# Germination inhibitors of fungal spores:

# identification and mode of action

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## Contents

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# Chapter 1

General Introduction

# INTRODUCTION

Fungal spores are important vehicles for colonisation of all kinds of substrates such as seeds, foods and plants. Spoilage fungi can cause severe economic losses and are potential hazards, since fungal outgrowth may result in the production of mycotoxins. There is a great interest among agricultural, food industrial, and medical disciplines to prevent or control fungal contamination. New antifungal agents are developed and current research is aimed at elucidation of mechanisms that underly germination of spores.

This Chapter describes fungal contamination, the germination process of fungal spores, mycotoxin production and methods to control fungal growth including the mode of action of antifungal agents. In addition, fluorescence techniques employed to study fungal germination are described.

G. S. Chitarra and Jan Dijksterhuis. Part of this chapter will be submitted

## FUNGAL SPORE GERMINATION

Fungi are known for their capability to produce sexual and/or asexual spores as agents of reproduction, dispersal and survival. The main classes of fungi including Zygomycetes (e.g. *Rhizopus*), Ascomycetes, (e.g. *Talaromyces* and *Neurospora*) and Basidiomycetes (e.g., *Agaricus* and *Puccinia*) form sexual spores called zygospores, ascospores and basidiospores, respectively. A variety of asexual spores are formed among different fungal groups. The Deuteromycetes (e. g. *Penicillium* and *Aspergillus*) are defined as those fungi, which only form asexual spores namely conidia, and no sexual stage is recognised till now.

Spores play an important role in the life cycle of fungi acting as dispersal or survival. Dispersal spores are produced in higher numbers than the survival spores and they are separated completely from the parent mycelium to facilitate migration to a new site. They have a moderate capacity for survival in the resting dormant state. They are also capable to germinate readily under favourable environmental conditions including the presence of nutrients (29). Conidia are the agent of dispersal of asexual spores, which are produced by several genera of fungi such as *Aspergillus, Penicillium* and *Fusarium,* which are important species in medical, industrial, and agricultural areas (68). Conidia can be formed in chains on the tips of specialised spore-forming cells, and are hydrophobic. Mature conidia have to survive in dry conditions during dispersion through the air (22). In contrast to dispersal spores, survival spores are produced in lower numbers and may not be separated from the parent mycelium (15). For example, thick-walled chlamydospores are produced by e.g. *Mucor racemosus, Fusarium culmorum* and *Peacylomyces varioti* within their hyphae or within their spores under unfavourable conditions (15).

Many fungi are able to produce more than one kind of spore, for example *Neurospora crassa*, which is able to produce two types of asexual spores called microconidia and macroconidia. Microconidia are tiny uninucleate spores, which germinate poorly on agar media and do not survive for a long time. It is suggested that microconidia are probably involved in the sexual process (16). On the other hand, macroconidia are multinucleate spores produced in abundance, easily capable to germinate on agar media and readily dispersed in to the air (3).

In almost all cases, fungal contamination of foods and food products, and colonisation and infection of plants and animals is usually initiated by contact of the host with spores (conidia), subsequently by germination. The germination process is the beginning of fungal growth in followed foods and of infections of plants or animals. It involves the initiation of biochemical activities, with an increase of metabolism and induction of morphological changes (21, 30). Germination of spores often requires environmental nutrients that trigger the spores, which can be designated as the activation event. *Fusarium culmorum* and *Rhizopus* spores require a carbon and nitrogen source, whereas, *Penicillium griseofulvum* and *Aspergillus nidulans* need glucose for germination (21, 58). However, water, oxygen, and carbon dioxide are known to be universal factors that activate spore germination. In addition, other low molecular weight

nutrients (e.g. C-source, specific amino acids and vitamins) and for example inorganic salts can activate germination (30).

Isotropic growth, also designated as swelling, is the first morphological change in spore germination of many fungal species, where the spore starts to swell and consequently increases its volume. Swelling is not merely water uptake, it is also characterised by changes in the composition of the cell, cell wall growth, and increase in dry weight (7). At the same time, swelling is accompanied by numerous metabolic activities including respiration, RNA and protein synthesis (57, 78), as well as trehalose breakdown (58).

Following swelling, cell wall deposition of polysaccharides (chitin) becomes polarized, and the extension of the fungal cell occurs at a restricted area at the tip of the cell (61). This results in elongation of a germ tube and the formation of a branching mycelium. Extensive studies have been carried out on the germination of unicellular spores, e.g. *Colletotrichum*, *Aspergillus*, *Penicillium* and *Rhizopus* (10, 12, 17, 52, 43), but hardly anything is known about germination of multicellular conidia. In the germination of multicellular conidia from *Fusarium culmorum* and *Magnaporthe grisea*, the germ tubes are produced preferably from apical cells and seldom from middle cells (3, 36).

At the end of vegetative growth, as a consequence of nutrient exhaustion, spores are produced allowing the fungus to be dispersed, to survive or to enter a dormant state (30). Nutrient limitation or inhibitory compounds may impose this reversible phenomenon called dormancy. In the case of so-called exogenous dormancy germination proceeds as soon as environmental conditions are favourable. In contrast, in endogenous dormancy, the spores do not germinate even under favourable conditions. Specific treatments to activate the germination process are needed such as heating or exposure to polyacetylenes as describes for *Puccinia carthamni* (41). Dormant spores contain a pre-existing pool of mRNA and ribosomes, primed for rapid activation and translation in the presence of nutrients (58).

## CONTAMINATION

Fungi can be found in a wide variety of environments, such as in seeds, plants, soil, water, insects, food and food products, and animal products. Phytopathogenic fungi can cause economic crop losses when they are associated with seeds and plants (33). Pre-emergence damping-off is an example of a common disease of seedlings in cotton, attributed to the presence of *Pythium spp*. and *Rhizopus oryzae* in the soil (33). *Colletotrichum* causes anthracnose disease in several fruits and vegetables such as banana, avocado, papaya, and tomato, decreasing their commercial values. Other fungi such as *Fusarium*, are economically important phytopathogens in plants and are capable of mycotoxin production. Scab of wheat, named Fusarium head (ear) blight, is caused by *F. graminearum*, which was responsible for epidemics in Canada and USA, and also *F. culmorum*, which caused the same disease in Poland and The Netherlands (63, 71). Fungal infection of grain, nuts and fruits is often preceded by physical damage caused by insect invasion or mechanical injury during harvest.

Food may also become contaminated during processing and handling operations. Processed food can be considered as a complex medium that fungi may colonise and spoil. Fungal species associated with particular foods correlate with the characteristics and properties of the product (22). The primary cause for the deterioration of breads, for example, rye breads are *Penicillium roqueforti, P. paneum, P. carneum* and *Paecilomyces varioti.* Contamination comes from the environment, e.g. air, water, walls and floors are considered to be main source of contamination of beef carcasses with *Penicillium*, *Aspergillus*, *Mucor* and *Cladosporium* species (34). Contaminated commodities, such as cereals, can deteriorate during storage, resulting in higher contamination levels of whole wheat flour (79).

Fungal growth reduces the nutritional value of storage grains and feed and may result in the production of mycotoxins and allergenic spores (23). Mycotoxins are poisonous, often carcinogenic secondary metabolites of fungi, which are associated with certain disorders in animals and humans (24, 66).

The natural occurrence of the mycotoxins produced by *Fusarium* species is in cereals in temperate countries, since these fungi require lower temperatures for growth and mycotoxin production than the *Aspergillus* species. Different toxigenic species and their mycotoxins are presented in Table 1.

**Table 1**: Some toxigenic species of fungi and their principal mycotoxins.

| **Fungal species** | **Mycotoxins** |
|---|---|
| *Aspergillus flavus; A. parasiticus* | Aflatoxins |
| *A. flavus* | Cyclopiazonic acid |
| *A. ochraceus; Penicillium verrucosum* | Ochratoxin A |
| *P. expansum; P. carneum; P. paneum* | Patulin |
| *P. roqueforti; P. carneum; P. paneum* | Roquefortine C |
| *P. roqueforti* | PR toxin |
| *Fusarium culmorum; F. graminearum; F. sporotrichioides* | Deoxynivalenol |
| *F. sporotrichioides; F. poae* | T-2 toxin |
| *F. sporotrichioides; F. graminearum; F. poae* | Diacetoxyscirpenol |
| *Fusarium culmorum; F. graminearum; F. sporotrichioides* | Zearalenone |
| *F. moniliforme* | Fumonisins |

## CONTROL OF FUNGAL DEVELOPMENT

Heat treatment is a technique commonly used processing in food industry. Spoilage fungi are inactivated by heat, such as in pasteurisation or sterilization. However, the ascospores of some fungi are heat resistant and they can survive at high temperatures (85ºC), for example those of *Talaromyces macrosporus* (22). Moreover, germination and growth are prevented by maintaining certain environmental conditions as the use of low oxygen pressure, low water

activities and organic acids or a combination of these methods. Low oxygen pressure and organic acids are used to preserve grass forage in silos with so-called ensilage process. The low oxygen concentration and the organic acids in silos lower the metabolism of fungi preventing their growth. Lowering the water activities (0.65- 0.86) prevents fungal growth as well. Nonetheless, some osmotolerant and xerophilic fungi are able to grow in the presence of high concentrations of sugar and salt and can cause spoilage in these conditions (22). However, for many commodities, such as seeds grains, beans and peas, their resistance towards fungal spoilage is due to their low water activity, provided they are properly dried and well stored.

**Food preservatives**

Fungi have specific pH requirements for growth. Therefore, by altering the hydrogen ion concentration, the development of spores can be controlled. The main antimicrobial food preservatives that have been extensively used are weak organic acids and esters (propionate, sorbate, benzoate and benzoate esters (parabens)), organic acid acidulants (lactic, citric, malic, and acetic acids), inorganic acid preservatives (sulfite), mineral acids (phosphoric and hydrochloric acids) and antibiotics (natamycin) (13, 27, 37). Propionic acid is a highly effective fungal inhibitor used in cheese and bakery products industries. Sec-butylamine is commonly used in its free form to preserve fruits against contamination and damage by storage fungi such as *Penicillium* and *Aspergillus.* In addition, sorbic acid prevents fungal growth and decreases mycotoxin biosynthesis by inhibiting the biological pathways responsible for their production (77).

**Antibiotics and target sites**

Despite the introduction of numerous agents with potent antifungal activity, little success has been seen in the treatment of several mycoses (62). Antifungal polyenes include three main compounds: natamycin, nystatin, and amphoteracin B. These compounds act by increasing the permeability of the fungal cell membrane, after binding to sterols (e.g. ergosterol) causing the death of the cell (31). Several in vitro susceptibility tests for antifungal agents have been developed in order to establish better chemotherapeutic approaches against fungal infections. Amphotericin B was tested against the zygomycetous, the genera *Mucor* and *Rhizopus*, and against *Aspergillus.* These are opportunistic fungi in immunocompromised hosts and may also cause mycoses in animals (53, 59). Amphotericin B has a broad spectrum of activity but it does not cover all the fungal pathogens. Nystatin is used in medical, veterinary and agricultural practices, but it is insoluble in water and is toxic, especially by its dose-dependent nephrotoxicity (18).

Other potential antifungal compounds that act at the level of the membrane are the iturins (A-E), produced by *Bacillus subtilis*. Iturins, as a group, are cyclic lipopeptides characterised by the presence of seven α-amino acids (65, 35, 40, 64). Other lipopeptides that belong the iturin group are the bacillomycins D, F, and L, and mycosubtilin (9). Recently, Moyne et. al (54) described the antifungal action of two bacillomycin D variants. Iturin A is efficacious as a seed treatment for many seed-borne fungi and stable over time on treated seeds, but it is not able to inhibit aflatoxin production of *Aspergillus flavus* (38). Nowadays, a wide range of antifungal agents is used in combating biodeterioration and in preventing or treating fungal diseases of plants and for treating diseases in animals and humans (Table 2).

**Table 2**: Antifungal compounds and their target sites.

| **Antifungals** | **Target sites** |
|---|---|
| Benzimidazoles; Griseofulvin | Mitosis |
| 5-Fluorocytosine | Nucleic acid synthesis |
| Acylalanines | RNA polymerase I |
| Kasugamycin; Sordarins | Protein synthesis |
| Carboxamides; Strobilurins | Respiration |
| Fosetyl-AL | Phosphate metabolism |
| Imidazoles; Triazoles; Thiocarbamates | Ergosterol synthesis |
| Nystatin; Amphoteracin B; Natamycin; | Plasma membrane |
| Polyoxin; Nikkomycins; Echinocandins | Cell wall synthesis |

**Antifungals from plants**

Many plants produce essential oils that have antifungal activity. These chemicals are secondary plant defence metabolites that may be produced in response to microbial attack. Essential oils can be extracted from plant materials such as spices, herbs, berries, and roots and stems (8). Oils with fungicidal activity are obtained from different plants genera including the *Ocimum*, *Thymus*, *Origanum*, *Anethum*, *Eucalyptus*, *Foeniculum* and C*itrus* (51). Crop protectants and food preservatives of natural origin are often considered as potentially safe for consumption, but their effective use in practice has only been established in a few cases. Essential oil components such as S-carvone, carvacrol, cinnamaldehyde, and thymol are known for their antimicrobial activities (70).

**Self-inhibitors**

Fungi produce substances during growth that influence their development. Several fungi, at high density of spores (crowding effect), produce inhibitory substances, named self-inhibitors, which inhibit germination and growth. Self-inhibitors have been characterised in *Puccinia*,

*Uromyces, Colletrotrichum, Dictyostelium, Fusarium* and *Aspergillus* and can be volatile or non-volatile (2, 5, 6). Various self-inhibitors have been isolated and identified after extraction from culture filtrates of fungi (Table 3). Self-inhibitors can also influence other fungal processes, for example mycosporine-alanine produced by *C. graminicola* prevents appressorium formation (44, 45). The self-inhibitors produced by *Glomerella cingulata* and *Dictyostelium discoideum* inhibit protein synthesis (4, 47).

**Table 3**: Self-inhibitors from fungi

| **Fungi** | **Chemical compound** | **References** |
|---|---|---|
| *Colletotrichum graminicola* | Mycosporine-alanine | (45) |
| *Colletotrichum gloeosporioides* | Gloeosporone | (42) |
| *Colletotrichum capsici* | Not identified | (48) |
| *Glomerella cingulata* | Not identified | (47) |
| *Dictyostelium discoideum* | N, N-dimethylguanosine | (5) |
| *Penicillium griseofulvum* | Not identified | (25) |
| *Fusarium oxysporum* | Nonanoic acid | (26) |
| *Syncephalastrum racemosum* | Nonanoic acid | (32) |
| *Aspergillus niger* | Not identified | (6, 39) |
| *Puccinia graminis* var *tritici* | Coumarins and phenolic acids; Methyl cis ferulate | (49, 75) |
| *Puccinia helianthi* | Methyl 3,4 dimethoxycinnamate | (50) |
| *Puccinia antirrhini* | Methyl 3,4-dimethoxycinnamate | (50) |
| *Uromyces phaseoli* var *typica* | Aspartic and glutamic acid | (80) |
| *Anisogramma anomala* | Not identified | (74) |
| *Geotrichum candidum* | Not identified | (72) |
| *Hemileia vastatrix* | Organic acid | (55) |
| *Blastocladiela emersonii* | Not identified | (1) |
| *Tiletia caries* | Trimethylalanine | (76) |
| *Peronospora tabacina* | 5-Isobutyroxy-β-ionone | (46) |
| *Microsporum gypseum* | Not identified | (60) |

Self-inhibitors inhibit spore germination reversibly, after removal of the compound from the spore or its environment, the germination is initiated. The major function of self-inhibitors is to prevent premature germination of spores directly after spore formation and before spore dispersion. This mechanism guarantees that spores only germinate after dispersal into environments that may favour outgrowth to establish a mycelium.

## FLUORESCENCE PROBES

The most common criteria used to measure fungal germination is the emergence of germ tubes. Fluorescence microscopy techniques, whether or not in combination with Flow cytometry (FCM) and Fluorescence ratio-imaging microscopy (FRIM), are technologies that allow rapid analysis of physiological parameters and have the potential of single cell analysis (14, 20). These technologies are also used to assess the impact of antimicrobials on different cellular characteristics including membrane permeability, membrane potential, respiration and intracellular pH (14, 19, 56). The fluorescent probe carboxyfluorescein diacetate (cFDA) is an enzyme activity probe that can pass through the spore membrane. Once inside the spores, it is cleaved by non-specific esterases to release the fluorescent carboxyfluorescein (cF), which is retained inside the spores. Therefore, the viability can be correlated with the ability of the spores to accumulate carboxyfluorescein (28). Propidium iodide (PI), a nucleic acid probe (mol. wt = 668 g $mol^{-1}$) and TOTO-1 (mol. wt. = 1303 g $mol^{-1}$), a yellow fluorescent dimeric cyanine dye, are supposed to cross only through damaged cell membranes. Fluorescence methods using probes such as cFDA and PI have been used to evaluate conidial viability (69). The pH dependent probe, 5(and 6-)-carboxyfluorescein succinimidyl ester (cFSE) is used to measure the intracellular pH of individual spores. The probe cFSE can form conjugates with aliphatic amines and is therefore better retained within the cell than non-conjugated probes such as cF (11). Other pH-dependent probes are also available, for example, BCECF, calcein, and SNARF-1 (61, 67). Changes in pH have been observed to be clearly associated with differentiation processes (73).

## OUTLINE OF THIS THESIS

Fungi and their toxic metabolites cause losses of food products, cause diseases in plants and animals, and may have adverse effects on human health. A crucial step in fungal colonisation and infection is the germination process, subsequently resulting in mycelial growth. The aim of the work described in this thesis is to study fungal germination and the effects of antifungal compounds, using *Penicillium paneum* conidia and *Fusarium culmorum* macroconidia as model organisms. Antifungal compounds from *Bacillus subtilis* as well as a newly discovered self-inhibitor of *Penicillium paneum* were used in this study.

**Chapter 2** describes the identification and characterization of an iturin-like antifungal compound produced by *Bacillus subtilis* YM 10-20. Fluorescence probes in combination with flow cytometry and scanning electron microscopy were applied to assess the action of the antifungal compound against spores. In **Chapter 3**, the sequence of events in the germination process of multicompartment *Fusarium culmorum* macroconidia and the effects of antifungal compounds on this process was investigated by measuring the intracellular pH employing fluorescence ratio imaging microscopy (FRIM). **Chapter 4** describes the identification and

characterization of 1-octen-3-ol, a volatile self-inhibitor of spore germination from *P. paneum.* The effect of this self-inhibitor on different developmental processes during fungal germination was studied. In **Chapter 5**, the mode of action of 1-octen-3-ol was investigated. It targets the membrane affecting intracellular pH, respiration, and protein synthesis. In **Chapter 6**, the proposed pathways involved in the formation of 1-octen-3-ol are discussed including a possible role for lipoxygenases in the breakdown of linoleic acid, a possible precursor in *P. paneum*. In addition, applications and perspectives are discussed in the prevention of fungal contamination and growth.

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# Chapter 2

An antifungal compound produced by *Bacillus subtilis* YM 10-20 inhibits germination of *Penicillium roqueforti* conidia

## SUMMARY

In the food industry there is a great interest in the use of antifungal compounds for biological control of fungi such as *Penicillium roqueforti*. In this study we investigated the production of an antifungal compound produced by *Bacillus subtilis* YM 10-20, isolated from maize. We investigated the inhibitory effect of the supernatant of *B. subtilis* on growth of various fungi and on germination of *P. roqueforti* conidiospores. A strong inhibitory effect was observed on the growth of *Fusarium culmorum, Mucor sp., Aspergillus niger, Aspergillus parasiticus, Aspergillus flavus* and *P. roqueforti*. The germination of *P. roqueforti* was completely inhibited in the presence of the *B. subtilis* YM 10-20 supernatant (50% supernatant and 50% Malt extracted Broth). The inhibitory compound was shown to be more effective in the early stage of the spore germination process. The active antifungal compound was isolated by acid precipitation (acidification to pH 2). Further analysis by High Performance liquid Chromatography (HPLC) and Mass spectrometry suggested that this compound is very likely to be a cyclic lipopeptide of the iturin group showing high similarity to iturin A, which the insertion in fungal membranes is mediated by cholesterol (20). In correspondence with this, the antifungal activity was reduced when cholesterol was added to the supernatant. The germination of conidiospores of *P. roqueforti* in the presence of the antifungal compound was totally inhibited. The criterion most frequently used to measure germination is the emergence of germ tubes as determined by microscopy. In this study, we also applied the fluorescent probes, carboxyfluorescein diacetate (cFDA) and propidium iodide (PI), to assess the viability of conidiospores of *P. roqueforti*. Conidiospores treated with the antifungal compound of *B. subtilis* could not be labeled by carboxyfluorescein (cF), where as they were labeled with PI. This indicates that the conidiospore membrane was permeabilised, resulting in killing of the spores.

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# INTRODUCTION

*Penicillium roqueforti* is a common fungal contaminant of bakery and silage products. It may produce several mycotoxins such as roquefortine C, isofumigaclavine A and B, PR toxin and mycophenolic acid which can cause economic losses and may affect human health (26).

The approaches that have been used to control fungal contamination in food are usually based on heat treatment or chemical treatment (26, 22). The antifungal food additives commonly used as preservatives are inorganic compounds e.g. sulphite and nitrite, or weak organic acids, such as acetic, propionic, sorbic and benzoic acid (11, 9, 29). Alternatively, antifungal agents produced by microorganisms may be used as biocontrol agents. In particular, *Bacillus subtilis* is known to produce a number of antifungal compounds including Alboleutin, Bacitracin, Botrycidin, Clorotetain, Fengycin, Iturins and Rhizocticins (31). These antifungal peptides inhibit the growth of a large number of fungi such as *Aspergillus, Penicillium* and *Fusarium* species (16), as well as yeasts, i.e. *Candida albicans* and *Saccharomyces cerevisiae* (3, 24, 12, 30). Whereas most of these antifungals have been tested against mycelial growth, very little information is available about their effect on fungal spore survival and germination.

*Penicillium* produces conidia as agents of reproduction, dispersal, and / or survival. Conidia can survive for a long time under environmental stress, and outgrowth in food can result in food spoilage and potentially in the production of mycotoxins. Since germination is the starting event of the asexual life cycle of this fungus, the antifungal compounds should preferably prevent germination.

Microscopic assessment of germination has for long been the standard method to determine the viability of fungal conidia, however such germination assays are time-consuming. Fluorescence methods using probes such as cFDA and PI have been used to evaluate conidial viability (27). Carboxyfluorescein diacetate (cFDA) is an enzyme activity probe that can pass through the membrane of the spores. Once inside the spores, it is cleaved by non-specific esterases to release the fluorescent carboxyfluorescein, which is retained inside the spores. Thus, the viability can be correlated with the ability of the spores to accumulate carboxyfluorescein (8). Propidium iodide (PI) is a nucleic acid probe that is supposed to cross only through damaged membranes in cells (6). Fluorescent labelling in combination with flow cytometry (FCM) allows fast measurement of individual cells for viability assessment (6, 7). Several methods have been developed using flow cytometry to assess the effects of antibacterial agents on different cellular characteristics or parameters, including membrane permeability, membrane potential and respiration (19).

We here describe the isolation, characterization and identification of an antifungal compound produced by *Bacillus subtilis* YM 10-20. HPLC and mass spectrometry analysis revealed the compound to be similar iturin A. Using fluorescence probes in combination with flow cytometry, and using scanning electron microscopy we show that this compound efficiently permeabilises and disrupts fungal spores.

## MATERIALS AND METHODS

### Bacterial culture

*Bacillus subtilis* strain YM 10-20 (LU 1004 – NRRL B 23189), isolated from preharvest maize (17), was maintained on Plate Count Agar (PCA) slants and stored at 4ºC. The inoculum for production of antifungal compounds was prepared by growing the cells in brain heart infusion (BHI) medium, on a rotary shaker at 130 rev $min^{-1}$ at 30°C for 48h.

### Fungal suspension

*Penicillium roqueforti* LU 510 was isolated from sugar beet press pulp (18), and was grown on malt extract agar medium (MEA, CM59, Oxoid Ltd) at 25°C for a maximum of 7 days. A spore suspension was prepared by adding 9 ml of peptone physiological salt solution (8.5 g $l^{-1}$ NaCl with 1 g $l^{-1}$ bacteriological peptone (Oxoid Ltd) + 0.1% Tween 80) to the agar slant. Subsequently, the suspension was filtered through a 17 µm nylon filter, centrifuged twice at 4000 X g for 3 min, and the pellet containing the spores was resuspended in malt extracted broth (MEB) medium (CM57, Oxoid), adjusted to pH 4.0 with lactic acid. The concentration of spores was determined using a haemocytometer and adjusted to 1.0 x $10^6$ conidia $ml^{-1}$.

### Production and isolation of antifungal compounds from *B. subtilis* YM 10-20

The method of Arrendale *et al.* (1) with some modifications was used to extract the antifungal compound produced by *B. subtilis* strain YM 10-20. *B. subtilis* cells grown in BHI were harvested after 48h of incubation, final $O.D._{600}$ approximately 3.5, and centrifuged at 4,000 X g at 20°C for 15 min. The supernatant was filtered through a sterile 0.2 µm pore size filter. Subsequently, the pH of the supernatant was adjusted to 2 with concentrated HCl. After centrifugation at 16,200 X g for 10 min at 20°C the precipitate was collected and dissolved in methanol/water (50:50 v/v) pH 8, filtered through 0.2 µm membrane filter, and the HCl precipitate was stored at -20°C.

### Antifungal activity of *B. subtilis* YM 10-20

The supernatant of *B. subtilis* was tested for antagonistic activity against *Penicillium roqueforti*, *Aspergillus niger, A. parasiticus, A. flavus, Mucor sp., Fusarium culmorum* and *Rhizopus sp*.. Agar plates were prepared by mixing 50 ml of MEA medium with spore suspensions to obtain final concentration of $10^4$ spores $ml^{-1}$ of each fungus. Two wells of 8

mm in diameter were made in each plate and filling with 80 µl of 2% bacteriological agar sealed the bottom of the wells. Subsequently, the wells were filled with 200 µl of *B. subtilis* strain YM 10-20 supernatant or HCl precipitate suspended in methanol/water (50:50 v/v). The methanol/water (50:50 v/v) solution was used as a control. The plates were incubated for 3 days at 30°C and subsequently the diameter of the inhibition zone was measured. All experiments were carried out with two replicates per fungus.

**Germination test**

A volume of 500 µl of the *P. roqueforti* LU 510 spore suspension of 1.0 x $10^6$ conidia per ml was centrifuged and the pellet containing the spores was resuspended in 500 µl of medium (MEB) pH 4 in the presence of 10, 25, and 50% of the supernatant of *B. subtilis* YM 10-20. As a control, the pellet was resuspended in 500µl of MEB with sterile distilled water (50:50 v/v) pH 4. After incubation at 25ºC for 8 h, 100 conidia of *P. roqueforti* were analysed with an Olympus Optical CO microscope (1000x magnification), Ltda BX40 (Tokyo, Japan) and the percentage of germinated conidia of each suspension was calculated. A conidium was considered germinated if the germ tube was longer than one-half of the diameter of the conidium. The HCl precipitate of *B. subtilis* was also used for the germination test at identical conditions and concentrations as presented above. The experiment was performed in triplicate, and the results of a typical experiment are presented.

**Identification by HPLC and Mass spectrometry analysis**

HPLC analysis was performed by injecting 50 µl of the extracted material of *B. subtilis* on a Spherisorp ODS-2 column, 4.6 mm ϕ x 250 mm (Chrompack, Bergen op Zoom, The Netherlands) and monitoring at 214 and 280 nm. Elution (0.9 ml $min^{-1}$) was performed in a linear gradient in methanol/water (50:50 v/v) during 0 to 20 min; methanol/water (80:20 v/v) during 20 to 60 min; 100% methanol during 60 to 65 min and methanol/ water (50:50 v/v) during 65 to 75 min. Individual fractions of the HCl precipitate of *B. subtilis* were collected manually and were subsequently tested for antagonistic activity against *P. roqueforti* as described previously. The mass spectrometry analysis was performed on a Perseptive Biosystems Voyager DE$^{TM}$-RP Matrix assisted laser desorption/ionization (MALDI)- Time of flight (TOF) to determine the molecular weight of the compounds. Three matrices were applied in this study, sinapinic acid (proteins and peptides Mw > 10000Da), α-cyano-4 hydroxy cinnamic acid (proteins and peptides Mw <10000 Da) and 2,4,6-trihydroxiacetophenone (THAP) (peptides). Purified iturin A was kindly provided by Prof. Dr. Françoise Peypoux (Laboratoire de Biochimie Analytique et Synthese Bioorganique, Lyon, France).

**Monitoring germination capacity**

The spore suspension of *P. roqueforti* at a concentration of $10^6$ spores ml $^{-1}$ was incubated in the presence of *B. subtilis* supernatant for 1, 2, or 3 h, and also in the presence of *B. subtilis* HCl precipitate for 2 h. Subsequently, the spores were harvested by centrifugation at 2,500 g for 3 min. The pellet was washed in MEB and incubated for 10 h. Each sample was observed every 2 h using the microscope, to determine the percentage of spore germination. The control used in the HCl precipitate solution consisted of spores suspended in MEB medium with methanol/water (50:25:25 v/v/v).

**Scanning Electron Microscopy (SEM) analysis**

Samples of fungal mycelium were removed from the border of the inhibition zone and transferred to a nuclepore polycarbonate filter with 1 μm pores (Costar, Cambridge, MA, USA). These filters are glued with Tissue Freezing Medium (Electron Microscopy Sciences Washington DC USA) on a brass specimen holder. The specimens were frozen in liquid nitrogen and subsequently transferred into the cryo-preparation chamber (Oxford CT 1500 HF, Oxford Instruments, High Wycombe, UK) which was dedicated to FESEM. Inside the cryo-preparation chamber the sample kept 3 minutes at -95°C to sublime water contamination. The samples were coated at -95°C with 5 nm platinum by magnetron sputtering and observed in an FESEM (JSM 6300F Tokyo, Japan) at -180°C and 5KV. Digital images were recorded.

**Fluorescence of conidiospores**

The fluorescent probes, carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) (Molecular Probes, Eugene, Oregon, USA) were applied to assess the viability of *P. roqueforti* conidiospores. Spores were labelled with cFDA and PI. A stock solution of cFDA was prepared in acetone (4.6mg ml$^{-1}$) and stored at -20°C in the dark. A stock solution of PI (1.0-mg ml$^{-1}$) was prepared in distilled water and stored in the refrigerator. Conidiospores of *P. roqueforti* LU 510 at a concentration of $10^6$ spores ml $^{-1}$ were incubated for 2 h in the absence and presence of 1.0 ml of HCl precipitate of *B. subtilis* YM 10-20. Samples containing 1.0 ml of spore suspension of *P. roqueforti* were incubated with cFDA and PI at a final concentration of 10 μM. The samples were incubated for 15 min at 25°C and subsequently, the staining of the conidiospores was determined microscopically. The Axioskop epifluorescence microscope was equipped with a 50-W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength of 450 to 490 nm, emission wavelength > 520 nm), and a Plan-Neofluar objective lens.

### Flow cytometric analysis

Analysis of individual cells was performed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with an air-cooled argon ion laser at 15-mW (excitation wavelenth 488-nm). Conidiospores of *P. roqueforti* LU 510 at a concentration of $10^7$ spores ml $^{-1}$ were incubated for 3 h in the absence and presence of 1.0 ml of HCl precipitate of *B. subtilis* YM 10-20. Subsequently, samples were diluted to $10^6$ spores ml $^{-1}$ in 50 mM $KP_i$ buffer (pH 7.0), and delivered at the low flow. The instrument was set up to collect 5 parameters: forward (FSC) and side scatter (SSC), and three fluorescent signals. A band pass filter of 530 nm (515 to 545 nm) was used to collect the green fluorescence (FL-1), a band pass filter of 585 nm (564 to 606 nm) was used to collect the yellow- orange fluorescence (FL-2), and a long pass filter of 670 nm was used to collect the red fluorescence (FL-3). FSC was collected with a diode detector. SSC and the three fluorescence signals were collected with photomultiplier tubes. All signals were collected by using logarithmic amplifications. Data were analysed with the CELLQuest program (version 3.1f; Becton Dickinson) and the WinMDI program (version 2.8; Joseph Trotter, John Curtin School of Medical Research, Camberra, Austria). The sample analysis time was 1 min. in approximately 5000 particles were analysed.

## RESULTS

### Inhibition of germination and fungal growth

The supernatant of *Bacillus subtilis* strain YM 10-20 inhibited growth of *Penicillium roqueforti, Aspergillus niger, Aspergillus parasiticus, Aspergillus flavus, Mucor* sp. and *Fusarium culmorum,* whereas growth of *Rhizopus* sp. was not affected. Analysis of the inhibition zone diameters revealed *F. culmorum* to be the most sensitive fungus, followed by *P. roqueforti* and *A. niger* (data not shown).

The germination efficiency of *P. roqueforti* spores after 8 hours incubation in the absence of *B. subtilis* strain YM 10-20 supernatant was 84% (Fig.1). In the presence of 10, 25, and 50% of supernatant, the percentage of germination decreased to 7, 1 and 0%, respectively. *P. roqueforti* was chosen as the model organism to test the inhibitory activity of the HCl precipitate of the *B. subitilis* YM 10-20 supernatant.

The diameter of the inhibition zone of the HCl precipitate, and that of the supernatant was 25 and 17 mm, respectively. The inhibitory zones remained the same for several days (data not shown).

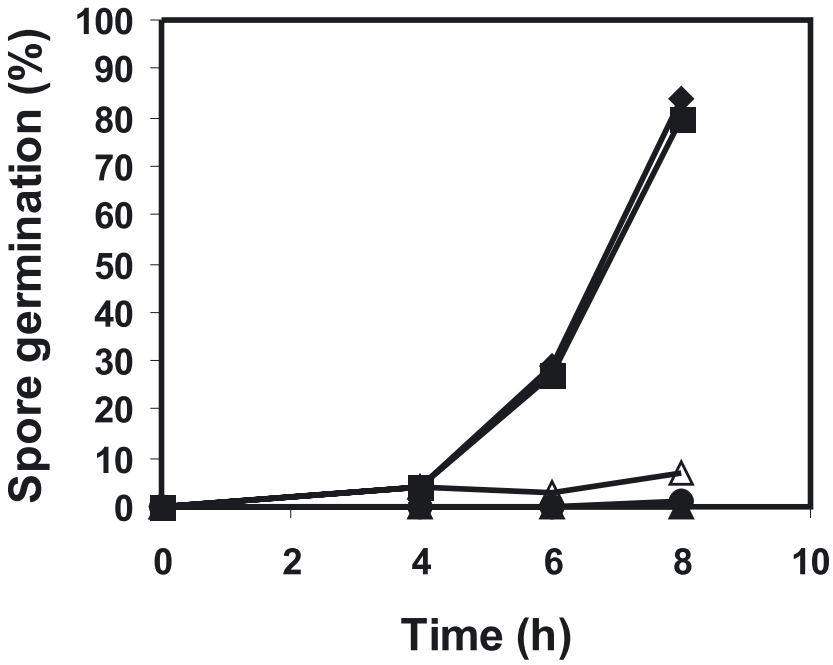



**Fig. 1** Germination of *P. roqueforti* in malt extract broth MEB (♦), in MEB/dest water (50:50 v/v) (■), and MEB containing 10% (Δ), 25% (●) and 50% (▲) of supernatant of *B. subtilis* YM 10-20.

**Effect of temperature and cholesterol on antifungal activity**

The antifungal compound produced by *B. subtilis* YM 10-20 was shown to be heat stable. After heat-treatment at 70ºC and 100ºC for 1 h, the growth of *P. roqueforti* was still inhibited and the inhibitory zones were 18 and 8 mm, respectively (data not shown). Furthermore, the antifungal compound was found to be resistant to proteolytic enzymes, including Pronase E, proteinase K and α chymotrypsin (data not shown).

The antifungal activity was also tested in the presence of cholesterol (Sigma, U.S.A.). After 9 hours of incubation, the percentage of spore germination of *P. roqueforti* in the control, (MEB/water (50:50 v/v)), was 90%, whereas it was completely inhibited in the presence of the antifungal compound (MEB/supernatant (50:50 v/v)). However, addition of cholesterol (15 µg $ml^{-1}$) resulted in the latter case in 61% of spore germination, which points to a significant reduction of the antifungal activity under these conditions. Germination of *P. roqueforti* in the control was not affected by cholesterol (data not shown).

**Identification of the HCl precipitate**

Analysis of the HCl precipitate by High Performance Liquid Chromatography showed one fraction that produced a clear inhibition zone against growth of *P. roqueforti*. Comparison of the HPLC profile of the HCl precipitate of strain YM 10-20 with that of purified iturin A revealed similar retention times and peak heights (data not shown).

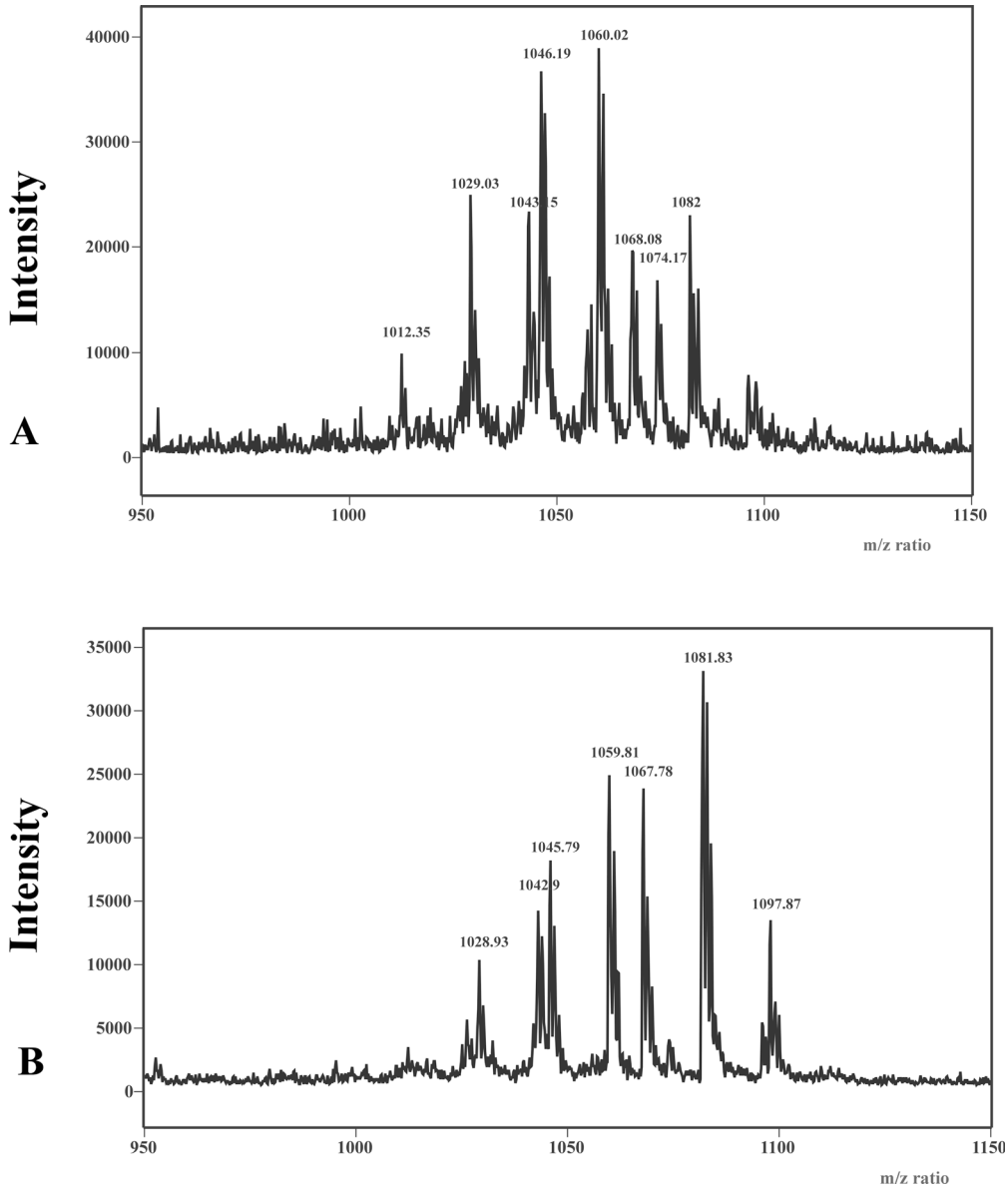


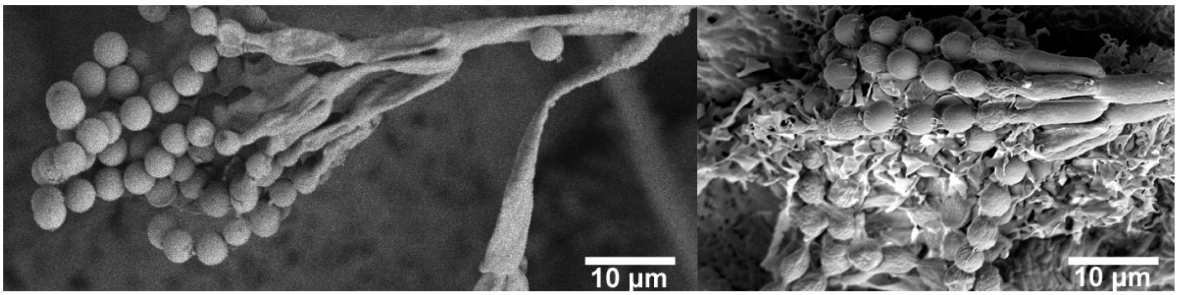

**Fig. 2** Mass spectrometry analysis (Maldi-Tof) of Iturin A (A) and the HCl precipitate of *B. subtilis* YM 10-20 (B).

The mass spectrum of the compound of *B. subtilis* YM 10-20 showed homologous molecular ion peaks differing by 14 mass units at m/ z 1029 and 1043. Other homologous peaks were observed at 1068 and 1082. The difference of 22 mass units in the molecular ion peaks at m/ z

1046 and 1068 and peaks at 1060 and 1082 indicates the presence of salt i.e. sodium ions. A difference of 17 mass units in the molecular ion peaks at m/ z 1029 and 1046 and peaks at 1082 and 1098 was found as well. Comparison of the mass spectrum of the HCl precipitate with that of purified iturin A showed identical molecular ion peaks (1029, 1043, 1046, 1060, 1068, 1074 and 1082) (Fig. 2), from which we conclude that the antifungal compound of *B. subtilis* strain (HCl precipitate) is highly similar to iturin A.

**Scanning electron microscopy and fluorescent probes**

SEM analysis showed major damage of the hyphae and the spores of *P. roqueforti* in the presence of the HCl precipitate (Fig. 3). Furthermore, germination of *P. roqueforti* was not observed after pre-incubation of the conidiospores with the HCl precipitate for 2 h, followed by transfer to fresh MEB/methanol/water (50:25:25 v/v/v), suggesting irreversible damage of the conidiospores (data not shown).




**A** **B**

**Fig. 3** SEM of *P. roqueforti* grown on malt extract agar in the absence (A) and presence (B) of B. subtilis YM 10-20 supernatant.

To study the effect of the antifungal compound in more detail, fluorescent probes were applied to assess the membrane permeabilisation and viability of the conidiospores of *P. roqueforti.* Untreated, control conidiospores could be stained with cF, but not with PI. In contrast, conidiospores treated with the HCl precipitate for 2 h could not be stained with cF, whereas, they were stained with PI. This indicates that the spores were permeabilised after treatment with the HCl precipitate. Indeed, FCM results show a distinct population of PI labeled spores (Fig.4).

## DISCUSSION

In this study the antifungal compound produced by *Bacillus subtilis* YM 10-20 was shown to inhibit germination and growth of *Penicillium roqueforti* conidiospores. The antifungal compound is very heat stable, its activity is reduced in the presence of cholesterol, and it is resistant to proteolytic degradation. These characteristics indicate that the antifungal compound may belong to the iturin group of antibiotics, which are known to interact with sterols of the cytoplasmic membrane of fungi (12, 13, 14).

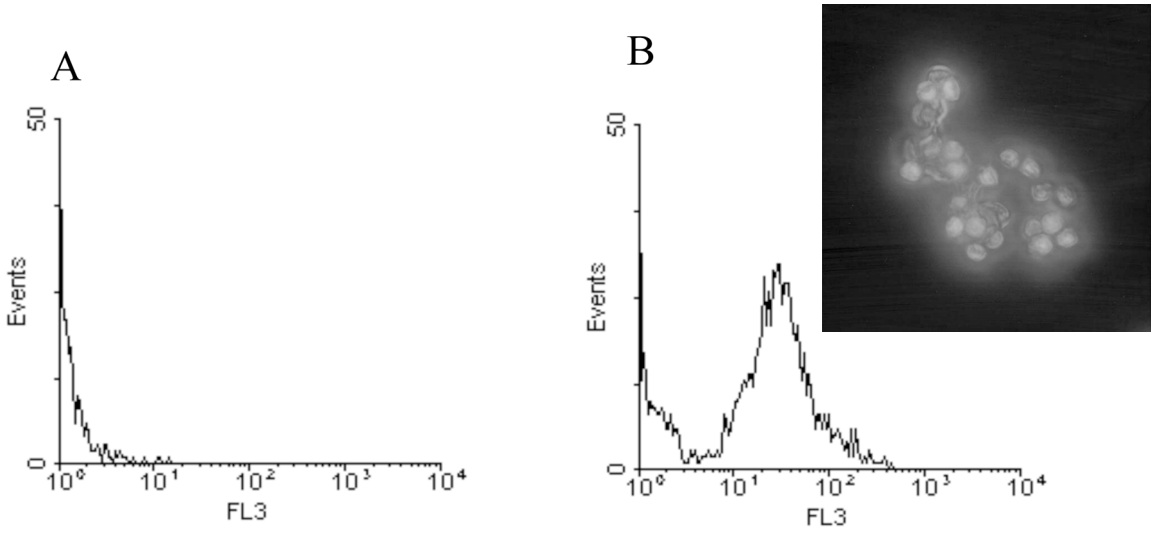



**Fig**. **4** Histograms of PI-stained control spores (A) and HCl precipitate- treated spores (B). The insert in fig B shows a fluorescence microscopy picture of PI labelled spores.

Most of the known antifungal agents produced by *B. subtilis* are polypeptides (2, 4, 23, 16), including iturins A- E, bacillomycins D, F, and L, and mycosubtilin (5). Recently, Moyne *et al.,* (15) described the antifungal action of two bacillomycin D variants. Iturins are cyclic lipopeptides characterised by the presence of seven α-amino acids (21, 20, 10, 12). Iturin A has been shown to increase the permeability of lipid membranes of fungal cells by pore formation resulting in the loss of essential macromolecular compounds (30).

HPLC analysis of the HCl precipitate from YM 10-20 showed that this compound is similar to iturin A. In addition, mass spectra analysis of the *B. subtilis* compound and that of purified iturin A, revealed a high similarity between these compounds i.e. identical molecular ion peaks were identified, suggesting that the compound produced by *B. subtilis* YM 10-20 to be iturin A.

The HCl precipitate of strain *B. subtilis* YM 10-20 totally inhibited the germination of *P. roqueforti* conidiospores. Scanning electron microscopy analysis of *P. roqueforti* conidiospores exposed to the *B. subtilis* supernatant revealed destruction and morphology

changes of spores. Germination of *P. roqueforti* was also inhibited in the presence of purified iturin A (data not shown). It appears that the HCl precipitate also affects the permeability of the membrane of conidiospores preventing germination. Under these conditions the spores will lose the ability to initiate biochemical activities, to increase metabolism and to initiate morphological changes. Notably our studies reveal that these compounds may also act on spores.

Spore inactivation was also assessed by fluorescence techniques. Fluorescence microscopy and FCM revealed PI labelling of damaged cells, indicating permeabilisation of membrane of *Penicillium roqueforti* conidiospores after exposure to the HCl precipitate. FCM analysis is a rapid, reliable and sensitive method to assess viability (25, 7, 19).

According to our studies, the antifungal compound may be effective in the control of fungal growth, however, *Rhizopus* sp. was not sensitive (23). This may be explained by the low ergosterol content of *Rhizopus* sp. membrane (28, 3).

In conclusion, fluorescence staining in combination with FCM and SEM are efficient tools for assessing the action of antifungal compounds against spores. Iturin-like compounds may permeabilise fungal spores and inhibit their germination. This is the first study that shows that iturin-like compounds may permeabilise fungal spores and inhibit their germination.

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# Chapter 3

Differentiation inside multicelled macroconidia of *Fusarium culmorum* during early germination

## SUMMARY

Multicelled conidia are a common phenomenon in many species in the Fungal Kingdom, but hardly any attention has been given in the past to the germination process of these structures. We investigated germination and the effects of antimicrobials on multicompartment macroconidia of *F. culmorum* by measuring the intracellular pH ($pH_{in}$) employing fluorescence ratio imaging microscopy (FRIM). The macroconidia were labelled with the pH dependent probe 5(and-6)-carboxyfluorescein succinimidyl ester (cFDA-SE). The results showed that an increase in the intracellular pH of macroconidia occurred simultaneously with their swelling. Then, the separate compartments of macroconidia showed different $pH_{in}$ values. Cells that were forming a germ tube had higher internal pH values than not germinating cells. Germ-tube formation was mostly from apical compartments with germ tubes showing higher $pH_{in}$ values compared to the cells of the macroconidium. Interference of the germination process with the antifungals, nystatin and nonanoic acid, affects $pH_{in}$ of the conidia and their differentiation. The pH gradients in the macroconidial compartments dissipated very fast in the presence of nystatin at levels of 1 and 4 µg/ml. At sublethal doses (0.3 µg/ml) of nystatin, the apical compartments appeared to be preferentially targeted, resulting in lower $pH_{in}$ values in these compartments. Strikingly, the reduced germination capacity of apical compartments under these conditions was compensated by an increased germination capacity of middle compartments. This study provides evidence for differentiation inside macroconidia during early germination. The compartments of the conidium may not be separated cellular entities, but communicate during germination. Multicellular fungal spores may still have the possibility to germinate after adverse conditions, while unicellular conidia have had their change.

G. S. Chitarra, Jan Dijksterhuis, P. Breeuwer, F. M. Rombouts and T. Abee. Differentiation inside multicelled macroconidia of *Fusarium culmorum* during early germination. Submitted

# INTRODUCTION

A relative large number of fungal species form multicelled conidia that serve for survival and dispersal. These include genera such as *Fusarium* sp., which are important plant pathogenic fungi and mycotoxin producers, *Alternaria* sp. that cause allergic reactions and produce mycotoxins; *Helminthosporium solani,* which cause silver scab on potatoes and *Magnaporthe,* the agent of rice blast and the most important pathogen in this common crop plant.

Germination starts with an isotropic growth phase, designated as "swelling", which is followed by polarised growth. Swelling is certainly not merely water uptake alone, but is characterised by active incorporation of compounds into the cell wall (Bartinicki-Garcia and Lippman, 1977), and by the synthesis of protein and RNA (Van Etten et al., 1983; Ojha and Barja, 2003). During polarised growth, extension of the fungal cell occurs at a restricted area at the tip of the hypha (Parton et al., 1997), and results in elongation of germ tube. Extensive studies have been carried out on the germination of unicellular spores e.g. *Colletotrichum, Aspergillus, Penicillium* and *Rhizopus* (Bourret, 1986; Marin et al., 1998; Breeuwer et al., 1997; Chaky et al., 2001; Leandro et al., 2001), but hardly anything is known about germination of multicelled conidia. Why do fungi produce relatively large and multicellular conidia? Can these conidia be considered as a group of independent cells that are only connected physically, do they act individually, and/or do the compartments communicate with each other? Some evidence about functional differentiation in multicelled conidia has been obtained in the case of *Magnaporthe grisea.* In this organism, germ tubes arise most commonly from the apical cell, less frequently from the basal cell, and rarely from the middle cell (Jelitto et al., 1994).

The multicelled conidia of *Fusarium culmorum* were chosen as a model system. *Fusarium culmorum* is a fungal pathogen in many plants (Persson et al., 1997; Gang et al., 1998). In addition, the fungus produces mycotoxins such as zearalenone, trichothecenes and culmorim (Gang et al., 1998; Doohan et al., 2000). A hallmark of this fungus is the banana-shaped multicellular macroconidia. The usual number of septa is five, but it can range from 3 to 8. Each conidium possesses a more or less pointed apex and a distinct footcell. Each compartment is uninucleate, and one or more are able to form a germ tube. Note that macroconidia do not serve for long-term survival; that is the function of the chlamydospores, a second type of spore produced by these organisms.

The intracellular pH is associated with many cellular processes such as DNA transcription, protein syntesis, and regulation of the enzyme activities and cell wall synthesis (Breeuwer et al., 1997). Changes in pH have been observed to be clearly associated with differentiation processes (Stewart et al., 1988; Inouye, 1985).

The aim of this study was to investigate germination and the effects of antimicrobials on multicompartment macroconidia of *Fusarium culmorum* by measuring the intracellular pH employing fluorescence ratio imaging microscopy (FRIM). In this work evidence is presented

that the separate compartments of macroconidia have difference in pH and germination capacity. Multicellular spores may be more versatile in their responses to adverse conditions. We show evidence that communication may exist between the separate compartments.

## MATERIALS AND METHODS

### Preparation of macroconidia suspension

*Fusarium culmorum* VTT D-80148 was isolated from barley and supplied by A. Laitila, (VTT, Biotechnology and Food Research, Espoo, Finland). The fungus was incubated for 7 days on Potato Dextrose Agar medium (PDA, Difco Laboratories, Detroit, Michigan) at 25$^{o}$C and then kept at 4$^{o}$C. *F. culmorum* was inoculated in 1% CMC broth medium containing (per L): 10 g carboxymethylcellulose, 1 g $NH_4NO_3$, 1 g $KH_2PO_4$, 0.5 g $MgSO_4$ x 7 $H_2O$ and 1 g yeast extract (Booth, 1977) and was incubated at 25$^{o}$C (120 rpm) for 5 days to induce macroconidium formation. The culture was diluted 1:1 with 0.005% Tween 80 and the mycelium was fragmented by glass beads using a vortex and filtered through glass wool. Subsequently, the resulting suspension was centrifuged for 3 minutes at 2,500 g and macroconidia were resuspended in 50 mM of potassium phosphate ($KP_i$) buffer adjusted to pH 7.0. The spore density was determined using a haemocytometer and adjusted to 1.0 x $10^7$ and 1.0 x $10^6$ conidia/ml respectively and used in the perfusion of the antifungal experiments. Germination was defined as the production of one or more germ tubes. Each experiment was performed in triplicate.

### Effects of antifungal compounds on germination

Macroconidia were resuspended in malt extract broth (MEB) medium, (CM57, Oxoid) adjusted to pH 4.0 with lactic acid. Spore density was determined using a hemocytometer and adjusted to 1.0 x $10^6$ conidia/ml. Macroconidia were incubated at 25ºC for 6 hours, in the presence of antifungal compounds namely, nystatin (from 0.3 to 4.0 μg/ml) and nonanoic acid (from 0.1 to 1 mM). For the experiment in which we investigated which compartment starts germination, the incubation time of macroconidia were shorter (4 hours and 30 minutes). A stock solution of 0.1 mg/ml (0.11 mM) of nystatin was prepared in ethanol. At least 100 macroconidia were analysed microscopically with an Olympus Optical CO microscope (1000x magnification), Ltda BX40 (Tokyo, Japan). All experiments were performed in triplicate.

**Labelling of spores**

The internal pH of fungal spores was estimated by using 5(-6)-carboxyfluorescein succinimidyl ester (20 μM cFDA-SE, in 50 mM $KP_i$ buffer pH 7.0, Molecular Probes Europe B.V., Leiden, The Netherlands). The fungal suspension was incubated with cFDA-SE for 1 hour at 35$^{o}$C. After washing the dye away the cells were kept in 50 mM $KP_i$ buffer pH 7.0 with glucose (10 mM) for 30 minutes at 30$^{o}$C to eliminate nonconjugated cFSE. The efficiency of the fluorescent labelling of the spores was observed by epifluorescence microscopy (Zeiss Axioskop, Oberkochen, Germany). The microscope was equipped with a 50-W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength, 450 to 490 nm; emission wavelength > 520 nm) a 100 x Plan-Neofluar objective lens (n.a. = 1.3, Carl Zeiss, Oberkochen, Germany).

**Immobilization of spores and perfusion system**

Macroconidia were suspended in 50 mM potassium citrate phosphate buffer pH 4 ($CKP_i$ 4), and immobilized on glass slides as previously described (Mozes et al., 1987; Breeuwer and Abee, 2000). Briefly, the glass slides were immediately sealed and held in place by rubber O rings in the perfusion chamber. The chamber consisted of a water thermostated aluminium holder with two round 25 mm glass slides, separated by a 0.3 mm spacer. The total volume of the chamber was 125 $mm^3$. Two small stainless steel tubes, serving as the inlet and the outlet, gave access to the inside of the chamber, which was connected to a waterbath. The spores were fixed on the upper glass slide of the chamber. Different solutions were passed through the chamber by means of syringes and a stepping motor. During the germination study, MEB (5 times diluted in distilled water pH 4.0) was perfused at a flow rate of approximately 0.5 ml/hour and at 25ºC. During the study with antifungal compounds, the perfusion started with diluted MEB for 20 minutes and subsequently the antifungal compounds were added.

**Image analysis system**

The fluorescence ratio image microscopy (FRIM) system used consisted of an upright Zeiss Axiophot fluorescence microscope 1000W Xenon monochrometer (VisiTech International Limited, Sunderland, UK) at 490 and 470 wavelength excitation, adjusted at 20 bandwidth. The emission light was collected by Plan Neofluar (n.a. =1.3) 100 X oil objective, a 500-560 nm dichroic mirror, and a 515 nm emission bandpass filter. Fluorescence emission was collected with an extended CCD Camera (VisiTech International Limited, Sunderland, UK) and the images were recorded by the image processing unit (X-Windows on an interactive UNIX system) supplied with the Quanticell 900 software package from Applied Imaging. The resolution of the images was 512 X 512 pixels for calibration and 256 X 256 pixels during

studies on germination and the effect of antifungal compounds. The fluorescence intensity and the ratio values of the pixels in the images were expressed on a scale from 0 to 4096. The time interval between acquisition of the 490 nm and the 470 nm images was 5800 ms. The images were saved as Tiff files, imported to Photopaint to improve the images for contrast enhancement and Coreldraw 9 was used to process the images (Corel Corporation, Ontario, Canada). Ratio imaging was initiated at time zero of a solution through the chamber. Regions were drawn along the perimeter and within the separate compartments of the macroconidium.

**Calibration of $pH_{in}$**

Calibration curves of cFDA-SE labelled macroconidia at different $pH_{in}$ values were determined by dividing the fluorescence intensity at 490 nm by the fluorescence intensity at 470 nm ($R_{490/470}$) for each pixel of a spore image. The calibration was determined in vitro and inside cell for the range from pH 4.0 to 9.0. The buffers used were 50 mM potassium citrate phosphate buffer from pH 4.0 to 7.0 and 50 mM sodium borate from pH 8.0 to 9.0. For in vitro calibration cFDA-SE (20 µM final concentration) was used in buffers adjusted to various pH values with NaOH. Labelled macroconidia were treated with ethanol (63%, v/v, 30º C, 30 minutes) to permeabilize the membrane and thus equalize the pH of the buffer and the cell interior, $pH_{in}$ and the external $pH_{ex}$ of the spores. This is designated as the in vivo treatment (Budde and Jakobsen, 2000). Subsequently, the suspension was centrifuged and the spores were resuspended in appropriate buffers. In each experiment at least 10 spores were analysed with 2 repetitions. The calibration curve was fitted according to a four parameter sigmoid function $y = a + b/\{1+\exp [-(x-c)/d\}]$ and the parameters a to d were determined. The $pH_{in}$ was calculated using the formula $pH_{in} = -[\ln ((b- (ratio-a))/(ratio-a)) \times d] + c$

**Effect of antifungal compounds on $pH_{in}$**

For the $pH_{in}$ analysis on labelled macroconidia with perfusion experiments either antifungals were added at time 0 of the perfusion (nystatin at sublethal dose 0.3 µg/ml), or added at a later stage i.e. after 20 minutes of the perfusion (nystatin at 1 and 4 µg/ml and nonanoic acid at 1mM in 5 times diluted MEB). Ratio $_{(490/470)}$ imaging was initiated at time 0 of perfusion, and the images were recorded with intervals of 10-20 minutes.

**Statistical analysis**

The results were statistically analysed by ANOVA using the statistical program SPSS software version 10.1 for Windows 95/98/NT/2000. Fluorescence values were obtained from triplicate experiments and the means were compared in a paired Student's *t* test (Tukey's Studentized range (HSD) at 0.05 confidence interval. Measurements were done targeting

either the entire macroconidia or separate compartments within macroconidia. Measurements on compartments were classified by their location, at apical compartments and middle compartments. The mean values of the fluorescence at apical and middle compartments were compared within each event of germination, namely time of starting the experiment (time 0), swelling, germ tube formation and germ tube elongation. Another comparison of the fluorescence values of apical and middle compartments was done among the events of macroconidia germination. The null hypothesis was that there were no significant differences between means of fluorescence values at apical compartments and middle compartments, and no significant difference between means of the fluorescence values during the events of germination.

## RESULTS

### Differential germination of macroconidia

The events in the germination of *Fusarium culmorum* macroconidia are shown in Fig. 1A. Macroconidia at the start of the experiment (I) were suspended in malt extract broth. Swelling (II) occurred in all compartments within 1 hour and the emergence of the germ tube (III) started after 2 hours. The germ tubes emerged mostly from apical compartments and subsequently elongated (IV). The stages are illustrated by light microscopy in figure 1C. Ungerminated macroconidia are represented in Fig. C1, the swelling stage in fig. C2 and germ tube formation is illustrated in Fig. C3. Analysis of macroconidia after 4 hours incubation showed that 77% of the population of spores germinated from the apical compartment, 3% germinated from the middle compartment and 22% did not germinate (Fig. 2). Germination of more than one compartment of the spore was commonly observed. In the majority of cases macroconidia germinated from two compartments, which were located apically. Germination of a single compartment was common, and germination of three or more compartments rare (Fig. 3). After a longer period (12 hours) germination of three or more compartments was achieved in case 24% of the macroconidia (data not shown).

In another series of experiments we investigated the effect of antifungal agents on germination of *F. culmorum* macroconidia. Table 1 shows the effect of both, the polyene antibiotic nystatin and nonanoic acid, compounds. Nystatin at 0.7 µg/ml delayed germ tube emergence markedly. In the presence of 1µg/ml nystatin, only 2 % of the macroconidia had germinated after 6 hours. At a concentration of 4µg/ml germination was completely inhibited. In the presence of nonanoic acid (0,2-0,25 mM), germination of spores had decreased to 10% and at higher concentration (1 mM) no germination occurred within 6 hours of incubation. Macroconidia that were not treated with antifungal agents showed 85% of germination after 6 hours of incubation time. However, macroconidia had clearly formed germ tubes after 24 hours in the presence of 1 mM nonanoic acid (data not shown).

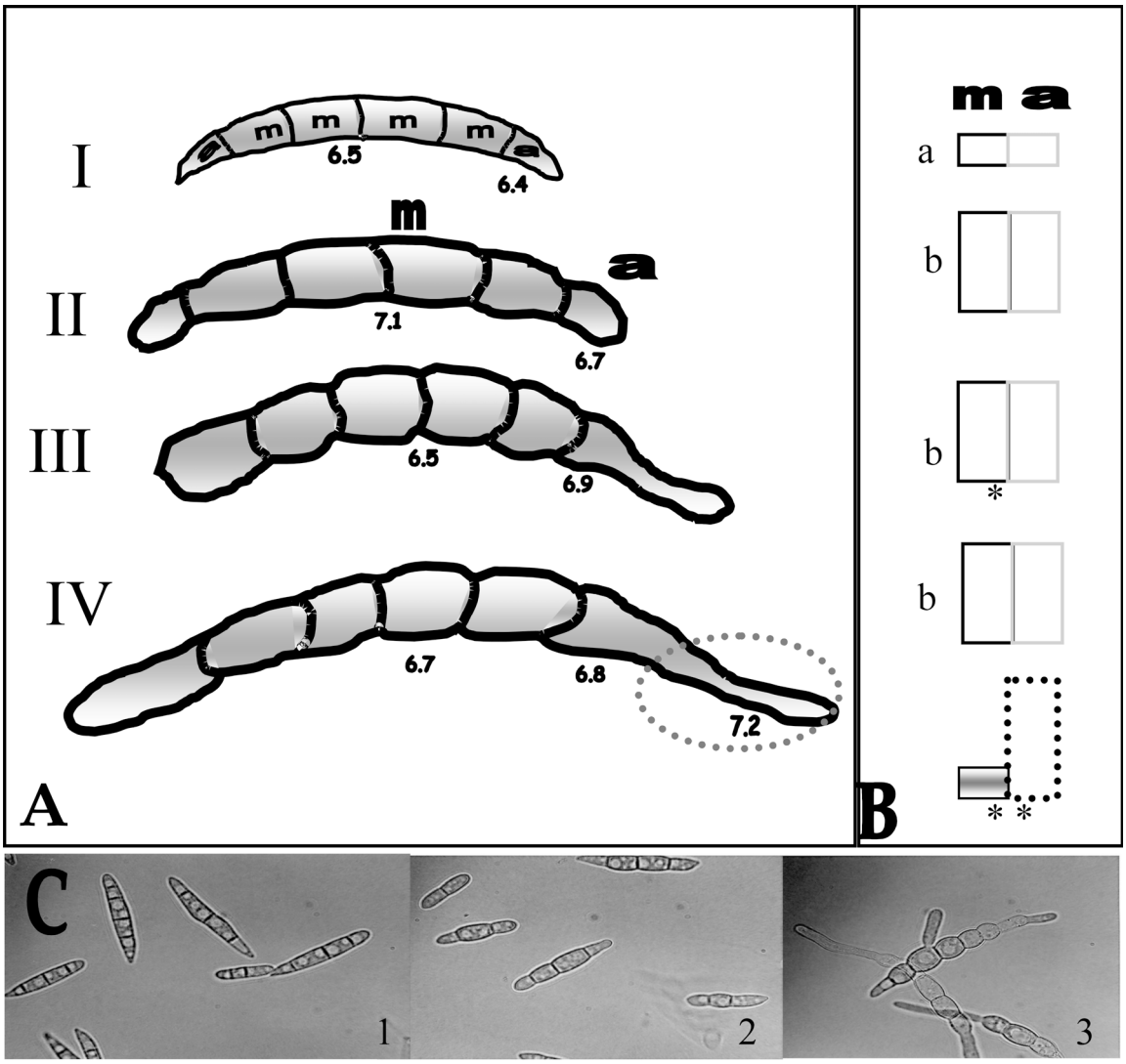


**Fig. 1** Germination of *Fusarium culmorum* macroconidia and associated $pH_{in}$ events. (A) Events: T=0 (I), swelling (II), germ tube formation (III) and elongation (IV), the of apical (**a**) and middle (**m**) compartments. (B) Statistical representation of the comparison of the $pH_{in}$ between (**a**) and (**m**) compartments within each event in the germination process and among events. Note that comparison of the $pH_{in}$ values between (**a**) and (**m**) compartments within of the event marked with an asterisk (*) are significantly different at $P \leq 0.05$. Lower case letters (a, b) represent comparisons among events. Black squares represent average of $pH_{in}$ of middle compartments and gray squares represent average of $pH_{in}$ of apical compartments. The full gray square represents the average of $pH_{in}$ of macroconidia and dot square represents the $pH_{in}$ of new germ tube. The $pH_{in}$ values of ungerminated cells, Time=0 (I) is statistically different at $P \leq 0.05$ from other events. Stage IV, the $pH_{in}$ of elongated tube (circle of the dotted line) designated by asterisks (**) in figure 1B. (C) Illustration of the stages of spore germination of *Fusarium culmorum* in malt extract broth (MEB) pH 4.0, 25ºC, ungerminated macroconidia (C1), and swelling stage (C2) and germ tube formation (C3). Germ tube formation can occur in one, two or three compartments of fusiform macroconidia. Means of $pH_{in}$ values are based on three experiments.

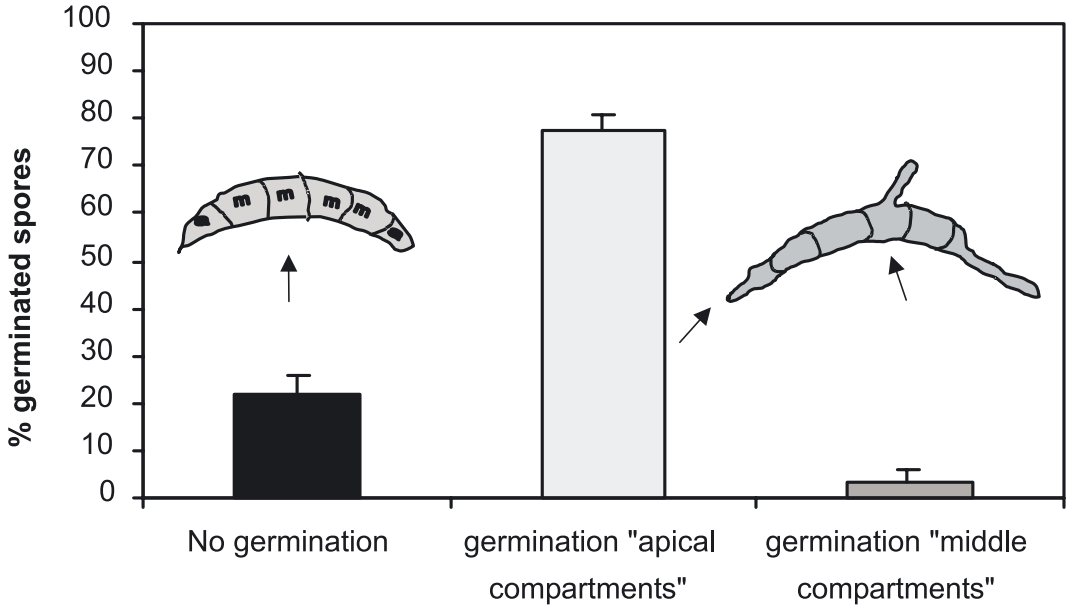


**Fig. 2** Germination of different compartments of macroconidia of *Fusarium culmorum* in MEB incubated for 4 hours. Macroconidia that did not germinate (■), that started germination at apical (■) and at middle compartments (■). Means of three independent experiments, and the error bars indicate s.d..

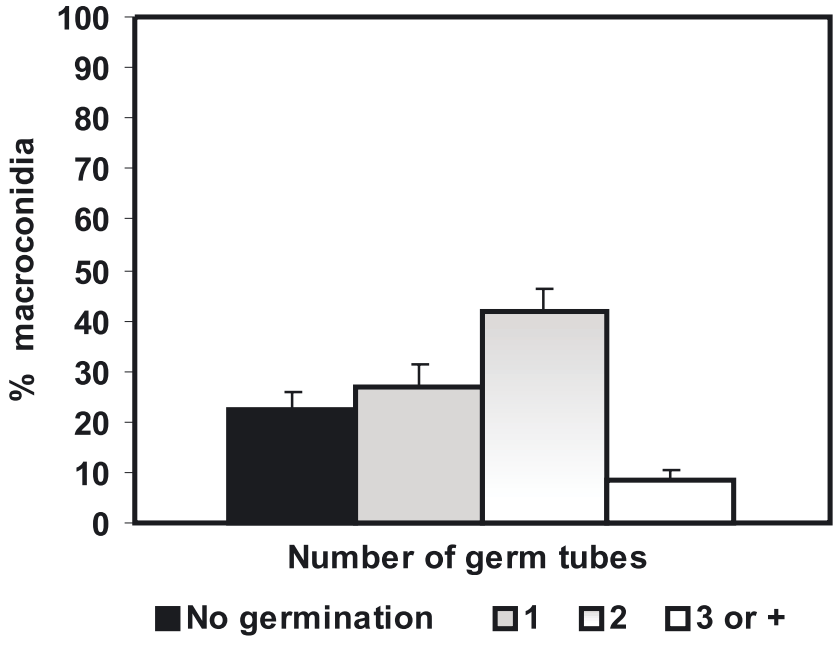


**Fig. 3** Macroconidia of *Fusarium culmorum* incubated for 4 hours in MEB pH 4.0, 25º C. Macroconidia that did not germinate (●), that produced 1 germ tube (●), 2 germ tubes (●), and 3 or more germ tubes (○). Means of three independent experiments, and the error bars indicate s.d..

**Table 1**. Effects of antifungal agents on germination efficiency of *Fusarium culmorum* macroconidia.

| Nystatin | Percentage germination after 6 h of incubation |
|---|---|
| 0 | 85 |
| 0.3 µg/ml | 73 |
| 0.7 µg/ml | 45 |
| 1 µg/ml | 2 |
| 4 µg/ml | 0 |
| Nonanoic acid | Percentage germination after 6 h of incubation |
| 0 | 85 |
| 0.1mM | 78 |
| 0.20 mM | 11 |
| 0.25 mM | 7 |
| 1mM | 0 |

*Control macroconidia were incubated in malt extract broth pH 4.0 for 6h, 25ºC
The percentage of germination values are based on three experiments.

**Calibration curve**

The relation between pH and the ratio at two wavelengths ($R_{490/470}$) of cFSE in vitro (of the immersing buffer) and inside cell are shown in Figure 4A. There was a clear difference between the values of the $R_{490/470}$ when the probe was in buffer alone (in vitro) or inside cell of macroconidia permeabilised with ethanol. This was most notable in the range between pH 5.0 and 6.0. The $R_{490/470}$ in the macroconidium was found to be higher within this pH range. This clearly shows that the microenvironment inside the macroconidium influences the spectroscopic properties of the probe. The sensitivity of the probe is greatest between pH 6.0 and 8.0, while values below pH 5.0 are difficult to distinguish. Image analysis showed that the macroconidia were able to maintain an average internal pH of 6.5 at an extracellular of pH 4.0. The internal pH was slightly higher and remained constant (pH 6.8 to 7.1) over a range of extracellular pH ($pH_{ex}$) from 5.0 to 8.5 (Fig. 4B).

**Intracellular pH and germination**

The internal pH was calculated for whole macroconidia at different stages of the germination process. At the start of germination the $pH_{in}$ was 6.5 $\pm$ 0.07 and increased during the first 30 minutes to 6.8 $\pm$ 0.13 (Fig. 4C). From that moment on no significant net major change of the

internal pH was observed. However, the standard deviation of the calculated $pH_{in}$ values of macroconidia during germination was highly variable. At two stages during the germination, the internal pH of the spores varied markedly. Samples taken at 90 and 180 minutes after beginning of the experiment had s.d. of 0.41 and 0.90 respectively, while in all other cases, the s.d. ranged from 0.09 to 0.29 (see Fig. 4C).

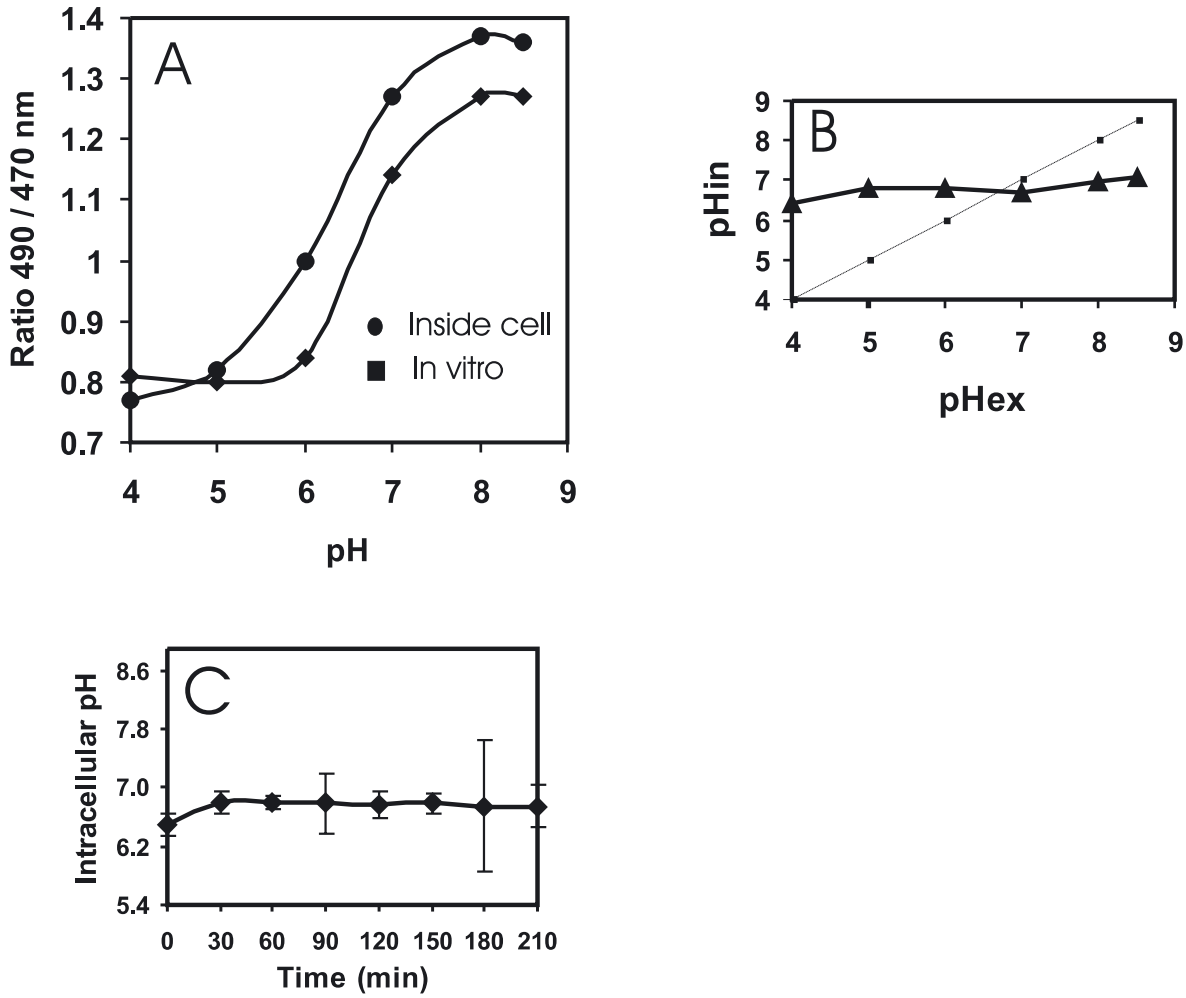


**Fig. 4** Relationship between the pH and the fluorescence ratio (490 / 470 nm) of cFSE in vitro (buffer) and inside cell of macroconidia. Inside cell, $pH_{in}$ was equilibrated to $pH_{ex}$ by incubation with 63% (v/v) ethanol **(A).** The $pH_{in}$ values of *Fusarium culmorum* macroconidia at different extracellular pH. The dashed line is the line for the equation $pH_{in} = pH_{ex}$. The buffer used in A and B was 50 mM potassium citrate phosphate (pH 5.0 to 7.0) and 50 mM sodium borate (pH 8.0 to 8.5). The $pH_{in}$ values are averages based on approximately twenty individual macroconidia (**B).** Mean of $pH_{in}$ of *F. culmorum* macroconidia during germination. Immobilised cFDA-SE labelled macroconidia were perfused at 25ºC in MEB 5X diluted in distilled water pH 4.0 **(C).** The $pH_{in}$ values are averages of eighteen individual macroconidia.

In subsequent experiments we assessed the internal pH inside the individual compartments of the conidium. Before germination, the average internal pH of the whole macroconidium was

6.5 (see Fig. 1A I). In apical compartments of the conidium the pH was 6.5 and in the middle compartments, 6.4. During swelling, the internal pH of the macroconidia increased; middle compartments showed the greatest increase, reaching to pH 7.1, while apical compartments reached pH 6.7. In the course of germ tube formation, the $pH_{in}$ of apical compartments rose further to pH 6.9 whereas the internal pH of middle compartments decreased to pH 6.5. The pH inside of the newly formed germ tubes was higher than the compartments of the conidium, namely, pH 7.2 (see Fig. 1A).

Statistical evaluation of the mean pH of apical and middle compartments was done using Tukey's Studentized range (HSD) test (Fig. 1B). No significant difference in $pH_{in}$ between apical and middle compartments was found at stage I (beginning of the experiment), II (swelling) and IV (elongation of germ tube). However, at stage III (germ tube formation), the apical compartments were significantly less acid than middle compartments ($P< 0.05$, see the asterisk (*) in figure 1B. In a second analysis, the pH values were compared between different stages of germination (I, II, III, IV). The $pH_{in}$ values at swelling, germ tube formation and elongation were significantly different compared to ungerminated cells ($P< 0.05$, differences are designated as **a** and **b** in figure 1B). During stage IV, elongated tube (circle of the dotted line), the pH of the new germ tube is significant different of the whole macroconidia ($P< 0.05$, designated by asterisks (**) in figure 1B).

**Antifungal compounds and changes in internal pH**

Subsequently, macroconidia were exposed to the polyene antibiotic nystatin. All macroconidia had initial $pH_{in}$ values between 6.5 and 6.8. Exposure to nystatin (1 µg/ml) at a $pH_{ex}$ of 4.0 resulted in dissipation of the pH-gradient within 20 minutes. When a higher concentration of nystatin (4 µg/ml) was added to macroconidia, the pH gradient collapsed instantaneously indicating cell death (Fig. 5). The intracellular pH of macroconidia exposed to 1 mM of nonanoic acid showed large fluctuations between 5.4 and 6.5 during a period of 90 minutes. A decrease of the internal pH was observed twice followed by its restoration (data not shown). As a control, when macroconidia were treated with 63% of ethanol, no pH gradient and no germination were observed (data not shown).

Only 3% of macroconidia that were not treated with antifungal compounds germinated from the middle compartments and 74% from apical cells (after 4.5 hours). Remarkably, macroconidia showed an increased germination from the middle compartments, reaching 26% of the cells in the presence of a sub-lethal dose (0.3 µg/ml) of nystatin. Still 46% of the macroconidia germinated from apical cells (Fig. 6A). The intracellular pH of the middle compartments of macroconidia that formed germ tubes was 6.4. (Fig. 6B). The $pH_{in}$ of apical compartments in these conidia was strongly lowered to pH 5.4. The insert in Fig. 6B shows the morphology of macroconidia under these conditions.

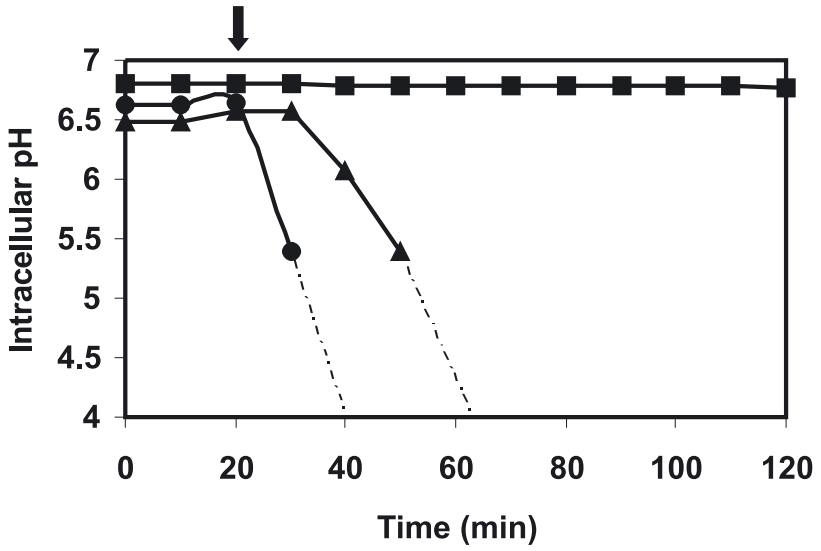


**Fig. 5** The $pH_{in}$ of macroconidia of *Fusarium culmorum* in MEB (■), and in the presence of 1µg/ml (○) and 4 µg/ml of nystatin (Δ). The arrow indicates the time at which perfusion with nystatin started. The ratio values are averages based on fifteen individual macroconidia. It is assumed that $pH_{in}$ and $pH_{out}$ (pH 4) equilibrate. Note that in vivo pH values below pH 5 cannot be determined (see Fig 4).

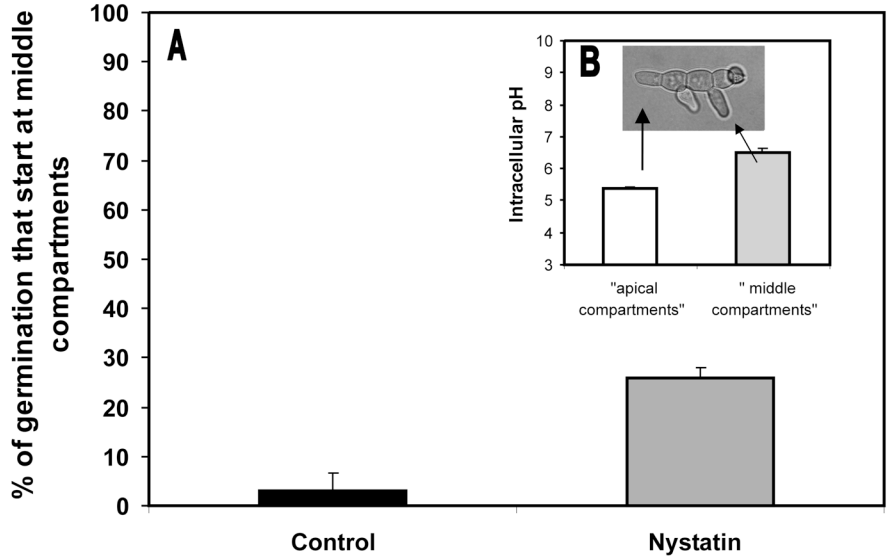


**Fig. 6** Macroconidia germination starting at middle compartments in MEB (control) and in the presence of 0.3 µg/ml of nystatin, after 4 hours and 30 minutes of incubation. The percentage of germination values are based on three independent experiments, error bars indicate s.d. **(A).** The $pH_{in}$ values of germinated macroconidia from apical and middle compartments in the presence of nystatin at 0.3µg/ml **(B).** The insert in Fig. 6B shows the changes in morphology of macroconidia.

Apical cells were swollen and showed reduced germination or distorted germ tubes. When the apical cells succeeded to germinate the $pH_{in}$ was 6.5. Generally, these macroconidia showed

shorter germ tubes than the untreated macroconidia, namely approximately three-quarters of the length of controls.

## DISCUSSION

### Differentiation of macroconidial compartments

In this research, we studied the germination process of multicompartment *Fusarium culmorum* macroconidia. We found clear evidence for differentiation of and communication between the compartments of the macroconidium during early germination. Such a differentiated development inside multicelled conidia was suggested by the observation that germ tubes developed preferably from apical cells and only seldom from middle cells. This differentiation is also observed with *Magnaporthe grisea* conidia and Jelitto et al. (1994), which displays a similar proportion of germinated macroconidia from the apical cell as we found in macroconidia of *F. culmorum*.

We monitored the internal pH inside individual compartments to study the process of differentiation in more detail. Non-germinated spores generally show a lower $pH_{in}$. This seems to be a general trend with fungal spores, also observed in ungerminated sporangiospores of *Rhizopus oligosporus* (Breeuwer et al., 1997; Breeuwer and Abee, 2000) and *Penicillium paneum* (unpublished). During the first stage of germination, swelling, the internal pH increased in all compartments of the conidium. At that moment, the internal pH measurements obtained from individual compartments of the macroconidia showed no significant differences. Subsequently, the internal pH of apical cells that formed germ tubes increased further, while $pH_{in}$ of non-germinating compartments (in most cases the middle ones) declined. The internal pH values of germinating and non-germinating cells were significant different. Although these results suggest that intracellular pH is associated with germination, they do not establish whether an increase in $pH_{in}$ occurs prior to or is a product of the metabolic changes inside the cell. Newly formed germ tubes had the highest internal pH of all cell types observed in this study. The new germ tubes have higher $pH_{in}$ (>7.2) than macroconidia. The figures observed for these cells are comparable to the cytoplasmic pH found in hyphae of *Neurospora crassa* (pH 7.57) with dextran-conjugated dye (Parton et al., 1997), and in germ tubes of *Magnaporthe grisea* (pH 7.4 ± 0.1; (Jelitto, 1999). The $pH_{in}$ of germinated compartments of the macroconidia of *F. culmorum* was lowered to $pH_{in}$ 6.8 after outgrowth.

*Is there communication between compartments?*

After early germination, between isotropic growth and germ tube formation, the cells of the macroconidium start to differentiate. The inclination of the apical cells to germinate over middle cells may be the result of the difference in the surface-to-volume ratio of apical cells compared to middle cells. This may facilitate differences in e.g. transport processes and

henceforth introduce an asymmetry in development of the macroconidium. Subsequently, the non-germinating cells (middle compartments) exhibit a lower internal pH, which is probably associated with an increase in the size and number of the vacuoles (data not shown). Vacuole formation inside non-germinating compartments has recently been reported in conidia of *M. grisea*. In these compartments spherical vacuoles were stained with the vacuole-staining dye cDFFDA (Atkinson et al., 2002).

The consistent pattern of germination in macroconidia was changed after treatment with sublethal doses of nystatin. Nystatin, a polyene antibiotic derived from *Streptomyces noursei* and *Streptomyces aureus*, forms channels in the plasma membrane by interaction with ergosterol; these increase membrane permeability and kill the cell (Groll et al., 1998; Pauw, 2000; Charvalos et al., 2001). At sublethal doses, the apical cells appear to be preferentially targeted compared to middle cells. The collapse of the pH gradient in these cells and the distortion of germination were compensated by an increased germination capacity of middle compartments (associated with higher internal pH). This shift of germ tube formation towards the middle cells suggests that communication between compartments of multicelled conidia exist. Middle compartments also germinate in macroconidia that are not treated with nystatin, but often at a later stage (after 6 hours) when the germ tubes of apical cells are much elongated. This may suggest that germ tube forming cells exhibit an “apical dominance” over middle cells, which is alleviated at later stages of development.

*Why do so many fungi form multicelled conidia?*

Multicellular conidia may be more versatile in their responses to adverse conditions compared to unicellular spores. When a first wave of germination fails, a second one may be successful when conditions improve. If not, the middle cells of the macroconidium may differentiate into the very resilient chlamydospores. The fungi *F. oxysporum* and *F. sulphureum* are able to form chlamydospores out of middle compartments during prolonged nutrient, poor conditions (French and Nielsen, 1966; Schneider and Seaman, 1974). During this process, cellular contents of the apical compartments are transported to the middle compartments (resistant ones), which increase in size, and then form lipid inclusions and thick walls, characteristic of chlamydospores.

In conclusion, we found difference inside macroconidia monitored by FRIM. The consistent pattern of germination in multicelled conidia changed after exposure to antifungals, which suggests communication between compartments of macroconidia. Multicellular conidia may be more versatile in their responses to adverse conditions.

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# Chapter 4

Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor

## ABSTRACT

*Penicillium paneum* is an important contaminant of cereal grains, which is able to grow at low temperature, low pH, high levels of carbon dioxide, and in acid conditions. *P. paneum* produces mycotoxins, which may be harmful to animals and humans. We found that conidia in dense suspensions showed poor germination, suggesting the presence of a self-inhibitor. Volatile compound(s) produced by these high-density conditions also inhibited mycelial growth of different species of fungi belonging to a variety of genera, suggesting a broad action range. The heat stable compound was isolated by successive centrifugation of the supernatant obtained from spore suspensions with a density of $10^9$ conidia $ml^{-1}$. Using static headspace analyses two major peaks were distinguished, with the highest production of these metabolites after 22 h of incubation at 25ºC and shaking at 140 rpm. Gas chromatography coupled with mass spectra analysis (GC-MS) revealed the compounds to be 3-octanone and 1-octen-3ol. Notably, only the latter compound appeared to block the germination process at different developmental stages of the conidia (swelling and germ tube formation). In this study 1-octen-3-ol influenced different developmental processes during the *P. paneum* life cycle including induction of microcycle conidiation and inhibition of spore germination. The compound henceforth may act as a fungal hormone during development of the fungal organism.

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# INTRODUCTION

The spore is an important vehicle for distribution or long-term survival of fungi. Conidia are airborne spores, which can be produced in very large numbers by fungi belonging to the order Eurotiales. This order, which includes the genera *Aspergillus* and *Penicillium* (13), contains many species of food related fungi. Conidia are dispersed into the air, but do not germinate before they reach a suitable substrate. Invariably, germination is inhibited when spores are present in high densities, an effect observed for example in *Aspergillus niger* and *Syncephalastrum racemosum* (2, 18). This is designated as the crowding effect. Also, in the formation of an adhesive bud on spores of the nematophagous fungus *Drechmeria coniospora,* there is a clear crowding effect (34). These observations suggest that intercellular signaling prevents premature germination. The mechanism however has not been elucidated.

Acervuli of *Colletotrichum* form large numbers of conidia embedded in mucilage, but not germinating there. From the mucilage that surrounds the conidia of this fungus a self-inhibitor of germination named mycosporine-alanine, has been identified and characterised, (24). Germination is also retarded in pustules of rust fungi (Basidiomycetes) where cis-ferulic acid methyl ester and cis-3,4-dimethoxycinnamic acid methyl ester have been identified (33).

Self-inhibitors have been characterised in *Puccinia, Uromyces, Colletrotrichum, Dictyostelium, Fusarium oxysporum* and *Aspergillus* and can be volatile or non-volatile (1, 2). They also can influence other fungal processes for example, mycosporine-alanine produced by *Colletotrichum graminicola* prevents appressorium formation (23, 24). The self-inhibitors produced by *Glomerella cingulata* and *Dictyostelium discoideum* inhibit protein synthesis (1, 25).

Self-inhibitors inhibit spore germination reversibly. After removal of the compound from the spore or its environment germination is initiated. The major function of self-inhibitors is stated as prevention of premature germination of spores directly after spore formation and before dispersal. This mechanism guarantees that spores only germinate after dispersal into environments that favour outgrowth to establish at mycelium. Self-inhibitors can be localised outside the conidial cells, for instance inside the mucilage of acervuli. The compound also can be localised associated with the cell wall of the spore (27), and can be removed by washing with water (16, 32).

*Penicillium paneum* has been assigned to the *Penicillium roqueforti* group on the basis of morphological studies, genetic characterisation and secondary metabolite profiles (6). This fungus is an important contaminant of cereal grains and is able to grow at low oxygen levels, low pH and, high levels of carbon dioxide, as well as in acid conditions (5, 30). *P. paneum* can produce mycotoxins such as roquefortine C and patulin, which are harmful to animals and humans (6). Studies of self-inhibitors are of interest in understanding the regulatory mechanisms involved in germination. Investigation of self-inhibitors is relevant to the

development of techniques that prevent food spoilage and crop diseases. Here we report the identification of 1-octen-3-ol as a volatile self-inhibitor in *P. paneum*.

## MATERIALS AND METHODS

### Preparation of conidial suspensions

The strain used in this work was recently classified after morphological studies and genetic characterisation (β- tubulin) as *P. paneum*, and previous described as *P. roqueforti* LU 510 (10). *P. paneum* was grown on malt extract agar medium (MEA, CM59, Oxoid Ltd, Hampshire, UK) inside glass tubes at 25°C for 7 days. Spore suspensions were prepared by addition of 9 ml peptone physiological salt solution (8.5 g $l^{-1}$ NaCl with 1 g $l^{-1}$ bacteriological peptone [Oxoid], supplemented with 0.1% Tween 80) to the culture. Suspensions were prepared from 25 tubes and filtered through a 17 µm nylon filter, reaching a final volume of 180 $ml^{-1}$ suspensions. Subsequent the suspension was centrifuged (4,000 X g) for 3 min. Conidia were resuspended in 2 ml of malt extract broth (MEB) medium (CM57, Oxoid, Hapmpshire, UK), and the suspension was adjusted to pH 4.0 with lactic acid. The spore suspensions were adjusted to densities of $10^9$, $10^8$, $10^7$, $10^6$ conidia $ml^{-1}$ and cells were counted in haemocytometer (Bürker Türk).

### Germination of conidia on different media

For germination studies, conidia were inoculated in MEB (pH 4.0) and incubated in a water bath at 25ºC shacking at 140 rpm) for 10 h. High density spore suspensions with $10^9$ conidia $ml^{-1}$ were then diluted in MEB to $10^6$ conidia $ml^{-1}$and incubated for another 10 h. As a control, germination of *P. paneum* at $10^6$ conidia $ml^{-1}$ was also studied. Germination was also analyzed on solid medium. Droplets (5µl) of conidia suspensions containing $10^6$ to $10^9$conidia $ml^{-1}$ were placed on microscopic slides coated with a very thin layer (0.5 mm) of MEA medium and the slides were incubated for 24 h at 25°C in a Petri dish containing wet filter paper. Germination was determined by examination of at least 100 conidia harvested with adhesive tape from the surface of separate colonies, using an Olympus Optical CO microscope (1000 x magnification, Ltda BX40, Tokyo, Japan). The criterion used to measure germination was the emergence of germ tubes (17). The experiments were performed in triplicate.

**Activity of volatile metabolites on radial growth**

The method described here for study of the inhibition of fungal radial growth by volatile metabolites was developed by Lisa Fredman (Swedish University of Agricultural Sciences, Uppsala, Sweden, personal communication). The technique uses one Petri dish containing two layers of agar, a top and a bottom layer (Fig.3). The top side contain the inoculated spore suspension. In our study, this consisted of $10^6$ or $10^9$ conidia $ml^{-1}$ on 3% MEA (w/v). For this, a volume of 50 µl of the *P. paneum* spore suspension was lined onto the surface of the agar using a spiral plater (I.K.S. BV, Leerdam, The Netherlands). On the bottom side of the plate, a mycelial plug was placed that had been obtained from the periphery of a 5 days-old MEA culture. The plate was incubated for 7 days at 25º C. As a control, a plate lacking conidia on the top layer was incubated. The diameters of colonies on the bottom layer (excluding the central plug) were measured. The effect of volatile compounds produced by *P. paneum* conidia at $10^6$ conidia $ml^{-1}$ was tested on the other fungal species including *Penicillium roqueforti* strain LU 513, *Aspergillus niger*, *A. parasiticus*, *A. flavus*, *Fusarium culmorum* VTT D-80148 and *Rhizopus oligosporus* LU 575. Experiments were done in sixfold replication.

**Extraction of the volatile compounds**

For extraction of the inhibitor(s), a modified version of the procedure of Leite and Nicholson (24) was used. The compound(s) was extracted from a suspension of $10^9$ conidia $ml^{-1}$ immediately after preparation of the suspension (0 h), after 22 and 45 h of incubation at 25º C. We tested three different solutions for incubation of conidia suspension for further extraction: MEB pH 4.0, MEB pH 6.0 and distilled water. Under all conditions, the percentage of inhibition was the same (data not shown). From that point on, the experiments were done using extracted compound(s) in MEB pH 4.0 since the germination of spores was also assessed in the same medium. The suspensions were centrifuged at 4,000 X g for 4 min at 20º C to remove the conidia. The supernatant was centrifuged at 7,000 X g for 10 min at 15º C and the supernatant obtained was centrifuged at 13,000 X g for 20 min at 4º C and filter sterilised through a 0.2 µm filter (FP 030/03 Schleicher and Schuell GmbH, Dassel Germany). This extract solution was tested against fresh conidia of $10^6$ *P. paneum* conidia $ml^{-1}$ and the germination of conidia was assessed after 7 h. As a control fresh conidia with a density of $10^6$conidia $ml^{-1}$ in MEB pH 4 were used. The results are the means of three independent experiments.

**Detection and identification of the volatile compound(s)**

Volume consisting of 2 ml of $10^9$ conidia $ml^{-1}$ suspensions was each transferred to a headspace vial, which was closed with a Teflon/butyl seal and a magnetic crimp cap.

Samples were analysed by static headspace analysis using a Fisons Instruments autosampler HS 800 (Interscience, Breda, The Netherlands) gas chromatography (GC). The column was a 30 m x 0.54 mm id df = 1.0 µm, fused silica DB-WAX column (J&W Scientific), 30 KPa for detection of the volatile compounds. The oven temperature was held at 60º C for 5 min, and then programmed to 110º C at a rate of 3º C / min, 170º C at 4º C / min and at 200º C for 3min isotherm. The control was MEB medium at pH 4.

For dynamic headspace analysis, 2 ml of $10^9$ conidia $ml^{-1}$ suspensions that had been incubated for 22 h, was added to 8 ml of distilled water and transferred to the purge & trap (P&T). Different suspensions were incubated in polypropylene tubes (Greiner GmbH, Frickenhousen, Germany) or in glass tubes. Nitrogen gas at a flow rate of 40 ml/min was passed through spore suspensions held at 25º C in a water bath for 15 min, to trap the volatile compounds in a glass tube (160 mm x 4 mm ID) filled with 90 mg 20/35 Tenax TA mesh (Chrompack, Breda, The Netherlands). For subsequent analysis, the Tenax trap was heated 10 min in a thermodesorption cold trapping injector (Chrompack, 16200) at 250º C with a helium flow rate of 15 ml/min. Upon ballistic heating of the cold trap, the compounds were transferred to the connected DB5 capillary column (60m, 0.25 mm ID, 0.25 µm film thickness) programmed from 60º C to 280º C at a rate of 6 º C /min. The column was connected to a Finnigan MaT95 mass spectrometer, operated in the 70 eV EI ionization mode, and scanning from molecular mass ranging from 24 to 300 at 0.7 sec/degrees. Compounds were identified by matching the spectra against those in the NIST98 library and the Wageningen Library of mass spectra, and by checking their Kovats (DB5) indices. Three different experiments were performed for identification of the compound(s).

**Inhibitory assay**.

Spore suspensions were incubated in the presence of 4 mM of 3-octanone (Fisher Scientific BV, Hertogenbosch, The Netherlands) and 1-octen-3-ol (Janssen, Geel, Belgium). After 3 and 7 h, 100 conidia were analyzed under the microscope and the percentage of germinated spore was calculated, as described above. The control used consisted of spores suspended in MEB medium. The results are the means of three independent experiments.

## RESULTS

### Effect of spore density on germination efficiency

Suspensions of $10^6$ *P. paneum* conidia per ml spores (controls) showed 90% germination within 10 h of incubation in MEB. However, at $10^9$ conidia $ml^{-1}$ only 7% germinated was observed. In order to test if this reduction was a consequence of the increased spore density, a dense spore suspension was diluted to $10^6$ conidia $ml^{-1}$ with MEB pH 4.0. Indeed, after an additional 10 h of incubation in MEB, 96% of the conidia had germinated (Fig 1). When 5 µl

volumes of suspensions ranging from $10^6$ to $10^9$ conidia $ml^{-1}$ were placed on a very thin (0.5 mm) agar layer for 24 h, all spores germinated in inoculum densities of $10^6$ to $10^8$ conidia $ml^{-1}$, but only 10% of conidia germinated in case of $10^9$ conidia $ml^{-1}$ (Fig. 2).

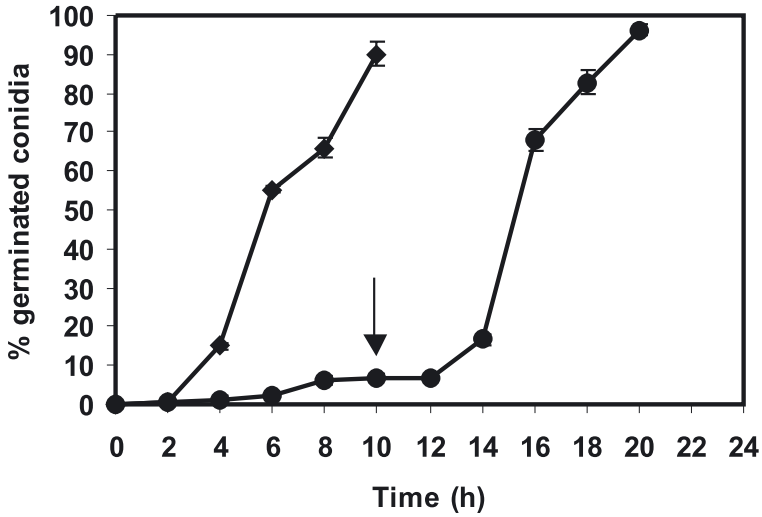


**Fig. 1** Germination of *Penicillium paneum* conidia at 25º C. Densities tested were $10^6$ (■) and $10^9$ spores $ml^{-1}$ (●). The arrow represents the time (10 h) at which the spore suspension was diluted to $10^6$. Three independent experiments were performed and the error bars show the standard deviation.

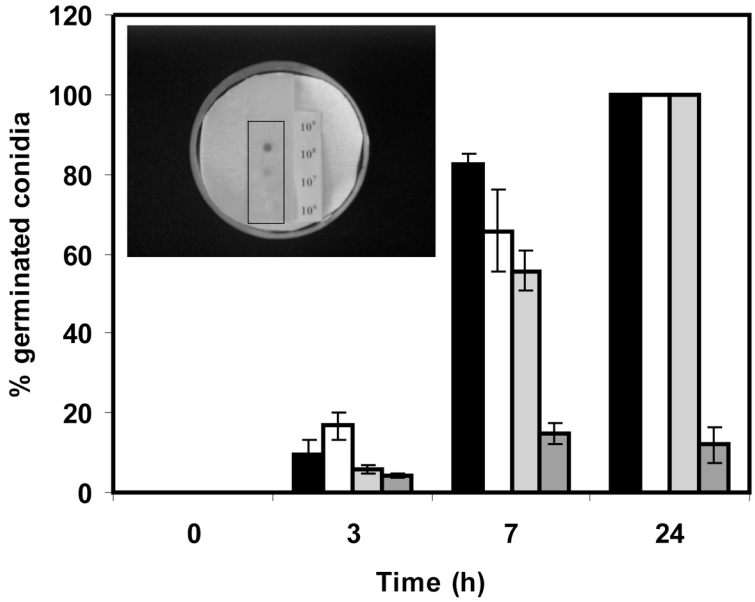


**Fig. 2** Germination efficiency at different concentrations of *P. paneum* conidia on MEA placed on microscope slides and harvested at 3, 7 and 24 h as a conidial concentration of $10^6$(■), $10^7$ (□), $10^8$ (▒) and $10^9$ spores/$ml^{-1}$ (▓). The insert shows conidia suspensions on the microscopic slide. Three independent experiments were performed and the error bars show the standard deviation.

**Inhibition of radial mycelial development by *P. paneum* conidia**

To investigate whether a volatile was involved in the effect noted above, mycelial growth was analysed in a system where the conidia suspension was placed on the upper part of plate while a mycelial plug was kept in the bottom part (Fig. 3). At density levels of $10^6$ conidia $ml^{-1}$ on the top side of the plate, the radial growth of *P. paneum* was already restricted, suggesting that the inhibitory compound(s) was volatile. At $10^9$ conidia $ml^{-1}$ mycelial growth was completely. Different fungal species were tested for analysis of the inhibitory spectrum. The mycelial growth of *P. paneum, P. roqueforti, A. niger, A. parasiticus, A. flavus, F. culmorum,* and *R. oligosporus* was restricted by *P. paneum* spore suspensions of $10^6$ conidia $ml^{-1}$ that had been distributed by spiral plating on the upper part of the plates (Table 1). These results show that volatile compound(s) inhibits hyphal growth in different fungal species.

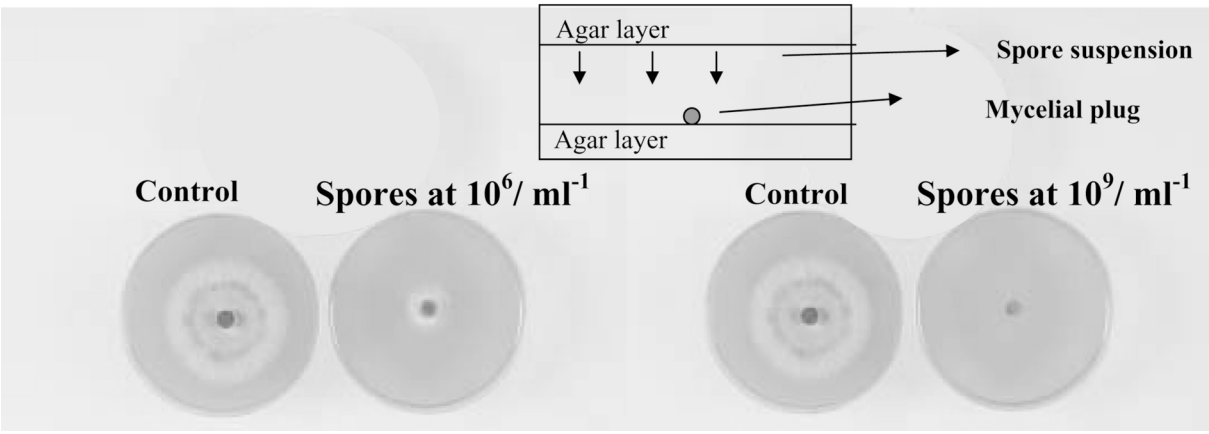


**Fig. 3** Inhibition of mycelial development by *P. paneum*. The plates contained MEA medium with 3% agar on the top and bottom sides. On the bottom side of the plate a mycelial plug of *P. paneum* was placed in the centre. On the upper layer, the spore suspensions with $10^6$ or $10^9$ conidia $ml^{-1}$ were distributed by spiral plating. The plates were incubated for 7 days at 25º C. As a control, plates were incubated without *P. paneum* spore suspensions.

**Effect of the extracted compounds on germination**

The effect of the extracted volatile compound(s) on fresh conidia of *P. paneum* in $10^6$conidia $ml^{-1}$ suspensions in MEB pH 4.0 was measured (Fig. 4). Germination efficiency in the control was 71% within 7 h. Conidia exposed to compounds that were extracted from freshly prepared spore suspension (0 h) germinated with an efficiency of 84% (data not shown). Exposure to compounds that were extracted after 22 and 45 h incubation showed only 17 and 57%, germination, respectively. Apart from inducing the highest inhibition of germination the 22 h extract also induced microcycle conidiation (Fig. 4A). This phenomenon is characterized by the rapid sporulation after germination, bypassing the vegetative growth phase that usually

follows germination. The extracted compounds that inhibited germination were heat stable since exposure to 100ºC for 5 mim did not affect their activity (data not shown).

**Table 1**: Inhibition of mycelia radial development of several fungi by a spore suspension of *Penicillium paneum* with a density of $10^6$ conidia $ml^{-1}$ on the upper side of the plate. The plates were incubated for 7 days at 25º C. As a control, plates without *P. paneum* spore suspensions were incubated. The diameter of the growth zone of the related fungi inoculated in the middle of the plates was measured. Each fungus was tested in triplicate and the experiment was performed twice.

| **Fungi** | **Mycelial growth diameter (cm)** | |
|---|---|---|
| | Control * | + Inhibitor |
| *Penicillium paneum* | 4.2 | 2.2 |
| *Penicillium roqueforti* 513 | 7.1 | 2.6 |
| *Fusarium culmorum* D-80148 | 5.8 | 2.7 |
| *Aspergillus parasiticus* | 5.6 | 3.8 |
| *Aspergillus flavus* | 7.6 | 3.6 |
| *Aspergillus niger* | 2.7 | 1.5 |
| *Rhizopus oligosporus* 575 | 8.5 | 3.3 |

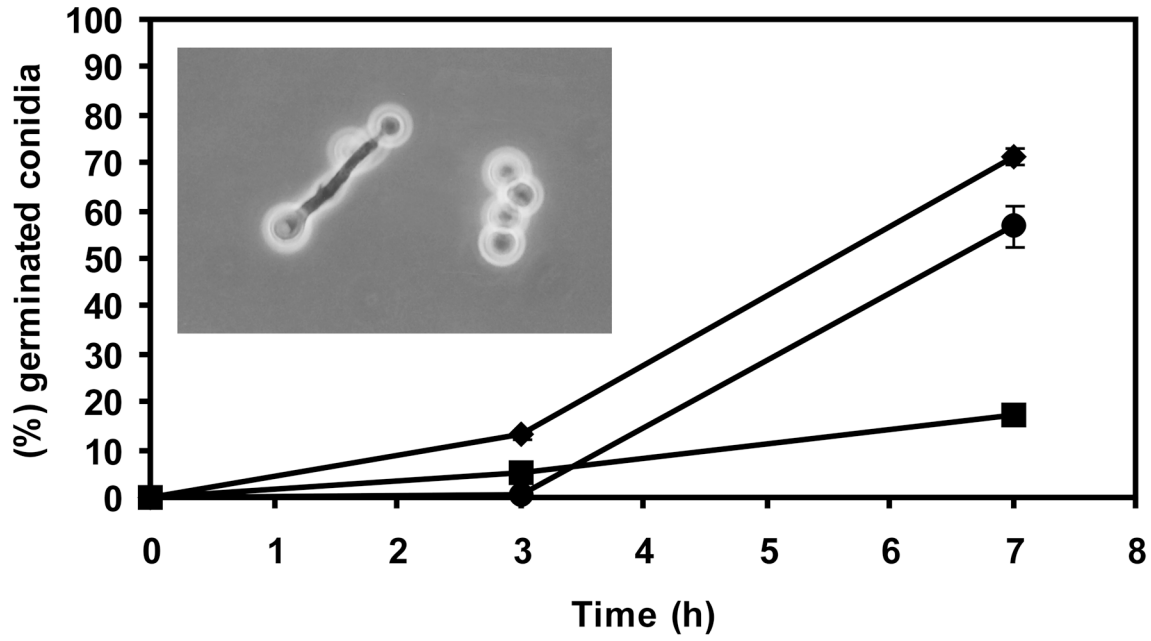


**Fig. 4** Germination of fresh conidia of *P. paneum* exposed to Extracted Compounds (**EC)** from *P. paneum.* Fresh conidia in the absence (▲) and in the presence of EC collected at 22 h (■), and 45 h incubation (●). The insert picture shows microcyclic conidiation of the primary conidia in the presence of EC collected at 22 h. Three independent experiments were done and the error bars show the standard deviation.

**Identification of the volatile compounds**

The static headspace samples of high density conidial suspensions, showed two distinct peaks of volatile compounds produced at retention times (RT) of 10.875 and 19.875 minutes (data not shown). The area measurements for peaks of the volatile compounds in samples obtained at 0, 22, and 45 h, correlate strongly with the degree of inhibition of spore germination seen with the same extracts. The mass spectrometric identification of the volatile compounds found in the sample collected at 22 h of incubation is given in the Table 2. The two dominant peaks were identified as 3-octanone and 1-octen-3-ol (see Fig. 5).

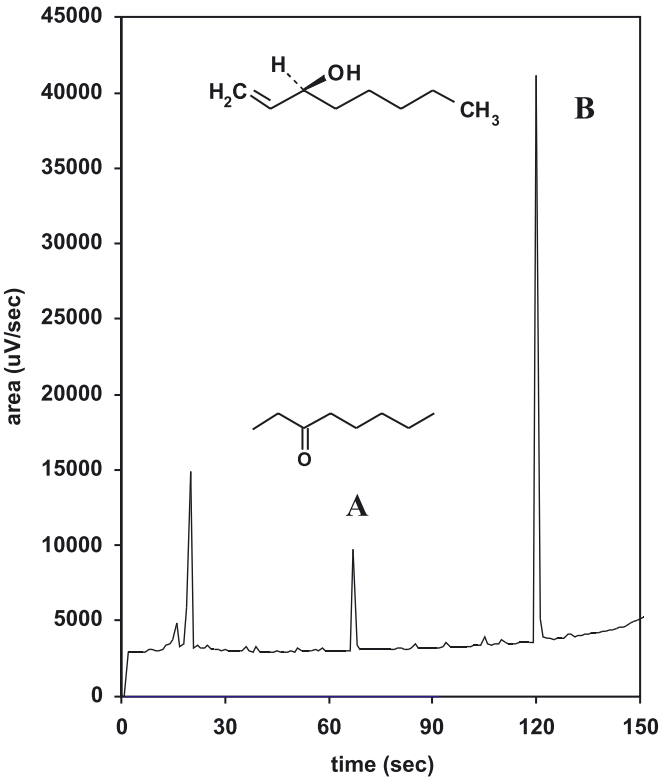


**Fig. 5** Headspace analysis chromatogram and chemical structures of the volatile compounds produced by *P. paneum* with $10^9$ conidia $ml^{-1}$ incubated in MEB pH 4 at 22h. Peaks of 3-octanone (A) and 1-octen-3-ol (B).

**Table 2**: Identification of the volatile compounds produced by spore suspension of *Penicillium paneum* incubated 22 h.

| Compounds | GC peak area |
|---|---|
| 1, 3-octadiene (cis and trans) | 270 |
| 1, 5-octadien-3-ol | 56 |
| 1-octen-3-ol | 3400 |
| 3-octanone | 900 |
| 5-octen-3-one | 80 |
| 3-octanol | 105 |
| nonanal | 48 |
| decanal | 104 |
| Sesquiterpene hydrocarbons | 43 |

**Action of 3-octanone and 1-octen-3-ol**

The effect of externally added pure 3-octanone and 1-octen-3-ol on *P. paneum* conidia was analysed (Fig. 6). In the presence of 4 mM 1-octen-3-ol, the germination frequency after 7 h was 2%, while after the same exposure to 3-octanone it was 75%. In the control, 83% of the conidia germinated. Different concentrations of 1-octen-3-ol were tested evaluate if a dose dependent effect on germination could be observed. A decrease in germination efficiency in comparison with the control level was observed in all concentrations tested and in the presence of 1, 2.5, 4.0 and 5.0 mM of 1-octen-3-ol, germination efficiency after 7 h was 83, 35, 13 and 1%, respectively (Fig. 7). Controls containing no inhibitor showed a germination efficiency of 88%.

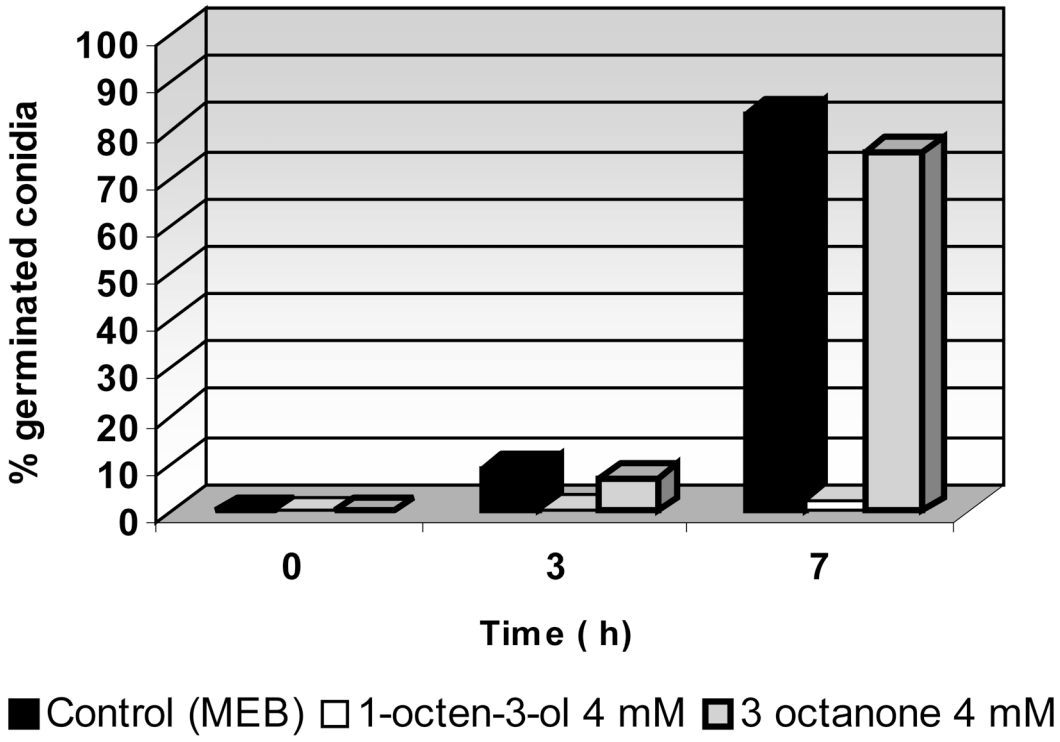


**Fig. 6** Action of 1-octen-3-ol and 3-octanone on *P. paneum* conidial germination. Percentage of germinated conidia in MEB (■), in the presence of 4 mM 1-octen-3-ol (■) and in the presence of 4 mM of 3-octanone (■). The results are the means of three independent experiments.

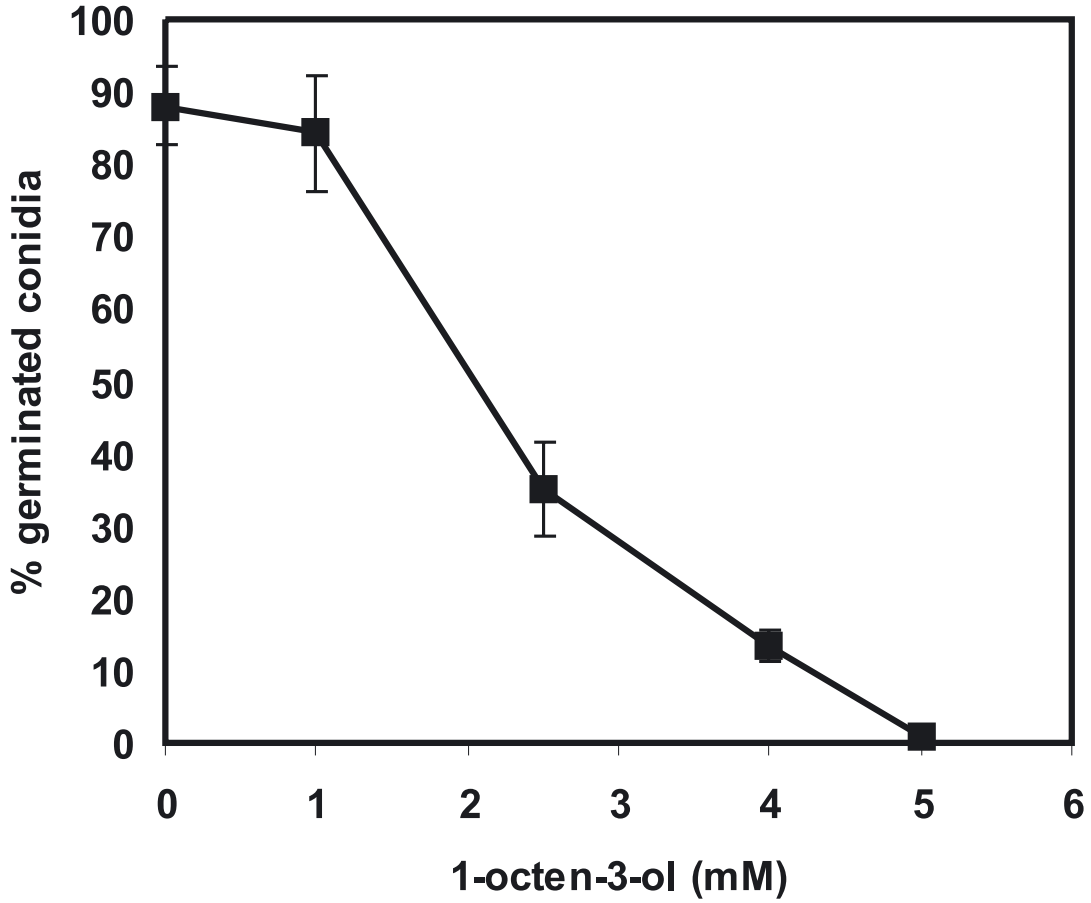


**Fig. 7** Effect of different concentrations of 1-octen-3-ol on germination efficiency of *P. paneum* with $10^6$ conidia $ml^{-1}$ incubated for 10 h in MEB pH 4 at 25ºC and shaking at 140 rpm. As a control, conidia were incubated without 1-octen-3-ol. Three independent experiments were performed and the error bars show the standard deviation.

To study the effect of the inhibitor on different stages of germination, 4 mM of 1-octen-3-ol was added to a conidial suspension directly after addition of the conidia to MEB and also after 2 and 4 h of incubation. These times roughly corresponds with the germination stages of isotropic growth (2h, swelling) and germ tube formation (4 h). Figure 8 shows that germination was blocked after addition of 1-octen-3-ol during swelling of the conidia, and no germ tubes were formed. Also after 4 h, the conidia that had not already formed germ tubes were blocked. The microcycle conidiation was also induced in the presence of 4 mM 1-octen-3-ol.

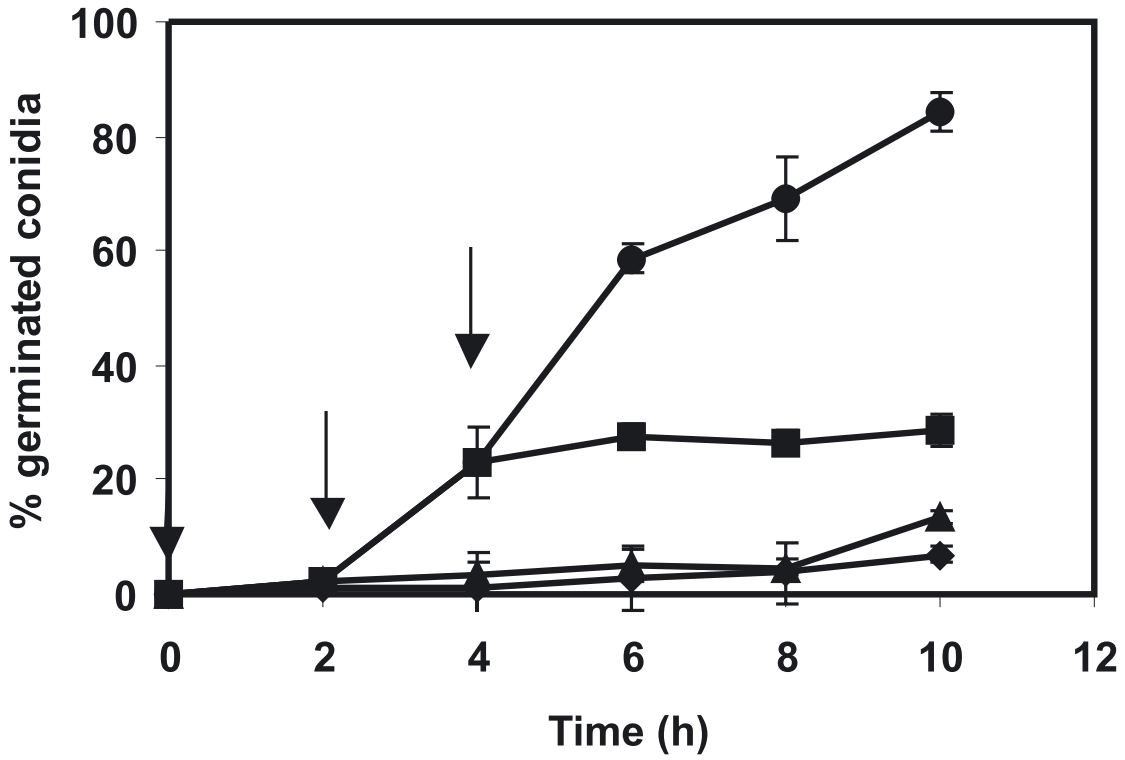


**Fig. 8** Action of 1-octen-3-ol at different germination stages of *P. paneum* conidia. The arrows represent the time of addition of the compound. Percentage of germination in MEB pH 4 (●), in the presence of 4 mM of 1-octen-3ol added at T=0 (◆), after 2h (▲), and after 4h of incubation (■). The results are the means of three independent measurements. Error bars show the standard deviation.

## DISCUSSION

The volatile compound, 1-octen-3-ol blocks the germination process of *Penicillium paneum* conidia. For the first time, this substance has been demonstrated to be a volatile germination self-inhibitor. The compound was measured in conidial dense suspensions and its effect was alleviated by dilution of the conidia. Also, the compound inhibited mycelial growth of various fungal from different genera, indicating an additional regulatory effect on the physiology and development of the fungal organism.

Different fungi and acrasiomycetous species show reduction of germination under crowding conditions including *Dictyostelium*, *Puccinia* and *Uromyces*. The compounds that are responsible for this effect have been studied in detail and a number of non-volatile self-inhibitors have been identified including N, N-dimethylguanosine, as well as a cis-ferulic acid methyl ester and a cis-3,4-dimethoxycinnamic acid methyl ester (1, 26). Within certain fungal fructification structures namely acervuli, non-volatile self-inhibitors prevent premature

germination (24, 22). Large numbers of conidia are also formed on conidiophores of *Penicillium* and *Aspergillus.* In these species the spores and the spore forming structures are directly exposed to the air. In these fungi, volatile self-inhibitors may be more efficient than non-volatiles (23). *Colletrotrichum graminicola* is able to produce a non-volatile self-inhibitor, mycosporine-alanine, and a volatile self-inhibitor, 3-hexen-1-ol (23, 24). Conceivably, the volatile 1-octen-3-ol of *P. paneum* is produced by conidia and released into the air in order to inhibit germination until appropriate environmental conditions prevail.

1-Octen-3-ol is well known as a major component of the odour of the mushrooms *Agaricus bisporus* and *Pleurotus* species (11, 28). 1-octen-3-ol though known from a wide range of fungi has never been recognised as a germination self-inhibitor. A wide selection of important food-related fungi including *Penicillium camemberti, P. chrysogenum, P. commune, P. tricolor, P. viridicatum, P. aurantiogriseum, P. citrinum, P. funiculosum, P. raistricki, Aspergillus niger, A. ochraceus, A. oryzae, A. parasiticus, Alternaria* species and *Fusarium* species have been shown to produce this compound (4, 20, 21). Interestingly, 1-octen-3-ol is not produced in dense suspensions of *P. brevicompactum* IBT 18329, *P. commune* CBS 468.84, *P. crustosum* CBS 101025, *P. chrysogenum* CBS 779.95 and *P. roqueforti* CBS 135.65 (data not shown).

In general, fungal volatiles have been investigated for different purposes. These compounds have been used as indicators of fungal growth on grains (31, 4), and as stimulators or inhibitors of plants, fungi and bacteria (14, 15). Beltran-Garcia et al. (3) studied antibacterial activities of mixtures of volatile compounds (1-octen-3-ol, 3-octanol, octanol, 3-octanone, and 2-octanone) at concentrations found in the mushroom *Pleurotus ostreatus,* and found that these mixtures inhibit growth of *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, and *Salmonella typhimurium*. Volatile metabolites have been also used as a taxonomic criterion for classification of *Penicillium* species (21).

In fungi, 1-octen-3-ol is a product of the enzymatic breakdown of linoleic acid, by lipoxygenase and a hydroperoxide lyase. The biosynthesis of 1-octen-3-ol is generally considered to make a major contribution to mushroom flavor. The compound is detected in raw mushrooms, especially when they are damaged (19, 28). The compound is present in higher concentrations in mushroom gills, where the spores are formed. Peak levels of 1-octen-3-ol detected over the course of development of *Agaricus bisporus* fruit bodies were found in young caps about 35 mm in diameter with closed veils (12, 28). The fact that 1-octen-3-ol is produced in areas were high density concentrations of propagules occur e.g. on mushroom and in conidial masses of *Penicillium* species, suggests that this compound has a common function as an inhibitor of premature spore germination.

Together with 1-octen-3-ol a non-volatile metabolite, 10-oxo-trans-8-decenoic acid (ODA) is formed during the oxidative breakdown of linoleic acid by fungi. This compound is considered to have an influence on the development of fungal structures in the mushroom. It stimulates growth of the mycelium, fruiting body initiation and stipe elongation. It has been

regarded as a growth regulating substance (GRS) produced by gills (9, 29). The, two substances originating from the degradation of linoleic acid by lipoxygenase may act in concert as a complex of hormones (growth regulators). In addition, 1-octen-3-ol was shown to induce microcyclic conidiation in *P. paneum*, suggesting an additional role as a factor driving the fungal colony to spore production and dispersal. We suggest that products of linoleic acid breakdown may play an important role in the regulation of transition between vegetative and sexually and asexually reproductive structures. ODA may also influence the production of spore-forming structures on hyphae, but evidence for the latter remains to be given.

Recently, the link between linoleic acid derivatives and asexual and sexual spore development was studied in *Aspergillus nidulans.* The authors describe the "Psi factor" a mixture of three hydroxylated linoleic acids. The proportion of the compounds designated as PsiA, PsiB and PsiC controls the ratio of asexual to sexual spore development. PsiB and PsiC promote development of cleistothecia and inhibit asexual conidiation while PsiA is an antagonist of PsiB and PsiC (7, 8).

Current investigation is focused on the mechanism of action of 1-octen-3-ol on conidia. Elucidation of this mechanism may give new insights into the regulation of germination. 1-Octen-3-ol is a promising compound to investigate for possible applications in the prevention of food spoilage and the control of crop diseases.

## ACKNOWLEDGEMENTS

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# Chapter 5

Mechanism of action of 1-octen-3-ol, a volatile self-inhibitor of spore germination in *Penicillium paneum*

## ABSTRACT

1-Octen-3-ol is a volatile germination self-inhibitor produced by *Penicillium paneum* that blocks the germination process and induces microcycle conidiation (Chapter 4). Here, we investigated its mode of action. The diameter of conidia treated with 1-octen-3-ol (4 mM) for 4 h was similar to that of freshly harvested condia (5 µm). Exposure to 1-octen-3-ol resulted in staining of 10-20% of the conidia with PI and TOTO, fluorescent DNA probes that cannot enter cells with an intact membrane, whereas only 3-5% of non-treated conidia were stained. Furthermore, addition of 1-octen-3-ol to germinating conidia resulted in a transient dissipation of the pH gradient. From this, we conclude that slight permeabilization of the fungal membrane occurs in the presence of the inhibitor. Two dimensional gel electrophoresis analysis of protein patterns revealed striking differences between non-germinated conidia, germinated conidia and 1-octen-3-ol-treated conidia. Firstly, eighteen proteins that appeared in the germinated conidia were present at lower levels in the 1-octen-3-ol-treated conidia. Secondly, eight proteins that were absent from the non-germinated conidia, were present during germination (swelling and germ tube formation), yet were not found in conidia exposed to 1-octen-3-ol. It is conceivable that these proteins play a role in germ tube formation, since this process is blocked upon exposure to 1-octen-3-ol. Finaly, sixteen unique proteins were found in 1-octen-3-ol-treated conidia. In conclusion, 1-octen-3-ol targets the plasma membrane interfering with essential metabolic processes thereby preventing swelling and germination of the conidia. The identification of the proteins that were blocked or induced in 1-octen-3-ol-treated conidia may add further to our understanding of the action of 1-octen-3-ol and the fungal response to it.

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# INTRODUCTION

Fungi can grow in all kind of foods including cereals, meat, milk, fruits, vegetables, and nuts. Fungal growth is accompanied by secretion of enzymes and release of many different primary and secondary metabolites (12). Fungal enzymes are widely used in food industry in different applications. For instance, glucose oxidase from *Aspergillus* and *Penicillium chrysogenum* is used to eliminate traces of oxygen in bottled fruit juice. Further, chymosin, the protease produced by *Rhizomucor miehi,* which gene is cloned and expressed in *Aspergillus oryzae*, is used in cheese manufacture to coagulate the milk proteins (9). Conidia of *Penicillium roqueforti* are known to convert fatty acid into methyl ketones, which are typical for the blue-cheese flavour and can be used in the manufacture of some salad dressings, soups, crackers and cakes (13). As a result of their metabolic versatility *Penicillium* species also can degrade many different compounds including food preservatives such as sorbate. Degradation of sorbate results in the formation of *trans*-1,3-pentadiene which has an unpleasant kerosene-like odor. Fungal contamination of coffee beans results in the release of a phenolic odor (musty) caused by 2,4,6-trichloroanisolethe (18). Fungal growth may result in the production of mycotoxins, which is of growing concern is the food industry (10). Mycotoxins are secondary metabolites produced by fungi that cause disorders in animals and humans (11, 17). Volatile compounds formed by fungi can have several biological effects on growth of other species of fungi or other microorganisms (1). The volatile compound 1-octen-3-ol is one of the most important flavour components found in mushroom and is used as a spoilage indicator in stored cereals (16, 4, 21). Recently, we provided evidence that conidia of *Penicillium paneum* in high density suspensions ($10^9$ conidia $ml^{-1}$) produce 1-octen-3-ol and that it functions as a self-inhibitor of germination. The compound blocked swelling (isotropic growth) and prevented germ tube formation (polarised growth), and induced microcycle conidiation (chapter 4).

In the present work, the mode of action of the volatile germination self-inhibitor 1-octen-3-ol produced by *P. paneum* conidia was studied. Its impact on different cellular parameters including membrane integrity, intracellular pH, respiration and protein synthesis was investigated.

# MATERIALS AND METHODS

### Preparation of conidial suspensions

*Penicillium paneum* was grown on malt extract agar medium (MEA, CM59, Oxoid Ltd, Hampshire, England) inside tubes at 25°C for a maximum of 7 days. A spore suspension was prepared by adding 9 ml of peptone physiological salt solution (8.5 g $l^{-1}$ NaCl with 1 g $l^{-1}$

bacteriological peptone (Oxoid Ltd, Hampshire, England) + 0.1% Tween 80) to the fungal culture. Suspensions were prepared from 25 tubes and filtered through a 17 μm nylon filter and centrifuged (4000 X g) for 3 min. Conidia were resuspended in 2 ml of malt extracted broth (MEB) medium (CM57, Oxoid Ltd, Hampshire, England), adjusted to pH 4.0 with lactic acid. Spore densities of $10^9$ to $10^6$ conidia $ml^{-1}$ were adjusted using a Bürker Türk haemocytometer.

**Chemicals and probes**

The chemicals used in this study were 3-octanone from Fisher Scientific BV (Hertogenbosch, The Netherlands); 1-octen-3-ol from Janssen (Geel, Belgium); TOTO 1,19 (4,4,7,7-tetramethyl-4,7-diazaundecamethyl-ene)-bis-4-[3-methyl-2,3dihydro(benzo-1,3-oxazole)-2-methylidene]-1-(39-tri-methylammoniumpropyl)-pyridinium tetraiodide; PI (Propidium iodide) and cFDA-SE (5- (and 6)-carboxyfluoresceindiacetate succinimidyl ester) were obtained from Molecular Probes Europe BV (Leiden, The Netherlands). The protease inhibitor cocktail appropriate for fungal extracts was purchased from Sigma Chemical Co., St. Louis, USA.

**Location of germination self-inhibitor**

To investigate if the self-inhibitor compound produced by *P. paneum* could be washed from the spores, a volume of 1 ml of spore suspension with a high density of $10^9$conidia $ml^{-1}$ was centrifuged every 10 min at 5000 g for 2 min at 25º C, and the pellet containing the spores was resuspended in 1 ml of MEB pH 4.0. Washing was repeated during a period of 4 h. As a control, a spore suspension of $10^9$ conidia $ml^{-1}$ was centrifuged and resuspended in the same medium i.e. the obtained supernatant. Another control was the spore suspension at $10^6$ conidia $ml^{-1}$ in the presence of 4 mM of 1-octen-3-ol, incubated for 4 h at 25ºC, without washing. After the washing experiment, all the samples were incubated at 25º C for 22 h, and the percentage of spore germination was determined under the microscope. Germination of conidia was analysed with an Olympus Optical CO microscope (1000 x magnification), Ltda BX40 (Tokyo, Japan) and was considered positive when a conidium produced a germ tube. The experiment was performed in triplicate.

**Measurement of respiration**

Experiments were performed in 4 ml MEB pH 4.0 in a stirred, water-jacketed vessel. The oxygen concentration was recorded continuously with an oxygen electrode (YSI model 5300 biological oxygen monitor, Yellow Springs Instruments Co. Inc., Yellow Springs, USA). The electrode was calibrated in distilled water and in MEB pH 4.0. The spore suspensions had a

density of $10^7$ conidia $ml^{-1}$. After temperature equilibration for 2 min, a total of 0.1 ml of pellet of *P. paneum* was added and oxygen consumption measured. Fresh harvest conidia and conidia during isotropic growth, after incubation of 2 h (swelling stage) at 25ºC, were used. After 10 min of measurement, 156 µl of 1-octen-3-ol at 40 mM was added to give a final concentration of 4 mM and its effect on respiration was assessed. Two independent experiments were carried out.

**Size determination of conidia**

The size of conidia was determined by using ProtoCOL System, micrometer in the microscope (Synbiosis Ltd, Cambrigde, UK). A conidial suspension of *P. paneum* with a density of 4 x $10^6$ conidia $ml^{-1}$ was prepared as described before. The size of conidia were measured at different developmental stages: directly after harvesting, after 2 h (swelling stage) and 4 h (germ tube formation) of incubation at 25º C, and also in the absence (MEB) and presence of 4 mM 1-octen-3-ol. At least 100 conidia were measured. The results are the means of three independent experiments.

**Fluorescence labelling of conidia**

To assess whether 1-octen-3-ol affected the membrane integrity of conidia, spore suspensions were incubated for 2h with 4mM of 1-octen-3-ol at 25ºC and stained with either TOTO or PI. TOTO is a membrane impermeant dye that binds to DNA forming a green-fluorescent complex. PI is an impermeant dye that binds to nucleic acids forming a red-fluorescent complex. A stock solution of 100 µl/$ml^{-1}$ of TOTO was prepared in dimethyl sulfoxide (DSMO) and a stock solution with PI (1.0 mg/$ml^{-1}$) was prepared in distilled water. Both solutions were kept in the refrigerator. For labelling conidia with TOTO, spore suspension of $10^6$ conidia $ml^{-1}$ were incubated with 1µM TOTO at 30ºC for 10 min and subsequently washed once with Kpi pH 7.0. For labelling conidia with PI, spore suspension of $10^6$ conidia $ml^{-1}$ were incubated with 10µM PI at 30ºC for 10 min and subsequently washed once with Kpi pH 7.0. As controls, heat-killed conidia (90ºC for 10 min) and freshly harvested conidia were used.

For the analysis of the internal pH, conidia were labelled with cFDA-SE (20 µM in 50 mM $KP_i$ buffer pH 7.0) during 1 hour at 35ºC (5). Subsequently, conidia were washed and the pellets were kept in 50 mM $KP_i$ buffer pH 7.0 with glucose (10 mM) for 30 minutes at 30ºC to eliminate nonconjugated cFSE. The efficiency of the fluorescent labelling of the spores was observed by upright Zeiss Axiophot fluorescence microscope 1000W Xenon monochrometer (VisiTech International Limited, Sunderland, UK). The emission light was collected by Plan

Neofluar (n.a. =1.3) 100 x oil objective, a 500-560 nm dichroic mirror, and a 515 nm emission bandpass filter.

**Immobilization of conidia and the image analysis system**

The fluorescence ratio image microscopy (FRIM) system was equipped by an upright Zeiss Axiophot fluorescence microscope 1000W Xenon monochrometer (VisiTech International Limited, Sunderland, UK). Conidia were suspended in 50 mM potassium citrate phosphate buffer pH 4.0 ($CKP_i$ 4), and immobilized on glass slides as described by Breeuwer and Abee (5). Ìmmobilized spores were studied in a perfusion chamber, which solutions were pumped. Two solutions, MEB pH 4.0 and 1-octen3-ol in MEB pH 4.0, diluted 5 times in distilled water were used. Ratio imaging was initiated at time zero of the perfusion of a solution through the chamber. The emission light was collected by Plan Neofluar (n.a. =1.3) 100 X oil objective, a 500-560 nm dichroic mirror, and a 515 nm emission bandpass filter**.** Emission was collected with an extended CCD Camera (VisiTech International Limited, Sunderland, UK) and images were acquired by the image processing unit (X-Windows on an interactive UNIX system) equipped with the Quanticell 900 software package from Applied Imaging (Joyce Loebl Instruments, Sunderland, UK). The resolution of the images was 256 X 256 pixels during the studies on germination and the effect of 1-octen-3-ol. The fluorescence intensity and the ratio values of the pixels in the images were expressed on a scale from 0 to 4096. The time interval between acquisition at 490 nm and 470 nm images, adjusted at 20 bandwidth, was 5800 ms. The images were saved as Tiff files, which were imported to Photopaint to improve the images for contrast enhancement and Coreldraw 9 was used to process the images (Corel Corporation, Ontario, Canada). For analysis, different conidia were selected on the computer screen by means of a computational drawing procedure.

**Calibration of $pH_{in}$**

To construct a calibration curve of labelled conidia with cFDA-SE, the pH-dependent fluorescence was determined in vitro (in buffer) and inside the permeabilized conidia (in vivo) at pH values ranging from pH 4.0 to 9.0. For this, the fluorescence intensity at 490 nm was divided by the fluorescence intensity at 470 nm ($R_{490/470}$) for each pixel of a spore image. The buffers used were 50 mM potassium citrate phosphate buffer from pH 4.0 to 7.0 and 50 mM sodium borate from pH 8.0 to 9.0. For in vitro calibration, cFDA-SE (20 µM final concentration) was used in the different buffers that were adjusted to various pH values with NaOH. In order to equilibrate the pH of the buffer ($pH_{ex}$) and the interior of the spores ($pH_{in}$), ethanol (63%, v/v) was added to the labelled spores to permeabilise the membrane. This is designated as the in vivo treatment (8). The suspension was incubated for 30 minutes at 30º C and after centrifugation, the spores were resuspended in buffer. In each experiment at least 13

spores were analysed with 2 repetitions. The calibration curve was fitted according to a four parameter sigmoid function y = a + b/{1+exp [-(x-c)/d}] and the parameters a to d were determined. The $pH_{in}$ was calculated using the formula $pH_{in}$ = -[ln ((b- (ratio-a))/(ratio-a)) x d] + c

**Effect of 1-octen-3-ol on $pH_{in}$**

The $pH_{in}$ was analysed on labelled immobilised conidia in a perfusion chamber. The measurements started at time 0 of perfusion with 5 times diluted MEB and after 20 minutes of perfusion 4 mM of 1-octen-3-ol in MEB were added. Ratio $_{(490/470)}$ imaging was initiated at time 0 of perfusion and the images were recorded with intervals of 10-20 minutes during a period of 120 min and at 420 min.

**Protein extraction**

A conidial suspensions with a density of 9.0 x $10^6$ conidia $ml^{-1}$ were collected from 6 tubes of *P. paneum*, grown on MEA at 25°C for 5 days, using peptone physiological salt solution as described above. Freshly harvested conidia, conidia incubated 5 h in MEB pH 4.0, and conidia incubated 5 h in the presence of 4 mM of 1-octen-3-ol were used. *P. paneum* suspensions were incubated at 25ºC, shaking at 140 rpm and centrifuged at 4,000 x g, at 25ºC for 4 min. The pellet was resuspended in potassium phosphate buffer, KPi pH 7.0, with protease inhibitor at 0.2 g $ml^{-1}$ and zirconium beads (diameter, 1mm; Biospec Products). Total cellular proteins were extracted from the conidial suspensions using a bead beater (B. Braun Biotech International, Melsungen, Germany), by four treatments of 3 min, after each homogenising treatment, and the preparations were cooled on ice (22). The preparations were centrifuged twice at 13000 g for 5 min at 4ºC, zirconium beads were allowed to sediment by gravity, and the supernatant containing the cellular proteins was analysed by two-dimensional gel electrophoresis (2D-E). The protein content of the conidial extract was determined using the bicinchoninic acid assay (Sigma, St. Louis, MO) with bovine serum albumin as the standard and equal amounts of protein were applied on the gel.

**Protein analysis by 2D-E**

2D-E was performed as previously described (22) using Pharmacia 2D-E system (Pharmacia Biotech, Uppsala, Sweden). Equivalent amounts of protein (30 µg of protein) extracted from freshly harvest conidia, and conidia incubated for 5h in MEB and conidia incubated for 5h in the presence of 4 mM of 1-octen-3-ol in MEB respectively were used. A sample solution of 15.1 µl (30µg of protein) of protein (germinated conidia) was treated with 15.1µl of lysis solution (9 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 3-10L [Pharmacia Biotech], 2%

Triton X-100, 6 mM Pefabloc SC [Merck, Darmstadt, Germany]). These preparations were kept on ice, and 32.2 µl of sample solution (8M urea, 2% 2-mercaptoethanol, 2% IPG buffer 3-10L, 0.5% Triton X-100, a few grains of bromofenol blue) was added to the protein solution. The total volume was loaded on the acidic end of a first-dimension IEF gel with a pI range of 4 to 7 (Immobiline Dry Strips; Pharmacia Biotech). For the second dimension, homogeneous sodium dodecyl sulfate (SDS) 12 to 14% polyacrylamide gels (PAGE) (ExcelGel; Pharmacia Biotech) were used. Two molecular mass markers were used, with band sizes of 97, 66, 45, 30, 20, and 14 kDa (Pharmacia Biotech, Sweden) and of 210, 125, 101, 56.2, 35.8, 29, 21.1, and 6.9 kDa (BioRad, Richmond, USA), respectively. The gels were silver stained and analysed (3). Representative gels obtained from triplicate experiments are shown below.

## RESULTS

### Association of the self-inhibitor with the conidium

Spore suspensions under crowding conditions ($10^9$ conidia $ml^{-1}$) that were successively washed, every 10 min in MEB (pH 4.0), only showed 1 % germination after 22h. Spore suspensions at $10^6$ conidia $ml^{-1}$ showed 4% germination in the presence of 4 mM 1-octen-3-ol germinated after 22 h of incubation (data not shown). Even when spores were continuously washed and resuspended for 4 h in MEB, no germination was observed. These data suggest that the self-inhibitor is continuously produced by the conidia and/or that it is very strongly associated with the conidia.

### Effect of 1-octen-3-ol on respiration of conidia

The respiration of conidia at $10^7$ conidia $ml^{-1}$increased 39% during a period of 2h ranging from harvesting of the cells to the swelling stage. Freshly harvested conidia in the presence of 4mM of 1-octen-3-ol exhibited a reduction of respiration of 12% compared to conidia in MEB alone. The same experiment was done with spores at the swelling stage (2 h after harvesting) and a reduction of the respiration of 5% was observed in the presence of the inhibitor. This indicates that spore respiration is not significantly affected by 1-octen-3-ol.

### Effect of 1-octen-3-ol on isotropic growth

The diameter of the conidia was measured at different stages of germination in the absence or presence of 1-octen-3-ol (Fig. 1). Untreated *P. paneum* conidia were smaller than 5 µm at the beginning of the experiment. After 2 h of incubation in MEB, 74% of the conidia had increased diameters ranging from 5 to 9 µm. After 4 h, conidia were further swollen and 81%

of the population of the conidia was between 5 and 9 µm and 3 % had a diameter between 9 and 13 µm. In the presence of 1-octen-3-ol, 78% and 67% of the conidia was smaller than 5 µm, after 2 h and 4 h, respectively. Conidia larger than 7µm were not observed at all in the presence of the inhibitor and germ tube formation was blocked (data not shown).

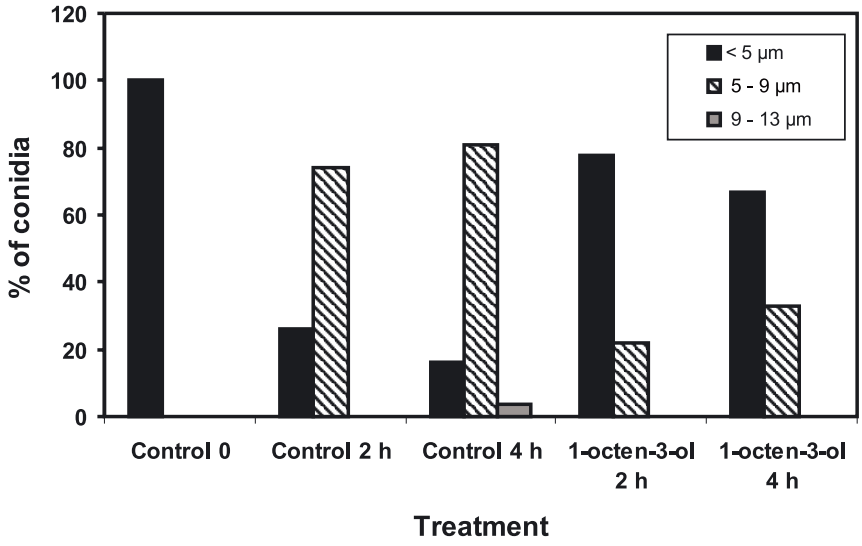



**Fig. 1** Sizes of conidia (µm) of *P. paneum*; freshly harvested (control), after 2 h and 4 h of incubation at 25ºC in MEB pH 4.0, and in the presence of 4 mM 1-octen-3-ol. Percentages of conidia with sizes of < 5 µm (■), between 5 and 9 µm (▨), and between 9 and 13 µm (■) are shown.

**Assessment of membrane integrity**

In order to study if a treatment with 4 mM 1-octen-3-ol for 2 h had an effect on membrane permeability, conidia were labelled with the fluorescent probes TOTO or PI. The results revealed that exposure to 1-octen-3-ol resulted in a slight permeabilization of the spore membrane. The number of fluorescent i.e. membrane damaged conidia labelled with TOTO was 10% in the presence of 1-octen-3-ol, 79% in heated-treated (10 min, 90ºC) conidia and 5% with freshly harvested conidia (Fig. 2). Staining with PI showed 20% fluorescent conidia after treatment with 1-octen-3-ol, 90% staining after heat-treatment and only 3% staining with untreated conidia. These results show that 1-octen-3-ol has a permeabilizing effect on the conidia membrane. Notably, with 1-octen-3-ol not all conidia are effected and the staining efficiency is significantly lower than that of the heat treatment.

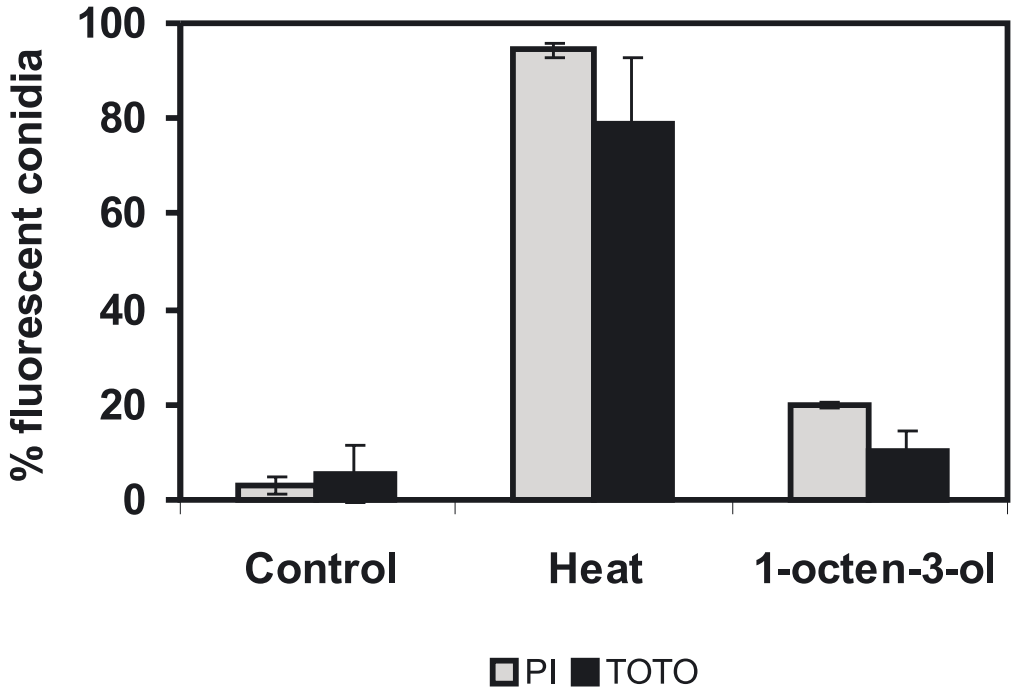



**Fig. 2** Effect of 4 mM 1-octen-3-ol on fluorescent staining of *P. paneum* conidia. Conidia incubated in MEB, heat treated at 90ºC for 10 min and exposed to 1-octen-3-ol for 2 h at 25ºC were labelled with fluorescent probes. Percentage of fluorescent PI (■) and TOTO (■) labelled conidia, monitored by epifluorescence microscopy.

**1-Octen-3-ol has a transient effect on the pH gradient**

Calibration curves with fluorescence ratios of cFSE at different pH values both in vitro and in vivo are shown in Figure 3. The fluorescence ratio values of the probe inside permeabilized conidia were in most cases lower than that of the values in buffer. Permeabilized conidia showed a larger range in which the internal pH could be measured namely between pH 5 and pH 8. The internal pH of viable conidia was calculated at different external pH values (6.0 to 8.5) and ranged from pH 6.4 to 7.0 (data not shown). The $pH_{in}$ of *P. paneum* conidia was pH 5.4 $\pm$ 0.1 in diluted MEB at pH 4.0. During initial stages of germination within 20 min an increase of the internal pH to 6.2 was observed. Then, the internal pH only slightly increased to 6.5 during a period of 90 min (Fig. 3B). In the presence of 4 mM of 1-octen-3-ol added after 20 min of perfusion, the $pH_{in}$ decreased from 6.2 to pH 5.3 within 40 minutes. Thereafter, a gradual increase of the $pH_{in}$ was observed in approximately 60 min finaly reaching $pH_{in}$ 6.2 again. This suggests that 1-octen-3-ol has a transient effect on the pH gradient, resulting in lowering of the $pH_{in}$ of conidia during the germination phase.

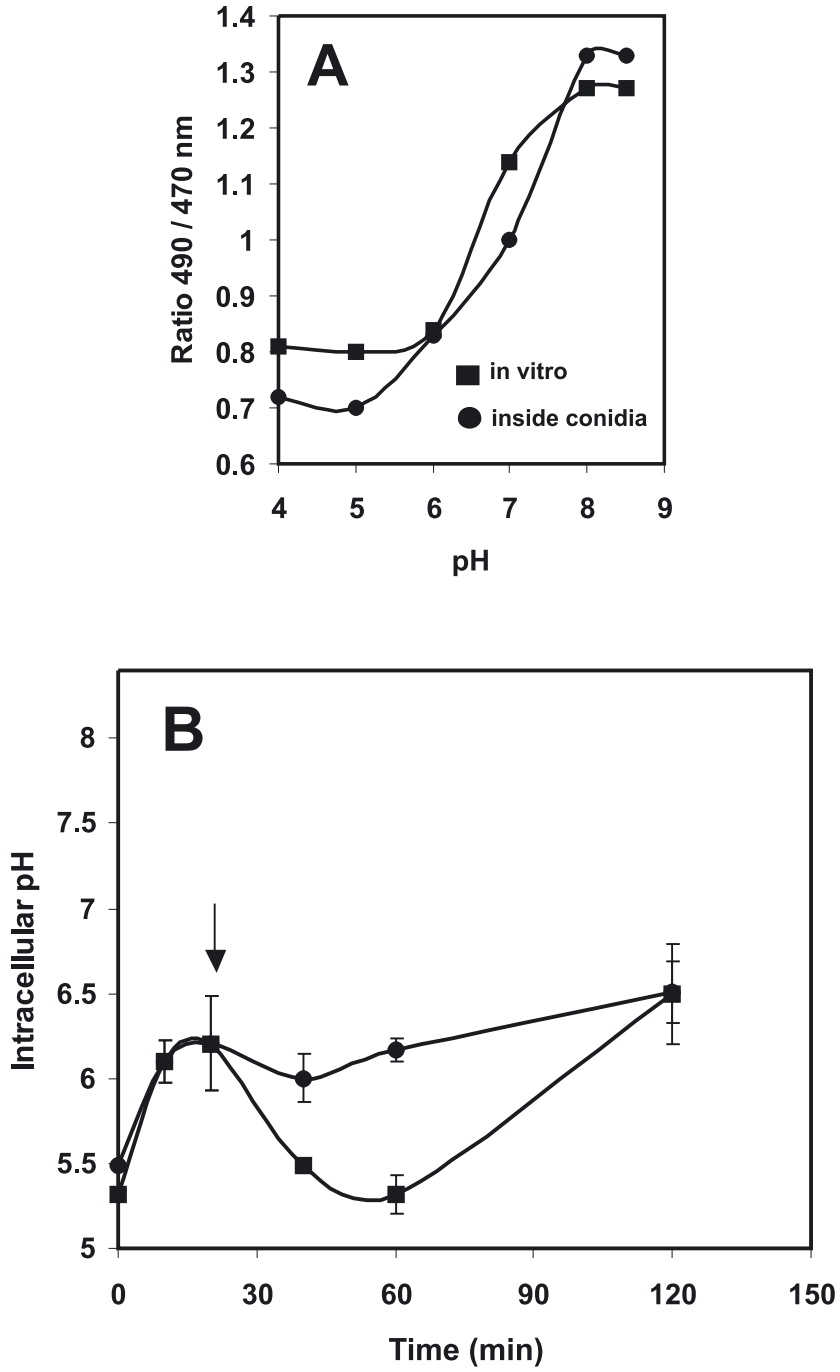



**Fig. 3** Relationship between the pH and the fluorescence ratio (490 / 470 nm) of cFSE in vitro (buffer) and inside conidia. With conidia, $pH_{in}$ and $pH_{ex}$ were equilibrated by incubation with 63% (v/v) ethanol. The buffers used were 50 mM potassium citrate phosphate (pH 5.0 to 7.0) and 50 mM sodium borate (pH 8.0 to 8.5) **(A).** Immobilised cFDA-SE labelled conidia were perfused at 25ºC in MEB 5X diluted in distilled water pH 4.0. The $pH_{in}$ of conidia of *P. paneum* in MEB (●), and in the presence of 4 mM 1-octen-3-ol (■) are shown. The arrow indicates the time at which perfusion with 1-octen-3-ol started. The ratio values are averages based on analysis of approximately thirteen conidia and the experiment was performed in duplicate **(B).**

**Protein profiles of non-germinated, germinated and 1-octen-3-ol treated conidia**

Protein profiles of cell free extracts of non-germinated conidia, germinated conidia (5 h) and 1-octen-3-ol-treated conidia (5 h) were analysed using two-dimensional gel-electrophoresis. Figure 4A shows the protein profiles from freshly harvested non-germinated conidia. Protein profiles of germinated conidia incubated for 5 h in MEB are represented in Fig. 4B and proteins of conidia which were kept for 5 h in the presence of the self-inhibitor, 1-octen-3-ol are presented in Fig. 4C. Conidia in the presence of 1-octen-3-ol showed reduced swelling and had not formed germ tubes, while 35% of conidia germinated after 5 h incubation in MEB in the absence of the inhibitor (see also Chapter 4). Selected proteins were labelled with numbers 1, 2, 3, etc. or letters, A, B, C, etc. Numbers designated all proteins specific for untreated conidia, and letters were used for new proteins that appeared in treated conidia. Freshly harvested conidia showed many proteins of low molecular mass (the majority of the proteins are smaller than 40 kDa [Fig. 4A]) and low pI (4.0 - 5.5). After 5 h of incubation in MEB many proteins appeared (Table 1[Fig. 4B]). Proteins extracted from these conidia showed a broader pI range and more proteins with a higher molecular mass, with most proteins between 20 and 66 kDa (Fig. 4B). Conidia that were kept in the presence of 1-octen-3-ol showed a very different protein pattern, with the majority of proteins carrying a molecular mass between 20 and 90 kDa and a pI ranging from 4-7 (Fig. 4C). Comparisons were made among protein patterns under these three different conditions by marking specific proteins. Gels were analysed and substantial differences in protein profiles are listed in Table 1. Eight proteins (18, 20, 22, 33, 34, 35, 36, and 38) that were not present in fresh conidia appeared in conidia during germination (including swelling and germ tube formation), while they were not found in conidia treated with 1-octen-3-ol. Another 26 proteins were observed in germinated and 1-octen-3-ol-treated conidia, although their levels were different in treated conidia, 23 of them decreased and 3 proteins showed increased levels in treated conidia (Table 1). Five proteins (14, 19, 24, 32 and 37) that were present in fresh conidia and in conidia during germination were not found in treated conidia. Notably, a group of 16 proteins was induced in the presence of 1-octen-3-ol (designated as A-P). All these proteins clustered together in an area with a relative high Molecular mass and pI.

## DISCUSSION

A wide range of fungi show the so-called crowding effect, meaning that germination is inhibited at high spore concentrations. They range from the class of Zygomycetes (*Rhizopus*) to Ascomycetes including the genera *Aspergillus* and *Penicillium*. However, information about the physiological state of the spores during crowding effects is scarce. In the present study we investigated different possible target sites of the self-inhibitor 1-octen-3-ol produced

by *Penicillium paneum.* The pathway involved in its production is not known, but 1-octen-3-ol is a product of the oxidative breakdown of linoleic acid and contributes to the flavour of mushrooms.

**Table 2:** Comparison of proteins patterns between non-germinated, germinated conidia in MEB and 1-octen-3-ol treated conidia incubated 5 h at 25ºC.

| Proteins | Non germinated spores | Germinated spores | |
|---|---|---|---|
| | | 5h | 5h with 1-octen-3-ol |
| 18, 20, 22, 33, 34, 35, 36, 38 | - | + | - |
| A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P | - | - | + |
| 14, 19, 24, 32, 37 | + | + | - |
| 1, 2, 4, 5, 6, 7, 8, 9, 10, 13, 15, 16, 17, 21, 23, 25, 26, 27, 28, 29, 30, 31, 39 | - | + | + [a] |
| 3, 11, 12 | - | + | + [b] |

**a –** Proteins that appeared in lower levels in treated conidia
**b -** Proteins that appeared in higher levels in treated conidia

The self-inhibitor in a concentration of 4 mM inhibited swelling and blocked germ tube formation of conidia in MEB. Their sizes were equivalent to untreated spores, which confirms that this compound is active during the early stages of germination. The mode of action of self-inhibitors of rust fungi such as *Uromyces phaseoli* and *Puccinia graminis,* named methylcisferulate and methyl 3,4 dimethoxycinnamate is completely different. These compounds prevent digestion of the pore plug where germination begins and therefore release of the germ tube from the uredospores is prevented (15).

We found a slight inhibitory effect (5-12%) of 1-octen-3-ol on oxygen consumption in freshly harvested and swollen conidia (incubated 2 h) in MEB. A stronger effect was observed in the case of an unidentified self-inhibitor of *Geotrichum candidum* arthrospores in suspensions with $10^7$-$10^8$ spores $ml^{-1}$, and here oxygen consumption decreased 12 fold (20). To study the

effect of 1-octen-3-ol, fluorescent probes were applied to assess its impact on the membrane integrity.

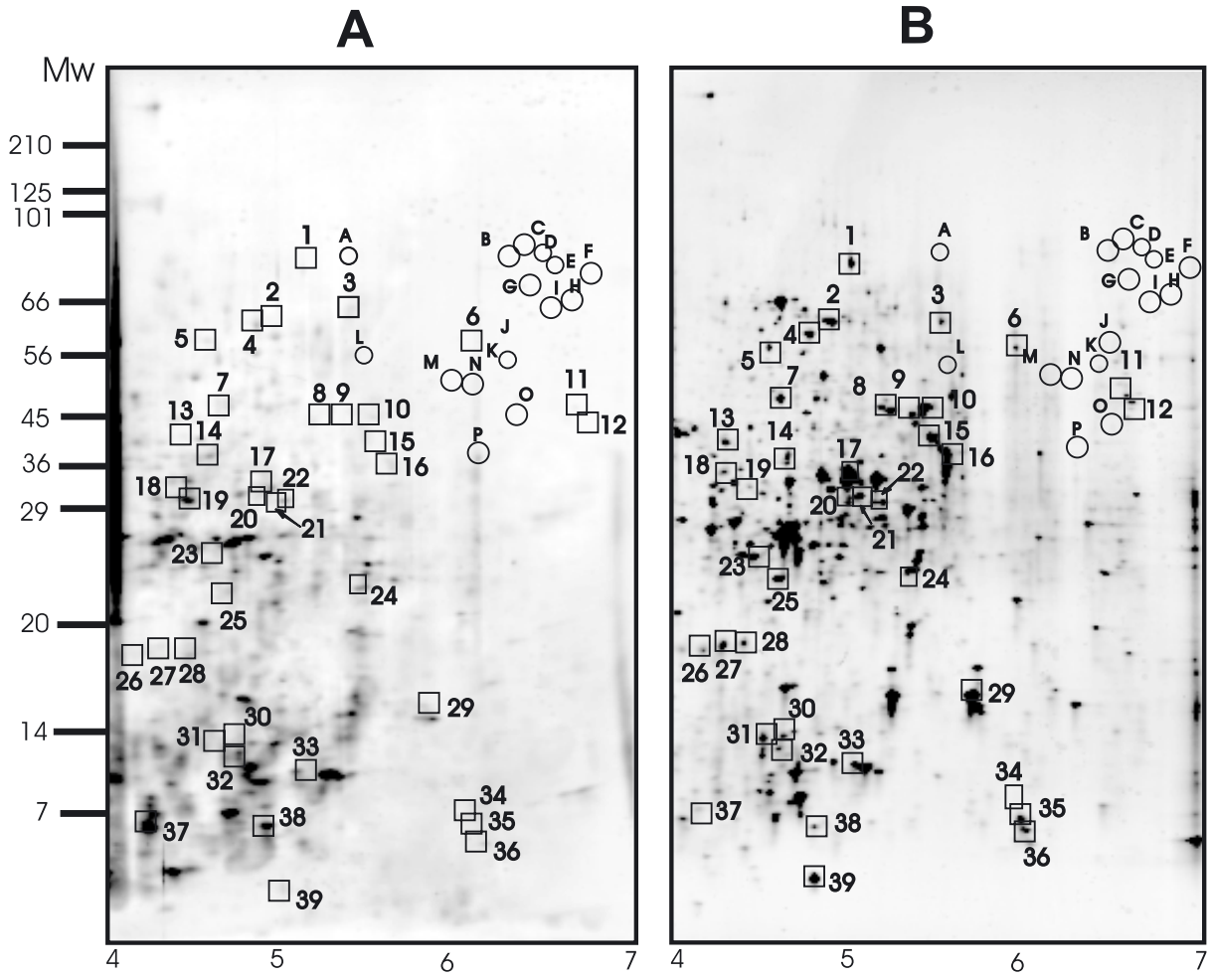
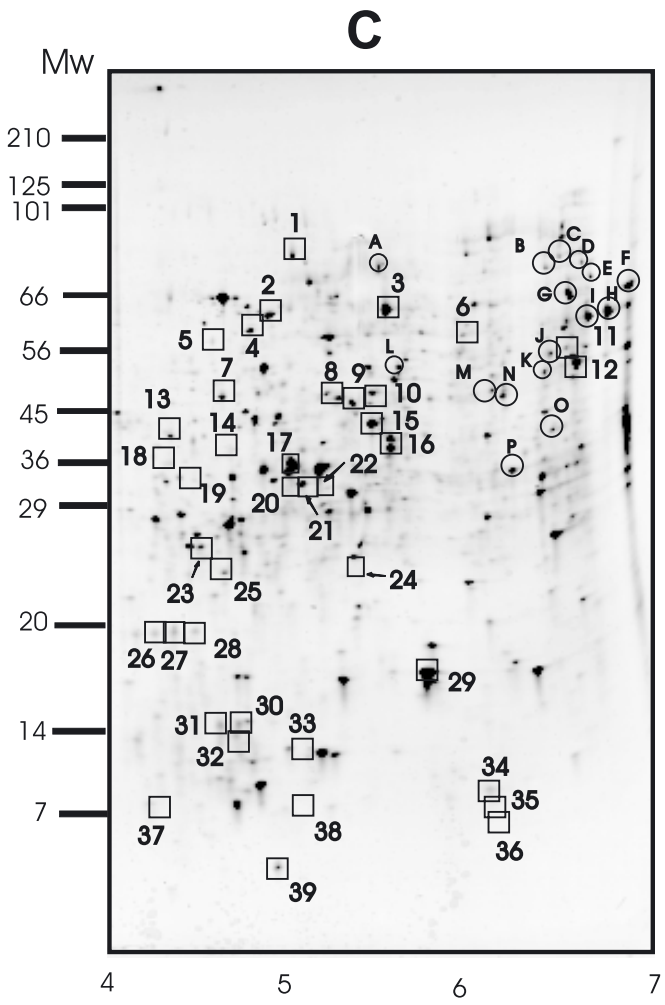



**Fig 4** Effect of 1-octen-3-ol on protein profiles of *P. paneum* conidia. Two-dimensional gel electrophoresis of cell free extracts of freshly harvested conidia (**A**), and germinated conidia incubated 5 h at 25ºC **(B)**, and conidia incubated in the presence of 4 mM 1-octen-3-ol at 25ºC for 5 h (**C**), using a pI ranging from 4 to 7.




We tested two different membrane impermeable fluorescent dyes, TOTO and PI. The probes, TOTO and PI were able to stain 10-20% of conidia-treated with 1-octen-3-ol, 79-90% of heated killed conidia and 3-5% of untreated conidia. These data indicate a small membrane permeabilizing effect of 1-octen-3-ol. TOTO has also been used as an indicator of viability of *Listeria monocytogenes* cells (14). PI has been used as an indicator of cell death of bifidobacterium cells during bile salt stress (2), for assessment of viability of fungal spores in combination with flow cytometry (7) and for assessment of membrane integrity of *Oenococcus oeni* cells after exposure to ethanol (19).

Exposure to 1-octen-3-ol was also observed to affect the intracellular pH of germinating conidia. At the beginning of germination *P. paneum* conidia had an intracellular pH of 5.4. During the first stage of germination (swelling) an increase of the intracellular pH to 6.2 was observed. When 1-octen-3-ol was added the internal pH of the spore dropped to pH 5.4, but

subsequently the $pH_{in}$ was restored to 6.2 within 60 min at $pH_{ex}$ of 4.0. This recovery of the $pH_{in}$ indicated that 1-octen-3-ol treated-conidia had sufficient energy to pump out $H^+$ of the cell and that ATPase activity was not affected. *F. culmorum* incubated with nonanoic acid (1mM) was shown to display similar behaviour, the intracellular pH fluctuated between 5.4 and 6.5 during a period of 90 min at an extracellular pH of 4.0 (Chapter 3). Sporangiospores of *Rhizopus oligospores* exposed to sublethal doses of nonanoic acid (1mM) show a similar restoration phenomenon (6).

Analysis of protein patterns revealed striking differences between freshly harvested conidia, germinated conidia and 1-octen-3-ol treated-conidia. Although the treated-conidia had not formed germ tubes, this protein pattern was very different from that of fresh conidia. Eight proteins that were absent from the non-germinated conidia, were present during germination (swelling and germ tube) yet were not found in conidia exposed to 1-octen-3-ol. Presumably, these proteins play a role in isotropic growth or germ tube formation, which is inhibited or blocked upon exposure to 1-octen-3-ol, respectively. Sixteen proteins were only present in 1-octen-3-ol treated-conidia. This suggests that these proteins are required for adaptation of the conidia to the inhibitor in order to overcome the inhibition action. Our results suggest that inhibition of spore germination by 1-octen-3-ol involves targeting the membrane resulting in mild effects on spore respiration and membrane permeabilization causing a transient lowering of the intracellular pH, finaly resulting in significant differences in the protein composition of self-inhibitor exposed conidia. The nature of the proteins which levels were affected or that were unique in 1-octen-3-ol treated-conidia is unknown. Identification of these proteins may add further to our knowledge of the action of 1-octen-3-ol and the fungal response to it.

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# Chapter 6

General Discussion

## INTRODUCTION

Fungi and their toxic metabolites cause losses of foods, diseases in plants and animals and may have adverse effects on human health. A crucial step in fungal contamination and infection or colonization is the germination process, subsequently resulting in mycelial growth. The work described in this thesis highlights the sequence of events in the germination process of fungal spores, notably conidia from *Penicillium paneum* and multicompartment macroconidia from *Fusarium culmorum*. Furthermore, fungal inhibitors including an iturin-like compound that is produced by *Bacillus subtilis* and a volatile self-inhibitor of germination of *Penicillium paneum,* which was identified as 1-octen-3-ol, and their mode of action investigated. These compounds appear to be affecting different target sites including membrane integrity, intracellular pH, respiration and protein synthesis. Detailed insight in the fungal germination process and action of antifungal compounds may contribute to a more efficient control of fungal infection of plants and animals, food spoilage and toxin production.

## SEQUENTIAL EVENTS IN GERMINATION

The germination of fungal spores was investigated in detail, using *Fusarium culmorum* macroconidia and *Penicillium paneum* conidia (Chapters 3 and 5). One approach was focused on the intracellular pH ($pH_{in}$). This parameter determines to a large extent the metabolic condition of the cell(s). Non-germinated spores generally showed a lower $pH_{in}$ than germinated conidia. The work described in Chapters 3 and 5 shows that the $pH_{in}$ of non-germinated unicellular conidia of *Penicillium paneum* is lower than that of *Fusarium culmorum* multicellular macroconidia. The average of the $pH_{in}$ observed for non-germinated spores of *F. culmorum* ($pH_{in}$ 6.4) and *P. paneum* ($pH_{in}$ 5.4) are within the pH range previously reported for *Rhizopus oligosporus,* which is between pH 5 to 6.5 (4). The germination of *P. paneum* conidia and *F. culmorum* macroconidia under optimal conditions is homogeneous in the isotropic growth (swelling stage). During swelling there is an increase in the intracellular pH of spores of *F. culmorum* and *P. paneum.* No significant difference ($P < 0.05$) in $pH_{in}$ between the different compartments of *F. culmorum* macroconidia at this stage could be observed (Chapters 3 and 5).

Notably, during germ tube formation of *F. culmorum* macroconidia, a clear differentiation between the compartments of the macroconidium was observed. Germ tubes develop preferably from apical cells and with low frequency from middle compartments. Such a differentiation has also been observed in *Magnaporthe grisea* conidia (2, 11). The non-germinated middle compartments of *F. culmorum* showed a low internal pH, which may be associated with an increase in the size and number of the vacuoles (Chapter 2). Similar results were found by Atkinson et al (2), who reported the formation of spherical vacuoles inside

non-germinated compartments of *M. grisea* conidia. In contrast, the apical cells showed higher pH values and subsequent germination. This suggests that there is a link between the internal pH and germ tube formation (Chapter 3). The $pH_{in}$ of newly formed germ tubes of *F. culmorum,* pH 7.2, is similar to the $pH_{in}$ of *M. grisea* cells, pH 7.32, as determined with the fluorescent pH-indicator SNARF-1 (10).

Macroconidia of *F. culmorum* proved to be a good model system to study germination. Because of its multicelled structure, differences in germination between compartments could be investigated. Changes in $pH_{in}$ that were associated with differentiation processes have also been observed for *Dictyostelium discoideum* and *Candida albicans* (9, 25). Although these results suggest that the intracellular pH is associated with the germination process, they do not establish whether an increase in $pH_{in}$ occurs prior to or whether it is a product of the metabolic changes associated with germination.

Notably, the middle compartments in macroconidia also germinate, but at a later stage (after 6 hours), when the germ tubes of apical cells are already very elongated (Chapter 2). This suggests that germ tube forming cells exhibit an “apical dominance” over middle compartments, which is alleviated at the later stages of cellular development. The preferential germination of apical cells may result from the difference in the surface-to-volume ratio of apical cells compared to that of middle compartment cells (Chapter 2). This larger surface-to-volume ratio will be reflected in a relative larger transport activity allowing increased accumulation of nutrients and henceforth introduce an asymmetry in the development of the macroconidium (22).

## INHIBITORY MECHANISMS ON GERMINATION

Antifungal agents that inhibit fungal germination and/or mycelial growth have been shown to have target sites in the microbial cells. In this thesis, we have identified an antifungal iturin-like lipopeptide, produced by *Bacillus subtilis* YM 10-20 (Chapter 2). This compound probably belongs to the iturin group (iturin A) of antibiotics, which is known to interact with sterols of the cytoplasmic membrane of fungi (15, 18). According to our studies, the antifungal compound is effective as an inhibitor of fungal growth as observed in the species of *Penicillium, Aspergillus* and *Fusarium*. However, the strain of *Rhizopus* sp. that we used was insensitive to the iturin-like compound (21)(Chapter 2). A possible explanation for this is the low ergosterol content of the *Rhizopus* sp. membrane (24, 3). Inhibition of fungal growth by iturin has been shown in stored grains (12), in postharvest peaches (8) and in mycotoxigenic species of fungi such as *Aspergillus parasiticus, A. flavus and A. ochraceus* (13).

The iturin-like compound was shown to inhibit the germination of *Penicillium paneum* conidiospores. Fluorescence microscopy and FCM revealed that the DNA probe propidium iodide could stain damaged cells, indicating the permeabilisation of the *P. paneum* conidiospore membrane, upon exposure to this compound (Chapter 2). This is in accordance

with the proposed action of iturin A. Iturin A is known to increase the permeability of lipid membranes of fungal cells by pore formation, which results in the loss of essential macromolecular compounds (26). This action is similar to that of the antifungal polyene Amphotericin B (20). Permeabilization of the spore membrane blocks biochemical activities and initiates morphological changes. Notably, our studies revealed for the first time that this iturin A like compound acts on spores.

Another compound used in this research is the antibiotic nystatin that interacts with ergosterol of macroconidia membrane of *F. culmorum* (Chapter 3). Nystatin, a polyene antibiotic derived from *Streptomyces noursei* and *Streptomyces aureus*, forms channels in the plasma membrane by interaction with ergosterol, consequently increasing the membrane permeability and causing cell death (6, 7). The pH gradients in the macroconidial compartments dissipated very fast in the presence of nystatin at levels of 1 and 4 µg/ml (Chapter 2). At sublethal doses (0.3 µg/ml) of nystatin, the apical compartments appeared to be preferentially targeted, resulting in low $pH_{in}$ values in these compartments.

Nystatin affects $pH_{in}$ of the conidia of *F. culmorum* during the germination process and differentiation such that preferential germination occurs from the middle compartments. Nonanoic acid, a fungal self-inhibitor also prevents the germination, e.g. swelling and germ tube formation, by dissipation of the pH gradient of *F. culmorum* macroconidia and *P. paneum* conidia (Chapters 3 and 5).

The volatile self-inhibitor 1-octen-3-ol efficiently blocked germination at a concentration of 4 mM. Inhibition of spore germination by 1-octen-3-ol involves multiple target sites. The self-inhibitor compound may inhibit spore germination as a result of the permeabilization of the membrane concomitant with perturbation of the intracellular pH effecting spore respiration and protein synthesis. The inhibition of swelling and germ tube formation by 1-octen-3-ol is dose dependent.

A detailed analysis of protein profiles of untreated and 1-octen-3-ol treated cells revealed significant differences. Eight proteins that are not present in the non-germinated conidia, but appeared in conidia during the germination (swelling and germ tube formation) process, were not found in conidia treated with 1-octen-3-ol. Presumably, these proteins play a role in the germ tube formation, which is blocked under the latter condition. Notably 16 proteins were induced in the presence of 1-octen-3-ol. Identification of these proteins may add further to our understanding of the action of 1-octen-3-ol and the fungal response to it. Notably, a self-inhibitor of germination of *Glomerella cingulata* conidia has been suggested to inhibit protein synthesis (17). Volatile compound(s) produced by *P. paneum* in dense suspensions inhibited mycelial growth of different species of fungi. 1-Octen-3-ol also inhibits spore germination of different fungal species belonging to a variety of genera suggesting a broad action range (Chapter 4). Therefore, the compound may find broader application in preventing outgrowth of fungal spores.

## RESPONSE TO INHIBITORS

Fungal spores respond differently under adverse conditions. For example, sublethal heat injury results in extended lag phase and increased sensitivity to preservatives, such as sorbic acid and butylated hydroxyanisole (23). Fungi may recover from sublethal injury and regain their capacity to grow. However, *P. paneum* conidia exposed to the iturin-like compound produced by *B. subtilis* was shown to affect the permeability of the conidial membrane resulting of the spore destruction (Chapter 2).

The antifungal nonanoic acid (1mM) inhibited swelling and germ tube formation of *F. culmorum* macroconidia and *P. paneum* conidia (Chapters 3 and 5). The intracellular pH of *F. culmorum* fluctuated between 5.4 and 6.5 in the presence of nonanoic acid during a period of 90 minutes at an extracellular pH of 4.0 (Chapter 3). Recovery of the $pH_{in}$ indicated that macroconidia had sufficient energy to pump out $H^+$ of the cell. Moreover, conidia of *P. paneum* incubated for 1 h with 1-octen-3-ol displayed the same behaviour, with the intracellular pH of 6.2 dropping to 5.4, similar to that of untreated conidia, and subsequent recovery to an intracellular of pH 6.2 (Chapter 4). Probably, the membrane $H^+$ ATPase was not affected, and the conidia were able to extrude protons and recover the capacity to maintain intracellular pH homeostasis in the presence of low concentration of 1-octen-3-ol. A similar homeostasis phenomenon was also observed in *Rhizopus oligosporus* sporangiospores exposed to sublethal doses of nonanoic acid (1 mM) (4).

The developmental processes during the *P. paneum* life cycle were blocked upon addition of 1-octen-3-ol at different stages. As a response, microcycle conidiation was induced. Microcycle conidiation, the formation of a new conidium on a germ tube, is observed when extracted compounds of very dense conidial suspensions or 4 mM 1-octen-3-ol were applied against fresh conidia. 1-Octen-3-ol may act on development of hyphae, germination of spores and formation of conidia on germ tubes. Our observations suggest that 1-octen-3-ol act as fungal hormone. 1-Octen-3-ol treated conidia of *P. paneum* showed different protein profiles compared to that of untreated conidia. Twenty-three proteins that appeared in the germinated conidia showed lower levels in the 1-octen-3-ol treated conidia. Strikingly, 16 new proteins appeared in these treated cells suggesting that they are required for the adaptation to 1-octen-3-ol since these proteins did not appear in the non-germinated and untreated conidia (Chapter 4).

*The hypothetical model of germination of multicelled macroconidia*

At sublethal doses of nystatin (0.3 μg/ml), the apical cells appear to be preferentially targeted, and the inhibition of apical germination is compensated by an increase in germination capacity of the middle compartments, which is associated with a higher internal pH of these cells (Chapter 3). On the other hand, the middle cells of the macroconidium may differentiate

into the very resilient chlamydospores, which can be seen as a survival strategy (Fig 1). The shift of the germ tube formation of *F. culmorum* towards the middle cells suggests that there is some form of communication between compartments of multicelled conidia. Such a communication between the compartments during germination also suggests that the compartments of the conidium may not be separate cellular entities.

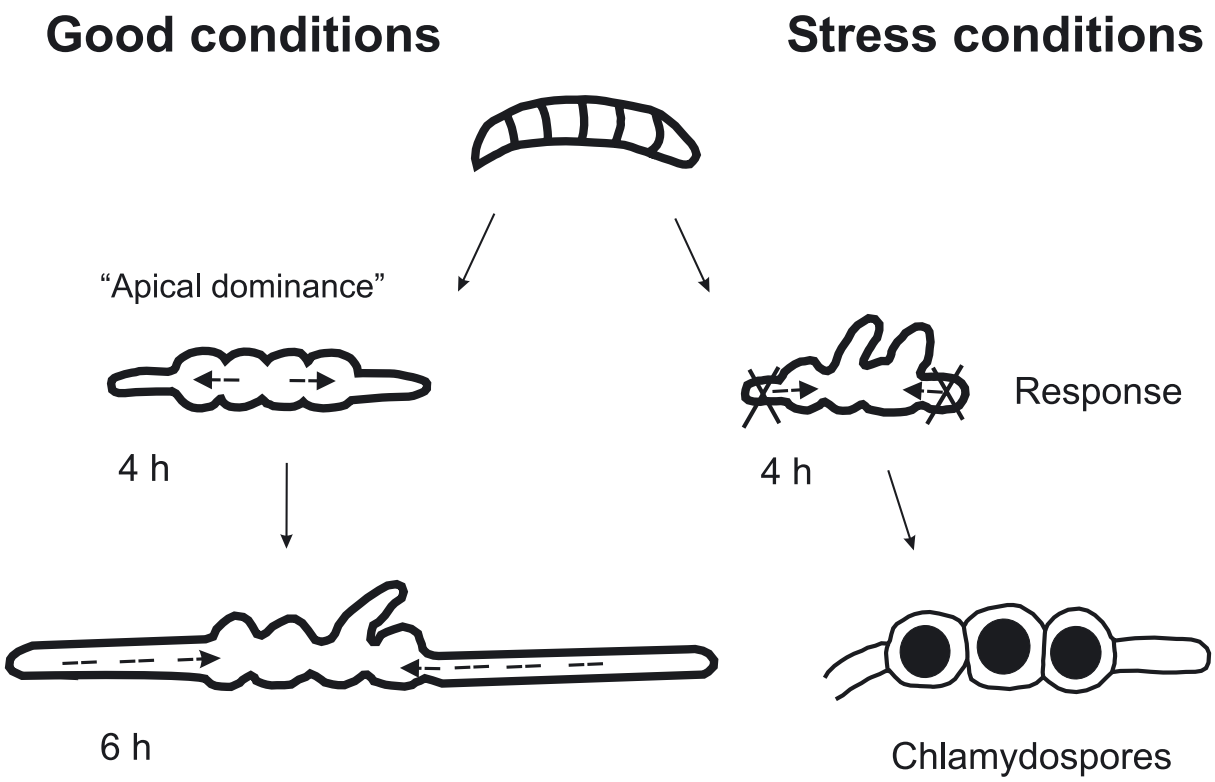


**Fig. 1** Germination of multicompartment *Fusarim culmorum* macroconidia in the absence and presence of 0.3 µg/ml of nystatin.

Overall, the shift of the germ tube formation shows that multicellular conidia are more versatile than unicellular conidia in their response to adverse conditions, which may enhance their survival chances (Chapter 2).

**APPLICATIONS AND PERSPECTIVES**

A fungal self-inhibitor is described in this thesis. Here we discuss the possibilities for practical application of these (volatile) antifungals. The first aim of this research was to detect and identify new/natural compounds with antifungal activity. The iturin-like compound produced by *Bacillus subtilis* YM 10-20 identified here was shown to be effective against spores. This compound may find potential application in agriculture as an inhibitor of fungal

plant pathogens. For example, iturins inhibit *Monilia fructicola* that causes brown rot disease in peaches (11).

*1-Octen-3-ol formation*

Mechanistic insight in self-inhibitor action of may be used to develop new compounds to control fungal spoilage of foods and crops. Further knowledge may be obtained from a deepening insight in the formation of 1-octen-3-ol (Fig.2). It is assumed that 1-octen-3-ol is a product of the enzymatic breakdown of linoleic acid by lipoxygenase and hydroperoxide lyase.

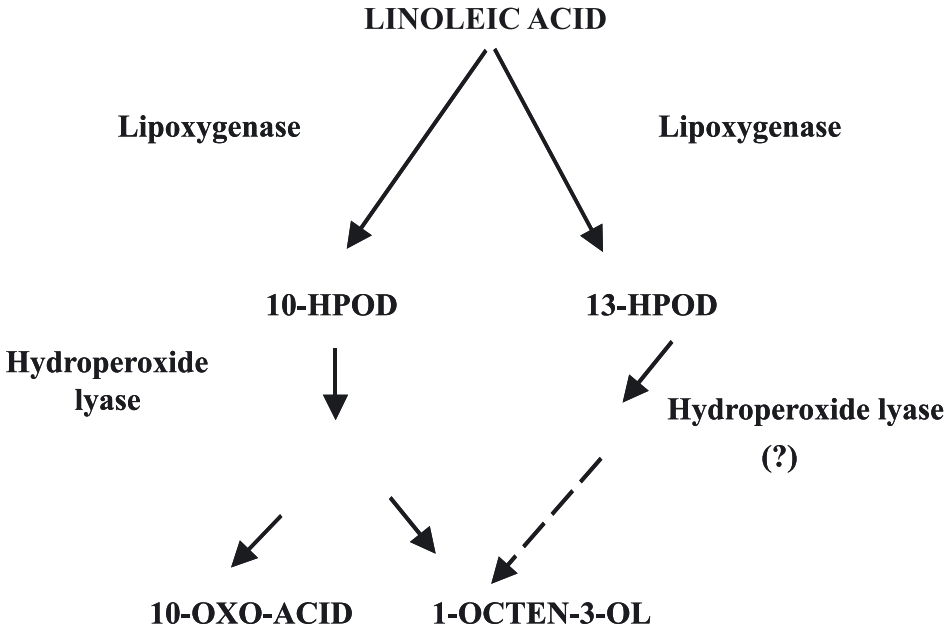


**Fig 2**: Proposed pathways for the formation of 1-octen-3-ol (11, 17).

The scheme shows two possible pathways for the oxidation of linoleic acid leading to 1-octen-3-ol: in *Pleurotus pulmonarius*, linoleic acid is oxidised to the hydroperoxy acids: 10-HPOD (10-s-hydroperoxy-trans-8,cis-12-octadecadienoic acid) and 13-HPOD (13-L-hydroperoxy-cis-9,trans-11-octadecadienoic acid). 10-HPOD can be split by hydroperoxide lyase into 1-octen-3-ol and 10-oxo-trans-8-decanoic acid (10-oxo acid, ODA) as proposed by Assaf et. al (1) . Another pathway is proposed by Kuribayashi et al (14), in which linoleic acid is oxidised into 10-HPOD and 13-HPOD that are involved in the conversion to 1-octen-3-ol, in *Pleurotus ostreatus*. Both lipoxygenase and hydroperoxide lyase are assumed to play a crucial role in the synthesis. Studies on lipoxygenases (LOX) in plants and animals have been reported, but less is known about fungal lipoxygenase, although at least five fungal lipoxygenases have been purified and characterized (16).

Together with 1-octen-3-ol, a non-volatile metabolite, 10-oxo-trans-8-decenoic acid (ODA) is formed during the oxidative breakdown of linoleic acid by lipoxygenase(s) in fungi. ODA is considered to affect growth in mushrooms influencing the development of fungal structures in the mushroom. It stimulates growth of the mycelium, stipe elongation, and fruiting initiation during mushroom development. Because of this, it is now recognized as a growth regulating substance (GRS) produced by gills (5, 19). The co-product 1-octen-3-ol is supposed to play a role in inhibition of mycelial growth and spore germination and thus also act as regulator of fungal development. 1-Octen-3-ol was shown to induce microcycle conidiation, indicating an additional effect on the developmental stages of fungi (Chapter 4). Could be of interest to study the effect of ODA without and with 1-octen-3-ol in fungal growth and differentiation of *P. paneum*. Certainly, more information on the possible role of lipoxygenase(s) and other enzymes, such as hydroperoxyde lyase and hydroperoxyde isomerase in the breakdown of linoleic acid in *Penicillium paneum* is needed, including the products formed from linoleic acid oxidation and the pathway involved in the formation of 1-octen-3-ol.

As a further research step we performed inhibition experiments to investigate the role of lipoxygenase(s) in 1-octen-3-ol formation. Three lipoxygenase activity peaks were detected in cell free extracts of conidia of *Penicillium paneum* (11µg of protein) (Figure 3). The highest activity was obtained at a pH 9.0, a second maximum was at pH of 6 and a third at pH 4.5. The activity of the enzyme(s) was completely inhibited in the presence of 300 µM of the antioxidant nordihydroguaiaretic acid (NDGA), which has been reported to inhibit lipoxygenase and hydroperoxide lyase, by converting the active Fe (III)-form of lipoxygenase into its inactive Fe (II)-form (27).

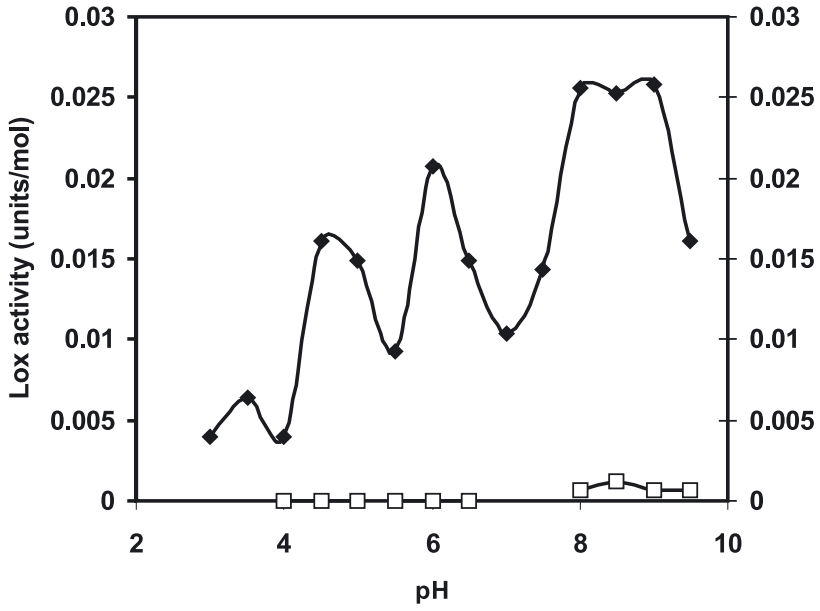


**Fig. 3** Effect of pH on lipoxygenase activity in cell extract of conidia from *Penicillium paneum* in the absence (♦) and in the presence ( ) of 300 µM of nordihydroguaiaretic acid (NDGA).

Future work should reveal whether blocking of lipoxygenase prevents formation of 1-octen-3-ol and subsequently loss of the self-inhibition phenomenon. A detailed understanding of the molecular events occurring during conidial germination could well lead to novel ways to control fungal spoilage. New research directions are emerging that will conceivably supply answers to solve basic questions regarding the fungal germination process and the control there of including the use of fungal self-inhibitors.

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# SUMMARY

Fungi can be found in a wide variety of environments, such as seeds, plants, soil, water, insects, and food products, both from plant and animal origins. Fungi cause severe economic losses and are potential hazards, because the infection or invasion process may result in plant diseases, spoilage of food and food products, production of mycotoxins and allergic reactions. On the other hand, fungi are indispensable in decay of organic matters and in the biotechnological production of e.g. organic acids, enzymes and antibiotics. In almost all cases, contamination of food and its products and infection of plants and animals is initiated by contact of the host with airborne conidia: in the host environment the conidia germinate. The germination process is a crucial step in fungal infection, which involves the initiation of biochemical activities, an increase of the metabolic rates, and morphological changes. The subsequent step is mycelial growth. There is a great interest in industrial, food industrial and medical areas to prevent and to control fungal contamination. For many years fungicides have been used to control pathogens in plants and seeds. Antibiotics that exhibit potential activity have been used against fungal infections in humans, animals and agricultural products. In order to prevent fungal spoilage in foods different practices are used such as heat, low oxygen pressure, low water activities and weak organic acids and esters, or a combination of these methods. New and emerging ways to control spoilage are based on the use of many of the antimicrobial compounds naturally produced by plants and by fungi. Plant antimicrobial compounds are amongst others the components of essential oils, and fungal antimicrobial compounds are the so called (self) inhibitors.

In this thesis the germination of fungal spores and the effects of antifungal compounds on this process are described taking *Penicillium paneum* conidia and *Fusarium culmorum* macroconidia as examples. In our work we used an antifungal compound produced by *Bacillus subtilis* as well as a newly discovered self-inhibitor of *Penicillium paneum.* We isolated and identified these compounds and described their mode of action.

In Chapter 2 the identification and characterization of an antifungal compound produced by *Bacillus subtilis* YM 10-20, which inhibits *Penicillium roqueforti* was described. The antifungal compound was isolated by acid precipitation with HCl. Identification by HPLC and mass spectrometry analysis showed it to be highly similar to iturin A. The iturins are cyclic lipopeptides characterised by the presence of seven α amino acids. Other lipopeptides that belong to the group of iturins are bacillomycins D, F, and L, and mycosubtilin. The iturin-like compound described in this thesis inhibited fungal germination and growth. Fluorescence probes in combination with flow cytometry and scanning electron microscopy were applied for assessing the mode of action of this antifungal compound against spores. Destruction and morphological changes in *P. roqueforti* conidia in the presence of the inhibitor were revealed. The iturin-like compound permeabilised fungal spore membranes and blocked germination.

In Chapter 3 the sequence of events in the germination process of multicompartment *Fusarium culmorum* macroconidia and the effects of antifungal compounds were investigated by measuring the intracellular pH ($pH_{in}$) employing fluorescence ratio imaging microscopy (FRIM). The macroconidia were labelled with the pH dependent probe 5(and-6)-carboxyfluorescein succinimidyl ester (cFDA-SE). An increase in the intracellular pH of macroconidia occurred simultaneously with their swelling. During swelling, the separate compartments of macroconidia showed different $pH_{in}$ values, however not statistically significant. Compartments that were forming a germ tube had statistically significant higher internal pH values than not germinating compartments. Notably, during germ tube formation of *F. culmorum* macroconidia, a clear differentiation between the compartments of the macroconidium was observed. Germ tubes develop preferably from apical cells and with low frequency from middle compartments, and compartments with newly formed germ tubes showed higher $pH_{in}$ values compared to other compartments. Addition of antifungals, nystatin and nonanoic acid during germination, affects $pH_{in}$ of the conidia and their differentiation. The pH gradients in the macroconidial compartments dissipated very fast in the presence of nystatin at levels of 1 and 4 µg/ml. At sublethal doses (0.3 µg/ml) of nystatin the apical compartments appeared to be preferentially targeted, resulting in lower $pH_{in}$ values in these compartments. Strikingly, the reduced germination capacity of apical compartments under these conditions was compensated by an increased germination capacity of middle compartments. This study provides evidence for differentiation inside macroconidia during early germination. The shift of the germ tube formation of *F. culmorum* towards the middle cells suggests that there is some form of communication between compartments of multicelled conidia, suggesting that the compartments of the conidium may not be separate cellular entities. Overall, the shift of the germ tube formation shows that multicellular conidia are more versatile than unicellular conidia in their response to adverse conditions, which may enhance their survival chances

In Chapter 4 the identification and characterization of a self-inhibitor produced by *P. paneum* was described. The heat stable compound was isolated by successive centrifugation of the supernatant obtained from spore suspensions of *P. paneum* with a density of $10^9$ conidia $ml^{-1}$. In static headspace gas chromatographic analysis two major peaks were distinguished: the highest production of these metabolites was after 22 h of incubation at 25ºC and shaking at 140 rpm. Gas chromatographic coupled with mass spectrometry analysis (GC-MS) revealed the volatile compounds to be 3-octanone and 1-octen-3ol. Notably, only the latter compound appeared to block the germination process at different developmental stages of the conidia (swelling and germ tube formation). 1-Octen-3-ol influenced different developmental processes during the *P. paneum* life cycle including induction of microcycle conidiation and spore germination. It inhibits growth of several fungal species indicating its broad action spectrum. The compound henceforth acts as a fungal hormone in the life cycle of the fungus.

In Chapter 5 the mode of action of the volatile germination self-inhibitor 1-octen-3-ol produced by *Penicillium paneum* was studied. Different target sites including membrane integrity, intracellular pH, respiration and protein synthesis were investigated. The diameter of conidia treated with 1-octen-3-ol (4 mM) for 4 h was similar to that of freshly harvest spores (5 µm). Inhibition of germination was associated with a small inhibitory effect on respiration. Exposure to 1-octen-3-ol revealed partial staining with PI and TOTO, a DNA fluorescent probes that cannot enter cells with an intact membrane. Slight permeabilization of the fungal membrane was observed. Furthermore, addition of 4mM of 1-octen-3-ol resulted in a transient dissipation of the pH gradient. Two dimensional gel electrophoresis analysis of protein patterns revealed striking differences between freshly harvested spores, germinated conidia and 1-octen-3-ol treated conidia. Firstly, twenty-three proteins that appeared in the germinated conidia were present in lower levels in the 1-octen-3-ol treated conidia. Secondly, nine proteins that were absent from the non-germinated conidia, were present during the germination (swelling and germ tube formation), yet were not found in conidia exposed to 1-octen-3-ol. Presumably, these proteins play a role in the germ tube formation, which is blocked upon exposure to 1-octen-3-ol. A final difference was that 12 new proteins appeared in 1-octen-3-ol treated conidia. This suggests that these new proteins are required for the adaptation in the presence of the inhibitor. However, this adaptation did not result in germ tube formation. Our results suggest that inhibition of spore germination by inhibitor 1-octen-3-ol involves multiple target sites. It has an effect on spore respiration, permeabilization of the membrane, perturbation of the intracellular pH and protein production. The nature of the proteins that were blocked and induced in 1-octen-3-ol treated conidia is unknown, but identification of these proteins may add further to our understanding of the action of 1-octen-3-ol and the fungal response to it.

In Chapter 6 an overview is presented of the main outcomes of this study, and the proposed pathways in the formation of 1-octen-3-ol are discussed. In addition, possible applications and perspectives are discussed in the prevention of fungal contamination and growth.

# SAMENVATTING

Schimmels zijn overal te vinden in de omgeving, bijvoorbeeld op zaden en planten, in grond en water, bij insecten en op voedingsmiddelen, zowel plantaardige als dierlijke. Schimmels veroorzaken grote economische verliezen en sommige zijn potentieel gevaarlijk, omdat ze, door infectie of invasie plantenziekten en voedselbederf kunnen veroorzaken. Ook produceren sommige schimmels mycotoxinen en kunnen ze allergische reacties opwekken. Overigens zijn schimmels ook onmisbaar voor de afbraak van organisch materiaal en bij de biotechnologische productie van bijvoorbeeld organische zuren, enzymen en antibiotica.

Vrijwel altijd wordt besmetting van voedsel en infectie van planten en dieren geïnitieerd door contact met sporen vanuit de atmosfeer waarna deze sporen ontkiemen. Het ontkiemingsproces is een kritische fase, waarin biochemische processen op gang komen, het metabolisme versneld wordt en morfologische veranderingen optreden. De volgende fase is groei van mycelium. Het voorkomen en beheersen van schimmelbesmetting is van groot industrieel en medisch belang. Jarenlang werden fungiciden gebruikt om schimmels bij planten en zaden te beheersen. Ook werden antibiotica gebruikt tegen schimmelinfecties bij mensen, dieren en landbouwproducten. Tegen schimmelbederf bij voedingsmiddelen werden nog verschillende andere werkwijzen gebruikt, zoals hittebehandeling, lage zuurstofspanning, lage wateractiviteit, zwakke organische zuren en hun esters, of combinaties van deze methoden. Nieuwe wegen om bederf te beheersen zijn gebaseerd op het gebruik van veel van de antimicrobiële stoffen die van nature door planten en schimmels worden geproduceerd. Plantaardige antimicrobiële stoffen zijn onder andere etherische oliën en bij schimmels zijn dat de zogenaamde (zelf)inhibitoren.

In dit proefschrift wordt de ontkieming van schimmelsporen en de effecten van antischimmel componenten op dit proces beschreven aan de hand van *Penicillium paneum* conidia en *Fusarium culmorum* macroconidia. We gebruikten een fungicide component geproduceerd door *Bacillus subtilis* en ook een door ons ontdekte zelfremmer van *P. paneum*. We isoleerden en identificeerden deze componenten en beschreven hun werkwijze.

In Hoofdstuk 2 beschrijven we de identificatie en de karakterisering van een stof geproduceerd door *B. subtilis* YM 10-20, die *Penicillium roqueforti* remt. Het fungicide werd geïsoleerd door zure precipitatie met HCl. Analyse met HPLC en massaspectrometrie toonde aan dat het een stof betrof die veel overeenkomst vertoont met iturine A. Iturines zijn cyclische lipopeptiden met zeven α-aminozuurresiduen. Tot deze groep van iturines behoren eveneens bacillomycine D, F en L en mycosubtiline. De iturine-achtige stof beschreven in dit proefschrift remt sporenkieming en groei. De werkzaamheid van deze component tegen sporen werd bestudeerd met behulp van fluorescente probes in combinatie met flow cytometrie en scanning elektronen microscopie. Structurele defecten en andere morfologische veranderingen werden geobserveerd bij *P. roqueforti* sporen in aanwezigheid van de remstof.

De iturine-achtige stof maakt de membraan van schimmelsporen doorlatend en blokkeert de ontkieming.

In Hoofdstuk 3 worden de opeenvolgende gebeurtenissen in het ontkiemingsproces van meercellige sporen (macroconidia) van *Fusarium culmorum* onderzocht, alsmede het effect van antischimmel componenten, door het meten van de intracellulaire pH ($pH_{in}$) met behulp van fluorescentie *ratio imaging* microscopie (FRIM). De macroconidia werden gelabeld met de pH-afhankelijke *probe* 5 (en 6)-carboxyfluoresceine succinimidyl ester (cFDS-SE). De intracellulaire pH nam gelijktijdig toe met de zwelling van de macroconidia. Gedurende het zwellen vertoonden de afzonderlijke geledingen (compartimenten, cellen) van macroconidia verschillende pH-waarden, echter deze verschillen waren niet statistisch significant. Geledingen die een kiembuis maakten hadden wel een statistisch significante hogere interne pH dan niet-ontkiemende cellen. Juist bij de kiembuisvorming van macroconidia van *F. culmorum* was er een duidelijk onderscheid tussen de compartimenten van de macroconidia. Kiembuizen ontstaan bij voorkeur bij apicale cellen en slechts in lage frequentie bij de binnencellen. Het gedurende de ontkieming toevoegen van schimmelremmende stoffen zoals nystatine en nonaanzuur heeft een effect op de $pH_{in}$ en de differentiatie van de conidia. De pH-gradient in de geledingen van de macroconidia dissipeerde zeer snel bij concentraties van nystatine van 1 tot 4 μg/ml. Bij subletale doses (0,3 μg/ml) worden bij voorkeur de apicale cellen getroffen, die dan een lagere $pH_{in}$-waarde vertonen. Het verminderde ontkiemingsvermogen van de apicale geledingen wordt onder deze omstandigheden opvallend gecompenseerd door een verhoogd ontkiemingsvermogen van de binnenste cellen. Deze studie duidt op differentiatie bij de cellen in macroconidia in de vroege ontkiemingsfase. De verschuiving van kiembuisvorming in macroconidia van *F. culmorum* naar de binnenste cellen suggereert dat er een of andere vorm van communicatie bestaat tussen de compartimenten van veelcellige conidia, en dat de compartimenten van de conidia niet onafhankelijk zijn van elkaar. Uiteindelijk laat het verschuiven van de kiembuisvorming zien dat meercellige conidia een groter aanpassingvermogen hebben dan eencellige, hetgeen hun overlevingskansen vergroot.

In Hoofdstuk 4 wordt de identificatie en de karakterisering beschreven van een zelfremmer geproduceerd door *P. paneum*. De hittestabiele stof werd geïsoleerd door herhaald centrifugeren van het supernatans verkregen van sporesuspensies van *P. paneum* met een concentratie van $10^9$ conidia per ml. Door *headspace* gaschromatografie konden twee belangrijke pieken worden onderscheiden. De maximale productie van deze metabolieten werd verkregen na 22 uur schuddend (140 toeren per minuut) incuberen bij 25°C. Met gekoppelde gaschromatografie-massaspectrometrie (GC-MS) kon worden aangetoond dat de vluchtige verbindingen 3-octanon en 1-octeen-3-ol waren. Alleen de laatste component bleek het ontkiemingsproces in verschillende stadia (zwelling van de conidia en kiembuisvorming) te blokkeren. 1-Octeen-3-ol had een effect op verschillende ontwikkelingsprocessen in de levenscyclus van *P. paneum*, waaronder de inductie van conidiavorming in een microcyclus

en spore-ontkieming. Het remt de groei van een aantal schimmelsoorten, hetgeen op een breed werkingsspectrum wijst. Deze stof werkt dus als een hormoon in de levenscyclus van de schimmels.

In Hoofdstuk 5 wordt de werkwijze bestudeerd van de vluchtige ontkiemingsremmer 1-octeen-3-ol, geproduceerd door *P. paneum*. Verschillende aangrijpingspunten, waaronder de membraan, de intracellulaire pH, de ademhaling en de eiwitsynthese werden onderzocht. De diameter van conidia, 4 uur lang behandeld met 1-octeen-3-ol (4 mM) kwam overeen met die van onbehandelde sporen (5 μm). Kiemremming ging gepaard met een geringe remming van de ademhaling. Door blootstelling aan 1-octeen-3-ol gingen de sporen gedeeltelijk fluoresceren met PI en TOTO, twee DNA-specifieke fluorescerende *probes* die in cellen met beschadigde membraan kunnen binnendringen. De sporenmembraan werd dus enigszins doorlatend. Bij 4 mM 1-octeen-3-ol trad er een tijdelijke dissipatie van de pH-gradiënt op. De eiwitpatronen lieten bij tweedimensionale gelelectroforese opvallende verschillen zien bij versgeoogste sporen, ontkiemende conidia en conidia behandeld met 1-octeen-3-ol. Ten eerste waren er 23 eiwitten in geringere mate aanwezig in de met 1-octeen-3-ol behandelde sporen dan in de verse sporen. Ten tweede waren er negen eiwitten te zien tijdens ontkieming (zwelling en kiembuisvorming) die er niet waren in ongekiemde sporen en ook niet in sporen behandeld met 1-octeen-3-ol. Waarschijnlijk spelen deze eiwitten een rol bij de kiembuisvorming, die door 1-octeen-3-ol geblokkeerd wordt. Een laatste verschil betrof 12 nieuwe eiwitten die in 1-octeen-3-ol behandelde conidia te voorschijn kwamen. Het lijkt er op dat deze nieuwe eiwitten nodig zijn voor aanpassing aan de remstof. Echter, deze aanpassing resulteerde niet in kiembuisvorming. Onze resultaten duiden er op dat er verschillende aangrijpingspunten zijn in de remming van sporenontkieming door de remstof 1-octeen-3-ol. Er is een effect op de ademhaling van sporen, de doorlatendheid van de membraan, de verstoring van de intracellulaire pH en de eiwitproductie. De aard van de eiwitten waarvan de synthese werd geremd en geïnduceerd in conidia behandeld met 1-octeen-3-ol, is onbekend. Identificatie van deze eiwitten kan leiden tot meer inzicht in de werking van 1-octeen-3-ol en de reactie daarop door de schimmel.

# Curriculum Vitae

**Gilma Silva Chitarra** was born on July 17, 1960 in Ribas do Rio Pardo, State of Mato Grosso do Sul (MS), Brazil. In 1978 she received her high-school diploma in Araçatuba, State of São Paulo (SP). In December 1984, she obtained her Bachelor Degree in Science in Agronomy the "Faculdade de Agronomia e Zootecnia Manoel Carlos Gonçalves" in Espírito Santo do Pinhal - SP. After that, she worked for privaties companies for six years. From 1992 to 1994, she worked at the Department of Phytopathology, Laboratory of Forest Pathology, at the Federal University of Viçosa (UFV), in Viçosa (MG), under supervision of Prof. dr. Acelino Couto Alfenas. Also in 1994, she started her Master Degree in Science of Phytopathology at the Federal University of Lavras (UFLA), in Lavras (MG), under supervision of Prof. dr. José da Cruz Machado. She was awarded with a master's degree in 1996. Between 1997 and 1999, she joined the Department of Agrotechnology and Food Sciences, Laboratory of Food Microbiology, of the Wageningen University (WUR), in Wageningen, The Netherlands, as part-time researcher. In August 1999, she was granded with a 4 year-fellowship by the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Brasil). She was a Ph.D fellow at the same Department of the Wageningen University, under supervision of dr. Tjakko Abee and Prof. dr. ir. F. M. Rombouts.

## List of publications

### Research papers

- **Chitarra, G.S., Breeuwer, P., Nout, M.J.R., van Aelst, A.C., Rombouts, F. M., and Abee, T.** 2003. An antifungal compound produced by *Bacillus subtilis* YM 10-20 inhibits germination of *Penicillium roqueforti* conidiospores. Journal of Applied Microbiology **94**: 159-166.
- **Chitarra, L.G., Machado, J.C., Chitarra, G.S. and Vieira, M.G.G.C.** 2002. Efeito do deslintamento químico de sementes de algodoeiro (*Gossypium hirsutum* L.) sobre o nível de ocorrência de *Colletotrichum gossypii* e desenvolvimento do fungo em exudado de sementes deslintadas. Fitopatologia Brasileira **27**: 128-133
- **Chitarra, G.S., Dijksterhuis, J., Breeuwer, P., Rombouts, F. M., and Abee, T.** Differentiation inside multicelled macroconidia of Fusarium culmorum during early germination. (Submitted)
- **Chitarra, G.S., Abee, T., Rombouts, F. M., Posthumus, M.A., and Dijksterhuis, J.** Germination of penicillium paneum conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. (Submitted)

- **Chitarra, G.S., Abee, T., Rombouts, F. M., and Dijksterhuis, J.** Mechanism of action of 1-octen-3-ol, a volatile self-inhibitor of spore germination in *Penicillium paneum*. (In preparation)

**Other scientific publications**

- **Chitarra, G.S., Dijksterhuis, J., Rombouts, F. M., and. Abee, T.** A volatile germination self-inhibitor of *Penicillium paneum*. Workshop I: Secondary metabolism at the 8th International Fungal Biology Conference 2002, 1st –5th December, Guanajuato, Gto, Mexico, p: 32. (Oral presentation).
- **Chitarra, G.S., Breeuwer, P., and Abee, T.** Assessment of intracellular pH of multicompartment Fusarium culmorum spores using image analysis. Scientific Spring Meeting 2002, "Fungi in food: Functional genomics & Physiology of food spoilage fungi" at the Congress in Papendal, Arnhen, The Netherlands, 9th –10th April. (Oral presentation).
- **Chitarra, G.S., Breeuwer, P., Dijksterhuis, J., Rombouts, F. M., and Abee, T.** Assessment of intracellular pH of multicompartment *Fusarium culmorum* spores using image analysis. Abstract book at the Symposium on Physiology of Yeasts and Filamentous Fungi 2001, 5th-8th July, Hindsgavl Castle, Denmark, p: 97. (Poster).
- **Chitarra, G.S., Breeuwer, P., Nout, M.J.R., Rombouts, F. M., and Abee, T.** Iturin A produced by *Bacillus subtilis* YM 10-20 permeabilises conidiospores of *Penicillium roqueforti.* Abstract book at the 7th International Fungal Biology Conference 1999, 22nd - 25th August, Groningen, The Netherlands, p: 45. (Poster).
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- **Passador, G.C.; Peters, I., Chitarra, G.S, and Alfenas, A.C.** Allozymes of genotypes of *Eucalyptus cloeziana* resistant to the rust (*Puccinia psidii*). Abstract book at the International Congress of Phytopathology, 1993, Montreal, Canada, p.181. (Poster).
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- **Nobrega, M.B.M**., **Peters, I., Chitarra, G.S. and Alfenas, A.C.** Análise de isoenzimas em doze espécies de *Eucaliptus.* **Revista Brasileira de Genética**, Ribeirão Preto, n.18, v.3, p.251, 1995. (Poster).

This work was carried out at Wageningen University, Department of Agrotechnology and Food Sciences, Laboratory of Food Microbiology, P.O. Box 8129, 6700 EV, Wageningen, The Netherlands.
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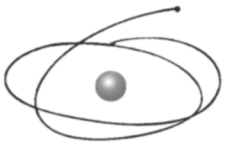

CAPES - Brasil

Gilma Silva Chitarra – Germination inhibitors of fungal spores: identification and mode of action – 2003

Front cover: Five pictures of fungal spores treated with antifungals. From top to bottom: SEM of *Penicillium paneum* conidia in the presence of *Bacilus subtilis* YM 10-20 supernatant (Chapter 2 and 4); fluorescence microscopy picture of *Penicillium paneum* conidia treated with iturin-like compound labelled with propidium iodide (Chapter 2 and 4), microcyclic conidiation of the primary conidiation of *Penicillium paneum* in the presence of extract compound collected at 22 h from *Penicillium paneum* conidia (Chapter 4), macroconidia of *Fusarium culmorum* in the presence of 0.25 mM nonanoic acid and macroconidia of *Fusarium culmorum* in the presence of 0.3 µg / ml nystatin (Chapter 3).