The ecological consequences of a mutualistic lifestyle: An Exploration of Reproduction in Termitomyces spp.

Franz Bertenbreiter

2023

Laboratory of Genetics

Droevendaalsesteeg 1 – 6708 PB Wageningen - The Netherlands

WAGENINGEN
UNIVERSITY & RESEARCH

The ecological consequences of a mutualistic lifestyle: An Exploration of Reproduction in Termitomyces spp.

Name of student: Franz Bertenbreiter

MSc study programme: Plant Sciences

Registration number: 1166115

Course code: GEN80436

Period: Period 5

Date: May 10th, 2023

Supervisor(s): Duur Aanen and Eric Bastiaans

Examiner(s): Duur Aanen and Eric Bastiaans

Abstract

Termitomyces is a genus of obligate mutualistic fungi that form edible mushrooms and have a symbiotic relationship with termites (Isoptera, Termitidae) of the Macrotermitinae subfamily. These fungi are grown as crops deep inside termite colonies in specialized structures that can be considered farms. Despite the ecological and scientific and cultural importance of this multitrophic interaction, several unknows exist about it, particularly the circumstances that bring about mushroom formation. Moreover, this basidiomycete genus is characterized by the production of nodules, structures carrying asexual spores that termites consume for nutrition, and serve for dispersal of the fungus within colonies. A main factor influencing the physiological processes initiating both sexual and asexual cycles of the fungus is nutrition. In this research, we explored the effects of different growth substrates, such as rabbit or black soldier frass, on the somatic growth, asexual, and sexual dispersal of *Termitomyces* and related fungal genera. Furthermore, we tested various abiotic conditions to analyze the behavior of the fungus in response to these, including the addition of activated charcoal as a casing layer and initial spore density. We found that none of the seven triggers applied initiated mushroom production of *Termitomyces* in vitro. Moreover, our experiments showed that initial spore density plays a critical role in the yield of asexual spores and somatic growth of the fungus. Specifically, we found that there is an optimal spore density for nodule production.

Table of contents

1. Introduction

Ecology of the fungus

Termitomyces (Lyophyllaceae) is a genus of basidiomycete fungi that are iconic for their edible sporophores occurring on top of termite colonies across Africa and Asia, some of which reach impressive sizes of up to 100 cm or more in diameter (Morris, 1986) (Fig. 1). *Termitomyces* mushrooms are highly regarded in the paleotropics for their taste and medicinal properties, constituting also a relevant income source for rural families (Koné et. al., 2013). Furthermore, this genus has cultural importance, being embedded in the folklore of different cultures, as in the case of the Yoruba people of Nigeria, that employ it in ritual practices (Oso, 1977). Besides these uses, likely tracing back to early humans in Africa, *Termitomyces* fungi have repeatedly awaken the interest of scientists ever since their description in 1942 (Heim; 1942), particularly, due to their intricate relationship with different species of termites. Despite all of the above, the ecology of the fungus inside of termite mounds, and perhaps more importantly, the factors bringing about sporocarp formation are still poorly understood. Consistently, it has been proven to be particularly challenging to grow basidiocarps in-vitro despite several attempts to develop optimized media for the fungus (Aryal 2020, Da Costa et. al., 2019). This report explores the ecology of the fungus from an evolutionary perspective, analyzing factors influencing fitness of the fungus as well as those that might initiate mushroom formation inside and outside of termite colonies.

Figure 1. Mushroom of Termitomyces Titanicus, retrieved from Wilson et. al., (2006-present).

Of termites and fungi, conflict and cooperation

Termitomyces fungi are characterized by their mutualistic symbiosis with of termites (Isoptera, Termitidae) of the Macrotermitinae subfamily. It is commonly said that the termites farm the fungus as a means of nutrition, much like we humans do with our crops. They do so in a specialized structure deep inside the

termites´ mounds, the fungus comb (Fig.2). Termites rely on their fungus gardens to digest the recalcitrant biomass they forage. Without their symbionts, termite colonies fail to develop combs and ultimately die (Johnson et. al., 1982; Korb & Aanen, 2003). Fungal symbionts break the complex molecular structures of foraged lignocellulosic material, making nutrients available for the colony that otherwise they would not be able to utilize. In order to start their fungus comb, worker termites first have to seek and bring fungal spores inside their starting colonies. *Termitomyces* spores will then sprout and grow in the young comb material, expanding their mycelial network and eventually forming nodular structures. Nodules are spherical-like structures that contain asexual spores; the latter are consumed by the termites for nourishment together with foraged biomass and then excreted on the comb, expanding the comb´s size in the process. In return, termites continuously feed and protect their symbionts, isolating them from competition and ensuring their development and dispersal.

As mentioned, the mutualistic symbiosis between *Termitomyces* fungi and their hosts insects is obligate, which implies that both partners not only benefit from their interaction but cannot live without each other at all (de Fine Licht et. al., 2005). Despite this co-dependency, both organisms face fundamental conflicts of interest as a result of their interaction. Obligate host-symbiont associations are characterized by the great control that hosts exert over most living processes of their symbionts, being symbiont reproduction a particularly critical factor (Frank, 1996). Generally, symbionts will be vertically transmitted from one generation to the next through one of the parents and not both (uniparental inheritance), i.e., the symbiont is transmitted from the host parent to their offspring directly during its own reproduction, generally via the female (Frank, 1996). This type of transmission generally aligns the interests of both partners, as the fitness of the one translates into the success of the other. That is not case for this symbiosis, as besides few exceptions, *Termitomyces spp.*, build sexual fruiting bodies outside of the termite´s mounds, reproducing independently from their hosts. The latter also means that termites actively have to forage for new spores in the wild to start new farms (horizontal transmission) (de Fine Licht et. al., 2006). It is immensely intriguing how such a symbiotic interaction emerged and how it may remain stable despite apparently conflicting interests.

Figure 2. A. Termite mound of a fungus-farming termite (Macrotermes natalensis). B. Close-up of the architecture of the underground fungus comb. C. Royal Chamber of the colony. Image of Poulsen (2015). D. Entire fungus comb. Photo by Lakshya Katariya (Butler, 2018).

The life-cycle of (free-living) basidiomycetes

Fungi have a characteristic life-cycle that not only differentiates them from other organisms but also delimitates the array of strategies they can use to efficiently utilize their resources, increase their dispersal success and survival chance. Even within the same order, the biology of fungi can vary considerably depending on their niche. Commonly, the lifecycle of the "average" basidiomycete is described as follows (Fig.3):

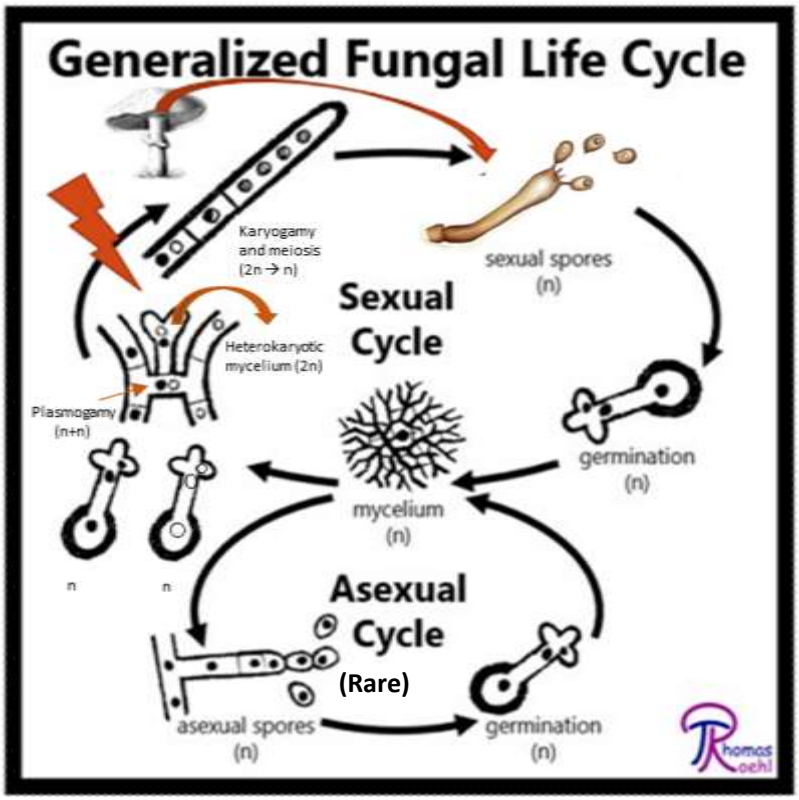

Figure 3. A spore of a "free-living" fungus undergoes a "generalized" life-cycle where it grows as a hypha, then as a network of monokaryotic hyphae (mycelium). Eventually this mycelium fuses with a compatible partner (plasmogamy), becoming heterokaryotic mycelium. Under the right conditions, this mycelium may trigger its sexual cycle (symbolized here as a thunder), forming basidiospore producing fruiting bodies. Depending on the fungus, mature mycelium may also trigger its asexual cycle, producing asexually developed spores, arising from differentiated mycelium from either homo- or heterokaryotic mycelium but this is rare for basidiomycetes. Image modified from Roehl (2006).

The common free-living basidiomycete begins its life as a uninucleate haploid basidiospore, which germinates in the substrate it lands on, acquiring resources and growing as an expanding homokaryotic (one type of nucleus) mycelium. This initial stage is generally short-lived, as monokaryotic hyphae will actively seek to fuse with another compatible monokaryon while growing. When two hyphae with compatible genotypes meet, the somatic fusion of two mating monokaryons, also known as plasmogamy, occurs. Both partners transfer their nuclei into the fusing hypha of the other partner, becoming heterokaryotic (more than one type of nucleus) as a result. Thus, the fused hyphae now contain two haploid nuclei per cell, one for from each precursor monokaryon. This stage, referred to as the heterokaryotic phase, is the main and longest-lived life stage of basidiomycetes. Heterokaryons will continue to proliferate in their substrate, fusing with other compatible hyphae, accumulating resources and expanding somatically (Moore-Landecker, 1996).

The area a fungus can colonize through somatic growth tends to be limiting, as substrates are finite and permanently contested upon countless organisms in time and space. Hence, once the mycelium has been able to reliably establish itself in the substrate and if the ecological and physiological conditions provide, it may trigger its sexual cycle. In order to colonize new substrate, fungi spread their genetic material through spores. Heterokaryotic mycelium is able to form specialized structures that develop and spread spores, we refer to them as basidiomata/basidiocarps or simply mushrooms. Inside of basidiomata, the nuclei of a dikaryotic mycelium can fuse into a single diploid nucleus; this process is known as karyogamy and occurs within basidia. Basidia are special sexual cells inside of basidiomata. Meiosis may then occur within the latter, commonly resulting in 4 uninucleate daughter cells, which we call basidiospores. These will be dispersed by different (a)biotic agents such as wind. Once the new spores have landed on a substrate, they are able to repeat the described cycle (Moore -Landecker, 1996).

Sexual reproduction is relatively costly in terms of resources and time, as it requires the encounter of a compatible partner and the development of differentiated tissue that not only does not attain nutrients but drains their reserves. To overcome these challenges, some fungi have developed the ability to disperse via the production of asexual spores, i.e., through cloning themselves. Asexual spores are cheaper to produce, being formed often shortly after the establishment of the early mycelium when hyphae differentiate into specialized structures, the conidiophores (Moore-Landecker, 1996). In some cases, monokaryons are able to produce asexual spores, hence eliminating the need for fusing to reproduce. Despite the advantages of this strategy, species relying uniquely on asexual reproduction do not benefit from genetic recombination happening during karyogamy and meiosis, consequently limiting their genetic resources and adaptability potential. It is important to mention that asexual reproduction is rather rare for (free living) basidiomycetes, only few species undergo this type of reproduction, in addition to sexual reproduction. In contrast, other fungi from other phyla such as several Ascomycetes perform asexual reproduction much more commonly than basidiomycetes.

A shared way of life and consequences for fungal fitness

Pringle & Taylor (2002), define fitness as: “the survival and reproductive success of an allele, individual or group”. The strategies such a biological unit can employ to increase its fitness may vary vastly depending on the context of their habitat. The aforementioned life cycle accurately describes the development of free-living, saprotrophic fungi, which commonly do not require intricate relationships with other organisms to complete their life cycles. In this case, fungi tend to depend on “choosing” a “selfish” strategy that will maximize their own success when compared to other organisms competing for the same niche. I.e., natural selection will favor strategies that increase the dispersal and establishment chances of the fungus itself in order to increase its survival and dispersal. These strategies include, firstly, somatic growth, then asexual or sexual reproduction.

In contrast, obligate mutualistic fungi, such as *Termitomyces* require a host organism to survive and disperse. *Termitomyces* differs from their free-living counterparts in several ways. Firstly, the performance of the fungus is directly related to the conditions termites provide for it, such as the amount of foraged and defecated biomass in the comb. Secondly, termites likely steer the growing behavior of the fungus towards their own benefit, as they are “interested” in the production of edible nodules of symbionts. To this effect, sexual fruiting bodies represent an unnecessary and costly investment that prevents the maximization of edible biomass (Aanen, 2006; Korb & Aanen, 2003). This creates a conflict, as the fungus is interested in

reproducing sexually outside the restricted space of its termite nest, in order to benefit from genetic recombination and a wider dispersion range to avoid competition (Wisselink et. al., 2020). It is highly likely that conflicts of interest like the latter, may derive in detrimental consequences for the stability of the mutualism, specially, fitness of the host is reduced due to virulent traits of symbionts. It is believed that several parasites developed from previously mutualistic relationships with conflicting interests (Hibbet et al., 2000). Hence, in order to stabilize a mutualism, it is probably useful to align interests of hosts and symbionts.

Some organisms, such as attine ants and a few species of farming termites, resolved this puzzle through vertical transmission of their symbionts, i. e., aligning the reproduction of both hosts and symbionts at the same time (Mikheyev et. al., 2006). As explained, since most *Termitomyces* species present a sexual reproduction independent from their hosts, other stabilizing mechanisms may be at play.

The domestication of *Termitomyces* likely has a single evolutionary origin, without reversing into a free-living state or turning into parasitism (Aanen et al., 2002). This underlines the stability of the symbiosis. Likewise, some degree of co-cladogenesis has been observed between both partners (Aanen et al., 2002; Da Costa et al., 2019; Nobre et al., 2011), meaning that *Termitomyces* clades associate with specific Termite clades. Hosts are able to switch their symbionts within these clades (Van de Peppel & Aanen, 2020), likely fostering the cooperative behavior of the fungal strains, as otherwise they will choose for a more cooperative symbiont instead. Yet, one of the main mechanisms in which termites enforce partner fidelity and restrict parasitic tendencies of their symbionts seems to be keeping a single fungal strain per comb, i.e., through monocultures (Aanen et. al., 2009). This way, termites reduce possible parasitic tendencies of symbionts arising from competition with each other. Instead, fungi benefit from substrates that will be exploited solely by themselves. Consistently, in-vitro experiments showed that monocultures produce considerably higher nodule yields than mixed cultures (Aanen et. al., 2009).

In order to increase its chances of survival, a fungal strain needs to ensure its compartmentalization inside a termite host colony. In the context of symbiont transmission, compartments refer to physical, specialized structures (inside) of hosts, used to control the development, reward cooperative tendencies, punish virulent tendencies, and/or reduce possible conflict among different strains of symbionts competing for a host (Chomiki et al., 2020). In the case of *Termitomyces*, the symbiont compartment is the fungus comb, which can be colonized by a single fungal strain (monocultures) at the time. Termites may reward cooperative behavior of *Termitomyces* by allowing most cooperative strains access to grow in the comb. To clarify, a cooperative symbiont would be one that maximizes the production of edible spores above other strategies. As *Termitomyces* symbionts obligatorily rely on host compartments for survival, the incentive for cooperation is high. In the same manner, non-cooperative tendencies of symbionts may be punished by hosts, by simply choosing more cooperative symbionts. This seems likely to be the case, as symbiont switching has been found to be relatively common for Macrotermitinae (Van de Peppel & Aanen, 2020).

In vivo development of *Termitomyces*: Toward the understanding of basidiocarp formation

Lyophyllaceae fungi, the family which *Termitomyces* belongs to, can vary greatly in their ecology, encompassing ectomycorrhizal, saprotrophic, parasitic and mutualistic species (Hofstetter et. al., 2014). Van de Peppel et. al. (2021), studied the phylogeny of *Termitomyces and* sister genera *Blastosporella* and *Arthromyces*, all of which are characterized by growing on fecal material from different insects. These phylogenetic studies showed that such associations are likely the result of a common ancestor developing an

affinity for growing on insect-fecal material. The latter, in addition to other complementing traits, may have likely predisposed the ancestor of *Termitomyces* toward its domestication, setting the stage for the primitive termite agriculture (Van de Peppel, 2021). The described domestication event has been estimated to have occurred 30 million years ago (Aanen et al., 2009; Nobre et al., 2011; Roberts et al., 2016), leaving plenty of room for both organisms to adapt further to their cohabitation. Fecal substrates tend to have a high C/N ratio and often contain species-specific metabolites (Elissen et. al., 2023) that may be recognized by fungi to trigger certain physiological responses, such as e.g., initiate sporocarp formation. Remarkably, *Blastosporella zonata*, a species discovered in the cloud forests of the neotropics (Baroni et. al., 2007; Van de Peppel et. al., 2022) developed basidiocarps when grown in a medium based on horse dung (not published). This sheds light on the possibility of also achieving the production of *Termitomyces* mushrooms utilizing fecal substrates in-vitro. Previous experiments have focused on determining the growth performance of *Termitomyces* on different substrates (Aryal, 2020; Da Costa et. al., 2019), finding mixed results and no development of mushrooms. To my knowledge, no previous study has utilized fecal material as a growth substrate for *Termitomyces.*

Substrate composition has an effect on the physiology of fungi; however, other factors such as environmental conditions are critical in the development of fungi as well. It still remains unknown what specific circumstances promote sporocarp formation. Meanwhile, *Termitomyces* species seem to form basidiocarps based on specific host-colony, and outside-colony ecological factors. In other words, not only do fungi or termite specific factors determine the onset of fruiting body formation but also processes occurring outside their host´s colonies. Koné et. al. (2011), performed one of the few studies in vivo investigating ecological factors linked to mushroom formation of *Termitomyces*, discovering an apparent correlation between mushroom formation, the occurrence of rainy periods and the alate dispersal of termite-hosts. The latter will be explained more extensively below.

Nutrient dynamics of the fungus comb

Fungal symbionts spend the biggest part of their lives growing in fungus gardens known as combs. The fungus comb is the specialized structure built inside of Macrotermitinae termite mounds and acts as the colonization site of their fungal symbionts. It is a brown-yellowish, brain shaped complex composed of foraged biomass, that passed through the termite's gut (Kusumawardhani et. al., 2021). The comb is built in the early stages of colony formation, being maintained by constant addition of plant material inoculated with spores (Nobre & Aanen, 2012). For the starting inoculum, it is inferred that Termites forage sexual *Termitomyces* spores in the wild, they ingest and excrete them on top of the comb. The initial spores grow into mature mycelium, forming nodules containing conidia. These asexual spores are then consumed by termites together with plant material, passing their gut, and resisting digestion, to be finally excreted on top of the comb again (Leuthold et al., 1989; Rouland-Lefèvre et. al., 2006). Throughout this process, termites control the flow of nutrients of the fungus. Thus, understanding these nutritional dynamics is critical to comprehend the life strategies of *Termitomyces*.

Termites are eusocial insects presenting individuals with different distinctive phenotypes and clearly defined tasks within the colony. There are for instance soldiers, whose sole duty is to defend the colony from potential threats. Alates, winged individuals that will start new colonies and are to become the new queens and kings. Also, all of these last ones, are the only casts capable of reproduction. Moreover, there are workers, which are in charge of the maintenance of the nest structure or foraging of nutritive plant material

(Crosland, 1996). In Macrotermitinae, workers also build the fungus combs (Rouland-Lefèvre et. al., 2006) and, depending on their age, they show further division of labor. On the one hand, old adult workers in the wild forage the biomass that will be consumed by the colony. On the other, young workers have the duty of building and maintaining the mound and its fungus garden. The latter feed on the forage of the old workers; this material passes their gut, being partially digested and then excreted on top of the comb. Contrarily, old workers feed exclusively on the mature fungus comb (Traniello & Leuthold, 2000) (Fig. 5). This means that the foraged material passes the gut of termites twice, not being further consumed afterwards (Chiu et. al., 2019; Liang et. al., 2020). In sum, there is a defined gradient in the comb, the bottom of the comb being the oldest, consisting of largely digested plant material and mycelium, and the top composed mostly out of fresh lignocellulosic substances inoculated with *Termitomyces* spores. The aforementioned nutritional dynamics of the fungus garden are complex but they appear to be in a sustainable balance. The latter may be controlled, among other factors, by the queen of the colony who determines the ratio of foraging workers to "idle" larvae and individuals of other castes (Roisin, 2000).

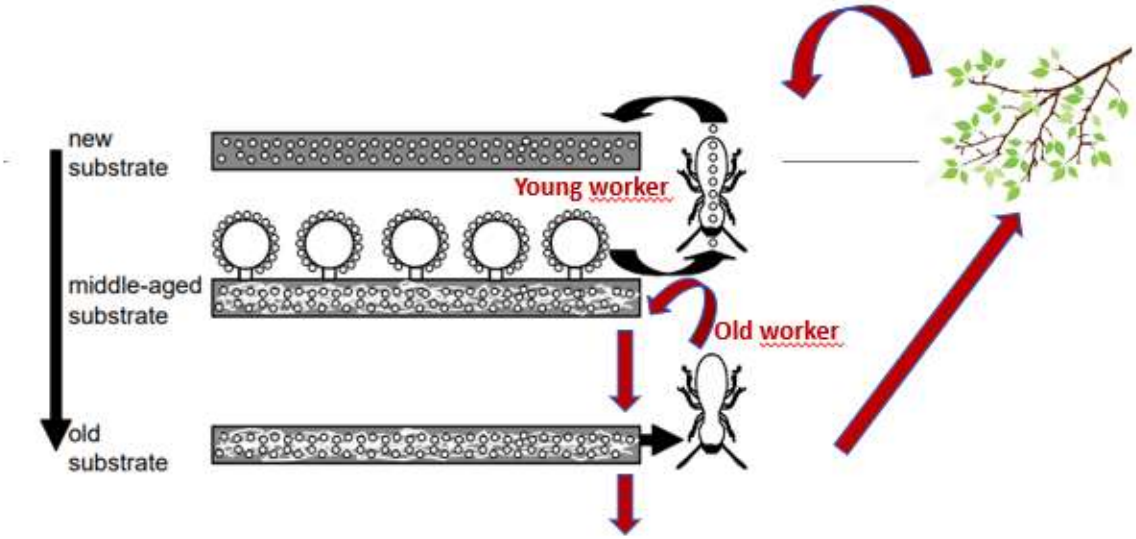

Figure 4. Nutrient flow of a fungus comb. Old workers forage fresh material for young workers and consume mature comb material, which is then excreted and not consumed anymore. Young workers consume fresh forage and excrete it on top of the comb. (Modified from Aanen, 2006).

Comb biomass status is also affected by seasonal, atmospheric, and host-related processes. Nonetheless, the rate of addition to consumption of comb biomass appears to remain balanced throughout the season (Johnson, 1981). Under these conditions, *Termitomyces* seems to stay vegetative throughout the year. However, during the onset of the rainy seasons basidiocarps tend to appear on top of termite mounds (Koné et. al., 2011). Curiously, the occurrence of rain also converges with the alate dispersal of termite hosts. During this period, a termite colony invests its resources in producing swarms of winged, sexually capable individuals that have the task of starting new colonies. In turn, new colonies require fungal spores for the construction of their fungus gardens.

Moreover, alate production should disrupt the mentioned dynamics of addition and removal of biomass in the comb. In early, non-reproductive stages of nest formation, most resources go into building the latter to support the imminent growing termite population. However, as the colony reaches sexual maturity, much of this energy is directed into the production of costly sexuals instead. It has been estimated that alate

production can be up to 7 times as expensive when compared to the production of workers (Lapage & Darlington, 2000). The trade-offs following alate can be split in two stages: firstly, a diminished rate of addition of new comb material, and secondly, a reduced foraging potential. The further comes as a result of fewer young workers being generated as a result of alate production, translating into proportionally less fresh biomass being consumed and excreted on the garden, hence, reducing the amount of new comb built. However, during this stage, the proportion of old workers consuming mature comb remains stable. Thus, the balance of addition and removal is disrupted, as in proportion there is more biomass being removed than added on the comb. The latter starts as time progresses, alate production ceases whilst worker population is re-established. The balance of addition and removal changes towards the addition of biomass, as now there are proportionally fewer old workers removing comb material when compared to the newer generations of young workers adding it (Fig. 3).

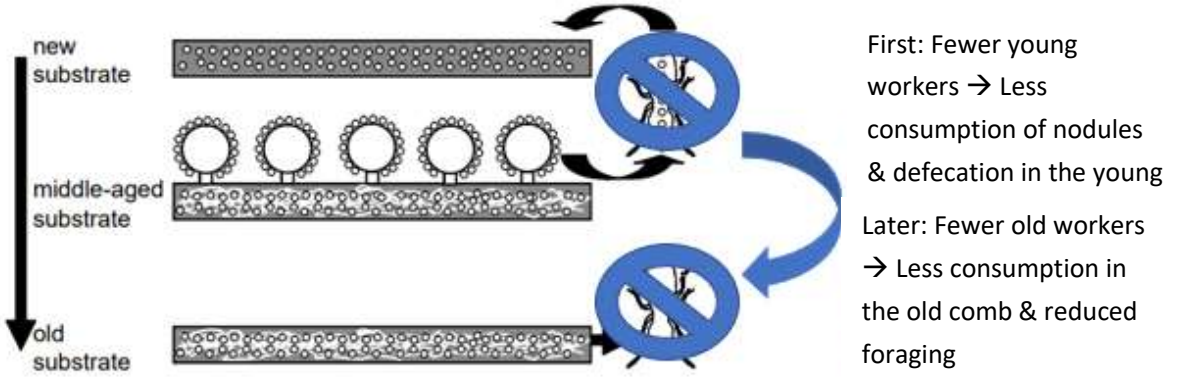

Figure 5. Comb dynamics after the production and swarming of alates. (Modified from Aanen, 2006).

When fungi trigger mushrooms

The co-occurrence of rain, alates and *Termitomyces* mushrooms, strongly hint towards some type of link between these factors. As it is often the case in the fungal kingdom, it can be challenging to distinguish specific factors responsible for inducing fruiting body formation. The requirements for optimal mycelial growth, and the ones needed for stimulating fruiting may vary significantly from one another, as can be concluded from the colony dynamics of fungus farming termites.

Moreover, empirical evidence of Basidiomycete development shows no one-fits-all solution for the enigma of fruiting body formation. Higher fungi differ greatly in their requirements for mushroom formation, some fungi, for instance, may require triggers that signalize a change in the benefit/cost ratio of mushroom formation, e.g., through exposure to light and high humidity levels as in the case of the porcelain mushroom (*Oudemansiella mucida*) (Lee et. al., 2007), or specific bacterial communities as in the case of Chanterrelles (*Catharellus cibarius*) (Bennett & Feibelman (2001). Kües & Liu (2000), elucidated various factors that may induce mushroom formation for several fungi; among the latter, they illustrated the critical role of a high C/N ratio. Various authors have studied the chemical composition of combs and nodules, suggesting that older comb is rich in carbon and nodules in nitrogen (Chiu et. al., 2019). Analogously, changes in the addition/removal of biomass in the fungus comb during/after the onset of alates likely changes the C/N ratio of the substrate: First lowering it, as there are proportionally more old workers consuming mature comb than

young workers defecating fresh rich material on it, and later, rising the C/N due to proportionally fewer old workers than young ones adding biomass. In other words, the substrate first gets depleted and then saturated with carbon. Unfortunately, there are no studies analyzing comb biomass status post alate production, but it is likely that *Termitomyces* symbionts answer to these changes in some way, such as through mushroom formation.

Potential triggers of mushroom formation

Mechanical stress

Mature mycelium of some fungi responds to mechanical damage by the aggregation and differentiation of the hyphae around the broken mycelium mushroom initials (Kües & Liu, 2000). Mechanical damage is constantly present in *Termitomyces* gardens through the removal of nodules; however, its proportion changes during and after the swarming of alates. E.g., one may consider that directly after the swarming of sexuals, when less nodules are being consumed. These may develop into fruiting bodies, i. e., the absence of nodule removal initiates the fruiting bodies. However, recent evidence suggests that production of asexual nodules and sexual mushrooms are two separate developmental pathways, and hence independent from each other (Vreeburg et. al., 2020a).

CO2

Changes in the carbon budget of the colony may have a significant effect on the development of their symbionts. In order to meet their nitrogen requirements, fungus-farming termites have to digest and excrete large amounts of plant material, hereby, releasing massive emissions of carbon dioxide (Vesala, 2019). The swarms of alates drastically reduce the number of individuals within colonies, and significantly lower emissions of carbon of colonies (Seiler et. al., 1984). Consistently, a reduction in carbon dioxide levels seems to be critical for fruiting body formation of various species (Kües & Liu, 2000), like with the button mushroom (*Agaricus bisporus*) (Noble et. al., 2003). In turn, low CO_2 concentrations are thought to diminish the accumulation of compounds that inhibit the formation of initials (Long & Jacobs, 1974). In industrial mushroom production, a tight regulation of the carbon budget of substrates is critical. For this reason, the cultivator may utilize a so-called casing. This is generally a layer of peat containing different microorganisms. The bacteria present and/or physical-chemical composition of the casing facilitates the exchange of gases, reduces carbon dioxide concentrations of the substrate, and metabolizes or adsorbs volatile compounds produced by the fungus that may inhibit its fructification (Bechara et. al., 2009; Noble et. al., 2009). The use of these microorganisms can be problematic due to potential contamination with pathogens. Therefore, an axenic layer of activated charcoal can be used instead to absorb the inhibitory compounds and foster the formation of mushrooms (Noble et. al., 2003). To my knowledge, there has been only one study attempting to induce basidiocarps on *Termitomyces* with a casing layer, which was unsuccessful (Botha & Eicker, 1992; Heim, 1942).

Carbon budget: C/N

The carbon balance inside of termite colonies varies along with its population, and also with the rate of addition of new substrate, but *Termitomyces* not only is affected by the quantity of carbon available but also by its composition. Comb biomass varies with the vegetation around colonies (Vesala et. al., 2022). Overall, termites forage material near to nests without much selection, among others, animal dung, rotten wood or

leaf litter (Chiu et. al., 2019). Nonetheless, colonies close to woody savannahs have shown to grow more abundantly and produce basidiocarps more consistently (Koné et. al., 2011). Consistently, studies in-vitro suggest that *Termitomyces* prefer specific complex carbon sources; when several C-sources were tested, rice, wheat-bran or cellobiose yielded the highest dry biomass when compared to several other simple and complex substrates (Da Costa et. al., 2019). Furthermore, it is expected that the C/N will go up over time, assuming that N is more limiting for growth, hence, being proportionally used more than C. Likewise, different C/Ns have been associated with specific physiological processes of fungi. E.g., high C/N ratios of up to 80/1 have been regarded as optimal for mushroom development in the button mushroom (Kües & Liu, 2000).

Fecal substrates

Fecal substrates contain large amounts of carbohydrates that support the growth of coprophilous fungi. Nonetheless, factors like structure, pH, moisture content or specific bacterial communities and metabolites vary depending on the origin of the dung, also, how digested lignocellulosic biomass is varies depending on the source (Amandeep et. al., 2015). In particular, bacterial communities in dung have been shown to hamper or promote fruiting (Safar & Cooke, 1988). Given that *Termitomyces* naturally grows on fecal material of termites, it may be well adapted to these types of substrates and to using specific cues of dung as signals to trigger growth responses.

Laccase activity associated with fruiting

The mycelial growth of *Termitomyces* has been established several times in-vitro, showing that growth occurs in a wide arrange of substrates (Aryal, 2020; Da Costa et. al., 2019). However, the metabolites produced by the fungus may vary depending on the substrate it is grown on (Gangwar et. al., 2016; Yang et. al., 2020). Studies suggest that different peroxidases and laccases are synthetized by this genus, most likely to degrade strong lignocellulosic material (Schalk et. al., 2021). Bose et. al. (2007), and showed that laccase was especially active in the late stages of mycelial growth for the species *Termitomyces clypeatus*, with other studies finding similar results for several species of this genus (Taprab et. al., 2005). Fungal laccases have a broad array of functions and are expressed more over along the life cycle of fungi (Thurston, 1994). Likewise, various basidiomycetes are characterized by increased laccase concentrations prior to fruiting body formation (Ross, 1982; Suguimoto et. al., 2001). Accordingly, laccase expressing genes have been associated with factors promoting fructification of higher fungi (Kües & Rühl, 2011; Kües & Liu, 2000; Zhang et. al., 2015). Hence, the activity of this enzyme has been hypothesized to play a major role for mushroom initiation of several fungi. There is not necessarily a correlation between laccase activity and fruiting in general. Eliciting laccase activity in a controlled environment requires the presence of polyphenols with structures similar to the ones present in lignocellulosic material. Tannic acid or veratryl alcohol have been used in previous studies with other fungi to successfully trigger laccase activity (Carbajo et. al., 2002; Suguimoto et. al., 2001).

Other stimuli

Kües & Liu (2000), compiled a summary of several conditions that lead to the development of basidiomata in basidiomycetes. Besides many factors mentioned above, they underline the role of light in fostering aggregation and differentiation of mycelium into mushrooms. In general, fungi seem to respond with the formation of mushrooms after the application of stress. The latter likely signalizes a change in the growing conditions which shifts the cost/benefit ratio of sexual reproduction towards the second.

Research questions

Based on the information presented above, the following research questions have been formulated to guide this report:

- How may the life strategies of a symbiotic fungus such as *Termitomyces spp*. vary from the ones of a free-living fungus?
- What triggers may initiate mushroom formation in *Termitomyces spp.* and how does the fungus react to different stimuli of mushroom formation in other agarics?
- How does the fungus behave when grown on fecal and other novel substrates in-vitro?
- How does *Termitomyces spp.* respond to changes in spore density in regard to its growth and nodule formation and what consequences could these responses have for in-vivo conditions of the fungus?

In order to answer these questions, I formulated the following goals:

(1) Investigate substrates potentially optimizing the vegetative growth and reproduction of *Termitomyces spp*. and sister genera by experimenting with different novel media with distinct nutritional properties. (2) Assess the effect of the aforementioned substrates as well as different abiotic and biotic external growing conditions on the development of sexual and asexual structures of *Termitomyces* spp. and sister genera. (3) Infer about the consequences that the outcomes of (1) and (2) may have for the in-vivo dynamics of the symbiosis of *Termitomyces spp*. based on the evolutionary ecology of the fungus.

2. Methods

General methodology

Literature research, fungi used and general materials

To support the bases of this research, a systematic literature search was done by using Google Scholar and the catalogue of the Wageningen University & Research library. Publications used to establish initials ideas include: Evolution of mutualisms in the basidiomycete genus *Termitomyces* (Nieuwenhuis at al., 2021), Fundamentals of the fungi (Moore-Landecker, 1996), Insect-fungal associations (Vega & Blackwell, 2005), On conflict and resolution in the termite-fungus symbiosis (Vreeburg, 2020b), The evolution of allorecognition and somatic fusion in ascomycete filamentous fungi (Bastiaans, 2015), On the origin of species in *Termitomyces* (Van de Peppel, 2021), Reproduction in fungi: genetical and physiological aspects (Elliot, 1994) and Sexual selection in fungi (Nieuwenhuis, 2012).

Fungi used across experiments are:

Termitomyces strains: *T. cryptogamus* (T88 and T89) and *Termitomyces sp*. (T73)

Blastosporella zonata

Arthromyces matolae

Unless otherwise specified, suspensions were made by scrapping the surface of petri dishes containing malt extract agar (MEA) (30g malt extract/L and 20g agar /L) twice with a spatula (2mm scrapping surface) until the surface was full of nodules. The mycelium was then placed in a 1ml Eppendorf tube containing 400ml of saline, the mycelium was crushed with a manual or electrical grinder and then filled with 600ml of saline. Suspensions where the number of spores is explicitly specified were counted with a hemocytometer.

All fungi were initially inoculated on malt-yeast-agar (MYA) (20g malt extract/L, 2g yeast extract/L and 15 g agar/L). When mycelium covered the plate, fungi were transferred to plates containing (MEA) and multiplied continuously into new (MEA) petri dishes to keep fresh stocks to utilize in all experiments. Moreover, all plates and bottles were grown in incubators at a temperature of 30°C, except *Blastosporella* treatments, which were grown at room temperature (ca. 25°C).

Asexual spore yield in plates and bottles was estimated by measuring the surface occupied by nodules with ImageJ (see below).

Image processing and analysis

All images were analyzed with ImageJ, first manually setting the scale of petri dishes to either 60 or 100mm, then, different thresholding options were used to analyze the particles. Particles were counted and their sizes were added to give the area covered by the regions of interest for every experiment.

For the first experiment, images were converted to 8-bit and applied the IsoData thresholder for black and white images. Due to the varying quality of images, threshold levels were tailored to every image, however the threshold levels ranged between 180-192, procuring to take the same portion of the threshold area. Images of the process can be found in the appendix (Appendix: Fig. 1; Appendix: Fig. 2). The regions of interest (inside of petri dishes) were manually selected with the freehand tool. All other experiments (spore

density experiments), had more uniform images, and hence were analyzed with the color threshold tool and the default method settings of the RGB color space, except for the upper threshold level of the green color which was set to 211. The oval tool was used to select the regions of interest, although sometimes the freehand tool was used to avoid counting colonies of contaminating organisms.

Nodules of *Termitomyces* are 3 dimensional structures but ImageJ analyses images as 2 dimensional, this implies that nodule area and yield estimations are underestimations of reality. The implications of these are explained further in the discussion section.

Plotting and statistical analysis

Almost all plots presented were made with the ggplot package in R, except the graphs of experiment 1 showing the effect C/N and nutrition for the growth of fungi, which were made in Excel. In addition, ANOVA analyses were performed with the aov() function, and post hoc turkey tests (HSD) with the TurkeyHSD() function in R, always using an alpha level of 5%.

Experiments

Experiment 1: Effects of C/N ratios and nodule removal on growth and sexual development of *Termitomyces spp.*

The goal of this experiment was to assess the effect of nutrition, C/N ratio and mechanical damage on the growing behavior and mushroom formation potential of *Termitomyces* and its sister strains. In the first phase of the experiment, the different fungi were inoculated in the 15 media mentioned below and let grow for 45 days. After this period, the comb can be considered mature (Liang et. al., 2020; Yang et. al., 2021). The strains were inoculated into agar plates containing a simplified version of the medium used by Shik et. al. (2016) and da Costa et. al. (2019), where glucose and starch are used as carbon sources and peptone is used as a nitrogen source, vitamin, trace elements and agar proportions can be seen in the Appendix (Tab. 1; Tab. 2).

Two treatments were distinguished during inoculation: one treatment with a sole inoculum in the center of the plate and one where inoculum will be spread systematically on the surface of the plate. The first treatment was used to estimate radial growth performance based on different concentrations (g/L) and diverse C/N ratios. The second treatment was used to test the effect of nodule removal/mechanical damage in conjunction with the different concentrations and C/N ratios as possible triggers of mushroom formation. During this phase of the experiment, the radial growth was assessed by photographing the plates of the treatments, once per week.

The second phase of the experiment consisted of testing triggers of mushroom formation. To this aim, the media of the plates, where inoculum was systematically spread, was allowed to develop for a further 85 days, following Koné et al., (2011) investigations, finding this period to be the longest time observed where fructification occurred in-vivo. During this phase, the media were carefully observed and photographed once per week to find signs of pinning or fruiting. As explained, the main triggers were:

1. Different C/N ratios that may simulate depletion of substrate, without any mechanical damage (nodule removal).

2. The same procedure as 1. but repeatedly removing nodules formed on the substrate every two weeks. In total the experiment lasts 130 days (45 days + 85 days).

The C/N ratios used were as follows: 12:1, 6:1, 3:1, 1:1 and 1:3 and the concentrations (g/L) were divided into 3 variants for each C/N ratio: 8g/L, 16g/L and 32g/L.

135 60mm plates were prepared for every strain. 9 plates for each C/N ratio of every concentration (9 plates x 3 concentrations x 5 ratios = 135 plates). The 9 plates are divided into 3 plates/replicates for the point inoculation treatment and 6 plates for the surface inoculation treatments; the latter being further divided into 3 plates/repetitions each for the treatment with and without nodule removal, respectively. 4 strains were tested, 135 plates per strain, adding up to a total of 540 plates. An overview of the set-up can be found in the appendix (Tab. 3). Every plate was prepared by adding 12ml of media, letting dry and applying 35µl of suspension. Point inoculation treatments were used to assess the effect C/N and nutrition on colony size, plots correspond to these treatments.

Experiment 2: Effects of different carbon sources and the addition of a casing layer on the growth and development of fruiting bodies of *Termitomyces spp.*

Two strains of *Termitomyces spp*. (T89 and T73 and one of *Blastosporella zonata* were initially inoculated on malt-yeast-agar (MYA) and multiplied into new petri dishes. Once these plates were fully colonized, they were gently scraped with a spatula to obtain spore containing nodules. The scratched material was placed into two Eppendorf tubes together with 1ml of saline; the suspension was grinded carefully until it became a cloudy suspension. Subsequently, the suspension of both tubes was transferred into a big (5ml) Eppendorf tube and diluted with 2,5 ml of saline. The suspension was completed by adding 150µl of PS antibiotics (Penicillin-Streptomycin) and vortexing it for a few seconds.

The aim of this experiment was to imitate the shape and composition of the fungus comb in nature by using structurally complex substrates, as well as investigate the effect of fecal substrates on the growth, development and fruiting body formation potential of the fungus. The strains were inoculated in a mixture based on a modified version of sporeslab.io´s MYCO-PRO™ Agar Petri Dish Formula, which consists of different proportions of bacteriological grade agar, malt extract and milled rye grain. The exact composition can be found in the appendix (Tab. 4). In some treatments, black soldier frass was added to the mix, in the proportions recommended by sporeslab.io (5g per 1000ml). The media were allowed to grow until the mycelium ended its vegetative phase, up to a maximum of 45 days, which is the time when under natural conditions the fungus comb is mature (Liang et. al., 2020; Yang et. al., 2021). The inoculation of the fungi was done in 250ml glass flasks containing the aforementioned media and, as well as in treatments with pure rye grains, pure insect frass with malt extract, and a mixture of insect frass and rye grains. 50µl of suspension was applied per bottle in agar-based media and 100µl in the other complex media.

The second phase of the experiments began once the fungi had reached maturity, in half of the treatments, 2cm of a so called “casing layer” was applied with the intention of triggering mushroom formation. Activated charcoal was utilized as a casing layer following the guidelines used by Noble et. al. (2003). Here, three treatments are used: 1. activated charcoal granules (4mm). 2. Activated charcoal powder. 3. Activated charcoal granules (4mm) with the addition of 2ml plastic tubes with holes in the lid, containing about 2g of soda lime, to reduce the concentration of carbon dioxide in the bottle (Noble et. al., 2003). After 85 days upon the application of the casing, the experiment is terminated. In total 180 bottles were used: 60 per

strain, divided into 12 bottles for each of the 5 media. Of these 12 bottles, 3 were no given any casing layer, while the remaining 9 were, 3 for each casing treatment. The set-up can be found in the appendix (Tab. 5).

Experiment 3: Effect of the presence rabbit feces and casing on the growth and development of fruiting bodies of *Termitomyces spp*.

Termitomyces symbiont spores pass the gut of termite hosts twice as has been explained above in the introduction of fungus comb dynamics. Other animals are also characterized by ingesting and digesting their food twice, e. g., ruminants such as cows, but also rabbits. In addition, rabbits feed predominantly on lignocellulosic material similar to plant material termites feed upon. For these reasons, a simplified version of experiment 2 was performed replacing black soldier frass with rabbit feces.

Analogous to experiment 2, a modified MYCO-PRO™ medium was used in two variants for the two *Termitomyces* strains T88 and T73. In the first variant, 5 grams of pure rabbit feces were crushed and mixed with the other ingredients, autoclaved and poured inside of 100ml glass flasks. In the second, the procedure was done with crushed rabbit feces and wooden pellets. A last treatment is prepared by mixing a bit of hay with uncrushed rabbit feces, wooden pellets and 15ml of water, then autoclaved and inoculated. Suspensions of both strains were made from plates containing mature nodules, scraping the latter with a spatula, crushing and adding the nodules to 1ml of saline, so creating a cloudy suspension that was finally vortexed and inoculated. All bottles were inoculated with 50µl of suspension. In total 20 bottles were used, 10 bottles for each strain. 4 of the 10 bottles were filled with agar with frass, 4 with agar with frass and wooden pellets and the last 2 bottles with hay and uncrushed rabbit feces and wooden pellets. Bottles were grown at 30°C

After 45 days, a 2 cm casing layer consisting of activated charcoal granules (4mm) was applied to some of the treatments, in others, the same casing and 1 soda lime filter were added, and in other samples 3 soda lime filters are put inside. The overview of the set-up can be found in the appendix (Tab. 6).

Experiment 4: Growth behavior of *Termitomyces spp.* and sister genera on different concentrations of frass mixed with water-agar or malt-containing agar

The rationale behind this experiment is to assess whether the presence of frass fosters the growth behavior observed for *Termitomyces* in pure frass during experiment 2. In the latter, isolated hyphal clusters were observed growing on top of frass powder. The goal of this experiment was to determine whether this growth is observed again, and at what concentration(s).

8 different media containing either 0, 1, 10 or 100 g crushed black soldier frass per liter water were prepared to test the effect of frass on the growth of fungi. 4 of these media were prepared by adding the respective concentration to 20g agar and 1 l of water and then autoclaving the mix. The remaining 4 media contained the same ingredients and 10% of the weight of the added frass in malt. Malt was used to determine if lack of absorbed nutrients caused the aforementioned growth behavior. Proportions can be found in the appendix (Tab. 7). 12 ml of the media are poured in 60mm petri dishes. Every strain had 4 replicates for every frass concentration, in either water-agar or malt-containing agar. I. e., there were 32 petri dishes per strain. In this experiment *Termitomyces* strains T73, T89, *Blastosporella* and *Arthromyces* were used. Hence, a total of 128 plates were prepared.

Experiment 5: Effect of the presence of tannic acid, exposure to light and cold in the development and fruiting body formation of *Termitomyces spp.*

Tannic acid has been reported to trigger the activity of laccases for other basidiomycetes, such as *Coriollopsis gallica* (Carbajo et. al., 2002). In turn, increased laccase activities have been associated with the formation of mushrooms for different agarics (Kües & Liu, 2000; Kües & Rühl, 2011). In order to assess the effect of laccases on the growth and development of mushrooms in *Termitomyces,* the following experiment was performed:

Termitomyces strains T73 and T89 were gown in 3 different MEA-based media. The first medium is plain MEA, the second is MEA with a concentration 100 µM of tannic acid, and the third is MEA is the same as the second but with the addition of 5g per liter water of black soldier frass. Exact recipes can be found in the appendix (Tab. 8). In total, 15 petri dishes were used per strain; every 5 plates contained one of the aforementioned media.

De (1983), published a curious study reporting successful production of basidiocarps of *Termitomyces microcarpus,* after exposing the inoculum to light for prolonged periods. No other study reporting such outcome in same conditions has been published, hence the feasibility of this approach to obtain *Termitomyces* mushrooms is doubtful. In order to test the effect of light on the mushroom formation potential of the fungi used in this experiment, 2 plates of each strain are exposed to light (37W) at 30°C, after 45 days from the inoculation. Likewise, temperature changes are reported to trigger mushroom formation in other fungi. In order to test the latter, 45 days after starting the experiment, 2 plates of each strain were exposed for 3 hours to 4°C and then put back on incubators at 30°C. Of every 5 plates containing one of the media mentioned, one was grown as a control. 20 days after the exposing the plates to light and cold stress, the experiments were terminated (due to time).

Experiment 6: Inoculation of fungus comb with 2 *Termitomyces* strains

To test the suitability of old fungus comb as substrate in-vitro, 2 little pieces of fungus comb of *Macrotermes Natalensis* termites (4 cm x 3 cm) were autoclaved in glass jars and then inoculated with 500µl of fungal suspensions, containing either T88 or T73 spores. The entire surface of the comb was evenly pipetted with the suspension. These suspensions were made by scraping the surface of mature mycelium with a spatula and adding the nodule mass to 1ml of saline until a cloudy suspension is formed. The liquid gathered in the bottom of the jar was pipetted out, and the jars were then closed.

Experiment 7: Effect of spore density on mycelial growth and asexual spore formation of *Termtitomyces* and sister genera *Arthromyces* and *Blastosporella*

In experiment 1, it was observed that petri dishes with the same nutrition and C/N differed noticeably in the production of nodules. Moreover, it was often observed that although established colonies had the potential to colonize the entire surface of plates, they did not do so even after months of incubation. In addition, phenotypes and nodule yields of point-inoculum and spread-inoculum plates differed greatly among each other. Bastiaans et al. (2015), showed in experiments with the ascomycete *Neurospora crassa* that spore density has a no effect on asexual spore yield of the fungus when fusion among spores is possible. In this experiment, it was systematically tested if spore density also resulted in the same nodule yields for

Termitomyces (see stages 1 and 2 below). Furthermore, in *Neurospora crassa* it has been found that spore density does not have a significant effect on the size of growing colonies, but only on the time to achieve a certain size. I.e., initially higher spore densities grow faster than lower ones (but all densities reach similar sizes eventually) (Richard et. al., 2012). The effect of spore density on colony growth was also investigated in *Termitomyces* (see stage 3 below).

These observations and studies gave rise to the question of what effect spore density has on the growth and development of asexual spores (nodules) of *Termitomyces* and sister genera. To test the latter, the following experiment is devised:

A first original suspension was made by scraping the surface of petri dishes containing mature mycelium and conidia for all 4 different fungi (T73, T88, *Arthromyces* and *Blastosporella*). The scraped material was added to a 1ml Eppendorf tube, crushed with an electrical grinder and vortexed with 1ml of saline. 10µl of every suspension is diluted with 90µl of saline in a new Eppendorf tube to use in counting plates. Each of the 4 diluted suspensions was pipetted into a hemocytometer to count the spore concentrations of suspensions. Once the diluted spore density had been established, the original suspension density was calculated. The original suspension of every strain was subsequently diluted 7 times in steps of 5x. In the end, every strain had 8 suspensions with decreasing spore numbers. Spore numbers for every suspension of each strain can be found in the appendix (Tab. 9).

This experiment was separated in 3 stages:

Stage 1 – inoculation of all 4 strains on the surface of 90mm petri dishes containing 1/5 MEA. Only T88 was analyzed.

Here, 5 petri dishes for every suspension were used, giving a total of 40 petri dishes per strain. Plates containing the medium are inoculated with 50µl of suspension, the liquid was spread with glass beads which were removed after inoculation.

Stage 2 – inoculation of T88 by mixing the spores in the agar of 60mm petri dishes containing 1/5 MEA.

Here, 3 petri dishes for every suspension were used, giving a total of 24 petri dishes. Plates containing the medium were inoculated with 35µl of suspension during the pouring of the agar at 42°C.

Stage 3 – point inoculation of T88 on the surface of 60mm petri dishes containing 1/5 MEA.

Here, 5 petri dishes for every suspension were used, providing a total of 40 petri dishes. Plates containing the medium were inoculated by pipetting 2µl of suspension in the center.

Stage 1 treatments were let grow for 14 days, other plates were let grow for 21 days, afterwards experiments were terminated. 1/5 MEA was used, as the relatively low nutrition available should promote mycelial growth, making comparisons in colony sizes more evident.

3. Results

High C/N´s foster coverage of the plate with mycelium and nodules while low ratios decrease growth and produce thin and elongated nodules

Poor (8g/l), medium (32g/L) and high (64g/L) nutrient availability all influenced the growing behavior of *Termitomyces* (Fig. 6; Fig 7). As a reference, 20g/L was considered to be a standard nutrient concentration. The growth behavior is analyzed in the next paragraphs below based on the results of T88 as: 1. all treatments grew. 2. results were consistent throughout all samples. Also, point inoculation samples were used for the analysis, unless stated otherwise. In contrast, for both T73 and *Arthromyces*, several treatments did not grow at all, making this analysis impossible (Appendix: Fig. 3). *Blastosporella zonata*, was also attempted to be grown, however, dire contamination and a late realization of it prevented the analysis for this strain (Appendix: Fig. 4).

For these results, the colony size of all point inoculation treatments with their respective replicates were estimated. While all plates showed growth, the latter changed with nutritional conditions. In poor nutrient availability conditions (8g/L) and in medium ones (32g/L), there is a clear trend: the higher the C/N the bigger the growth. For high nutrient availability conditions 64g/L this is not the case, as all but the treatment with the lowest C/N reached similar sizes (Fig. 6). Moreover, the lower the C/N ratio, the smaller the coverage of the plate with mycelium (Fig. 8). For the treatments with the two lowest nutritional conditions (8 and 32g/L), the highest carbon fraction treatment (12/1) produced noticeable more mycelium than any of both the 1/1 and 1/3 treatments. However, the trend was not so clear for rich nutrient availability (64g/L) treatments, where almost all treatments covered the surface of petri dishes with 1400-1600 mm^2, excepting the treatment where nitrogen had the biggest fraction (1/3). The last, grew considerably smaller, reaching an area of only around 600 mm^2. For their part, spread inoculation samples, all covered the whole surface of their plate with aerial mycelium regardless of the C/N ratio. In general, treatments with lower nutrition and higher C/N showed slightly lager growth, perhaps being more explorative to obtain nitrogen.

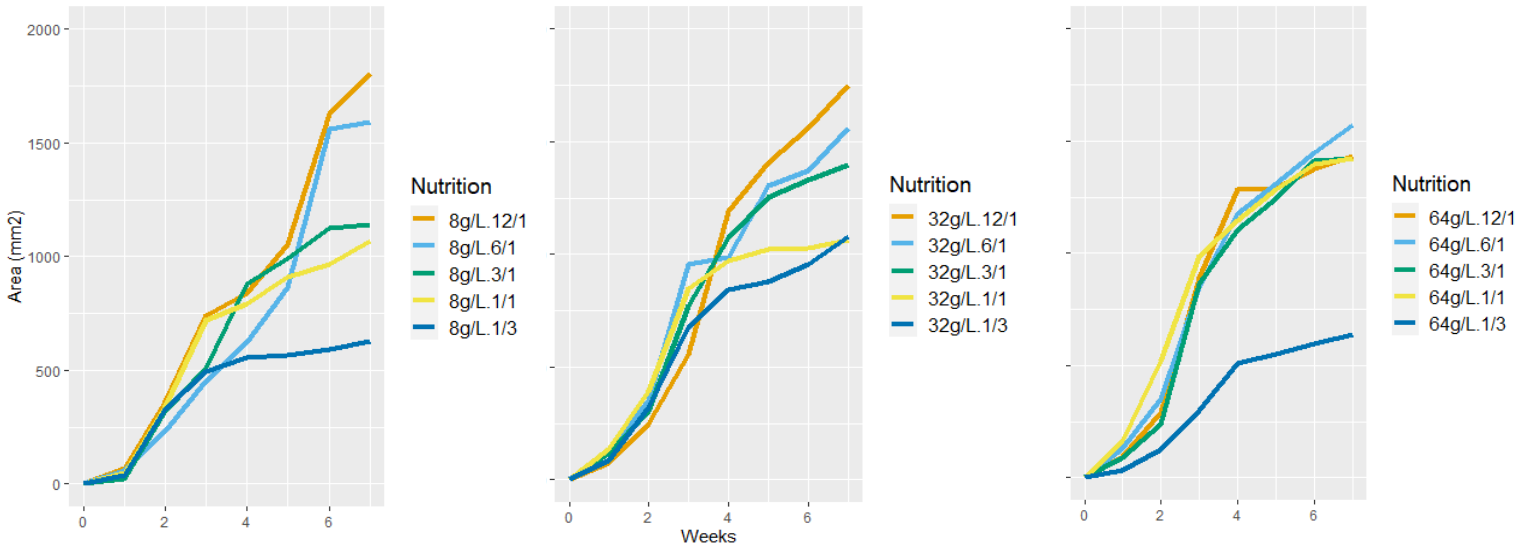

Figure 6. Growth response of single colonies of Termitomyces cryptogamus "T88" over 7 weeks based on nutrition availability and C/N. The plot corresponds to point inoculation treatments. Left: Treatments with media containing 8g/L (left), 32g/L (center) and 64g/L (right) of nutrition. X axis represents weeks while Y axis the area of colonies in mm2. C/N ratios of each treatment are indicated in the legend.

Figure 7. Reference of the effect that nutrition availability had on mycelial growth of individual colonies. The pictures correspond plates of Termitomyces "T88" with a C/N ratio of 1/3.Treatments with a nutrition availability of 64g/l (left), 32g/l (center) and 8g/l (right) are shown.

Regarding nodule production, poor nutritional conditions (8g/L) created fewer nodules when compared to treatments richer in nutrition, regardless of their C/N ratio. This was apparent in the phenotypes of point inoculation but also for spread inoculation samples (Fig. 9). Moreover, all treatments with 1/1 and 1/3 ratios produced thin and elongated nodules, as opposed to all other treatments, where packed masses of nodules with circular shapes were observed (Fig 7; Fig 9). Nodular mass was highly likely larger than observed, as nodules were growing in layers, in a "3-Dimensional" structure. Due to the nature of this analysis (pictures), images can only be analyzed in 2 dimensions. Thus, the observed nodular mass likely represents an underestimation of reality. Furthermore, the size of 3-D nodules plays a role in the number of spores that are contained inside of nodules. Bigger nodules contain more spores than little ones even if both cover the same surface area, simply because the volume of bigger nodules is larger (see discussion and appendix: Fig. 5).

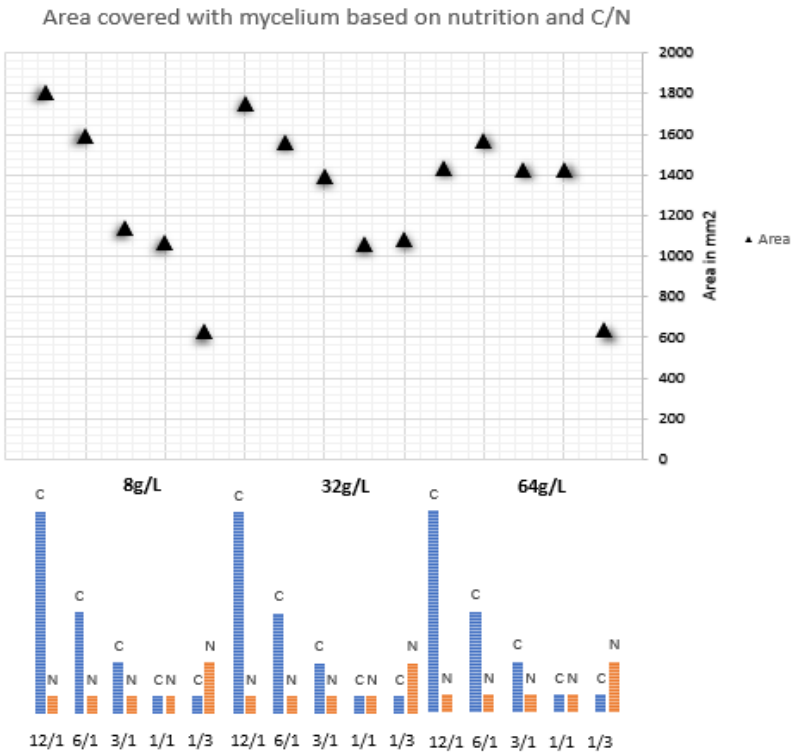

Figure 8. Effect of C/N and overall nutrition availability on the mycelial coverage (in mm2) of Termitomyces "T88" (point inocula). On the x-axis: all 5 C/N ratios (12/1; 6/1; 3/1; 1/1; 1/3) tested and each of the 3 nutrition levels used (8, 32 and 64 g/L). hence, in total 15 treatments were tested. C to N proportion is visualized in the bars below the chart. Blue bars represent carbon and orange bars nitrogen. On the y-axis: The area covered with mycelium in mm2. The black triangles represent the of each one of the 15 media/treatments used.

Mechanical damage is only limitedly offset by *Termitomyces* in terms of mycelial growth regardless of nutrition and C/N, in addition, nodules are not regrown.

For all 15 treatments, plates that were scraped saw little mycelial regrowth even after months of the stress application. It was evident that no nodule masses were formed, only packed hyphal networks were observed for some treatments, while others regrew only extremely slowly. Some scraped plates show nodules; however, the latter are remnants from the scraping process (Fig. 10). The mycelium removed was collected in a plate and observed for duration of the experiment, showing no signs of growth (Appendix: Fig. 6).

Figure 9. Nodules produces by Termitomyces "T88" based on different nutrition availability. 64g/l (left), 32g/l (center), 8g/l (right). The upper row shows point inoculum treatments, the lower row shoes spread inoculum treatments. Images shown correspond to a C/N of 6/1.

Nutrition level, C/N ratio and mechanical damage did not trigger mushroom formation

None of the tested factors in this experiment appeared to trigger the production of mushrooms even after months of observation.

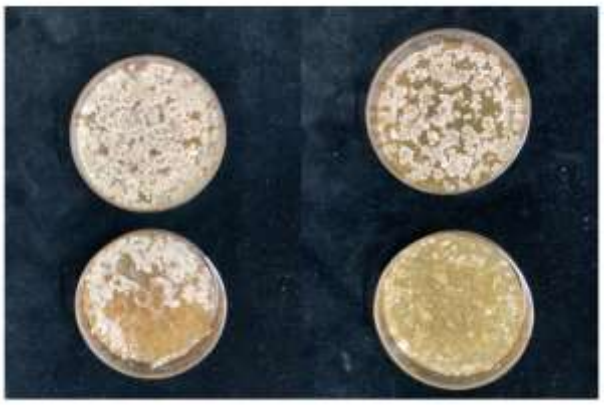

Figure 10. Difference between scraped (down) and untouched plates (up). It can be clearly seen where mycelium was scraped as the yellow coloration of the agar is visible

Different C-Sources result in distinct growing patterns in *Termitomyces* and *Blastosporella*.

After inoculation and during the first weeks, a few bottles of *Termitomyces* "T88" showed contaminants, which is why they were removed. Remaining bottles in the end of experiments can be found in the appendix (Tab. 10).

For T73, in both agar-based treatments, the area was evenly covered with mycelium. Interestingly, these treatments without frass yielded a noticeable higher biomass than the ones mixed with frass (Fig. 11). In "complex" media, none of the substrate was completely colonized either, and only mixed growth responses were observed. Pure frass treatments saw virtually no growth except for individual, little, long threads of hyphae that were observable in the surface (images not shown). At first, these structures were confused with salt crystals but a closer look revealed it was indeed mycelium that had grown. Samples were frass and rye were mixed showed no apparent growth. Moreover, bottles filled with rye colonized only little specific patches of the substrate, except for 1 bottle where growth was more evident. However, the latter did not colonize the whole substrate either (Fig. 11).

Figure 11. Growth of Termitomyces "T73" in different substrates. From left to right: MEA, frass MEA, pure frass, rye, rye mixed with frass

For T88, treatments showed similar growth for both agar-based treatments (Fig. 12). Here, a few individual colonies grew, never covering the entire surface. Pure frass "complex" treatments showed the same growing behavior as explained for T73. However, mycelium seemed to grow more abundantly as the clumps were more evident than for T73. Treatments where rye and frass were mixed displayed no growth either and rye treatments were colonized by mycelium more abundantly than for T73. Also, nodules were more apparent here, though this may not be appreciated in the pictures (Fig. 12).

Analogous to *Termitomyces,* bottles inoculated with *Blastosporella* were able to colonize all substrates, except for rye mixed with frass. Frass mixed in the agar fostered the growth of densely-packed mycelium and elevated structures (conidiophores), when compared to only agar treatments, where aerial mycelium was more prominent and only small patches of conidiophores were observed (Fig. 14). It was apparent that this fungus grew more abundantly in pure frass as compared to *Termitomyces,* since there were considerably more filamentous structures present than for the latter under like conditions (Fig. 13).

Figure 12. Growth of Blastosporella zonata in different substrates. From left to right: MEA, frass MEA, pure frass, rye, rye mixed with frass

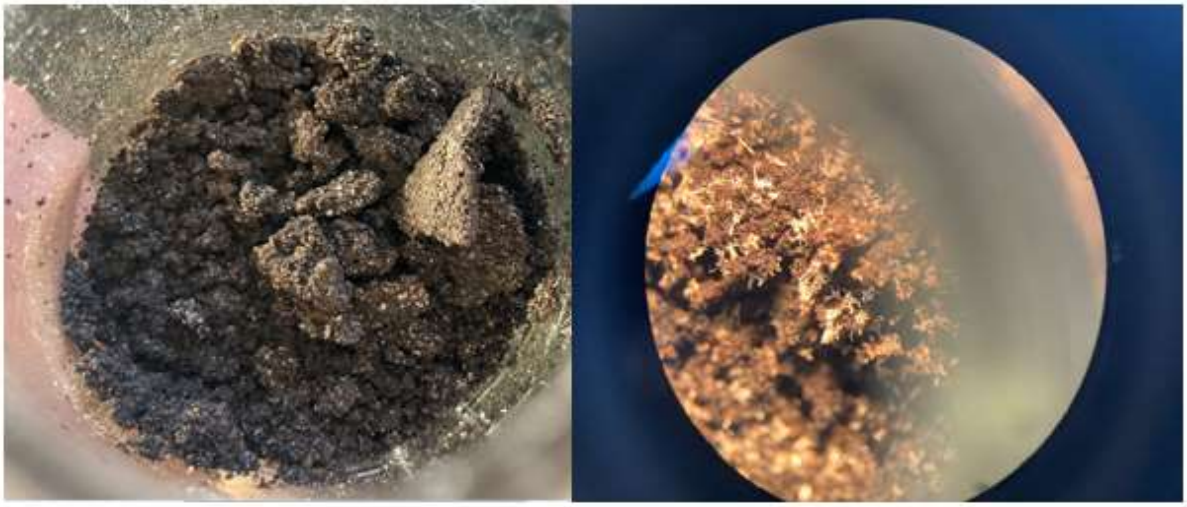

Figure 13. Inside a bottle of Termtiomyces "T88" containing pure frass as a substrate. White spots are clumps of hyphae (left). Binocular view of the filamentous clumps of mycelium for Blastosporella zonata (right).

Figure 14. Growth of Termitomyces "T88" in different substrates. From left to right: MEA, frass MEA, pure frass, rye, rye mixed with frass

Casing did not produce noticeable effects

Treatments where charcoal powder was applied did not elicit growth of the fungus, as none of these samples showed colonization of the casing layer with mycelium, except for one sole bottle where mycelium grew on top of rye and then apparently also on the charcoal powder (Fig. 15). The mycelium underneath the powder was not visible as it sticked to the agar and the mycelium, making it impossible to analyze these samples; however, no anomalies were seen apart from the one bottle that showed colonization of the casing.

Moreover, bottles where charcoal granules were applied, both with and without soda lime filters, did not produce any noticeable effects on the behavior of the fungus (Fig. 15). No signs of initials were observed.

Figure 15. Casing of bottles with activated charcoal. From left to right: Granules with soda lime filter on agar, powder on agar, granules and filter on frass agar, granules on frass, powder on rye in the only bottle where mycelium colonized the casing (visible white spots).

Termitomyces grew well on agar mixed with rabbit feces and other lignocellulosic material

Bottles inoculated showed mixed growth results for both strains. Some samples grew to cover the whole surface of the substrate, while others only developed a few small colonies, independently of whether there was only rabbit frass or also other lignocellulosic mass in the agar. Likewise, nodule formation ranged from only a few nodules produced to abundant nodule yields (Fig. 16). There was no observable difference between treatments with soda lime filters and ones without casing. Neither, did casing have an obvious effect on the growth or development of fungi from what could be seen by moving the casing. Finally, both strains grew and developed nodules poorly on bottles where pure rabbit feces were mixed with wooden pellets and hay (Fig. 16). There were no signs of mushroom initials.

Figure 16. Bottles containing different casing treatments and media based on rabbit feces and Termitomyces "T88". From left to right: frass-agar with wooden pellets and without casing, frass-agar with crushed wooden pellets and without casing, frass-agar with crushed wooden pellets and with soda-lime filters, frass-agar with activated charcoal granules, frass-agar with activated charcoal and a soda lime filter, different lignocellulosic material and complete rabbit feces (little nodules observable on the top of a straw).

Higher frass concentrations produced more fungal biomass on both water frass-agar and malt frass-agar (T88)

In the initial days, several plates were contaminated after pouring agar; in some cases, only one plate was left. Remaining/analyzed plates can be seen in the appendix (Tab. 11).

In general, T88 grew better on media containing higher frass concentrations than on ones poorer in frass. Also, malt containing plates produced substantially more spores than water agar treatments for this fungus (Fig. 17). Water-agar plates displayed similar growing behavior to malt treatments, except that samples containing 10 and 100g/l of frass showed smaller growth but thicker-packed nodules. These structures

resembled much the crystal-like hyphal structures observed in the bottle treatments with frass only (Fig. 18). Likewise, none of the treatments showed signs of mushroom formation.

For both T73 and Arthromyces, only small growth was observed. In both cases, one plate of the treatment with malt and 100g/L frass covered the entire surface with mycelium, though this could be a contaminating organism. These results were not plotted.

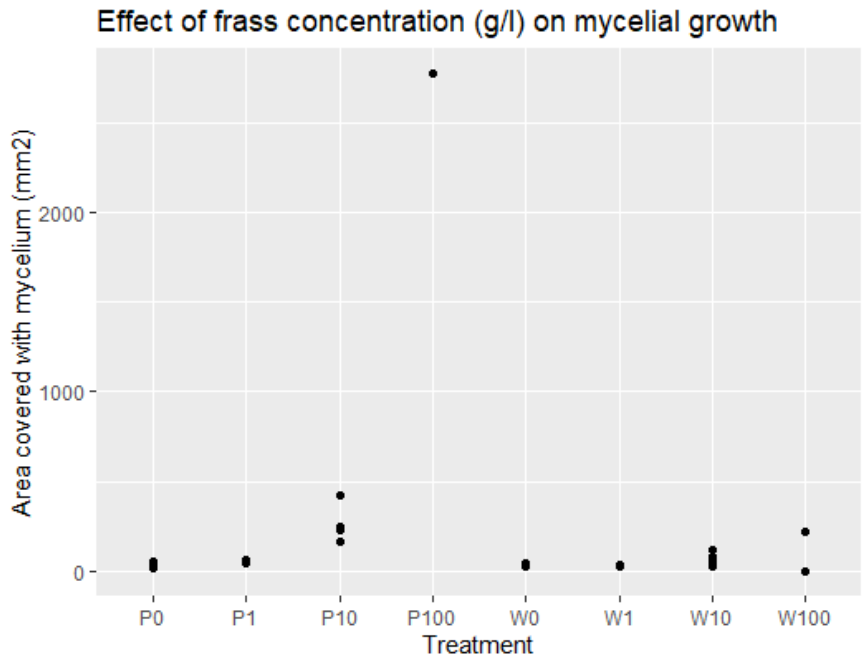

Figure 17. Growth of Termitomyces "T88" on different frass concentrations mixed with agar or agar and malt. P refers to treatments containing malt (10% of the frass grammage) and W refer to water-agar treatments. Numbers 0 to 100 show the amount of frass present in the medium in g/l. E.g., P10 refers to a medium containing 10 grams of frass per litter of water and 1g of malt extract.

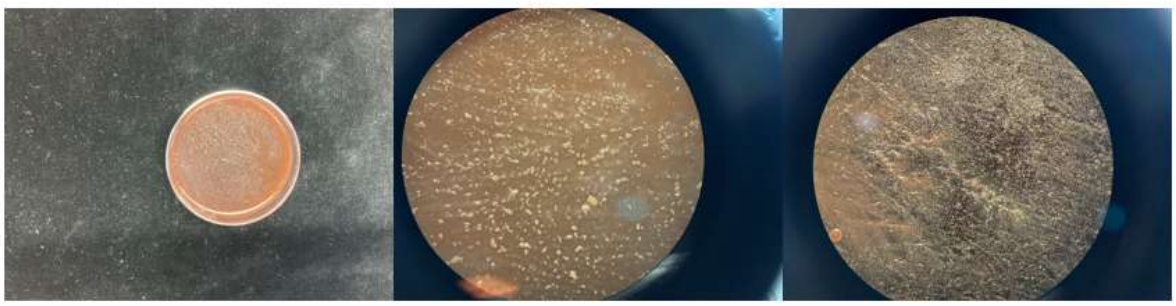

Figure 18. Termitomyces "T89" growing on water-agar plate of a medium containing 10g/l frass (first two pictures left). Plate of the same type of medium but containing 100g/l frass for the same strain.

Tannic acid presence produces less and focalized formation of nodules

All T88 treatments grew. The presence of tannic acid did not have a noticeable effect on the phenotype of the fungus. MEA treatments grew covering the whole surface of plates and producing thin but abundant nodular complexes. In contrast, samples containing tannic acid (TA) presented mixed growth covering the surface of plates, but with thinner mycelium and focal patches of nodules. However, the differences between both MEA and TA treatments were not so marked. Furthermore, treatments containing frass and TA covered

the whole substrate surface and generated abundant nodules, while also showing a browner coloration throughout the mycelium. These plates showed no evidence of mushroom formation. The plates presented got contaminated at a later stage, which is visible in the images, but nodules may still be appreciated (Fig. 19).

Similarly, T73 plates showed mixed results for all treatments, except for the frass with TA samples that were exposed to light and cold; these showed noticeable thick mycelium packed with nodules. Plates containing MEA grew only poorly; the reason for this is unknown (Fig. 19).

Treatments where light and cold stress was applied did not show signs of mushroom formation

The phenotype of plates that were exposed either to light or cold stress did not apparently vary from control treatments, except for those of T73 where mycelial biomass was thicker as compared to control frass/TA samples (Fig.19). Moreover, plates exposed to light were contaminated, as condensed water probably facilitated colonization of alien fungi. None of these treatments achieved mushroom formation either.

Figure 19. Phenotypes of treatments testing the effect of tannic acid, light and cold stress. The first row corresponds to "T88" and the lower row to "T73". Plate treatments from left to right: Frass and tannic acid mixed in MEA, Frass and tannic acid mixed in MEA and exposed to cold, Frass and tannic acid mixed in MEA and exposed to light, Tannic acid mixed in MEA, plain MEA. Green spots visible in treatments containing frass and tannic acid are contamination.

Fungus comb did not elicit any type of growth

Even though mycelium was visible prior to autoclaving the comb, none of both tested *Termitomyces* strains showed any growth in the comb fragments used (Fig. 20).

Figure 20. Fungus comb of Macrotermes Natalensis that was inoculated with "T88" showing no signs of growth. Left: Entire comb. Right: Close-up showing the architechture of the comb

There is an optimal spore inoculation density for spore yield

T. cryptogamus displayed different growing patterns based on the number of spores that were present in the substrate. These patterns were observed both when the spores were mixed in, or placed on the surface of the agar. In both cases, mycelium of treatments with low spore densities colonized small fractions of the plates only, while increasing spore density resulted in an increased coverage of plates until the plate was completely covered from middle high spore densities (2.48×10^5) on (Fig. 21; Fig. 22; Fig. 25). While mycelium coverage increased with higher spore numbers, nodule yield does so only up to an optimum spore density above which, reduced nodule formation was seen. That was evident both when spores were mixed in the agar and inoculated on top. As for the first, we found the optimal spore density for nodule yield to be $4.96*10^4$ and for the second between $1.24*10^6$ and $6.2*10^6$ spores (Fig. 23; Fig. 24). Moreover, in treatments where spores were mixed in the agar, for the two highest spore densities nodules were not observable at all.

Pairwise comparisons of all treatments confirmed the previously mentioned observations for both inoculation methods. In the case of superficial inoculation, the nodule area for both 1.24×10^6 and 6.2×10^6 varied significantly from the nodule areas obtained with low spore densities and with the highest spore density treatments. Additionally, the latter values did not significantly differ from each other in terms of the nodule area produced (Appendix: Tab. 12).

Likewise, pairwise comparisons of the treatments performed with spores mixed with agar indicate that the nodule area that resulted from the suspension with $4.96*10^4$ spores (yield optimum) differed significantly from all other spore density treatments, except the one with 9920 spores, which yielded a slightly smaller nodule area (Appendix: Tab. 12) (Fig. 24).

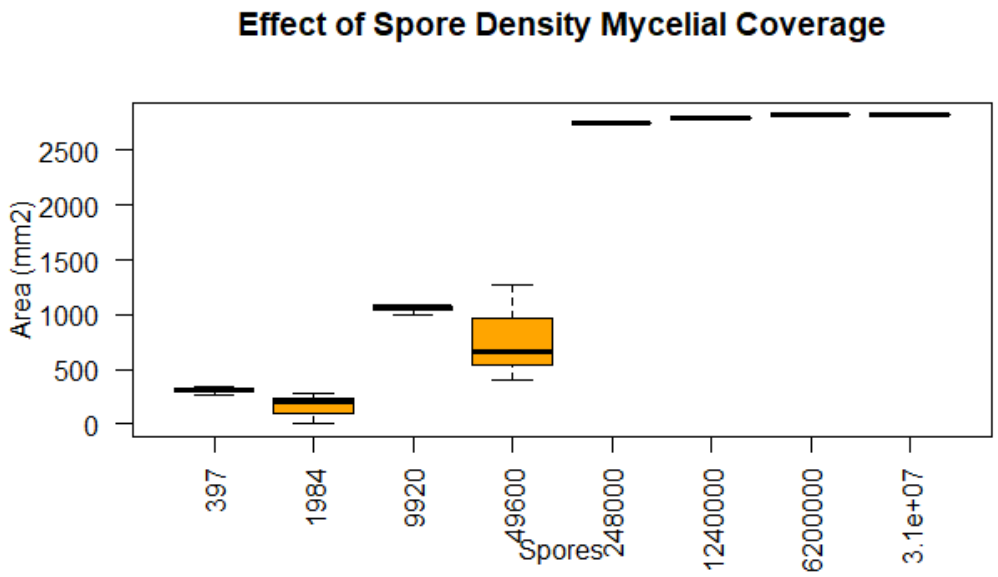

Figure 21. Boxplot showing the effect of spore density on the coverage of plates with mycelium when inocula are mixed with the agar being poured in plates, for "T88". The x-axis shows all 8 spore densities used, while the y-axis exhibits the area covered (in mm²) by mycelium. Boxplots show the range of values obtained by the respective spore density treatment. Whiskers and points above or below the boxes represent the maxima or minima observed for the respective treatment, whereas the marked line inside of boxes depicts the mean for the treatment.

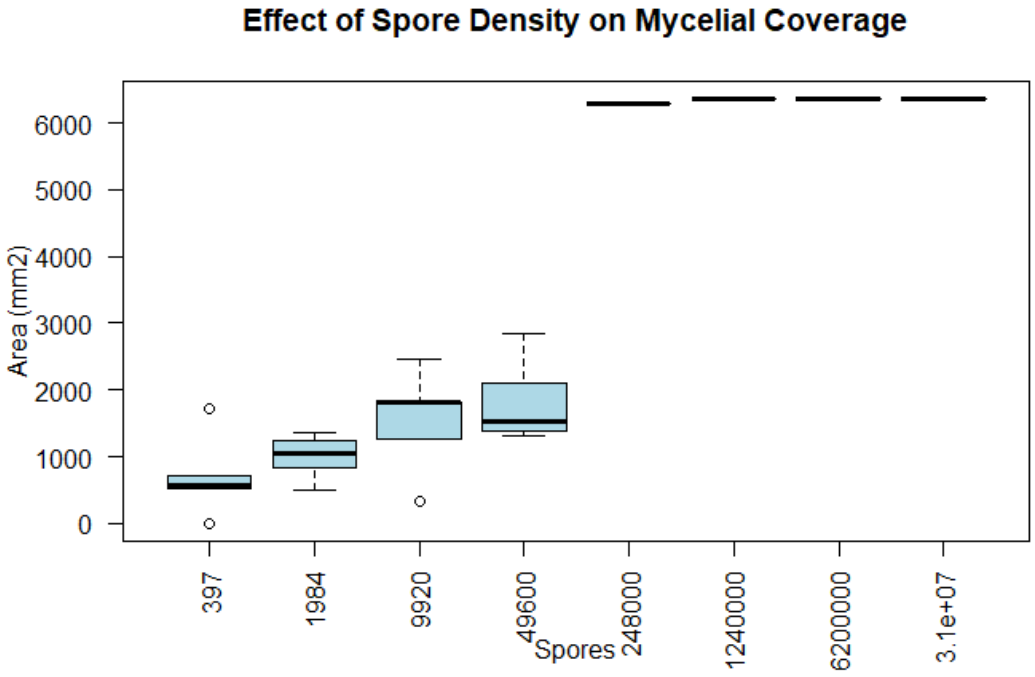

Figure 22. Boxplot showing the effect of spore density on the coverage of plates with mycelium when inocula are spread on the surface of plates, for "T88". The x-axis shows all 8 spore densities used, while the y-axis exhibits the area covered (in mm²) by mycelium. Boxplots show the range of values obtained by the respective spore density treatment. Whiskers and points above or below the boxes represent the maxima or minima observed for the respective treatment while the marked line inside of boxes depicts the mean for the treatment.

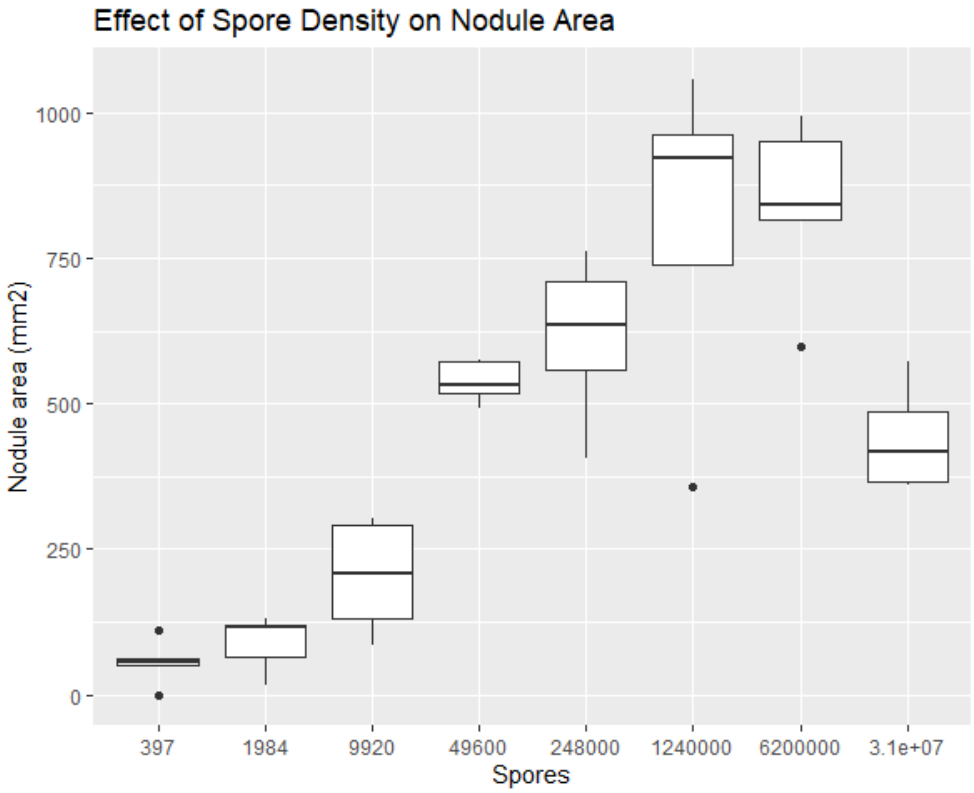

Figure 23. Boxplot showing the effect of spore density on nodulation of the fungus when inocula are spread on the surface of plates, for "T88". The x-axis shows all 8 spore densities used, while the y-axis exhibits the area covered (in mm^2) with nodules. Boxplots show the range of values obtained by the respective spore density treatment. Whiskers or points above or below the boxes represent the maxima or minima observed for the respective treatment while the marked line inside of boxes depicts the mean for the treatment.

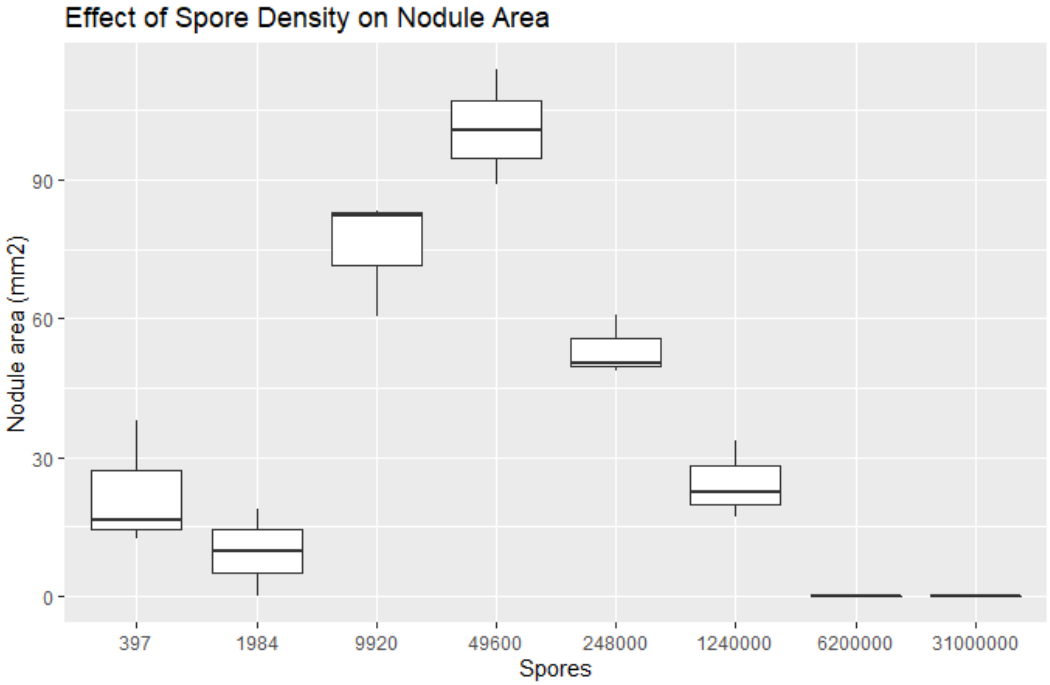

Figure 24. Boxplot showing the effect of spore density on nodulation of the fungus when inocula are mixed with the agar during the pouring of plates, for "T88". The x-axis shows all 8 spore densities used, while the y-axis exhibits the area covered (in mm^2) with nodules. Boxplots show the range of values obtained by the respective spore density treatment. Whiskers above or below the boxes represent the maxima or minima observed for the respective treatment, whereas the marked line inside of boxes depicts the mean for the treatment.

Colonies grow to a certain size and then cease to expand

In low and medium-low spore densities, one would have expected plates to be completely colonized, perhaps taking longer than treatments with more spores; nonetheless, this was not the case. It was observed that colonies tend to stop growing after reaching a certain size which changed depending on how many other colonies were found in their surroundings (Fig. 25).

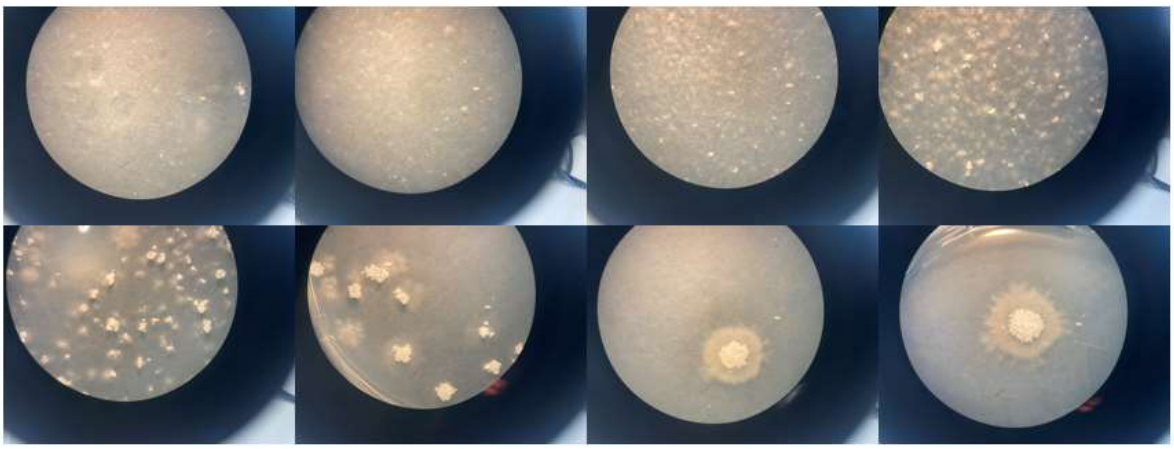

Figure 25. Close-up of phenotypes of "T88" when inoculated on the surface of plates, at 8 different spore densities. Descending spore densities from left to right and from the upper to the lower row. In other words, the plate in the first row left has the highest spore density and the one in the lower row right the lowest. Higher spore densities show little nodulation while the latter becomes more evident with decreasing spore number. Pictures in the second highlight the effect of neighboring colonies/spores on the growth of colonies, despite space for growth is vastly available, colony growth seem to tune to neighboring spore numbers.

For point inoculation treatments, spore density plays a relevant role in mycelial growth but not so much in nodule yield

Low spore numbers imply that there is a chance that spores do not germinate; in this experiment, this occurred for the two lowest densities. In analogy to the results shown above, in point inocula, if the spores did germinate, they did not colonize the complete surface of the petri dish, but stopped growing when reaching sizes of 400+ mm^2. Nevertheless, spore density apparently does have an effect on the size colonies reach. Low spore densities produce smaller colonies while higher ones produce bigger ones up to reaching a limit ($1.24*10^6$ spores). Thereafter, spore density does not make any difference in regard to colony size (Fig. 26). These observations are supported by pairwise Tukey tests, which show significant differences between subsequent increasing spore density treatments. Moreover, treatments with the three highest spore densities did not differ significantly from one another in the colony sizes they produced, i. e., spore number ceased to have an effect on fostering colony growth (Appendix: Tab. 14). In contrast, nodule yield did not vary significantly between the different spore densities that germinated (all except for the two lowest) (Fig. 27). That became evident in the pairwise post-hoc turkey tests performed, where none but one comparison (9920 vs $2.48*10^5$ spores) indicated significant differences (p-value 0.498) in the nodule yield produced by the different treatments (Appendix: Tab. 15).

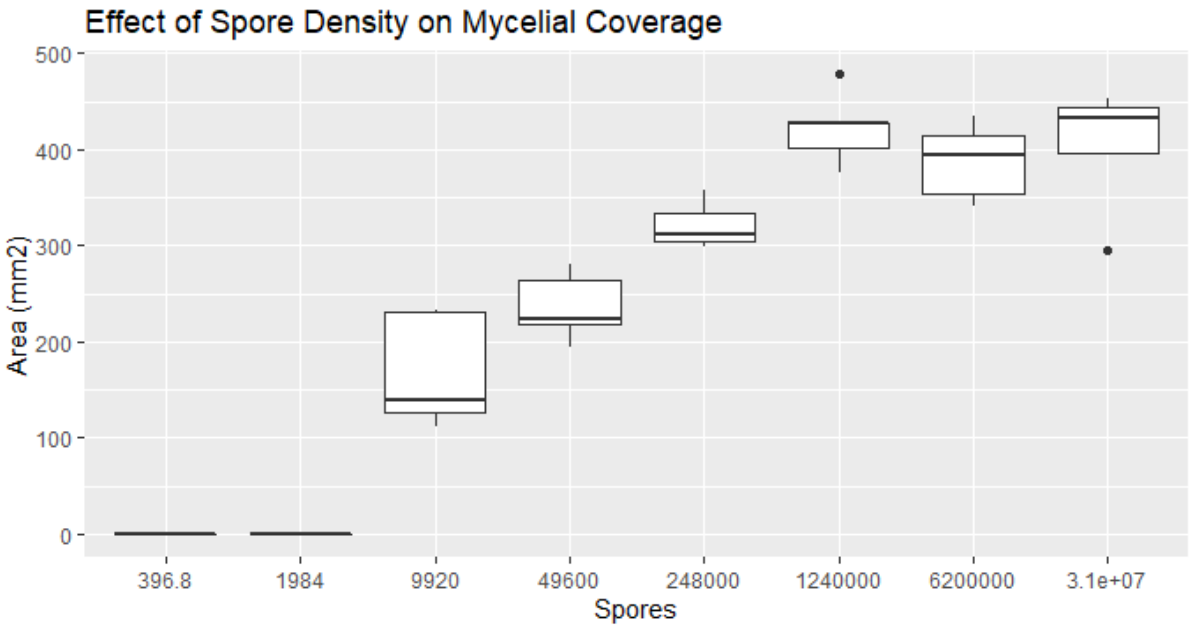

Figure 26. Boxplot showing the effect of spore density on the coverage of plates with mycelium, when point inoculation is used, for "T88". The x-axis shows all 8 spore densities used, while the y-axis exhibits the area covered (in mm2) by mycelium. Boxplots show the range of values obtained by the respective spore density treatment. Whiskers and points above or below the boxes represent the maxima or minima observed for the respective treatment, whereas the marked line inside of boxes depicts the mean for the treatment.

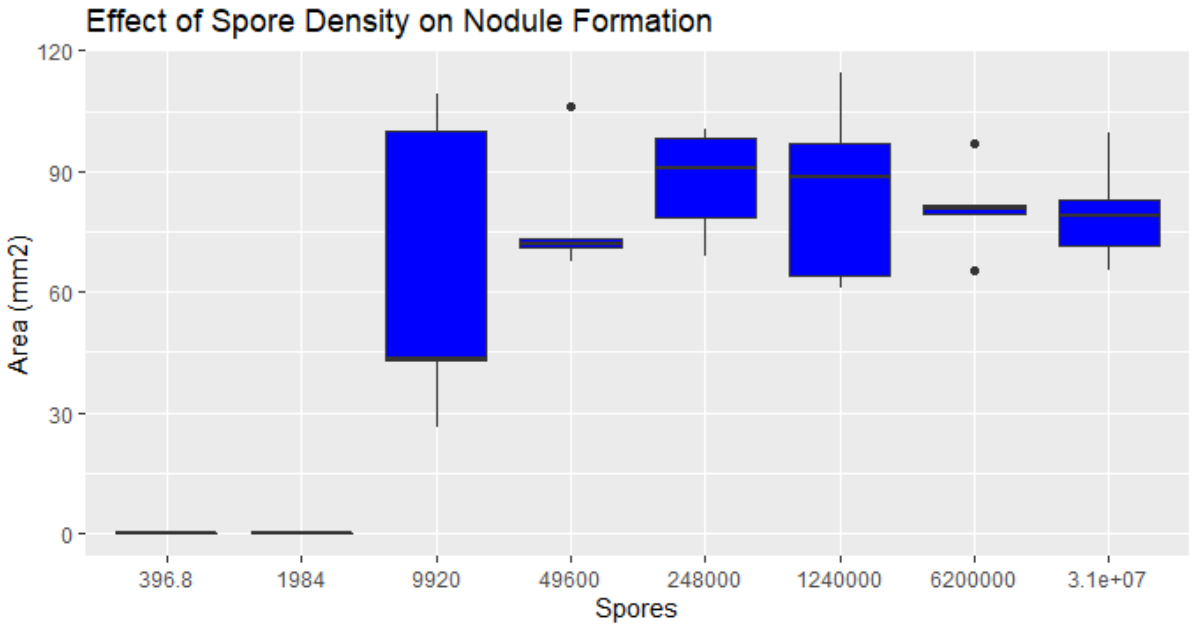

Figure 27. Boxplot showing the effect of spore density on nodulation of the fungus, when point inoculation is used, for "T88". The x-axis shows all 8 spore densities used, while the y-axis exhibits the area covered (in mm^2) with nodules. Boxplots show the range of values obtained by the respective spore density treatment. Whiskers or points above or below the boxes represent the maxima or minima observed for the respective treatment, whereas the marked line inside of boxes depicts the mean for the treatment.

4. Discussion

General Discussion

Several experiments have been conducted to assess the growth and development of *Termitomyces spp.* (and related genera) in and on different substrates and under different abiotic conditions. These experiments had the goals to elicit and expose trends of the somatic and asexual dispersal of the fungus in-vitro, as well as to attempt to trigger the initiation of mushrooms of the different strains grown. The relation between nutrition, spore density, growth and conidiophore formation in in-vitro conditions were studied, demonstrating that the first two play a critical role in the mycelial growth and nodule yield. In addition, despite the marked effect that nutrition and spore density had on the development of fungi, low spore densities and point inoculations revealed that *Termitomyces* single-spore colonies grow only to relatively small sizes even though space and nutrition for growth were available, and that these observations were highlighted under low to medium spore densities. Black soldier frass and rabbit feces were used as novel substrates, both in pure form and mixed in agar and in many cases, they supported growth and nodulation of *Termitomyces* or exposed a growth reaction where the fungus grew in isolated clumps of mycelium. Tannic acid did not elicit extreme growth responses of the fungus and neither did it produced marked tendencies besides a slightly brown coloration in some plates. Fungus comb was used as a substrate but failed to support mycelium growth at all.

Furthermore, no sexual fruiting bodies were obtained throughout the six months of this research despite several attempts to induce mushroom formation. *Termitomyces* is undoubtably particular in its ecological conditions and its patterns of mushroom formation. Mushroom production for this genus remains an enigma even after eight decades since its description by Heim (Heim, 1942). This research, both in the context of sexual and asexual reproduction sought to expand the knowledge about the ecology and fructification of *Termitomyces*. In the following, the results presented here will be linked to the ecology and evolution of the fungus and to the consequences that the phenotypes observed during these experiments may have for *Termitomyces* in nature.

Somatic growth in *Termitomyces a* result of nutrition, spore density and somatic fusion?

One would expect *Termitomyces* mycelium to be adapted to frequent mechanical damage, as nodules are recurrently removed by termites under natural conditions. Interestingly, our experiments showed that mycelium could not consistently regrow after being scraped away. Perhaps, termites compensate the damaged mycelium simply by inoculating new material much more effectively than the fungus would be able to do on its own via somatic growth. Hence, regrowth of mycelium after mycelial injury is likely not fostered by natural selection for *Termitomyces* as it may be the case for other fungi like *Phanerochaete velutina* (Donnelly & Boddy, 1998).

Termite hosts control the nutrient fluxes of their symbionts, providing them with complex foraged substrates such as grassy and woody biomass and with nutritious defecated material enriched in nitrogen by gut endosymbionts (Dangerfield & Schuurman, 2000; Sapountzis et. al., 2016). Based on our experiments, it is expected that high excrement content relative to other food sources limit the growth of mycelium, as it was observed in experiment 2 where rye mixed with frass and pure frass treatments produced no or minimal growth. Likely, high frass contents lack either essential nutrients or easy availability of the latter both of which are needed for mycelial growth. Alternatively, the nutritional composition of black soldier frass can

vary significantly from that of fungus-farming termite excrements, as both organisms feed on different substrates. Black soldier flies are generalists that feed on decaying waste without much selection (Harnden & Tomberlin, 2016), hence, their feces can vary significantly depending on their source of nutrition. Rabbit feces however, are expected to be more similar to termite frass, since the diets of both organisms are based on grassy and woody material, and also ingested food passes the gut of both twice. It would be worthy to design experiments testing the effect of feces of different origins on the growth and development of the fungus.

Moreover, the natural flow of nutrients inside of fungus combs is marked by changes in (foraged) biomass availability and by frequent fluctuations in the C/N ratio due to seasonality and/or colony cycles, e.g., alate production (Dangerfield & Schuurman, 2000; Lapage & Darlington, 2000; Mitchell, 2008). Our experiments testing different C/N ratios and overall nutrition availability, showed that high C/Ns foster mycelial growth, while low C/Ns noticeably limit the mycelial growth of *Termitomyces.* These observations are consistent with the results of Da Costa et al. (2019) that analyzed the effect of protein to carbon ratio on mycelial and biomass growth of *Termitomyces spp.,* finding that biomass production is dependent not only on carbohydrate and nutrition availability but on nitrogen (protein) availability and its proportion to the C sources. Moreover, several plates containing media with higher C/Ns did not colonize the entire surface of plates but ceased growing after reaching a specific size. As seen, low C/Ns may limit biomass production. This does not occur only for *Termitomyces* but also for other fungi that have similar, obligate mutualistic relationships but with attine ants. In the latter, experiments demonstrated that low carbohydrate to protein ratios (P/C), negatively influence biomass yield and mushroom production of fungal cultivars of ants (Shik et al., 2016).

Interestingly, not only does nutrition affect the growth and development of fungi but our experiments demonstrated that spore density also plays a role on both mycelial growth and nodule yield. When analyzing growth behavior based on spore density, our experiments showed that mycelium of *Termitomyces:* 1. grows only to a programmed size until it stops expanding somatically. 2. Inhibits its growth based on the number of spores around a starting colony. Hence, both low C/Ns and low spore density yield similar phenotypes, namely, colonies reaching a certain size that is not able to colonize a substrate even tough space is available for further growth. Growth gets restricted stronger with increasing number of spores, but at high spore densities the effect is irrelevant because the whole surface of petri dishes is colonized by all the germinating spores present regardless. The restricted growth of *Termitomyces* contrasts strongly with the behavior of other higher fungi such as *Neurospora crassa.* Experiments with this fungus, generalized the idea that higher spore densities are beneficial for fungal fitness as they increase the speed to produce asexual spore yield, but that on the long term, yield does not vary in mass based on the initial amount of spores present (Bastiaans et al., 2015; Richard et. al., 2012). This poses the question of how does *Termitomyces* behave inside combs, ultimately, spore densities are expected to be quite high due to termites constantly spreading new inoculum via defecation.

One of the studies cited above, determined that somatic fusion increased the fitness of the fungus as measured by the speed to reach a determined yield in monocultures of *Neurospora crassa* (the yield does not vary over the long term) (Richard et al., 2012). Moreover, it was observed that somatic growth was significantly faster when the starting spore's density was higher for monocultures. Our experiments with point inoculation for *Termitomyces,* showed similar patterns, as plates carrying the two most dense suspensions grew initially and reached their final size faster than treatments containing less pores (not published). This hints at the occurrence of somatic fusion in *Termitomyces* monocultures as well, perhaps

even with an increased rate in the initial states after inoculation. The phenomenon of somatic fusion is still poorly understood for this fungus (Nobre et. al., 2014; Hsieh et. al., 2017), but it is likely that the differences in growth observed in our experiments (Exp. 7) are the result of differences in somatic fusion and that the latter increases fitness of the fungus up to a threshold level, the point of optimal nodule yield. Nonetheless, high spore densities could hamper fungal fitness, as repeated fusion of spores lying in the same area, decreases the allocation of resources toward mycelial growth, and therefore, to hyphae collecting nutrients. Somatic fusion, thus, becomes relatively costly when compared to mycelial growth, hampering overall performance of the fungus. Ultimately, hyphal fusion is a costly trait that pays off through the production of asexual spores (Bastiaans et al., 2015). Richard et al. (2012), eloquently point out that the possible benefits of fusion are reliant on the habitat and spore densities present in the environment. Fusion tends to allow for larger growth which is beneficial in open environments where there is available substrate and space to growth. But also, it can slow the growth and limit the spread of fungi colonies with low spore densities, especially on substrates limited in size and competed among various organisms.

Nonetheless, a notion thinking that the size of mycelium correlates with its fitness and fecundity (Pringle & Taylor, 2002) is perhaps not the best to approach fitness in *Termitomyces.* The apparently programmed reduction and subsequent stop in the growth of mycelium is perhaps no coincidence but an adaptation to the symbiotic lifestyle of the fungus, specifically, to being spread by termites. Dispersion via termites might be much more effective than somatic growth, hence, natural selection could have potentially fostered a programmed stop in somatic growth to favor other traits such as indigestibility of spores inside nodules to facilitate spread through hosts. Furthermore, as explained, nodules of *Termitomyces* are 3-dimensional structures. The overall size of colonies/mycelium is not a strong indicator of yield if the size of nodules formed are not accounted for. Bigger nodules contain more spores than smaller ones that cover the same surface area, simply because the volume of the first is larger, thus contains more spores (Appendix: Fig. 5). Future studies must account for this when estimating asexual spore yield based on the surface area covered by nodules.

Sexual development of *Termitomyces*: a benefit/cost interplay

Many of the experiments conducted throughout the duration of this research had the intention to trigger mushroom production of *Termitomyces.* From the start, it was clear that this would be challenging given all the unknowns surrounding the process of fructification in *Termitomyces.* Sexual reproduction is often associated with (opportunity) costs for a fungus, e.g., hampering asexual reproduction (Chamberlain & Ingram, 1997). The general idea is that resources put into the production of sexual structures cannot be used for the formation of other tissue. In particular, the reduction of vegetative mycelium that scavenges for and accumulates resources could be costly to the point of dramatically risking the survival a fungus. To put it more precisely, natural selection fostered the development of mechanisms to tune the physiology of the fungus in function to their specific changing living conditions, i.e., that in a given context somatic growth increases the fitness of the fungus while in another one, sexual dispersion is best suited to increase its survival success. To adapt to their specific environment, a fungus “sets” a species dependent threshold that signals that the benefits of sexual reproduction surpass its costs. Species where sexual reproduction occurs more frequently as compared to other dispersal strategies such as asexual reproduction or somatic growth will have set this threshold lower than fungi dispersing sexually more seldom (Nieuwenhuis, 2012).

Respectively, given that *Termitomyces* reproduces in nature much less frequently sexually than asexually, the benefit/cost "switch" initiating mushroom production seems to be "set" high.

All triggers (see experiments 1-5) applied, obviously did not trigger the "switch" from the vegetative to the generative, sexual phase of the fungus. In this regard, much can be only speculated, as all applied triggers were supposed to change the nutritional, physical or metabolic circumstances of the fungus, attempting to elicit physiological changes in the fungus. Nonetheless, the exact responses of the fungus to these triggers in relation to the natural circumstances that would trigger mushrooms in-vivo are not known simply because the natural triggers of mushroom formation are unknown. However, an interesting result to discuss is the irrelevance of mechanical damage as a mushroom trigger. It was believed that termites likely suppressed fruiting of their symbionts by consuming the (asexual) nodules that otherwise would have developed into sexual primordia. However, Vreeburg et. al. (2020a), revealed that sexual and asexual reproductive structures are morphologically distinct from each other and that they result from different developmental pathways. Hence, removal of nodules is not directly related to fruiting of primordia. Moreover, our experiments suggest that mechanical damage/nodule removal does not foster mushroom formation via the aggregation of newly grown hyphae stemming from mature injured mycelium that differentiate into primordia, as observed in other fungi like *Schizophyllum commune* (Leonard & Dick, 1973).

Likewise, the presence of tannic acid in the growth substrate of *Termitomyces* did not trigger the initiation of basidiomata. We based our research on Carbajo et. al. (2002), which found tannic acid to elicit the production of laccases in *Coriolopsis gallica.* However, another publication suggests that tannic acid is not a trigger of laccase activity per se but only an indicator molecule for the presence of laccases (Kiiskinen et al., 2004). In the latter, it is explained that under the presence of laccases, tannic acid turns brown, which interestingly, was observed in plates containing tannic acid (Exp. 5).

Asexual development and fitness

Nutritional and abiotic conditions explored in this research were not standardized and assessed in the number of inocula/spores used in experiments. Therefore, the asexual reproduction of *Termitomyces* was specifically analyzed in experiment 7, using a determinate number of spores and a nutritional poor substrate, in order to clearly visualize nodulation patterns of the fungus. In this way, nodulation was limited, hence, nodules covering the entire surface was prevented, which would have made impossible to see differences in the surface area. Nonetheless, the effect of nutrition on the nodulation of fungi was evident, with low nutrient concentrations showing little nodulation and higher ones showing progressively more nodules. Similarly, high spore densities showed reduced nodulation when compared to lower spore densities. Thus, both low nutrition and high spore densities tend to limit the yield of asexual spores. Also, both lower nutrition levels tested (8 g/l and 32 g/l), and high spore densities resulted on an increase in the mycelial coverage of plates (Exp. 1; Exp. 7). In general, mycelial surface is a significant predictor of spore number, as seen in the free-living ascomycete *Aspergillus niger*, which could potentially be assessed as a measure of fitness (Pringle & Taylor, 2002). Similarly, the abovementioned experiments varying spore densities and degree of fusion for *Neurospora crassa* (Bastiaans et al., 2015; Richard et al., 2012), hint to increased fitness as a result of higher spore densities and increased somatic fusion. This is seen in a faster somatic growth and production of asexual spores. As explained in the somatic growth section of the discussion, this notion of fitness does not apply to *Termitomyces*. Higher spore densities, i.e., increased mycelial colonization does not

translate into an increase spore production. Nodule yield increases with spore density up to an optimum after decreasing again. High spore densities seem to have an inhibitory effect on nodulation. This phenomenon can be the result of the ecology of *Termitomyces*, which is a symbiotic fungus and that can rely on its host of dispersal. Interestingly a parasitic basidiomycete of grasses, *Puccinia graminis* has been observed to fine tune its sporulation based on the density of competing strains (Pringle & Taylor, 2002). The intended message of this statement is that symbiotic fungi may be more inclined to adapt their living strategies in response to exogenous competition that could occupy their symbiotic compartments (hosts) than free living fungi (which do not face this challenge). After all, in contrast to free living fungi, symbiotic fungi of Macrotermitinae or of attine ants do potentially face competition to occupy their symbiotic host compartment, which they need in order to survive (Chomiki et al., 2020; Shik et al., 2016). Moreover, *Termitomyces,* being an obligate mutualistic fungus, adapted to coexisting with its termite hosts has developed specialized methods of dispersion, such as having asexual spores that resist the digestion of termites. It is puzzling however, what consequences the adaptation of nodulation to spore density have, as it is expected that termites continuously inoculate new material, hence, spore densities inside of combs are likely high (though it is unknown how high). Studies in-vivo are needed to analyze the effect of spore number of the nodule yield of combs.

Ecology of the fungus: a crop and a mutualistic lifestyle

We have summarized three strategies that a fungus can take in order to disperse: 1. Via somatic growth. 2. Through the spread of asexual spores. 3. Through the spread of sexual spores. The predominant strategy that characterizes a fungus in a given moment, varies with its life stage and ecological niche and can be analyzed in terms of a cost/benefit ratio. In general terms, the interplay between somatic growth, asexual and sexual reproduction is species dependent and potentially controlled by genetic, seasonal, nutritional, physical and metabolic factors as well as from surrounding, potentially competing organisms (self, intra and interspecific interactions) (Chamberlain & Ingram, 1997). The experiments conducted throughout this research elucidated several of the abovementioned factors that can potentially influence the dispersal strategy of a fungus, in this case for *Termitomyces*. A marked outcome of the experiments and literature review conducted was an experimental exhibition of the cooperative tendencies and adaptations that *Termitomyces* has developed as a mutualistic fungus. Free-living fungi are adapted to different life circumstance and are characterized by individualistic life strategies that tend to increase their own survival and dispersal. In contrast, the life strategies of *Termitomyces*, are characterized not only by supporting their own growth and dispersal but by increasing the survival success of other organisms as well, namely, their termite hosts. This results in the alignment of both symbiont and host fitness.

This alignment of interests is perhaps most tangible at the level of asexual reproduction of symbionts. Contrary to free-living fungi, asexual sporulation of *Termitomyces* seems to be not only adjusted to the needs of the fungus but also to the ones of termites. This can be seen in the following: 1. Fungal nodules are consumed by termites and the spores inside of nodules remain viable even after passing the gut of termites. 2. The frequency of asexual reproduction vs sexual reproduction is much higher for the first. 3. Somatic growth of the fungus seems to be programmed to stop after reaching a certain size, likely as dispersion of the fungus inside colonies is much more effective via termite defecation.

Nonetheless, all fundamental interests of symbionts and hosts are not always aligned. Sexual reproduction of *Termitomyces* may be detrimental for termites, as the allocation of resources to the formation of sexual structures hampers the yield of termite's farms, ultimately decreasing the potential of termite colonies. Therefore, it is not in a "termite colonies' short-term interest" to allow the formation of mushrooms of their symbionts even though they might be reliant of sexual spores for the foundation of new colonies (Vreeburg et. al., 2020b). Especially if we consider that other nearby colonies are also potentially able to release sexual spores that can be used by a colony that has suppressed the mushroom formation of their symbionts. In theory, natural selection would foster circumstances that increase the performance of individual termite colonies, such as the suppression of costly mushrooms that hamper edible nodule production. Yet, *Termitomyces* mushrooms do occur in nature for most Macrotermitinae species (de Fine Licht, 2006). One could hypothetize then, that this is a case of kin altruism to prevent a tragedy of the commons, where cheater colonies progressively appear until the pool of mushrooms/sexual spores, needed for colony formation is reduced to an unsustainable minimum that impairs the survival of whole fungus farming termite kin(s). Besides a few species of two genera, most fungus-farming termite species are completely dependent on sexual spores of their symbionts in order to establish new viable colonies (de Fine Licht et al., 2006). Moreover, several fungus-farming termite species may release masses of alates from their colonies seeking new spores during the same period of the year (Koné et al., 2011), hence competition for spores may be a relevant factor determining colony establishment success. How most Macrotermitinae have maintained a stable mushroom production despite the given circumstances is truly extraordinary, this is a complex issue worth of further research.

Analogous to human agriculture, termites have developed controlled environments in order to deal with conflicts of interest with their crops/symbionts. One such adaptation is keeping their symbionts in monocultures, which prevents virulence of symbionts arising from interspecific competition inside combs (Aanen et. al., 2009). Moreover, cheater symbiont linages are prevented not only through monocultures but simply through choosing a more cooperative symbiont strain instead. Macrotermitinae hosts often switch symbionts within the same clade (Van de Peppel & Aanen, 2020). Termites reinforce partner fidelity by favoring cooperative symbionts over ones with virulent tendencies. Symbionts are selectively pressured to cooperate with termites in order to keep their symbiont compartment within hosts, otherwise the cost of keeping cheater symbionts inside of hosts will outweigh their benefits over time. If this happens, current symbionts will be replaced with others that provide benefits more effectively (Sachs & Simms, 2006).

Improving points

Even though results tended to be consistent for experiments using *Termitomyces cryptogamus* "T88", other fungi exhibited less coherent results. A possible explanation for the latter is human artifacts along the development of experiments. As a starting mycologist with limited laboratory experience, initial mistakes included contamination of plates with other microorganisms, which made the analysis of certain experiments impossible or required their replication. Moreover, during the inoculation of the first and second experiments, glass bead were used to spread the inoculum. In some cases, the spores were not properly spread across the surface of petri dishes as they might have been left resting too long after pipetting, which meant that the liquid started drying or penetrating the agar, thus, loosing dispersibility. Non-human error and different experimental conditions between repetitions exposed to the same treatment were present and made the standardization of results difficult. Non-human artifacts include: 1. An incubator stopping

functioning for a few days during experiments 4, 5 and 7. 2. Humidity and colonization area of complex “3d” substrates during experiments 2 and 3 varied significantly. Initially, it was estimated necessary to periodically water bottles containing casings based on the protocol of the literature used. However, as time passed by, it was observed that some bottles were quite wet and others dry even though all were handled in the same manner, hence, for the sake of standardization and simplicity casings were not watered. Furthermore, rye treatments seldom colonized big portions of rye (Exp. 2), this may have been due to not using enough inoculum for the amount of substrate. Furthermore, fungus combs used as substrate during experiment 6 did not grow at all, perhaps because of dryness that could have avoided by watering the substrates every few days.

As it has been mentioned, asexual spore yield was estimated with the area covered with nodules. However, we did not account for the fact that nodules are 3-dimensional structures. The volume of a single big nodule, and hence, the number of spores potentially inside of it is larger than the volume of 4 smaller nodules covering the same surface area (Appendix: Fig. 5). Hence, the estimates presented for yield are biased underestimations. Thus, in experiments where yield was estimated (1 and 7), larger nodules from treatments with a low C/N would have translated into more spores than initially expected when compared to other nutritional variants which produced abundant masses of small nodules. A way of checking the spore content of plates/nodules is described in the next paragraph.

Finally, an artifact of the analysis of images lies on the fact that images of plates are 2-dimensional, while in real life, mycelium and nodules have a significant 3-dimensional elevation. *Termitomyces* nodules tend to overlay on top each other, especially in rich media. Images do not account for nodules lying on top of other nodules, hence, the estimations of nodule yield for all plates analyzed with ImageJ are underestimations of reality. A way of dealing with this issue would have been to simply harvest the surface of plates containing nodules for each plate, then create a suspension and count the number of spores with a hemocytometer to get a more real estimation of the actual number of spores in every plate. However, this approach is not only highly time consuming but also involves the destruction of experiments which could have potentially yielded mushrooms after months of waiting.

Conclusions & Recommendations:

Throughout the experiments conducted in this research we attempted to mimic several conditions present in the natural habitat of *Termitomyces* fungi. Black soldier frass and rabbit excrements were used as novel substrates, showing that Termitomyces is able to grow on these media, even thriving if other nutritional sources are also present. Likewise, experiments with different C/N ratios and nutrition levels elucidated patterns of mycelial growth and nodulation of *Termitomyces* potentially present in nature, as a consequence of seasonal and cyclical fluctuations in foraging and food availability of termites. Similarly, inoculation frequency and thus, spore density, fluctuate with the number of workers present in combs over time. This has also consequences for termite colonies palpable at the nodule yield. As “Low” or “high” spore densities negatively impact the potential nodule yield of symbionts that can be attained at optimized spore numbers. As all other living organisms, fungal symbionts face trade-offs in allocating their limited resources and must “select” the most suitable strategy that can enhance their fitness in response to the prevailing ecological conditions over time. Being an obligate mutualistic fungus, highly adapted to co-exist with their host, we have hypothesized that natural selection has fostered cooperative strategies in *Termitomyces* species. i.a., in

an effort to keep their symbiont compartment from being occupied by other competing strains. We saw that Termitomyces grows somatically up to a threshold level, after this point, colonies stop growing and utilize resources in the formation of edible nodules containing asexual spores. We argued that the latter may be an adaptation to being spread via termites. Moreover, we explored sexual reproduction of *Termitomyces*, finding that sexual fruiting bodies require a significant investment of resources and are costly in terms of opportunity costs. Furthermore, we infer that if the benefits of sexual reproduction outweigh their costs, based on a given context, e.g., during substrate depletion, sexual spore production may pay off as a strategy to increase survival of the fungus. Likewise, we explored the implications of fruiting body formation for termite ecology. It would make sense for termite hosts to suppress fructification of their symbionts, because mushroom production implies a waste of resources that could otherwise have been used for the production of edible nodules. Nonetheless, termites rely completely on sexual spores for the formation of new colonies, what could dramatically drop the apparent costs of mushrooms. In addition, we conducted different experiments applying different stresses to *Termitomyces* mycelium, in an attempt to trigger mushroom formation by signalizing a positive switch in the benefit/cost ratio of mushroom formation. Unfortunately, our experiments did not achieve this goal, and hence, mushrooms were not formed.

Hypothesizing about the implications of the in-vitro results obtained for the in-vivo context of the fungus, was one of the main challenges of this research. To a large extent, this was because the knowledge about the ecology of *Termitomyces* is surrounded by several unknowns. This is partly due to the challenges implied in following the live development of a colony over longer periods of time. It is striking that, despite the relevance of *Termitomyces* in the paleotropics, and in spite of the fact that the genus was described over 8 decades ago, to my knowledge, only one study analyzing the ecology of fruiting in detail has taken place (Koné et. al., 2011). More research around fructification patterns of Termitomyces is needed in order to even contemplate the possibility of producing mushrooms in vitro, e.g., to profit from the nutritional benefits of this fungus (Botha & Eicker, 1992). Other insightful topics for in-vivo research include mound biomass changes occurring prior, during and post basidiocarp formation inside colonies, as well as studies of the metabolic changes in the mycelium during pinning and fruiting. Likewise, it is still unclear to what extend do termites allow the fructification of their fungal crops and if allowing mushrooms to fruit could be considered an example of kin altruism. Studies have identified potential indications of mushroom suppression for Termitomyces species with both uniparental/vertical, and horizontal symbiont transmission (de Fine Licht et al., 2006; Vreeburg et al., 2020). It would be informative to investigate further the potential mushroom suppression of hosts in detail for other Macrotermitinae species. The mentioned knowledge gaps require in-vivo studies over longer periods of time, for which collaboration with partners in the paleotropics is necessary, hence, forging research alliances with local scientists may be crucial to elucidate new aspects of the ecology of *Termitomyces* and their hosts.

Similarly, in-vitro research is needed to elucidate unclear physiological aspects of the fungus. Several publications to date have focused on finding substrates to enhance the growth of vegetative mycelium of Termitomyces spp., but not many have attempted to specifically trigger mushroom formation. We recommend testing other triggers that may potentially lead to mushroom formation, such as the effect of specific bacterial cohorts and/or their metabolites found inside of fungus combs, or the effect of specific termite chemicals such as pheromones. Furthermore, it would be worthwhile to try growing Termitomyces in a bioreactor setting to fine-tune changes in CO2/O2 levels, hence simulating the effect of alate dispersal. Lastly, we recommend further investigation into the effect of spore density on asexual spore yield, first with

even higher spore densities than the ones used here, and second with different nutritional levels to assess separately the influence of nutrition and spore density on asexual spore yield and somatic growth.

Acknowledgements

I want to foremost thank my friends for their constant support throughout the process of writing this thesis, in particular Kim and Abel that were often there helping me to find the motivation to complete this task. I want to thank my parents Hans and Eliana for their ongoing support throughout the years, with special thanks to my father for helping me with checking the layout of this text. I want to thank my rabbit Nilsson for contributing to this research with his poop which was used as a growth substrate for fungi. Also, big thanks to the GEN students for their company, friendship and good mood along the ride. Finally, I would like to expressly thank both of my thesis supervisors Eric Bastiaans and Duur Aanen for their valuable insights, for their guiding and support.

5. Literature

Aanen, D. K. (2006). As you reap, so shall you sow: coupling of harvesting and inoculating stabilizes the mutualism between termites and fungi. *Biology Letters*, *2*(2), 209-212.

Aanen, D. K., de Fine Licht, H. H., Debets, A. J., Kerstes, N. A., Hoekstra, R. F., & Boomsma, J. J. (2009). High symbiont relatedness stabilizes mutualistic cooperation in fungus-growing termites. *science*, *326*(5956), 1103-1106.

Aanen, D. K., Eggleton, P., Rouland-Lefevre, C., Guldberg-Frøslev, T., Rosendahl, S., & Boomsma, J. J. (2002). The evolution of fungus-growing termites and their mutualistic fungal symbionts. Proceedings of the National Academy of Sciences, 99(23), 14887-14892.

Amandeep, K., Atri, N. S., & Munruchi, K. (2015). Ecology, distribution perspective, economic utility and conservation of coprophilous agarics (Agaricales, Basidiomycota) occurring in Punjab, India. *Curr. Res. Environ. Appl. Mycol. J. Fungal. Biol*, *5*, 213-247.

Aryal, H. P. (2020). Optimization of cultural media for mycelial growth of Termitomyces Microcarpus (Berk and Broome) R. HEIM. TTPP, 269.

Bastiaans, E. (2015). On the evolution of allorecognition and somatic fusion in ascomycete filamentous fungi. Wageningen University. Retrieved 2023, from http://edepot.wur.nl/342494.

Bastiaans, E., Debets, A. J., & Aanen, D. K. (2015). Experimental demonstration of the benefits of somatic fusion and the consequences for allorecognition. *Evolution*, *69*(4), 1091-1099.

Baroni, T. J., Franco-Molano, A. E., Lodge, D. J., Lindner, D. L., Horak, E., & Hofstetter, V. (2007). Arthromyces and Blastosporella, two new genera of conidia-producing lyophylloid agarics (Agaricales, Basidiomycota) from the neotropics. mycological research, 111(5), 572-580.

Bechara, M. A., Heinemann, P. H., Walker, P. N., Demirci, A., & Romaine, C. P. (2009). Evaluating the addition of activated carbon to heat-treated mushroom casing for grain-based and compost-based substrates. *Bioresource technology*, *100*(19), 4441-4446.

Bennett, J. W., & Feibelman, T. (2001). Fungal bacterial interactions. In *Fungal Associations* (pp. 229-242). Springer, Berlin, Heidelberg.

Botha, W. J., & Eicker, A. (1992). Nutritional value of Termitomyces mycelial protein and growth of mycelium on natural substrates. Mycological Research, 96(5), 350-354.

Bose, S., Mazumder, S., & Mukherjee, M. (2007). Laccase production by the white-rot fungus Termitomyces clypeatus. *Journal of basic microbiology*, *47*(2), 127-131.

Butler, R. A. (2018). Farmer termites bury invaders alive to protect Fungus Farms. Mongabay. Retrieved November 26, 2022, from https://india.mongabay.com/2018/10/farmer-termites-bury-invaders-alive-to-protect-fungus-farms/.

Carbajo JM, Junca H, Terrón MC, González T, Yagüe S, Zapico E, González AE (2002). Tannic acid induces transcription of laccase gene cglcc1 in the white-rot fungus Coriolopsis gallica. Can J Microbiol. 48(12):1041-7. doi: 10.1139/w02-107. PMID: 12619815.

Chamberlain, M., & Ingram, D. S. (1997). The balance and interplay between asexual and sexual reproduction in fungi. In *Advances in Botanical Research* (Vol. 24, pp. 71-87). Academic Press.

Chiu, C. I., Ou, J. H., Chen, C. Y., & Li, H. F. (2019). Fungal nutrition allocation enhances mutualism with fungus-growing termite. Fungal Ecology, 41, 92-100.

Chomicki, G., Werner, G. D., West, S. A., & Kiers, E. T. (2020). Compartmentalization drives the evolution of symbiotic cooperation. *Philosophical Transactions of the Royal Society B*, *375*(1808), 20190602.

Crosland, M. W. J., Lok, C. M., Wong, T. C., Shakarad, M., & Traniello, J. F. A. (1997). Division of labour in a lower termite: the majority of tasks are performed by older workers. *Animal behaviour*, *54*(4), 999-1012.

da Costa, R. R., Vreeburg, S. M., Shik, J. Z., Aanen, D. K., & Poulsen, M. (2019). Can interaction specificity in the fungus-farming termite symbiosis be explained by nutritional requirements of the fungal crop?. Fungal Ecology, 38, 54-61.

Dangerfield, J. M., & Schuurman, G. (2000). Foraging by fungus-growing termites (Isoptera: Termitidae, Macrotermitinae) in the Okavango Delta, Botswana. *Journal of Tropical Ecology*, *16*(5), 717-731.

De, A. B. (1983). Basidiocarp production by Termitomyces Microcarpus (Berk. And Br.) Heim in culture. Current Science, 52(10), 494-495.

de Fine Licht, H. H., Andersen, A., & Aanen, D. K. (2005). Termitomyces sp. associated with the termite Macrotermes natalensis has a heterothallic mating system and multinucleate cells. *Mycological research*.

de Fine Licht, H. H., Boomsma, J. J., & Aanen, D. K. (2006). Presumptive horizontal symbiont transmission in the fungus-growing termite Macrotermes natalensis. Molecular ecology, 15(11), 3131-3138.

Donnelly, D. P., & Boddy, L. (1998). Repeated damage results in polarised development of foraging mycelial systems of Phanerochaete velutina. *FEMS microbiology ecology*, *26*(2), 101-108.

Elissen, H., van der Weide, R., & Gollenbeek, L. (2023). Effects of black soldier fly frass on plant and soil characteristics: a literature overview.

Elliot, C. G. (1994). Reproduction in fungi. Genetical and Physiological Aspects, Chapman and Hall: London.

Frank, S. A. (1996). Host control of symbiont transmission: the separation of symbionts into germ and soma. *The American Naturalist*, *148*(6), 1113-1124.

Gangwar, R., Rasool, S., & Mishra, S. (2016). Evaluation of cellobiose dehydrogenase and laccase containing culture fluids of Termitomyces sp. OE147 for degradation of Reactive blue 21. *Biotechnology Reports*, *12*, 52-61.

Ghorai, S. Impact of complex media on production of cellobiase and sucrase from filamentous fungus Termitomyces clypeatus. : 2020, 211.

Harnden, L. M., & Tomberlin, J. K. (2016). Effects of temperature and diet on black soldier fly, Hermetia illucens (L.)(Diptera: Stratiomyidae), development. *Forensic Science International*, *266*, 109-116.

Heim, R. (1942). Nouvelles études descriptives sur les agarics termitophiles d'Afrique tropicale. *Archives du Muséum national d'Histoire naturelle, 6ème série*, *18*(1), 107-166.

Hibbett, D. S., Gilbert, L. B., & Donoghue, M. J. (2000). Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature*, *407*(6803), 506-508.

Hofstetter, V., Redhead, S. A., Kauff, F., Moncalvo, J. M., Matheny, P. B., & Vilgalys, R. (2014). Taxonomic revision and examination of ecological transitions of the Lyophyllaceae (Basidiomycota, Agaricales) based on a multigene phylogeny. *Cryptogamie, mycologie*, *35*(4), 399-425.

Hsieh, H. M., Chung, M. C., Chen, P. Y., Hsu, F. M., Liao, W. W., Sung, A. N., ... & Ju, Y. M. (2017). A termite symbiotic mushroom maximizing sexual activity at growing tips of vegetative hyphae. *Botanical studies*, *58*, 1-14.

Johnson, R. A., Thomas, R. J., Wood, T. G., & Swift, M. J. (1981). The inoculation of the fungus comb in newly founded colonies of some species of the Macrotermitinae (Isoptera) from Nigeria. *Journal of Natural History*, *15*(5), 751-756.

Johnson, R. A. (1981). Colony development and establishment of the fungus comb in Microtermes sp. nr. usambaricus (Sjostedt)(Isoptera: Macrotermitinae) from Nigeria. *Insectes sociaux.= Social insects*.

Kiiskinen, L. L., Rättö, M., & Kruus, K. (2004). Screening for novel laccase-producing microbes. *Journal of applied microbiology*, *97*(3), 640-646.

Koné, N. A., Dosso, K., Konaté, S., Kouadio, J. Y., & Linsenmair, K. E. (2011). Environmental and biological determinants of Termitomyces species seasonal fructification in central and southern Côte d'Ivoire. Insectes sociaux, 58(3), 371-382.

Koné, N'GA., Yéo, K., Konaté, S., & Linsenmair, K. E. (2013). Socio-economical aspects of the exploitation of Termitomyces fruit bodies in central and southern Côte d'Ivoire: Raising awareness for their sustainable use. *Journal of Applied Biosciences*, *70*, 5580-5590.

Korb, J., & Aanen, D. K. (2003). The evolution of uniparental transmission of fungal symbionts in fungus-growing termites (Macrotermitinae). *Behavioral Ecology and Sociobiology*, *53*, 65-71.

Kusumawardhani, D., Nandika, D., Karlinasari, L., Arinana, A., & Batubara, I. (2021). Architectural and physical properties of fungus comb from subterranean termite Macrotermes gilvus (Isoptera: Termitidae) mound. Biodiversitas Journal of Biological Diversity, 22(4).

Kües, U., & Liu, Y. (2000). Fruiting body production in basidiomycetes. Applied microbiology and biotechnology, 54(2), 141-152.

Lee, G. W., Jaysinghe, C., Imtiaj, A., Shim, M. J., Hur, H., Lee, M. W., ... & Lee, T. S. (2007). The Artificial Cultivation of Oudemansietta mucida on the Oak Sawdust Medium. Mycobiology, 35(4), 226-229.

Leonard, T. J., & Dick, S. (1973). Induction of haploid fruiting by mechanical injury in Schizophyllum commune. *Mycologia*, *65*(4), 809-822.

Lepage, M., & Darlington, J. P. (2000). Population dynamics of termites. In Termites: evolution, sociality, symbioses, ecology (pp. 333-361). Springer, Dordrecht.

Leuthold, R. H., Badertscher, S., & Imboden, H. (1989). The inoculation of newly formed fungus comb with Termitomyces in Macrotermes colonies (Isoptera, Macrotermitinae). *Insectes Sociaux*, *36*(4), 328-338.

Liang, S., Wang, C., Ahmad, F., Yin, X., Hu, Y., & Mo, J. (2020). Exploring the effect of plant substrates on bacterial community structure in termite fungus-combs. Plos one, 15(5), e0232329.

Long, P. E., & Jacobs, L. (1974). Aseptic fruiting of the cultivated mushroom, Agaricus bisporus. *Transactions of the British Mycological Society*, *63*(1), 99-107.

Mikheyev, A. S., Mueller, U. G., & Abbot, P. (2006). Cryptic sex and many-to-one coevolution in the fungus-growing ant symbiosis. *Proceedings of the National Academy of Sciences*, *103*(28), 10702-10706.

Mitchell, J. D. (2008). Swarming flights of the fungus-growing termite, Macrotermes natalensis (Haviland)(Isoptera: Macrotermitinae), and the environmental factors affecting their timing and duration. *African Entomology*, *16*(2), 143-152.

Moore-Landecker, E. Kingdom fungi: Division Basidiomycota In: Fundamentals of the Fungi, 1996. Chapter 6.

Morris, B. (1986). Notes on the genus Termitomyces heim in Malawi. *The Society of Malawi Journal*, *39*(1), 40-49.

Nieuwenhuis, B. P. S. (2012). Sexual selection in Fungi. [internal PhD, WU, Wageningen University]. https://edepot.wur.nl/222420

Nieuwenhuis, M., Nieuwenhuis, M., Zwaan, B. J., Aanen, D. K., Bakker, F. T., Zwaan, B. J., Aanen, D. K., & Bakker, F. T. (2021). Evolution of mutualisms in the basidiomycete genus termitomyces (dissertation). Wageningen University.

Noble, R., Fermor, T. R., Lincoln, S., Dobrovin-Pennington, A., Evered, C., Mead, A., & Li, R. (2003). Primordia initiation of mushroom (Agaricus bisporus) strains on axenic casing materials. *Mycologia*, *95*(4), 620-629.

Noble, R., Dobrovin-Pennington, A., Hobbs, P. J., Pederby, J., & Rodger, A. (2009). Volatile C8 compounds and pseudomonads influence primordium formation of Agaricus bisporus. *Mycologia*, *101*(5), 583-591.

Nobre, T., Fernandes, C., Boomsma, J. J., Korb, J., & Aanen, D. K. (2011). Farming termites determine the genetic population structure of Termitomyces fungal symbionts. *Molecular Ecology*, *20*(9), 2023-2033.

Nobre, T., & Aanen, D. K. (2012). Fungiculture or Termite Husbandry? The Ruminant Hypothesi. *Insects*, *3*(1), 307-323.

Nobre, T., Koopmanschap, B., Baars, J. J., Sonnenberg, A. S., & Aanen, D. K. (2014). The scope for nuclear selection within Termitomyces fungi associated with fungus-growing termites is limited. *BMC Evolutionary Biology*, *14*, 1-12.

Oso, B. A. (1977). Mushrooms in Yoruba mythology and medicinal practices. *Economic Botany*, *31*(3), 367-371.

Poulsen, M. (2015). Towards an integrated understanding of the consequences of fungus domestication on the fungus-growing termite gut microbiota. *Environmental Microbiology*, *17*(8), 2562-2572.

Pringle, A., & Taylor, J. W. (2002). The fitness of filamentous fungi. *Trends in microbiology*, *10*(10), 474-481.

Richard, F., Glass, N. L., & Pringle, A. (2012). Cooperation among germinating spores facilitates the growth of the fungus, Neurospora crassa. *Biology Letters*, *8*(3), 419-422.

Roberts, E. M., Todd, C. N., Aanen, D. K., Nobre, T., Hilbert-Wolf, H. L., O'Connor, P. M., ... & Stevens, N. J. (2016). Oligocene termite nests with in situ fungus gardens from the Rukwa Rift Basin, Tanzania, support a Paleogene African origin for insect agriculture. *PLoS One*, *11*(6), e0156847.

Roehl, T. (2016, November 8). #009: Overview of the Fungal Life Cycle. Fungus Fact Friday. Retrieved March 9, 2023, from https://www.fungusfactfriday.com/009-overview-of-the-fungal-life-cycle/

Roisin, Y. (2000). Diversity and evolution of caste patterns. In Termites: evolution, sociality, symbioses, ecology (pp. 95-119). Springer, Dordrecht.

Ross, I. K. (1982). The role of laccase in carpophore initiation in Coprinus congregatus. Microbiology, 128(11), 2763-2770.

Rouland-Lefèvre, C., Inoue, T., & Johjima, T. (2006). Termitomyces/termite interactions. In *Intestinal microorganisms of termites and other invertebrates* (pp. 335-350). Springer, Berlin, Heidelberg.

Rühl, M., Majcherczyk, A., & Kües, U. (2013). Lcc1 and Lcc5 are the main laccases secreted in liquid cultures of Coprinopsis cinerea strains. *Antonie van Leeuwenhoek*, *103*(5), 1029-1039.

Sachs, J. L., & Simms, E. L. (2006). Pathways to mutualism breakdown. *Trends in ecology & evolution*, *21*(10), 585-592.

Safar, H. M., & Cooke, R. C. (1988). Interactions between bacteria and coprophilous Ascomycotina and a Coprinus species on agar and in copromes. *Transactions of the British Mycological Society*, *91*(1), 73-80.

Schalk, F., Gostinčar, C., Kreuzenbeck, N. B., Conlon, B. H., Sommerwerk, E., Rabe, P., ... & Beemelmanns, C. (2021). The termite fungal cultivar Termitomyces combines diverse enzymes and oxidative reactions for plant biomass conversion. *Mbio*, *12*(3), e03551-20.

Sapountzis, P., De Verges, J., Rousk, K., Cilliers, M., Vorster, B. J., & Poulsen, M. (2016). Potential for nitrogen fixation in the fungus-growing termite symbiosis. *Frontiers in Microbiology*, *7*, 1993.

Seiler, W., Conrad, R., & Scharffe, D. (1984). Field studies of methane emission from termite nests into the atmosphere and measurements of methane uptake by tropical soils. *Journal of atmospheric Chemistry*, *1*(2), 171-186.

Shik, J. Z., Gomez, E. B., Kooij, P. W., Santos, J. C., Wcislo, W. T., & Boomsma, J. J. (2016). Nutrition mediates the expression of cultivar–farmer conflict in a fungus-growing ant. *Proceedings of the National Academy of Sciences*, *113*(36), 10121-10126.

Sporeslab.io. (n.d.). *Sporeslab.io*. PNWM. Retrieved November 2, 2022, from https://www.sporeslab.io/shop/MYCO-PRO%E2%84%A2-Agar-Petri-Dish-Formula-p307299387.

Sporeslab.io. (n.d.). *Sporeslab.io*. PNWM. Retrieved November 2, 2022, from https://www.sporeslab.io/shop/MYCO-PRO%E2%84%A2-Insect-Frass-p307289598.

Suguimoto, H. H., Barbosa, A. M., Dekker, R. F., & Castro-Gomez, R. J. (2001). Veratryl alcohol stimulates fruiting body formation in the oyster mushroom, Pleurotus ostreatus. FEMS Microbiology letters, 194(2), 235-238.

Taprab, Y., Johjima, T., Maeda, Y., Moriya, S., Trakulnaleamsai, S., Noparatnaraporn, N., ... & Kudo, T. (2005). Symbiotic fungi produce laccases potentially involved in phenol degradation in fungus combs of fungus-growing termites in Thailand. *Applied and Environmental Microbiology*, *71*(12), 7696-7704.

Thurston, C. F. (1994). The structure and function of fungal laccases. *Microbiology*, *140*(1), 19-26.

Traniello, J. F., & Leuthold, R. H. (2000). Behavior and ecology of foraging in termites. In *Termites: evolution, sociality, symbioses, ecology* (pp. 141-168). Springer, Dordrecht.

van de Peppel, L. J., & Aanen, D. K. (2020). High diversity and low host-specificity of Termitomyces symbionts cultivated by Microtermes spp. indicate frequent symbiont exchange. *Fungal Ecology*, *45*, 100917.

van de Peppel, L. J. J. (2021). On the origin of species in Termitomyces. [internal PhD, WU, Wageningen University]. Wageningen University. https://doi.org/10.18174/550803.

van de Peppel, L. J., Nieuwenhuis, M., Auxier, B., Grum-Grzhimaylo, A. A., Cárdenas, M. E., de Beer, Z. W., ... & Aanen, D. K. (2021). Ancestral predisposition toward a domesticated lifestyle in the termite-cultivated fungus Termitomyces. Current Biology, 31(19), 4413-4421.

van de Peppel, L. J., Aime, M. C., Læssøe, T., Pedersen, O. S., Coimbra, V. R., Kuyper, T. W., ... & Baroni, T. J. (2022). Four new genera and six new species of lyophylloid agarics (Agaricales, Basidiomycota) from three different continents. *Mycological Progress*, *21*(10), 1-14.

Vega, F. E., & Blackwell, M. (2005). Insect-fungal associations: ecology and evolution. Oxford University Press.

Vreeburg, S. M., de Ruijter, N. C., Zwaan, B. J., da Costa, R. R., Poulsen, M., & Aanen, D. K. (2020a). Asexual and sexual reproduction are two separate developmental pathways in a Termitomyces species. Biology letters, 16(8), 20200394.

Vreeburg, S. M. E. (2020b). *On conflict and resolution in the termite-fungus symbiosis*. [internal PhD, WU, Wageningen University]. Wageningen University. https://doi.org/10.18174/527068

Vesala, R. (2019). Diversity and ecology of Termitomyces symbionts in Macrotermes mounds of the Tsavo Ecosystem, Kenya. *Dissertationes Schola Doctoralis Scientiae Circumiectalis, Alimentariae, Biologicae*.

Vesala, R., Rikkinen, A., Pellikka, P., Rikkinen, J., & Arppe, L. (2022). You eat what you find–Local patterns in vegetation structure control diets of African fungus-growing termites. Ecology and evolution, 12(3), e8566.

Wilson, N., Hollinger, J., et al. 2006-present. Mushroom Observer. https://mushroomobserver.org

Wisselink, M., Aanen, D. K., & van't Padje, A. (2020). The longevity of colonies of fungus-growing termites and the stability of the symbiosis. *Insects*, *11*(8), 527.

Yang, G., Ahmad, F., Liang, S., Fouad, H., Guo, M., Gaal, H. A., & Mo, J. (2020). Termitomyces heimii associated with fungus-growing termite produces volatile organic compounds (VOCs) and lignocellulose-degrading enzymes. Applied Biochemistry and Biotechnology, 192(4), 1270-1283.

Yang, G., Ahmad, F., Zhou, Q., Guo, M., Liang, S., Gaal, H. A., & Mo, J. (2021). Investigation of Physicochemical Indices and Microbial Communities in Termite Fungus-Combs. *Frontiers in microbiology*, *11*, 581219.

Zhang, J., Chen, H., Chen, M., Ren, A., Huang, J., Wang, H., ... & Feng, Z. (2015). Cloning and functional analysis of a laccase gene during fruiting body formation in Hypsizygus marmoreus. Microbiological research, 179, 54-63.

6. Appendix

Figures

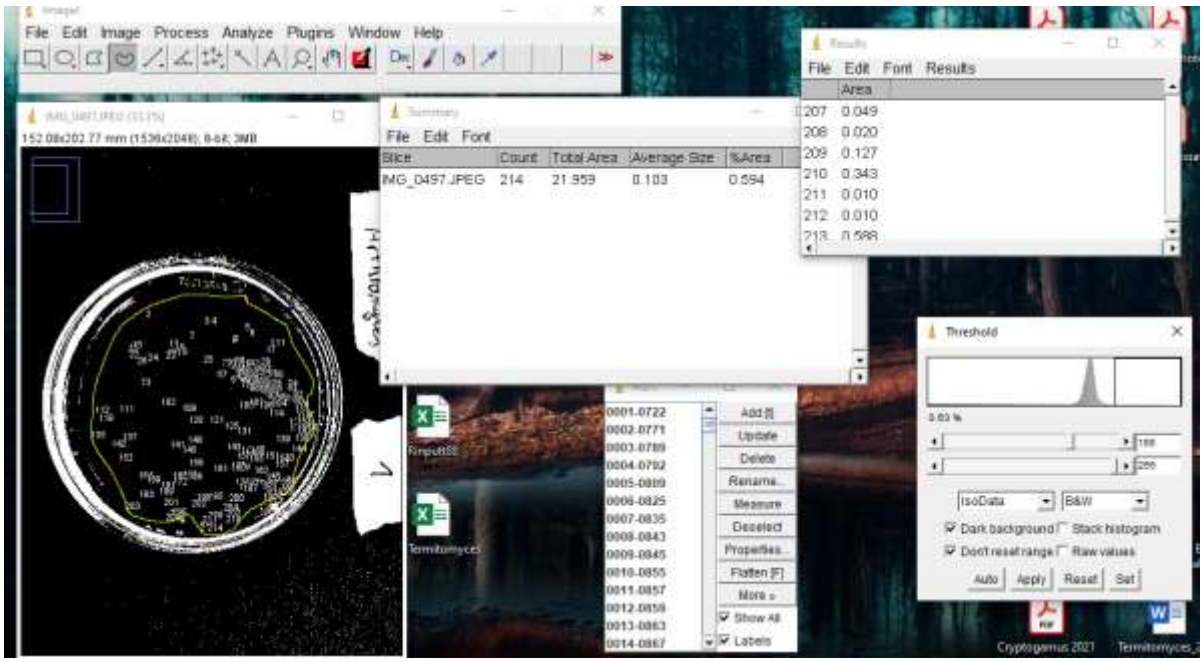

Fig. 1 Image Processing with Image J, example of an analysis done for plates of experiment 1. The isoData threshold method and the black and white threshold color option were used. In this case images were converted to 8-bit and then tresholded with the Treshold tool (Image, Adjust, Treshold), we see a threshold upper value of 188. Particles were then analysed with the Analyse Particles tool (Analyse, Analyse Particles).

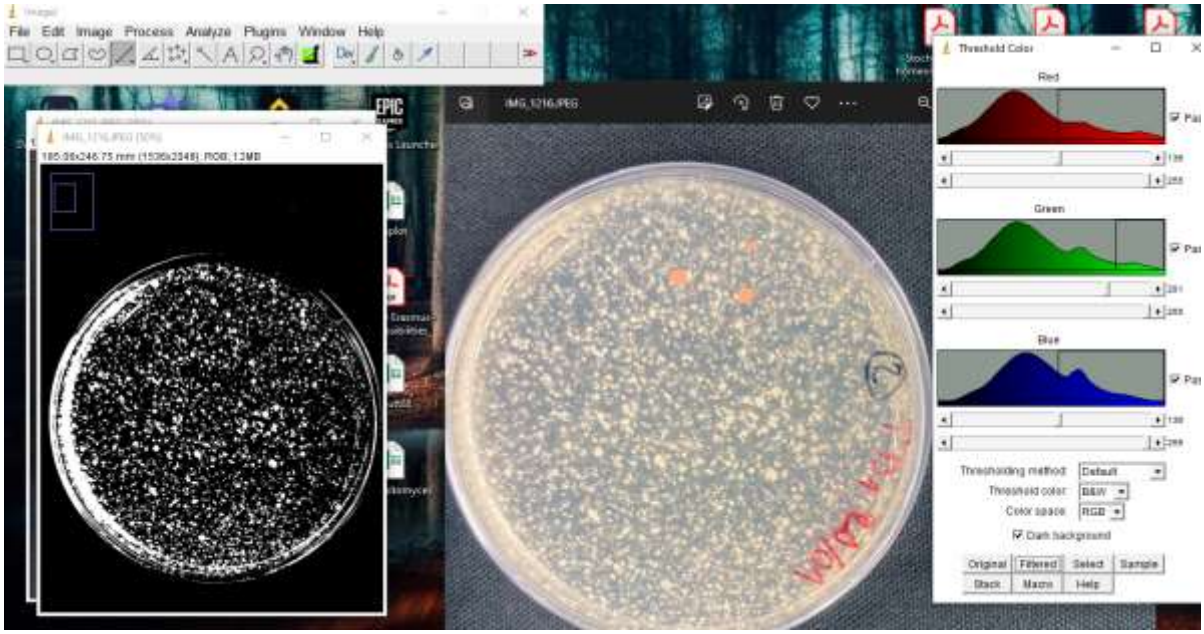

Fig. 2 Image Processing with Image J, example of an analysis done for plates of experiment 7. Images were tresholded with the Color Treshold tool (Image, Adjust, Color Treshold). The default threshold method, RGB color space and the black and white threshold color option were used. Only the green upper threshold values were changed, in this case setting the value to 201. Particles were then analysed with the Analyse Particles tool (Analyse, Analyse Particles).

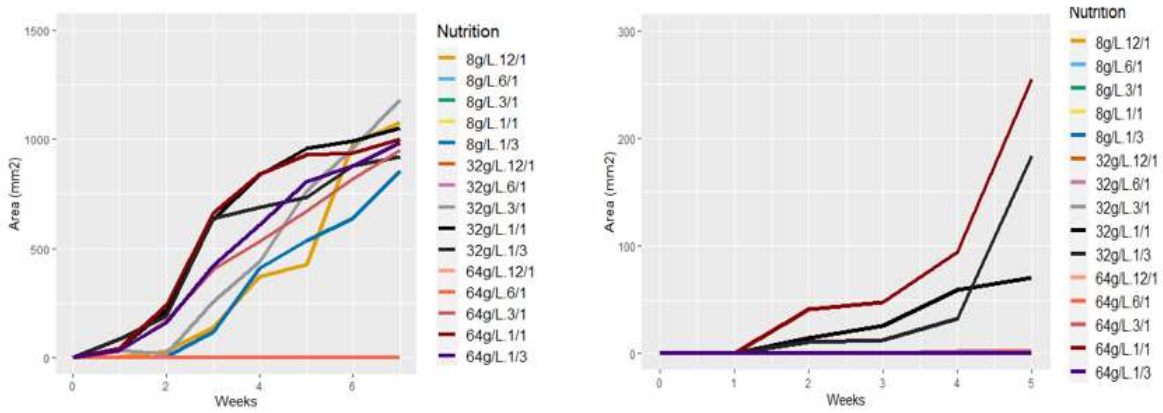

Fig. 3 Treatments of Termitomyces T73 (left) and Arthromyces (right) that grew in experiment 1. On the right of plots: the nutrition level and C/N for each treatment.

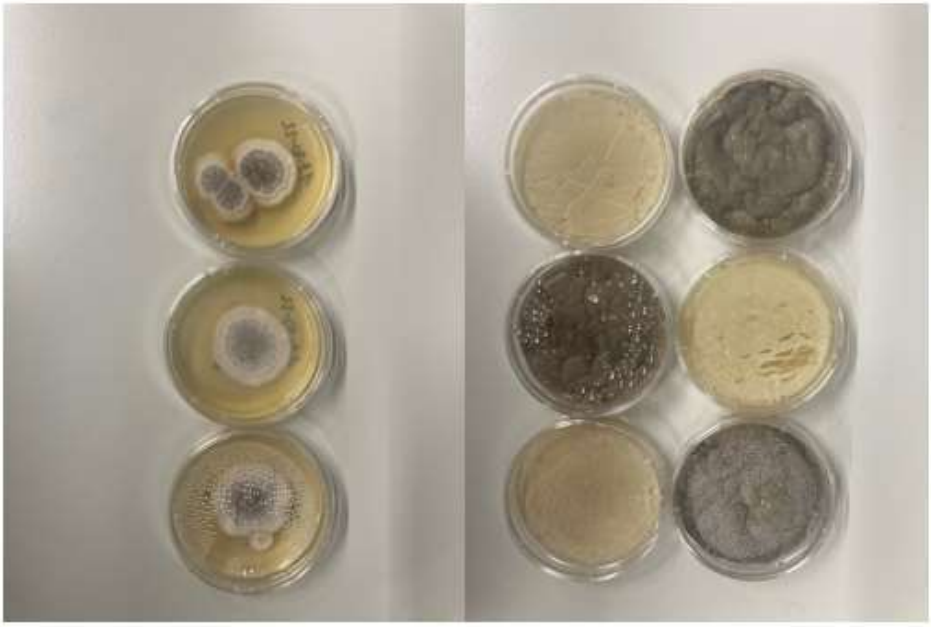

Fig. 4 "Blastosporella zonata" treatments were grown for weeks before realizing that they were actually an Asperaillus species (Ascomycota).

Fig. 5 Representation of 1 big sphere beside 4 smaller spheres that cover the same surface area. Assuming that the spheres have uniform density and are made of the same material, the one big sphere has a larger volume than 4 smaller spheres that cover the same surface area. This is due to the fact that the volume of a sphere is proportional to the cube of its radius, while its surface area is proportional to the square of the radius.

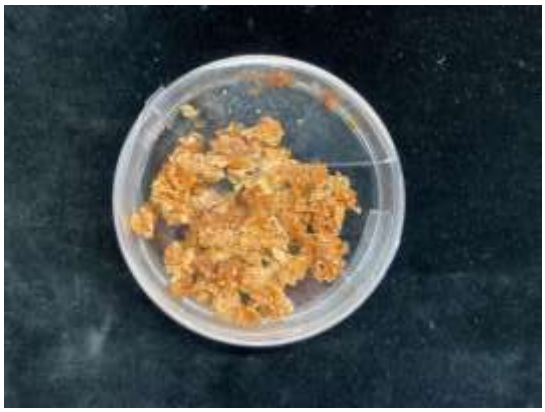

Fig. 6 Pile containing all the nodules removed during experiment 1 for Termitomyces "T88".

Tables

Table 1. Media and proportions for the different treatments based on concentrations (g/L) and C/N ratios. (Modified from da Costa et. al., 2016)

This is the reference for 1 L of distilled water.

C/N	Peptone	Starch	Glucose	Glucose monohydrate	Vitamin (L)	Trace Elements (L)	Agar
Concentration: 8g/L							
12/1	0.62	3.69	3.69	4.06	0.001	0.002	16.0
6/1	1.14	3.43	3.43	3.77	0.001	0.002	16.0
3/1	2	3	3	3.3	0.001	0.002	16.0
1/1	4	2	2	2.2	0.001	0.002	16.0
1/3	6	1	1	1.1	0.001	0.002	16.0
Concentration: 32g/L							
12/1	2.48	14.76	14.76	16.24	0.001	0.002	16.0
6/1	4.56	13.72	13.72	15.09	0.001	0.002	16.0
3/1	8	12	12	12.12	0.001	0.002	16.0
1/1	16	8	8	8.8	0.001	0.002	16.0
1/3	24	4	4	4.4	0.001	0.002	16.0
Concentration: 64g/L							
12/1	4.96	29.52	29.52	32.47	0.001	0.002	16.0
6/1	9.12	27.44	27.44	30.18	0.001	0.002	16.0
3/1	16	24	24	24.24	0.001	0.002	16.0
1/1	32	16	16	17.6	0.001	0.002	16.0
1/3	48	8	8	8.8	0.001	0.002	16.0

Table 2. Vitamin and trace element solutions used in experiment 1

Vitamin – solution for CM for *Aspergilli*
• **10 mg thiamine (A43)** • **125 mg riboflavin-5- phosphat Na (R19)** • **10 mg p – aminobenzoic acid (A58)** • **100 mg niacinamide (N34)** • **50 mg pyridoxin – HCl (P17)** • **0.2 mg D(+)- biotin (B10)** • **10 mg D – pantothenic acid (P14)** • **100 ml Demin** **filter sterilise**
Use 1:500

***Podospora anserina* Trace-elements** for *Podospora anserina* • **500 mg** **citric acid.H_2O** • **500 mg** **$ZnSO_4.7H_2O$** • **100 mg** **$Fe(NH_4)_2SO_4.6H_2O$** • **25 mg** **$CuSO_4.5H_2O$** • **5 mg** **$MnSO_4$** • **5 mg** **H_3BO_3** • **5 mg** **$Na_2MoO_4.2H_2O$** • **100 ml** **Demin** **filter sterilise**
Use 1:1000

Table 3. Overview of experiment set-up used in experiment 1

Concentration	C/N	# Of plates
8g/L	12/1	9
	6/1	9
	3/1	9
	1/1	9
	1/3	9
16g/L	12/1	9
	6/1	9
	3/1	9
	1/1	9
	1/3	9
32g/L	12/1	9
	6/1	9
	3/1	9
	1/1	9
	1/3	9
Total per strain		**135**
Strain	# Of plates	
T88	135	
T73	135	
BZ011	135	
Arthromyces	135	
Total	**540**	

Table 4. Overview of media and casing materials used in experiment 2

Agar based medium for 1 L (Modified from sporeslab.io´s MYCO-PRO™): 20g of bacteriological agar 19g malt extract 1g milled rye grain 5g insect frass (for treatments with frass)
Rye-grain medium: 20 grams of cooked and autoclaved rye 25 ml of water
Solid frass medium: 35 g of frass 35 ml of water 0.7 g of malt extract
Rye and frass medium: 20 g of rye 5 g of frass 30 ml of water
Casing layer: Activated charcoal, with a layer of about 2 cm (Noble et. al., 2003), other publications suggest 3-5cm for peat-based casing. 10 ml of water sprayed on top Soda lime filters made by: 1. Making holes on top of 1.5ml plastic tubes 2. Filling tubes with 2g of soda lime.

Table 5. Experimental set-up of experiment 2

Media	Casing	# Of bottles
Agar	-	3
	AC granules	3
	AC powder	3
	AC granules and soda lime	3
Agar with frass	-	3
	AC granules	3
	AC powder	3
	AC granules and soda lime	3
Rye	-	3
	AC granules	3
	AC powder	3
	AC granules and soda lime	3
Frass and malt	-	3
	AC granules	3
	AC powder	3
	AC granules and soda lime	3
Frass and rye	-	3
	AC granules	3
	AC powder	3
	AC granules and soda lime	3
Total per strain		**60**
Strain	# Of bottles	

Termitomyces T73	60	
Termitomyces T89	60	
Blastosporella zonata	60	
Total	**180**	

AC= Activated charcoal

Table 6. Set-up and casing of experiment 3

Treatment	Substrate Type	# of Bottles per Strain
Charcoal granules	Agar with frass	1
	Agar with frass and wooden pellets	1
Charcoal granules and soda lime filters	Agar with frass	0
	Agar with frass and wooden pellets	1
Soda lime filters	Agar with frass	0
	Agar with frass and wooden pellets	1
Control	Agar with frass	2
	Agar with frass and wooden pellets	2
Lignocellulosic uncrushed material	Hay, wooden pellets and rabbit feces	2
Total	-	10

Table 7. Media used in experiment 4 and their respective proportions

Media	Ingredients	# of Plates per Strain
Water agar 0g of frass	20g agar; 1 L water	4
Water agar 1g of frass	20g agar; 1 L water; 1g black soldier frass	4
Water agar 10g of frass	20g agar; 1 L water; 1g black soldier frass	4
Water agar 100g of frass	20g agar; 1 L water; 1g black soldier frass	4
Malt-containing agar 0g of frass	20g agar; 1L water	4
Malt-containing agar 1g of frass	20g agar; 1L water; 1g black soldier frass; 0.1g malt extract	4
Malt-containing agar 10g of frass	20g agar; 1L water; 1g black soldier frass; 1g malt extract	4
Malt-containing agar 100g of frass	20g agar; 1L water; 1g black soldier frass; 10g malt extract	4
Total		32

Table 8. Media used in experiment 5 (for 1 Liter)

MEA: 20g of agar 30g of malt extract 1l of water
MEA (Tannic acid 100µM): 20g of agar 30g of malt extract 1ml of a 100mM tannic acid stock 1l of water
MEA (Tannic acid 100µM and black soldier frass 5g/L): 20 g of agar 30g of malt extract 1ml of a 100nM tannic acid stock 1l of water
100 mM tannic acid stock: 340 g of tannic acid 100ml of water

Table 9. Spore numbers of every suspension used in experiment 7

Strain	# of Spores in the Suspension
Termitomyces cryptogamus "T88"	Suspension 0: $3.1*10^7$ Suspension 1: $6.2*10^6$ Suspension 2: $1.24*10^6$ Suspension 3: $2.48*10^5$ Suspension 4: 49600 Suspension 5: 9920 Suspension 6: 1984 Suspension 7: 397
Termitomyces sp. "T73"	Suspension 0: $3.1*10^7$ Suspension 1: $6.2*10^6$ Suspension 2: $1.24*10^6$ Suspension 3: $2.48*10^5$ Suspension 4: 49600 Suspension 5: 9920 Suspension 6: 1984 Suspension 7: 397
Blastosporella zonata	Suspension 0: $1.2*10^7$ Suspension 1: $2.4*10^6$ Suspension 2: $4.8*10^5$ Suspension 3: 96000 Suspension 4: 19200 Suspension 5: 3840 Suspension 6: 768 Suspension 7: 154

Arthromyces matolae	Suspension 0: $3.5*10^6$ Suspension 1: $7*10^5$ Suspension 2: $1.4*10^5$ Suspension 3: 28000 Suspension 4: 5600 Suspension 5: 1120 Suspension 6: 224 Suspension 7: 45

Table 10. Remaining bottles after experiment 2 was completed

Fungus	Type of medium	# of bottles remaining (per casing treatment)
Termitomyces cryptogamus (T88)	Agar	No casing: 3 AC granules: 3 AC granules and SLF: 3 AC powder: 3 Total: 12
	Agar with frass	No casing: 3 AC granules: 3 AC granules and SLF: 3 AC powder: 3 Total: 12
	Rye	No casing: 3 AC granules: 3 AC granules and SLF: 3 AC powder: 3 Total: 12
	Frass	No casing: 2 AC granules: 3 AC granules and SLF: 3 AC powder: 3 Total: 11
	Rye and frass	No casing: 1 AC granules: 3 AC granules and SLF: 3 AC powder: 3 Total: 10
Termitomyces sp. (T73)	Agar	No casing: 3 AC granules: 3 AC granules and SLF: 3 AC powder: 3 Total: 12
	Agar with frass	No casing: 3 AC granules: 3 AC granules and SLF: 3 AC powder: 3

		Total: 12
	Rye	No casing: 1 AC granules: 2 AC granules and SLF: 2 AC powder: 2 Total: 7
	Frass	No casing: 2 AC granules: 3 AC granules and SLF: 0 AC powder: 3 Total: 8
	Rye and frass	No casing: 0 AC granules: 2 AC granules and SLF: 1 AC powder: 2 Total: 5
Blastosporella zonata	Agar	12
	Agar with frass	12
	Rye	12
	Frass	12
	Rye and frass	12

AC= Activated Charcoal; SLF: Soda Lime Filter

Table 11. Reaming plates after experiment 4 was completed

Fungus	Type of medium	# of plates remaining
Termitomyces cryptogamus (T88)	Water agar with frass	0g/l:4 1g/l:4 10g/l:4 100g/l:1
	Malt agar with frass	0g/l:4 1g/l:4 10g/l:4 100g/l:4
Termitomyces sp. (T73)	Water agar with frass	0g/l:4 1g/l:4 10g/l:2 100g/l:2
	Malt agar with frass	0g/l:4 1g/l:4 10g/l:4 100g/l:2
Arthomyces matolae	Water agar with frass	0g/l:4 1g/l:4 10g/l:4 100g/l:3

	Malt agar with frass	0g/l:4 1g/l:4 10g/l:3 100g/l:4

Table 12. Pair-wise comparisons using an HSD-turkey test (alpha=0.05) for the treatments of experiment 7, inoculated by spreading the suspensions on the surface of plates, analyzing the effect of spore density on nodule yield.

#ofSpores

	diff	lwr	upr	p adj
1984-396.8	34.3332	-239.055999	307.7224	0.9998938
9920-396.8	147.7216	-125.667599	421.1108	0.6559364
49600-396.8	482.4774	209.088201	755.8666	0.0000618
248000-396.8	558.5418	285.152601	831.9310	0.0000048
1240000-396.8	751.8942	478.505001	1025.2834	0.0000000
6200000-396.8	784.6842	511.295001	1058.0734	0.0000000
3.1e+07-396.8	384.4414	111.052201	657.8306	0.0016559
9920-1984	113.3884	-160.000799	386.7776	0.8750171
49600-1984	448.1442	174.755001	721.5334	0.0001974
248000-1984	524.2086	250.819401	797.5978	0.0000151
1240000-1984	717.5610	444.171801	990.9502	0.0000000
6200000-1984	750.3510	476.961801	1023.7402	0.0000000
3.1e+07-1984	350.1082	76.719001	623.4974	0.0050300
49600-9920	334.7558	61.366601	608.1450	0.0081600
248000-9920	410.8202	137.431001	684.2094	0.0006914
1240000-9920	604.1726	330.783401	877.5618	0.0000010
6200000-9920	636.9626	363.573401	910.3518	0.0000004
3.1e+07-9920	236.7198	-36.669399	510.1090	0.1293049
248000-49600	76.0644	-197.324799	349.4536	0.9837989
1240000-49600	269.4168	-3.972399	542.8060	0.0557409
6200000-49600	302.2068	28.817601	575.5960	0.0219529
3.1e+07-49600	-98.0360	-371.425199	175.3532	0.9369492
1240000-248000	193.3524	-80.036799	466.7416	0.3291347
6200000-248000	226.1424	-47.246799	499.5316	0.1658813
3.1e+07-248000	-174.1004	-447.489599	99.2888	0.4586651
6200000-1240000	32.7900	-240.599199	306.1792	0.9999221
3.1e+07-1240000	-367.4528	-640.841999	-94.0636	0.0028821
3.1e+07-6200000	-400.2428	-673.631999	-126.8536	0.0009829

Table 13. Pair-wise comparisons using an HSD-turkey test (alpha=0.05) for the treatments of experiment 7, inoculated by pipetting suspension during pouring of the agar, analyzing the effect of spore density on nodule yield.

#ofSpores

	diff	lwr	upr	p adj
1984-396.8	-1.270200e+01	-39.302457	13.898457	0.7143658
9920-396.8	5.313600e+01	26.535543	79.736457	0.0000753
49600-396.8	7.892733e+01	52.326876	105.527791	0.0000004
248000-396.8	3.111167e+01	4.511209	57.712124	0.0162526
1240000-396.8	2.181667e+00	-24.418791	28.782124	0.9999885
6200000-396.8	-2.226100e+01	-48.861457	4.339457	0.1381491
3.1e+07-396.8	-2.226100e+01	-48.861457	4.339457	0.1381491
9920-1984	6.583800e+01	39.237543	92.438457	0.0000051
49600-1984	9.162933e+01	65.028876	118.229791	0.0000001
248000-1984	4.381367e+01	17.213209	70.414124	0.0006715
1240000-1984	1.488367e+01	-11.716791	41.484124	0.5473960
6200000-1984	-9.559000e+00	-36.159457	17.041457	0.9062412
3.1e+07-1984	-9.559000e+00	-36.159457	17.041457	0.9062412
49600-9920	2.579133e+01	-0.809124	52.391791	0.0608275
248000-9920	-2.202433e+01	-48.624791	4.576124	0.1455790
1240000-9920	-5.095433e+01	-77.554791	-24.353876	0.0001237
6200000-9920	-7.539700e+01	-101.997457	-48.796543	0.0000008
3.1e+07-9920	-7.539700e+01	-101.997457	-48.796543	0.0000008
248000-49600	-4.781567e+01	-74.416124	-21.215209	0.0002570
1240000-49600	-7.674567e+01	-103.346124	-50.145209	0.0000006
6200000-49600	-1.011883e+02	-127.788791	-74.587876	0.0000000
3.1e+07-49600	-1.011883e+02	-127.788791	-74.587876	0.0000000
1240000-248000	-2.893000e+01	-55.530457	-2.329543	0.0281277
6200000-248000	-5.337267e+01	-79.973124	-26.772209	0.0000713
3.1e+07-248000	-5.337267e+01	-79.973124	-26.772209	0.0000713
6200000-1240000	-2.444267e+01	-51.043124	2.157791	0.0838446
3.1e+07-1240000	-2.444267e+01	-51.043124	2.157791	0.0838446
3.1e+07-6200000	4.736952e-15	-26.600457	26.600457	1.0000000

Table 14. Pair-wise comparisons using an HSD-turkey test (alpha=0.05) for point inoculum treatments of experiment 7, analyzing the effect of spore density on mycelial coverage.

#ofSpores

	diff	lwr	upr	p adj
1984-397	5.684342e-14	-80.943303	80.94330	1.0000000
9920-397	1.681410e+02	87.197727	249.08433	0.0000035
49600-397	2.361776e+02	155.234308	317.12091	0.0000000
248000-397	3.211701e+02	240.226825	402.11343	0.0000000
1240000-397	4.218071e+02	340.863770	502.75038	0.0000000
6200000-397	3.874958e+02	306.552453	468.43906	0.0000000
3.1e+07-397	4.037542e+02	322.810853	484.69746	0.0000000
9920-1984	1.681410e+02	87.197727	249.08433	0.0000035
49600-1984	2.361776e+02	155.234308	317.12091	0.0000000
248000-1984	3.211701e+02	240.226825	402.11343	0.0000000
1240000-1984	4.218071e+02	340.863770	502.75038	0.0000000

6200000-1984	3.874958e+02	306.552453	468.43906	0.0000000
3.1e+07-1984	4.037542e+02	322.810853	484.69746	0.0000000
49600-9920	6.803658e+01	-12.906722	148.97988	0.1524198
248000-9920	1.530291e+02	72.085795	233.97240	0.0000193
1240000-9920	2.536660e+02	172.722740	334.60935	0.0000000
6200000-9920	2.193547e+02	138.411424	300.29803	0.0000000
3.1e+07-9920	2.356131e+02	154.669823	316.55643	0.0000000
248000-49600	8.499252e+01	4.049213	165.93582	0.0340753
1240000-49600	1.856295e+02	104.686159	266.57276	0.0000005
6200000-49600	1.513181e+02	70.374842	232.26145	0.0000235
3.1e+07-49600	1.675765e+02	86.633242	248.51985	0.0000037
1240000-248000	1.006369e+02	19.693642	181.58025	0.0069450
6200000-248000	6.632563e+01	-14.617674	147.26893	0.1741269
3.1e+07-248000	8.258403e+01	1.640725	163.52733	0.0428767
6200000-1240000	-3.431132e+01	-115.254620	46.63199	0.8625036
3.1e+07-1240000	-1.805292e+01	-98.996220	62.89039	0.9956317
3.1e+07-6200000	1.625840e+01	-64.684903	97.20170	0.9977204

Table 15. Pair-wise comparisons using an HSD-turkey test (alpha=0.05) for point inoculum treatments of experiment 7, analyzing the effect of spore density on nodulation.

#ofSpores

	diff	lwr	upr	p adj
1984-396.8	-4.263256e-14	-37.26042	37.26042	1.0000000
9920-396.8	6.441548e+01	27.15506	101.67590	0.0000862
49600-396.8	7.802745e+01	40.76703	115.28787	0.0000030
248000-396.8	8.741003e+01	50.14961	124.67045	0.0000003
1240000-396.8	8.493020e+01	47.66978	122.19062	0.0000006
6200000-396.8	8.081380e+01	43.55338	118.07422	0.0000015
3.1e+07-396.8	7.967650e+01	42.41608	116.93692	0.0000020
9920-1984	6.441548e+01	27.15506	101.67590	0.0000862
49600-1984	7.802745e+01	40.76703	115.28787	0.0000030
248000-1984	8.741003e+01	50.14961	124.67045	0.0000003
1240000-1984	8.493020e+01	47.66978	122.19062	0.0000006
6200000-1984	8.081380e+01	43.55338	118.07422	0.0000015
3.1e+07-1984	7.967650e+01	42.41608	116.93692	0.0000020
49600-9920	1.361196e+01	-23.64846	50.87238	0.9308931
248000-9920	2.299455e+01	-14.26587	60.25497	0.4980691
1240000-9920	2.051472e+01	-16.74570	57.77514	0.6350347
6200000-9920	1.639832e+01	-20.86210	53.65874	0.8387261
3.1e+07-9920	1.526102e+01	-21.99940	52.52144	0.8818026
248000-49600	9.382588e+00	-27.87783	46.64301	0.9909231
1240000-49600	6.902758e+00	-30.35766	44.16318	0.9986347
6200000-49600	2.786352e+00	-34.47407	40.04677	0.9999969
3.1e+07-49600	1.649058e+00	-35.61136	38.90948	0.9999999
1240000-248000	-2.479830e+00	-39.74025	34.78059	0.9999986

```
6200000-248000  -6.596235e+00 -43.85666  30.66418 0.9989798
3.1e+07-248000  -7.733530e+00 -44.99395  29.52689 0.9972008
6200000-1240000 -4.116405e+00 -41.37683  33.14401 0.9999553
3.1e+07-1240000 -5.253700e+00 -42.51412  32.00672 0.9997700
3.1e+07-6200000 -1.137295e+00 -38.39771  36.12312 1.0000000
```