaran	++	+	++	++	++	++
Cardlan	±	±	±	±	-	-
Pustlan	±	±	±	+	-	±
CMC	±	±	±	±	-	-
Cellulose	±	±	±	+	±	±
Starch	++	+	++	++	++	++
Dextrin	+	±	++	++	++	++

Experimental Procedure

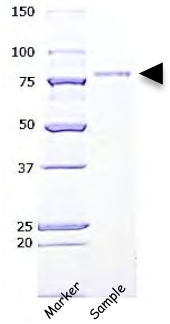
T. matsutake NBRC30605 was inoculated to 20ml GY medium (Glucose 20 g, Yeast extract 3 g, distilled water 1 L; pH5.1) and cultured without shaking at 24℃ for 60 days.

Purification

Crude enzyme

- Dialyzed (20mM acetate buffer)
- CM 650C (eluted with 0~0.5M of NaCl)
- Superdex 200

Purified enzyme



The N-terminal amino acid sequence of the enzyme showed

L-V-T-F-P-D-C-C-N-G-P-L-K-N.

A BLAST search of the protein database (JGI *T.matsutake*) indicated that the N-terminal amino acid sequence of the enzyme was identical with that of beta-xylosidase gene (Protein ID 1392972).

```
T.matsutake   1:ML-----RLFFLSLDLYSVFALVYTFPDCVNGPLKNNAVCDTTKDPATRAKAVIQLFTVEELINNTVNLSPGVPRLGLPSYQWWSEALHGVASSPGVTFA 96
S.lacrymans   1:MLTTRAR--FLP-LVLTAGLASAYSFPDCTNGPLAQNAICDTSLDPISRATAVVDLFTIDELINNTVSTSPGVPRLGLPPYQWWSEGLHGVADSPGVNFS 97
P.placenta    1:MTVLLA-----LSCLALAGQAFGA--FPDCANGPLITNTVCDTSATPLERATALISLFTLEEKINNTGNTAPGVPRLGLPAYQWWDEALHGVAESPGVTFA 94

T.matsutake  97:PSGNFSYATSFPEPTSMGAAFDDPLTKAVATVVSTEARAFNNFGRAGIDFWTPNINPFKDPRWGRGAETPSEDPYHVSQYVVNLIQGLQGGLDPKPYFKV 196
S.lacrymans  98:ASGEFSYATSFPDPITMGAAFDDELIKSVGAIVGMEGRSFNNYGRAGLDFWTPNINPFKDPRWGRGDETPGEDPYHLAQYVVNLVQGLQGGLDPKPYYQV 197
P.placenta   95:PSGEYSYATSFPDPILMGAAFDDALINHVATIVSTEARAFNNANRSGIDFWTPNINPFKDPRWGRGDETPGEDPFHLQSYVVNLITGLQGGLDPE-YKRI 193

T.matsutake 197:VADCKHFAAYDMENWEGNIRMAFDAKVTIQDLSEYYLPAFDSCVRDAKVASIMCSYNSVNGIPSCANSYLLQTILRDLWGFNDDR-WVTSDCDAIDNTYS 295
S.lacrymans 198:ISTCKHFAAYDLEDWDGNYRYGFDAIVTTDDLSEYYLPSFDSCVRDAKVGAAMCSYNAVNGIPSCANTYLLQSILRDFWGFAEDR-WVTSDCDAVDNIYD 296
P.placenta  194:VATCKHFAAYDLNWEGNVRYGFDALVSLQDLSEFYTRSFRTCARDANVGSFMCSYNAVNGVPSCANSYLLQDILRDHWGWTNEDQYITSDCDAIQNIYE 293

T.matsutake 296:THHYTSTYAQAVADALKAGTDLDCGTTYAQYLPAAFNQSLITRSDLERALTRQYTSLIRLGYFDPASRQPYRQLDWSNINTPDADNLAHQAAVEGTVLLK 395
S.lacrymans 297:PHNYTKTPEEAVADALKAGTDIDCGTFYSEYLPGAYNQSLITETELRQALIRQYASLVRLGYFDPTDIQPYRQYNWNNVDTPDADQLAYQAAAEGIVLLK 396
P.placenta  294:PHYTTATRAETVADALNAGTDLDCGEYYPENLGAAYDQGLFTESTLNRALIRQYAALVKLGYFDPADTQPYRQIGWANVSTPEAEELAYTAAVEGITLLK 393

T.matsutake 396:NDGVLPLKKTIKKVALTGPWANATTQMQGNYQGIAPFLVSPLQGAMNAGYQVSFALGTNISGTSTTQFAAALSAAQAADAVIFAGGIDQSVERESLDRTS 495
S.lacrymans 397:NDGTLPLSSDIKNIALIGPWGNATGEMQGNYYGVAPYLISPLMGAVATGYNVTYVFGTNITSNDTSGFAAAIAAADGADAVVFAGGIDETVESEGNDRNY 496
P.placenta  394:NDGTLPLSPSIKTIALIGPWANATTQMQGNYYGVAPYLISPLMAAEELGFTVYYSAGPGVDDPTTSSFPAAFAAAEEAADAIIYAGGIDITVEAEAMDRYT 493

T.matsutake 496:IAWPGNQLDLIAALGEGTGKPLVVVQFGGGQVDDTVCKANKTVNAIVWGGYPGQSGGDALFETLSGKAAPAGRLPITQYPAEYAGQIPMTNMNLRPDNNSP 595
S.lacrymans 497:ITWPGNQLDLVGELAAVGKPLVVVQFGGGQVDDTSLKANSTVNALLWAGYPGQSGGSALFDIISGKVAPAGRLPVTQYPADYVYEIPMTDMDLRPNATSP 596
P.placenta  494:LDWPGVQPDFIDQLSLLGKPLIVLQFGGGQIDDSALLPNPGVNALVWIGYPGQSGGKAIMDILVGNAAPAGRLPITQYPLDYVYQVAMTDMSLRPSPTNP 593

T.matsutake 596:GRTYKWYQGTPPFPFGHGLHYTTFDVTWHHNPPAQYDTSSLLGSSHSSGHQYPDLAPFYVFNIDVKNTGQTLSDYVAL-LFLDGKAGPAPYPNKQLISYT 694
S.lacrymans 597:GRTYKWYTGTPIYDFGYGLHYTTFSYKWAKAPSSTYNLQTLVQSGNLYS--YLDLAPFDTFTVNVTNTGNVTSDFASL-LFVNGTYGPSPYPNKSLITYA 693
P.placenta  594:GRTYMWYTGTPIVEFGFGLHYTTFTASLSQPSAPSYDIATLVSLCSGVAHP--DLCPEASYTANVTNTGSSVTSDFVSL-LFLAGEHGPAPYPNKVLVAYD 691

T.matsutake 695:RLHDTNPGSTSSAQLPVTLGSIARADAQGNLWIYAGSYRLSIDTTPALTHNFVLRGQSVQLTSWPQDT--SI-                          764
S.lacrymans 694:RLHDIASGDTASVALGVTLGSIARADTYGNMWLYPGTYQVTLDTLGVLTYQFDLTGSATQITEFPQDNDAPPS                          766
P.placenta  692:RLHAIAPLASQTTTLNLTLGSLSRVDDYGNTILYPGEY----------TLIFDVDAKS---TS----------                          741
```

	% ID
glycoside hydrolase family 3 protein [*Serpula lacrymans* var. lacrymans S7.3]	67%
β-xylosidase [*Postia placenta* Mad-698-R]	61%

2,295-bp, Amino acid: 764

Enzyme properties

Substrate specificities

substrate	relative activity (%)
1% Xylan (beechwood)	100
1% Xylan (oat-spelt)	0
1% Arabinogalactan	0
1% Cellulose	0
1% Avicel	0
0.2% Locust Bean Gum	0
p-N-β-Xylopyranoside	100
p-N-β-Glucopyranoside	47.9
p-N-β-cellobiopyranoside	3.3
p-N-β-Galactopyranoside	0
Xylobiose	100
Xylotriose	94
Xylotetraose	44
Xylopentaose	35

The polysaccharide-degrading activity was determined using the DNS method. The *p*NP substrates activities were determined to using *p*NP substrates and *p*-nitrophenol (*p*NP) measured at 405 nm. The xylo-oligosaccharide-degrading activity was determined to using 1 mM xyl_n (n=2-6) and a D-xylose Assay kit.

Kinetic parameters

substrate	K_m	V_{max}	V_{max}/K_m
p-N-β-Xylopyranoside	1.28	14.45	11.29
xylobiose	0.88	22.94	26.11

Effects of compounds

Chemical reagent (10mM)	Relative activity(%)	Chemical reagent (10mM)	Relative activity(%)
control	100	Mn^{2+}	0
Mg^{2+}	182	K^+	282
Li^+	277	Co^{2+}	114
Fe^{2+}	168	Ca^{2+}	67
Hg^{2+}	141	Ni^{2+}	29
Ag^+	173	Cu^{2+}	160
Al^{3+}	105	DTT (5mM)	128
Zn^{2+}	27	PMSF (5mM)	74
Na^+	277	EDTA (5mM)	13

Comparative characterization of fungal β-xylosidases

Strain	M.M.	Spec. Act.(U/mg)	Temperature(℃)		pH	
			Opt.	stability	Opt.	stability
T. matsutake	81,000	17.5	55	<30	4.0	3.0-4.0
A. carbonarius	100,000	3.3	60	-	4.0	3.5-6.5
A. ochraceus	137,000	41.4	70	<75	3.0-5.5	3.0-6.0
F. verticillioides	94,500	57.0	65	<50	4.5	4.5-6.0
T. harzianum	60,000	3.4	70	-	4.0-4.5	-
T. reesei	100,000	27.9	60	<55	4.0	4.0-6.0
S. thermophile	45,000	83.2	50	<50	7.0	6.0-8.0

Specific activities were measured with *p*NP substrate

Conclusion

Beta-xylosidase might be the main component of the xylan degrading enzyme of *T. matsutake*. The purified xylosidase releases xylose not only from xylooligosaccharides but from wood xylan. Based on this properties of the enzyme, xylan should be one of new candidates as growth carbon source for long term culture in artificial cultivation.

Acknowledgement: This study was supported in part by a grant of Strategic Research Foundation Grant-aided Project for Private Universities from Ministry of Education, Culture, Sport, Science, and Technology, Japan (MEXT), 2015-2017 (S1512004).

Expression analysis of protease genes of Lentinula edodes in culture periods

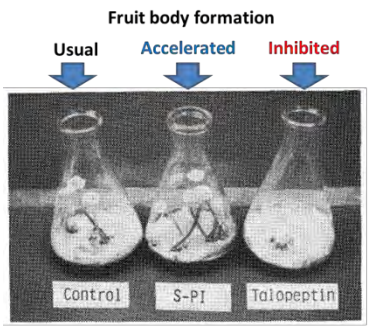
KINKI UNIVERSITY

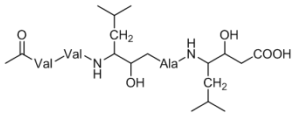
○Yasuhisa FUKUTA, Norifumi SHIRASAKA

Div. Apl. Chem., GradSch. Agr. Kindai Univ., 3327-204 Nakamachi, Nara 631-8505, Japan

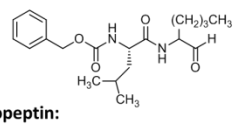
【Introduction】

We have been reported that the role of proteinases in the fruit-body formation of Basidiomycetes. The opinion was based on our observations that the fruit-body formation of Basidiomycetes such as *Lentinula edodes* was accelerated by the addition of Pepsin inhibitor from *Streptomyces naniwaensis*, S-PI (Pepstatin Ac). (Fig. 1)





S-PI (*Streptomyces*-pepsin inhibitor):
Aspartic protease (Pepsin) Inhibitor



Talopeptin:
Metalloprotease Inhibitor

Fig 1. Effect of protease inhibitors for fruit body formation in mushrooms.

There are found that: 1) Protease system of *L. edodes* was composed of three acidic (in culture filtrate, vegetative mycelium, and fruit-body) and two metalloproteases (in vegetative mycelium and fruit-body). 2) A dramatic change of protease activities in the mycelium occurred when S-PI was added in the culture medium at the time of induction of fruit-body formation. 3) The acidic proteinase in the mycelium had the highest sensitivity to S-PI among the carboxyl proteinases reported so far. This phenomenon is of interest concerning the promoting mechanism of fruit-body formation by S-PI.

However, there is no information regarding gene and amino acid sequences of these proteinases, and their expression time in culture periods of *L. edodes*.



Fig 2. Primordium formation of *L. edodes*.

Table 1. Putative protease genes analyzed in this study.

Transcript ID	BLAST Result (Best Hit protein)	Best Hit of KEGG-annotated protein	Pfam Hit(s)
g7269	aspergillopepsin [Aspergillus fumigatus Af293]	mucin-7 precursor [Homo sapiens]	Peptidase A4 family Enterobacterial Ail/Lom protein Domain of unknown function (DUF1869)
g7534	acid proteinase [Cryphonectria parasitica]	-	Peptidase A4 family
g7597	glutamic protease [Pholiota nameko]	-	Peptidase A4 family Extensin-like protein C-terminus
g10028	glutamic protease [Pholiota nameko]	-	Peptidase A4 family Protein of unknown function (DUF2817) Domain of unknown function
g866	putative aspartic protease [Lentinula edodes]	pepsin A-like [Equus caballus]	Eukaryotic aspartyl protease Retroviral aspartyl protease
g867	putative aspartic protease [Lentinula edodes]	Pepsinogen 4, group I (pepsinogen A) [Homo sapiens]	Eukaryotic aspartyl protease Retroviral aspartyl protease
g2120	family A1 protease [Phanerochaete chrysosporium]	pepsin A-like isoform 2 [Ailuropoda melanoleuca]	Eukaryotic aspartyl protease
g5195	hypothetical protein SCHCODRAFT_65201 [Schizophyllum commune H4-8]	similar to predicted protein [Ciona intestinalis]	Eukaryotic aspartyl protease
g5196	hypothetical protein SCHCODRAFT_65201 [Schizophyllum commune H4-8]	similar to predicted protein [Ciona intestinalis]	Eukaryotic aspartyl protease
g7258	putative aspartic protease [Lentinula edodes]	similar to predicted protein [Ciona intestinalis]	Eukaryotic aspartyl protease
g10056	hypothetical protein SCHCODRAFT_65201 [Schizophyllum commune H4-8]	cathepsin E isoform 4 [Macaca mulatta]	Eukaryotic aspartyl protease
g584	hypothetical protein SERLADRAFT_360310 [Serpula lacrymans var.lacrymans S7.9]	glutamate carboxypeptidase 2 [Canislupus familiaris]	Transferrin receptor-like dimerisation domain Peptidase family M28 PA domain
g1220	hypothetical protein SCHCODRAFT_255134 [Schizophyllum commune H4-8]	prostate specific membrane antigen variant F [Homo sapiens]	Peptidase family M28 Transferrin receptor-like dimerisation domain PA domain Nicastrin
g2267	aspartyl aminopeptidase [Coprinopsis cinerea okayama7#130]	-	Aminopeptidase I zinc metalloprotease (M18) M42 glutamyl aminopeptidase
g3115	hypothetical protein SCHCODRAFT_255134 [Schizophyllum commune H4-8]	glutamate carboxypeptidase 2 [Canislupus familiaris]	Peptidase family M28 PA domain Nicastrin
g4055	predicted protein [Laccaria bicolor S238N-H82]	neprilysin [Gallus gallus]	Peptidase family M13
Refference gene			
g2113 (gpd)	glyceraldehyde-3-phosphate dehydrogenase [Lentinula edodes]		

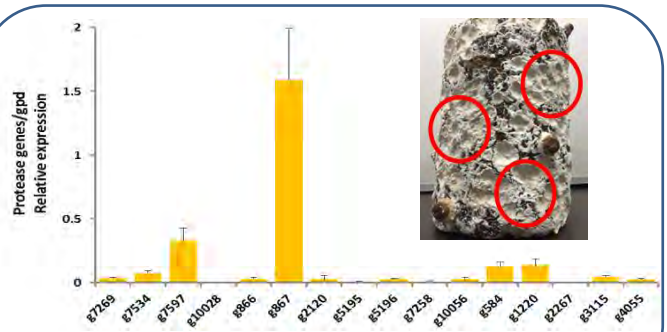


Fig 3. Relative expression of protease genes from *L. edodes* in mycelia.

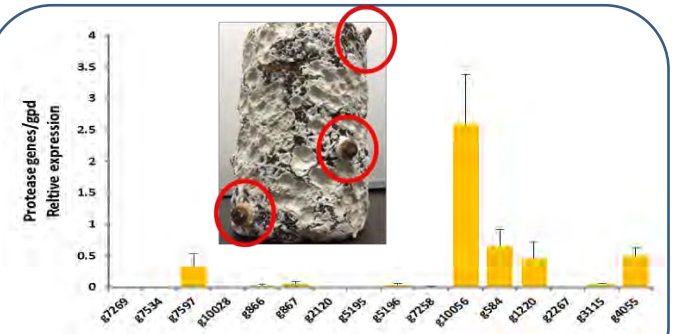


Fig 4. Relative expression of protease genes from *L. edodes* in primordium.

Summary

We analyzed expression level of protease genes based on ForestGEN *L. edodes* genome database in mycelia and primordium. In mycelia, g866 (Pepsin-A like protein) was showed high level expression compared with others, and g10056 (Eukaryotic aspartic protease), g584, g1220(peptidase family M28), and g4055(peptidase family M13) changed expression level in primordium. It seemed these genes have some roles for fruit-body formation of the fungus.

Understanding the nutritional requirements of oyster mushroom (*Pleurotus* sp.) grown in axenic culture

THE UNIVERSITY OF SYDNEY
FACULTY OF AGRICULTURE AND ENVIRONMENT

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Research Findings

(1) *Pleurotus sp.* strain PO13 grown on solid and liquid media showed different preferences for inorganic and organic nitrogen

(2) Nitrogen consumption by *Pleurotus* sp. depends on form, availability and combination of N sources in the medium

Introduction

Nitrogen is an essential element in substrates used for mushroom cultivation and its utilization by fungi needs to be understood to maximise productivity. The use of axenic culture can facilitate our understanding of the biology and physiology of cultivated fungi. Techniques available include agar plate and liquid media cultures.

Spent coffee grounds are becoming popular for mushroom cultivation. It is not well known how biologically efficient this substrate is or if compounds such as caffeine become toxic to fungi. To improve our understanding of caffeine metabolism and assimilation by fungi our hypotheses are: (1) the addition of caffeine will have an effect hyphal growth and, (2) when provided at a certain concentration will prevent fungal growth. In addition, (3) the type of nitrogen available (inorganic or organic) will affect fungal growth and mycelium development differently when added to a defined medium.

Methods

Growing conditions: Cultures using agar plates and liquid medium were kept at 25 °C. Liquid culture was maintained on a rotary shaker at 150 rpm. **N sources:** Inorganic and organic sources, including caffeine, were added after autoclaving the medium. **Coffee extracts:** Spent coffee grounds (SCG) and fresh coffee grounds ((FCG). **Growth measurement:** For agar plates – radial growth as the mean of two perpendicular measurements of colony size (mm day^{-1}); for liquid media – fungal biomass as wet weight (mg ml^{-1}). **Statistical analysis:** One-way ANOVA and Tukey's post-hoc test (p <0.05). Bars represent mean values (n = 5) and error bars are standard deviation, lower case letters represent differences among means.

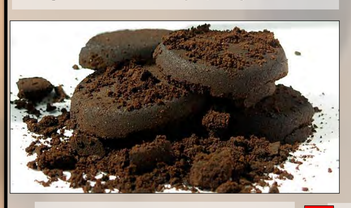


Isolation of pure culture



Agar culture *Experiments (1-4)*

Experiment (1, 2)

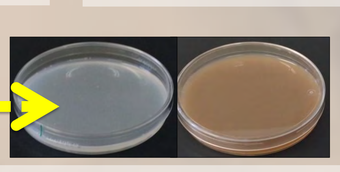


Spent coffee grounds (SCG)

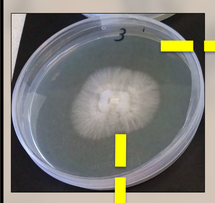


Fresh coffee grounds (FCG)





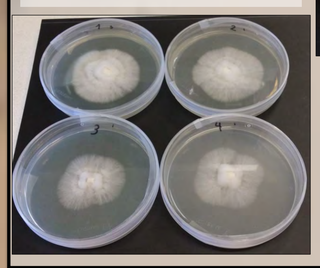
Minimal medium (MM) Potato dextrose agar (PDA)



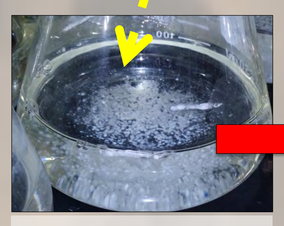
Experiment (3)
Caffeine 0-100 mM N



Experiment (4)
N sources 10 mM N



Liquid culture *Experiment (5)*

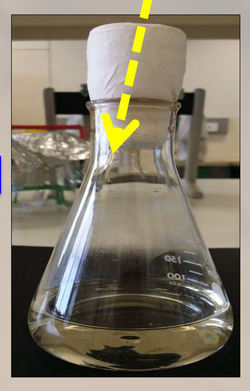


Liquid fungal culture



Inoculum 2%

N sources



This series of experiments provided information about:

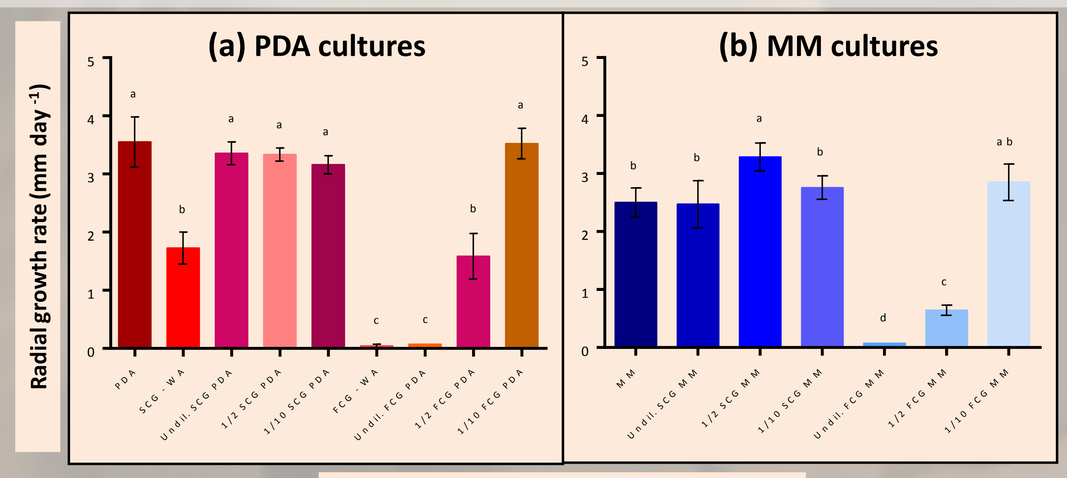
*** The optimum number of days for growth on culture based on radius of colony (7 days) or mycelia biomass (14 days)**

*** Nitrogen concentration (10 mM N) suitable for fungal cultivation on liquid medium**

*** Selection of best pH of medium (5.5, experiment not showed)**

Results

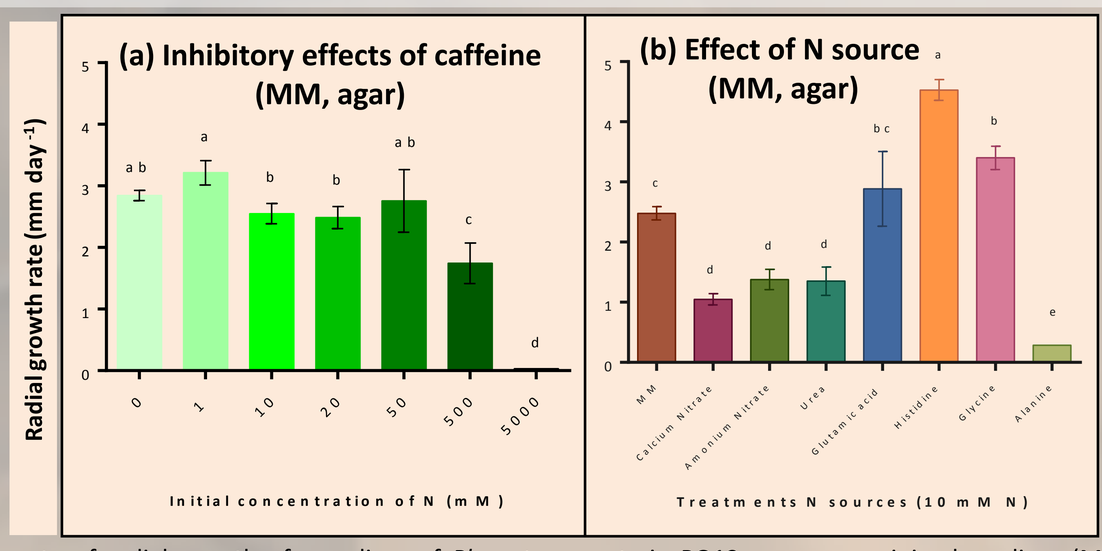
*** Extracts of SCG and FCG enhanced fungal growth, particularly when grown on MM**



Mean rate of radial growth of mycelium of *Pleurotus* sp. strain PO13 grown on **(a)** potato dextrose (PDA), **(b)** minimal medium (MM) and water agar (WA) supplemented with extracts of spent coffee grounds (SCG) and fresh coffee grounds (FCG) at three dilutions (undiluted, 1:1 and 1:10 extract:distilled water).

*** Low concentrations of caffeine as N source supported fungal growth, whereas high concentration inhibited and suppressed growth**

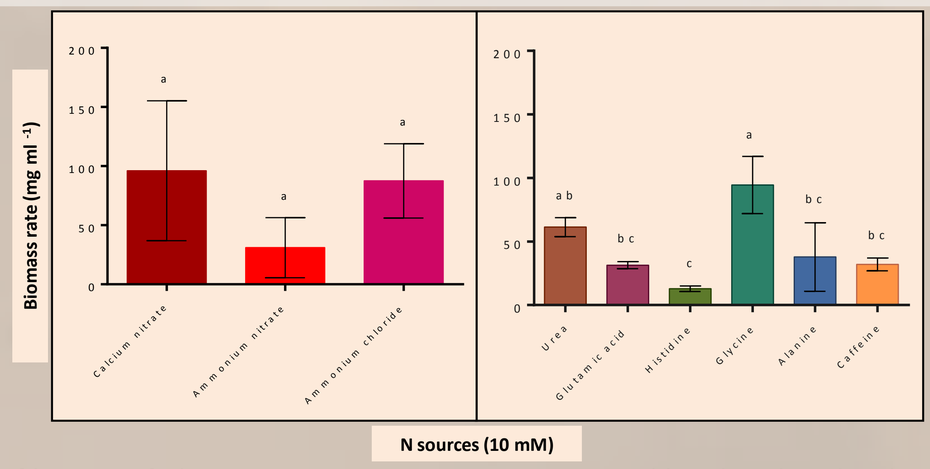
*** Organic N sources support better fungal growth**



Mean rate of radial growth of mycelium of *Pleurotus* sp. strain PO13 grown on minimal medium (MM) supplemented with **(a)** different concentrations of caffeine as N source (0-100 mM), **(b)** different N sources (10mM N).

N sources effect (including caffeine) on liquid MM

*** *Pleurotus sp*. utilized organic N differently**



Mean rate of biomass of *Pleurotus* sp. strain PO13 grown on minimal liquid medium (MM) supplemented with different N sources (10 mM N)

Future work: Caffeine metabolism and nitrogen utilization will be determined via detection of degradation products

Acknowledgments

We thank the government of Chile, for my **PhD funding *Becas Chile***, the Faculty of Agriculture and Environment for ***Thomas Pawlett Top Up*** Scholarship, and **Dr. Noel Arrold** from Li Sun Exotic Mushrooms in Mittagong NSW, Australia, who kindly supplied the fungal strain.

Effects of NO and Trehalose on Heat Stress Responses in *Pleurotus eryngii* var. *tuoliensis*

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Introduction

Pleurotus eryngii var. *tuoliensis* is a favored mushroom of rich nutrition and medicinal value. Now it keeps growing in demand and cultivation scale. However, the spawning of the mushroom cultivated in agricultural facilities in China are usually in naturally high temperature period, which always lead to "burnt mycelium" and declined production. Therefore, to explore the mechanism of mycelial heat stress response would establish a theoretical basis for a new species breeding and have both scientific and industrial significances.

In organisms, nitric oxide and trehalose, as a signal molecular and protective metabolite respectively, have important roles in response to abiotic stresses. The research with respect to the functions of NO and trehalose in response to heat stress attracted more and more attentions.

Methods and Materials

P. eryngii var. *tuoliensis* (CCMSSC 00489) was provided from the China Center for Mushroom Spawn Standards and Control. For all experiments, mycelia were grown in the potato-dextrose agar (PDA) medium for 10 days. Then ten pieces (a diameter of 5 mm punch) of mycelia from solid medium were transferred to the 100 mL liquid potato-dextrose (PD) medium (250 mL flask). The cultures were incubated with shaking at 25°C and 140 rpm for 6 days. Mycelia were subjected to heat stress at 37°C for various time. 100μM of SNP or 0.2g/ml trehalose (the final concentration) were added to the mycelia before heat stress if needed. TBARS (Kong et al., 2012) , SOD (Beauchamp et al.,1971), CAT (Aebi et al., 1974), POD (Velikova et al., 2000) and GR (Hasanuzzaman et al., 2012) were assayed according to the previous published researches.

Results

Thiobarbituric acid reactive substances (TBARS, an indicator of cell membrane damage) is an indicator of cell membrane oxidative damage. After the *Pleurotus eryngii* var. *tuoliensis* mycelium was treated at 37°C for several days, more TBARS was detected significantly compared to the untreated sample (Fig.1). The result revealed that heat stress caused oxidative damage to the *P. eryngii* var. *tuoliensis* cell membrane.

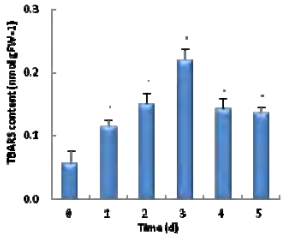


Fig.1 Effect of heat stress on TBARS content in mycelia of *P. eryngii* var. *tuoliensis*

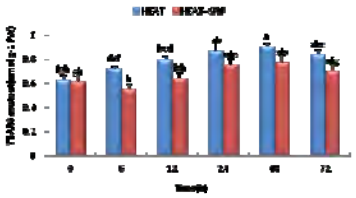


Fig.2 Effect of NO on TBARS content in mycelia of *P. eryngii* var. *tuoliensis*.

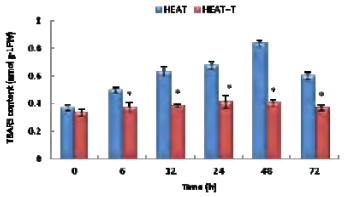


Fig.3 Effect of trehalose on TBARS content in mycelia of *P. eryngii* var. *tuoliensis*.

The antioxidant enzymes in mycelia of *P. eryngii* var. *tuoliensis* changed significantly, while each responded differently under heat stress (Figs. 4 and 5). The activity of SOD had no significant change in 48 h, but increased significantly after that with 1.55 fold of that in control(0 h); the activity of CAT increased significantly after 24 h and reached the maximum with 5.02 fold of that in control(0 h); the activity of POD expressed significant increase after 12 h and reached the maximum with 2.82 fold of that in control (0 h). Unlikely, GR activity increased first and then decreased, reached the maximum at 24h. All of these showed that CAT was the most sensitive enzyme when *Pleurotus eryngii* var. *tuoliensis* responded to the heat stress.

Exogenous NO had different effects on the activities of antioxidant enzymes (Fig. 4). The application of exogenous NO increased the activities of SOD, CAT and GR, but inhibited POD activity. Consequently, exogenous NO enhanced heat-resistance of *P. eryngii* var. *tuoliensis* mycelia by improving SOD, CAT, GR activities. Especially, the activity of CAT was measured by $mM \cdot min^{-1} \cdot mg^{-1}$ of protein, and it played the most important role in eliminating the reactive oxygen species and mitigating the oxidative damage.

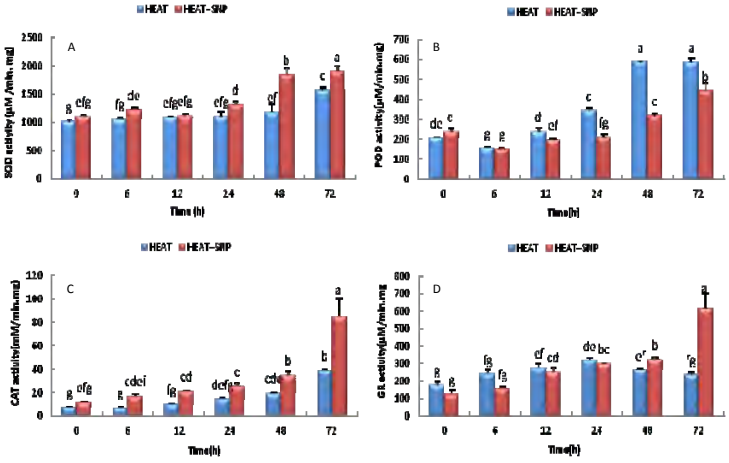


Fig. 4 Effect of exogenous NO on the four oxidative enzymes activities in the mycelia of *P. eryngii* var. *tuoliensis* after heat stress

Exogenous trehalose had different effects on the antioxidant enzymes (Fig. 5). The application of exogenous trehalose increased the activities of SOD, CAT and POD, but inhibited GR activity. Exogenous trehalose relieved oxidant damage, enhanced heat-resistance of *P. eryngii* var. *tuoliensis* by improving the activities of SOD, CAT and POD.

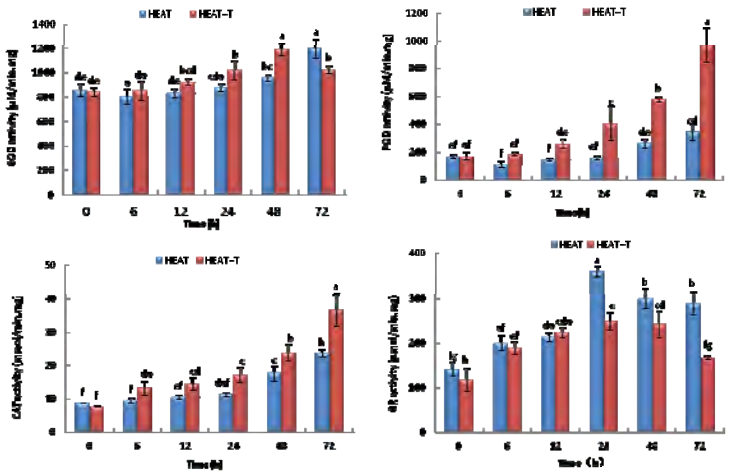


Fig. 5 Effect of exogenous trehalose on the four oxidative enzymes activities in the mycelia of *P. eryngii* var. *tuoliensis* after heat stress

Conclusion and Discussion

The study found heat stress caused oxidative damage to the *P. eryngii* var. *tuoliensis* mycelia and exogenous NO and trehalose could alleviate the damage. Thus, we detected the effects of exogenous NO and trehalose on the oxidative enzymes activities in the mycelia after heat stress and found the mechanisms seemed different. The common pathway of exogenous NO and trehalose mitigating the oxidative damage is that they increase the activities of SOD and CAT. And the different way is that the exogenous NO increased GR activity while trehalose increased POD activity. Besides, NO could also directly work as antioxidant substance to eliminate the $O_2^{\cdot-}$(Besson-Bard et al., 2008). But the detailed signal transduction pathway remains unclear.

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Temporal and spatial heterogeneity of oyster mushroom lignocellulolase activities during large-scale production

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Introduction

Oyster mushroom (*Pleurotus ostreatus*) is one of the market-leading edible mushroom. As a saprotrophic, white rot fungi it is grown on composted and pasteurized wheat straw (substrate).

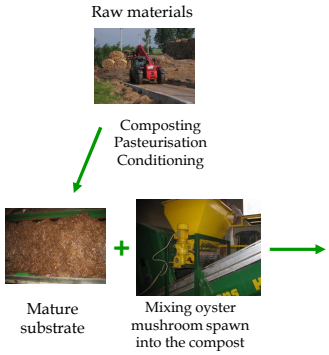
During its production, *Pleurotus* sp. colonize the substrate, and degrade the plant cellulose and lignin polymers.

Aims

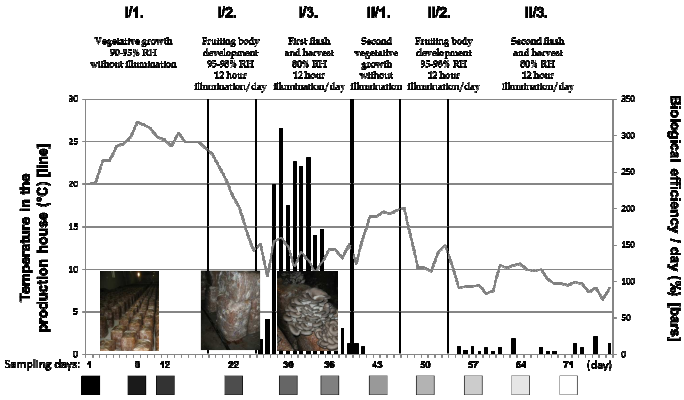
- Monitor the substrate colonization and fruiting body production of oyster mushroom by its lignocellulose degrading enzyme activities in a large-scale production facility.
- Analyse the temporal and spatial heterogeneity of these enzyme activity.

Materials

Substrate production



Sensu stricto mushroom production with two cultivation cycles



Methods

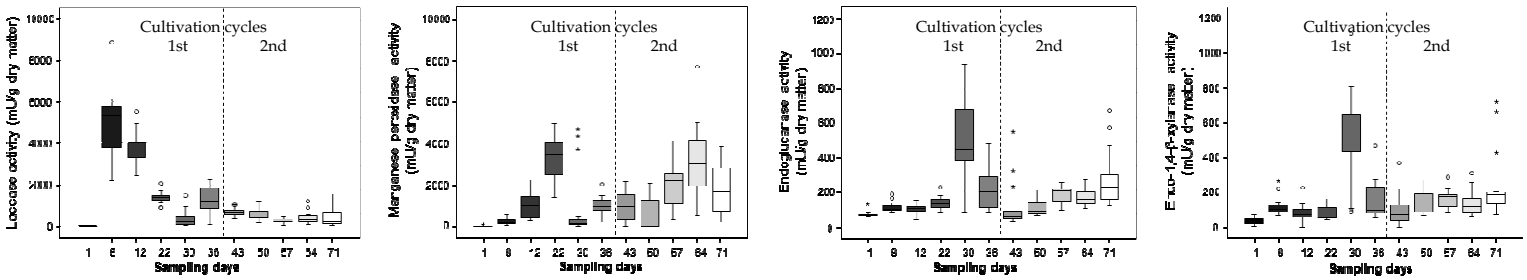
Each time 5 parallel blocks were sampled

Measurement of enzyme activities:

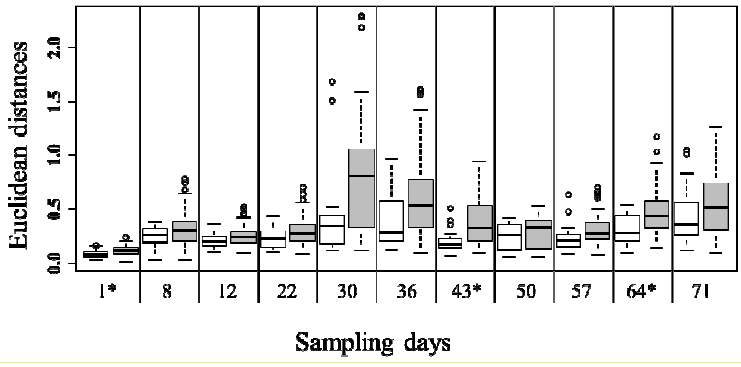
- endocellulase, endoxylanase (Azo-CM-Cellulose, Azo-Xylan substrate)
- laccase (ABTS substrate)
- manganese peroxidase (DMAB and MBTH substrate)
- exocellulase, β-glucosidase, xylosidase, exochitinase (p-nitrophenyl labelled substrate)

Results

P. ostreatus lignocellulase activity



Temporal and spatial heterogeneity in the substrate



The within (white boxes) and among (grey boxes) block variability of enzyme activities based on Euclidean distances.

The distances were calculated using range standardised enzymatic data at each sampling date among the three random samples within each substrate block and the average of the five distances are displayed. Bars represent standard deviations.

Conclusions

- Oyster mushroom enzymatic activities showed a clear succession during the two cultivation cycles.
- At the first stages of the cultivation the substrate blocks were not different from each other, as the within block variation had the same magnitude as the among block variation. During the first flush and harvest both kind of variabilities increased. But later this difference disappeared, which could be partially related to the relatively uniform physicochemical parameters in the production house.
- It is important information for the growers that a relatively stable environment contributes to the quite uniform physiological status of the blocks.
- The outlined approach is suitable for checking the homogeneity of mushroom substrate colonization, which can improve production efficiency.

This research was supported by the Hungarian Scientific Research Found (OTKA K 83764) and by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/-11-1-2012-0001 'National Excellence Program' (A1-MZPD-12-0166).

Session Germplasm & Taxonomy

Establishment of *Lentinula edodes* core collections in China

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Introduction

- ***Lentinula edodes*** is one of the most important commercial edible mushroom in China.
- Estimating **genetic diversity** of these germplasm resources is the prerequisite for effective protection and rational utilization.
- **Core collection** was introduced and defined as a subset of accession presenting the maximum possible genetic diversity contained in the entire collection with minimum repetition.

Objectives

- To investigate the **genetic diversity and relationships** of Chinese *L. edodes* cultivars in a large scale.
- To create the **core collection** of *L. edodes* in China.
- To **compare and evaluate** the feasibility of the two different methods in generating the core collections in *L. edodes.*

Working Programme

- **Eighty-nine shiitake strains** currently cultivated in the main production area of China.
- **9 ISSR and 9 SRAP** analysis.
- **Data analysis** for genetic diversity.
- **Construction** of the preliminary core collections.

Results

- **Polymorphism of the ISSR and SRAP markers**

Both ISSR and SRAP PCR experiments were repeated twice. Only unambiguous and reproducible DNA bands were manually recorded. Characteristics of the ISSR and SRAP markers were listed in Table 1.

Table 1 Characteristics of ISSR and SRAP markers used in this study

Primers	N	NPL	PPL (%)	Ne	H	I
P2	13	13	100	1.422	0.242	0.372
P4	10	10	100	1.438	0.261	0.401
P5	18	16	88.89	1.347	0.204	0.317
P6	12	11	91.67	1.254	0.165	0.268
P7	9	9	100	1.356	0.219	0.348
P8	19	19	100	1.375	0.228	0.359
P16	8	8	100	1.561	0.325	0.487
P20	16	16	100	1.200	0.140	0.242
P27	21	21	100	1.098	0.082	0.164
ME1+EM2	14	14	100	1.483	0.296	0.457
ME1+EM4	11	9	81.82	1.467	0.266	0.399
ME3+EM8	14	8	57.14	1.202	0.117	0.180
ME5+EM2	23	23	100	1.206	0.151	0.260
ME5+EM4	11	9	81.82	1.402	0.237	0.359
ME6+EM4	9	6	66.67	1.446	0.255	0.376
ME6+EM6	15	14	93.33	1.332	0.206	0.303
ME6+EM7	14	11	78.57	1.248	0.159	0.260
ME7+EM1	18	14	77.78	1.219	0.141	0.228
Total	255	231				
Average	14.17	12.83	90.59	1.312	0.193	0.307
ISSR average	14	13.67	97.62	1.311	0.193	0.309
SRAP average	14.33	12	83.73	1.312	0.193	0.304

- **Genetic diversity and relatedness among Chinese shiitake cultivars based on ISSR and SRAP markers**

For the 89 *L. edodes* cultivars, the GS estimated by the SM coefficient varied from 0.576 to 0.988, with an overall mean of 0.820. A dendrogram constructed from the SM coefficient matrix using the UPGMA method was shown in Fig. 1.

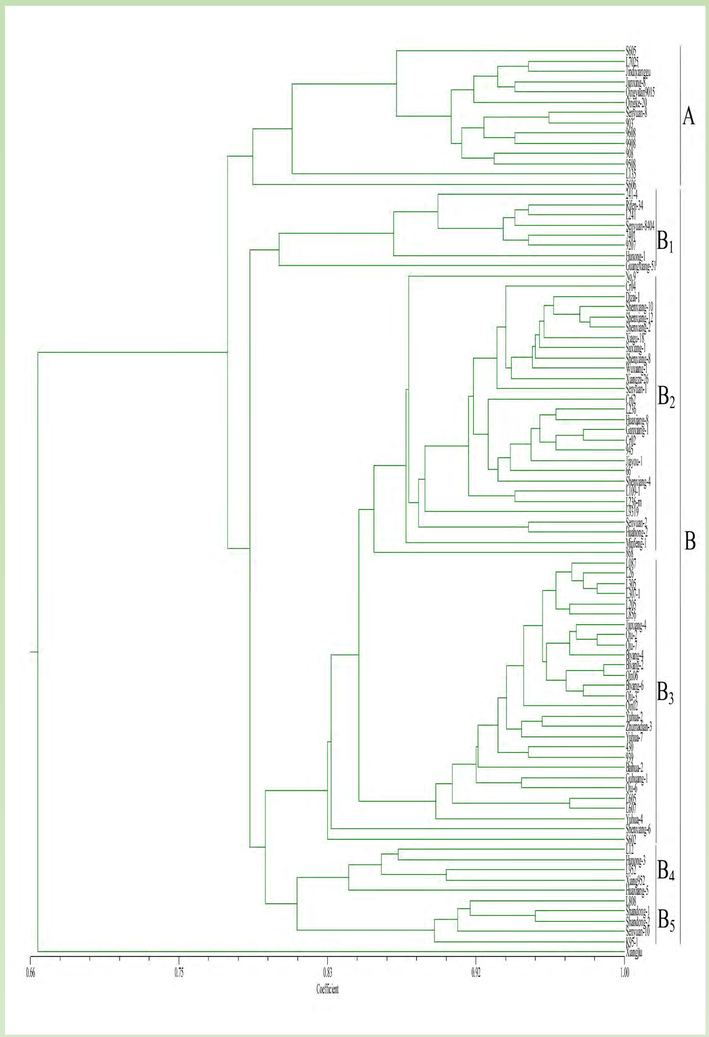


Fig. 1. UPGMA dendrogram of 89 *L. edodes* strains constructed using genetic similarity coefficients based on ISSR and SRAP data.

Except for Xiangjiu, all the *L. edodes* strains were clustered into two main groups (A and B) and group B was further delineated into five subgroups (B1–B5). The UPGMA clustering results indicated a positive correlation with the traits of the tested strains in most cases, and that the strains with similar traits tended to cluster together into the same groups or subgroups.

- **The preliminary core collections of *L. edodes* cultivars in China**

Core1 was constructed by the advanced M(maximization) strategy using the PowerCore version 1.0 software and contained 21 strains, whereas Core2 was created by the allele preferred sampling strategy using the cluster method and contained 18 strains (Table2).

Table 2 Comparisons of the two preliminary core collections with the original germplasm

Group	Number of samples	Number of loci retention	NPL	PPL (%)	Na	Ne	H	I
Original germplasm	89	255	231	90.59	1.906	1.312	0.193	0.307
Core1	21	254	230	90.55	1.906	1.366	0.224*	0.352*
Core2	18	249	217	87.15	1.872	1.366	0.226*	0.354*

* Indicates significant difference in genetic diversity index at 0.05 level between the core collection and the original germplasm.

Conclusions

- Both core collections were highly representative of the genetic diversity of the original germplasm. Moreover, Core1 contained strains with more types of agronomic traits those in core2. Consequently, Core1 was chosen as *L. edodes* cultivars core collection.

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DIVERSITY AND DOMESTICATION OF WILD EDIBLE SPECIES OF GENUS *LENTINUS* FROM NORTH WEST HIMALAYAS

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ISMS Congress 19th International Society for Mushroom Science, Amsterdam 2016, 29th May - 2nd June

ABSTRACT

The genus *Lentinus* Fr. belongs to the family *Polyporaceae* and is represented by 597 legitimate species the world over in comparision from India the status of this genus being represented by 30 species is very poor. In the study area altitude varies between 196 m - 3,600 m, the Himalayan belt is a mega hotspot centre having rich floristic diversity, represented by well demarcated vegetational zones, comprising of pure coniferous to mixed coniferous to broad leaved trees whereas in the plains the heaps of organic matter, open pastures, road side plantations, gardens, etc. provides perfect habitat for rich macrofungal diversity. In this region the monsoon season starts in June and ends up in mid October. This is the best season for the collection of these mushrooms. The average temperature varies between 18°C to 44°C. The fungal forays to the North West Himalayas were undertaken for the collection of *Lentinus*. As a result, number of collections of this genus were made worked out, identified and described taxonomically along with their ethanomycological data with respect to their edibility status gathered from the local people. Of the described taxa *L. transitus* sp. nov. and *L. kashmirinus* sp. nov. are reported new to science whereas, *L. adhaerens* var. *inadharens* Melencon & Bertault and *L. nigroosseus* Pilát are reported for the first time from India. *L. nigroosseus, L. velutinus, L. connatus, L. tuberregium* and *L. tigrinus* are known wild edible species which are collected by the local people from the forests for cooking. *L. kashmirinus* sp. nov. has good edibility potential being collected by the local. The biochemical analysis of the wild edible taxa along with their domestication trials will be discussed.

INTRODUCTION

- Genus *Lentinus* Fr. belongs to the family *Polyporaceae* Fr. ex Corda.
- Known by 597 species the world over (Mycobank, 2016) while only represented by 30 species from India
- *Lentinus* usually occurs on dead tree stumps in gregarious caespitose habit very rarely it is graminicolous
- Carpophores are medium to large, fleshy, with central to lateral stipe
- Lamellae decurrent, gill edges entire, denticulate to serrate
- Spore print white to cream
- Basidiospores oblong to cylindrical, inamyloid
- Gill edges always sterile
- Cystidia either metuloid or in the form of gleocystidia, cheilocystidia or skeletocystidia
- Pleurocystidia and hyphal pegs are rarely present
- Pileus cuticle usually undifferentiated forming a repent epicutis of radially parallel hyphae, sometimes trichodermial
- Carpophore context with dimitic hyphal system
- While on mycofloristic survey to the North West Himalayas a number of *Lentinus* species were encountered of these 10 species viz. *L. kashmirinus*, *L. transitus* sp. nov., *L. tuberregium*, *L. squarrosulus*, *L. tigrinus*, *L. connatus* , *L. adhaerens* var. *inadhaerens*, *L. velutinus* ,*L. ponderosus and L. detonsus* Fr. were identified
- The ethnomycological deleberations reveal the first 6 species to be edible, as these are collected from the wild by the native people as source of food
- Thus nutritional and nutraceutical studies were taken up for these 6 species with an aim to domesticate them

MATERIAL AND METHODS

Diversity:
- Collection of material
- Macroscopic characters
- Spore deposit
- Drying and preservation
- Microscopic observations
- Identification

Nutritional Evaluation:
- Proteins (Lowry *et al.*, 1951)
- Fats (AOAC,1995)
- Carbohydrates (Hedge and Hofreiter, 1962)
- Crude fibers (Maynard, 1970)

Nutraceutical Evaluation:
- Alkaloids (Maxwell *et al.*, 1995)
- β– carotene and Lycopene (Barros *et al.*, 2007)

VARIOUS HABITATS



AREA SURVEYED



TAXONOMIC OBSERVATIONS

L. kashmirinus Munruchi Kaur and Nazir Ahmad Malik



- Carpophore 12 x 8.2cm, convex to applanate, cap white ($30A_1$), light orange ($5A_5$) squamules more concentrated near the centre, flesh up to 2 cm broad
- Lamellae decurrent, edges serrate, grayish orange (5B6)
- Spore print greyish yellow (2B$_6$)
- Basidiospores 8 - 13 x 3.32- 4.15 µm, (Q= 2. 4), oblong to cylindric, inamyloid, bi-tetra sterigmata
- Hymenophoral trama descending
- Hyphal pegs absent
- Pleurocystidia cylindric, deeply seated in the trama
- Cheilocystidia metuliodal, sinous to fusoid

Edibility: As per the Ethnomycological data collected from local population it is an edible fungus.

Collection Examined: Jammu and Kashmir, Damhal Hanji Pora, Village Khull (2300m, N33^0 35.727 E074^{0}59.727) growing solitary on the dead stump of *Cedrus deodara* towards the end of spring season on 3rd of May 2013, Nazir Ahmad Malik, PUN6595 (**Holotype**).

Remarks: Based upon some of its diagnostic features including denticulate lamellae, metuloidal cheilocystidia, thick walled unbranched skeletal hyphae and hymenophoral trama descending the present collection has been put under subgenus *Panus*, section *Pulverulenti* (Pegler 1983). Further with this section, the presently examined collection falls close to *L. adharens* (Alb. & Schewein.) Fr. in possessing clamps, docurrent gills running 1-2 cm down the stipe and presence of some cheilocystidia with metuloidal apices. However, the Indian collection lacks resinous amber coloured secretion in the carpophore. The basidiospores of the presently examined species are much larger i.e. 8 – 13 x 3.32–4.15 µm in comparison to 6-10 x 2.5 – 3.5 µm of *L. adharens*. In view of the above the present collection is published as new species.

L. transitus sp. nov.



- Carpophore 6 x 9.5 cm broad, convex; cap white ($2A_1$) to grayish orange ($5B_6$) to brownish orange ($6C_6$) appressed fibrillose with squamose scales in centre, flesh upto 1.8cm broad
- Lamellae adnodecurrent, edges dentate, white ($2A_1$) to Pale orange ($5A_2$)
- Spore print creamish white
- Basidiospores 6.64 - 9.96 x (2.49) 3.32 – 4.15 µm, (Q=2), cylindric, inamyloid
- Hymenophoral trama descending
- Hyphal pegs present
- Cheilocystidia metulloid
- Pleurocystidia - Gloeocystidia fusoid ventricose

Edibility: Ethnomycological data reveals it is an edible fungus.

Collection Examined: Jammu and Kashmir, Damhal Hanji Pora, village Khull (2300m, N33^0 35.987 E074^{0}59.765), growing caespitose clusters on the dead stump of *Cedrus deodara* in summer season on 11th of July, 2013, Nazir Ahmad Malik, PUN7548 (**Holotype**), 2550m, N33^{0}35.827' E074^{0}59.407, growing caespitose clusters on the stump of *Cedrus deodara* during summer season on 2nd of April, 2014, Nazir Ahmad Malik, PUN7554 (**Holotype**).

Remarks: The carpophores of this collection are thick fleshed, lamellae are not furcated, the skeletolgiative hyphae are unbranched and gleocystidioid, pleurocystidia and cheilocystidia are present. Thus as per Pegler (983) it falls under subgenus *Panus*. Further, this collection has hyphal pegs, emergent tramal hyphae at the gill edges. The basidiospores are less than 10um long and it lacks pseudocystidium at the stipe base. In those characters it seems nearer to subgenus *Lentinus*. With these overlapping characters of the subgenera *Panus* and *Lentinus* the present collection is unique in having deeply dentate gill edges, it seems to be a link between the two subgenera. Thus, a new species *L. transitus* is proposed to adjust this collection.

L. connatus Berk



- Carpophore 4 x 7 cm, infundibuliform, red brown ($8D_8$) to white ($8A_1$); reddish brown ($8D_8$) appressed fibrillose scales; flesh upto 0.2 cm broad
- Lamellae decurrent, light yellow ($4A_4$), gill edge serrate
- Spore print white ($3BA_1$)
- Basidiospores (4.98) 5.81- 7.47 (9.13) x 2.49- 3.32 µm, (Q= 2) , cylindric, inamyloid
- Hymenophoral trama regular
- Pleurocystidia cylindric
- Cystidial hairs crowded, fusoid, thin to thick walled interspersed with clavate cheilocystidia

Edibility: It is an edible species (Zhishu, 1993)

Collection Examined: Jammu and Kashmir, Damhal Hanji Pora, (1800m, 33^{0}31.612 N – 074^{0}51.231E), growing gregarious form on the stump of *Populus alba* during the spring end on 3rd of May 2013, Nazir Ahmad Malik, PUN7549.

Remarks: Although the present collection falls under the section *Tuberregium* (Pegler, 1983) and close to *L. tuberregium* (Fr.) Fr. but it differs in always arising from a sclerotum which is not the case for *L. tuberregium*. Further our collection also relates to *L. anthocephalus* but differs from it as in *L. anthocephalus* the lamellae are densely crowded, sublinear, the stipe is comparatively longer and spores are narrower (Pegler, 1983). Our collection falls under *Lentinus connatus* Berk. having typical habit growing in gregarious clusters on dead decaying wood, possessing central to lateral stipe and the presence of emerging cystidicial hairs at the gill edges with the absence of hyphal pegs.

L. tuberregium (Fr.) Fr.



- Carpophore 7.7 x 6.5 cm, convex, creamy white ($3A_2$), squamulose, light brown ($6D_5$) towards centre; flesh upto 0.2 cm broad
- Lamellae decurrent, lamellulae unequal, brownish orange ($5C_5$), gill edge dentate
- Spore print greyish orange ($6B_5$)
- Basidiospores 6.64 – 9.96 x 3.32- 4.15µm, (Q= 2), cylindric, inamyloid
- Hymenophoral trama slightly descending
- Pleurocystidia cylindric, deeply seated
- Cheilocystidia clavate

Edibility: It is medicinally important edible species (Boa, 2004; Kumar and Kaviyarasan, 2012).

Collection Examined: Jammu and Kashmir, Panzalla Takya (2150m, 34^{0}16.792 N – 074^{0}16.308E), caespitose clusters on living stump of *Pinus wallichiana* in Autumn season on 8th of September, 2012; Naseema Aqbar Wani, PUN7553.

Remarks: The present collection falls under subgenus *Panus* as it has unbranched, thick walled skeletal hyphae in the context, the hyphal pegs are lacking while the cheilocystidia are present. Further, under this subgenus it matches well with *L. tuberregium* in possesing robust carpophores, whitish cap with ocharaceous brown squamules, crowded narrow lamellae, cylindric basidiospores without oil guttules. Further, there is absence of metuloid cystidia. In the context the generative hyphae are not inflated.

L. squarrosulus Mont.



- Carpophore 3.5 x 8 cm broad, convex, off white, pale orange ($5A_2$) to light brown($6D_5$) squamulose; flesh upto 0.2 cm broad
- Lamellae decurrent, orange white ($5A_2$), lamellulae unequal, gill edge dentate.
- Basidiospores 5.81 – 8.3 x 3.32- 4.15µm, (Q= 1.7), elliptical, inamyloid.
- Hyphal pegs present in clusters

Edibility: It is an edible species (Boa, 2004)

Collection examined: Jammu and Kashmir, Bandipora, Village Malangam (2100m, 34^0 23.698 N – 074^{0}29.707E), growing in caespitose clusters on the stump of *Populus indica* during summer season on 17th of June, 2015, Naseema Aqbar, PUN7553; Himachal Pradesh, Bhadrol (850m) growing on dead stump of *Mangifera indica* on 5th of November, 2009, Sapan Kumar, PUN4710; Palampur (1200m) growing on stump of *Albizia chinensis* on 10th of November, 2009, Sapan Kumar, PUN415; Kotla Barog (3000m) growing on dead stump of *Juglens regia* on 20th of July, 2006, N. S. Atri, PUN4127.

Remarks: The above examined collection matches in its morphology and anatomy to the details provided for *L. squarrosulus* by Pegler (1977, 1983 and 1986). The carpophore is found growing in caespitose cluster, typically the colour of the fruiting body is white bearing squamules on both the palal and stipe surfaces. Stipe is almost having a evanescent annulus and the gill edges are denticulate.

L. tigrinus (Bull.: Fr) Fr.



- Carpophores 3.9 x 6.6 cm, sub infundibuliform to applanate, creamy white ($1A_2$), squa mulose brown ($6E_6$) to grayish ($6D_5$)
- Lamellae docurrent, creamy white ($1A_2$), gill edge deeply serrate
- Spore print creamish white ($1A_2$)
- Basidiospores 5.81 – 8.8 x 3.32- 4.15µm, (Q=1.7), elliptical, inamyloid.
- Hymenophoral trama regular
- Pleurocystidia cylindric, sinous, deeply seated
- Hyphal pegs moderately abundant
- Cystidial hairs crowded, fusoid, thin to thick walled

Edibility: It is an edible species (Boa, 2004).

Collection examined: Jammu and Kashmir, Jagurpur (1615m, 34^{0}27.645N – 074^{0}16.248E) growing caespitose clusters on stump of *Pinus wallichiana* in the mixed forest in Spring season on 3rd of May, 2014, Naseema Aqbar Wani, PUN7546, PUN7555; Damhal Hanji Pora, (2000m, 33^{0}31.0133N – 074^{0}51.121E), growing caespitose clusters on the dead stumps of *Populus alba* in ending of spring season on 13th of June 2015, Nazir Ahmad Malik, PUN7552.

Remarks: The present collection matches and fits well in the description provided for *L. tigrinus* by Pegler (1983). It is characterized by presence of partial veil forming annulus which is soon eroded at maturity. Further it consist hyphal pegs, cystidial hairs and presence of generative hyphae in the trama.

L. detonsus Fr.



Locality: Jammu and Kashmir, village Khull
Altitude: 2134m
GPS location: N33^{0}35.987' E074^{0}59.423
Date of collection: 11th of July, 2013
Accession No.: PUN7551

L. adhaerens var. *inadhaerens* Melencon & Bertault



Locality: South Kashmir, village Khull
Altitude: 2127m
GPS location: 3^{0}34.682 N – 074^{0}58.507E
Date of collection: 11th of July, 2013
Accession No.: PUN7550

L. ponderosus O.K.Miller



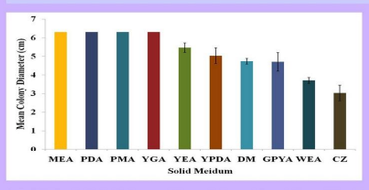
Locality: South Kashmir, village Baregiward
Altitude: 2700m
GPS location: 33^{0}34.432' N – 074^{0}51. 322E.
Date of collection: 20th of August, 2015.
Accession No.: PUN7864

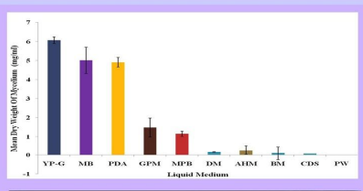
L. velutinus Fr.



Locality: South Kashmir, village Nagam
Altitude: 2233m
GPS location: 33^{0}34.904 N – 074^{0}51.731E
Date of collection: 11th of July, 2013
Accession No.: PUN7863

CULTURE STUDY OF *LENTINUS TIGIRINUS*







Vegetative growth of *Lentinus tigrinus* on different Solid media



Vegetative growth of *Lentinus tigrinus* on different Liquid media Solid media



Primordia

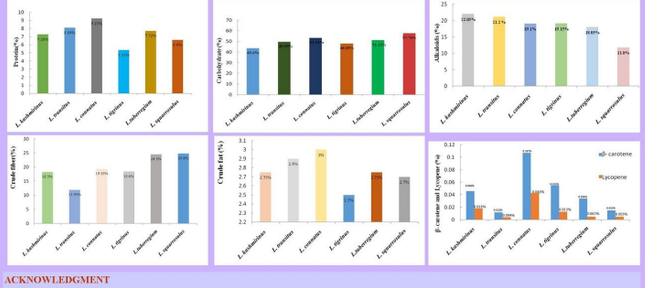


Fruit body

DISCUSSION

In the present investigation of the 17 field collections falling under 10 species of *Lentinus*, 6 are known edible species from the ethnomycological data collected and correlated with literature. Further nutritional and nutraceutical studies on these six species reveals that for the vegetative growth of the mycelium Yeast Glucose (YP-G) is the best liquid media and Malt Extract Agar (MEA) is the best solid media especially in case of *Lentinus tigrinus*, *L. connatus* possess maximum percentage of proteins, ß-carotene, lycopene and crude fat. *L. kashmirinus* consists minimum carbohydrate and maximum alkaloid composition. High content of crude fiber was observed in *L. tuberrigium*. Moreover, Pure culture of *L. tigrinus* have been raised and maintained through repeated subculturing.

NUTRITIONAL AND NUTRACEUTICAL ANALYSIS



ACKNOWLEDGMENT

I thank UGC and DST for providing financial support under SAP I, II and III of DRS and FIST respectively.

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CONSERVATION METHODOLOGY DEVELOPMENT AND CREATION OF A FUNGAL GENETIC RESOURCE COLLECTION OF WILD FUNGI IN LA RIOJA (SPAIN)

Mª Luisa Tello-Martín, Margarita Pérez Clavijo.

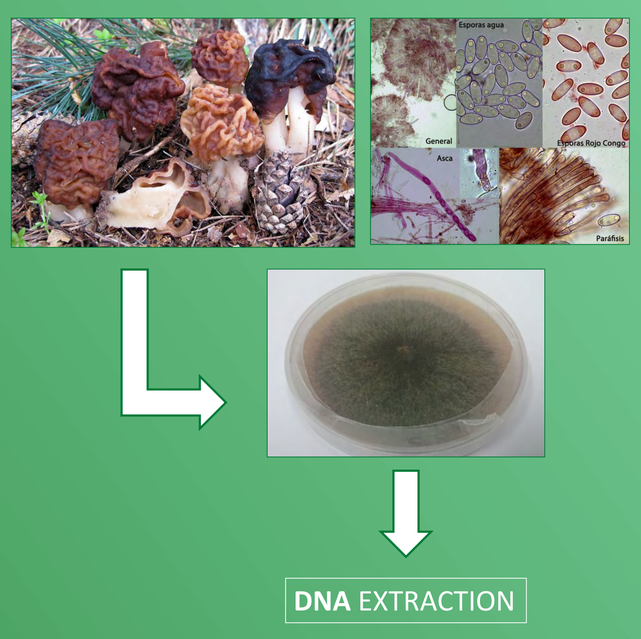
Mushroom Technological Research Center of La Rioja (CTICH), Autol (La Rioja), Spain.

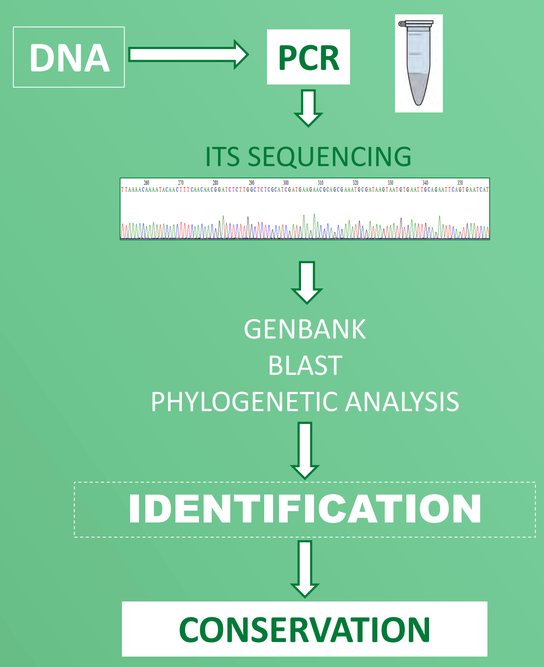
INTRODUCTION: The most extraordinary feature of life is its diversity. Scientists estimate that species loss rate has increase from 1,000 to 10,000 times the background rate, with literally dozens of species going extinct every day. In the case of fungi, biodiversity loss is unpredictable because the overall number of species remains still unknown though estimates set this value ranging from 0.5–9.9 million. Currently, only 70,000 species have been described, thus representing a mere 5-10% of the total diversity. Biodiversity loss is a problem with difficult solution; therefore, in an effort to conserve biodiversity, gene banks have been created to store and conserve the genetic resources.

OBJECTIVE: The main objective was to create a fungal genetic resource collection which allows the conservation of wild fungal biodiversity in the region of La Rioja (Spain) through the development of different fungal conservation techniques.

METHODS:

A study of fungal species of interest was made to establish a catalogue of selected species that should be included in the collection. Through field trips to different locations in La Rioja region, selected species in the catalogue were located and collected. Collected fungi were identified morphologically. The mycelium of each fungi was isolated in petri dishes with Rose Bengal Chloramphenicol Agar medium. To confirm the isolates identification DNA from each sample was extracted with a commercial kit and regions ITS (Internal Transcribed Spacer) was amplified. ITS sequences then were identified using similarity searches in BLAST. Identity confirmed samples were conserved under different methods to search the best in each case. Combinations of substrates (PDA, wood), preservation solutions (distilled water, glycerol), and temperatures (4, -86 ˚C) were evaluated for each isolate.

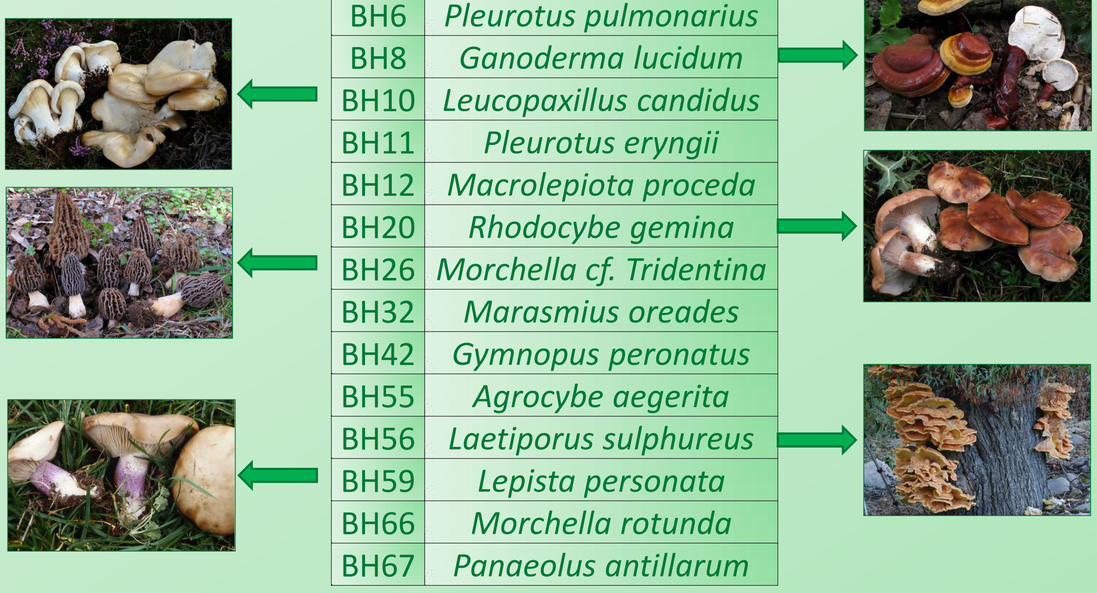




RESULTS AND DISCUSSION

A study of fungal species of interest was made to establish a catalogue of 103 selected species that should be included in the collection. Through field trips to different locations in La Rioja region, 67 selected species in the catalogue were located and collected. Collection place and morphological description of the collected fungi were thoroughly detailed in individual files compiled in a recording book. The mycelium of each fungi was isolated in petri dishes with Rose Bengal Chloramphenicol Agar medium. From the 55 isolates that were able to develop in petri dishes with artificial media, molecular analysis was performed to confirm the isolates identification. DNA from each sample was extracted with a commercial kit and regions ITS (Internal Transcribed Spacer) was amplified. ITS sequences then were identified using similarity searches in BLAST. Identity confirmed samples were conserved under 5 different methods to search the best in each case. Combination of substrates (PDA and wood), preservation solutions (distilled water and glycerol), and temperatures (4˚C and -86˚C) were used in four of the methods. The fifth method was based on the perlite protocol developed by Homolka, L. 2010. A total of 15 species was successfully conserved (Table 1), among them, species with medicinal properties like *Ganoderma lucidum*, protected species like *Rhodocybe gemina*, also edible mushrooms like *Leucopaxillus candidus* and toxic mushrooms like *Panaeolus antillarum*.

Table 1. Species successfully conserved.



COD.	SPECIES
BH5	*Pleurotus ostreatus*
BH6	*Pleurotus pulmonarius*
BH8	*Ganoderma lucidum*
BH10	*Leucopaxillus candidus*
BH11	*Pleurotus eryngii*
BH12	*Macrolepiota proceda*
BH20	*Rhodocybe gemina*
BH26	*Morchella cf. Tridentina*
BH32	*Marasmius oreades*
BH42	*Gymnopus peronatus*
BH55	*Agrocybe aegerita*
BH56	*Laetiporus sulphureus*
BH59	*Lepista personata*
BH66	*Morchella rotunda*
BH67	*Panaeolus antillarum*

Table 2. Sample recovery results.

METHOD	M1		M2		M3		M4		M5		CT
MEDIA	PDA	MEA	PDA	MEA	PDA	MEA	PDA	MEA	PDA	MEA	
BH5											9
BH6											9
BH8											9
BH10											8
BH11											9
BH12											9
BH20											4
BH26											9
BH32											7
BH42											7
BH55											9
BH56											9
BH59											7
BH66											7
BH67											2
SR%	86		46		66		73		60		

Method 1: Substrate: PDA, Preservation solution: distilled water, Conservation temperature: 4˚C.
Method 2: Substrate: PDA, Preservation solution: glycerol, Conservation temperature: -86˚C.
Method 3: Substrate: perlite, Preservation solution: MYA medium, Conservation temperature: -86˚C.
Method 4: Substrate: wood, Preservation solution: distilled water, Conservation temperature: 4˚C.
Method 5: Substrate: wood, Preservation solution: glycerol, Conservation temperature: -86˚C.
CT: Conservation time in months.
SR: Sample recovery in percentage.

After conservation methods the sample recovery (SR) was evaluated through mycelium growth development. A sample was considered recovered when mycelial growth was noted after 5 days. Mycelium growth recovery was evaluated in two different media (PDA and MEA). Table 2 shows the results of mycelium recovery; in dark green colour, strong and fast mycelium growth; in light green colour, good mycelium growth; in yellow colour weak mycelium growth and in red colour no mycelium growth. Right column shows the conservation time evaluated for each sample.

CONCLUSIONS: Sample recovery results showed that not every method is valid for every species. Thus, the most accurate approach to conserve fungal samples would be to use several methods to ensure a safety and successful recovery. In this study best results were obtained with methods 1 and 4, both based on distilled water and conservation temperature of 4 ˚C, where 86 and 73% of the samples were recovered. For *Rhodocybe gemina, Marasmius oreades* and the two *Morchella* samples (*Morchella tridentina* and *Morchella rotunda*) cryopreservation seems to be an unsuccessful treatment for conservation but positive results were obtained using the simplest method M1. This study is a small contribution to the knowledge of fungal biodiversity conservation. Further research should be done in this area to enhance the protection of fungi kingdom, a very important but sometimes forgotten source of biodiversity.

Acknowledgments: This research was financed by the Economic Development Agency of La Rioja (ADER) (2012-I-IDD-00124).

First record and molecular Identification of *Amanita manginiana* in Jordan.

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Sample collection

Amantia spp Collection and Pure Culture Preparation

Amantia spp isolates were collected in 2014 from Tall Al-Rumman in Jordan. The mycelium was isolated from fruiting body by aseptically removing a small piece of fruiting body of mushroom samples and transferring it to potato dextrose agar (PDA). After four days of incubation, a pure culture was prepared.

Molecular identification

DNA Extraction

Genomic DNA was extracted using E.Z.N.A. SP Fungal DNA Mini Kit. DNA concentrations were visualized and estimated by gel electrophoresis. DNA stock solutions were adjusted to 30 ng/µl with sterile distilled water and stored at -20 °C for PCR reaction.

ITS Sequence Analysis

The ITS regions (ITS1, 5.8S rRNA gene and ITS2) and Partial sequences of the large subunit nLSU gene, containing rRNA coding regions were amplified using ITS1 and ITS4 forward and reverse primers. ITS1-F (5'-TCC GTAGGTGAACCTGCG- 3') which is specific for the higher fungi and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC- 3') and primers LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR16(5'TTCCACCCAAACACTCG-3'). Hot start PCR was performed to amplify above ITS region in a 25 µL total volume reaction under the following program conditions: 94 °C for 5 min for initial genomic DNA denaturation, 40 cycles at 94 °C for 40 s, 55 °C for 30 s (annealing) and 72 °C for 1 min (DNA amplification), and a final elongation (extension) at 72 °C for 5 min. The ITS amplified products were purified using E.Z.N.A Gel Extraction Kit-Spin Protocol and analyzed to test the integrity and concentration on 1.5% agarose gel.

Sequencing Analysis

ITS PCR products were directly sequenced in both directions. Sequencing reactions were performed and the sequences were automatically determined in the genetic analyzer.. Sequences were used to query GenBank via BLAST (http://blast.ncbi.nlm.nih.gov) and a multiple sequence alignment was carried out using Clustal X.

DNA Extraction:

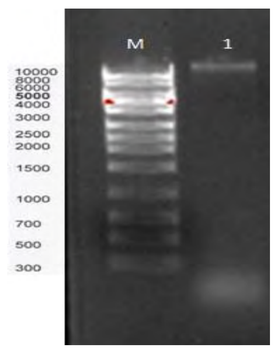


Figure1 . Genomic DNA isolated from ***Amanita* spp** from Tall Al-Rumman location. Lane 1(M): DNA ladder 1KB

ITS Sequence Analysis

The ITS region and part of the large subunit LSU of rRNA genes were amplified using ITS1, ITS4 and LROR primers using PCR hot start program. Results revealed that the length of the sequence for ITS region was between 600-650bp and it was achieved as single sharp band (Figure 2). While for large subunit LSU the product size was around 600bp (Figure 3).

Results

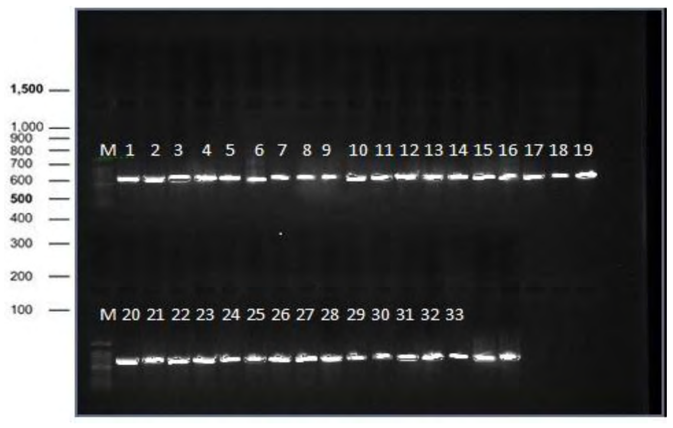


Figure2. PCR amplification of r DNA IT'S from ***Amanita* spp** isolate. PCR were conducted as 5µl was loaded onto a 1.5% agarose gel and bands were visualized by red safe ethedium bromide staining Isolates were ordered from R (1-11); D (12-22) and Q (23-33) ***Amanita* spp** (34+35). Lane 1(M): DNA ladder 100bp.



Figure3. PCR PCR amplification of rDNA large subunit from ***Amanita* spp** isolates. PCR were conducted as 5µl was loaded onto a 1.5% agarose gel and bands were visualized by red safe ethedium bromide staining Isolates were ordered from R (1-11); D (12-22) and Q (23-33). ***Amanita* spp** (34+35)

PCR product Sequencing Analysis

PCR products were gel purified, run in 1% agarose gel and processed for nucleotide sequencing. The partial nucleotide sequences sample were obtained and analyzed for BLAST (Basic Local Alignment Search Tool) search program using NCBI- National Center for Biotechnology Information Site) against the whole GenBank data base of nucleotide sequences (Using Clustural W software) for identification. Each PCR product was amplified using forward and reverse primers and both were used for Blast that based on identity% for both ITS region and LSU rRNA genes. As shown below in Table 1 and 2.

Table 1: Molecular Identification of *Amanita manginiana* for LSU rRNA Gene.

Best Relative strain			***Amanita manginiana* similarity**		
Taxon	**ID%**	**GeneBank Accession No.**	**Taxon**	**ID%**	**GeneBank Accession No.**
Amanita manginiana voucher HKAS56933	91	KJ466438.1	*Amanita manginiana*	95	KP161281.1

Table 2: Molecular Identification of *Amanita manginiana* for ITS Region.

Best Relative strain			***Amanita manginiana* similarity**		
Taxon	**ID%**	**GeneBank Accession No.**	**Taxon**	**ID%**	**GeneBank Accession No.**
Amanita manginiana voucher HKAS56933	91	KJ466438.1	*Amanita manginiana*	91	AF024463.1

Session Casing

COMPOSITION OF COMPOSTED WASTE PAPER CASING IN *COPRINUS COMATUS* CULTIVATION

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INTRODUCTION

The initiation and growth of mushroom fruiting bodies depends not only on genetic capability of the mycelium to yield in fruit, also on environmental, physical, chemical and microbiological factors. **Casing material** is crucial element of obtaining **high quality yield** in cultivation of ***Coprinus comatus*** (O.F. Mull). Pers. In commercial cultivation, fruiting bodies appear on the surface of casing material covering the compost after germination phase. **Casing material triggers conversion from vegetative to productive growth**. Peat, as the most common casing material is "slow-renewable" factor and its geographic availability is limited. Recently the use of **alternative cheap and easily available casing material is investigated.**

Objective: applicability of casing material from composted waste office paper supplemented with AD liquid fraction as an addition to peat and it impact on yield, carpophores dry matter content and morphological features and chemical composition of fruiting boddies and casing material.

Results: Composted waste paper could be used as alternative casing material in cultivation of ***Coprinus comatus*.**





Anaerobic digestion





Straw 18%
Paper 36%
Digestate 40%

Peat + paper 15%

Peat + paper 25%

Peat + paper 35%

compost

Straw
Chicken
Manure

peat



	Fruiting boddies mg/kg	casing
Na	2417-2796	2754-10825
K	25040-32530	16446-21540
Ca	260-416	3273-25820
Zn	32-81	21-32
Cu	26-91	12.4-16.1
Pb	1.6-4.2	9.7-13.8
Fe	34-105	405-1670
Cd	0.38-1.86	0.53-0.68
DM	6-16 %	21-37%
ASH	3-19%	59%
C	-	14-25%
N	-	0,28-0,7%
Organic matter	-	27-47%
yield g/3kg of cultivated substrate)	250-370 (97 control)	-

Scientific work financed from the funds for science in 2012-2015 granted to an international co-funded project No 179/CIPUE/2012 and PULS Young Researchers Subsidy No 507.649.68

Comparative Tests of Casing Soils of Different Origins

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[2]*Mushroom Spawn Plant and Strain Research Laboratory, Biokékes Nonprofit Ltd., H-3395 Demjen, Top. Nr. 0287/8. Hungary*

The importance of casing materials in button mushroom cultivation is increasing. The limitations on peat mining, the growing prices and the different cultivation techniques require a regular evaluation of casing materials that are currently available on the market. In our experiments we examined two casing soils of different origins (Dutch and Hungarian) in the laboratory and in small scale cultivation.

Material and method

In laboratory tests we have determined the capillary rise, permeability, the mineral and lime content, the pH and the EC value and the residue on ignition of the two samples. In the cultivation experiment 5 cm of casing was placed on top of the boxes containing phase III compost. The standard cultivation technique was followed. The same amount of CAC and irrigation was applied in case of the two soils. Samples were taken every 5 days to determine EC and pH values.

Results

The tests showed that the Hungarian soil have higher water absorbing and retaining capacity (*Table 1* and *Figure 1*). It contains four times more magnesium and five times more sulfate, while the phosphorus level of Dutch casing soil proved to be eight times higher (*Table 2*).

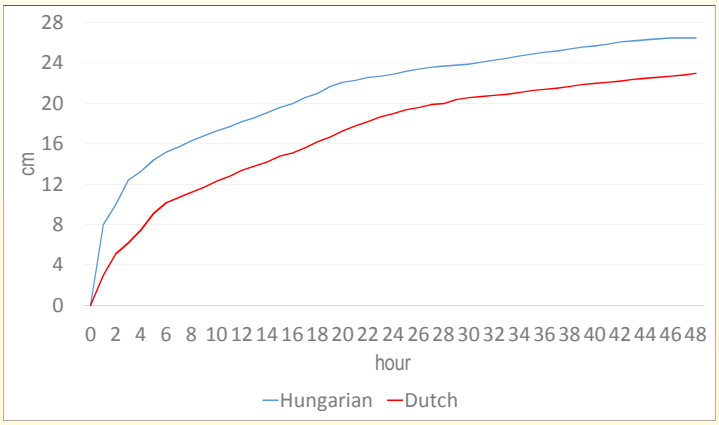


Figure 1. Result of the capillary rise test

Table 2. Chemical characteristics of the casings

	Hungarian	Dutch
pH and EC values		
pH	6,55	7,51
EC (µS/cm)	300,7	79,6
Ammonium (mg/l)		
NH_3	0,8	0,5
NH_4^+	1,0	0,5
Nitrate (mg/l)		
NO_3^-	3,0	18,5
Phosphorus (mg/l)		
PO_4^{3-}	6	49
P_2O_5	4,5	-
Potassium (mg/l)		
K_2O	3	3,8
Other components		
Ca^{2+}	35	30
Mg^{2+}	20	5
SO_4^{2-}	35	7
$CaCO_3$	34,1 g/l	8,2 g/l
Organic materials (%)		
	8,1	9,5

The reason could be the higher organic matter content, which was also proven by the residue on ignition results. The carbonate level of the Hungarian casing soil was four times as high. Concerning the pH level, the mycelium free Hungarian casing soil had a lower pH than the Dutch. The EC value of the Hungarian casing was four times as high.

Table 1. Water retaining capacity

	Hungarian	Dutch
Duration (sec)	78,5	123,5
Water permeability (cm^3)	29,3	42,5
Total retained water (cm^3)	20,7	7,5

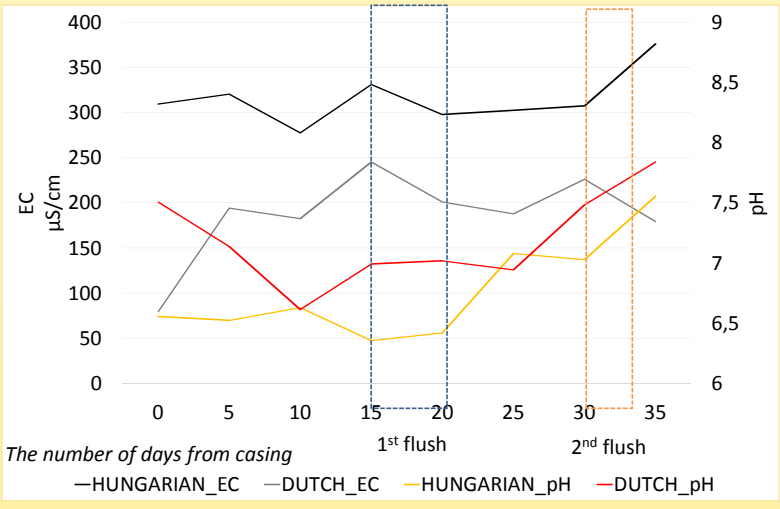


Figure 2. The pH and EC values under the cultivation

The pH of the Hungarian casing varied a bit during the cultivation period and only increased significantly after the second flush. The EC was initially higher in case of the Hungarian soil, and fluctuated only moderately after the spawn run (*Fig.2*). At first the pH of the Dutch soil was higher, and then it decreased slightly, though by the end of the second flush it reached pH 8. The EC of the Dutch casing was lower initially. It increased by the end of case-running, but still did not reach the EC value of the Hungarian sample. The salinity increased by the end of the second flush in case of both samples. The dry matter content was growing until after the first flush, then it started to decrease.

19th ISMS Congress International Society for Mushroom Science Amsterdam 2016 29th May - 2nd June

SZENT ISTVÁN UNIVERSITY
FACULTY OF HORTICULTURAL SCIENCE, BUDAPEST

Session Mycorrhiza’s

Influence of some management practices on the fruiting of *Amanita caesarea* in a chestnut grove

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2 Oficina Comarcal Agraria de Aracena (Huelva)
3 Universidad de Alcalá, Alcalá de Henares (Madrid)

European Union
European Regional Development Fund

IFAPA

INTRODUCTION

In the Natural Park of Sierra de Aracena y Picos de Aroche (SW Spain) chestnut groves occupy 5000 ha and constitute an emblematic landscape identified as the landmark of the Park. However in recent years it has been detected a progressive abandonment of this groves probably due to its low profitability. The Park has a large variety of mushrooms, many of which are ectomycorrhizal fungi (ECM), that is, they are associated with the tree roots and play an important role in plant nutrition. In fact, are considered good indicators of forest health. Several ECM are among the most precious and prized edible fungi, and their harvesting could represent an important source of income. *Amanita caesarea* is an EMC often associated to chestnut trees whose edible sporocarps are well prized. However the production of fruiting bodies depends on many factors, so that, depending on the year it may be little or even null which hinders their exploitation.

To date the artificial reproduction of these fungi to the fruiting stage continues to elude the efforts of researchers. So we have studied the evolution of *A. caesarea* mycelium in soil throughout the year and it has highlighted the importance of temperature on fruiting. The aim of this study was also to determine on the one hand, if irrigation and/or the superficial plowing in productive areas, and secondly, if the mycelium of *A. caesarea* applied in adults chestnut trees could increase the occurrence of this appreciated fungus. If so would add value to the chestnut groves.

MATERIAL AND METHOD

Study site and plots

The study was conducted in a chestnut grove located in the Natural Park Sierra de Aracena y Picos de Aroche.
Four plots (500 m² each) were established in a chestnut grove for applying the following treatments: irrigation, plow and irrigation+plow. Irrigation was applied (5 l/m² per day) from late August until the first rains of September. One of the irrigated plots was also plowed in spring.
In a non productive area five chestnut trees were inoculated in four equidistant points located 2 m of each tree with *A. caesarea* mycelium entrapped in polymerized alginate beads.

Mushroom harvest

Specimens of *A. caesarea* were collected twice a week during the fruiting period (September-November).

DNA extraction from soil and ARISA (Automated Ribosomal Intergenic Spacer Analysis) profiles

Soil cylinders at 0–20 and 20–40 cm depth were taken in productive areas with a 3 cm diameter soil auger from spring to autumn.
Determinations of the amount of *A. caesarea* mycelial DNA in soil samples were made by analysis of fragments ARISA (Automated Ribosomal Intergenic Spacer Analysis). DNA extractions were performed with the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlbad, CA, USA) according to manufacturer´s instructions. The obtained DNA was amplified by a first PCR with primers ITS4 ITS1F and then another PCR with fluorescently labeled primers using FAM (6-carboxyfluorescein) as fluorochrome. They were then processed by automated ribosomal intergenic spacer analysis. The software converted fluorescent data into electrophoregrams (ARISA profiles); the peaks represented fragments of different sizes and the heights of the peaks were the relative proportion of the fragments in the total product.
Soil samples were taken monthly at different depths and the amount of mycelium was measured by fluorescence.
Samples taken from suitable specimens and from *A. caesarea* mycelial pure culture (Daza et al., 2006) were selected for DNA extraction and were processed in the same way to be used as controls.

RESULTS

Number of specimens of *A. caesarea* collected per plot

Year	Treatment			
	Plowed	Irrigation	Plowed + Irrigation	Control
2014	10	40	63	10
2015	2	14	16	3

Although the number of specimens collected varies greatly from year to year, the effect of irrigation was reflected even in the worst year, but no effect was observed in plowed plots.
Two years after mycelial inoculation no effect on *A. caesarea* fruiting has been observed.

ARISA is a very effective and sensitive method for detecting differences between complex fungal communities at various spatial scales. On soil samples generate complex profiles with bands of between approximately 300 and 1,100 bp. When only the true fungi (Ascomycota, Basidiomycota, Zygomycota, and Chytridiomycota) were considered, the length of the region ranged from 390 bp to 882 bp (Ranjard et al., 2001).
The DNA isolated from fruiting bodies and from mycelial pure cultures of *A. caesarea* shows a peak at 644 bp and were used as a reference to identify *A. caesarea*. In general fluorescence values increased in soil samples where *A. caesarea* fruited. The amount of mycelium in soil varied throughout the year and with depth. ARISA data indicate that the mycelium of *A. caesarea* takes refuge deeper in the ground when the temperature drops in the coldest months. Mycelium production of *A. caesarea* seems to respond to a seasonal pattern.

Fluorescence intensity of peaks (644 bp) corresponding to *A. caesarea* in soil samples taken at different depths (0, 20 and 40 cm)

Sample	Depth	Month						
		March	April	May	June	September	October	November
U	0	-	-	-	nd	400	56	-
	20	739	484	260	148	104	4921	-
	40	-	357	98	56	-	8759	-
W	0	-	174	-	nd	8541	227	-
	20	739	198	616	1036	993	-	527
	40	-	-	-	2761	609	8760	-
X	0	-	74	423	nd	7135	112	-
	20	614	317	3365	731	286	52	168
	40	-	173	1902	3323	206	382	-
Y	0	-	132	117	nd	496	101	-
	20	1040	1505	3189	2131	392	495	1948
	40	1318	3329	5240	4219	394	1578	4095
Z	0	-	-	-	nd	-	184	-
	20	521	549	7500	10503	377	231	-
	40	5089	4039	6037	1940	499	1373	6851

-, undetected; nd, not determined

CONCLUSION

Irrigation may be recommended to increase the fructification of *A. caesarea*. We can not discount the effect of the plow or mycelial inoculation because maybe two years is a short time to make conclusions.

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Ranjard L, Poly F, Mougel C, Thioulouse J, Nazaret S. (2001). Applied and environmental microbiology 67 (10):4479-4487.

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Instituto de Investigación y Formación Agraria y Pesquera
CONSEJERÍA DE AGRICULTURA, PESCA Y DESARROLLO RURAL

On the Road of Terfess and Truffles in Morocco

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SUMMARY

The first Mycotour in the world which covers both truffles as terfess as well as Morocco's rich culture, gastronomy and nature is organized by Prof. Khabar and Judith Evenaar MSc. In December 2015 a partnership has been launched between the Wageningen Truffle Center and the University Mohamed V of Rabat. The partnership aims to raise awareness for the importance of fungi for the resilience of ecosystems and to develop a sustainable truffle chain.

INTRODUCTION

Morocco is a divers country. Nature, gastronomy and culture are rich and divers. Morocco counts more than 10 species of truffles of the genera *Tuber, Terfezia and Tirmania.* In recent years European truffles like the summer truffle have been found in the wild in Morocco. Climate and soil conditions are excellent for cultivation of these European truffles which attract the highest prices. Three black truffle orchards were started in Middle Atlas in Debdou (2000) and Immouzer Kandar (2008 and 2014). However, indigenous desert truffles are also worth more attention.

THE DESERT TRUFFLE

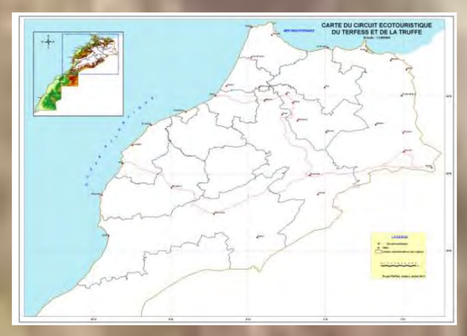
The desert truffle is of great cultural, economical and ecological value. The desert truffle is local food and is exported to the Middle East and is therefore also an important revenue. Desert truffles are very important to combat desertification. However production is rapidly decreasing by overexploitation (poor regulation and wrong harvest methods) and damage by increasing number of cattle.

DEVELOPMENT OF A SUSTAINABLE TRUFFLE CHAIN

Valorization of the desert truffle will be instrumental in the development of a sustainable chain. Establishment of a sustainable agro-industry for desert truffles and gourmet truffles will supply high value food, promote rural development, provide income, restore degraded areas and incorporate traditional knowledge.

THE MYCOTOUR

The 8 day Mycotour (next one April 2017) will visit the most important truffle areas of Morocco. These areas include the Mamora Forest, Jabaa Forest, Middle Atlas and Sahara. The tour will give insight into the diversity of the mycoflora, harvest of truffles and truffle markets, as well as many technical aspects of truffle cultivation. The cultural, economical and ecological values of truffles will be shown.



CONCLUSION

Mycotourism will offer extra income and raise awareness for the importance of fungi for Morocco.







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2. Khabar L., 2014. Chapter 10, Mediterranean Basin: North Africa in V. Kagan Zur et al. (eds), Desert Truffles, Soil Biology 38, Springer- Verlag Berlin Heidelberg , pp. 143-158.
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DESERT TRUFFLES AND TRUFFLES IN MOROCCO

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ABSTRACT

Morocco counts more than ten species of "Terfess" (*Terfezia*, *Delastria*, *Picoa* and *Tirmania* genera) and five species of truffles (genus *Tuber*). The terfess are harvested usually in semi-arid or arid climate in sandy-silty light soils. The forest of Mamora in the North West of Morocco, the plains of the highlands to the east and the Sahara in the South and South East are very favourable environments for the presence of these kinds of truffles. On the other hand three truffle species (*Tuber rufum* Pico, *T. excavatum* Vitt. and *T.uncinatum*/*T. aestivum* Vitt.) were collected in the Middle Atlas between 1600 and 2000 meters above sea level under sub-humid and humid climate and in calcareous soils. In addition to the chain of the Middle Atlas, many other areas in Morocco, namely the High Atlas chain in central Morocco, the Rif mountains in the north chain of the uplands and the massive Debdou in eastern Morocco are areas potentially suitable for truffles given their climate (humid and sub humid) and the nature of their land (generally calcareous). The presence of truffles (genus *Tuber*) in these areas is likely and planting truffle seedlings seems promising. By the way the results from the first outplanting of oaks mycorrhized with *Tuber melanosporum* in the region of Debdou and in middle Atlas were positive.
The aim of the Poster is to present the different species, how to identify and harvest as well as areas of their distribution.

DESERT TRUFFLES

Morphology of "Terfess" : various (forme, color, etc..)



Hostplants of "Terfess"



Methodes de récolte des "Terfess"



Delastria rosea (Tulasne, 1843)



Terfess amère de taïda

Terfezia arenaria Trappe, 1971

(=*Tuber arenarium* Moris, 1829)
(=*Terfezia leonis* Tulasne, 1851)



Terfess rose de la foret de la Mamora

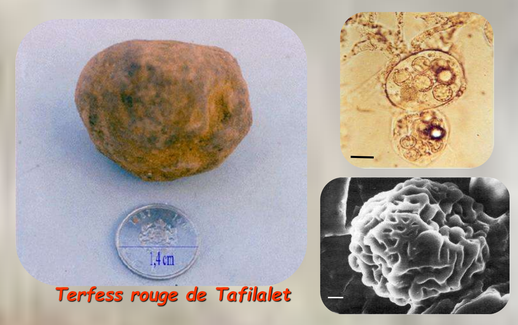
Terfezia boudieri Chat. 1891

(= *T. boudieri* var. *arabica* Chat. 1892)
(=*T. deflersii* Pat. 1894)



Terfess blanc de Tafilalet

Terfezia claveryi Chatin, 1891



Terfess rouge de Tafilalet

Terfezia leptoderma Tulasne, 1851

(= *Terfezia olbiensis* Tulasne, 1851)



Terfess lisse de la foret de la Mamora

Picoa juniperi Vittadini, 1831

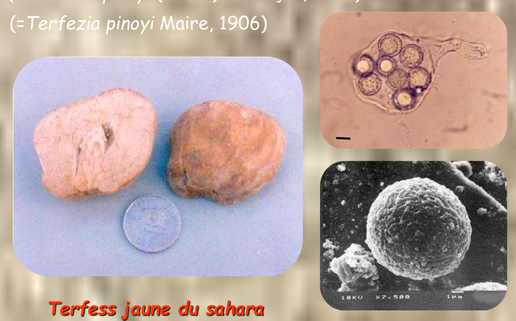
(=*Picoa juniperina* Tulasne,1851)



Ezzouber

Tirmania pinoyi (Maire) Malençon, 1973

(=*Tirmania pinoyi* (Maire) Malençon, 1973)
(=*Terfezia pinoyi* Maire, 1906)



Terfess jaune du sahara (zoubaïdi)

Tirmania nivea (Desf. Ex. Fr.) Trappe, 1971

(=*Tuber niveum* Desf. ex Fr., 1823) (=*Terfezia ovalispora* Patouillard, 1890)
(=*Tirmania ovalispora* (Pat.) Pat., 1892) (=*Tirmania africana* Chatin, 1892)
(=*Terfezia africana* (Chat.) Maire, 1916)
(=*Tirmania camboni* Chatin, 1892)



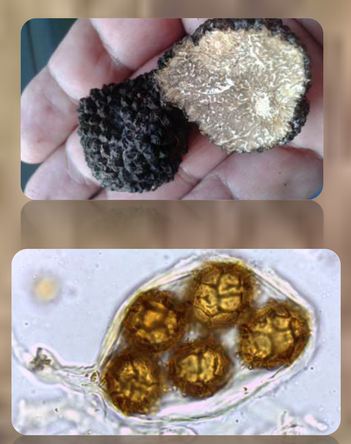
Terfess blanc du sahara (zoubaïdi)

TRUFFLES

Tuber melanosporum



Tuber uncinatum/aestivum



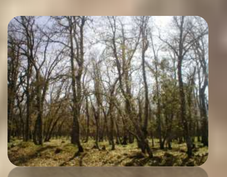
Tuber rufum



Tuber excavatum



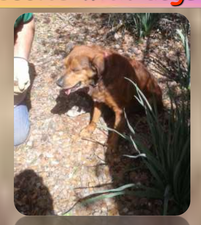
Quercus faginea



Quercus ilex

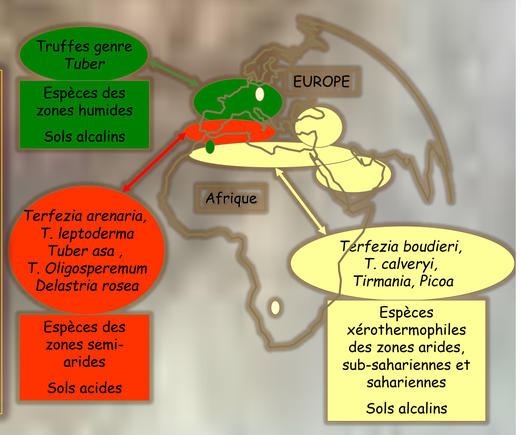


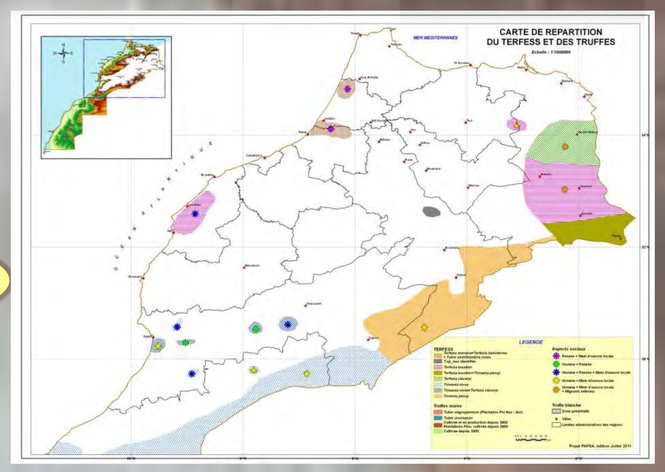
Recolte with dogs



CONCLUSION

There are strong analogies between the species which develop in the Mediterranean countries. The species found in Morocco, like *Terfezia arenaria, T leptoderma, Tuber asa, T. oligospermum, Delastria rosea* are found in the south of Spain, south of France and in the south of Italy. These species must be found in all countries in North Africa.
On the other hand, the species like *Picoa juniperi, Terfezia claveryi, T. boudieri, Tirmania pinoyi, T. nivea*, collected in the south and south east of Morocco and in the deserts of Tunisia, Algeria but also in the Middle-East, are collected in the south of some Mediterranean countries (southern of Spain: Estrémadure; south of Italy: Sicily, Sardinia). *Tuber melanosporum, T.aestivum/uncinatum, T. rufum, T. escavatum* commun in all Europe, are localised in Morocco, in zone of mountain (Middle Atlas).





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Mycorrhization, myceliar growth and basidiospores germination of some species of Boletaceae

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INTRODUCTION

Boletaceae is a family of fungi characterized by fruit-bodies which produce an high number of sexual spores for the reproduction of the species. Nowadays, only few studies focused their attention on spore germination processes and many aspects are already yet to understand. A large member of species belonging this family live in symbioses with trees and they are called ectomycorrhizal fungi: these close relations makes the plants more stronger and resistant to many types of biotic and abiotic stress. In particular the symbioses can increase the ability of plants to absorb micronutrients from the soil and increase resistance to pathogens; on the other hand, plants provide nutrients to fungi like glucose, fructose and other elements important for the fungal growth and the production of fruit-bodies. The aim of this study was evaluate the ability of some fungal species to grow into *in vitro* condition, estimate their spore germination rate and produce mycorrhizal plants in sterile condition.

Fig.1



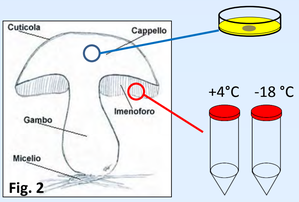
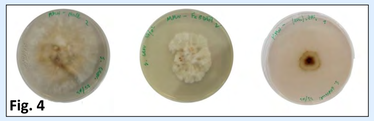


Fig. 2


Fig. 4

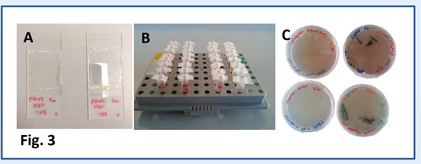


Fig. 3


Fig. 5


Fig. 6

MATERIALS AND METHODS

In this study we focused the attention on six different species, collected in Val Grande, Piemonte, Italy.: *Boletus edulis, Leccinum duriusculum, Suillus granulatus, S. luteus, S. grevillei* and *Xerocomus badius* (Fig.1). From each fruit-bodies, a plug of mycelium was removed and plated on different media (MMN, FDA, Hagem, Fries and Gamborg), as showed in fig.2. Moreover, spores were collected and storaged in distilled sterile water at 4°C and -18°C into a skim-milk/glycerol suspension to prevent damages to cells during the freezing process (Fig. 2). The following methods were used in order to performed out study:

-to evaluate the spore germination. three different systems have been used: A- germination on microscope slides , B- germination in liquid culture with different media and C- germination on standard agar media and on agar media containing fragment of root (Fig.3);

-to understand which elements can improve/affect the growth of the fungi tested, the mycelia were plated in different culture media each delete of a single nutrient (Fig.4);

-to recreate a soil-like substrate optimized for the fungal growth, culture media, peat and vermiculite have been mixed together in the same proportion and all the species of mushrooms were inoculated in this artificial substrate to evaluate their mycelium growth.

-for the production of *in vitro* mycorrhizas, fungi were growth on agar media in micro-propagation tubes. Plant seeds were collected from the seed bank of "Corpo Forestale dello Stato" and surfed sterilized before growing on agar medium (Fig.5). After 30 days of incubation at 23°C , seedlings were inoculated in micro-propagation tubes with a substrate of peat and vermiculite (1:1) to allow the formation of mycorrhizas (Fig.6). Morphological and structural analysis of mycorrhizas has been carried out in stereomicroscope, optical microscope and a scanning electron microscope (SEM), cutting down longitudinal and transversal sections of lateral roots.

RESULTS

Spore germination: fresh spores showed higher rates of germination compared to spores stored for 2 weeks and 1 months at 4°C in distilled water, but no differences were highlighted between spores stored at 4°C and -18°C. The germination percentage reached in all test were under 1%, but many spores showed a pronounced germinative pore, in particular *Xerocomus badius* spores (Fig.7). The long-term conservation of spores were reached using cryoprotectants like skim-milk and glicerol (Fig.8), but their germination ability were compromised after few months. However, this results were similar to all previously studies and in particular many authors agreed about that the germination process is a combination of different factors that couldn't been replicate under laboratory condition. Flavonoids are a class of plant secondary metabolites producted from roots and they can help basidispore germination. Nevertheless, no additional germination rate were observed in Petri dishes with root fragments inside the agar medium, probably because there were not available components to stimulate the germination process. However, it's possible that spores have an intrinsic dormancy that could be maintained for very long periods; today, the right mechanism that regulate dormancy and germination are yet to be understand.

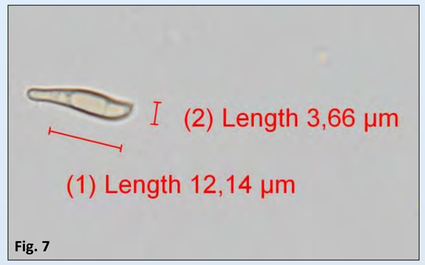


Fig. 7

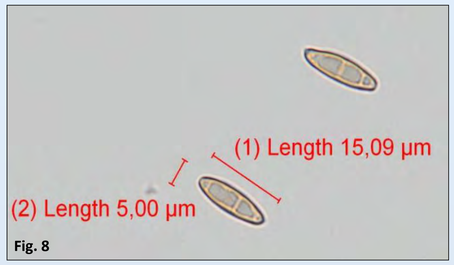


Fig. 8

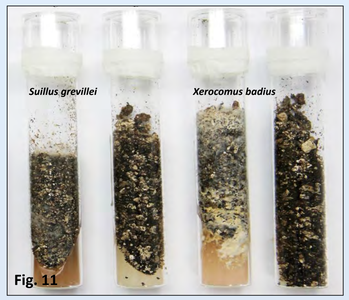


Fig. 11

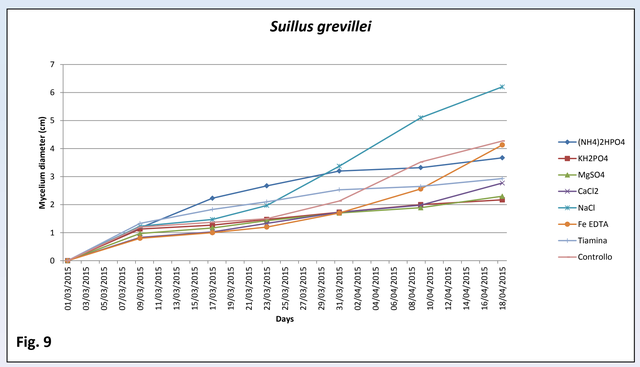


Fig. 9

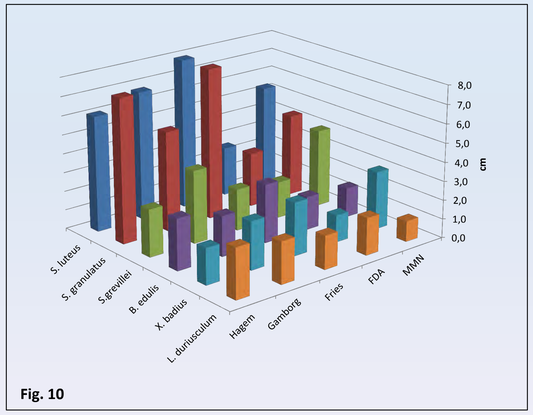


Fig. 10

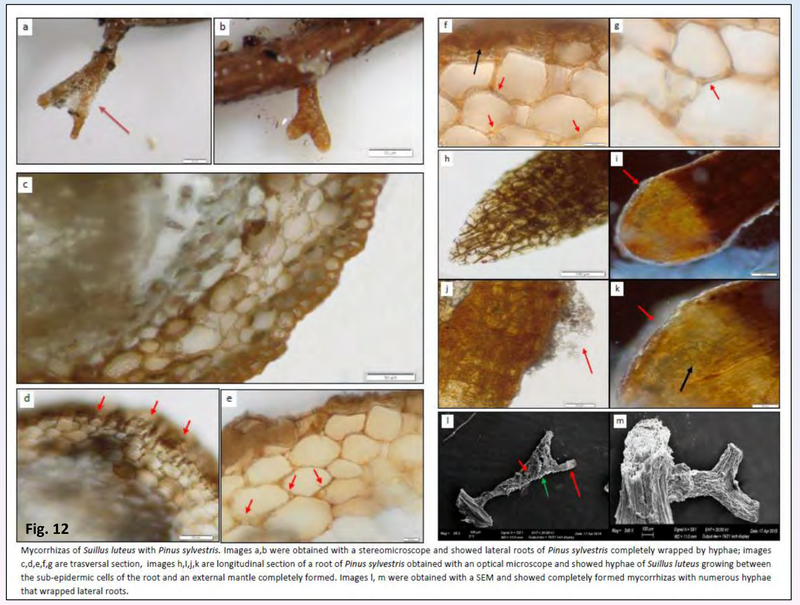


Fig. 12

Mycorrhizas of *Suillus luteus* with *Pinus sylvestris*. Images a,b were obtained with a stereomicroscope and showed lateral roots of *Pinus sylvestris* completely wrapped by hyphae; images c,d,e,f,g are trasversal section, images h,i,j,k are longitudinal section of a root of *Pinus sylvestris* obtained with an optical microscope and showed hyphae of *Suillus luteus* growing between the sub-epidermic cells of the root and an external mantle completely formed. Images l, m were obtained with a SEM and showed completely formed mycorrhizas with numerous hyphae that wrapped lateral roots.

Mycorrhization: interesting results were observed with different species: Best results were obtained between *Suillus luteus* and *Pinus sylvestris* (Fig. 12) but also *Boletus edulis* and *Suillus grevillei* showed a good ability to form mycorrhizas with the lateral roots of very young plants after only 2 month of incubation at 20°C with 16 hours of photoperiod. Usually, in nature this fungal species are called «late stage fungi» because they form symbiosis only with mature plants; however, this study underline that in an environment without antagonistic organisms these fungi can form mychorrizae at all stages of growth of the plant. Plants like *Pinus sylvestris, Picea abies* and *Larix decidua* showed a better development of mycorrhizae respectivly with *Suillus luteus*, *Boletus edulis* and *Suillus grevillei*. On the other hand, there were not evidence of mycorrhizas formation between *Leccinum duriusculum* with *Betula pendula* and *Xerocomus badius* with *Picea abies*. The reasons for this lack of mycorrhizas symbiosis may be due to ecological, chemical or morphological problems between plants and fungus or to a low development of root system after few month of incubation. Despite encouraging results, the mycorrhization remains a difficult process and further studies will be necessary. More future effort could lead to a less time consuming process for mycorrhizal plants production and maximize the mycorrhizas formation. In this way, mycorrhizal plants could be used for many forestry and biotechnological applications like bioremediation and restoration of depredated or polluted areas.

Myceliar growth: the species used in this study showed a high rate of myceliar growth on agar medium and it was possible to analyze the role of each element of all media tested and (Fig. 9, 10). On medium without carbon or nitrogen source the myceliar growth is reduced compared to the growth on the optimal medium. High concentration of NaCl and CaCl2 had a negative impact on the growth of the fungi, probably because they negatively affect the membrane potentials of mushroom cells and the osmotic processes limiting the hyphal growth. The carbon sources used showed different growing rates. In particular, glucose was the optimal source for the myceliar growth probably because it's easier to assimilate compared to the malt extract. Vitamins are also important compounds for the myceliar development: many species showed low growth rates and a limited number of hyphae on thiamine-free media. Regarding the artificial substrate made using peat and vermiculite, *Xerocomus badius* and *Suillus grevillei* showed a good colonization after only 2 month (Fig. 11). The optimization of this system could be used for a large scale production of inocula and mycorrhizal plants.

Session Miscellaneous

Production of *Bjerkandera adusta* 's ligninolytic enzymes on lignocellulosic substrates by solid state fermentation



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Introduction

Lignin is extremely recalcitrant to degradation.The white-rot fungi produce a array of extracellular oxidative enzymes that synergistically and efficiently degrade lignin such as laccase (Lac), manganese - peroxidase (MnP), and lignin peroxidase (LiP). The demand of these enzymes has increased in the recent years due to their potential in several industrial and biotechnological processes (detoxification of industrial effluent that contains dyes, phenols and others xenobiotics, biobleaching of Kraft pulp, denim washing, bioremediation). Production of enzyme from microbial sources is a costly affair and the only alternate to minimize the production cost in the use of inexpensive raw materials. The utilization of agro-industrial residues in this aspect is much appreciated due to their low cost and ease in availability. Adopting solid-state fermentation for enzymes production may add to the benefit of reducing the production costs. This work was carried out to study the ability of a fungus, isolated from an Algerian forest, to produce extracellular ligninolytic enzymes.

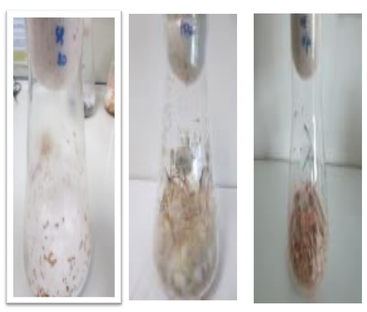
Methodology

Solid-State Fermentation













STRAIN

Bjerkandera adusta

PDA

10 days / 28°C

INOCULUM

20 agar discs with mycelia

Nutritive medium

(Elisashvili et *al*.,2008)

28°C / 5 days

INOCULATION

12 ml of l'inoculum/

45 g de substrat

Incubation at 28°C

EXTRACTION

10 g / 50 ml water

Agitation /1H

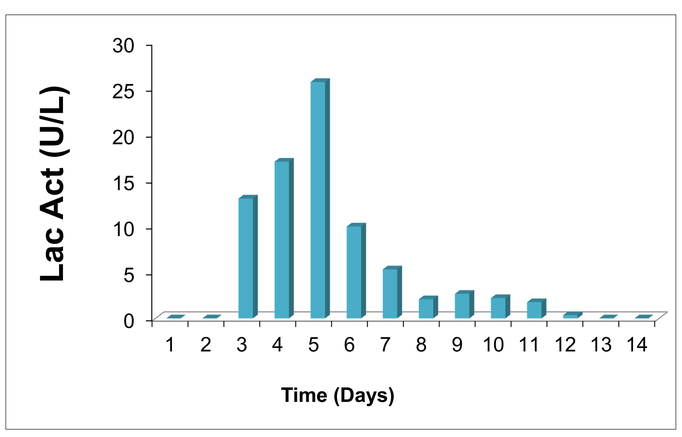
Centrifugation

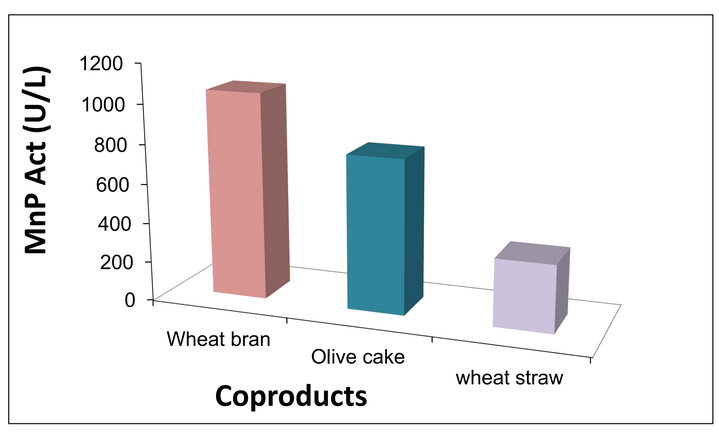
Enzymatic Extract

Enzymatic assays

- MnP(Paszczynski et *al,* 1986)
- Lac (Lomascolo et *al*. 2002)
- LiP (Tien and Kirk ,1984)

Results & Discussion





Monitoring of Laccase (wheat bran) and MnP secretion during the solid fermentation of *B. adusta* on agricultural coproducts

Lac activity was detected only on wheat bran (25.70 U/L) after 5 days of culture. Some authors have been reported that laccase is not present in *B. adusta (Belcaz et al., 2005; Romero et al., 2007). In* addition, Tripathi et *al.(2012)* indicate that Lac activity, on static conditions, is inducible in *B. adusta* and requires nutrient rich medium for its secretion. This result shown that wheat bran can induce laccase secretion in the selected strain.

Wheat bran shows the greatest levels of MnP activity during 4 days of cultivation (1049 U/L). The maximum MnP activity was showed the 15th day of the incubation on olive cake (about 780 U/L), whereas the maximum activity was found the 5th day with wheat straw (341 U/L).

Regarding LiP enzyme, no activity was detected. This result is in disagreement with some studies (Romero et *al*., 2006). Indid, LiP produced by *Bjerkandera sp.* strain BOL13 showed about 70 U/L of enzyme activity was recorded in wheat husk substrate. Recently, Solarska et *al.* (2009*)* revealed a lower LiP activity in *B. adusta* (1.3 U/L). However, several strains of *Trametes versicolor and Bjerkandera adusta* are known as LiP producers but do not always produce lignin peroxidase depending on strain or culture conditions (Martinez et *al*., 2005).

Conclusion

Wheat bran is a very common worldwide agricultural residue that could act as an inducer of Lac activity and a good substrate for MnP enzyme in *Bjerkandera adusta* BRFM 1916 culture, which is efficient lignin-degrading specie and can be useful in bioprocessing application for saccharification of agro-residues.

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MYCELIAL GROWTH OF SOME STRAINS OF THE *WUTZ ANIM* (DEAD-EYE) MUSHROOM

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Abstract:
The Tacaná Volcano Biosphere Reserve is a very important area for the Soconusco zone, in southern Mexico; it includes different ecosystems with a variety of fauna, flora and mushrooms such as the *Wutz anim* group (word of the *Mam* ethnic group). The latter are macromycetes belonging to the Agaricaceae family characterized by being edible and have haemostatic properties. After collecting several specimens of this group and identify them by molecular means, this study aims to assess their mycelial growth in a controlled environment. The genera *Lycoperdon*, *Holocotylon* and *Bovista* are part of the mushroom group known as *Wutz anim.* The isolated strains presented a mycelial growth in the six media evaluated, with an average radial extension rate of 0.57±0.04, 0.66±0.06 and 0.77±0.2 mm per day respectively. It was observed that each genus develops better in different culture media, although no basidiomata could be obtained. This study constitutes the first report of the genus *Holocotylon* in the region.
Keywords: Puffballs, Agaricales, *Lycoperdon*, *Bovista*, *Holocotylon*.

INTRODUCTION

The Biosphere Reserve of the Tacana Volcano (TVBR) is located in southern mexico, at the frontier with Guatemala. In the TVBR there are human settlements principally the *Mam* ethnic group, which is direct descendant of the Mayas established in the region since prehispanic times. Among the mushrooms this ethnic group gather, the group called *Wutz anim* (dead eye) takes advantage for its edible and medicinal properties (hemostatic). It is assumed that these mushrooms belong to the former group Gasteromycetes (currently Agaricomycetes) characterized by the angiocarpic structure of the fruitbody, commonly known as “puffballs”. These include among other genera, *Calvatia, Bovista*, *Holocotylon* and *Lycoperdon.*

There is no record on the cultivation of species of *Holocotylon.* Therefore, the aim of this research was to asses the mycelial growth ability on solid culture of several strains of mushrooms belonging to the *Wutz anim* group.



MATERIALS AND METHODS

Strains and culture media

The strains studied were isolated from basidiomes collected in the Biosphere Reserve of the Tacana Volcano by regular mycological methods. Six culture media were used to test the growth capability of each strain: Malt extract agar (MEA), MEA complemented with Kirk (K) media (López et al. 2012), potato dextrose and yeast extract agar (PDYA), Melin-Norkrans culture media (MCM) (Riquelme 2011), Czapeck-Dox (Cz-D) media (Becard and Fortín 1988) and Hagem (Hg) media (Helmholz et al. 1999).

Mycelial growth

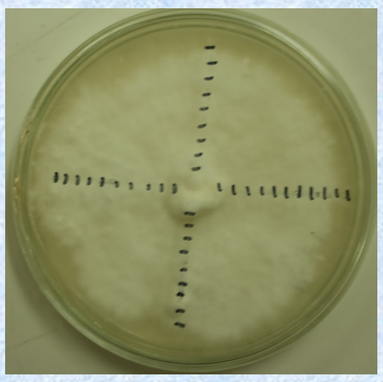
Three Petri dishes per media were inoculated (repetitions) with a 30 days 0.5 cm agar mycelium fragment of the strains under study. They were incubated at 24°C and mycelial growth was recorded every third day for four weeks. The radial extension rate (RER) was determined by the equation y=Krx +c (y=independent variable, Kr=slope value taken as RER, x=dependent variable and c=intersection). With the data obtained an analysis of variance and a mean comparison test (Tukey, p≤ 0.05) using R statistical package (Fox 2005) was performed.



Holocotylon



Lycoperdon



RESULTS

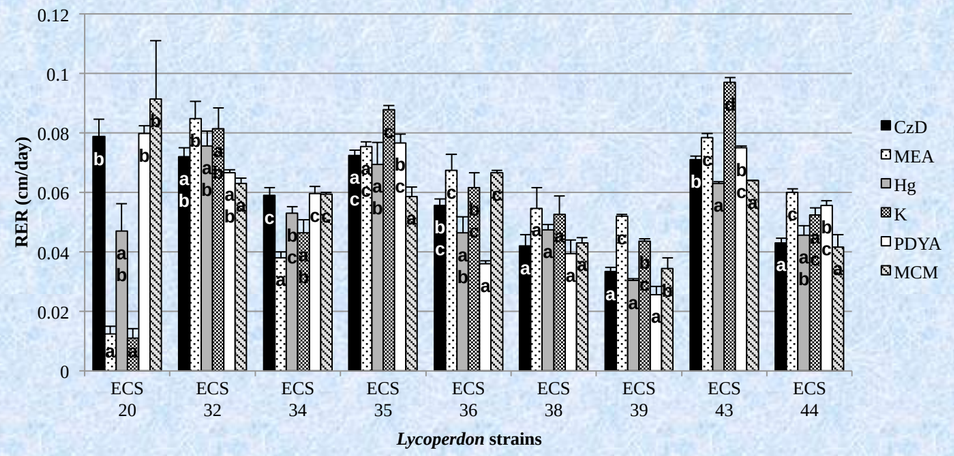
Molecular identification

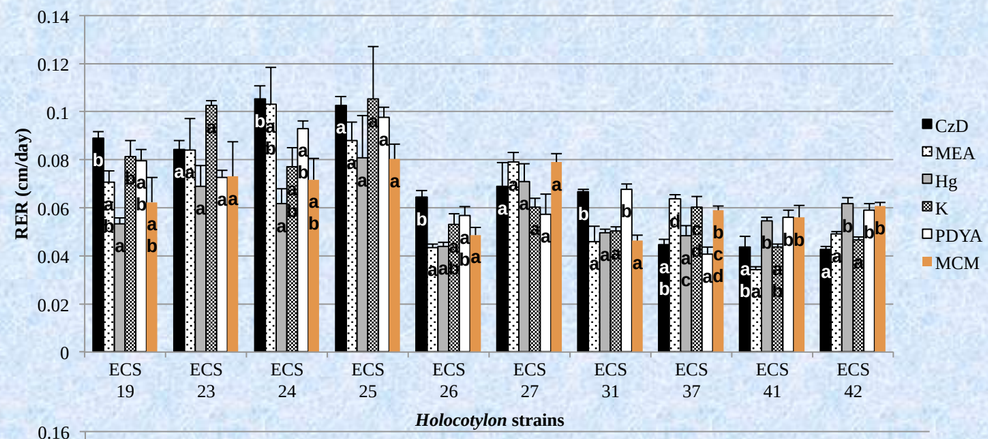
The 22 strains isolated comprising the referred *Wutz anim* group in the TVBR belong to the genera *Lycoperdon*, *Bovista* and *Holocotylon* as indicated on Table 1.

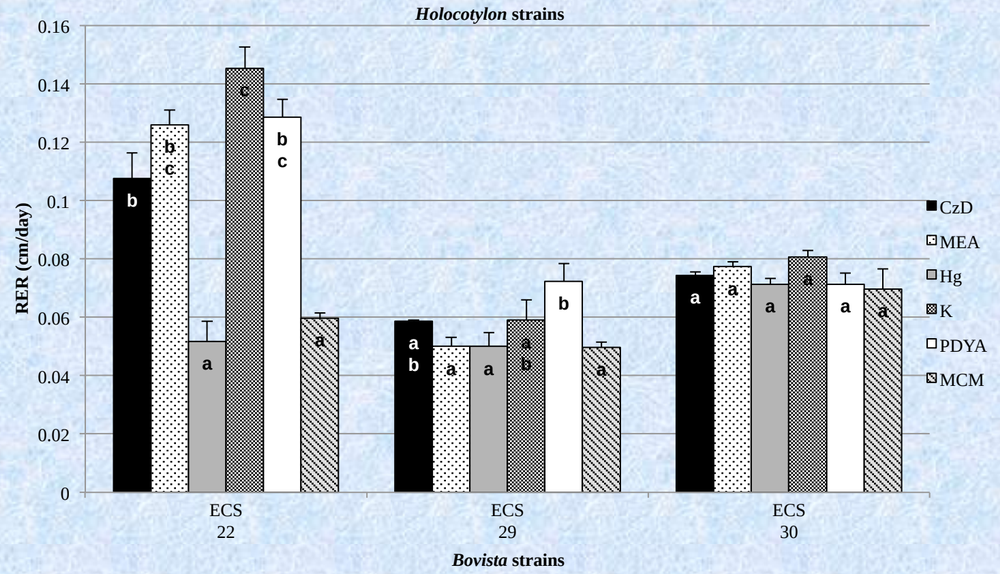
Nb of strains	Species	Strains
1	*Lycoperdon perlatum*	(ECS-20)
4	*L. ericaeum*	(ECS-32, ECS-36, ECS-38 and ECS-44)
4	*L. rupicola*	ECS-34, ECS-35 ECS-39 ECS-43)
10	*Holocotylon brandegeeanum*	(ECS-19, ECS-23, ECS-24, ECS-25, ECS-26, ECS-27, ECS-31, ECS-37, ECS-41 and ECS-42)
2	*Bovista graveolens*	(ECS-29 and ECS-30)
1	*B. nigrescens*	(ECS-22)

Mycelial growth in culture media

The strains of the genera *Lycoperdon*, *Holocotylon* and *Bovista* presented a mycelial growth in the six media evaluated, with an average radial extension rate of 0.57±0.04, 0.66±0.06 and 0.77±0.2 mm per day respectively.







Figs. Radial extension rate of the isolated strains in six culture media, evaluated for 30 days. Same letters indicate no significant difference between the media of each strain, according to the Tukey test (α=0.05). **1)** genus *Lycoperdon* **2**)genus *Holocotylon* **3**) genus *Bovista*

DISCUSSION

In MEA media colonies of 0.8-2.6, 1.4-3.9 and 1.1-3.2 cm in diameter were recorded for the strains of the genus *Lycoperdon*, *Bovista* and *Holocotylon* respectively, during four weeks of incubation. Similar to that described by Bulmer and Beneke (1961; 1964), it has been reported that an increase in the concentration of these carbohydrates promotes greater mycelial growth. Meanwhile, yeast extract has been reported as the best source of nitrogen for fungal growth (Chang et al. 2006; Jo et al. 2009; Torres-Lopez et al. 2011), in addition to being an excellent source of thiamine (Tortora et al. 2007), which plays a very important role in the development of these organisms as it contributes in the generation of energy through the breaking of carbohydrates (Kavanagh 1942; Sedlmayr et al. 1961, Chung and Tzeng 2009). Yeast extract together with that of malt are also complex substrates that contains a wide variety of proteins, fats, minerals, vitamins, sugars and salts in necessary and sufficient amounts that promote fungal mycelial growth (Torres-López et al. 2011).

ACKNOWLEDGEMENTS

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Characterization of Commercially and Wild White *Pleurotus* Based on Fruiting Body Production and Protein Content

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Introduction

White oyster mushrooms are an only one in a group of oyster mushrooms that have good economic prospects in Indonesia. They are easy to cultivate, good adaptation with hot climate of Indonesia and good acceptable by people due to their own white color. The farmers use different unknown sources of commercially isolates to cultivate without knowing of isolate capacity in fruiting body production and the nutritional contents. The objective of this research is to give the farmers the information about isolates which are best on production and nutrition.

Methods

The commercially isolates of AMD, BBR, BNK, CSR, MTR, PGN, TBN, USX obtained from farmers and different locations were grown on 1 kg of substrates containing 82% of *Paraserianthes falcataria* sawdust, 15% of rice bran, 2.5% of lime and 2.5% of gips in the 17x35cm sized polypropylene bags. The nutrition of fruiting bodies were proximate analyzed following the method of AOAC (1980).

Results

The cultivation patterns of 8 white oyster mushroom isolates (Figures 1, 2, 3, 4, 5, 6)

- Total Fresh Weights 200 g and more
- BE more than 75%
- Fresh Weights of (1st+2nd+3th harvest) 200 g and more
- FW percentage of (1st+2nd+3th harvest) 80% and more
- VP less than 30 days
- RP less than 100 days
- GDP less than 120 days
- Harvest number 5 and less
- Total Pileus number 40 and more
- Pileus number of 1st harvest 20 and more
- Diameter pileus of 1st harvest 11 cm and less

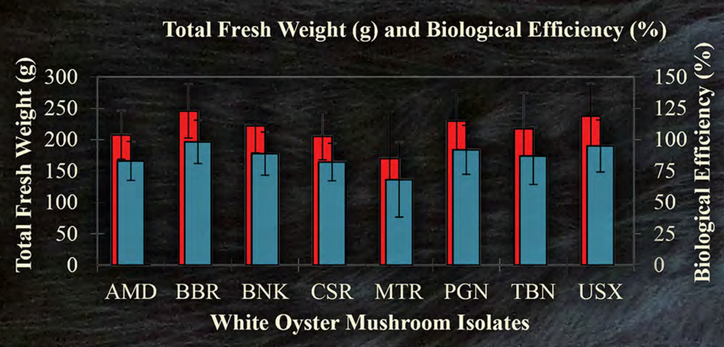


Figure 1. All mushroom isolates produced total fruiting body fresh weights of more than 200 g/1kg substrate except MTR isolate (red bar)
All mushroom isolates (including wild HS isolate, not shawn) had more than 75% of biological efficiency except MTR isolate (blue bar).

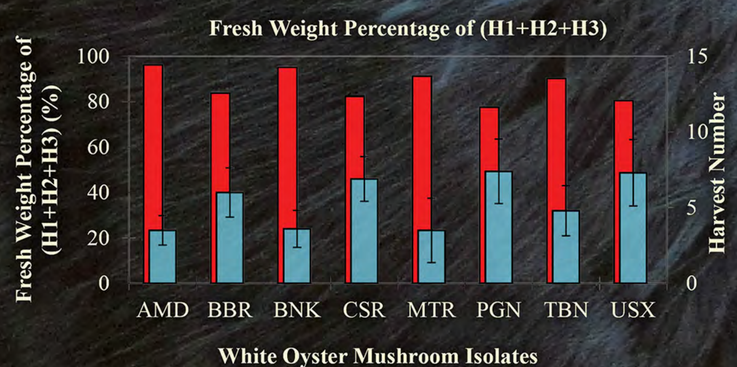


Figure 2. Almost all mushroom isolates had percentages of fruiting body fresh weights from 3 harvests (H1+H2+H3) of more than 80%, there were AMD, BBR, BNK, CSR, MTR, TBN, and USX isolates (red bars).

Only AMD, BNK, MTR and TBN isolates (blue bars) had harvest number of 5 and less



Figure 3. Only AMD, BNK, and TBN isolates (red bars) had total pileus number of 40 pieces and more.

Pileus diameter of all mushroom isolates were in a range of 4.6-6.0 cm (blue bars).

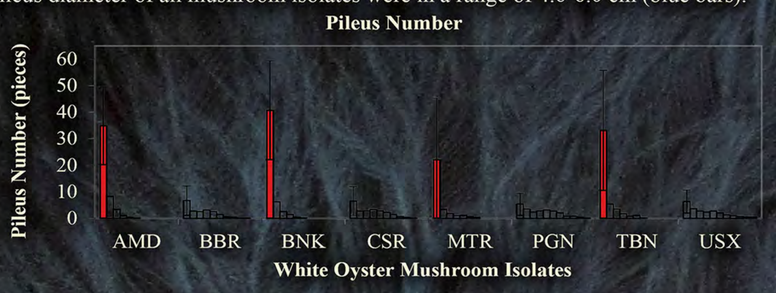


Figure 4. Only AMD, BNK, MTR, and TBN isolates had 1st harvest pileus number of 20 pieces and more (red bars).

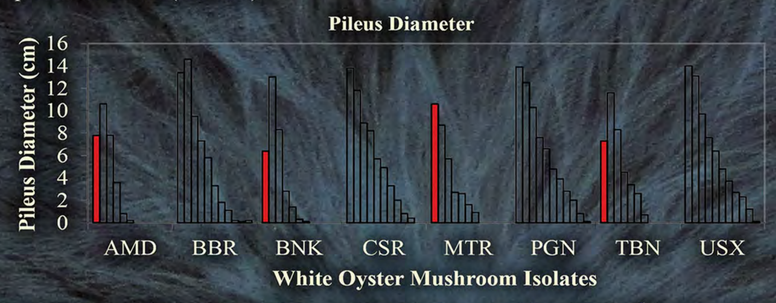


Figure 5. Only AMD, BNK, MTR, and TBN isolates (red bars) had 1st harvest pileus diameter of 11 cm and less.

There is a tendency that the 1st harvest pileus diameters were smaller when less harvest number.

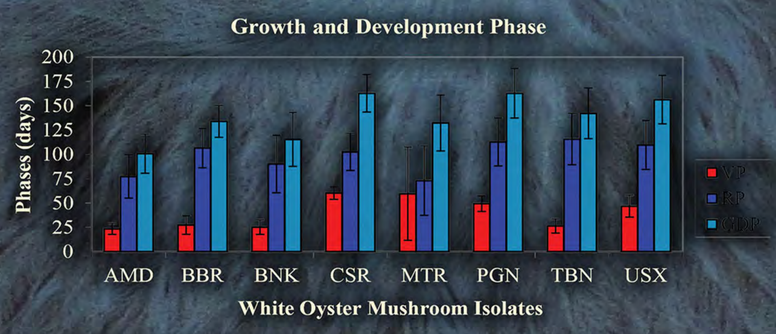
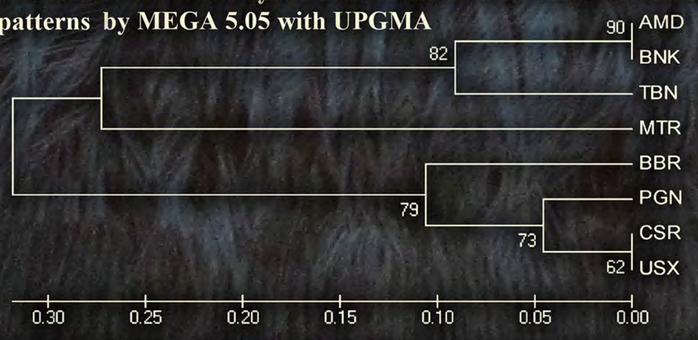


Figure 6. Only AMD, BBR, BNK, and TBN isolates (red bars) had vegetative phases of 30 days and less.

Only AMD, BNK, and MTR isolates (dark blue bars) had reproductive phases of 100 days and less

Only AMD, and BNK isolates (light blue bars) had growth and development phases of 120 days and less.

The cluster of 8 white oyster mushroom isolates based on the cultivation patterns by MEGA 5.05 with UPGMA



The dendogram showed 3 separated clusters, there were of 1st cluster of AMD, BNK, TBN isolates; 2nd cluster of MTR isolate and 3th cluster of BBR, PGN, CSR, and USX isolates.

AMD and BNK isolates were in the same cluster with similar cultivation patterns. As well as for CSR and USX isolates.

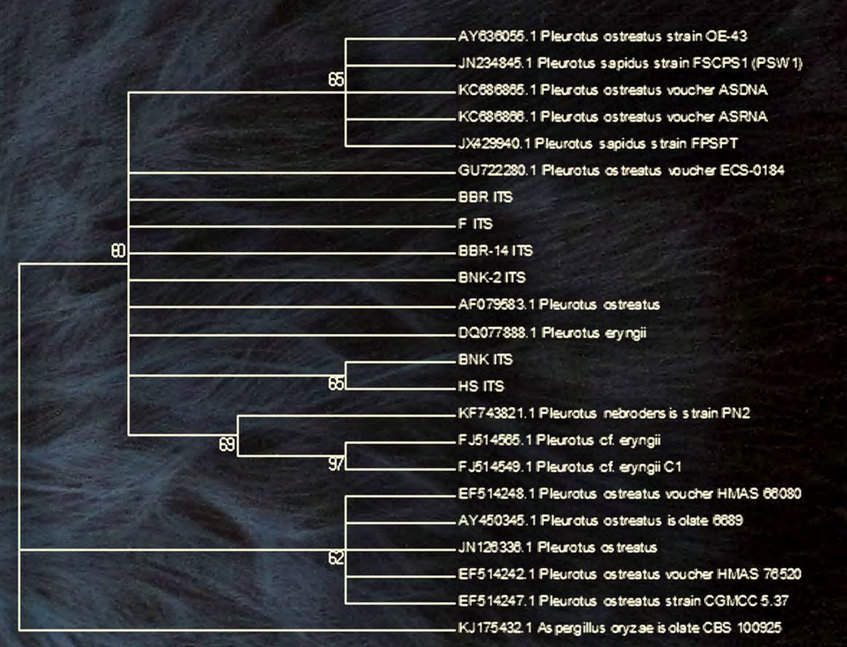


Only AMD, CSR, MTR, PGN, TBN, USX, and wild HS isolates (dark blue bars) had protein contents of 20% and more. USX isolate was the highest (28%). F is hybrid isolate of BBR and BNK isolates.

Only TBN and USX had crude fiber of 10% (light blue bars).

All isolates had low fat contents, less than 2% (red bars).

Molecular character of BBR, BNK, F, HS isolates



Based on ITS sequences data of isolates, they did not match closely with any species presently lodged in GenBank but there were phylogenetic relationships among isolates with some selected species of *Pleurotus*.

Conclusions

- Based on cultivation patterns, AMD and BNK isolates were the best yield and the fastest growth and development.
- Based on protein and crude fiber contents, TBN and USX isolates were the best.
- TBN isolate was recommended to cultivate due to the best yield, high protein and crude fiber contents. But HS isolate had an high antioxidant activity (not shown)
- The research is in progress for molecular characterization of isolates and screening isolates which are adaptable to high temperatures in order to breeding program.

Acknowledgements

The authors wish to record its appreciation to Department of Culture and Education of Indonesia for financial support of this research in the project of Hibah Bersaing. Further, I am grateful to my colleagues and my assistant who were involved and helped in this research: Dr. Sri Listiyowati, Dr. Dedy duryadi solihin, Sepriyadi Rihi, Iwa and Engkus.



Assessment of cultivation and quality parameters for exotic mushrooms production on olive residues and wastes

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Introduction

The cultivation of olive trees and the olive-oil industry generate huge quantities of by-products which pose significant problems as regards their environmentally-acceptable handling and disposal. Various microorganisms have been tested for their biodegradation, including white rot fungi with promising results[1,2]. However, a very limited amount of information is available on the use of such materials as substrates for mushroom production[3].
The aim of this study was to evaluate the suitability of olive tree pruning residues (OLPR, including wood and leaves) and two-phase olive mill waste (TPOMW) for the cultivation of *Agrocybe cylindracea*, *Hericium erinaceus* and *Pleurotus ostreatus*, and to examine their possible effects on selected dietetic properties of the mushrooms produced.

Methodology









- **Cultivation parameters**: Incubation time; Earliness; Cultivation period; Yield per flush; Biological efficiency; Productivity; Mushroom size
- **Crude composition**: Crude protein; Crude fat; Crude fibers; Total carbohydrates; Ash
- **Quality properties**: Total polyphenols; Reducing power; Antiradical activity; α-glucans; β-glucans; Total glucans







Results and Conclusions

- All three species formed mushrooms primordia in all substrates tested with the exception of *H. erinaceus* on substrates containing TPOMW.
- The addition of TPOMW had a negative impact in earliness for *P. ostreatus* and *A. cylindracea* by slowing down primordia formation by up to two weeks.
- However, TPOMW-based substrates supported high mushroom yields reaching 181% for *A. cylindracea* and 192% for *P. ostreatus* of the values achieved by the control (wheat straw).
- Only when TPOMW was supplemented in high ratios (>50% w/w) then yields were lower than the control.

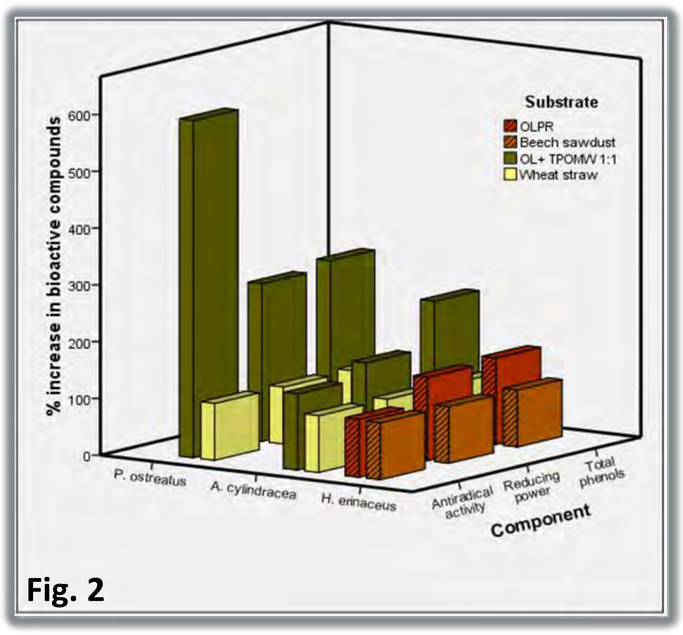


Fig. 2

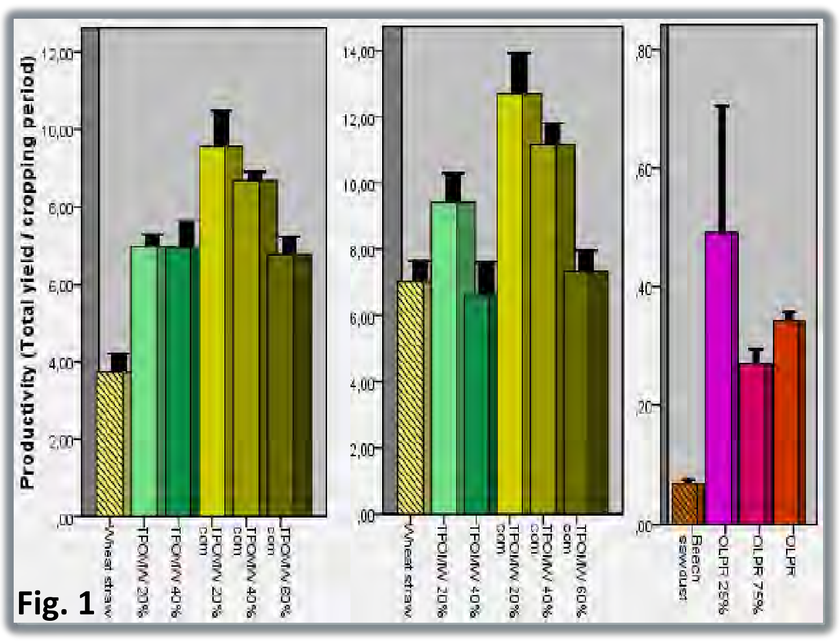


Fig. 1

- For *H. erinaceus,* best results were obtained on OLPR-based substrates achieving biological efficiency values 150% higher than the control. As regards productivity, the highest values were exhibited on the OLPR 25% w/w substrate, whereas a much inferior productivity was recorded in the control (Figure 1).
- For *P. ostreatus* and *A. cylindracea*, the highest productivity values were obtained when composted TPOMW 20% and 40% (w/w) media were used (Figure 1).

- The crude composition of the mushrooms produced did not differ significantly among species and substrates examined, except of the increased concentration in total lipids observed when higher OLPR and TPOMW amendments were used for *H. erinaceus* and *A. cylindracea* respectively, and in crude protein for *P. ostreatus.*
- Total phenolic content showed an increase of up to 189%, 139% and 52% for *P. ostreatus*, *A. cylindracea* and *H. erinaceus* respectively on substrates containing high proportion of olive waste, as compared to the control (Figure 2).
- Radical scavenging activity, (DPPH assay) and metal chelating capacity (FRAP assay) in methanolic extracts demonstrated a significant increase in mushrooms produced on TPOMW and OLPR substrates (Figure 2).
- Significant correlation was observed between total phenols and metal chelating capacity (r^2=0.93, P<0.01) confirming thereby that total phenols are a major antioxidant component in edible mushrooms.
- β-Glucans accounted for the far largest part of total glucans present in *H. erinaceus* fruit-bodies (94.4–95.9%, depending on the substrate), whereas the content in α-glucans was very low.
- β-Glucans and total glucan contents in *H. erinaceus* mushrooms cultivated on OLPR were significantly higher (23–24%) than those deriving from the control substrate.

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"Standardization of parameters for cost effective quality mushroom spawn production"

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ABSTRACT

For mushroom spawn production polypropylene bags (PP) of various sizes & gauges without respiratory filters are being used in India. Out of three sizes and gauges used, PP bags of 16"(L) X 7"(B) size of 110 gauge found best in terms of effective autoclaving & shaking during incubation. Plugging of spawn substrate bags with polyfill (polymer) in place of non absorbent cotton reduced the cost of spawn production by three percent. Immediate washing of boiled *Triticum aestivum* (wheat) grains with cold water retained the gluten in *Triticum aestivum* (wheat) grains vis a vis when grains were not washed after boiling. Colonization of grains by mushroom mycelium was faster when these were treated with 2% $CaSo_4$ + 2% $CaCo_3$ + 0.5% plaster of paris after boiling as compared to when treated with 2% $CaSo_4$ & 0.5% $CaCo_3$. It took only 12 days to colonize one kg grains when incubated at 25°C.



Fig.1: *A.bisporus* grain spawn.

INTRODUCTION

Mushroom spawn is a basic genetic material for propagation and production of mushroom. It is a vegetative mycelium on a carrier material used for colonization of substrate which is a prerequisite for any mushroom production [1]. During 1800, growers obtained spawn by collecting mycelium from natural habitat and this virgin mycelia colonize substrate like horse manure. Methods were seriously needed for production of quality, purity and mass production of mushroom mycelium. In 1932, Dr. James Sinden done significant work in spawn making using cereal grains especially as mycelium carrier. The advantage of grain spawn are increased number of inoculation sites, easy to handle and distribute well over the compost. Grains like *Triticum aestivum* (wheat), *Secale cereale* (rye), *Pennisetum glaucum* (millet) as well as *Sorghum bicolor* (sorghum) are being used for spawn production. However *Secale cereale* (rye) and *Triticum aestivum* (wheat) has been the most common grain employed all over the world [2,3]. The grain spawn was further improved by Stoller in 1962 [4]. Further other than carrier grains, studies have been done to find out suitable packing material like glucose bottles, polypropylene bags, it's size and thickness to put the carrier material into it for autoclaving and incubation. It has been observed that spawn suppliers are using polypropylene bags with or without respiratory sac. For good and uniform growth of mycelium calcium sulphate and calcium carbonate are being widely used to treat boiled grains. Keeping importance of mushroom spawn in view, an experiment was laid to find out suitable size of bag, optimum dose of $CaSO_4$, $CaCO_3$ and POP and nutrient content of grain.

METHODOLOGY

Preparations were made to produce wheat grain spawn. Bold wheat grain of WH711 variety were taken for spawn production. These were thoroughly washed and boiled to soften the grains and raise the moisture content. After this following treatments were given:

i) Use of different Polypropylene bag sizes

Boiled grains → sieved to drain water → immediately washed with cold water → surface dried under fan → coated with $CaSO_4$, $CaCO_3$ and POP → treated grains filled in Polypropylene bags of different sizes → autoclaved.

ii) Different treatment on wheat grains

a) Washed → boiled → sieved to drain water → surface dried under fan → coated with $CaSO_4$, $CaCO_3$ and POP → treated grains filled in Polypropylene bags → autoclaved.

b)Washed → soaked → boiled → sieved to drain water → surface dried under fan → coated with $CaSO_4$, $CaCO_3$ and POP → treated grains filled in Polypropylene bags → autoclaved.

c) Washed → boiled → sieved to drain water → immediately cold water treatment to the boiled grains → surface dried under fan → coated with $CaSO_4$, $CaCO_3$ and POP → treated grains filled in Polypropylene bags → autoclaved.

d) Washed → soaked → boiled → sieved to drain → immediately cold water treatment to the boiled grains → surface dried under fan → coated with $CaSO_4$, $CaCO_3$ and POP → treated grains filled in Polypropylene bags → autoclaved.

iii) Coating with different doses of $CaSO_4$, $CaCO_3$ and POP

Boiled grains → sieved to drain water → surface dried under fan → coated with $CaSO_4$, $CaCO_3$ and POP in different doses → treated grains filled in Polypropylene bags → autoclaved.

All above treatments were autoclaved for 90 minutes at 121°C and 15 lbps pressure and kept for overnight for cooling. All autoclaved bags were inoculated with master spawn of *A.bisporus* of U-3 strain @ 1%. After inoculation, inoculated bags were incubated at 24±2°C and shaking was done at 4th day. Observations were recorded regularly.

RESULTS AND DISCUSSION

❖ It was observed that mycelial colonization on wheat grains filled in 16" X 7" of 110 gauge thick polypropylene bags (PP) was faster (12 days) followed by 16" X 9" bags (14 days) as well as 14.5" X 8" sizes (14 days) of 110 gauge PP bags. It was further observed that colonization of grains by mycelium was faster in bags narrow as compared to wider bags. It was found that bags of the size 16" X 7" were better in effective autoclaving, handling and shaking after inoculation.

S.No	Bag sizes	pH	Mois-ture (%)	Spawn run (days)	*Quality of spawn (U-3)
TE	16" (L) X 7" (B)	7.5	52	12	++++
TF	16" (L) X 9" (B)	7.5	52	14	++
TG	14.5" (L) X 8" (B)	7.5	52	14	++

* Quality of spawn: + = Poor, ++ = Ok , +++ = Good, ++++ = Excellent.

Table 1: Effect of polypropylene bag size on mycelial growth of *A.bisporus*.



Fig .2: Effect of polypropylene bag size on mycelial growth of *A.bisporus*.

❖ For cost effective spawn production, we have replaced non- absorbent cotton with a porous polymer (Polyfill) for plugging the spawn bags using plugs made up of polymer (Polyfill-Rayon) which reduce the cost to 1/3rd vis a vis cost of non-absorbent cotton and gave equivalent results as compared to non-absorbent cotton.

❖ Wheat grains of WH-711 variety were used as substrate for spawn production. After boiling & autoclaving, it was observed that growth & time taken in colonization of substrate by mycelium was minimum on grain substrates which were cooled with cold water immediately after boiling. We got tested the samples of both treatment and found that gluten content (combination of protein and starch) was more in grains which were first boiled and then immediately washed with cold water as compared to boiled grains not cooled/washed with cold water. It was further observed that pre-soaked grains (8hrs) took less time to boil as compared to when grains were directly boiled.



Fig. 3: Effect of different treatment of wheat grains on mycelial growth of *A.bisporus*.

S.No.	Treatment	pH	Moisture (%)	Spawn run (days)	*Quality of spawn (U-3)
TA*	Washed+Boiled+Packed	7.5	52	12	+++
TB	Washed+Soaked+Boiled+Packed	7.5	52	12	+++
TC	Washed+ Soaked+Boiled+Cold water treatment+Packed	7.5	52	10	++++
TD*	Washed+Boiled+Cold water treatment+Packed	7.5	52	10	++++

*TA gluten = 30500 ppm and TD gluten = 101760 ppm (tested by FARE LABS Pvt. Ltd., India.)
* Quality of spawn: + = Poor, ++ = Ok , +++ = Good, ++++ = Excellent.

Table 2: Effect of different treatment of wheat grains on mycelial growth of *A.bisporus*.

❖It was further observed that boiled grains, when treated with 2% $CaSO_4$, 2% $CaCO_3$ & 0.5% Plaster of Paris proved as good substrate for faster colonization by button mushroom mycelium i.e. 12 days in comparison to grains treated with 2% $CaSO_4$ and 0.5% $CaCO_3$ in mycelium which took 17 days to colonize one kilogram substrate. It was concluded that growth of mycelium was good/vigorous on wheat grains treated with plaster of paris (0.5%) along with commercial grade $CaSO_4$ (2%) and $CaCO_3$ (2%) and it was mainly due to retention of moisture in grains.

Treatments	$CaSO_4$ (%)	$CaCO_3$ (%)	POP (%)	pH	Moisture (%)	Spawn run (days)	*Quality of spawn (U-3)
T1	2	0	0	7.2	49	30	+
T2	2	0.5	0	7.6	49	25	+
T3	2	1	0	7.7	49	23	+
T4	2	2	0	7.81	49	17	++
T5	2	2.5	0	8.0	49	17	++
T6	2	0	0.5	7.0	49	17	++
T7	2	0.5	0.5	7.2	49	17	++
T8	2	1	0.5	7.3	49	16	++
T9	2	2	0.5	7.5	49	12	++++
T10	2	2.5	0.5	7.7	49	14	+++
T11	2	0	1	7.2	49	15	++
T12	2	0.5	1	7.3	49	15	++
T13	2	1	1	7.4	49	14	+++
T14	2	2	1	7.6	49	14	+++
T15	2	2.5	1	7.6	49	14	+++
T16	2	0	1.5	7.2	49	15	++
T17	2	0.5	1.5	7.4	49	15	++
T18	2	1	1.5	7.6	49	13	+++
T19	2	2	1.5	7.8	49	15	++
T20	2	2.5	1.5	7.9	49	15	++

* Quality of spawn: + = Poor, ++ = Ok , +++ = Good, ++++ = Excellent.

Table 3: Effect of different combination of $CaCO_3$ and POP on mycelial growth of *A.bisporus*.

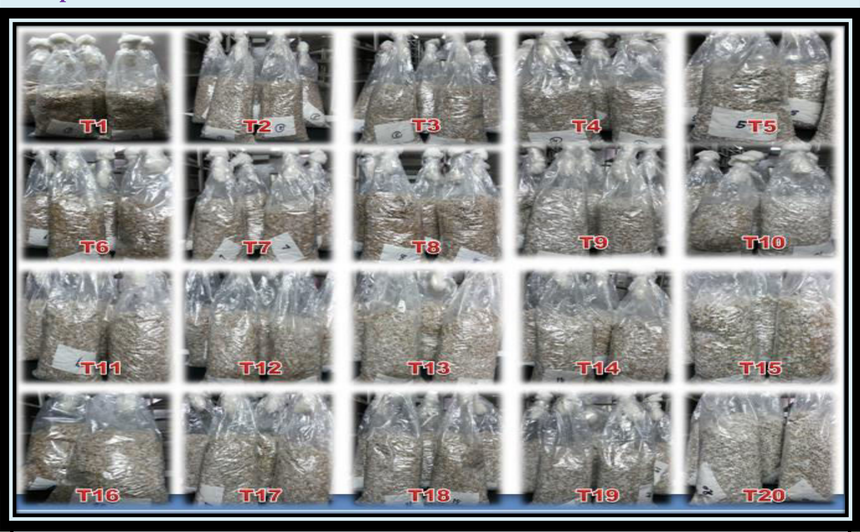


Fig.4: Effect of different combination of $CaCO_3$ and POP on mycelial growth of *A.bisporus*.

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Optimising Culture Conditions for *Ganoderma applanatum*, *G. cupreolaccatum* and *G. resinaceum*

SZENT ISTVÁN EGYETEM
KERTÉSZETTUDOMÁNYI KAR, BUDAPEST

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Introduction

Species of the *Ganoderma* genus are well known for its medicinal values. The taxonomical status of this genus is not settled due to the weak morphological identification keys. Samples examined in a study from available "*Ganoderma lucidum*" products content showed a great variety of different *Ganoderma* species. For its medical effects *G. lucidum* is the most researched of the genus, although other members of the genus were also proven to have the biologically active compounds.

Materials and methods

To obtain information about the mycelial culture conditions of three *Ganoderma* species (*G. applanatum, G. cupreolaccatum* and *G. resinaceum*), in our *in vitro* experiment we looked for the ideal media and optimal temperature needed for the three *Ganoderma* isolates made from fruiting bodies found in the Hungarian forests. The isolate cultures were placed on petri-dishes with two different culture media (potato dextrose agar /PDA/ and compost agar (Rainey, 1989)). The cultures were evaluated at three different temperatures: 20°C, 25°C and 30°C with four replicates in each treatment. The average diameter of the colony were measured on the 7th and the 11th day of their growth.

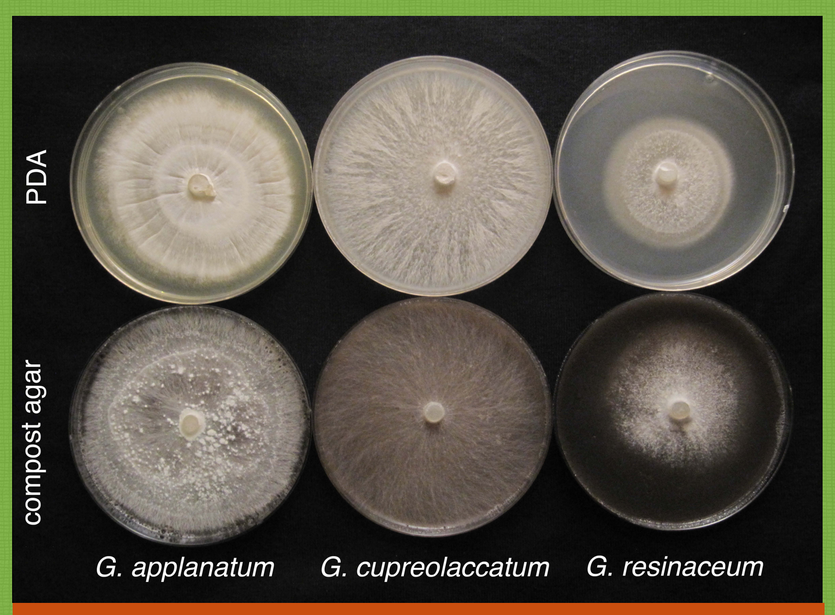


fig. 1. Ganoderma colonies on the 11th day, evaluated at 30°C

Results

The results showed that the compost agar media – which is usually used to maintain coprophilous fungi cultures with high nitrogen need – suited all the strains better compared to the PDA media we used (*fig. 1.*). 30°C was found to be the ideal temperature for all the species in the experiment (*fig. 2., 3.*). *G. cupreolaccatum* had the most vigorous mycelium growth in all the media and temperatures tested in our experiment (*fig. 5.*). *G. applanatum* mycelia shown faster colonisation on compost agar compared to PDA at every tested temperatures (*fig. 4.*). *G. resinaceum* mycelial growth was the slowest, but the compost agar suited also this strain better than the PDA media (*fig. 6.*).

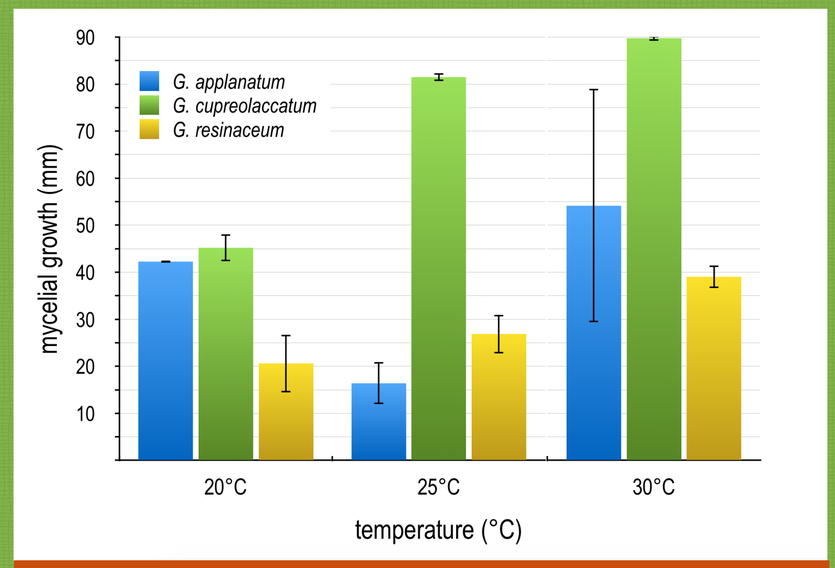


fig. 2. 11th day colony diameters on PDA

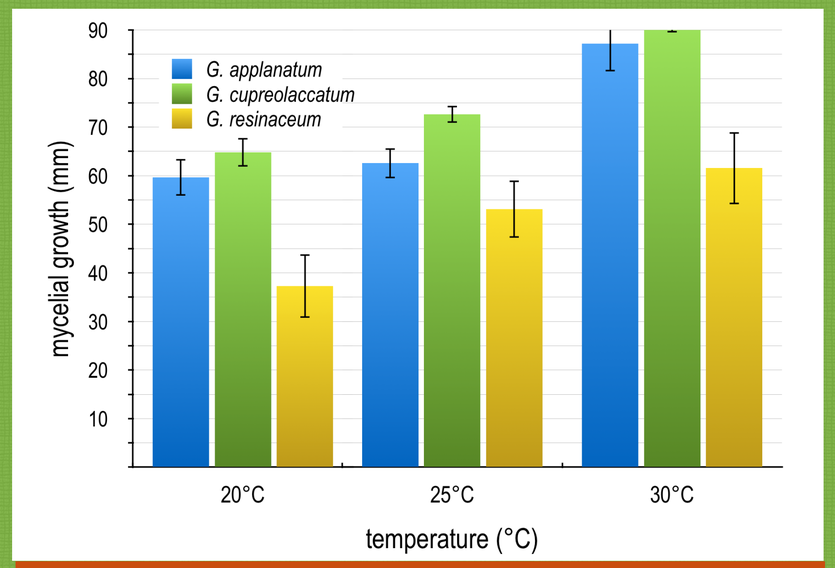


fig. 3. 11th day colony diameters on compost-agar

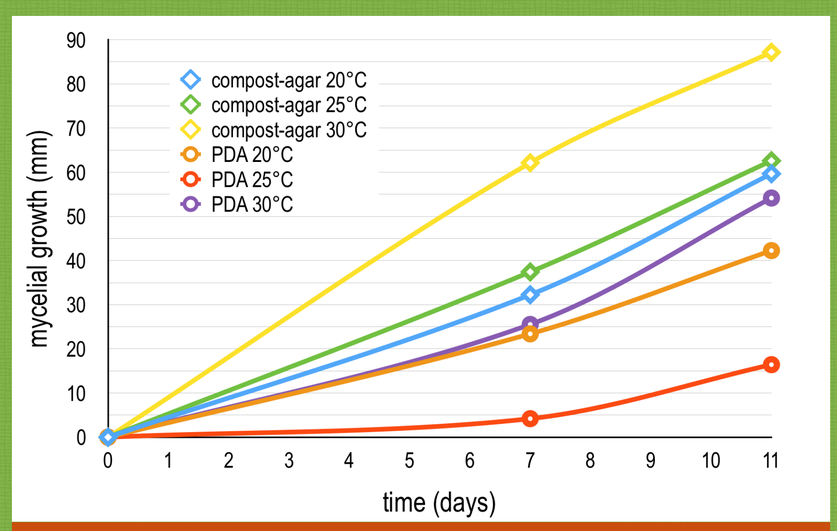


fig. 4. Mycelial growth of *Ganoderma applanatum*

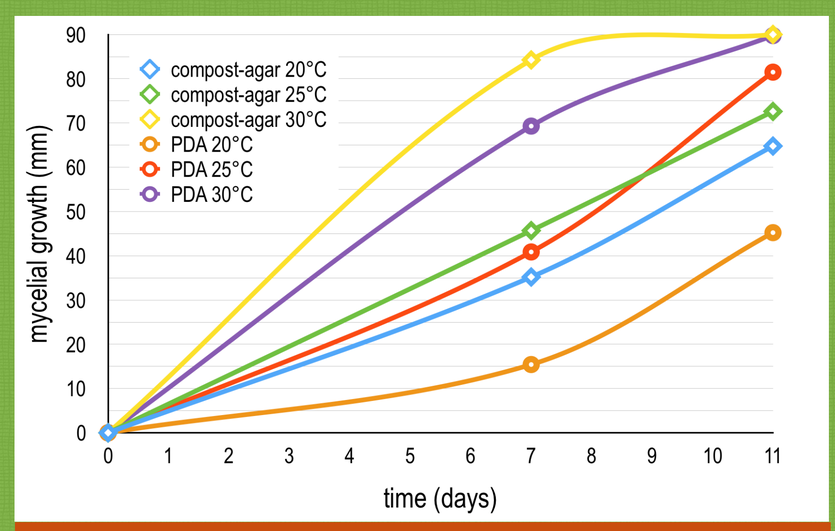


fig. 5. Mycelial growth of *Ganoderma cupreolaccatum*

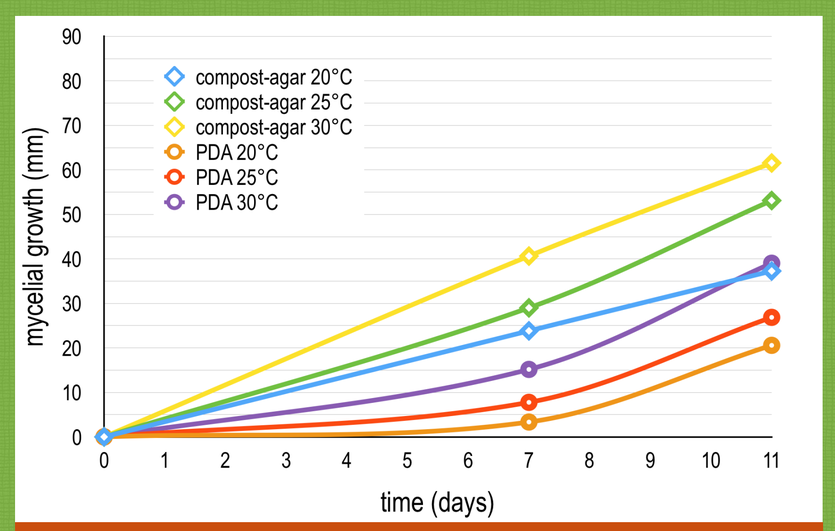


fig. 6. Mycelial growth of *Ganoderma resinaceum*

Materials and methods

For our experiment to cultivate *G. applanatum* with the *G. lucidum* cultivation methods we made grain spawn from two *G. applanatum* isolates. We inoculated grain and gypsum enriched sawdust substrate bags, sterilised at 121°C and 100 kPa for 4 hours. This substrate mixture is used commercially to grow *G. lucidum* mushrooms. The bags were 10 liter and weighted 3 kg. In total, 30 bags – applied with a gas exchange filter – were inoculated with 5 m/m% grain spawn. Full colonisation of the bags took 38 days at 25-27°C. After colonisation the bags were opened to promote primordia formation.



fig. 7. Primordia of *Ganoderma applanatum*

Results

15 days after introducing fruiting conditions, white primordia (familiar to *G. lucidum* primordia) formed at the top of the bags (*fig. 7.*). When the primordia reached the 2-3 cm height the development of the fruiting bodies stopped possibly due to the dropping air temperature of the cultivation room and lack of nutrients in the substrate.

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