Dry bubble disease of the white button mushroom

Ecology and control of *Lecanicillium fungicola*

Roeland Lucas Berendsen

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Droge mollenziekte van de champignon

Ecologie en bestrijding van *Lecanicillium fungicola*
(met een samenvatting in het Nederlands)

Proefschrift

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Content

General introduction

Fungi are cultivated worldwide for the production of edible mushrooms. The common white button mushroom, *Agaricus bisporus* (Lange) Sing, represents 40% of the production (http://www.isms.biz/edibles.htm). The white button mushroom is low in fat and rich in fibre. Moreover, it contains vitamins, minerals, linoleic acid and its derivatives, and bioactive compounds such as anti-cancer polysaccharides. The white button mushroom is considered a good source of digestible proteins. It has a protein content higher than most vegetables and only somewhat less than most meat products and milk. In view of increasing demand of high quality food with an increasing world population, mushrooms will be an important source of proteins that can replace meat for a major part (Kurtzman, 1997; Chiu *et al.*, 2000; Wani *et al.*, 2010).
Although professional cultivation of mushroom species has a long history in Asia, cultivation of *A. bisporus* stems from France and was first described by Tournefort in 1707. He used old horse manure, on which *A. bisporus* had grown, as inoculum for the production of mushroom on fresh horse manure (Van Griensven, 1988). In the past century, cultivation of mushrooms in general and *A. bisporus* in particular has developed into an industry of economic importance. Especially the Netherlands became an important mushroom producing country. In 2008, the Netherlands was the largest mushroom producing country in Europe and the third largest in the world with an annual production of 240,000 tons, following China (1,608,219 tons per year) and the United States (363,560 tons per year) (http://faostat.fao.org).

CULTIVATION OF *A. BISPORUS*

The common white button mushroom is grown commercially on a composted mixture of horse manure, wheat straw, chicken manure and gypsum. The preparation of this substrate takes place in two phases. In the Netherlands, compost preparation takes place in bulk quantities on specialized composting factories. In phase I or composting phase, the main ingredients are mixed, wetted and regularly turned for three weeks during which easily degradable compounds are consumed by the microflora in the substrate. Due to uncontrolled self-heating during this phase, temperatures can rise to 75˚C. The second phase takes place in large aerated tunnels. Temperature is controlled in this phase and kept at 56-60˚C for the first 6 hrs (pasteurization of the compost) and subsequently lowered to 45 ˚C for 8- 9 days (conditioning of the compost). The composting process results in a substrate that is selective for *A. bisporus.* This selectivity is partly based on the chemical composition of the compost. The lignin-humus complex that is formed is resistant to degradation by most microorganisms. However, it can be decomposed by members of the Basidiomycota, among which *A. bisporus* (Gerrits, 1988). The composition of the microflora also aids in the selectivity of the substrate. Because of the high temperature during com-

posting, the mesophilic microflora in the compost is largely replaced by a thermophilic one. This latter microflora is inactivated at the end of the composting process, when the compost temperature is lowered. By introducing an excess of *A. bisporus* mycelium to the compost through the addition of spawn (millet grains colonized by the fungus), this basidiomycete has a head start over competing microorganisms in replacing the thermophilic compost microflora (Gerrits, 1988; Beyer, 2003). The thermophilic community in the compost is dominated by the fungus *Scytalidium thermophilum*. It was found that the linear growth rate of *A. bisporus* on sterilized compost is strongly stimulated if the compost was preinoculated with *S. thermophilum.* The growth promoting effect of *S. thermophilum* is further illustrated by the correlation between the mushroom yield *of A. bisporus* and the density of *S. thermophilum* in non-sterilized compost. *S. thermophillum* also protects *A. bisporus* against negative effects of bacteria that reside in the compost. It was therefore postulated that *S. thermophilum* provides compost selectivity (Straatsma *et al.*, 1989).
After spawning it takes about 14 to 17 days for *A. bisporus* to fully colonize the compost (Beyer, 2003; Gerrits, 1988). The colonized compost is delivered to the mushroom growers, loaded on shelves and covered with a 5 cm thick casing layer. The casing layer typically consists of peat mixed with lime and is a requirement for the formation of mushrooms. The mycelium colonizes the casing layer during the first 8-10 days (figure 1). Mushroom formation is subsequently stimulated by increasing the ventilation and lowering the temperature and relative humidity in the growth facility. In response to these changed conditions the vegetative hyphae start aggregating and form primordia, some of which will develop into mature mushrooms.

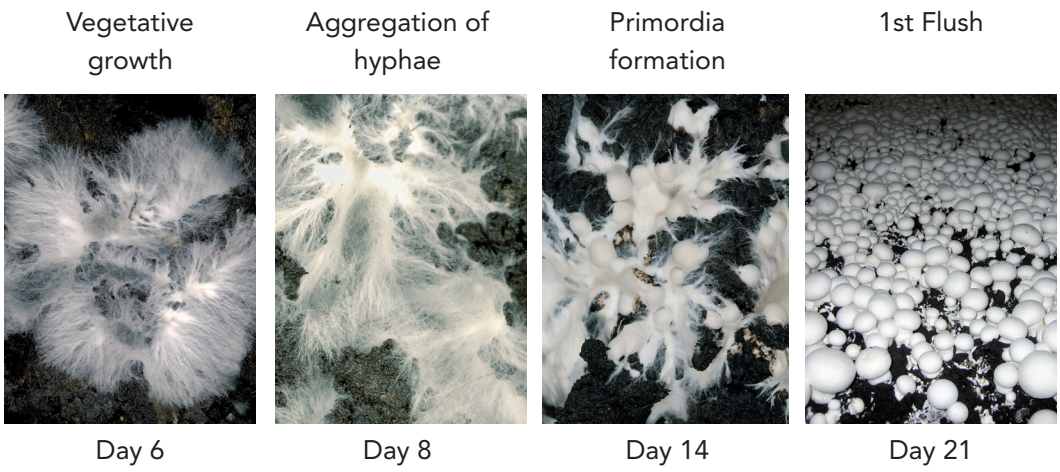

Figure 1. Colonization of the casing by *Agaricus bisporus* mycelium and subsequent development of mushrooms. Pictures: Hans van Pelt.

Initiation of mushroom formation is not well understood. Abiotic factors such as CO_2 concentration, temperature, and relative humidity are known to be of influence, but the casing microflora is thought to play a key role (Van Gils, 1988; Van Griensven, 1988). The microbial community and *Pseudomonas* spp. in particular, have been implicated in stimulation of fruiting body formation by degradation of a mushroom-formation-inhibiting factor (Grewal and Rainey, 1991; Miller *et al.*, 1995; Van Gils, 1988). *A. bisporus* fruiting body formation is inhibited in sterilized casing. It can be restored by addition of activated carbon (Eger, 1961) or by thorough ventilation (Noble *et al.*, 2009). These treatments have been suggested to remove inhibitors. The volatile 1-octen-3-ol may be such an inhibitor. This eight-carbon volatile is recognized as the typical mushroom smell and is produced in large quantities by *A. bisporus* (Combet *et al.*, 2009). It is known to inhibit mushroom formation. Of interest, 1-octen-3-ol is consumed by the casing microflora, which would explain how these microbes initiate mushroom initiation (Noble *et al.*, 2009).
The first mushrooms of a crop are picked 17 to 24 days after application of the casing layer and new flushes appear at 6 to 12 days intervals. The first two flushes are most productive. The third flush will typically produce half the quantity of mushrooms produced in one of the first two flushes and subsequent flushes will produce progressively less mushrooms. Commercial mushroom growers harvest only the first 2-4 flushes as subsequent flushes are considered uneconomic. Mushrooms are affected by a wide range of pests and pathogens that usually increase with each flush. There is a trend to take only two flushes, which helps to prevent epidemics and to keep pathogen levels on farms low (Beyer, 2003; Fletcher and Gaze, 2008).

DRY BUBBLE DISEASE

As in agricultural crops, the commercial production of mushrooms is hampered by a variety of organisms that negatively affect crop yield. The most commonly encountered biotic disturbances include insect pests, mites, nematodes, pathogenic fungi, antagonistic fungi, and viruses (Fletcher and Gaze, 2008). Pests and pathogens are problematic in all stages of mushroom crop development and can affect the colonization of compost by *A. bisporus*, the formation and quality of its mushrooms and their post-harvest deterioration. Most pests and pathogens can be managed through measures of hygiene that aim to exclude pathogens from the farm or contain them when already present. Nonetheless, some pathogens remain problematic (Largeteau and Savoie, 2010). Especially *Lecanicillium fungicola* causes severe infections that result in significant crop losses (Chapter 2; Berendsen *et al.*, 2010). *L. fungicola* is a mycoparasite that attacks *A. bisporus* during its generative period. The symptoms of infection depend on the developmental stage at which *A. bisporus* becomes infected (Holmes, 1971; North and Wuest, 1993). Irregularly

shaped, light brown necrotic lesions are found on mushrooms that are infected relatively late in their development. On the other hand, the characteristic malformed mushrooms, known as dry bubble, are formed when the infection occurs at early stages of mushroom development.

The impact of *L. fungicola* is expected to increase significantly in the near future. To date chemicals are used to protect against pathogenic fungi. Not many fungicides can be used for the control of dry bubble disease as often also the mushroom mycelium is affected. The few fungicides that have been used are either no longer available or are becoming increasingly ineffective because of the development of fungicide resistance in the pathogen (Bollen and van Zaayen, 1975; Fletcher and Yarham, 1976; Gea *et al.*, 2003; Wuest *et al.*, 1974). The only fungicide that is still effective and does not severely affect mushroom yield is Sporgon (active compound: prochloraz-manganese). However, sensitivity of *L. fungicola* to Sporgon has decreased and therefore increasing concentrations of the fungicide are necessary to effectively control dry bubble disease (Gea *et al.*, 2005; Grogan, 2008; J. Baars, Plant Research International (PRI), personal communication). Moreover, it is expected that in the future Sporgon and other fungicides will be banned from commercial mushroom growing. Development of alternative control measures is therefore urgently needed. A thorough understanding of the pathogen is indispensable for the development of such measures. So far, information about the parasitic interaction is limited (Chapter 2; Berendsen *et al.*, 2010). It has been suggested that *L. fungicola* can only infect the fruiting bodies of *A. bisporus* and not the vegetative mycelium (Bernardo *et al.*, 2004; Calonje *et al.*, 1997; Calonje *et al.*, 2000a). As a consequence, infection of mushroom cultures by *L. fungicola* must take place in the casing.

Intriguingly, 1-octen-3-ol, the compound that is involved in self-inhibition of mushroom formation (Noble *et al.*, 2009) was also found to inhibit the spore germination of several ascomycetes (Chitarra *et al.*, 2005; Chitarra *et al.*, 2004). Likely, *L. fungicola* spores are also sensitive to 1-octen-3-ol. If so, the decrease in 1-octen-3-ol levels in the casing that initiates *A. bisporus* fruiting body formation could simultaneously signal the presence of infectable stages of *A. bisporus* to the pathogen. Nonetheless, even without *A. bisporus* or 1-octen-3-ol, germination of spores of *L. fungicola* is inhibited in the casing, but not when the casing is sterilized (Cross and Jacobs, 1968). This implies that the casing microflora is responsible for this inhibition. This sensitivity of *L. fungicola* to microbial antagonism could be used to control dry bubble disease. Another interesting observation is that spores of *L. fungicola* do readily germinate in the casing once it has been colonized by *A. bisporus* (Cross and Jacobs, 1968; Thapa and Jandaik, 1987b). This indicates that *A. bisporus* releases a component(s) that induces germination of spores of the pathogen. Such a component could also be a target to develop effective control of the pathogen.

BIOLOGICAL CONTROL

Antagonistic microorganisms in soils have been exploited for effective control of fungal plant pathogens and this has resulted in a number of commercial products (Fravel, 2005; Weller *et al.*, 2002). Similarly, biological control of dry bubble disease could prove effective and this would support the mushroom industry's efforts to minimize the use of chemicals. Biocontrol bacteria that are introduced into a substrate (e.g. the soil) have to compete with the indigenous microflora. Often, the abundance of the introduced biocontrol bacterium decreases in time, resulting in reduced protection against the pathogen (Mazzola *et al.*, 1992; Raaijmakers *et al.*, 1995; Lugtenberg and Dekkers, 1999). Thus, the screening for biocontrol agents for the dry bubble disease should focus on microorganisms that naturally occur in the casing soil, and preferably are associated with *A. bisporus*. Fluorescent *Pseudomonas* spp. form a dominant group within the bacterial casing microflora. The relative population densities of these pseudomonads increase when the casing is colonized by *A. bisporus* (Doores *et al.*, 1986; Samson, 1986; Miller *et al.*, 1995; Fermor *et al.*, 2000; Pardo *et al.*, 2002). *Pseudomonas* species have many traits that make them effective biocontrol agents of plant pathogens (Weller, 2007). These traits also make them interesting candidates for biocontrol of *L. fungicola*. Pseudomonads can grow on a wide variety of substrates, are abundant in nature, have a high growth rate, can grow at relatively low temperatures and have a variety of mechanisms to antagonize other microorganisms. Mechanisms that are involved in plant disease suppression by these bacteria have been studied in detail over the last decades and include consumption of pathogen stimulatory compounds (Van Dijk and Nelson, 2000), siderophore-mediated competition for iron (Duijff *et al.*, 1999), and production of antifungal compounds (Chin-A-Woeng *et al.*, 2003; Haas and Defago, 2005) or lytic enzymes (Shapira *et al.*, 1989). All these modes of action may also be effective to control *L. fungicola* infections. Increased competition for iron in the casing could suppress *Lecanicillium* while the mushroom, capable of transporting nutrients from the compost, would likely be unharmed. Since *L. fungicola* and *A. bisporus* belong to different phyla (the Ascomycota and Basidiomycota, respectively) that diverged a few hundred million years ago (Berbee and Taylor, 2006), it is reasonable to expect that anti-fungals or lytic enzymes produced by certain biocontrol bacteria inhibit the pathogen but leave the host unaffected.

INDUCED RESISTANCE

Besides direct effects on pathogens, many biocontrol agents elicit induced systemic resistance (ISR) in plants (Bakker *et al.*, 2007; Van Wees *et al.*, 2008). Induced resistance is a state in which plant defense is potentiated against a wide range of pathogens. As mentioned, induced resistance can be triggered by the presence of beneficial microorgan-

isms, but can also be triggered by pathogens themselves (Durrant and Dong, 2004). After detection of a pathogen, defense is enhanced at the site of attack but also in distant plant parts. In this way, plants acquire a systemic resistance (systemic acquired resistance (SAR)) against subsequent attack. Moreover, wounding of plant tissue, such as occurs when plants are fed upon by insect herbivores, can similarly result in the systemic potentiation of defenses and is known as wound-induced resistance (Van der Ent *et al.*, 2009). The immune systems of animals can also adapt to a first attack of a pathogen. As a consequence, it will respond faster and stronger upon a second attack. This phenomenon is the basis for vaccination and, in humans and other vertebrate animals, typically involves an adaptive immune system that allows for recognition and memory of specific pathogens (Medzhitov and Janeway Jr, 1998). For mushrooms not much is known about defense against pathogens or pests (Largeteau and Savoie, 2010; Chapter 2). Moreover, systemic responses such as ISR or SAR have not been investigated. Nonetheless, the presence of functionally similar systems of potentiated responses to secondary attack in both plant and animals indicate that there is a strong adaptive advantage for such mechanisms. Uncovering induced resistance in mushrooms would allow for the development of new disease management strategies.

OUTLINE OF THIS THESIS

Dry bubble disease is a persistent problem in the cultivation of the white button mushroom *A. bisporus*. There is a pressing need for innovative ways to control spread and development of *L. fungicola* in mushroom cultivation as currently disease management relies heavily on one chemical (Sporgon) for which a reduced sensitivity of the pathogen has been reported. The research described in this thesis aims to supply targets for such innovative ways.

In order to develop effective control of *L. fungicola*, a thorough understanding of its ecology is crucial. Therefore in **chapter 2**, current knowledge about this mycopathogen is reviewed. The ecology of the pathogen is discussed with emphasis on host range, dispersal and primary source of infection. In addition, insights in the infection process and mushroom defense mechanisms are reviewed.

In **chapter 3**, the ecology of *L. fungicola* in the casing is investigated. It was found that *Lecanicillium* spores remain dormant until *A. bisporus* colonizes the casing. It was shown that the casing microflora is involved in this dormancy and mechanisms were investigated. It appears that the casing microflora produce antifungal compounds that impose a nutrient dependency on *L. fungicola* spores that otherwise germinate independent of nutrients.

Chapter 4 describes the search for antagonists that effectively suppress dry bubble disease. Possible mechanisms of antagonisms towards *L. fungicola* were investigated *in vitro* using well characterized *Pseudomonas* strains. Subsequently, a collection of bacteria that were isolated from colonized casing was screened for *in vitro* antagonism. *In vitro*, *L. fungicola* was inhibited by certain isolates through competition for iron and antibiosis. However, these isolates could not effectively suppress dry bubble disease. We conclude that biological control of dry bubble disease is not feasible.

Control of plant pathogens often functions not only through direct antagonism of the biocontrol agent on the pathogen, but also through induction of systemic resistance in plants. Induced resistance in both plants and animals is mostly triggered upon pathogenic attack. In **chapter 5**, it is shown that mushrooms of *A. bisporus* do not exhibit induced systemic resistance upon attack by *L. fungicola*.

In **chapter 6** application of 1-octen-3-ol to control dry bubble disease is investigated. It was found that 1-octen-3-ol treatment reduced dry bubble disease in inoculated mushroom cultures to levels resembling Sporgon treatment.

In **chapter 7** the results described in this thesis are discussed in view of possibilities to control dry bubble disease.

Pathogen profile: *Lecanicillium fungicola*, causal agent of dry bubble disease in white button mushroom

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SUMMARY

Lecanicillium fungicola causes dry bubble disease in commercially cultivated mushroom. This review summarizes current knowledge on the biology of the pathogen and the interaction between the pathogen and it's most important host, the white button mushroom, *Agaricus bisporus*. The ecology of the pathogen is discussed with emphasis on host-range, dispersal and primary source of infection. Also, current knowledge on mushroom defense mechanisms is reviewed.

Taxonomy: *Lecanicillium fungicola* (Preuss) Zare and Gams: kingdom fungi, phylum Ascomycota, subphylum Pezizomycotina, class Sordariomycetes, subclass Hypocreales, order Hypocreomycetidae, family Cordycipitaceae, genus *Lecanicillium*

Host range: *Agaricus bisporus*, *Agaricus bitorquis* and *Pleurotus ostreatus*. Although its pathogenicity for other species has not been established, it has been isolated from numerous other basidiomycetes.

Disease symptoms: Disease symptoms vary from small necrotic lesions on the caps of the fruiting bodies to partially deformed fruiting bodies called stipe blow-out or totally deformed and undifferentiated masses of mushroom tissue, dry bubble. The disease symptoms and severity depend on the time point of infection. Small necrotic lesions result from late infections on the fruiting bodies, whereas stipe blow-out and dry bubble are the result of interactions between the pathogen and the host in the casing layer.

Economic importance: *Lecanicillium fungicola* is a devastating pathogen in the mushroom industry and it causes significant losses in the commercial production of its main host, *Agaricus bisporus*. Annual costs for mushroom growers are estimated at 2 - 4% of the total revenue. Reports on the disease originate mainly from North-America and Europe. Although China is the main producer of white button mushrooms in the world, little is known in the international literature about the impact of dry bubble disease in this region.

Control: Control of *L. fungicola* relies on strict hygiene and the use of fungicides. Few chemicals can be used for control of dry bubbles because the host is also sensitive to fungicides. Notably, development of resistance of *L. fungicola* has been reported against the fungicides that are used to control dry bubble disease. In addition, some of these fungicides may be banned in the near future.

Useful websites:
http://www.mycobank.org
http://www.isms.biz
http://www.cbs.knaw.nl

INTRODUCTION

Lecanicillium fungicola (Preuss) Zare and Gams (synonyms: *Verticillium fungicola* (Preuss) Hassebrauk, *Verticillium malthousei* (Preuss) Ware) is the causal agent of dry bubble disease, which represents one of the biggest problems in the commercial production of the white button mushroom, *Agaricus bisporus*. Upon infection, *L. fungicola* can cause symptoms that range from small necrotic lesions on fruiting bodies to partial disruption of tissue in the stipe and cap causing stipe blow-out, or totally deformed and undifferentiated masses of mushroom tissue, the so called dry bubbles. As diseased mushrooms are unmarketable, infection by *L. fungicola* leads to significant losses in yield. The genus *Lecanicillium* consists of hyaline, phialidic hyphomycetes and contains both entomogenous and fungicolous species. *L. fungicola* can be distinguished by erect distinct conidiophores with very unequally sized conidia aggregated in large, slimy globose heads. Conidophores are verticilate with 2-5 whorls of 3-7 phialides (Zare and Gams, 2008).
Although *L. fungicola* has been shown to infect other basidiomycetes (see below), *A. bisporus* is considered its main host. Worldwide, 40% of the commercially produced mushrooms belong to this species (http://www.isms.biz/edibles.htm). *A. bisporus* is generally grown on a composted mixture of straw and manure. When this compost is fully colonized by *A. bisporus,* it is covered with a casing layer. This casing layer instigates the formation of fruiting bodies and typically an alkalized peat soil is used (Visscher, 1988).
Control of *L. fungicola* relies on strict hygiene and the use of fungicides. Few chemicals can be used for control of dry bubbles since the host is also negatively affected by many fungicides. The few fungicides that have been used are either no longer available or are becoming increasingly ineffective because of the development of fungicide resistance in the pathogen (Wuest *et al.*, 1974; Bollen and van Zaayen, 1975; Fletcher and Yarham, 1976; Gea *et al.*, 2005). Currently, control of dry bubble disease relies heavily on the use of prochloraz-manganese (i.e. Sporgon), but reduced sensitivity to this fungicide has been reported (Gea *et al.*, 2005; Grogan, 2008).

GENETIC DIVERSITY

Dry bubble disease was reported first by Constantin and Dufour (1892) who described all bubble diseases then known and referred to it as "la môle" disease. Derivations are

still used to name dry bubble disease in French, Spanish, German and Dutch ("môle sèche", "mole seca", "trockene Molle" and "droge mol", respectively). The word Môle was presumably derived from the latin word "moles" for "mass". Constantin and Dufour suggested that all bubble diseases were caused by one fungus, *Hypomyces perniciosae*, which could appear in different forms; one bearing two types of spores: a chlamydospore and big *Verticillium*-like conidia and a second form bearing only small *Verticillium*-like conidia.

In 1924, Smith distinguished dry bubble from wet bubble disease and described two different fungi as causal agents. Smith proposed the name *Cephalosporium constantinii* for the fungus that resembled the *Verticillium*-like fungus with small conidia and that caused dry bubble disease. Ware (1933) also described dry bubble disease and named the causal agent *Verticillium malthousei*, assuming the isolated species was not *Cephalosporium constantinii*, but similar to a fungus described in 1901 by Malthouse. Also in 1901, but independently, Preuss isolated and described a fungus from the cap of an unidentified mushroom, and named it *Acrostalagmus fungicola* (Gams, 1971). Hassebrauk (1936) isolated a similar fungus from *Puccinia corofinera* and renamed it *Verticillium fungicola*.

According to Gams (1971) *Verticillium fungicola, Cephalosporium constaninii* and *Verticillium malthousei* belong to the same species. Gams and Van Zaayen (1982) distinguished three varieties of *Verticillium fungicola*: var. *fungicola,* var. *aleophilum* and var. *flavidum*. Recently, it was concluded that based on ITS-region and SSU rDNA sequences, *V. fungicola* was more closely related to the often insect-pathogenic species of the genus *Lecanicillium,* than to the plant-pathogenic species of the genus *Verticillium* (Zare and Gams, 2008). *Verticillium fungicola* and its varieties *fungicola* and *aleophilum* were therefore renamed *Lecanicillium fungicola*. *Verticillium fungicola* var. *flavidum* was redefined as a separate species: *Lecanicillium flavidum*. The latter species differs from *L. fungicola* in ITS-sequence, in its optimum and maximum temperatures for growth, and morphologically in repeated branching of its conidiophores (Zare and Gams, 2008). The two remaining varieties of *L. fungicola* differ from each other mainly in a higher growth rate at 24˚C and a higher maximum temperature for growth for var. *aleophilum*. As a consequence, on *A. bitorquis,* which grows at a higher temperature than *A. bisporus*, var. *aleophilum* is mostly found, although both *L. fungicola* varieties can infect both species of *Agaricus* (Gea *et al*., 2003; Zare and Gams, 2008). In general, it is var. *aleophilum* that affects crop in Canada and the USA, while, in Europe, var. *fungicola* is the main causal agent of the disease (Collopy *et al*., 2001; Largeteau *et al*., 2004a). Bonnen and Hopkins (1997) studied morphology, virulence, fungicide resistance and RAPD grouping of a large collection of *L. fungicola* var. *aleophilum* isolates. It was shown that, although initial isolates from the U.S. were genotypically and phenotypically diverse, more recent isolates were much more similar. Based on RAPD and AFLP, it was concluded that European isolates of var.

fungicola are also genetically homogenous, although some polymorphisms exist and the population was less homogenous than var. *aleophilum* (Largeteau *et al.*, 2006). In this study, three French isolates appeared more polymorphous than a group of 15 isolates collected over a period of 27 years in the Netherlands, France and the U.K., likely due to the diverging culture conditions of some French growers. Genetic homogeneity is most likely linked to common culture practices, including the casing material used (peat moss) and selection pressure due to fungicide use (Bonnen and Hopkins, 1997; Largeteau *et al.*, 2006). The origin of the development of pseudoclones in America and Europe remains unsolved. Juarez del Carmen *et al.* (2002) concluded that the two varieties might be regarded as geographically isolated pathotypes.

HOST RANGE

L. fungicola can infect mushrooms other than *A. bisporus* and *A. bitorquis.* The parasitic fungus has been isolated from *Pleurotus ostreatus*. Upon inoculation of healthy *P. ostreatus*, *A. bitorquis*, and *A. bisporus*, these isolates caused disease and could be reisolated, thereby fulfilling Koch's postulates (Marlowe, 1982; Gea *et al.*, 2003). *L. fungicola* has also been mentioned as a pathogen of *Coltricha perennis* and *Pleurotus sapidus* (Marlowe, 1982). In addition, *L. fungicola* has been isolated from the basidiomycetes *Marasmiellus ramealis, Thelephora terrestris, Henningsomyces candidus, Hypholoma capnoides* and *Laccaria laccata* (Gams *et al.*, 2004; Zare and Gams, 2008). Although its pathogenicity to these mushroom forming basidiomycetes has not been demonstrated experimentally, its presence on their sporocarps makes it plausible that *L. fungicola* can infect a range of mushroom species. However, *L. fungicola* is not often found on wild mushroom and the *Telephora terrestris* samples used by Zare and Gams (2008) were decaying, indicating that *L. fungicola* does not have a wide host-range and might more often infect already decaying mushroom.

Since *L. fungicola* is closely related to a variety of insect pathogens, it has been suggested that it is able to infect insects (Collopy *et al.*, 2001; Yokoyama *et al.*, 2006; Amey *et al.*, 2007). The closely related *Lecanicillium psalliotae* was originally described as a mushroom pathogen (Treschow, 1941), but is now reported more often for its ability to infect nematodes (e.g. Pirali-Kheirabadi *et al.*, 2007). If *L.fungicola* is pathogenic to mushroom pests, such as the phorid fly *Megaselia herata* or the nematode *Ditylechus myceliophagus*, this would have significant consequences for our understanding of the pathogen's ecology. Yet, experimental evidence for *L. fungicola* being a pathogen of insects is lacking. Bidochka *et al.* (1999b) found that two isolates of *L. fungicola* were not pathogenic to *Galleria mellonella* larvae, even though one of the two isolates came

from *Lymantria dispar* larvae (gypsy moth) and the lytic enzyme activity was similar to the activity of related insect pathogens.

DISPERSAL

Already in 1933, Ware observed that dry bubble disease was associated with the presence of insects. Mites and springtails got stuck on dry bubbles, because their movement was impeded by mucilage and globules of spores adhering to their legs. Cross and Jacobs (1968) showed that a mixed population of *Megasalia halterata* and *Leptocera heteroneura* were effective in spreading spores over agar surfaces and that mixed fly populations from an infected farm could effectively transmit dry bubble disease. The vector competency of mushroom sciarid flies was suggested to depend on tibia morphology (Shamshad *et al*., 2009a). Air collected on a mushroom farm did contain spores of *L. fungicola* (Wong and Preece, 1987). However, wind does not seem to be important for spore dispersal as no effective dispersal was observed at wind speeds up to 10.75 m/s (Cross and Jacobs, 1968). Dispersal by splashing water was found to be very effective (Cross and Jacobs, 1968) and also dispersal by employees and equipment are reported to be important (Fekete, 1967; Wong and Preece, 1987). White (1981) showed that the initial percentage of dry bubbles in the first break of mushrooms was correlated to the initial population density of flies carrying *L. fungicola* spores, but the exponential spread of the disease in subsequent breaks was mainly due to watering.

PRIMARY SOURCE OF INFECTION

Spores of *Lecanicillium* remain viable for more than a year in soil (Cross and Jacobs, 1968), and if present on a mushroom farm, *L. fungicola* spores can survive for 7-8 months under dry conditions (Fekete, 1967). Therefore, once *L. fungicola* has occurred on a farm, there is likely to be a reservoir of inoculum on the site and this inoculum will serve as a source of infection for following crops as a result of poor hygiene or wind-blown dust and soil. However, the primary source of dry bubble infections is still under debate.

It is unlikely that *L. fungicola* is present in the compost delivered to the farms, since the spores die at 40˚C and will thus not survive the composting process where temperatures reach at least 70 - 80˚C (Gerrits, 1988). Because of this, it was mentioned already early on that the casing was more likely to be a source of infection (Ware, 1933). In support of this, Wong and Preece (1987) detected *L. fungicola* spores in 10% of the arriving peat batches on a British mushroom farm over a period of three years. However, it is not likely that *L. fungicola* proliferates saprophytically in peat, as its growth is inhibited by the mi-

crobial community in this substrate (Cross and Jacobs, 1967). Moreover, the anaerobic conditions and the low pH are unfavorable for *L. fungicola*. In the U.K. in 1988, surface peat was used mostly (Visscher, 1988) and *L. fungicola* may survive on basidomycete species that grow in and on such peat. Nowadays, in commercial farms, black peat is mostly used, which is taken from lower peat layers that seem to be a less likely habitat for *L. fungicola*. Indeed, replacing casing mixtures of clay, loam and humus by mixtures of sphagnum peat, sand and carbonate resulted in a considerable reduction of *Verticillium* incidence in the mushroom industry of Denmark (Bech and Riber-Rasmussen, 1967). Infected mushrooms, either in the wild or growing on adjacent mushroom farms, could be an important source of primary inoculation (Gams *et al.*, 2004; Zare and Gams, 2008). *Megaselia halterata* phorid flies are attracted by compost colonized by *A. bisporus* (Tibbles *et al.*, 2005) and during the filling of mushroom production cells indeed large numbers of flies are present (J. Hooijmans, mushroom grower, Kerkdriel, the Netherlands, personal communication). These flies may carry *L. fungicola* spores from external infections and thus act as the primary source. Contaminated equipment may also be a source of infection. An outbreak of dry bubble disease in 2004 on a Mexican mushroom farm was caused by the European *L. fungicola* variety *fungicola*, which is generally not found in North-America. It was suggested that this variety was introduced to the North-American continent through the import of materials or machines from Europe (Largeteau *et al.*, 2004a).

ECOLOGY OF *L. FUNGICOLA* IN THE CASING

Infection by *L. fungicola* most likely takes place in the casing, as it appears that *L. fungicola* cannot infect *A. bisporus* vegetative mycelium in the compost (Cross and Jacobs, 1968; Calonje *et al.*, 2000a; Bernardo *et al.*, 2004). In the casing, *L. fungicola* spores do not immediately germinate. Cross and Jacobs (1968) found that in natural soil and peat most spores had not germinated after 7 days, and the few germinated spores had short germ tubes. In sterilized soil and peat, however, the spores readily germinated and after 7 days extensive mycelium and sporulation was visible. The phenomenon that germination and growth of fungal propagules is inhibited by active soil microorganisms is general for most soils and is known as soil fungistasis (Lockwood and Filonow, 1981). Cross and Jacobs (1968) suggested that germination of *L. fungicola* spores requires an external nutrient source. In casing, *L. fungicola* spores did not germinate except in the immediate vicinity of *Agaricus* hyphae. After germination, the pathogen grew alongside the hyphae of *Agaricus*. These results suggest that nutrients leaking from *Agaricus* hyphae had instigated the *L. fungicola* spore germination. In agreement, Thapa and Jandaik (1987b) demonstrated that although spores of the pathogen can germinate in sterile water, ger-

mination and germ tube growth are greatly stimulated by the addition of nutrients. It was suggested that carbon is the stimulating factor. Fungistasis is not only caused by nutrient depletion, the production of inhibiting compounds also contributes to inhibition of spore germination. In the case of *L. fungicola*, it was demonstrated that volatiles from compost inhibited spore germination (Wuest and Forer, 1975). It appears that *L. fungicola* spores are dormant and germinate only when *Agaricus* colonizes the casing, thus awaiting conditions that favor proliferation of the pathogen.

MACROSCOPIC SYMPTOM DEVELOPMENT

Three types of symptoms are generally described after infection of *A. bisporus* with *L. fungicola*:

- Necrotic lesions (figure 1b): brown, light brown or grey discolorations on the cap or stipe, that can develop into warty outgrowths of the mushroom surface
- Stipe blow-out (figure 1c): fruiting bodies are partially deformed. Deformation of the stipe is often accompanied by splitting or peeling of the stipe tissue.
- Dry bubble (figure 1d): undifferentiated amorphous mass of mushroom tissue either white, heterogeneously discolored or homogenously discolored.

Figure 1. Fruiting bodies of *Agaricus bisporus* infected by *Lecanicillium fungicola* displaying different symptoms . **a)** healthy mushroom **b)** necrotic lesions **c)** stipe blow-out **d)** dry bubble

The time point of infection affects the type and severity of diseases symptoms. Holmes (1971) harvested mushrooms from beds that were inoculated with *L. fungicola* at different time points after applying the casing layer. Disease incidence was lowest when the pathogen was inoculated during casing application and increased to a maximum when inoculating 14 days after casing. At the latter time point, *Agaricus* hyphae had reached the casing surface, but had not yet formed primordia. It was suggested by Holmes (1971) that *L. fungicola* spores introduced into the casing before colonization by *Agari-*

cus were deprived of nutrients by soil fungistasis leading to reduced viability. Compared to inoculation at 14 days after casing, inoculation at 21 and 28 days after casing, when mature fruiting bodies are present, resulted in lower numbers of mushrooms with symptoms. This suggests that *Agaricus* is most susceptible to infection prior to the formation of mushrooms. However, a symptomless mushroom is not necessarily an uninfected mushroom as *L. fungicola* hyphae and conidia can be present on the cap surface before discoloration develops (North and Wuest, 1993). In fact, *L. fungicola* was detected on 25% of the symptomless mushrooms on a farm with high disease incidence (Wong and Preece, 1987).

North and Wuest (1993) investigated the effect of infection at different stages of the developing fruiting bodies. They showed that the time point of infection is decisive for the development of the different symptoms. In their experiments, dry bubble developed when primordia were inoculated, stipe blow-out developed when young pilei or primordia were inoculated, whereas necrotic lesions could develop following infection at any developmental stage of the mushroom. In agreement with the finding of Holmes (1971), at low inoculum density symptoms became visible only after a post-harvest incubation period (North and Wuest, 1993).

The contribution of *A. bisporus* DNA to the total DNA of *Lecanicillium*-infected-fruiting bodies is lower in infected primordia than in young bubbles, indicating that in a developing bubble the *Agaricus* mycelium expands faster than the mycelium of *L. fungicola* (Largeteau *et al.*, 2007). There is no tissue differentiation in bubbles and stipe-blow outs can show hymenial cavities without gills or with sterile gills. This suggests that *L. fungicola* infection interferes with tissue differentiation of the host (Largeteau *et al.* 2007). Results by Largeteau *et al.* (2010) confirmed this. They described that 6 genes were differentially expressed in healthy mushrooms at different developmental stages. During the development of a dry bubble, however, these genes maintained the expression level of the developmental stage at which they were infected. After primordium formation the expression of these genes did not change in a dry bubble, whilst in a developing mushroom most of these genes did. This also concurs with the finding of North and Wuest (1993) that symptoms depend on the time point of infection. Infection at the primordium stage, in which tissue is not yet differentiated, would then lead to an amorphous mass of undifferentiated mushroom tissue, whilst infection at later stages, when tissue differentiation has begun, would lead to (partially) deformed mushrooms. Largeteau *et al*. (2007) also proposed that *L. fungicola* has no effect on the growth rate of undifferentiated hyphae, because there was no correlation between the weight of the bubble and the quantity of host DNA. Indeed, the total weight of a mushroom crop is not affected by inoculation with *L. fungicola*, but bubbles are much smaller than normally developed mushrooms (Largeteau and Savoie, 2008). They tested the effect of six different isolates of *L. fun-*

gicola var. *fungicola* on its host and found that three isolates produced more diseased fruiting bodies than the others. Whereas these more aggressive isolates caused higher number of bubbles, other symptoms did not discriminate the level of aggressiveness. None of the isolates had an effect on total crop weight, indicating that the ability of *A. bisporus* to feed on its substrate was not affected. However, the three more aggressive isolates significantly increased the total numbers of mushrooms formed. These results suggest that infection with *L. fungicola* causes more primordia to develop. This could be due to the fact that these extra primordia develop into bubbles, which are smaller than healthy mushrooms, leaving more space and nutrients for other primordia to develop. Alternatively, *L. fungicola* may stimulate fruiting body initiation in the casing (Largeteau and Savoie, 2008). An explanation for this might be that some infected primordia, that would normally have aborted, keep on developing because the tissue differentiation is stopped by *L. fungicola.*

INFECTION

After spore germination, germlings grow alongside the hyphae of *A. bisporus* (Cross and Jacobs, 1968). Infection is initiated by attachment to the hyphae. Hyphae of *L. fungicola* can attach both to the vegetative mycelium of *A. bisporus* and to the mycelium of its developing fruiting bodies (Dragt *et al.*, 1996; Calonje *et al.*, 1997; Calonje *et al.*, 2000a; Shamshad *et al.*, 2009b). It is generally accepted that the *A. bisporus* vegetative mycelium is resistant to infection by *L. fungicola*. The integrity of *A. bisporus* vegetative hyphae is not affected by *L. fungicola*, as observed in dual culture on agar medium (Calonje *et al.*, 2000a; Shamshad *et al.*, 2009b) and in casing directly under an infected mushroom (Cross and Jacobs, 1968). Only Gray and Morgan-Jones (1981) reported that *L. fungicola* overgrew and caused severe necrosis in a colony of *A. bisporus* on an agar medium. However, this should be interpreted with care, since the medium was not specified.

Attachment of *L. fungicola* to hyphae of *A. bisporus* might be initiated through specific and aspecific interactions between surface molecules of both fungi. Fungal hydrophobins are small secreted proteins that self assemble at hydrophobic-hydrophilic interfaces into surface active amphipathic membranes (Wösten, 2001). They allow fungi to escape their aqueous environment, confer hydrophobicity to fungal surfaces in contact with air and mediate attachment of hyphae to hydrophobic surfaces. The outer surface of fruiting bodies of *A. bisporus* are lined with the hydrophobin ABH1, whilst the vegetative mycelium is covered by the hydrophobin ABH3 (Lugones *et al.*, 1996; Lugones *et al.*, 1998). These hydrophobins render the surfaces of fruiting bodies and hyphae in contact with air or a hydrophobic surface hydrophobic. *L. fungicola* also produces a hydrophobin and the outer surface of the hyphae shows a rodlet structure typical for hydrophobins (Calonje *et*

al., 2000b; Calonje *et al.*, 2002). Thus, it is likely that the pathogen and the host attach to each other by hydrophobic interactions between the hydrophobin layers. In *Magnaporte grisea,* the hydrophobin MPG1 has been shown to be involved in the formation of appressoria and is required for full pathogenicity (Soanes *et al.*, 2002).

A more specific attachment mechanism has been suggested by Bernardo *et al.* (2004), who isolated a glucogalactomannan from *L. fungicola* that specifically binds to the cell walls of *A. bisporus* fruiting body hyphae, but not to the cell walls of vegetative hyphae. Indeed germinated spores of *L. fungicola* showed agglutination in the presence of cell walls of fruiting body hyphae of *A. bisporus*. Purified lectins of cell walls of fruiting body hyphae of *A. bisporus* did agglutinate sheep erythrocytes, and this hemagglutinating activity was inhibited in the presence of glucogalactomannan of *L. fungicola.* The authors concluded that binding of the glucogalactomannan of *L. fungicola* to the lectins in the fruiting bodies of *A. bisporus* attaches *L. fungicola* to its host and is the first step in the infection of hyphae of *A. bisporus.* It can not be excluded that hydrophobins play a role in this process. Previously, it was shown that hydrophobins of *S. commune* have lectin-like activitites (Van Wetter *et al.*, 2000). The glucogalactomannan-lectin binding could serve *L. fungicola* in recognizing its host which leads to subsequent steps in the infection process (Collopy *et al.*, 2010). Interestingly, *Lfmpk-1,* a pmk1-like map-kinase of *L. fungicola*, is upregulated in cap lesions. Such map-kinases play an important role in interactions between fungal pathogens and plants. However, *Lfpmk1* mutants did not have reduced virulence. This indicates that it is not involved in the infection process (Collopy *et al.*, 2010). Perez Cabo and Garcia Mendoza (2008) found similar effects of the glucogalactomannan of *L. fungicola* on the hemagglutinating activity of a lectin of *P. ostreatus*. This implies that this mechanism might function in other susceptible mushroom species, but direct evidence for the necessity of lectin-glucogalactomannan recognition in the infection process is lacking.

After initial attachment, *L. fungicola* can grow inter- and intra-cellularly on *A. bisporus* fruiting body hyphae. Invasion of *A. bisporus* takes place through a combination of weakening of the cell wall by the production of lytic enzymes and by mechanical pressure through the formation of appressoria and peg penetration structures (Dragt *et al.*, 1996; Calonje *et al.*, 1997). *L. fungicola* produces a wide range of extracellular lytic enzymes. (Trigiano and Fergus, 1979; Kalberer, 1984; Calonje *et al.*, 1997; St Leger *et al.*, 1997; Bidochka *et al.*, 1999a; Bidochka *et al.*, 1999b; Calonje *et al.*, 2000a; Mills *et al.*, 2000; Juarez del Carmen *et al.*, 2002). Calonje *et al.* (1997) identified a number of exopolysaccharidase, endopolysaccharidase and protease activities. Growth in minimal medium with lyophilized *A. bisporus* cell walls increased the activity of most of these enzymes, which were often also present in lower amounts when grown in minimal medium with simple carbon sources like glucose, sucrose or fructose. 1,4-βglucanase was only pro-

duced in the presence of cell walls and not when grown on a simple carbon source medium. Electron microscopy showed that cell walls of *A. bisporus* were more efficiently digested by an *L. fungicola* enzyme extract taken from a cell-wall medium than from a fructose medium. Apparently, *A. bisporus* cell walls trigger the secretion of the appropriate enzymes by *L. fungicola* for the digestion of its host. Electron microscopy showed *A. bisporus* cell-wall degradation at the site of interaction with the pathogen in fruiting body hyphae, but not with vegetative hyphae (Calonje *et al.*, 1997; Calonje *et al.*, 2000a). However, *in vitro*, purified cell walls of vegetative hyphae of *A. bisporus* were digested by an enzyme extract from *L. fungicola*. The *in vivo* resistance of vegetative *A. bisporus* could be explained by differences in cell wall composition between vegetative and fruiting body hyphae of *A. bisporus* or by *in vivo* inhibition of secretion or activity of the lytic enzymes of *L. fungicola.*

L. fungicola produces a chemotrypsin protease with narrow specificity and large amounts of broad spectrum substilisin-like proteases (Bidochka *et al.*, 1999b; Kalberer, 1984; St Leger *et al.*, 1997), however, their involvement in the infection process has as yet not been studied. The involvement of VFGLU1, a predicted 1,6-β-glucanase, in the infection process by *Lecanicillium* has been shown (Amey *et al.*, 2003). *Vfglu1* mutants of the pathogen were less successful in growth on chitin amended medium and caused smaller lesion when inoculated on a mushroom cap. This suggests that *VfGlu1* is, remarkably, involved in the uptake of chitinous substrates and that the gene has a significant effect on the infection process.

BROWNING

Infection by *L. fungicola* is often accompanied by browning. This is caused by the formation of melanins in the infected mushroom tissue. Melanins result from enzymatic oxidation of phenolic substrates into quinones, which subsequently auto-polymerize into melanins. The formation of quinones is catalyzed by polyphenol oxidases. Laccase and tyrosinase are the only polyphenol oxidases formed by *A. bisporus*, in which laccase is the main polyphenol oxidase present in the vegetative mycelium. In mature fruiting bodies only tyrosinase activity was found (Savoie *et al.*, 2004). However, in a recent study expression of three laccase genes in the mature fruiting body was reported (Largeteau *et al.*, 2010).

Formation of melanins does not often take place in healthy tissue of commercial mushroom strains, evidenced by their white appearance. This is explained by the fact that most of the mushroom tyrosinase (99%) is present in an inactive form and kept separated from its phenolic substrates through cellular compartmentalization. Upon decompartmentalization, melanin precursors contact active tyrosinase, which leads to the formation

of melanins (Jolivet *et al.*, 1998). Decompartmentalization can result from bruising, senescing, extreme environmental conditions and infection by pathogens. Infections by *L. fungicola* can result in the lysis of mushroom hyphae, and thus decompartimentilization, by the combined effect of mechanical pressure and lytic enzymes (Dragt *et al.*, 1996). In this respect, it is interesting to note that dry bubbles can also be white. In this case, the *A. bisporus* hyphae are apparently still intact.

Soler-Rivas *et al.* (2000) investigated mushroom discoloration after pathogen infection. All pathogens tested provoked a discoloration of the mushroom tissue, but the discolorations caused by *L. fungicola* and *Pseudomonas tolaasii* were the most evident. Infection by *L. fungicola* resulted in paler and more yellow browning compared to that after *P. tolaasii* infection. In both cases, infections resulted in a higher tyrosinase activity. In contrast, other pathogens caused a reduction in the tyrosinase activity. It was proposed that the proteinases of both pathogens led to degradation of tyrosinases in mushroom and that active tyrosinase is an intermediate formed during the degradation of the tyrosinases by the proteinases (Soler-Rivas *et al.*, 2000).

Laccase activity has been detected in vegetative mycelium and primordia of *A. bisporus.* Laccase activity was also found in dry bubbles, but not in healthy mushrooms. The electophoretic profile of the laccases in dry bubbles differed from the laccase found in vegetative mycelium of *A. bisporus.* This difference could be brought about through alteration of the host laccases by the pathogen or through *de novo* induction of laccases in response to the pathogen (Savoie *et al.*, 2004). This might be a defensive response of the mushroom to infection by the pathogen or an unintended reaction of the mushroom and a manifestation of how *L. fungicola* affects the developmental program of the mushroom in the dry bubble. The difference in color of *L. fungicola* lesions compared to lesions caused by other mushroom pathogens that was found by Soler-Rivas *et al.* (2000) might also be explained by the laccase activity found in dry bubbles.

The function of melanins in mushrooms has not been determined. It has been suggested that melanins are involved in the defense against pathogens. In support of this, expression of a tyrosinase gene, *AbPPO2,* was up-regulated in fruiting bodies of *A. bisporus* after inoculation with *P. tolaasii* or after treatment with tolaasin, a toxin produced by *P. tolaasii.* Whether this induction is caused by direct recognition of the pathogen's toxin or by recognition of tolaasin related cell-damage could not be ascertained (Soler-Rivas *et al.*, 2001). On the other hand, young mushroom pins infected by *L. fungicola* showed a down-regulation of *AbPPO2* compared to healthy mushrooms pins. This suggests that *AbPPO2* is not involved in active defense of the mushroom against *L. fungicola* (Largeteau *et al.*, 2010). Also, a negative correlation was found between the percentage of host DNA and the intensity of discoloration in dry bubbles. This indicates that the formation of melanins does not inhibit the growth of *L. fungicola* (Largeteau *et al.*, 2007).

DISEASE RESISTANCE

It may well be that fungi invest in the defense of their fruiting bodies apart from producing melanin (see above). Fruiting bodies of many mushroom species contain toxins that are harmful to humans and other mammals (Spiteller, 2008). However, it has not been addressed whether this toxicity has an adaptive advantage for these mushroom species or that the toxins arise simply as byproducts (Sherratt *et al.*, 2005). It has been shown that opossums learn to avoid eating poisonous mushrooms (Camazine, 1983), which can be seen as an argument for mushroom toxins as weapons against fungivory. Also, it was found that cystidia have a defensive role against collembola in *Russula bella* and S*trobilurus ohsimae* fruiting bodies (Nakamori and Suzuki, 2007). It is tempting to speculate that mushroom species also invest in defense against microbial attacks. As dry bubble disease affects the ability of *A. bisporus* to form fertile fruiting bodies and consequently its ability to reproduce, one would expect selective pressure favoring those individuals with resistance to infection. However, commercial *A. bisporus* strains have been selected for their ability to produce mushrooms and not for their defensive traits. Attempts to find *A. bisporus* strains resistant to *L. fungicola* have been undertaken but only revealed strains with partial resistance (Dragt *et al.*, 1995; Wuest and Harvey, 1978; Wuest and North, 1988; Largeteau *et al.*, 2004b; Savoie *et al.*, 2004; Savoie and Largeteau, 2004). Dragt *et al.* (1995) studied necrotic lesions on the cap surface of a brown partial resistant cultivar. Less hyphae and sporulation of *L. fungicola* were found in the lesions on this cultivar and brown pigmented *A. bisporus* hyphae were observed underneath the necrotic hyphae. This may reflect a response of *Agaricus* similar to the hypersensitive response in plants where cells in infected tissue die and incapsulate the infection (Greenberg *et al.*, 1994). Like plants, also toxic molecules seem to take part in the defense. Savoie and Largeteau (2004) tested 17 strains of *A. bisporus* and found a negative correlation between susceptibility of *A. bisporus* strains to *L. fungicola* and hydrogen peroxide levels in dry bubbles of these strains, whereas there was no such correlation in healthy sporocarps. This indicates that hydrogen peroxide is involved in the defense against *L. fungicola*, confirming results presented by Thapa and Jandaik (1987a). White rot fungi, such as *A. bisporus,* are relatively well able to cope with high levels of hydrogen peroxide, since they use extracellular peroxidases and oxidases to degrade lignin (Jansen *et al.*, 2000). In addition to peroxide, also antibiotics that are produced by *A. bisporus* may play a role in the defense against *L. fungicola* (Mamoun *et al.*, 1995; Largeteau *et al.*, 2006). The role of antibiotic production has been demonstrated in the interaction of *A. bisporus* with the green mold *Trichoderma harzianum*. An unidentified metabolite extracted from *A. bisporus* vegetative mycelium and fruiting bodies was able to inhibit growth of two biotypes of *T. harzianum*, but stimulated the growth of biotype *Th2* (Mumpuni *et al.*, 1998).

Antibacterial effects of extracts of *A. bisporus* fruiting bodies have also been demonstrated (Tambekar *et al.*, 2006). A double layer test has been developed to assess effects of antibiosis by *Agaricus* on spore germination of *L. fungicola*. However, the inhibitory effect of *A. bisporus* strains in this test did not correlate with their dry bubble resistance (Mamoun *et al.*, 1995).

Also, susceptibility of different *A. bisporus* strains to *L. fungicola*, *P. tolaasii* and *Trichoderma aggressivum* did not correlate (Largeteau *et al.*, 2004b). This suggests that different pathogens are differentially recognized or affected by different defense mechanisms. The different biochemical mechanisms of microbially induced diseases of *A. bisporus* have been recently reviewed by Largeteau and Savoie (2010). However, (partial) resistance is not necessarily a result of active defense. Non-defense related characteristics of *A. bisporus* strains can also play a role. A significant correlation between the time needed by *A. bisporus* strains to form their first fruiting bodies and the susceptibility to *L. fungicola* was reported, earlier fruiting strains were significantly less diseased in a casing inoculation experiment (Largeteau *et al.*, 2004b).

Thomas *et al.* (2007) studied the interaction between *A. bisporus* and *L. fungicola* at the molecular level using suppressive subtractive hybridization and cDNA libraries of *A. bisporus.* They identified 80 genes of *A. bisporus* and 50 genes of *L. fungicola* that were differentially regulated in infected mushroom tissue compared to healthy tissue. A Chitin deacetylase gene of *A. bisporus* was strongy upregulated in infected tissue, however, RNAi hairpin-mediated gene silencing did not lead to increased susceptibility in infection trials. Also suppression of a gene encoding 3-deoxy-7-phosphoheptulonate synthetase, an enzyme known to play a role in plant-pathogen interactions, did not lead to changes in *L. fungicola* lesions on mushroom caps. This considerable effort shows that identifiying defense related genes is likely difficult, since *L. fungicola* has been shown to affect the *A. bisporus* developmental program in which a myriad of genes is involved. Largeteau *et al.* (2010) investigated expression levels of *hspA*, encoding a heat-shock protein of the HSP70 family in *A. bisporus.* It was found that the expression of this gene changes during the development of healthy fruiting bodies, but that in a developing bubble the *hspA* expression remains at the level found in a healthy primordium. They compared the expression of the *hspA* gene in three strains of *A. bisporus* that were relatively resistant to *L. fungicola* with its expression in three more susceptible strains. It was shown that *hspA* was more highly expressed in the primordia of the three more resistant strains. In the young bubbles and in vegetative mycelium expression of *hspA* was comparable in all strains. However, in primordia *hspA* was upregulated in resistant strains and down regulated in susceptible strains. This indicates that *hspA* is involved in resistance of mushroom primordia, but probably plays an indirect role (Largeteau *et al.*, 2010). Resistant and susceptible strains differed in the number of bubbles, but not in the amount of tissue

infected in the bubble. It was therefore proposed that *hspA* affects pathogen infection in primordia but not the further growth of the pathogen, .

FUTURE PROSPECTS

Currently, control of *L. fungicola* mainly relies on prevention and hygienic measures on the mushroom farms. Active control of the disease is difficult because the chemicals used become less effective, as the pathogen develops resistance and legislation restricts their use. Therefore, dry bubble disease is likely to remain one of the more devastating pathogens in commercial mushroom growing. A better understanding of the ecology of *L. fungicola* and its interaction with *A. bisporus* will lead to innovative ways to control dry bubble disease, e.g. biological control using antagonistic bacteria.
Research on the *L. fungicola - A. bisporus* interaction has been seriously hampered by the inability to efficiently transform *A. bisporus* and create single gene knock-outs. Future studies should focus on such a system, especially since the genome of *A. bisporus* will become available in the near future. This will facilitate detection of differentially expressed genes in infected mushrooms and, with that, perhaps genes involved in resistance against or susceptibility to *L. fungicola*. Methods to transform and make knock-outs in *L. fungicola* are available, facilitating research into the pathogen side of the interaction (Amey *et al.*, 2002; Amey *et al.*, 2003). Besides new means to combat dry bubble disease, future research may also elucidate the way in which *L. fungicola* disrupts the developmental program of *A. bisporus* and stops tissue differentiation. The elucidation of mechanisms through which the pathogen exerts this effect might also lead to manipulation of mushroom development in a way beneficial to the grower.

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Microbial inhibition of *Lecanicillium fungicola* in the mycosphere of *Agaricus bisporus*

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ABSTRACT

Dry bubble disease is a major problem in the commercial cultivation of the white button mushroom *Agaricus bisporus*. This disease is caused by the ascomycete *Lecanicillium fungicola*. Here, the ecology of the pathogen was investigated in the casing layer, in which the interaction between *A. bisporus* and *L. fungicola* takes place. In casing, germination of *L. fungicola* spores was inhibited by the microflora, a phenomenon known as fungistasis. The fungistasis is annulled when the casing is colonized by *A. bisporus* hyphae. We demonstrated that addition of *A. bisporus*-associated sugars to casing, similarly annulled the casing fungistasis. However, casing fungistasis does not seem to be based on competition for resources as *L. fungicola* spores were shown to germinate regardless of nutrient availability. Pseudomonas bacteria are a dominant group of bacteria in the casing and have previously been implied to be essential for the development of fungistasis in soils. Antibiotics produced by Pseudomonas bacteria inhibited *L. fungicola* spore germination. However, the addition of glucose desensitized spores of *L. fungicola* which resulted in germination in the presence of antibiotics. We conclude that fungistasis in the casing layer of mushroom cultures is caused by antibiotics produced by the microflora and that it postpones germination of *L. fungicola* until the mushroom host is present. Our observation that introducing single antibiotic producing *Pseudomonas* spp. strains could not reinstate casing fungistasis indicates that a consortium of bacteria causes fungistasis.

INTRODUCTION

Lecanicillium fungicola (Preuss) Zare and Gams (synonyms: *Verticillium fungicola* (Preuss) Hassebrauk, *Verticillium malthousei* (Preuss) Ware) is the causal agent of dry bubble disease. This disease represents one of the biggest problems in the commercial production of the white button mushroom, *Agaricus bisporus* (Berendsen *et al.*, 2010). Disease symptoms vary from small necrotic lesions on the cap of the fruiting bodies to partially deformed fruiting bodies called stipe blow-out or totally deformed and undifferentiated masses of mushroom tissue, known as dry bubble. Annual costs of dry bubble disease for mushroom growers are estimated at 2 - 4% of the total revenue. The control of *L. fungicola* relies on strict hygiene and the use of fungicides. Few chemicals can be used for the control of dry bubble as the host is also negatively affected by many fungicides. The few fungicides that have been used are either no longer available or are becoming increasingly ineffective because of the development of fungicide resistance in the pathogen (Bollen and van Zaayen, 1975; Fletcher and Yarham, 1976; Gea *et al.*, 2005; Wuest *et al.*, 1974). Currently, the control of dry bubble disease relies heavily on the use of prochlorazmanganese (i.e. Sporgon), but reduced sensitivity to this fungicide has been reported (Gea *et al.*, 2005; Grogan, 2008). Therefore, new ways to combat dry bubble disease are urgently needed. Antagonistic microorganisms in soils have been exploited for effective control of fungal plant pathogens and this has resulted in a number of commercial products (Fravel, 2005; Weller *et al.*, 2002). Similarly, biological control of dry bubble disease could prove effective and this would support the mushroom industries efforts to minimize the use of chemicals. Understanding *L. fungicola*'s ecology in the casing layer allows for sensible development of effective control of this pathogen.

The white button mushroom is grown on a composted mixture of horse and chicken manure. Fruiting body formation is initiated when compost that is fully colonized by *A. bisporus* is covered with a layer of casing soil. The casing typically consists of black peat mixed with spent lime and/or marl. It has been postulated that the microflora of the casing layer is necessary for fruiting body formation because it consumes a metabolite of the mushroom mycelium that is inhibitory to mushroom formation (Visscher, 1988; Noble *et al.*, 2003; Noble *et al.*, 2009).

Infection most likely takes place in the casing layer, as *L. fungicola* cannot infect *A. bisporus* vegetative mycelium in the compost (Bernardo *et al.*, 2004; Calonje *et al.*, 2000a; Cross and Jacobs, 1968). When compost colonized by *A. bisporus* is added to a plate colonized by *L. fungicola*, the pathogen is overgrown by *A. bisporus* (Berendsen and Schrier, unpublished observations).

The primary source of infection is debated. It is unlikely that *L. fungicola* is present in the compost delivered to the farms, since the spores will not survive the composting process

(Gerrits, 1988). Spores can survive in peat, the main ingredient of casing soil, for more than a year (Cross and Jacobs, 1968). However, it is not likely that *L. fungicola* proliferates saprophytically in peat, as its growth is inhibited by the microbial community in this substrate (Cross and Jacobs, 1968). Moreover, the low pH makes it an unlikely habitat for *L. fungicola*. Primary infection of the casing is therefore expected to take place on the farm. Insects, workers and equipment can carry spores of *L. fungicola* into mushroom cultivation facilities (Berendsen *et al.*, 2010; Cross and Jacobs, 1968; Shamshad *et al.*, 2009a; White, 1981; Wong and Preece, 1987). After *L. fungicola* spores have been introduced to the casing, spore germination is a crucial step in the infection process.

In most soils, germination and growth of fungi is restricted compared to that *in vitro* under similar conditions (temperature, moisture, pH etc.), a phenomenon known as soil fungistasis. As sterilization of soils removes fungistasis, it is thought to be brought about by the active microbiota in soils. The magnitude of the fungistatic effect differs between soils, but also different fungal species can be differentially sensitive to fungistasis. Soilborne plant-pathogenic fungi appear to be especially sensitive to soil fungistasis (de Boer *et al.*, 1998), but do germinate in the vicinity of their hosts. Therefore fungistasis is considered to be advantageous to pathogenic fungi, it prevents germination and growth under unfavorable conditions, postponing it until their host is present (Lockwood, 1977; Termorshuizen and Jeger, 2008).

Spores of *L. fungicola* appear to be sensitive to fungistasis in the casing (Cross and Jacobs, 1968). Moore and Wuest (1973) found that pre-treatment of casing with steam at 98˚C led to dramatic increases in dry bubble disease incidence of *L. fungicola*-inoculated mushroom cultures. Apparently, casing fungistasis is important in the ecology of the disease, but mechanisms through which casing fungistasis affects *L. fungicola* have not been studied. This research aimed at revealing factors that influence spore germination of *L. fungicola* in the casing, as it is considered a crucial first step in the infection of the mushroom. We found that fungistasis in the casing is lifted when the casing is colonized by *A. bisporus*, moreover, the addition of saccharides associated with *A. bisporus* hyphae lifted fungistasis in uncolonized casing. Antibiotics produced by the casing microflora are postulated to inhibit *L. fungicola* spore germination and addition of nutrients make the spores less sensitive to the inhibitory action of the antibiotics.

MATERIALS AND METHODS

FUNGAL CULTURES

Lecanicillium fungicola strain V9503 (Largeteau *et al.*, 2006), *Fusarium oxysporum* f.sp. *raphani* strain WCS600 (Leeman *et al.*, 1995) and *Botrytis cinerea* strain B0510 (Leon-Reyes *et al.*, 2010) were stored in glycerol at -80˚C. The fungi were grown on potato dextrose agar (PDA; Difco, Lawrence, USA) for 5 (*L. fungicola) or* 14 (*B. cinerea* and *F. oxysporum)* days at 24˚C. Spore suspensions were obtained from these cultures by adding 10 ml of demineralized water (DEMI) to each plate and filtering over sterile glass wool to remove mycelial fragments. Densities of the suspensions were set at $2 \cdot 10^4$ spores / ml after counting in a haemocytometer.

QUANTIFYING SPORE GERMINATION

Spores were fixed to black Cyclopore membranes (diameter: 25 mm, pore size: 0.2 µm Whatman, Florham Park, USA) by filtering 5 ml of the spore suspension over the membrane, using a syringe and a plastic filter holder (Whatman, Florham Park, USA). The resulting membrane was cut into 4 parts. These parts were incubated under the different experimental conditions. At the end of each experiment, membranes were lifted from the substrate and adhering soil particles were removed. Membranes were mounted on a microscope slide and spores were stained with 100 µl of a Calcofluor white solution (per liter: 1 g fluorescent brightener 28 (Sigma-Aldrich, Steinheim, Germany), 50 g KOH, 50 ml glycerol). The percentage of spore germination was determined by observation of the stained membranes using a Zeiss Axioskope fluorescence microscope equipped with a 70 W mercury lamp and the Zeiss filter set 02 (excitation 365 nm, emission 420 nm).

CASING FUNGISTASIS EXPERIMENTS

As the interaction between *L. fungicola* and *A. bisporus* takes place in the casing layer, *L. fungicola* spore germination was investigated on this substrate. The casing soil was a mixture of fresh black peat and air dried black peat (2:1, v: v) with spent lime (160 kg/ m^3). It was provided by CNC (Milsbeek, the Netherlands) and stored at 4˚C. To study the effect of the casing microflora, 200 g casing was mixed with 100 ml water and subsequently autoclaved. Colonized casing was taken from a mushroom production culture of *A. bisporus* strain A15 two weeks after application of the casing layer, and at this stage the first pins were present. To study effects of exogenous carbon sources, samples of 200 g of casing were mixed with 100 ml water, 100 ml of a 30mM solution of one of the following carbon sources in water (fructose, galactose, glucose, mannitol, mannose, rham-

nose, sucrose , trehalose or xylose) or with 100 ml of a 1 % glycogen solution. All treated casings were placed in sterile Petri dishes and the membranes containing *L. fungicola* spores were placed on top of the casing. The Petri dishes were subsequently incubated at 24˚C for 18 hrs.

UTILIZATION OF CARBON SOURCES BY *L. FUNGICOLA*

It was examined if the saccharides that annulled casing fungistasis could be used as a carbon source by *L. fungicola*. One hundred ml flasks with 40 ml of 1 % solutions of the following carbon sources (fructose, galactose, glucose, mannitol, mannose, rhamnose, sucrose , trehalose or xylose) in minimal medium(per liter: 2.6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 2.5 g $MgSO_4$, trace elements (Vishniac and Santer, 1957), pH 6.0) were inoculated with 10^7 *L. fungicola* spores and incubated at 24˚C for 1 week. Subsequently, the cultures were filtered over Whatman paper (No. 1; 12.5 cm diameter). The filters were dried at 60˚C for 3 days and dry weight of the fungal biomass was determined. Fungal growth on each saccharide was tested in triplicate.

NUTRIENT DEPENDENCY OF *L. FUNGICOLA* SPORES

To study if *L. fungicola* spore germination was affected by the nutrient availability, membranes containing *L. fungicola* spores were prepared as describe above. As an additional treatment the spores were washed twice by centrifugation (10 min, 4000 rpm) in sterile DEMI prior to membrane preparation. Spore membranes were cut in 4 parts. These parts were placed on 100 µl DEMI or 100 µl 10 mM glucose and incubated for 24 hrs at 24˚C.

LEACHING SPORE EXUDATES

It was examined if spores of *L. fungicola* were affected by continuous discharge of nutrients leaching from the spores, as was described for several fungal species (Hsu and Lockwood, 1973). Membranes containing spores of *L. fungicola, B. cinerea* or *F. oxysporum* f.sp. *raphani* were prepared as described above, but kept in the sterile plastic filter holders. Fifty ml of sterile DEMI was washed over the membranes and the system was filled with 1 ml of sterile DEMI. A modification of the leaching system described by Hsu and Lockwood (1973) was constructed by subsequently attaching the filter holders to a peristaltic pump (type 2005, Skalar) that pumped 25 ml of sterile DEMI per hr over the membranes. Control filter holders were not attached and sealed with parafilm. Additionally, filter holders containing membranes were filled with 1 ml Potato dextrose broth (PDB, Difco). Filter holders were incubated for 16 hrs at room temperature and subsequently membranes were prepared for microscopy.

DENSITY- DEPENDENT SELF-INHIBITION OF *L. FUNGICOLA* SPORE GERMINATION

Spores were harvested as described before and washed twice by centrifugation (10 min, 4000rpm) in sterile DEMI. The spore suspensions were set at 10^7, 10^6 and 10^5 conidia/ml. 200 µl of each suspension was incubated on a microscope slide in quadruplicate. Microscope slides were placed in closed plastic containers (20 cm x 30 cm x 10 cm) to prevent evaporation. Spore germination was assessed after 18 hrs using phase-contrast microscopy (Axioskope, Zeiss, Jena, Germany).

INHIBITION OF *L. FUNGICOLA* SPORE GERMINATION BY BACTERIA ISOLATED FROM CASING

The bacterial strains used in this study were isolated by Baars *et al.* (2003) from casing fully colonized by *A. bisporus* (Table 1). These bacterial isolates were yellow fluorescent on King's medium B agar (KBA) (Per liter: 10 g Proteose peptone (Difco), 1.5 g $MgSO_4$.7 H_2O, 1.2 g KH_2PO_4, 10 g glycerol and 15 g agar(Difco)) and were therefore considered *Pseudomonas* spp. Also, effects of *P. fluorescens* CHA0 on *L. fungicola* spore germination were investigated. This strain has been well characterized for its production of the antibiotics 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin and cyanide. To study the involvement of these antibitiotics, a *gacA* mutant of CHA0, CHA89, was used that lacks production of these compounds. The bacterial strains were grown on KBA at 28˚C for 24 hrs. Cells were suspended in 1 ml $MgSO_4$ of which 100 µl was plated on KBA and incubated for 24 hrs at 28˚C. Cells were harvested in 10 ml 10 mM $MgSO_4$ and washed twice by centrifugation (10 min, 4500 rpm).The pellet was resuspended in sterile 10 mM $MgSO_4$. Suspensions were diluted to a density of 10^9 colony-forming units (cfu) per ml. Spore germination was assessed by incubating the spore containing Cyclopore membranes on 200 µl bacterial suspension for 18 hrs at 28˚C in quadruplicate.

Table 1. Relevant characteristics of *Pseudomonas* strains used in this study

Strain(s)	Relevant characteristics	References
Ab03003 - Ab03067	Isolated from casing colonized by *A. bisporus*	(Baars *et al.*, 2003)
P. fluorescens CHA0	Isolated from tobacco rhizosphere; produces DAPG, PLT, PRN and HCN; produces iron regulated PSB, PCH and SA	(Laville *et al.*, 1992)
P. fluorescens CHA89	*gacA* mutant of CHA0; lacks production of DAPG, PLT, PRN and HCN ; produces PSB, PCH and SA	(Laville *et al.*, 1992)

EFFECTS OF CARBON SOURCE ON INHIBITION OF SPORE GERMINATION OF *L. FUNGICOLA* BY CHA0

Suspensions of *P. fluorescens* CHA0 and CHA89 were prepared as described above and set at 1.1· 10^9 cfu / ml. 900 µl of the bacterial suspension was mixed with 100 µl of a 100 mM solution of one of the following carbon sources (glucose, galactose, fructose, mannitol, trehalose, mannose, rhamnose, sucrose or xylose), to a 1% solution of glycogen, or to 100 µl sterile demineralized water. Spore containing Cyclopore membranes were incubated on 200 µl of the bacterial suspension for 18 hrs at 28˚C in quadruplicate and percentage of spore germination was determined.

UTILIZATION OF CARBON SOURCES BY *P. FLUORESCENS* CHA0

One hundred ml flasks with 10 ml of 10 mM solutions of the following carbon sources (fructose, galactose, glucose, glycogen, mannitol, mannose, rhamnose, sucrose, trehalose or xylose) in minimal medium were inoculated with *P. fluorescens* CHA0 and incubated at 24˚C and 220 rpm. Growth was determined by visually assessing turbidity of the solution after 4 days.

REINTRODUCTION OF BACTERIA TO STERILIZED CASING

It was investigated if the bacterial strains that could inhibit *L. fungicola* spore germination in a suspension could similarly do so in the casing. Therefore, full-gas microboxes (110x100x80mm; Eco2box, Ophasselt, Belgium) containing 200 g of casing (CNC, Milsbeek, the Netherlands) were sterilized by autoclaving 4 times on 4 subsequent days. Bacterial suspensions were prepared as described before and diluted to a density of 3·10^5 cells/ml. 100ml of bacterial suspension was added to each box of sterilized casing and thoroughly mixed. Control casings were prepared by adding 100 ml of sterile 10 mM $MgSO_4$. All treatments were compared to non-autoclaved casing with 100 ml sterile 10 mM $MgSO_4$. In an additional treatment the casing microflora was transferred to sterilized casing by mixing a suspension of 2 g of non-autoclaved casing in 100 ml $MgSO_4$ to sterilized casing. Each casing was then distributed over small Petri-dishes and incubated at 24 ˚C in a plastic tray with closed lid. On t= 0, and after 1 and 7 days, *L. fungicola* spore germination was determined on 4 Petri dishes of each casing using spore membranes as described above. Culturable bacterial populations in the casing were determined by dilution plating. 1 g of casing was suspended in 10 ml 10 mM $MgSO_4$, and dilution series were plated on 10 % Tryptic Soy Agar (TSA; per liter: 3 g tryptic soy broth (Difco), 15 g granulated agar(Difco)). Colonies were counted after 120 hrs incubation at 24 °C.

RESULTS

CASING FUNGISTASIS

Germination of *L. fungicola* spores on casing was investigated. On control casings approximately 20 % of the *L. fungicola* spores germinated within 18 hrs (figure 1). This low germination percentage on the control casing was persistent as after 90 hrs only 28% of the spores had germinated. On casing that had been autoclaved 99% of the spores germinated within 18 hrs. Close to 100% of the spores incubated on casing colonized by hyphae of *A. bisporus* germinated within 18 hrs, as did spores incubated on casing with 10mM glucose. Spores that were incubated on control casing for 18 or 90 hrs remained viable (data not shown).

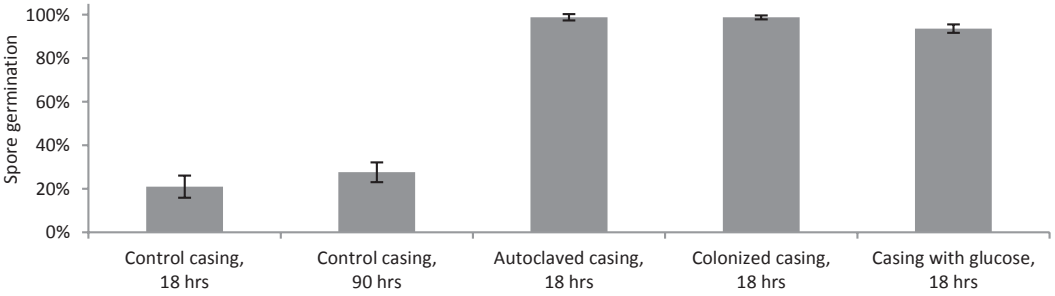

Figure 1. *L. fungicola* spore germination after incubation on: casing for 18hrs, casing for 90 hrs, autoclaved casing, casing fully colonized by hyphae of *A. bisporus* and casing with a glucose solution. Bars represent average germination (%) of 4 replicates. Error bars denote standard deviation.

CASING FUNGISTASIS IS LIFTED BY THE ADDITION OF MUSHROOM SACCHARIDES

Individual saccharides, reported to be present in *A. bisporus* (Domenech *et al.*, 2002; Grewal and Rainey, 1991; Wannet *et al.*, 2000), were added to casing. All saccharides with the exception of rhamnose stimulated spore germination on the casing (figure 2). In line with these results *L. fungicola* could use all saccharides, except rhamnose, as a sole carbon source for growth in minimal medium (table 2).

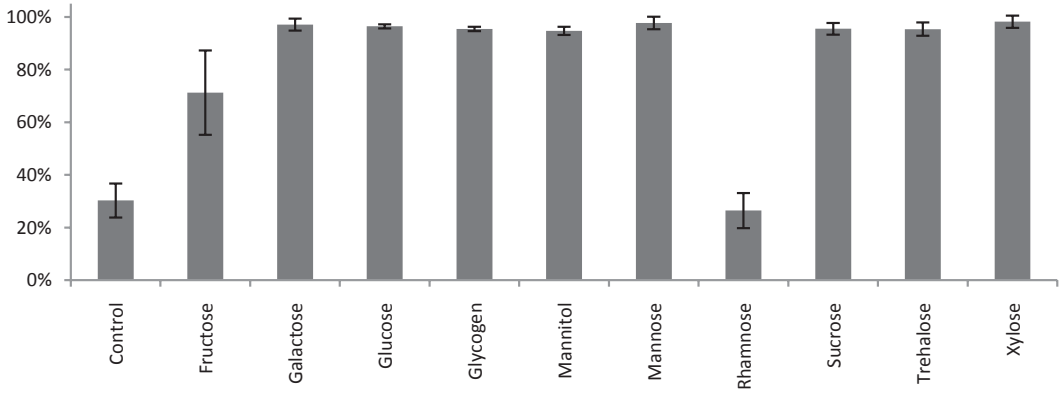

Figure 2. Effect of addition of saccharides on L. fungicola spore germination on casing. Bars represent average spore germination of 4 replicates. Error bars denote standard deviation.

Table 2. Growth of *L. fungicola* and *P. fluorescens* CHA0 in minimal medium on mushroom-associated saccharides as sole carbon source

Carbon source	***L. fungicola* growth**	***P. fluorescens* CHA0**
No carbon source	–	–
Fructose	+	+
Galactose	+	–
Glucose	+	+
Glycogen	+	–
Mannitol	+	+
Mannose	+	+
Rhamnose	–	–
Sucrose	+	+
Trehalose	+	+
Xylose	+	–

+ growth observed, – no growth observed

NUTRIENT DEPENDENCY OF *L. FUNGICOLA* SPORES

In a time course experiment the effect of washing or addition of glucose on spore germination of *L. fungicola* was investigated. Almost all spores of *L. fungicola* germinated within 24 hrs (figure 3). Washing of spores did neither affect the percentage of spores that germinated, nor the dynamics of germination. Addition of glucose also did not affect germination of unwashed spores.

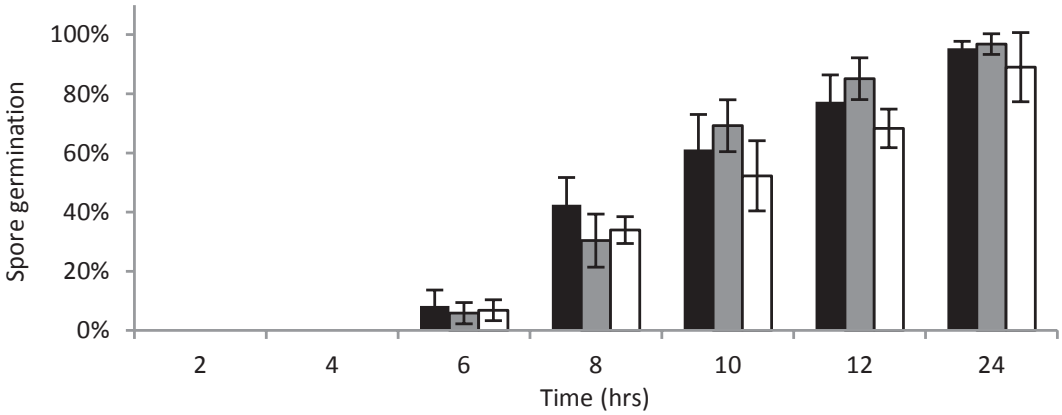

Figure 3. Nutrient dependency of *L. fungicola* spores on Cyclopore membranes. Spores were washed and placed on water (black bars) or not washed and placed on either water (grey bars) or 10 mM glucose (white bars). Bars represent average germination (%) of 4 replicates. Error bars denote standard deviation.

LEACHING OF NUTRIENTS FROM SPORES

The nutrient dependency of *L. fungicola* spores was compared to that of spores of *B. cinerea* and *F. oxysporum*. Most spores of *B. cinerea* germinated in PDB, but in water only 4% germinated within 16 hrs (figure 4a). Likewise the majority of conidia of *F. oxysporum* germinated in PDB, however, also in water 92 % of the spores germinated (figure 4b). The same was observed for germination of *L. fungicola* spores. Both in PDB and water most of them germinated within 16 hrs (figure 4c). Continuous leaching with DEMI at a rate of 25 ml per hr reduced germination of Fusarium spores to 26%, the *B. cinerea* spores did not germinate at all, and germination of *L. fungicola* was not affected by this treatment. Increasing the volume with which exudates of *L. fungicola* spores were leached to approximately 65 ml per hr also did not affect *L. fungicola* spore germination (data not shown).

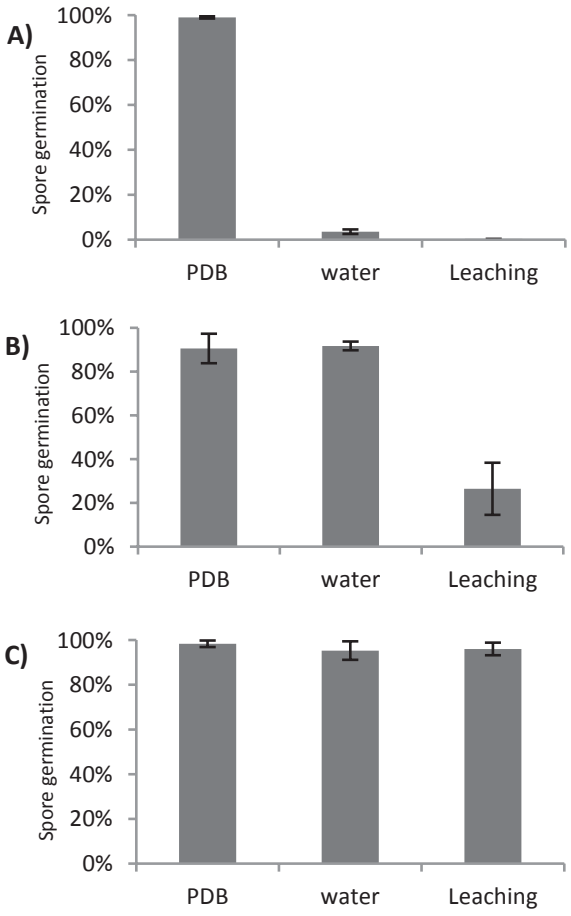

Figure 4. Spore germination of [A)] *Botrytis cinerea* [B)] *F. oxysporum* or [C)] *L. fungicola* on Cyclopore membranes in a leaching system. Membranes were incubated in PDB, water, or attached to the leaching system in which spore exudates were continuously discharged (25 ml/ hr). Bars represent average germination (%) of 3 replicates. Error bars denote standard deviation.

EFFECT OF SPORE DENSITY ON GERMINATION

Spore germination was affected by the density of the *L. fungicola* spore suspension (figure 5). Incubation in DEMI of washed spores, at a density of 10^5 spores/ml, resulted in 97 % germination. Increasing the density to 10^6 spores per ml did not affect germination, however, at 10^7 spores/ml germination was significantly reduced to 47%. The reduced germination at 10^7 spores/ml was partially annulled by the addition of 10mM glucose resulting in 80% of the spores germinating.

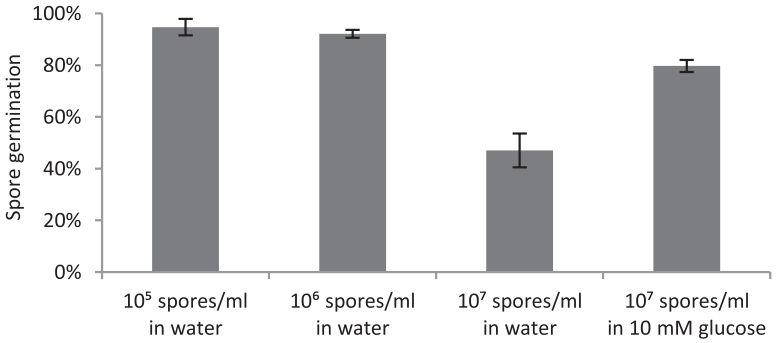

Figure 5. Germination of *L. fungicola* spores at different densities. Bars represent average spore germination (%) of 4 replicates. Error bars denote standard deviation.

INHIBITION OF *L. FUNGICOLA* SPORE GERMINATION BY BACTERIA ISOLATED FROM THE CASING

Bacteria, that were isolated from *A. bisporus* hyphae in the casing, were tested for their ability to inhibit germination of *L. fungicola* spores. When spore membranes were placed on high-density (10^9 cfu /ml) bacterial cell suspensions, spores of *L. fungicola* were differentially affected by bacterial strains from colonized casing (figure 6). Some strains did not affect germination, while others reduced the percentage of spores that germinated overnight to 20%.

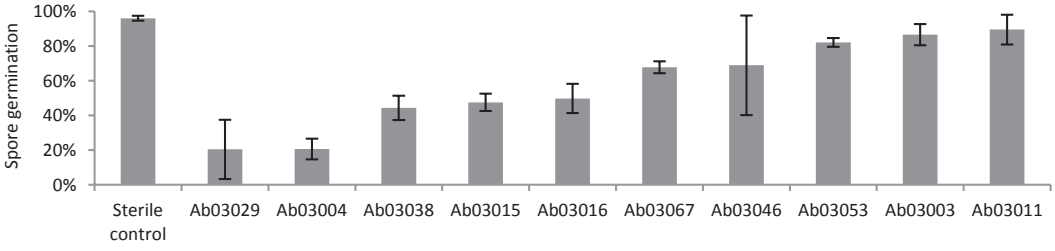

Figure 6. Effect of bacterial strains isolated from casing on *L. fungicola* spore germination. Bacteria were suspended in 10mM $MgSO_4$ and spores were applied on Cyclopore membranes floating on top of the suspension. Bars represent average spore germination (%) of 4 replicates. Error bars denote standard deviation**.**

PUTATIVE FUNGISTATIC ROLE OF ANTIBIOTICS PRODUCED BY BACTERIA

P. fluorescens CHA0 is well studied and known to produce a range of antibiotics, while its derivative, the *gacA* knock-out *P. fluorescens* CHA89 does not produce antibiotics (Laville *et al.*, 1992). At 10^9 cfu/ml, CHA0 reduced spore germination of *L. fungicola* spores to 32%, whereas most (86%) spores germinated on a suspension of CHA89 at similar densities. When transferred to 10mM glucose after 18 hrs incubation on the CHA0 suspension, 95 % of the spores germinated, indicating that CHA0 does not kill the spores (figure 7a). With the exception of rhamnose, addition of any of the mushroom-associated saccharides, increased *L. fungicola* spore germination on a suspension of CHA0 (10^9 cfu / ml) (figure 7b). CHA0 could grow in minimal medium with 10 mM of glucose, mannitol, mannose, trehalose or fructose as the sole carbon source, whereas galactose, glycogen, rhamnose, sucrose, and xylose did not support growth (table 2).

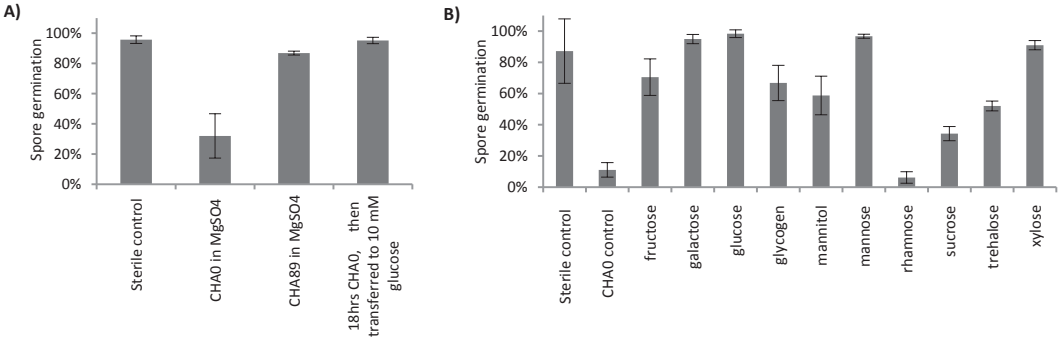

Figure 7. Effect of *P. fluorescens* CHA0 and its *gacA*-deficient-derivative CHA89 on *L. fungicola* spore germination. **A)** Germination of *L. fungicola spores* after 18 hrs incubation on suspensions of CHA0 and CHA89. Bacteria were suspended in 10 mM $MgSO_4$ or 10 mM glucose at 10^9 cfu/ml. **B)** Effects of mushroom-associated saccharides (10mM) on the inhibition of *L. fungicola* spore germination by *P. fluorescens* CHA0 in $MgSO_4$ at 10^9 cfu/ml. Bars represent average spore germination (%) of 4 replicates. Error bars denote standard deviation.

REINTRODUCTION OF BACTERIA TO STERILIZED CASING

Bacterial isolates were able to inhibit *L. fungicola* spore germination *in vitro* through the production of antibiotics. It was investigated if the same isolates could also suppress spore germination in the casing. Casing was sterilized by autoclaving and single bacterial isolates were introduced at a density of 10^5 cells / g casing. Effects on *L. fungicola* spore germination (figure 8) were determined at 1, 2, and 8 days after introduction of the bacteria. In non-sterilized control casing germination of *L. fungicola* spores was low (20-30 %) at all three time points, whereas on sterilized control casing the spores germinated freely (>98% germination). When 1% of the original microbial population was reintroduced, spore germination was not inhibited on the first day after reintroduction. 2 days after reintroduction, spore germination was slightly reduced (89% germination) and 8 days after reintroduction of the original population spore germination was reduced to 18% and comparable with germination on the untreated control casing. Introduction of *P. fluorescens* CHA0 or *P. fluorescens* CHA89 did not affect spore germination in any of the time points. Also, isolates that were previously effective inhibitors *in vitro,* were unable to affect spore germination. Casing bacterial populations were quantified during the experiment (not shown). On day 1, the number of bacteria in casings to which 1% of the original population was reintroduced was comparable to untreated casing (approximately 10^8 cfu/ml) and significantly higher than the casings with single isolates introduced (10^6-10^7 cfu/g casing). From day 2 onwards, the number of bacteria in casings of different treatment did not differ significantly and remained above 10^8 cfu/ g. casing for all treatments, with the exception of sterilized control casing which remained sterile.

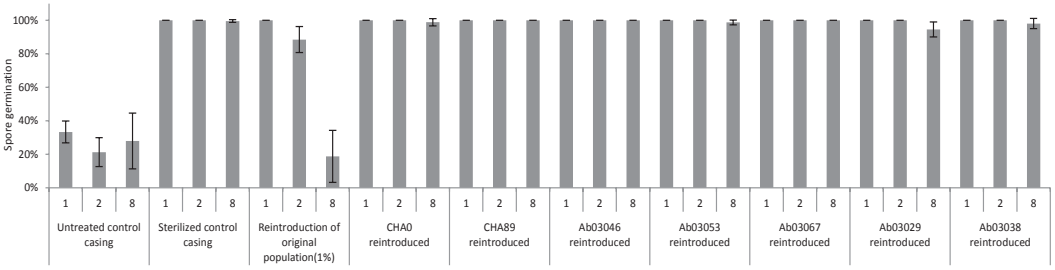

Figure 8. *L. fungicola* spore germination on sterilized casing 1, 2 and 8 days after reintroduction of 1% of the original population or of single *Pseudomonas spp.*. Bars represent the average spore germination on 4 replicate casings after 15 hrs of incubation. Error bars represent standard deviation.

DISCUSSION

Germination of *L. fungicola* spores is inhibited in microbially active casing. Such inhibition of fungal propagules is common to most soils, a phenomenon known as soil fungistasis (Lockwood, 1977). Both competition for nutrients and production of fungistatic compounds have been suggested as the mode of action behind this phenomenon (Lockwood, 1977; Garbeva *et al.*, 2011a). Addition of energy-yielding nutrients most often temporarily reliefs fungistasis, an argument for nutrient deficiency as the mechanism of fungistasis. Germination of fungal spores that do not depend on an exogenous nutrients supply to germinate, could be inhibited by the continuous removal of spore exudates (Hsu and Lockwood, 1973). It was proposed that soil microbiota bring about fungistasis by acting similarly as a nutrient sink. On the other hand, fungistatic compounds, including volatiles, were detected in soils (Hora and Baker, 1972; Xu *et al.*, 2004; Zou *et al.*, 2007; Garbeva *et al.*, 2011a). This implies that fungistasis can also be based on inhibition. We demonstrated that for spores of *L. fungicola,* casing fungistasis is lifted upon sterilization of the casing. Also, when the casing is colonized by *A. bisporus,* the fungistatic effect is eliminated and the *L. fungicola* spores readily germinate. These results confirm the preliminary observations by Cross and Jacobs (1968). Colonisation of the casing by *A. bisporus* likely results in increased carbon availability to microorganisms in the casing. Grewal and Rainey (1991) showed that in exudate of *A. bisporus* mycelium three simple sugars (glucose, mannose and rhamnose) and 17 amino acids were present. Besides carbon sources secreted by the mycelium, available carbon in the casing may be increased by (poly)saccharides shed from cell walls, or cell contents that is released when hyphae are damaged. Especially glycogen, mannitol and trehalose are abundantly present in generative hyphae of *A. bisporus*, and can make up to 20%, 30% and 18% of the total dry weight, respectively (Hammond and Nichols, 1979; Wannet *et al.*, 1999). In this study, we tested 10 saccharides that were previously detected in *A. bisporus* exudate (Grewal and Rainey, 1991), cell content (Wannet *et al.*, 2000), or cell wall (Domenech *et al.*, 2002) for their effects on casing fungistasis. Fructose, galactose, glucose, glycogen, mannitol, mannose, sucrose, trehalose and xylose clearly annulled the inhibition of spore germination on casing. The only saccharide that did not cancel casing fungistasis, rhamnose, could also not be used by *L. fungicola* as a sole carbon source in minimal medium. This indicates that addition of any carbon source that can be used by *L. fungicola* annuls casing fungistasis and makes it unlikely that a particular stimulant of *A. bisporus* specifically stimulates *L. fungicola* spore germination. Annulment of fungistasis after colonization of the casing by hyphae of *A. bisporus* is thus likely a result of nutrients leaching from the hyphae of *A. bisporus*. This seems to implicate that fungistasis is based on a lack of energy for spores of *L. fungicola* in a carbon-limited casing. Although Cross and Jacobs (1968) reported reduced germination of washed

spores on water, we observed high germination rates on water even after rigorous washing of the spores suggesting there is no need for external nutrients to germinate (figure 3). Hsu and Lockwood (1973) showed that the germination of nutrient-independent spores can be inhibited by continuous removal of spore exudates. In a similar system, we demonstrated that germination of *F. oxysporum* microconidia was indeed reduced when exudates were continuously drained (figure 4b). Spores of *L. fungicola*, however, were unaffected by the removal of exudates (figure 4c). Since *L. fungicola* spores do not need external nutrients to germinate, it seems unlikely that the casing fungistasis is caused by nutrient deprivation alone. We did observe reduced germination of *L. fungicola* on water at high spore densities and attributed this to self-inhibition. Such a density-dependent inhibition of *L. fungicola* spore germination was previously reported by Fekete (1967) and is likely involved in preventing early spore germination on the sporophore. Density-dependent inhibition could add to the fungistasis in the casing, but is also lifted when more nutrients become available (figure 5).

De Boer *et al.* (2003) demonstrated that for a coastal dune soil, fungistasis of different fungi could not be fully explained by nutrient deprivation and that microbial community composition was more consistently associated with fungistasis. They concluded that the presence and activity of pseudomonads might be essential for the development of fungistasis in a coastal dune soil. The fluorescent pseudomonads are dominant bacteria in the casing, they are involved in initiation of mushroom formation and their populations increase during colonization of the casing by the mushroom hyphae (Grewal and Rainey, 1991; Miller *et al.*, 1995; Noble *et al.*, 2003). Therefore we investigated the possible inhibitory effect of strains of *Pseudomonas* spp. that were isolated from casing. Some Pseudomonas strains, but not all of the strains we tested, inhibited *L. fungicola* spore germination *in vitro*. We hypothesized that this was correlated with the production of antifungal compounds. *P. fluorescens* strain CHA0 produces a range of antifungal compounds under control of the *gacA/gacS* regulatory system, and its *gacA* knock-out mutant, CHA89, lacks production of these antifungal compounds (Laville *et al.*, 1992). Indeed strain CHA0 suppressed spore germination of *L. fungicola* much better than mutant CHA89, demonstrating that spore germination of this fungus is sensitive to microbial production of antifungal compounds. However, CHA0 did not inhibit spore germination in the presence of 10mM glucose. Thus, production of antibiotics by *P. fluorescens* CHA0 can have a fungistatic effect on *L. fungicola* spores similar to casing fungistasis as it is annulled by addition of glucose. The availability of carbon sources is known to affect production of antibiotics in bacteria (Sanchez *et al.*, 2010). Carbon sources affect antibiotic production mostly through carbon catabolite repression, the repression of genes in the presence of a favored carbon source. This allows microorganisms to rapidly take up a preferred carbon source from a mixture of carbohydrates (Deutscher, 2008; Rokem *et al.*, 2007). Duffy and Defago (1999)

studied the influence of carbon sources on production of the antibiotics 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT) and pyrrolnitrin (PRN) by *P. fluorescens* CHA0. They found that glucose stimulated the production of DAPG and PRN, while it repressed production of PLT. Fructose, glycerol, and mannitol stimulated production of all three antibiotics, whereas sucrose did not affect the production of antibiotics. Because all carbon sources that could be utilized by *L. fungicola* also lifted inhibition by CHA0, and some of these saccharides could not be utilized by *P. fluorescens* CHA0 (figure 6b and table 2), we conclude that these carbon sources lift inhibition by affecting the *L. fungicola* spores. This is in line with Garbeva *et al*. (2011a), who proposed that nutrient-availability affects the sensitivity of fungi to fungistatic compounds. Fungi have ways to deal with fungistatic compounds, for instance by activating efflux pumps or by secreting enzymes (Schoonbeek *et al*., 2002; Duffy *et al*., 2003; Schouten *et al*., 2008), but these are activities that require energy. Addition of energy-yielding nutrients to soils could therefore annul casing fungistasis through the desensitization of fungal propagules.

Sterilization of casing annulled fungistasis (figures 1 and 7). Reintroducing the microflora by mixing in 1% non-sterilized casing, reinstated fungistasis within 2 days, confirming the microbial nature of this phenomenon. However, the introduction of selected strains of *Pseudomonas spp.* that inhibit spore germination *in vitro* did not result in effective fungistasis, suggesting that consortia of micro organisms are required for fungistasis. Indeed studies have indicated that microbial diversity is important for fungistasis (Wu *et al*., 2008; Toyota *et al*., 1996). Bacterial isolates that lack *in situ* activity when applied alone may act synergistically when they are part of microbial consortia (Garbeva and de Boer, 2009; Garbeva *et al*., 2011b). Microbial diversity could also result in a higher collective efficiency of nutrient removal and so cause fungistasis (Garbeva *et al*., 2011a). Consequently, this study does not present conclusive evidence that fungistasis in casing is exclusively based on inhibition. We would like to emphasize that the mechanism of fungistasis is dependent on the fungus under investigation. It is clear that spores that cannot germinate without the presence of external nutrients, such as the spores of *B. cinerea* in this study, will not germinate in soil as a result of nutrient deficiency. *F. oxysporum* spores that become nutrient-dependent when spore exudates are discharged will also be inhibited in soils by the nutrient-sink created by the competing microflora. In both cases, the effect of inhibitory compounds is superimposed on the nutrient deficiency and adds to soil fungistasis. However, inhibitory compounds are prone to play a more prominent role in the fungistasis of spores that germinate independent of external nutrients, such as the spores of *L. fungicola*. Inhibition by fungistatic compounds can provoke nutrient dependency in spores of *L. fungicola* that, without inhibition, can germinate on their endogenously stored reserves. Future research should conclusively demonstrate if this mechanism similarly works in the casing.

Fungistasis can be manipulated to reduce disease incidence. Such manipulation aims to either boost the fungistatic effect or temporarily lift it (Garbeva *et al.*, 2011a). However, for control of dry bubble disease both strategies likely will not work. Primary infection of the casing by *L. fungicola* likely takes place on the mushroom farm (Chapter 2; Berendsen *et al.*, 2010) and temporary relief of fungistasis in the presence of Lecanicillium's host is more likely to aggravate than cure disease. It was demonstrated that steam treatment of the casing prior to *L. fungicola* inoculations, which will lift fungistasis, leads to increased disease incidence (Moore and Wuest, 1973). This indicates that *L. fungicola* can luxuriate in the casing without competition. In this respect, it is interesting to note that formaldehyde treatment of casings just before application of the casing layer, which is meant to clear the casing of mushroom pathogens, is not uncommon. As formaldehyde treatment affects the entire casing microflora, it could possibly elevate casing fungistasis and aggravate dry bubble disease.

A different strategy would be to strengthen casing fungistasis by stimulating the saprophytic community. In plant-pathogenic interactions this is thought to function as it shortens the residence time of nutrients that stimulate fungal growth and so reduce the zone around the plant roots in which the fungistasis is annulled (Garbeva *et al.*, 2011a). In the interaction between *L. fungicola* and *A. bisporus*, this would not likely work as the casing is completely colonized by *A. bisporus*.

For other host-pathogen interactions, it has been shown that the pathogen and consequently the disease can be effectively suppressed through specific interaction with one or a limited number of microbial species (Weller *et al.*, 2002). Such specific suppression can develop naturally during successive crops of the host plant or can be imposed upon a soil by addition of antagonistic bacteria. Some attempts to identify biological control agents effective against *L. fungicola* have been reported (Bhatt and Singh, 2000; Singh *et al.*, 2000; Bhat *et al.*, 2010) , but serious follow up of these studies have not appeared. Dry bubble disease remains a problem for the mushroom industry and new ways to control the disease are needed. Biological control could provide this, however new screenings for biological control agents should take into account that high nutrient availability desensitizes *L. fungicola* spores.

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Effects of fluorescent *Pseudomonas* spp. isolated from mushroom cultures on *Lecanicillium fungicola*

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ABSTRACT

Dry bubble disease, caused by *Lecanicillium fungicola,* is a serious economic problem in the cultivation of the white button mushroom. Biological control of the disease would meet the mushroom industry's efforts to minimize the use of chemicals. A total of 160 bacterial strains were isolated from colonized casing and screened for *in vitro* antagonism of *L. fungicola*. Fifty three isolates inhibited *L. fungicola in vitro*. Using BOX-PCR, the 53 antagonistic isolates were grouped in 18 unique genotypes. Further characterization based on the 16S rDNA identified all isolates as *Pseudomonas spp.*. Using previously characterized Pseudomonas isolates and their mutants it was determined that *L. fungicola* is sensitive to both siderophore-mediated competition for iron and production of antibiotics. However, when tested for disease suppression, none of the *Pseudomonas* spp. strains isolated from colonized casing effectively controlled dry bubble disease. The insensitivity of dry bubble disease to direct biological antagonism and possibilities to implement other modes of action of biological control agents are discussed.

INTRODUCTION

Lecanicillium fungicola (Preuss) Zare and Gams (synonyms: *Verticillium fungicola* (Preuss) Hassebrauk, *Verticillium malthousei* (Preuss) Ware) causes dry bubble disease and is a serious and economically important problem in commercial production of the white button mushroom, *Agaricus bisporus* . Depending on the time point of infection, disease symptoms can vary. Small necrotic lesions on the caps of the fruiting bodies develop when mushrooms are infected late in development. Infections at earlier time points lead to partially deformed fruiting bodies called stipe blow-out or totally deformed and undifferentiated masses of mushroom tissue, the so called dry bubble (North and Wuest, 1993). Annual costs of *L. fungicola* infections for mushroom growers are estimated at 2 - 4% of the total revenue. Control of *L. fungicola* relies on strict hygiene and the use of fungicides. Only few chemicals can be used for control of dry bubble as the fungal host is also negatively affected by many fungicides. The few fungicides that have been used are either no longer available or are becoming increasingly ineffective because of the development of fungicide resistance in the pathogen (Bollen and van Zaayen, 1975; Fletcher and Yarham, 1976; Gea *et al.*, 2005; Wuest *et al.*, 1974). Currently, the control of dry bubble disease relies heavily on the use of prochloraz-manganese (i.e. Sporgon), but reduced sensitivity of the pathogen to this fungicide has been reported (Gea *et al.*, 2005; Grogan, 2008). Therefore, new ways to combat dry bubble disease are urgently needed. *A. bisporus* is generally cultivated on a composted mixture of horse and chicken manure, but will not form fruiting bodies unless the compost is covered with a casing layer. In the casing layer vegetative hyphae of *A. bisporus* aggregate and eventually form mushrooms. Different materials have been used for the casing layer, but mostly peat is the main ingredient (Noble *et al.*, 2003). Mushrooms are not formed on axenic casing, and so it was recognized that the casing microflora is important for the initiation of mushroom formation by *A. bisporus* (Visscher, 1988). Addition of activated carbon can reinstate mushroom formation in axenic casing. Mushroom formation can also be restored by thorough ventilation of axenic casing (Noble *et al.*, 2009). It is therefore assumed that the casing microflora consumes volatile self-inhibiting compound(s) produced by *A. bisporus*. Removal of such compounds by the microflora seems at least partially responsible for the on-set of mushroom formation. Bacterial isolates that stimulated mushroom formation have been identified as *Pseudomonas putida* or close relatives thereof (Hayes *et al.*, 1969; Rainey *et al.*, 1990). However, these single isolates were never as effective in stimulating mushroom formation as the natural microflora.

As the vegetative mycelium of *A. bisporus* is not affected by *L. fungicola*, it is assumed that infection by *L. fungicola* takes place in the casing after the hyphae start aggregating (Cross and Jacobs, 1968; Calonje *et al.*, 2000a; Bernardo *et al.*, 2004) . Germination of *L.*

fungicola spores is effectively inhibited in microbially active casing (Chapter 3;Cross and Jacobs, 1968). Thus, spore germination is postponed until the casing is colonized by *A. bisporus,* resulting in germination of *L. fungicola* just at the right moment, that is when the host is present in a susceptible stage (Chapter 3). The sensitivity of *L. fungicola* to microbial antagonism may be exploited for control of the pathogen. Antagonistic microorganisms in soils have been exploited for effective control of fungal plant pathogens and this has resulted in a number of commercial products (Weller *et al.*, 2002; Fravel, 2005). Mechanisms that are involved in plant disease suppression by bacteria, fluorescent *Pseudomonas* spp. in particular, have been studied in detail over the last decades and include siderophore-mediated competition for iron (Duijff *et al.*, 1999), consumption of pathogen stimulatory compounds (Van Dijk and Nelson, 2000), production of antifungal compounds (Chin-A-Woeng *et al.*, 2003; Haas and Defago, 2005) or lytic enzymes (Shapira *et al.*, 1989), and induction of systemic resistance (Van Loon *et al.*, 1998; Bakker *et al.*, 2007). All these modes of action may also be effective to control *L. fungicola* infections.
Biocontrol bacteria that are introduced into the environment have to compete with the indigenous microflora. Often, the abundance of the introduced biocontrol bacterium decreases in time, resulting in reduced protection against the pathogen (Mazzola *et al.*, 1992; Raaijmakers *et al.*, 1995; Lugtenberg and Dekkers, 1999). Thus the selection of possible biocontrol agents for the dry bubble disease should focus on microorganisms that naturally occur in the casing soil in high densities, and preferably associated with *A. bisporus*. In the compost and the casing soil bacteria are in close contact with the *Agaricus* mycelium (Masaphy *et al.*, 1987; Miller *et al.*, 1995). Fluorescent *Pseudomonas* spp. form a dominant group within the bacterial casing microflora and population densities of these pseudomonads increase when the casing is colonized by *A. bisporus* (Doores *et al.*, 1986; Samson, 1986; Miller *et al.*, 1995; Fermor *et al.*, 2000; Pardo *et al.*, 2002). The increase in populations of *Pseudomonas* spp. is comparable to the superior ability of fluorescent pseudomonads to colonize the rhizosphere (Lugtenberg and Dekkers, 1999) and may be explained by the fact that *A. bisporus* secretes sugars, amino acids and volatiles (Grewal and Rainey, 1991; Noble *et al.*, 2009) that stimulate growth and activity of these bacteria. Interestingly, *Pseudomonas* spp. are the most extensively studied group of biocontrol bacteria (Chin-A-Woeng *et al.*, 2003; Bakker *et al.*, 2007; Weller, 2007; Höfte and Altier, 2010) because they have many traits that make them effective biocontrol agents of plant pathogens. They can grow on a wide variety of substrates, are abundant in nature, have a high growth rate, grow at relatively low temperatures and have a variety of mechanisms to suppress growth of other microorganisms (Weller, 2007). These bacterial species are thus also interesting candidates for biocontrol of *L. fungicola*.
Some attempts to identify biological control agents effective against *L. fungicola* have been reported (de Trogoff and Ricard, 1976; Bhatt and Singh, 2000; Singh *et al.*, 2000;

Bhat *et al.*, 2010), but serious follow up has not been published. Moreover, culture tests are necessary to confirm the efficacy of the bacteria in controlling the dry bubble disease (Largeteau and Savoie, 2010). This study aimed at identifying bacteria in the mycosphere of *A. bisporus* that are antagonistic to *L. fungicola* and that can effectively suppress dry bubble disease. Known biocontrol agents of plant pathogens were used to identify possible mechanisms for biocontrol of dry bubble disease. It was found that, *in vitro, L. fungicola* was sensitive to competition for iron and to antibiosis. *In vitro* antagonistic isolates that were isolated from the colonized casing and compost were further characterized and screened for their ability to control the dry bubble disease in mushroom cultures. The effectiveness of the putative biocontrol agents was compared to that of prochloraz-manganese.

METHODS

FUNGAL CULTURES

Spore suspensions (10^6 spores /ml) of *L. fungicola* strain V9503 were stored in phosphate buffer (0.21 M NaH_2PO_4, pH 7.2) with 10 % glycerol at -80 ˚C. *L. fungicola* strain V9503 was cultured on potato dextrose agar (PDA) (Difco, Lawrence, USA) by plating 100 µl of spore suspension and incubating for 5 days at 24 ˚C. Spore suspensions were prepared from these cultures by adding 10 ml of demineralized water (DEMI) to each Petri dish and filtering over sterile glass wool to remove mycelial fragments. Densities of the suspensions were determined using a haemocytometer.

ISOLATION OF BACTERIA

Bacteria were isolated from aggregated hyphae of *A. bisporus* taken from colonized casing. Colonized casing was sampled from mushroom production cultures of *A. bisporus* when the first pins appeared from the casing. Aggregated hyphae were isolated from the casing using tweezers and they were incubated for 20 min in 10 mM MgSO4 to remove loosely-adhering bacteria. Firmly attached bacteria were brought in suspension through sonication as described by Cho *et al*. (2003). Suspensions were plated on King's medium B agar (KB agar (King *et al.*, 1954)), 10% tryptic soy agar (TSA; per litre: 3 g tryptic soy broth (Difco), 15 g granulated agar (Difco)), Casing extract agar (Rainey, 1991) and *A. bisporus* mycelial exudate agar (mycelial exudates as described by Grewal and Rainey (1991), 15 g granulated agar (Difco) per liter) and incubated at 28˚C for 48 hrs. From each of these media, 40 colonies were selected randomly and streaked to confirm purity. To exclude *Pseudomonas tolaasii* from the bacterial collection the so called white-line test, that is indicative for this bacterial mushroom pathogen (Wong and Preece, 1979), was used. Bacteria were stored in KB with 20% glycerol at -80 ˚C.

SCREENING FOR IN VITRO ANTAGONISM TO *L. FUNGICOLA*

The bacterial isolates were point inoculated on Pseudomonas agar F (Difco) and incubated for 2 days at 24 ˚C. Subsequently, a *L. fungicola* spore suspension (10^6 conidia / ml) was atomized over the plates using a test tube atomizer (Desaga GmbH, Wiesloch, Germany). Plates were sealed with Parafilm and incubated at 24 ˚C. The bacterial colony diameter and the diameter of the zone in which growth of *L. fungicola* was inhibited were determined after 3 days. The effect of siderophore and antibiotic production on *L. fungicola* was determined with well-studied biocontrol strains and mutant derivatives (table 1).

Table 1. Biocontrol strains and their mutant derivatives that were used in this study

Strain	Relevant characteristics	References
P. fluorescens CHA0	Isolated from tobacco rhizosphere; produces DAPG, PLT, PRN and HCN; produces iron regulated PSB, PCH and SA	(Laville *et al.*, 1992)
CHA89	*gacA* mutant of CHA0; lacks production of DAPG, PLT, PRN and HCN ; produces PSB, PCH and SA	(Laville *et al.*, 1992)
CHA631	*phlA* mutant of CHA0, does not produce DAPG	(Schnider-Keel *et al.*, 2000)
CHA638	*phlF* mutant of CHA0, constitutively produces DAPG	(Schnider-Keel *et al.*, 2000)
P. putida WC-S358r	Spontaneous rifampicin-resistant mutant of WCS358. Wild type isolated from potato rhizosphere, produces PSB	(Geels and Schippers, 1983; Glandorf *et al.*, 1992)
WCS358-JM213	Tn*5* mutant of WCS358r that does not produce PSB	(Marugg *et al.*, 1985)
WCS358::phl	Transformant of WCS358 constitutively producing DAPG	(Bakker *et al.*, 2002a)
WCS358::phz	Transformant of WCS358r constitutively producing PCA	(Bakker *et al.*, 2002a)
P. fluorescens WCS374r	Spontaneous rifampicin-resistant mutantof WCS374. Wild type isolated from potato rhizosphere; produces PSB, (PSM) SA	(Geels and Schippers, 1983)
WCS374- 02	Psb^-, Psm^+, SA^+, Tn*5* transposon mutant of WCS374	(Weisbeek *et al.*, 1986)
WCS374- 4A1	Psb^+, Psm^-, SA^+, *pmsA* mutant of WCS374r obtained by site-directed mutagenesis	(Djavaheri, 2007) De Vleesschauwer et al., 2008
WCS374- AT12	Psb^-, Psm^-, SA^+, Tn*5* transposon mutant of 4A1	(Djavaheri, 2007) De Vleesschauwer et al., 2008
WCS374- 4B1	Psb^+, Psm^-, SA^-, *pmsB* mutant of WCS374r obtained by site-directed mutagenesis	(Djavaheri, 2007) De Vleesschauwer et al., 2008
WCS374- BT1	Psb^-, Psm^-, SA^-, Tn*5* transposon mutant of 4B1	(Djavaheri, 2007) De Vleesschauwer et al., 2008
P. fluorescens WCS417r	Spontaneous rifampicin-resistant mutant of WCS417. Wild type was isolated from wheat rhizosphere	(Lamers *et al.*, 1988; Van Wees *et al.*, 1997)
WCS417-S634	Tn*5* mutant of WCS417 that does not produce PSB	(Duijff *et al.*, 1993)
RS111-a	Unspecified antifungal compound	(de Boer *et al.*, 1999; Bakker *et al.*, 2002b)

(DAPG)2,4-diacetylphloroglucinol (PLT) pyoluteorin (PRN) pyrrolnitrin (PSB) pseudobactin (PCH) pyochelin (SA) salicylic acid (PCA) phenazine-1-carboxylic acid (PSM) pseudomonine

BOX-PCR ANALYSIS OF ANTAGONISTIC ISOLATES

Isolates were cultured on KBA at 28 ˚C for 1 day. Single colonies were transferred to 20 µl sterile lysis buffer (0.05 M NaOH, 0.25 % SDS) and heated to 95 ˚C for 20 minutes. Lysates were quickly cooled on ice and 200 µl cold sterile water was added. Debris was pelleted by brief centrifugation (12.000 x *g*) and lysates were stored at -20 ˚C.
BOX-PCR was performed with primer BOX A1R (5' CTA CGG CAA GGC GAC GCT GAC G) (Versalovic *et al.*, 1994). 0.5 µl of the colony lysate was added to a total volume of 25 µl BOX-PCR-reaction mix (1x Stoffel buffer, Applied Biosystems, Foster City, Ca, USA), 3.75 mM $MgCl_2$, 600 µM of each dNTP, 1 µM primer BOX A1R and 2.5 U AmpliTaq Stoffel fragment DNA polymerase (Applied Biosystems, Foster City, Ca, USA). PCR conditions used in the thermocycler (Hybaid, Ashford, UK) were 7 min at 95 ˚C, followed by 30 cycles of 30 s 90 ˚C, 1 min 95 ˚C, 1 min 52 ˚C and 8 min 65 ˚C, and a final extension of 16 min at 65 ˚C. Amplified PCR fragments were analyzed by electrophoresis on 1.5 % agarose gels in 1x TAE buffer (40 mM Tris-acetate/1 mM EDTA, pH 8).

IDENTIFICATION OF ANTAGONISTIC ISOLATES

The V6-V8 region of the *16S rRNA* gene was amplified with primers 968f_GC and 1401R (Nübel *et al.*, 1996). 1 µl of the colony lysate (see above) was added to a total volume of 50 µl PCR reaction mixture (1x PCR buffer 2, Roch Diagnostics, Mannheim, Germany), 250 µM of dNTPs, 200 nM of each primer, and 2.5 U Expand Long Template enzyme (Roch Diagnostics). PCR conditions used in the thermocycler (Hybaid, Ashford, UK) were 5 min at 94 ˚C, followed by 35-40 cycles of 1 min 94 ˚C, 1 min 66 ˚C and 3 min 72 ˚C, and a final extension of 10 min at 72 ˚C. Presence of PCR product was checked by electrophoresis on 1.5 % agarose gels in 1x TAE buffer (40 mM Tris-acetate/1 mM EDTA, pH 8). PCR-products were sequenced by Baseclear (Leiden, the Netherlands). Ribosomal database project (RDP) Classifier was used to assign taxonomic lineage to the genus level (http://rdp.cme.msu.edu).

BIOASSAY FOR SUPPRESSION OF DRY BUBBLE DISEASE

Isolates were grown on KBA overnight at 28 ˚C. Cells were harvested in 10 ml $MgSO_4$ and washed twice by centrifugation (10 min, 4500 rpm) The pellet was resuspended in sterile 10 mM $MgSO_4$ and cells were added to casing soil (CNC, Milsbeek, the Netherlands) at a density of 10^6 colony forming units (cfu)/g of casing. Mushrooms were cultured in Economic premium climate chambers (Snijders scientific, Tilburg, the Netherlands, dimensions, 99 x 63 x 134 cm). For each isolate, three plastic containers (20 x 30 x 22.5cm) were filled with 3.5 kg of compost colonized by *A. bisporus* strain A15 (CNC, Milsbeek,

the Netherlands) and covered with 1 kg of casing mix (CNC, Milsbeek, the Netherlands). Five isolates were tested in each experiment and 3 control containers were taken along in which casing soil was treated with sterile 10 mM $MgSO_4$.
Conditions were set to 24 ˚C and 95 % relative humidity(RH) for the first 8 days and subsequently set to 20 ˚C and 88 % RH until the end of the experiment. On day 8, casing was inoculated with *L. fungicola* V9503 by spreading 50 ml of a conidial suspension (6.10^4 conidia/ ml) on the casing of each box, resulting in an inoculum density of 10^6 conidia/ m^2 of casing. As a positive control, containers were treated with 200 ml 0.1 % Sporgon (BASF, Arnhem, the Netherlands) immediately after inoculation with *L. fungicola*. The first two flushes were harvested and scored in 4 categories: healthy, spotted cap, stipe blow-out and dry bubble (Berendsen *et al.*, 2010). Weight and number of mushrooms in each category was determined.
Isolates *Ab03038* and *Ab3040* (table 2) were tested as described before but with 18 replicates per treatment evenly distributed over 3 climate chambers. Six replicates per treatment were used in experiments to compare effects of *P. fluorescens* CHA638 and CHA631, mutant derivatives of *P. fluorescens* CHA0 that either overproduces (CHA638) or no longer expresses (CHA631) DAPG, were those of their wild type CHA0.

SURVIVAL OF BACTERIA IN THE CASING AND ATTACHMENT TO MUSHROOMS.

The survival of the rifampicin-resistant *P. fluorescens* WCS417r and WCS374r, and *P. putida* WCS358r was tested during the bioassay for suppression of dry bubble disease. Additionally, bacterial survival was investigated by incubating casing containing the bacteria (added as described above) in Petri dishes. Plates were sealed with Parafilm and incubated at 24 ˚C. Approximately 1 g of casing was taken from the mushroom cultures and non-colonized casings 0, 7, 14 and 22 days after addition of bacteria. Casing samples of mushroom cultures and non-colonized casings were suspended in 10 ml 10 mM $MgSO_4$. Dilution series of the suspensions were plated on 10 % TSA for quantification of the total culturable bacteria and on KBA supplemented with 150 µg rifampicine and 100 µg Delvocid (DSM Food Specialties, Delft the Netherlands) per ml for quantification of the rifampicin-resistant pseudomonads.
To determine population densities of the introduced bacteria on the mushroom caps, healthy mushrooms were harvested at day 23, and bacteria were suspended in 20 ml 10 mM $MgSO_4$ by shaking for 2 hrs at 4 ˚C and sonication for 2 times 30 seconds in a sonication bath (Julabo). Dilution series of the suspensions were plated as described previously.

ASSAY FOR ANTAGONISM ON MUSHROOM CAPS.

Fresh mushrooms of strain Sylvan A15 (Hooijmans Champignons, Kerkdriel, the Netherlands) were harvested and the stipe was removed. Bacterial cell suspensions of selected isolates (10^9 cfu/ml) and a conidial suspension of *L. fungicola* (10^6 conidia/ml) were prepared as describe above. The suspensions of *L. fungicola* conidia were mixed with each bacterial cell suspension in a 1:1 (v:v) ratio. Fifteen mushrooms were inoculated for each isolate, by placing 2 µl of the mixture on the surface of a mushroom cap, halfway in between the centre of the mushroom cap and its edge. As a control, 15 mushroom caps were inoculated with a suspension of *L. fungicola* mixed with 0.1 % Sporgon (BASF, Arnhem, the Netherlands). Moreover, each mushroom cap was inoculated at the opposite side with a suspension containing *L. fungicola* conidia only. The inoculated mushrooms were incubated in an Economic premium climate chamber (Snijders scientific, Tilburg, the Netherlands) at 24 ˚C and 95 % relative humidity. Diameters of the lesions were determined after 4 days of incubation.
Antagonism against *L. fungicola* was tested for two isolates on mushrooms that were not harvested. Mushroom of strain Sylvan A15 were cultivated as described above. Mixtures of bacterial cells and *L. fungicola* conidia were prepared for the isolates Ab03128 and Ab03038 (table 2) as described above. Eight mushrooms of 0.5 – 1 cm in diameter were inoculated with a mixture of bacterial cells and *L. fungicola* conidia on one side of the cap and with conidia of *L. fungicola* on the other side. Lesion diameters were scored after 4 days.

STATISTICS

Statistical analysis was performed with SPSS 16.0. Significant differences in the bioassay for suppression of dry bubble disease were analyzed by analysis of variance (ANOVA) with a Tukey post-hoc test ($p < 0.05$) of the treatments in one climate chamber. The bioassay of isolates Ab03038 and Ab3040 with 18 replicates was performed in a randomized block design with each climate chamber as a block. Lesion size of *L. fungicola* infections on a mushroom cap were compared to control lesions with a paired Students T-test. Bacterial plate counts were $^{10}\log$ transformed before ANOVA with a Tukey post-hoc test ($p < 0.05$).

RESULTS

ISOLATION OF BACTERIA AND SCREENING FOR *IN VITRO* ANTAGONISM

In total 160 bacterial strains were isolated from casing colonized by *A. bisporus*. The isolates were screened for their abilities to inhibit spore germination and hyphal growth of *L. fungicola* on KBA. The majority of the isolates (107) did not affect germination and growth of *L. fungicola* (figure 1a). The remaining 53 isolates did inhibit *L. fungicola* in the zone surrounding their colony. Growth of *L. fungicola* was slightly reduced by 37 of these isolates (figure 1b), whereas 16 isolates produced clear zones of inhibition (figure 1c).

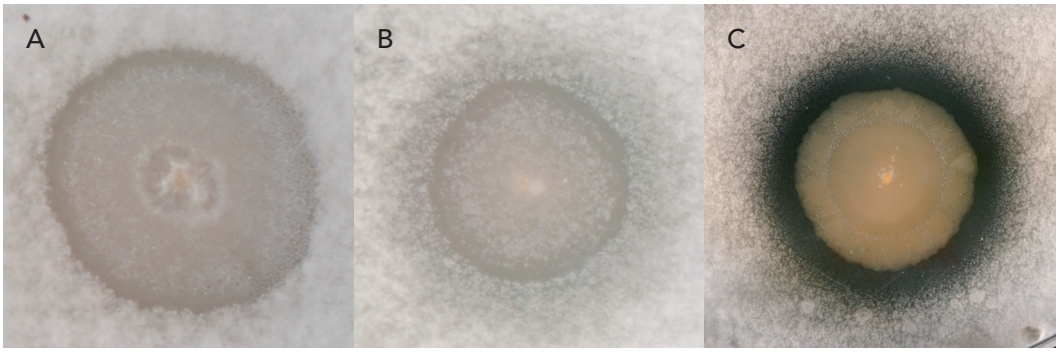

Figure 1. *In vitro* antagonism of bacterial isolates to *L. fungicola* after 72 hrs. In a zone around their colony, bacteria: [A)] did not affect growth of *L. fungicola*, [B)] reduced growth of *L. fungicola*, [C)] inhibited growth of *L. fungicola*.

The genotypic diversity of the antagonistic isolates was determined using BOX-PCR (table 2). Fifteen out of the 53 box-fingerprints were unique. The remaining 38 BOX-fingerprints comprised 3 different BOX-types, consisting of 12, 12 and 14 isolates, respectively. 16S rDNA was amplified for at least 1 isolate of each BOX-type and sequenced (Baseclear, Leiden, the Netherlands). RDP Classifier was used to assign taxonomic lineage to genus level. All sequences were assigned to the genus *Pseudomonas* with an estimated reliability of 100%.

Table 2. Overview of the *Pseudomonas* spp. selected for *in vitro* antagonism against *L. fungicola*

BOX-type	Frequency	Type of *in vitro* antagonism (figure 1)	Selected isolate	Taxonomy as assigned by RDP classifier
I	1	B	Ab03023	*Pseudomonas*
II	1	B	Ab03037	*Pseudomonas*
III	12	B	Ab03040	*Pseudomonas*
IV	1	C	Ab03046	*Pseudomonas*
V	1	C	Ab03053	*Pseudomonas*
VI	14	B	Ab03038	*Pseudomonas*
VII	12	C	Ab03029	*Pseudomonas*
VIII	1	B	Ab03056	*Pseudomonas*
IX	1	C	Ab03067	*Pseudomonas*
X	1	B	Ab03083	*Pseudomonas*
XI	1	B	Ab03084	*Pseudomonas*
XII	1	B	Ab03091	*Pseudomonas*
XIII	1	C	Ab03108	*Pseudomonas*
XIV	1	B	Ab03119	*Pseudomonas*
XV	1	B	Ab03125	*Pseudomonas*
XVI	1	B	Ab03155	*Pseudomonas*
XVII	1	B	LDab0012	*Pseudomonas*
XVIII	1	B	LDab0017	*Pseudomonas*

IN VITRO EFFECTS OF BACTERIAL ANTIBIOTIC AND SIDEROPHORE PRODUCTION ON *L. FUNGICOLA*

In vitro antagonism of known biocontrol agents against *L fungicola* was investigated on KBA and KBA with 200 µM $FeCl_3$. Sensitivity of *L. fungicola* to antibiosis was investigated using strains of fluorescent pseudomonads that are known producers of antifungal compounds. *P. fluorescens* RS111a, CHA0, CHA89, CHA631 and CHA638 and *P. putida* WCS358, WCS358::phl and WCS358::phz all inhibited the growth of *L. fungicola* on KBA (figure 2a). Addition of $FeCL_3$ abolished the inhibition by CHA89 and WCS358. Both strains do not produce antifungal compounds. CHA89 is *gacA* knock-out of CHA0 impaired in the production of antibiotics. The inhibition zone produced by CHA0 was not affected by the iron concentration of the medium. Mutants of WCS358, that were generated to constitutively produce either PCA or DAPG, produced a bigger inhibition zone under iron-rich conditions than the wild type. It can be concluded that on iron-rich medium, *L. fungicola* inhibition is mediated by antibiotics.

Mutants defective in the production of siderophores have been generated for *P. fluorescens* strains WCS374r and WCS417r, and *P. putida* strain WCS358r. The three wild-type strains all produce pseudobactin. Analysis of the pseudobactins of WCS358 and WCS374

revealed that their structures are different (Fuchs *et al*., 2001; Djavaheri, 2007). In addition to pseudobactin, WCS374r also produces the siderophores pseudomonine and salicylic acid (SA) (Mercado-Blanco *et al*., 2001). Siderophore knock-out mutants of these three strains were compared to the parental strains for their ability to inhibit *L. fungicola*. The wild type strains all inhibited *L. fungicola* on KBA, whereas the pseudobactin knock-out mutants were less (WCS374r) or not (WCS358r and WCS417r) inhibitory (figure 2b). WCS374r-4A1, in which the production of pseudomonine is knocked out and which consequently produces more pseudobactin (Djavaheri, 2007) showed more inhibition of *L. fungicola* than the wild-type. This additional inhibition is not observed for WCS374r-4B1, which only produces pseudobactin and not SA. WCS374r-AT12, in which production of both pseudobactin and pseudomonine is knocked out, did not inhibit *L. fungicola*. It can be concluded that on iron poor medium, *L fungicola* inhibition is mediated by siderophores and especially pseudobactin.

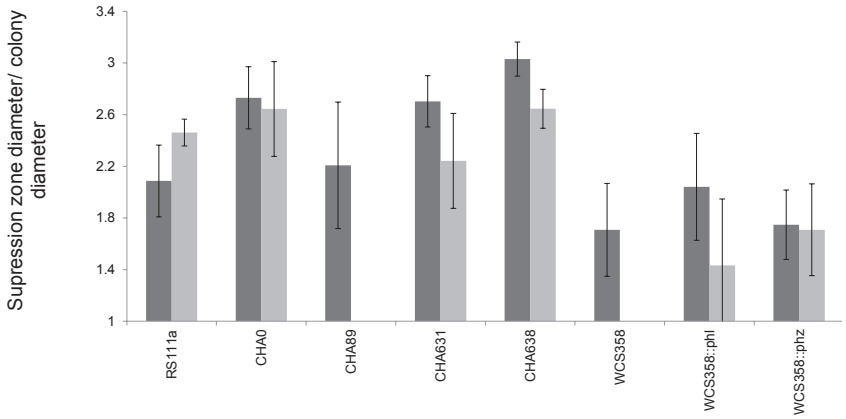

Figure 2a. Quantification of *in vitro* antagonism of RS111a, CHA0, WCS358r and various mutant strains to *L. fungicola*. CHA0 produces 4 antibiotics (DAPG, PLT, PRN and HCN). Its mutant derivatives are affected in the production of antibiotics: CHA89(ß*gacA*) produces no antibiotics; CHA631(ß*phlA*) does not produce DAPG, whilst CHA638(ß*phlF*) overproduces DAPG. WCS358r produces no known antibiotics but its mutant derivatives constitutively produce DAPG (WCS358::phl) or PCA (WCS358::phz). Bars represent the average diameter of the inhibition zone around three bacterial colonies on KB (dark bars) or on KB amended with $FeCL_3$ (light bars). Error bars represent standard deviations.

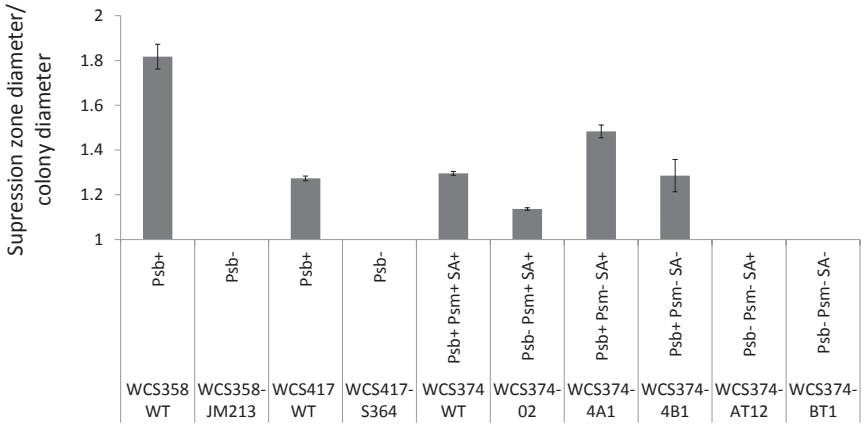

Figure 2b. Quantification of *in vitro* antagonism of WCS358r, WCS417r, WCS374r and various mutant strains to *L. fungicola*. Mutants derived from WCS358 and WCS417 do not produce siderophores. Mutants derived from WCS374r have the following characteristics: 02 (Psb-, Psm+, SA+), 4A1 (Psb+, Psm-, SA+), AT12 (Psb-, Psm-, SA+), 4B1 (Psb+, Psm-, SA-), and BT1 (Psb-, Psm-,SA-), where Psb = pseudobactin and Psm = pseudomonine. Bars represent the average diameter of the inhibition zone around three bacterial colonies on KB. Error bars represent standard deviations.

EFFECTS OF SELECTED BACTERIAL ISOLATES ON DRY BUBBLE DISEASE

Unique BOX-PCR fingerprint representatives of the *Pseudomonas* spp. isolates that were antagonistic to *L. fungicola in vitro*, were tested for their ability to control dry bubble disease in mushroom cultures (figure 3). The bacteria were mixed through the casing at a density of 10^6 cfu/g casing before applying the casing layer to the compost. The pathogen was inoculated on the casing 8 days after applying the casing. Within 2 - 3 weeks after casing application a first flush of mushrooms appeared

Sporgon reduced the dry bubble disease significantly. Addition of antagonistic bacteria to the casing of mushroom cultures never significantly reduced dry bubble disease compared to the untreated control. Disease incidence in cultures treated with bacteria ranged from 55 % of the disease in the control of the same experiment for isolate Ab3038 to 114 % of the disease in the control for Ab3108. Isolates Ab03038, Ab03040, Ab03037, Ab03046, Ab03023 and WCS358 were found to reduce disease incidence with more than 20 % in the first experiments and were therefore tested again in subsequent experiments. Only Ab3038 and Ab3040 reduced disease consistently with more than 20 %, but never statistically significant. However, this bioassay was performed with three replicates.

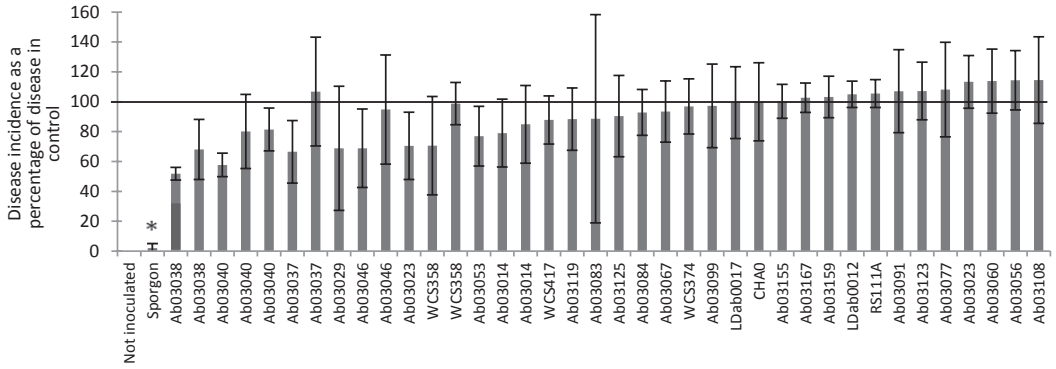

Figure 3. Effect of selected *Pseudomonas* isolates on dry bubble disease. Bars represent the average percentage of disease in 3 treated mushroom cultures relative to disease in the control-treatment which was set to 100 %. Error bars represent standard deviation. * denotes significant difference from control treatment.

To increase the power of the bioassay, it was therefore performed with 18 replicates for each of these strains. Compared to the control treatment, disease was slightly, but not significantly, reduced to 91 % by Ab03038 and 97 % by Ab03040 (figure 4). This experiment was repeated with similar results.

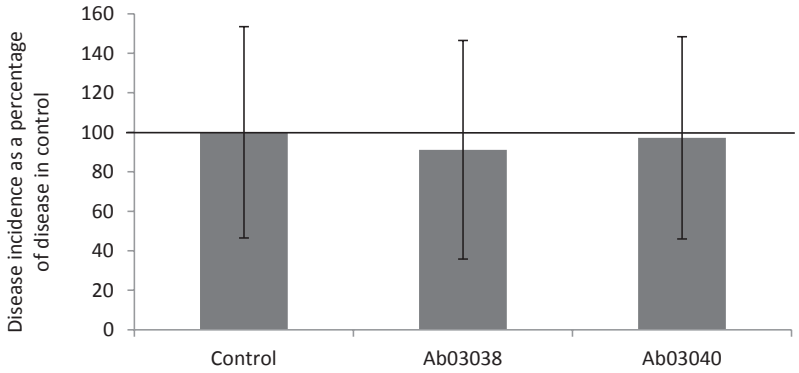

Figure 4. Effect of *Pseudomonas* isolates Ab03038 and Ab03040 on dry bubble disease. Bars represent the average percentage of disease in 18 treated mushroom cultures relative to disease in the control-treatment, which was set to 100 %. Error bars represent standard deviation.

P. fluorescens CHA0 was the most effective antagonist *in vitro*, but *in vivo* did not affect disease incidence (figure 3). To exclude the possibility that the effective antibiotic

DAPG was not produced by CHA0 in the casing, a mutant that constitutively produces DAPG (CHA638, *phlF knock-out)* was included in the bioassay, and as a control mutant CHA631 (*phlA knock-out)* that does not produce DAPG. Both the wild type CHA0 and the mutants CHA638 or CHA631 (figure 5a and 5b) did not affect disease incidence significantly making it unlikely that bacterially produced DAPG can contribute to control of dry bubble disease.

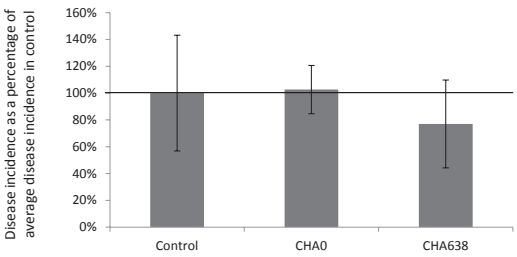

Figure 5a. Effect of *P. fluorescens* CHA0 and CHA638 on dry bubble disease. Bars represent the average percentage of disease in 6 treated mushroom cultures relative to disease in the control-treatment, which was set to 100 %. Error bars represent standard deviation.

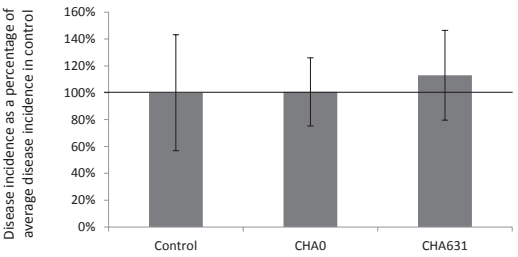

Figure 5b. Effect of *P. fluorescens* CHA0 and CHA631 on dry bubble disease. Bars represent the average percentage of disease in 6 treated mushroom cultures relative to disease in the control-treatment, which was set to 100 %. Error bars represent standard deviation.

ASSAY FOR ANTAGONISM ON THE MUSHROOM CAPS

To study interactions between the bacterial isolates selected in this study and *L. fungicola* on the fruiting body of Agaricus, suspension of the pathogen, in which the bacteria were also suspended were prepared. Development of lesions was measured after inoculation with a suspension of the pathogen, in which the bacteria were also suspended (treatment) or not (control). Two µl droplets of these suspensions were inoculated on the caps of harvested mushroom. Lesions appeared within 2 days and their diameters were measured after 4 days (figure 6a). None of the bacterial isolates significantly affected lesion diameter. The treatment with Sporgon drastically reduced lesion diameter.

Because harvested mushrooms start decaying rapidly after they have been detached from the mycelium (Brennan *et al.*, 2000) and thus may have increased levels of nutrients available to the pathogen that can interfere with bacterial antagonism (Garbeva *et al.*, 2011a), two isolates were also tested on mushrooms still attached to the mycelium. The effect of the isolates Ab03038 (most effective in bioassay) and Ab03128 (not antagonistic *in vitro*) and Sporgon was tested on growing mushrooms (figure 6b). Sporgon completely

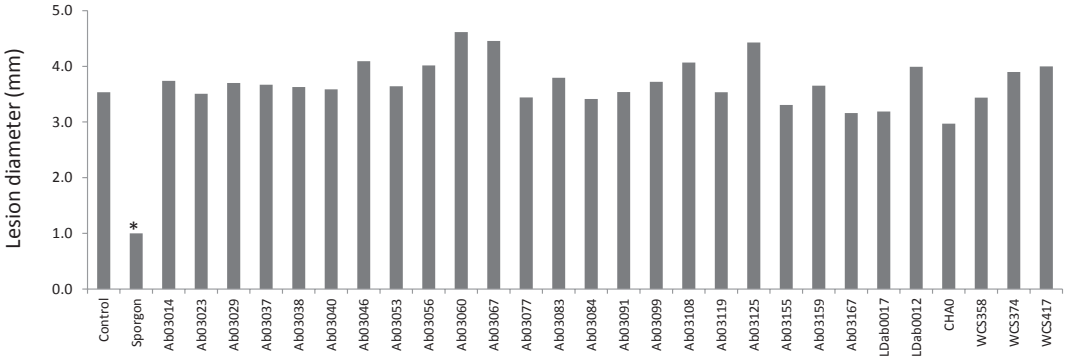

Figure 6a. Lesion diameter on harvested mushroom caps 4 days after inoculation with a suspension of *L. fungicola* spores and selected bacterial strains. Asterisk denotes significantly smaller lesion diameter than the control treatment.

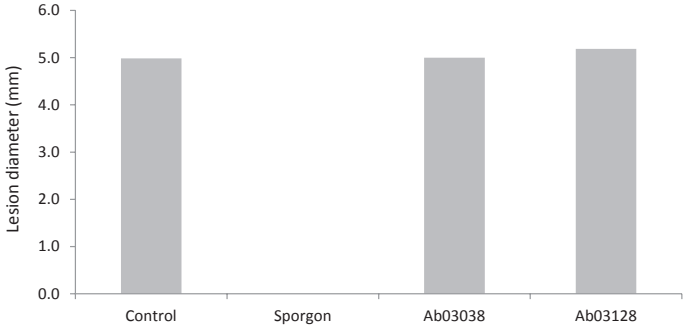

Figure 6b. Lesion diameter on mushroom caps that were still attached to the mycelium, 4 days after inoculation with a suspension of *L. fungicola* spores and selected bacterial strains.

SURVIVAL OF BACTERIA IN THE CASING

The ability of strains of fluorescent *Pseudomonas* spp. to maintain relatively high population densities is an important trait in relation to their abilities to control diseases. Therefore, the survival of rifampicin-resistant derivatives of *P. fluorescens* WCS374r and WCS417r, and *P. putida* WCS358r was tested in the casing in the bioassays (figure 7a). In the first week population densities of total bacteria, as determined on 1/10 TSA, increased 71 fold. In this week the casing was colonized by vegetative hyphae of *A. bisporus*. In the 2 weeks that followed, total number of bacteria decreased again. During the experiment, populations of WCS358r followed a pattern similar to those of the total culturable bacteria. Populations of WCS417r remained stable throughout the experiment, while the population of WCS374r decreased from 10^6 cfu/g casing at the start of the experiment to approximately 10^4 cfu/g casing at day 22. On day 23 of the bioassay, presence

of bacteria on mushrooms of the second flush was investigated. For each treatment, the total number of culturable and the number of rifampicin-resistant bacteria on the caps of healthy mushrooms were determined (figure 7b). The total number of bacteria did not differ significantly between the 3 treatments and ranged between 10^5 and 10^6 cfu/g of mushroom cap. The numbers of WCS374r on mushrooms were significantly lower than of WCS417r and WCS358r. The numbers of WCS358r and WCS417r on mushrooms did not differ significantly. The proportions of the selected bacteria on the mushrooms were comparable to the proportions in the casing on day 22 with less cfu of WCS374r than the other two bacteria.

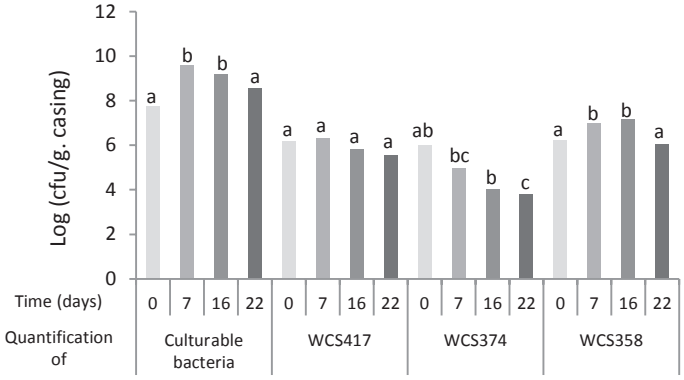

Figure 7a. Numbers (log cfu / g casing) of total culturable bacteria and rifampicine-resistant strains in the casing during a bioassay. Letters indicate significant differences between time points. Bars represent the average number of 3 samples for the 3 rifampicine-resistant strains and of all 9 samples for the culturable bacteria.

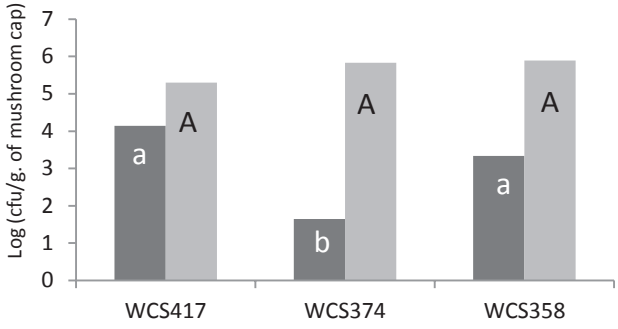

Figure 7b. Numbers (log cfu / g casing) of total culturable bacteria (light bars) and rifampicine-resistant strains (dark bars) on mushroom caps during a bioassay. Letters indicate significant differences between treatments. Bars represent the average number of 3 samples.

Survival of WCS417r, WCS374r and WCS358r was also determined in casing that was not colonized by mycelium of *A. bisporus* (figure 8). None of the bacteria were able to proliferate in this casing soil, and the population densities of all strains declined significantly in time. Again, population densities of WCS374r were lower than those of WCS417r and WCS358r.

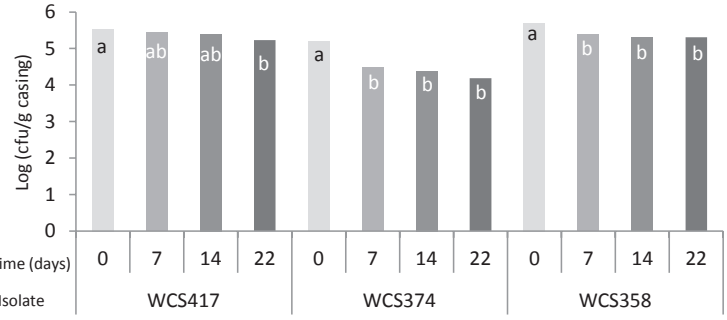

Figure 8. Numbers (log cfu /g casing) of rifampicine-resistant strains in the casing without hyphae of *A. bisporus*. Letters indicate significant differences between time points within each treatment. Bars represent the average number of 3 samples for the 3 rifampicine-resistant strains and of all 9 samples for the culturable bacteria.

DISCUSSION

Biological control of fungal pathogens using antagonistic bacteria has proven its merits in the management of plant diseases (Penyalver *et al.*, 2000; Raaijmakers *et al.*, 2009; Bailey *et al.*, 2010). We therefore investigated the potential of antagonistic bacteria to control *L. fungicola,* the causal agent of dry bubble disease of the white button mushroom.

One hundred and sixty bacterial isolates were obtained from casing colonized by *A. bisporus*. We identified 53 isolates that can inhibit *in vitro* growth of *L. fungicola.* These isolates comprised 18 genotypes as determined with BOX-PCR, all of which belonged to the genus *Pseudomonas*. This is not a surprising result because this genus is a dominant part of the microbial community of casing, especially after colonization of *A. bisporus* (Doores *et al.*, 1986; Samson, 1986; Miller *et al.*, 1995; Fermor *et al.*, 2000; Pardo *et al.*, 2002). The potential of *Pseudomonas* spp. to control plant diseases has been widely recognized (Weller, 2007) and this has led to the development and registration of commercial products (Haas and Defago, 2005; Fravel, 2005; Stockwell and Stack, 2007; Höfte and Altier, 2010).

Most of the antagonistic isolates that inhibited *L. fungicola* on KBA produced a yellow fluorescent pigment, a pseudobactin/pyoverdine type siderophore (Fuchs *et al.*, 2001). These siderophores have been implicated to play a role in disease suppression by effective competition for iron with the pathogen (Duijff *et al.*, 1993; Duijff *et al.*, 1999; Loper and Henkels, 1999). It appears that *L. fungicola* is sensitive to siderophore-mediated competition for iron since well characterized siderophore negative mutants of *P. fluorescens* WCS374r and WCS417r, and *P. putida* WCS358r did not inhibit the pathogen or inhibited to a lesser extent compared to the parental strain (figure 2b). Under iron rich conditions, strains WCS417r, WCS358r and WCS374r no longer or to a lesser extent inhibited *L. fungicola.* However, the pathogen is also sensitive to other metabolites produced by fluorescent pseudomonads since *P. fluorescens* RS111a and CHA0 also inhibited *L. fungicola* when iron was in abundance (figure 2a). CHA0 produces the antifungal metabolites 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, and cyanide, under the control of the *gacA/gacS* regulatory system (Dubuis and Haas, 2007; Laville *et al.*, 1992). Whereas CHA0 inhibited growth of *L. fungicola* both under iron deficient and iron rich conditions, mutant CHA89, a *gacA* knock-out derivative of CHA0 that does not produce these antibiotics, only inhibited *L. fungicola* when iron was deficient (figure 2a), suggesting that the fungus is sensitive to at least one of the antifungal metabolites of CHA0. Further evidence for the sensitivity of *L. fungicola* to 2,4-diacetylphloroglucinol was obtained using derivatives of CHA0 and *P. putida* WCS358 that constitutively produce this compound and using a similar approach it was found that the fungus is sensitive to phenazine-1-carboxylic acid (figure 2a). Thus, *L. fungicola* is sensitive to siderophore-mediated

competition for iron and to antibiosis by pseudomonads, suggesting that application of strains that produce effective siderophores and antibiotics can potentially control the dry bubble disease caused by this fungal pathogen.

The isolates that were antagonistic to *L. fungicola in vitro,* were tested in a bioassay for their ability to suppress dry bubble disease *in vivo* (figure 3). In this bioassay, treatment with Sporgon (active ingredient: Prochloraz-manganese), the only effective chemical available to Dutch mushroom growers, significantly reduced dry bubble disease. Whereas dry bubble disease incidence in the treatments with antagonistic bacteria was most often lower than in the control treatment, differences were never statistically significant. The power of the bioassay to detect differences was increased by testing the most promising bacterial strains (Ab03038 and Ab03040) using 18 replicates, compared to 3 replicates in the initial screening. In two independent experiments both Ab03038 and Ab03040 did not control the disease. Therefore we conclude that the antagonistic bacteria that were isolated in this study are not able to reduce dry bubble disease, certainly not to the level of control of Sporgon. To study interactions between the bacterial isolates and *L. fungicola* on the fruiting body of *Agaricus*, development of lesions was measured after inoculation with the pathogen on the cap, either with or without the *Pseudomonas* isolates (figure 6). Also under these conditions none of the bacterial isolates affected the development of *L. fungicola*, neither on harvested mushrooms nor on mushrooms that were still attached to the mushroom mycelium.

For effective biological control of plant pathogens by *Pseudomonas* spp., effective colonization of the plant roots by these bacteria is a prerequisite (Raaijmakers *et al.*, 1995). The lack of effectiveness of the bacterial isolates to control dry bubble disease in this study thus may have been due to too low population densities of the introduced pseudomonads. We investigated colonization of the casing by 3 *Pseudomonas* strains that are well studied in plant-soil systems. When introduced into casing, the population densities of strains WCS358r, WCS417r and WCS374r all declined over a three week period. However, when the casing was colonized by *A. bisporus* population densities of strains WCS358r and WCS417r were much higher (10 – 100 fold) compared to non-colonized casing. Strain WCS374r behaved differently and decreased during the two weeks *A. bisporus* needed to colonize the casing and form two flushes of mushrooms. This differential behavior of the WCS strains confirms observations on their abilities to colonize the rhizosphere of *Arabidopsis thaliana* (Doornbos *et al.*, 2009; Doornbos *et al.*, 2011). The total culturable fraction of bacteria in the casing increased during the first week, in which the casing was colonized by *A. bisporus* vegetative hyphae, and decreased slowly to pre-colonization levels during the next two weeks, in which the vegetative hyphae aggregated and formed fruiting bodies. We assume that during colonization of the casing by *A. bisporus* exudates from the hyphae stimulate growth of bacteria in the casing (Grewal

and Rainey, 1991). Apparently, also WCS358r and to a slightly lesser extend WCS417r are stimulated by these exudates. The effectiveness of a biocontrol agent can be correlated with its population size (Bull *et al.*, 1991). For control of fungal plant pathogens, it was found that a minimum density of 10^5 bacteria/g root is required (Raaijmakers *et al.*, 1995; Raaijmakers *et al.*, 1999). Population densities of both WCS417r and WCS358r remained above this threshold level. However, these strains were originally isolated from plant roots (Geels and Schippers, 1983), and they may be less adapted to survival in the casing compared to the strains isolated in this study. However, none of the strains isolated from colonized casing significantly reduced dry bubble disease, despite their effective *in vitro* antagonism. *In vitro*, we found that *L. fungicola* is sensitive to siderophores and antibiotics produced by strains of fluorescent *Pseudomonas* spp. For the control of plant pathogens by fluorescent pseudomonads, both siderophore and antibiotic production can fully explain the disease suppressive abilities of selected strains of these bacteria (Duijff *et al.*, 1993; Laville *et al.*, 1992). The lack of control of dry bubble disease observed in this study may have been due to the possibility that the selected antagonists do not produce the *L. fungicola* inhibitory compounds *in vivo*. However, the constitutive DAPG producer *P. fluorescens* CHA638 did also not reduce dry bubble disease significantly. Apparently, dry bubble disease is not susceptible to the action of antagonistic bacteria in the casing layer. In chapter 3, we described that spores of *L. fungicola* are sensitive to casing fungistasis. We concluded that the inhibition of spore germination in uncolonized casing is likely due to production of fungistatic compounds and that the spores are desensitized by nutrients leaking from *A. bisporus* hyphae when they colonize the casing. Possibly, colonized casing is such a nutrient-rich environment that addition of antagonistic bacteria cannot prevent *L. fungicola* spores from germinating.

Gilligan (1985) introduced the concept of the pathozone. For a fungal pathogen, it is the zone around an organ of its host in which a propagule of the pathogen must be if it is to have a chance at infecting its host. Probabilities of successful infection, i.e. (infection efficacy) of a pathogen propagule decline with increasing distance from its host. Around plants, it overlaps and mostly exceeds the rhizophere and it is limited by the saprophytic capabilities of a pathogen in a soil. Addition of antagonistic bacteria would aim to reduce the distance a pathogen can grow and so reduce the pathozone. In this respect, we observed that *A. bisporus* colonizes the casing totally and very densely. Contrasting to when plants and soil-borne pathogens interact, there are no large parts of the casing left uncolonized by the mushroom mycelium and the pathozone consequently covers all of the casing. It can therefore be argued that, although the antagonistic bacteria in our assay might reduce the distance *L. fungicola* can grow saprophytically, a suitable host is always nearby and infection efficacy is not significantly affected.

Besides reducing saprophytic growth capabilities of a pathogen, biocontrol agents of plant pathogens can also interfere with the infection process by eliciting induced systemic resistance (Bakker *et al.*, 2007; Van Wees *et al.*, 2008). Induced systemic defense responses are commonly found in both plant and animals and it is generally believed that induced defenses have evolved to save energy under conditions where few enemies are present. Such benefits could likewise be beneficial to fungi, however, to our knowledge, induced systemic defense responses in mushrooms have not been investigated. Elucidating induced defense in mushrooms against fungal pathogens may enable us to control dry bubble disease either by selection of disease resistant strains of *A. bisporus*, or selection of bacterial strains that can elicit induced systemic resistance.

ACKNOWLEDGEMENTS

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Induced resistance in *Agaricus bisporus* against infection by *Lecanicillium fungicola*

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ABSTRACT

Lecanicillium fungicola **causes dry bubble disease and is an important problem in the cultivation of** ***Agaricus bisporus*****. Little is known about the defense of mushrooms against pathogens in general and** ***L. fungicola*** **in particular. In plants and animals a first attack by a pathogen often induces a systemic response that results in an acquired resistance to subsequent attacks by the same pathogen. The development of functionally similar responses in these two eukaryotic kingdoms indicates that they are important to all multi-cellular organisms. We investigated if such responses also occur in the interaction between the white button mushroom and** ***L. fungicola*****. A first infection of mushrooms of the commercial** ***A. bisporus*** **strain Sylvan A15 by** ***L. fungicola*** **did not induce systemic resistance against a subsequent infection. Similar results were obtained with the** ***A. bisporus*** **strain MES01497, which was demonstrated to be more resistant to dry bubble disease. Apparently, fruiting bodies of** ***A. bisporus*** **do not express induced resistance against** ***L. fungicola*****.**

Dry bubble disease, caused by the ascomycete *Lecanicillium fungicola,* is a serious problem in cultivation of the white button mushroom *Agaricus bisporus*. *L. fungicola* can only infect the generative stage of *A. bisporus* and, depending on the time of infection, causes different macroscopic symptoms (Chapter 2; North and Wuest, 1993; Bernardo *et al.*, 2004). Interactions between *A. bisporus* and *L. fungicola*, including activation of defenses, have not been characterized in detail and in general such information on host pathogen interactions is lacking for mushrooms (Chapter 2). In contrast, a wealth of information is available on interactions between plants or animals and microbial pathogens.

To prevent or reduce damage caused by microbial pathogens, animals and plants have a basal defense formed by constitutively present physical and chemical barriers. During evolution, specialized pathogens developed that could overcome this defense. In response, both plants and animals have evolved an innate immune system that are activated upon recognition of a pathogen (Jones and Dangl, 2006; Martinon *et al.*, 2009). Inducible defenses are advantageous because activity of defenses under enemy-free conditions can reduce the fitness of an organism (Van Hulten *et al.*, 2006; Heil and Baldwin, 2002; Heil *et al.*, 2000).

A prerequisite for induced defense is the ability to recognize a pathogen. To this end, plants and animals have evolved pathogen recognition receptors (PRRs). PRRs recognize conserved features of pathogens, such as flagellin or chitin, which are known as pathogen-associated molecular patterns (PAMPs). Upon PAMP recognition, defenses are stimulated and infection by a pathogen will be prevented or halted. Thus, recognition of PAMPs leads to PAMP-triggered immunity. Pathogens have evolved ways to suppress induced defense by injecting molecules. These so called effectors interfere with host defense reactions and cause effector-triggered susceptibility. In reaction, plants have evolved a wide variety of resistance (*R*) genes that are pathogen-specific and recognize effectors directly or indirectly. Upon recognition of effectors, defense is activated and this results in effector-triggered immunity (Jones and Dangl, 2006). Animal PRRs seem to be limited to the recognition of highly conserved pathogen-associated molecular patterns. Vertebrate animals have evolved an additional system of acquired immunity that is triggered when the innate immune system is overwhelmed. The innate immunity of plants and animals share several characteristics despite the fact that they seem to have evolved independently (Ausubel, 2005; Jones and Dangl, 2006; Nürnberger *et al.*, 2004). For instance, an important resemblance between PRRs of plant and animals is the occurrence of extracellular leucine-rich repeat domains. It is not yet established whether other eukaryotes, such as the fungi, also have developed an innate immune system. It has been shown that genes encoding leucine-rich repeat domain containing receptors are relatively rare in fungi (Soanes and Talbot, 2010).

Most studies of fungi focused on their competitive interactions or on fungal attacks of plants or animals rather than their defenses against specialized pathogens. Indeed, fungi can react to the presence of hosts or antagonists, as exemplified by pathogenic fungi that produce mycotoxins in response to host-derived signals (reviewed by Reverberi *et al.*, 2010). Rohlfs *et al.* (2007) showed that, upon attack by the fungivorous springtail *Folsomia candida,* secondary metabolites produced by the soil mould *Aspergillus nidulans* are advantageous to the fungus. Silar (2005) demonstrated that *Coprinopsis cinerea* and *Podospora anserina* produce peroxide in response to other fungi. Reactive oxygen species (ROS) are involved in defense of both plants and animals upon pathogen recognition. They function as toxic compounds as well as in signaling for defense activation (Torres, 2009). Silar (2005) proposed a similar role for ROS in defense signaling in some fungi. The possible involvement of ROS in defense of *A. bisporus* against *L. fungicola* was studied by Largeteau (2004), who reported a negative correlation between the susceptibility of *A. bisporus* strains and hydrogen peroxide levels in dry bubbles of these strains. Characterization of defense of *A. bisporus against L. fungicola* at the molecular level has led to the identification of genes that were differentially regulated upon infection. Thomas *et al*. (2007) identified 80 genes of *A. bisporus* that were differentially regulated in infected mushroom tissue compared with healthy tissue. However, identification of defense related genes is difficult, a*s L. fungicola* affects developmental programming *A. bisporus,* in which a myriad of genes is involved (North and Wuest, 1993; Largeteau *et al.*, 2010).

Upon attack, plants induce their defense not only at the site of infection but also systemically. Induced resistance in plants is effective against a wide range of pathogens and can sometimes be maintained for the lifetime of the plant (Durrant and Dong, 2004). Often the induction of systemic resistance does not involve direct activation of defense, but results in a quicker and stronger defense response upon a second attack. This potentiation of defense responses in induced plants was termed priming as described in animals and humans (Conrath *et al.*, 2006; Conrath *et al.*, 2002; Ahmad *et al.*, 2010). In plants, the phytohormones jasmonic acid (JA), ethylene (Et), and salicylic acid (SA) are well established key regulators of defense signaling (Van der Ent *et al.*, 2009). Furthermore, lipid-derived molecules seem to be important in the signaling of induced resistance in both plants and animals (Jung *et al.*, 2009; Maldonado *et al.*, 2002; Pan *et al.*, 1998; Schultz, 2002; Grechkin, 1998).

Although the mechanisms of the acquired resistance phenomena in plants and animals are quite distinct, functionally they are quite similar. Ultimately, it matters if a host is naive to a pathogen or that the host has had past experience with a pathogen. The development of functionally similar systems of acquired resistance in both plants and animals indicates that such systems are important to all multi-cellular organisms. Here,

it was assessed whether *A. bisporus* develops an induced resistance upon attack by *L. fungicola*. To this end, the commercial and susceptible *A. bisporus* strain Sylvan A15 was used as well as the *A. bisporus* strain MES01497, which is partially resistant to infection of *L. fungicola*. The results indicate that the white button mushroom does not develop an induced resistance to *L. fungicola.*

MATERIALS AND METHODS

FUNGAL STRAINS AND GROWTH CONDITIONS

The fungi used in this study are listed in table 1. *L. fungicola* strain V9503 was stored at -80 ˚C in phosphate buffer (0.21 M NaH_2PO_4, pH 7.2) with 10 % glycerol. *L. fungicola* was cultured on potato dextrose agar (PDA) (Difco, Lawrence, USA) for 5 days at 24 ˚C. Spore suspensions were prepared from these cultures by taking up these reproductive structures in 10 ml of sterile demineralized water. After filtering the suspension over sterile glass wool, densities of the spore suspensions were determined using a haemocytometer.
Pieces of PDA colonized by *A. bisporus* strain MES01497 were stored at -80 ˚C. *S*train MES01497 was cultured on compost extract agar (CEA, per liter: extract of 100 g of compost fully colonized by *A. bisporus* strain Sylvan A15; 15 g granulated agar (Difco)) for 2 weeks at 24 ˚C.

Table 1. Fungi used in this study

***Lecanicillium fungicola* var. *fungicola* V9503**	Isolated from a mushroom farm in Noordhoek, the Netherlands in 1995 (Plant Research International (PRI), Wageningen, The Netherlands)
***Agaricus bisporus* Sylvan A15**	Commercially available strain (CNC, Milsbeek, the Netherlands)
***A. bisporus* MES01497**	Wild type from Dutch PRI collection selected for resistance against *L. fungicola* (PRI, Wageningen, The Netherlands)

SPAWN PREPARATION AND SPAWN RUN OF MES01497

Sorghum bicolor granules (400 g) were autoclaved in an equal amount (w/v) of water, inoculated with half a PDA plate fully colonized by MES01497 and incubated at 24 ˚C for 2 weeks in sterile full-gas microboxes (110x100x80mm; Eco2box, Ophasselt, Belgium). Conditioned compost (CNC, Milsbeek, The Netherlands) was mixed with 0.1 % (w/w) fully colonized sorghum granules and incubated at 24 °C and 95 % humidity for two weeks.

CULTIVATION OF *A. BISPORUS*

Mushrooms were cultured in controlled-climate incubators (Snijders scientific, Tilburg, the Netherlands, dimensions 99 x 63 x 134 cm). Plastic containers (17,5 x 27,5 x 22,5cm) were filled with 3,5 kg of compost colonized by *A. bisporus* strain Sylvan A15 (CNC, Milsbeek, the Netherlands) or by *A. bisporus* strain MES01497 (see above) and covered with

1 kg of casing mix for cutting companies (CNC, Milsbeek, the Netherlands). The cultures were incubated at 24 ˚C and 95 % relative humidity (RH) for 8 days and subsequently at 20 ˚C and 88 % RH for the duration of the experiment.

L. FUNGICOLA LESION DEVELOPMENT ON CAPS

Mushrooms of *A. bisporus* strain Sylvan A15 (cultured as described above) were placed in a closed tray on moist paper towels after removing their stipes. The caps of the mushrooms were point inoculated with a 2 µl droplet of a *L. fungicola* spore suspension, containing $5{\cdot}10^3$, $5{\cdot}10^4$, $5{\cdot}10^5$, $5{\cdot}10^6$ or $5{\cdot}10^7$ spores/ml. The inoculated area of the mushroom cap was sampled to a depth of 4 mm with a cork borer (12 mm diameter) immediately after inoculation or after 72 hrs incubation at 24 ˚ C. The sampled material was immediately frozen in liquid nitrogen and stored at -20˚C. The amount of *L. fungicola* DNA in the sample was determined by qPCR as described later.

To determine if L. fungicola was restricted to the discolored lesion that developed after infection, the cap of harvested mushrooms was inoculated with a 2 µl droplet of a L. fungicola spore suspension (107 spores/ml) and incubating for 72 hrs at 24 ˚C. Subsequently, material was collected by cutting 4 mm deep, 4 x 8 mm pieces from infected mushroom caps, starting from the center of the lesion (figure 1). The material was collected such that the second piece was directly adjacent to the lesion, the third piece 4 - 8 mm from the lesion and the fourth piece 8 – 12 mm. Samples were immediately frozen in liquid nitrogen and stored at -20˚C. The amount of L. fungicola DNA in the sample was determined by qPCR.

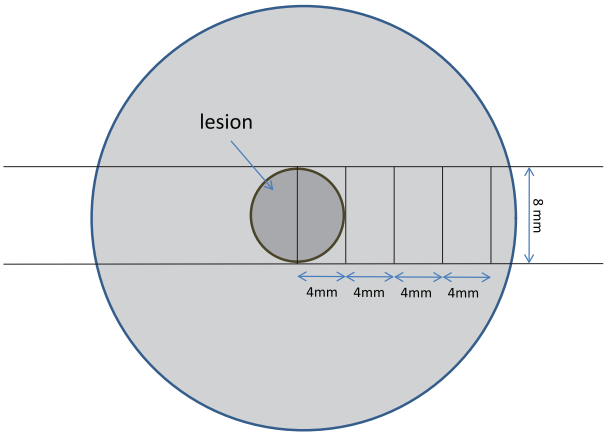

Figure 1. Schematic top view of mushroom cap with *L. fungicola* lesion. Lines indicate blocks (8 x 4 mm) excised to determine if *L. fungicola* was restricted to the discolored lesion.

EFFECTS OF PRIMARY *L. FUNGICOLA* INFECTIONS ON DISEASE CAUSED BY SECONDARY INFECTIONS

Mushrooms were cultivated as described above. In the first flush, mushrooms were pre-treated by placing 2 µl of a *L. fungicola* spore suspension ($1 \cdot 10^7$ spores/ml) or 2 µl sterile demineralized water on one side of the cap. After 24 hrs, the other side of all mushroom caps was inoculated with 2 µl of a fresh *L. fungicola* suspension ($1 \cdot 10^7$ spores/ml). Mushrooms were cultivated for another 72 hrs and lesion diameters were measured and material was sampled for qPCR quantification of *L. fungicola*.
Alternatively, mushrooms (strain Sylvan A15 and strain MES01497) were cultivated in boxes containing 2 kg of fully colonized compost and 0.75 kg casing layer. For each strain, 30 boxes were placed in controlled-climate incubators (conditions see above), and half of the boxes were inoculated with *L. fungicola* on day 1 by evenly wetting the casing with a *L. fungicola* spore suspension (final density corresponded to 10^6 conidia/m^2 of casing). When symptoms of dry bubble disease were visible in all inoculated boxes, 5 symptomless mushrooms were inoculated with 2 µl *L. fungicola* spore suspension ($1 \cdot 10^7$ spores/ml) in the center of the cap in all boxes. Mushrooms were cultivated for 3 (Sylvan A15) or 4 (MESO1497) more days during which lesions developed. Mushrooms were harvested and lesion diameters were measured. For each box the average lesion diameter was calculated.

SUSCEPTIBILITY OF A15 AND MESO1497 TO DRY BUBBLE DISEASE

To study differences in susceptibility to dry bubble disease, cultures of *A. bisporus* strains Sylvan A15 and MES01497 were infected with *L. fungicola* strain V9503. Immediately after application of the casing, the mushroom cultures were inoculated with V9503 by evenly wetting the casing with 50 ml of a spore suspension (corresponding to 10^6 conidia /m^2). The first 3 flushes were harvested and scored in 4 disease classes: Healthy, spotted cap, stipe blow-out and dry bubble (Berendsen *et al.*, 2010). Disease incidence was scored as the percentage of mushrooms with symptoms of dry bubble disease. Development of lesions on mushrooms was also studied for both strains. Cultures of Sylvan A15 and MES01497 were inoculated with 2 µl of a *L. fungicola* spore suspension on the caps of 10 small mushrooms (1- 1,5 cm cap diameter) still attached to the mycelium or 10 larger mushrooms (3-4 cm cap diameter) that had been harvested and placed in a closed tray on moist paper towels. Lesion diameters were determined after 96 hrs.

EFFECTS OF METHYL JASMONATE AND SALICYLIC ACID ON *L. FUNGICOLA* DEVELOPMENT

The effect of defense-associated phytohormones on *L. fungicola* was investigated using a modified method of Braaksma *et al.* (2001). 5 freshly harvested mushrooms were placed in 10 ml 0.1 mM or 1 mM methyl jasmonate (MeJA) in 1% (v/v) ethanol, 1 mM or 10 mM salicylic acid (SA) in 1% (v/v) ethanol or in 1% (v/v) ethanol (control treatment). Mushrooms were incubated at room temperature for 24 hrs and subsequently point inoculated with *L. fungicola* (see above). Lesion diameters were measured after 72 hrs.

DNA EXTRACTION FROM *L. FUNGICOLA* LESIONS

Total DNA was extracted from infected mushroom samples as described by Henrion *et al*. (1994) with slight modifications. Samples were lyophilized and subsequently disrupted with the Tissuelyser II (Qiagen, The Netherlands) for 1 min at 1500 shakes per min. The disrupted tissue was taken up in 700 µl of CTAB extraction buffer (100 mM Tris-HCL pH 8, 20 mM EDTA, 2 % w/v CTAB and 1.4 M NaCl), and incubated at 65 ßC for 30 min with gently mixing every 10 min. After adding 700 µl phenol : chloroform : isoamylalcohol (24 : 24 : 1) samples were centrifuged (5 min, 11,700 *g*) and the aqueous layer was transferred to a clean tube. An equal volume of chloroform: isoamylalcohol (24: 1) was added and samples were centrifuged (5 min, 11,700 *g*). The aqueous layer was transferred to a new tube and an equal volume of cooled (4˚C) isopropanol was added and DNA was pelleted by centrifugation (15 min, 11,700 *g*). The DNA was gently washed by adding 1 ml 70 % ethanol. After centrifugation (5 min, 11,700 *g*), the pellets were dried, resuspended in 100 µl sterile demineralized water and stored at -20 ßC.

QUANTIFICATION OF *L. FUNGICOLA* DNA BY QUANTATIVE POLYMERASE CHAIN REACTION (QPCR).

The Eurogentec qPCR core kit (Eurogentec, the Netherlands) was used according to the instructions of the manufacturer with primers and probe targeting the ribosomal 18S-rDNA of *L. fungicola*. Sequences of primers and probes were provided by Carolien Zijlstra (PRI, Wageningen, the Netherlands). The amplification mixture consisted of 300 nM of probe, 300 nM forward and reverse primer and 2 µl of DNA in a final volume of 30 µl. Real-time PCR amplifications were performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems, The Netherlands). Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Data were analyzed with SDS 2.2.2 software. C_t values of samples were converted to *L. fungi-*

cola DNA concentrations by calibration to DNA from a pure culture of *L. fungicola* at a concentration of 10 ng/ml (measured with Nanodrop ND-1000 [Isogen life science, de Meern, the Netherlands]). The calibration sample was used throughout this study.

STATISTICS

Data analysis was performed with SPSS 13.0, using Student's *t*-test if 2 variables were compared and analysis of variance (ANOVA) if more than 2 variables were compared.

RESULTS

DETECTION OF *L. FUNGICOLA* WITH QPCR

QPCR was used to detect *L. fungicola* on the surface of mushroom caps that were point inoculated with several densities of spores of the pathogen (figure 2a). The amount of *L. fungicola* DNA on mushrooms that were inoculated with ≤10^4 spores was below the limits of detection immediately after inoculation. In contrast, approximately 0.32 pg DNA/mm^2 was detected on mushrooms inoculated with 10^5 spores. After 72 hrs, *L. fungicola* DNA was detected in all inoculated mushrooms. Amounts of *L. fungicola* DNA increased with the inoculum density and ranged from 0.16 pg DNA/mm^2 when 10 spores were inoculated to 74 pg DNA/mm^2 when 10^5 spores were inoculated. Most *L. fungicola* DNA was detected within the lesion area on the mushroom cap, as investigated for inoculation with 10^4 spores (figure 2b). At 72 hrs after inoculation, *L. fungicola* DNA was detected in cap tissue directly adjacent to the lesion but it was a 1000 fold lower than inside the discolored tissue.

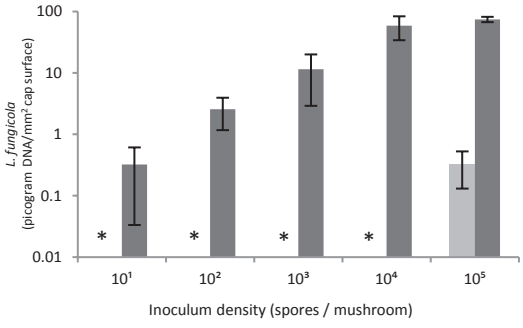

Figure 2a. Quantification of growth of *L. fungicola* on a mushroom cap. *L. fungicola* DNA was quantified immediately after inoculation (light grey bars) or after 72 hrs incubation (dark grey bars). Bars represent average *L. fungicola* DNA (pg/ mm^2 cap surface) of three samples as determined by qPCR. Error bars represent standard deviation. * below detection limit

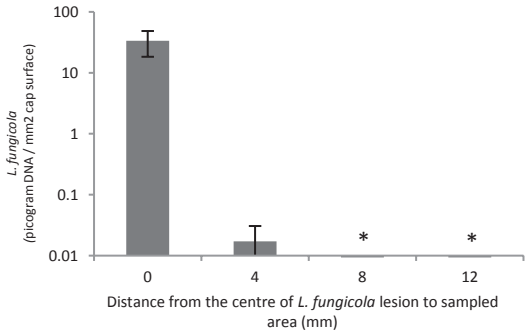

Figure 2b. Quantification of growth of *L. fungicola* on a mushroom cap. DNA was isolated from small blocks from concentric rings around an infected mushroom cap 72 hrs after inoculation (see figure 1). Bars represent average *L. fungicola* DNA (pg/ mm^2 cap surface) of three samples as determined by qPCR. Error bars represent standard deviation. * below detection limit

INDUCED RESISTANCE IN MUSHROOMS OF *A. BISPORUS* STRAIN SYLVAN A15

To test whether *A. bisporus* develops defense responses upon infection by *L. fungicola* small mushrooms of strain Sylvan A15 were infected with *L. fungicola* on two subsequent days on opposite sides of the mushroom cap. 72 hrs after the second infection, lesion size of the secondary infections was compared with that of lesions that were obtained on mushrooms that had not been infected before. No significant differences in lesion size were obtained between the samples (figure 3a). The amount of *L. fungicola* DNA as determined with qPCR also did not differ between the treatments (data not shown).
For induced resistance to be effective it may require more than one day between the primary and the challenge infection. To create a bigger time difference between the infections the following experimental setup was used. *A. bisporus* was cultured and half of the mushroom cultures were inoculated with a spore suspension of *L. fungicola* (10^6 spores/m^2) on the first day of the experiment. When disease symptoms were visible in the inoculated cultures (20 days after primary inoculation), 5 healthy mushrooms were point inoculated with 2 µl of *L. fungicola* spore suspension in all boxes. After 4 days, lesion size was determined. No significant differences in lesion size on mushrooms in healthy and diseased cultures were detected (figure 3b). qPCR analysis confirmed these results (not shown).

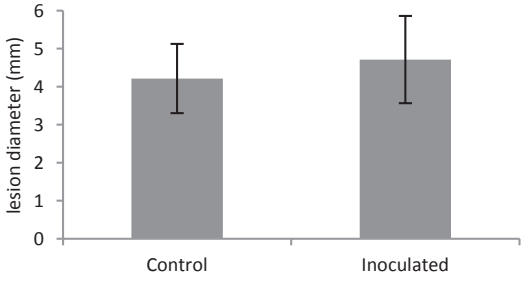

Figure 3a. Absence of induced resistance upon secondary infection of caps of *A. bisporus*. Development of lesions on mushroom caps of *A. bisporus* strain A15 that had or that had not been previously inoculated with *L. fungicola*. Bars represent average lesion diameter of 8 mushrooms. Error bars denote standard deviation.

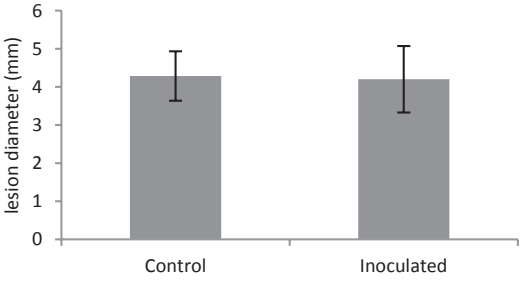

Figure 3b. Infection of healthy mushrooms in diseased and healthy mushroom cultures of *A. bisporus* strain Sylvan A15. Average lesion diameter 3 days after inoculation of healthy mushrooms of *A. bisporus* strain Sylvan A15 in mushroom cultures that had or had not been inoculated on the first day of the experiment with a spore suspension of *L. fungicola* in the casing. Inoculated containers displayed several mushrooms with symptoms typical of dry bubble disease. Bars represent average lesion diameter per treatment. Error bars represent standard deviation.

EFFECT OF PLANT-ASSOCIATED CHEMICAL INDUCERS OF RESISTANCE

In plants application of the phytohormones SA and JA induce systemic resistance against a wide range of pathogens (Durrant and Dong, 2004; Van der Ent *et al.*, 2009). Furthermore, JA derivatives were shown to affect cap opening of *A. bisporus* mushrooms (Braaksma and Schaap, 2005). Using an *in vitro* assay adopted from Braaksma *et al.* (2001) it was tested whether SA and MeJA, a functionally active derivative of JA, can induce resistance against *L. fungicola* infection. Harvested mushrooms were allowed to absorb solutions of MeJA (1 mM and 0.1 mM) and of SA (1 mM or 10 mM) for 24 hrs and were subsequently point inoculated with 2 µl of a *L. fungicola* spore suspension. The size of lesions that developed in 72 hrs following infection by *L. fungicola* was not significantly affected by pre-treatment with either MeJA or SA (figure 4).

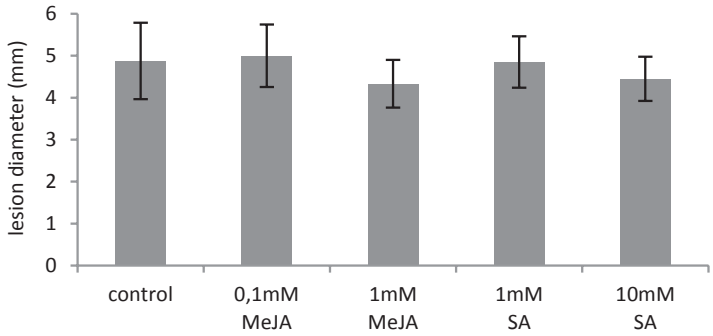

Figure 4. Effect of SA and MEJA application on *L. fungicola* lesion development. A spore suspension of the pathogen was point inoculated on harvested mushrooms. Bars represent average lesion diameter on 5 mushrooms. Error bars represent standard deviation.

A. BISPORUS STRAIN MES01497 IS MORE RESISTANT TO *L. FUNGICOLA* THAN STRAIN A15

Disease development by *L. fungicola* in the partially resistant *A. bisporus* strain MES01497 (Johan Baars, unpublished results) was compared to that of the commercially *A. bisporus strain* Sylvan A15. Both strains were grown and the casings were inoculated with a spore suspension of *L. fungicola* (10^6 spores/m^2) immediately after the casings had been applied to the compost. Fifteen days after the start of the experiment mushrooms were harvested for both strains and subsequently every week a new flush of mushrooms was harvested. The percentage of mushrooms with symptoms of dry bubble disease

was higher for A15 than for MES01497 in all flushes (figure 5a). In the first flush around 20% of the mushrooms of strain A15 showed symptoms of disease whereas MES01497 was completely healthy. In the second flush, 81% of the A15 mushrooms were diseased, whereas MES01497 displayed only 8% of dry bubble disease. In the third flush, A15 was completely diseased, compared to 32% disease in MES01497. The numbers of fruiting bodies produced by A15 and MESO1497 differed considerably, with A15 being much more productive in the first two flushes (figure 5b). However, in the third flush the number of mushrooms of strain A15 could not be determined as there were no healthy mushrooms and dry bubbles appeared in a cauliflower-like structure of which it was impossible to determine the number of primordia from which they originated.

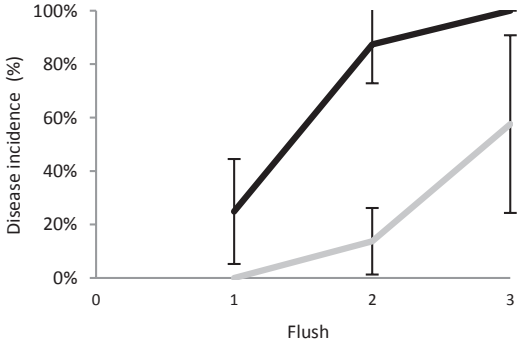

Figure 5a. Dry bubble disease incidence in mushrooms of *A. bisporus* strains A15 (black line) and MES01497 (grey line). The average number of diseased mushrooms as a percentage of total mushrooms is depicted for each flush. Bars represent the average of 9 containers. Error bars represent standard deviation

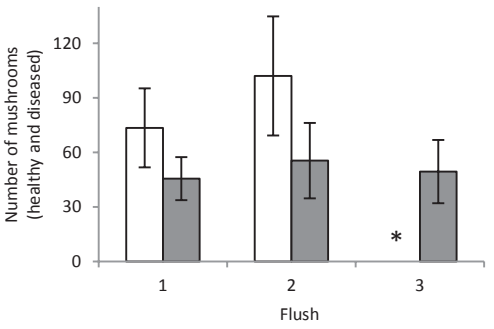

Figure 5b. Number of mushrooms produced by *A. bisporus* strains A15 (white bars) and MES01497 (dark grey). Bars represent the average of 9 containers. Error bars represent standard deviation. * could not be determined.

It was also investigated if after point inoculation on the caps, *L. fungicola* lesions would develop less on MES01497 than on Sylvan A15. Mushroom of both strains were inoculated immediately after being harvested or while still growing in culture. After 4 days, *L. fungicola* lesion diameters were determined (figure 6). Lesion diameters were largest on harvested mushroom of strain Sylvan A15. They were significantly bigger than lesions on harvested mushrooms of strain MES01497. Lesion diameters on mushrooms that were still in culture were significantly smaller than the lesions on harvested mushrooms. In this case, no differences between the two strains were observed.

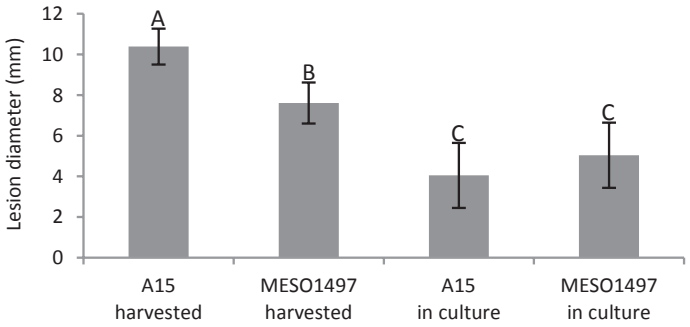

Figure 6. Lesion diameter on caps of harvested mushrooms and mushrooms in culture of *A. bisporus* strains MES01497 and Sylvan A15 72 hrs after inoculation with *L. fungicola.* Different letters indicate significant differences as determined by ANOVA and Tukey post-hoc test ($p<0.05$).

INDUCED RESISTANCE IN MUSHROOMS OF *A. BISPORUS* STRAIN MES01497

As MES01497 is more resistant to *L. fungicola* infections upon casing inoculation than Sylvan A15, it was investigated if this strain shows an effective induced resistance response upon a primary infection. Induced resistance in MES01497 was investigated using the approach described for Sylvan A15. Neither a primary infection of the same mushroom cap (figure 7a), nor development of dry bubble in neighboring fruiting bodies (figure 7b) could inhibit lesion development upon challenge inoculation with *L. fungicola* on mushroom caps.

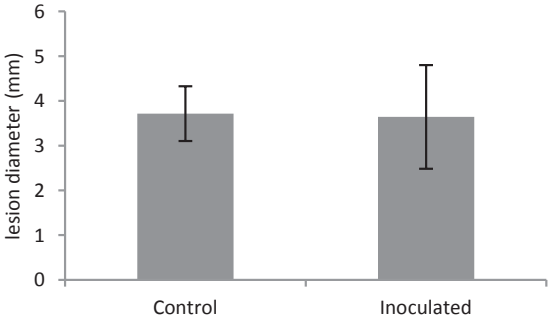

Figure 7a. Cap assay for induced defense. Development of lesions on mushroom caps of *A. bisporus* strain MES01497 that had either (inoculated) or not (control) been previously infected. Bars show average lesion diameter of 10 mushrooms.

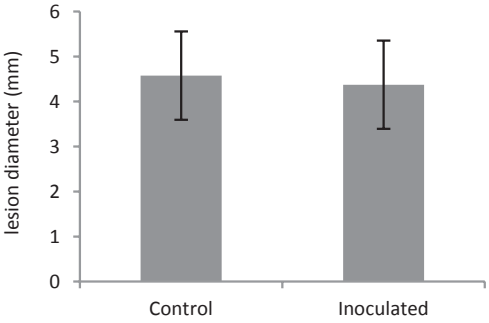

Figure 7b. Infection of healthy mushrooms in diseased (Inoculated) and healthy (Control) mushroom cultures of *A. bisporus* strain MES01497. Average lesion diameter 4 days after inoculation of healthy mushrooms of *A. bisporus* strain Sylvan MES01497 in mushroom cultures that had either or not been inoculated on the first day of the experiment with a spore suspension of *L. fungicola* in the casing. Inoculated containers displayed several mushrooms with symptoms typical of dry bubble disease. Bars represent average lesion diameter per treatment. Error bars represent standard deviation.

DISCUSSION

A. bisporus is an economically important mushroom species that is cultivated worldwide. Production of *A. bisporus* is affected by microbial pathogens such as *L. fungicola*. This pathogen is the causal agent of dry bubble disease, which causes significant yield losses (Chapter 2 / Berendsen *et al.*, 2010). Possibilities to increase resistance of white button mushroom to *L. fungicola* infection were investigated. It was hypothesized that the fungal response to pathogen infection is similar to that in other eukaryotes. Plants respond to pathogen infection by systemically priming their defenses resulting in a faster and stronger response to a subsequent infection (Conrath *et al.*, 2006; Conrath *et al.*, 2002; Ahmad *et al.*, 2010; Durrant and Dong, 2004). Also in animals, it matters if an individual has previously encountered a pathogen (Roth *et al.*, 2009; Pham *et al.*, 2007; Little and Kraaijeveld, 2004). Here, it is shown that *A. bisporus* mushrooms do not show an enhanced resistance response to *L. fungicola* infection upon a primary stimulus. Point inoculation of *Agaricus* mushroom caps with *L. fungicola* resulted in formation of lesions. The pathogen was mainly present in the discolored tissue of the lesion based on detection of *L. fungicola* DNA (figure 2). A primary infection with *Lecanicillium* on the cap of strain A15 did not affect lesion development of a second infection, suggesting that an induced systemic resistance response was not elicited on the cap. However, in this experiment there was only 24 hrs between the first and the second infection. For the induction of resistance in plants it is known that it takes several days to a week to reach the induced state (Van Loon *et al.*, 1998). Since mushrooms grow rapidly it is not possible to establish such a long time period between the two infections on the fruiting bodies themselves. Therefore, mushroom cultures were inoculated with *L. fungicola* immediately after application of the casing. Healthy mushroom that were surrounded with diseased mushrooms were subsequently challenged with the pathogen approximately 7 days after symptoms of dry bubble disease had appeared. Also in these experiments, a primary infection did not result in reduced disease symptoms caused by a challenge infection. Apparently *L. fungicola* infection does not lead to a systemic resistance response in *A. bisporus* strain A15.

The evidence for hormonal signaling in higher basidiomycetes is inconsistent and fragmentary (Moore, 1991). However, mycorrhizal fungi form a connecting network between plants that can be crucial for induced resistance upon pathogen attack in neighboring plants (Song *et al.*, 2010). This indicates that fungi can transport defense-related signals. Song *et al.* (2010) demonstrated that in this mycorrhiza mediated system, defense-related genes were induced in the plants neighboring an attacked plant that are regulated by SA as well as by JA dependent signaling cascades. Both SA and JA can enhance pathogen resistance in plants when applied exogenously (Pieterse et al., 1998). Little

is known about a role of SA and JA in mushrooms. However, application of JA derivatives or the plant hormone cytokinin can affect post-harvest development of *A. bisporus* fruiting bodies (Braaksma and Schaap, 2005; Braaksma *et al.*, 2001), indicating that *A. bisporus* is responsive to plant hormones. Application of SA or MeJA to harvested mushrooms did not result in enhanced resistance against *L. fungicola* infection (figure 4). Our preliminary data indicate that application of MeJA to growing mushrooms cultures also could not reduce dry bubble incidence or severity (unpublished results). Taken together, it is concluded that the highly susceptible *A. bisporus* strain Sylvan A15 does not express systemic defense responses against *Lecanicillium*. However, in plants, expression of systemic induced resistance can depend on the level of disease resistance in cultivars of the same species. For example, resistance against Fusarium wilt in carnation could be induced in a moderately resistant cultivar, but less efficient and less consistent in a susceptible cultivar (Van Peer *et al.*, 1991). Similarly more resistant strains of *A. bisporus* may be more effective in expressing induced resistance against *L. fungicola*.

In this study, mushroom cultures of *A. bisporus* strain MES01497 were demonstrated to be more resistant to *L. fungicola* infections than cultures of strain Sylvan A15 (figure 5), confirming the selection of MES01497 as being partially resistant to this pathogen (Johan Baars, unpublished results). However, no differences between the strains were observed when *L. fungicola* was inoculated on the cap of growing mushrooms. Foulongne-Oriol *et al*. (2011) screened 89 *A. bisporus* hybrids derived from an intervarietal cross and found that resistance was under polygenic control. As the percentage of bubbles formed in these hybrids was not correlated to the percentage of mushrooms with lesion, they postulated that different tolerance mechanisms might be involved. Our results seem to concur with these findings. When inoculated on the caps of harvested mushrooms *L. fungicola* caused larger lesions than on mushroom that were still attached to the mushroom mycelium. Also lesions were larger on harvested A15 mushrooms compared to MESO1497 (figure 6). These results suggest that differences in post-harvest deterioration of mushroom tissue may explain the observed differences in lesion development on the harvested mushrooms of the two Agaricus strains. Also, non-defense related characteristics might explain the differences in disease susceptibility between the strains. E.g. a correlation was found between the time needed by *A. bisporus* strains to form their first fruiting bodies and the susceptibility to *L. fungicola*: earlier fruiting strains were significantly less diseased in a casing inoculation experiment (Largeteau *et al.*, 2004b; Foulongne-Oriol *et al.*, 2011). Nonetheless, the first flush of mushrooms of MES01497 and Sylvan A15 were harvested on the same day.

Whereas we expected the partially resistant strain MESO1497 to express an enhanced level of disease resistance after appropriate stimulation, primary infections with *L. fun-*

gicola did not affect development of secondary infections in any of our experimental approaches (figure 7).

To our knowledge, this study has been the first investigation into induced resistance in fungi. In contrast to what is described for animals and plants, we found no evidence for an acquired resistance response in *A. bisporus.* In a comparative genomics approach, Soanes and Talbot (2010) reported that PRR's with a leucine-rich repeat domain, that have evolved convergently in both plants and animals to detect pathogens, are largely absent in the fungal kingdom. It was proposed that fungi might have evolved different classes of PRR's or that fungi have distinct ways of dealing with pathogens that is not based on pathogen-recognition. If mushrooms do not recognize pathogens as such, it is a logical consequence that pathogenic attack does not induce a systemic resistance in mushrooms. A lack of pathogen recognition would imply that mushrooms rely on constitutive and wound-induced defenses. Mushrooms are known to boast a wide array of constitutively present compounds that are toxic to animals, and many have a fungicidal activity. Although it has been demonstrated that opossums learn to avoid eating poisonous mushrooms (Camazine, 1983), for most toxic or fungicidal compounds it is unknown if they confer an adaptive advantage. Based on their biocidal activity their functions have been assumed to be defense related (Sherratt *et al.*, 2005; Spiteller, 2008). Often, mushrooms respond to damage by changing color, taste or odor, based on enzymatic transformation of an inactive precursor molecule to a biological active compound (Spiteller, 2008). In *A. bisporus,* a wound-activated response is the formation of melanins (Jolivet *et al.*, 1998; Soler-Rivas *et al.*, 2001). However, a defense related function of melanin formation has not been demonstrated and it appears that more intensely discolored dry bubbles contain more *L. fungicola* DNA (Largeteau *et al.*, 2007).

In conclusion, this research showed that systemic responses in mushrooms of *A. bisporus* are not present or are ineffective in resisting secondary infections by *L. fungicola*. Recently, the genome of *A. bisporus* has become available (http://genome.jgi- psf.org) and this will facilitate the study of differential gene expression upon infection of *A. bisporus* mushrooms. Because *L. fungicola* affects the developmental program of *A. bisporus,* identification of defense-related genes in *Lecanicillium*-infected mushroom tissue is difficult (Thomas *et al.*, 2007). Other pathogens of *A. bisporus* might be better suited to identify defense-related responses of *A. bisporus.* The discovery of *A. bisporus* defense responses might help explain the differences in susceptibility between Sylvan A15 and MES01497. Future research should, however, take into account that mushrooms possibly have evolved immune systems different from those of plants and animals and do not rely on recognition and induction of defense related genes.

ACKNOWLEDGMENTS

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Control of dry bubble disease with 1-octen-3-ol, a volatile produced by *Agaricus bisporus*

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ABSTRACT

Dry bubble disease caused by *Lecanicillium fungicola* is a persistent problem in the cultivation of the white button mushroom (*Agaricus bisporus*). Control is hampered by chemicals becoming less effective. Therefore, new ways to control dry bubble disease are urgently required. 1-octen-3-ol is a volatile that is produced by *A. bisporus* and many other fungi. In *A. bisporus* it has been implied in self-inhibition of fruiting body formation while it was shown to inhibit spore germination in ascomycetes. Here we show that 1-octen-3-ol inhibits germination of *L. fungicola*. Although concentrations of 1-octen-3-ol that normally occur in mushroom cultures do not seem to affect dry bubble disease, it is demonstrated that enhanced levels of 1-octen-3-ol can effectively control the malady. A potential role of 1-octen-3-ol in the casing ecology of *L. fungicola* is discussed.

Cultivation of the white button mushroom (*Agaricus bisporus*), the commercially most valuable mushroom species, is affected by a wide variety of pests and pathogens (Fletcher and Gaze, 2008; Largeteau and Savoie, 2010). A major and persistent problem in the mushroom industry is *Lecanicillium fungicola,* the causal agent of dry bubble disease (Berendsen *et al.*, 2010). Symptoms caused by this pathogen range from small necrotic lesions to amorphous masses of mushroom tissue. On a farm the disease can quickly spread with devastating results. Control of *L. fungicola* relies on strict sanitation and the use of fungicides. The few fungicides that have been used are either no longer available or are becoming increasingly ineffective due to development of fungicide resistance in the pathogen (Bollen and van Zaayen, 1975; Fletcher and Yarham, 1976; Gea *et al.*, 2005; Wuest *et al.*, 1974). Currently, control of the dry bubble disease relies heavily on the use of prochloraz-manganese, however, reduced sensitivity to this fungicide has been reported (Gea *et al.*, 2005). Therefore, new ways to combat the dry bubble disease are needed.
The white button mushroom is grown on a composted mixture of horse and chicken manure. After colonization of the compost by vegetative mycelium of *A. bisporus*, it is covered with a casing soil. The casing layer typically consists of black peat mixed with spent lime and/or marl. The microbial community in the casing layer, *Pseudomonas* spp. in particular, has been implicated in stimulation of fruiting body formation through the consumption of a mushroom-formation-inhibiting factor (Grewal and Rainey, 1991; Miller *et al.*, 1995; Visscher, 1988). In sterilized casing, *A. bisporus* fruiting body formation is inhibited (Eger, 1961), however, it can be reinitiated by addition of activated carbon (Eger, 1961) or by thorough ventilation (Noble *et al.*, 2009), which both would lead to removal of the inhibitors.
L. fungicola is unable to infect vegetative *A. bisporus* mycelium and can only infect the fruiting bodies (Bernardo *et al.*, 2004; Calonje *et al.*, 2000a; Cross and Jacobs, 1968). Infection must therefore take place in the casing layer. *L. fungicola* spore germination is inhibited in the casing through antibiotics produced by the casing microflora (Chapter 3). When the casing is colonized by *A. bisporus*, this inhibition is lifted by nutrients leaching from *A. bisporus* hyphae, which causes the spores to germinate. The time point of infection by *L. fungicola* is therefore linked to the formation of mushrooms.
Volatiles from compost can inhibit spore germination of *L. fungicola* (Wuest and Forer, 1975). The volatile organic compounds produced by *A. bisporus* are predominantly 8-carbon molecules (Noble *et al.*, 2009; Combet *et al.*, 2006; Grove and Blight, 1983). One of the major components is 1-octen-3-ol, causing the typical mushroom smell. In *A. bisporus,* enzymatic cleavage of linoleic acid leads to the formation of 1-octen-3-ol and 10-oxo-*trans*-8-decenoic acid (ODA) (Wurzenberger and Grosch, 1982; 1984). It has been suggested that 1-octen-3-ol production is a wound-activated chemical defense of mushrooms (Spiteller, 2008; Okull *et al.*, 2003), because damaged mushrooms produce

more 1-octen-3-ol (Combet *et al.*, 2009; Wu and Wan, 2000). In *Penicillium paneum,* 1-octen-3-ol is a self-inhibitor of spore germination (Chitarra *et al.*, 2004; Chitarra *et al.*, 2005). Conidia of this fungus were shown to self-inhibit germination at high densities. This mechanism is thought to prevent premature germination of spores before dispersal. This so called crowding effect has also been described for germination of *L. fungicola* spores (Fekete, 1967; Chapter 3). The volatiles produced by *P. paneum* inhibited the radial growth of fungi from different genera indicating that the inhibitory effect of 1-octen-3-ol is rather general (Chitarra *et al.*, 2004).

As 1-octen-3-ol is an inhibitor of spore germination in other ascomycetes, we hypothesized that *L. fungicola* spore germination is also affected by this compound. In this study, we explored the use of 1-octen-3-ol to control the dry bubble disease. Application of 1-octen-3-ol reduced dry bubble disease to a comparable extent as did Sporgon (effective compound: prochloraz-manganese), the only fungicide available for mushroom growers. Although, 1-octen-3-ol also affected *A. bisporus* vegetative growth, negative effects on mushroom yield could be prevented through timing of application. Also, a potential role of 1-octen-3-ol in the ecology of *L. fungicola* is discussed.

MATERIALS AND METHODS

FUNGAL CULTURES

L. fungicola strain V9503 was stored at -80 ˚C in phosphate buffer (0.21 M NaH_2PO_4, pH 7.2) supplemented with 10% glycerol. Spore suspensions were prepared from cultures that had grown on potato dextrose agar (PDA) (Difco, Lawrence, USA) for 5 days at 24 ˚C. Spores were harvested with demineralized water (DEMI) and mycelial fragments were removed by filtering over sterile glass wool. The spore density was determined using a haemocytometer and was set at 2.10^4 conida / ml.

QUANTIFYING SPORE GERMINATION

Five ml of spore suspension was filtered over a Cyclopore membrane (diameter: 25 mm, pore size: 0.2 µm, Whatman, Florham Park, USA) that was supported by a plastic filter holder (Whatman, Florham Park, USA). The membrane was cut into 4 equal parts and incubated under different experimental conditions. At the end of each experiment, membranes were lifted from the substrate and adhering soil particles were removed. Membranes were mounted on microscope slides and spores were stained with 100 µl Calcofluor white solution (per liter: 1 g. fluorescent brightener 28 (Sigma-Aldrich, Steinheim, Germany), 50 g KOH, 50 ml glycerol). The percentage of spore germination was determined by using a Zeiss Axioskope fluorescence microscope equipped with a 70 W Mercury lamp and Zeiss filter set 02 (excitation 365 nm, emission 420 nm).

SELF INHIBITION OF SPORE GERMINATION IN *L. FUNGICOLA*

L. fungicola strain V9503 was inoculated on PDA in a biological duplicate in a compartment of a 2-compartment Petri dish (Greiner bio one, Alphen aan de Rijn, the Netherlands), the culture was incubated for 96 hrs at 24˚C. Subsequently, *L. fungicola* spore containing membranes (see above) were placed on 100 µl of sterile phosphate buffer (0.1M KH_2PO_4, pH 7) in the second compartment. Plates were sealed with Parafilm and spore germination on the membranes was quantified after 18 hrs incubation at 24˚C.

EFFECT OF 1-OCTEN-3-OL ON *L. FUNGICOLA* SPORE GERMINATION

A saturated solution of 1-octen-3-ol (Sigma-aldrich, Steinheim, Germany) was prepared in DEMI (2.6 g / liter (de Beaufort and Voilley, 1995)). *L. fungicola* spores on Cyclopore membranes were incubated on 100 µl of demineralized water or 10 mM glucose solution, or on potato-dextrose agar in a two-compartment Petri dish (Greiner bio one, Alphen

aan de Rijn, the Netherlands). One ml of the saturated 1-octen-3-ol solution and 10, 50 and 100-fold dilutions (corresponding to 20, 2, 0.4 or 0.2 µmol 1-octen-3-ol, respectively) was added to the second compartment of the Petri dishes. The dishes were sealed with Parafilm and incubated at 20˚C. Spore germination was quantified as described above. Four independent replicates were used for each treatment.

EFFECT OF VOLATILES PRODUCED BY COLONIZED MUSHROOM COMPOST ON *L. FUNGICOLA* SPORE GERMINATION

L. fungicola spores on Cyclopore membranes were placed on 100 µl of demineralized water in a 2 compartment Petri dish. The second compartment was filled with compost colonized by *A. bisporus* strain Sylvan A15, or was left empty (control treatment). Four independent replicates were used for each treatment.

EFFECT OF 1-OCTEN-3-OL EXPOSURE ON DRY BUBBLE DISEASE

Mushrooms were cultured in controlled climate incubators (Snijders scientific, Tilburg, the Netherlands, dimensions 99 x 63 x 134 cm). Plastic containers (17,5 x 27,5 x 22,5cm) were filled with 3,5 kg of compost colonized by *A. bisporus* strain A15 (CNC, Milsbeek, the Netherlands) and covered with 1 kg of casing mix for cutting companies (CNC, Milsbeek, the Netherlands). The mushroom cultures were incubated at 24 ˚C and 95% relative humidity (RH) for 8 days and from day 9 at 20 ˚C and 88% RH. Effects of 1-octen-3-ol were studied either by exposing the cultures to the volatile or by applying it as a suspension in water. The mushroom cultures were inoculated with 10^6 conidia/ m^2 of *L. fungicola* strain V9503 by evenly wetting the casing with 50 ml of a spore suspension (10^3 conidia per ml demineralized water) on day 1.

Continuous exposure of mushroom cultures to volatile 1-octen-3-ol was established by placing 3 Petri-dishes, containing 2 ml of pure 1-octen-3-ol, in one incubator. The Petri-dishes were replaced every other day to have a continuous exposure to 1-octen-3-ol. A second (control) incubator was not subjected to 1-octen-3-ol treatment. Mushroom cultures were also temporarily exposed to 1-octen-3-ol for the first 6 days and then transferred to the control incubator.

In a separate experiment, a suspension of 1-octen-3-ol was applied to inoculated mushroom cultures. On day 3, cultures were treated with 200 ml of a 1.25% or a 0.125% (v/v) of 1-octen-3-ol in water. The control cultures were treated with water in a separate incubator. Mushroom containers treated with 1-octen-3-ol were moved to the control incubator on day 6.

In all experiments mushrooms larger than 1 cm of the first two flushes were harvested. The first two flushes were harvested and scored in 4 categories: Healthy, spotted cap, stipe blow-out and dry bubble (Berendsen *et al.*, 2010). Disease incidence was scored as the percentage of stipe-blow out and dry bubble.

EFFECT ON SPORE GERMINATION

On day 3 of the experiment in which mushroom cultures were exposed to 1-octen-3-ol as a volatile, the effect on *L. fungicola* spore germination was determined. Membranes containing spores of *L. fungicola* were prepared as described above. ¼ membranes were placed on 100 µl demineralized water, casing soil or PDA in an opened Petri dish and put in both climate chambers with 8 replicates. After 18 hrs, spore germination was quantified as described previously. The remaining membranes were replaced on fresh PDA and were incubated for 6 hr at 24˚C before quantification of spore germination.

QUANTIFICATION OF BACTERIAL POPULATIONS IN CASING

Casing soil was sampled from mushroom cultures on day 5, 12 and 19 of the experiment in which mushroom cultures were exposed to 1-octen-3-ol as a volatile. One g of casing soil was suspended in 10 ml 10 mM $MgSO_4$. Dilution series of the suspensions were plated on 1/10 tryptic soy agar (TSA; per liter: 3 g tryptic soy broth (Difco), 15 g granulated agar(Difco), 100 mg Delvocid (DSM, Delft, the Netherlands) for total culturable populations and on King's B medium agar$^+$ (KB agar (King *et al.*, 1954) supplemented with 13 µg/ml chloramphenicol, 40 µg/ml ampicilin, and 100 µg/ml Delvocid (DSM, Delft, the Netherlands)) for the population of *Pseudomonas* spp. Colony-forming units (cfu) were determined after 5 days of incubation at 28 C.

EFFECT OF 1-OCTEN-3-OL ON DRY BUBBLE DISEASE IN A COMMERCIAL SETTING

The effect of 1-octen-3-ol application on dry bubble disease was also studied in a large scale set up that closely resembles commercial mushroom growing. Containers (0.2 m^2) were filled with 15 kg of compost colonized by *A. bisporus* strain Sylvan A15 (CNC, Milsbeek, the Netherlands) and covered with a layer of casing mix (CNC, Milsbeek, the Netherlands). To enhance colonization of the casing, colonized compost was subsequently mixed through the casing at a density of 200 g / m^2. The cultures were subsequently inoculated with *L. fungicola* V9503 (see above). On day 3, 600 ml of a 1.25% or a 0.125% (v/v) 1-octen-3-ol suspension was added to the cultures. As a positive control, cultures

were treated with 600 ml 0.4 % (w/v) Sporgon (Active ingredient: Prochloraz-manganese, BASF, Arnhem, the Netherlands) in water. Six hundred ml of water was added to control cultures. Half of the control treated cultures were placed in the chamber with the 1-octen-3-ol treated cultures, and the other half in a separate chamber. A control treatment that was not inoculated with *L. fungicola* was kept in the treatment room. Ten replicates were used per treatment. The mushroom cultures were incubated at 23 ˚C and 95 % relative humidity (RH) for 9 days during which CO_2 concentrations were above 3000 ppm. On day 10, ventilation was increased and conditions were changed to 20 ˚C, 88 % RH and 1000-1200 CO_2 ppm. Mushrooms were harvested for 3 weeks, and numbers of diseased mushrooms and weight of healthy mushrooms were scored.

EFFECT OF 1-OCTEN-3-OL ON RADIAL GROWTH

Small blocks of PDA colonized by *A. bisporus* strain Sylvan A15 or *L. fungicola* were excised from the edge of growing colonies of both fungi. The blocks were inoculated on PDA in a two-compartment Petri dish (Greiner bio one, Alphen aan de Rijn, the Netherlands). Thirty µl 1-octen-3-ol, 1 ml saturated 1-octen-3-ol solution or 10- and 100-fold dilutions was placed the second compartment of the Petri dishes (corresponding to 200, 20, 2, or 0.2 µmol 1-octen-3-ol, respectively). The dishes were sealed with Parafilm and incubated at 20˚C.

STATISTICS

Data were analyzed using analysis of variance (ANOVA) with a Tukey post-hoc test (SPSS 16.0). When data did not fit a normal distribution or homogeneity of variance, non-parametric multiple comparison with tied ranks was performed (Zar, 1999). Bacterial counts were log transformed before analysis. Experiments were repeated with similar results except for the experiment performed in a commercial setting which was performed only once.

RESULTS

SELF INHIBITION OF SPORE GERMINATION IN *L. FUNGICOLA*

The effect of volatiles produced by a colony of *L. fungicola* on germination of its own spores was investigated. Spores of *L. fungicola* germinated within 18 hrs on phosphate buffer (figure 1). In the presence of a growing culture of *L. fungicola* on PDA, germination of spores was significantly reduced by 45 %.

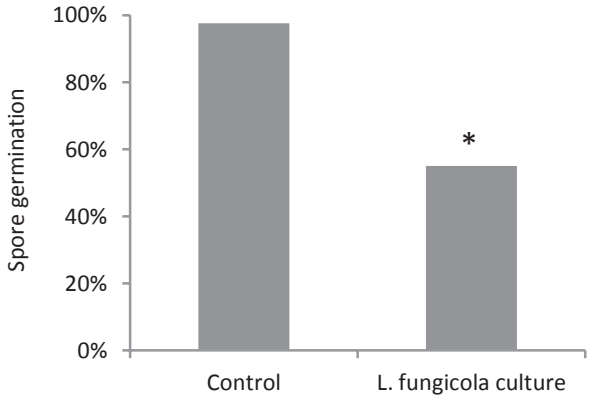


Figure 1. Effect of colony volatiles on germination of *L. fungicola* spores. Spore germination was determined with Cyclopore membranes incubated for 18 hrs on phosphate buffer next to sterile PDA or PDA colonized by *L. fungicola.* Bars represent average of 4 replicates. * indicates significant difference as determined with a Mann-Whitney test ($p<0.05$).

Effect of 1-octen-3-ol on *L. fungicola* spore germination

In a 2 compartment Petri dish, 20 µmol of 1-octen-3-ol completely inhibited spore germination on water and 10 mM glucose, while on PDB 2% of *L. fungicola* spores germinated (figure 2). Non-germinating spores of *L. fungicola* that were exposed to 20 µmol 1-octen-3-ol for 18hrs in the presence or absence of glucose were dead, since they did not germinate after transfer to fresh PDA (data not shown).Two µmol of 1-octen-3-ol reduced *L. fungicola* spore germination on water 5-fold. Spore germination was reduced to a lesser extend (55% and 67%, respectively) on 10 mM glucose or PDB (figure 2). Lower amounts of 1-octen-3-ol did not affect germination of *L. fungicola* spores.

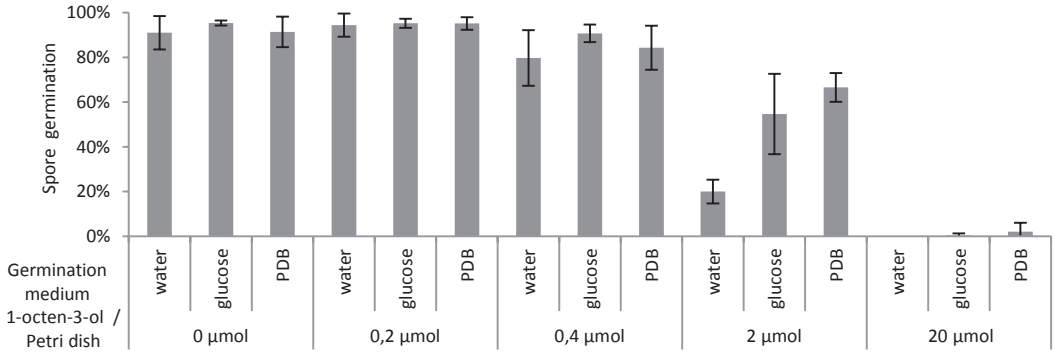


Figure 2. Effect of 1-octen-3-ol on spore germination of *L. fungicola* on different media. Spore germination was determined after 18 hrs on sterile water, 10 mM glucose or potato dextrose broth (PDB) in the presence of different quantities of 1-octen-3-ol. Bars represent average germination after 18 hrs of 4 replicates. Error bars represent standard deviation.

CONTROL OF DRY BUBBLE DISEASE WITH 1-OCTEN-3-OL AS A VOLATILE

The effect of exposure of mushroom cultures to volatile 1-octen-3-ol (47 mmol/ m^3 / 48 hrs) on dry bubble disease was investigated. In non-exposed containers, disease incidence was low (6.8 %) in the first flush, but increased to 58% in the second flush. Consequently, infection by *L. fungicola* did not significantly reduce the weight of healthy mushrooms in the first flush, but did reduce the weight of healthy mushrooms in the second flush (figure 3b). In both the first and the second flush, continuous or temporal exposure to 1-octen-3-ol completely suppressed disease (figure 3a). In the first flush, cultures that were continuously exposed to 1-octen-3-ol produced a significantly lower weight of healthy mushrooms than the non-exposed, non-infected containers. Weight of healthy mushrooms in temporarily exposed containers did not differ from non-exposed controls. In the second flush, the weight of healthy mushrooms from both temporarily and continuously exposed containers did not differ significantly from the non-infected, non-exposed treatment (figure 3b). However, the infected non-exposed treatment produced significantly less healthy mushrooms than the exposed containers. The total number of mushrooms (both healthy and diseased) per container did not differ significantly between treatments, although a trend towards a reduced number of mushrooms in the continuously exposed containers was observed in the first flush (figure 3c).

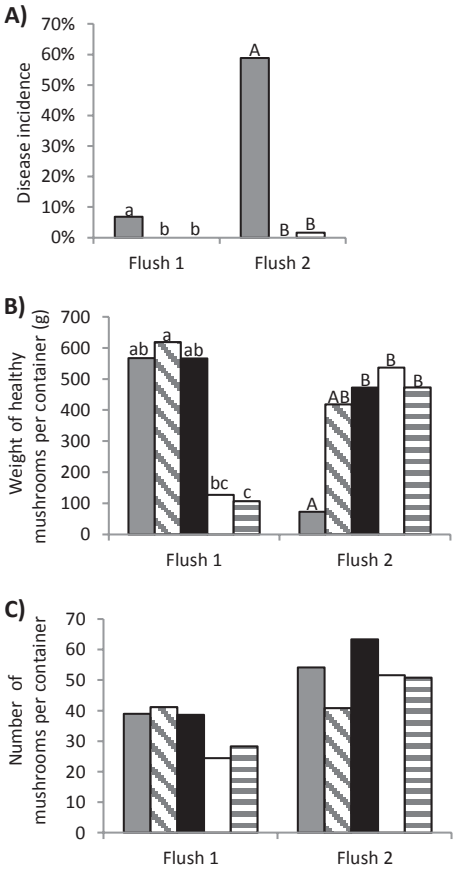


Figure 3. Effect of exposure to volatile 1-octen-3-ol on a) disease incidence, b) weight of healthy mushrooms or c) the total (diseased and healthy) number of mushrooms. Mushroom cultures were not exposed and inoculated with *L. fungicola* (grey bars), or not (diagonal stripes), temporarily exposed and inoculated (black bars) or continuously exposed and either (white bars) or not (horizontal stripes) inoculated with *L. fungicola*. Bars represent the average of 6 replicates. Significant (α=0.05) differences were determined by non-parametric multiple comparison. Letters indicate significant differences in each flush. The number of mushrooms did not differ significantly between treatments.

During the experiment the effect of exposure to volatile 1-octen-3-ol on *L. fungicola* spore germination was investigated. Whereas 92 % of the *L. fungicola* spores germinated within 18 hrs in the not exposed incubator, exposure to 1-octen-3-ol significantly reduced spore germination to 3 % (figure 4). Exposure to 1-octen-3-ol did not affect germination when the spores were incubated on PDA. As expected, spore germination was low (10 %) on casing soil. When exposed to 1-octen-3-ol, spore germination on casing was completely inhibited. To study if the inhibition by 1-octen-3-ol is reversible, membranes containing the spores were transferred to PDA medium after 18 hrs exposure to the volatile. Approximately 50 % of the spores germinated when filters incubated on water or casing in the 1-octen-3-ol exposed incubator were transferred to PDA, suggesting that part of the spores were killed.

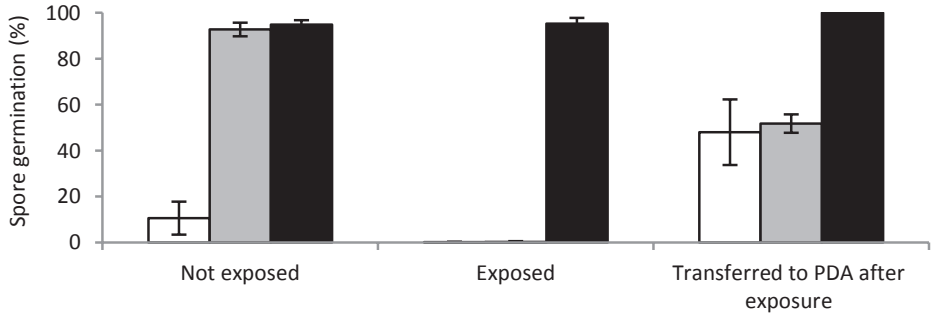


Figure 4. Germination of *L. fungicola* spores on casing (white bars), water (grey bars) or PDA (black bars) in incubators subjected to exposure to volatile 1-octen-3-ol. Bars represent the average of 4 membranes. Error bars represent standard deviation.

No effect on the total number of culturable bacteria in the casing were observed after 5 days of exposure to 1-octen-3-ol (figure 5a). Continuous exposure for 12 days significantly increased population densities and increased populations were sustained after 19 days. In mushroom cultures that were exposed to raised 1-octen-3-ol levels for the first 5 days only, population densities had also significantly increased after 12 days, but were significantly lower than after continuous exposure. After 19 days, population densities of the temporarily exposed casings were no longer significantly higher than those of non-exposed controls.

Population densities of fluorescent pseudomonads were significantly increased by both continuous and temporary exposure to volatile 1-octen-3-ol (figure 5b). The effect of continuous exposure was significant on all sampling dates and reached a maximum 100-fold increase over the control treatment after 12 days of exposure. Temporarily exposure significantly increased population densities of fluorescent pseudomonads on day 5 and day 12, but after 19 days the effect was no longer significant.

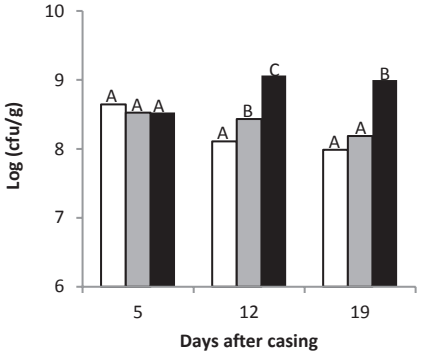


Figure 5a. Effect of 1-octen-3-ol exposure on population densities of total culturable bacteria. Bars represent average log of colony forming units (cfu) /g. of casings that were not (white bars), temporary (grey bars), or continuously (black bars) exposed to 1-octen-3-ol. Letters represent significant (α=0.05) differences between the treatments within a time point as determined through ANOVA.

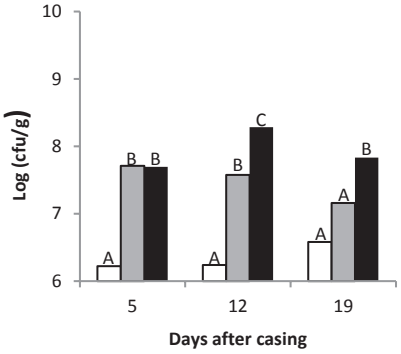


Figure 5b. Effect of 1-octen-3-ol exposure on population of *Pseudomonas* spp. Bars represent average log of colony forming units (cfu) /g. of casings that were not (white bars), temporarily (grey bars), or continuously (black bars) exposed to 1-octen-3-ol. Letters represent significant (α=0.05) differences between the treatments within a time point as determined through ANOVA.

APPLICATION OF 1-OCTEN-3-OL IN SUSPENSION TO CONTROL DRY BUBBLE DISEASE

Because saturating large mushroom growth cells with volatile 1-octen-3-ol would require copious amounts of 1-octen-3-ol, it was tested if dry bubble disease could be controlled by applying 1-octen-3-ol in water (1.25 % and 0.125 % w/v) in small incubators. In the first flush, disease incidence was low and although 1-octen-3-ol treatment reduced disease incidence the effects were not significant (figure 6a). In the second flush, disease incidence was 30 % in the control treatment. Treatment with 1.25 % 1-octen-3-ol significantly reduced disease incidence. Whereas disease incidence after treatment with 0.125 % 1-octen-3-ol was much lower than the control, this effect was not significant.
In the first flush, treatment with 1.25 % 1-octen-3-ol reduced the weight of healthy mushrooms significantly, whereas in the second flush, weight of healthy mushrooms was significantly increased. Effects of 0.125 % 1-octen-3-ol on weight of healthy mushrooms was in none of the cases significant (figure 6b). In both flushes, the total numbers of mushrooms (healthy and diseased) were not significantly affected by 1-octen-3-ol treatments (figure 6c).

Figure 6a. The effect of 0 – 1.25 % 1-octen-3-ol in water on dry bubble disease incidence. Mushroom cultures are treated with water (grey bars), 0.125 % 1-octen-3-ol (black bars) or 1,25% 1-octen-3-ol (white bars). Bars represent an average of 6 replicates. Significant (α=0.05) differences were determined by non-parametric multiple comparison. Letters indicate significant differences in each flush.

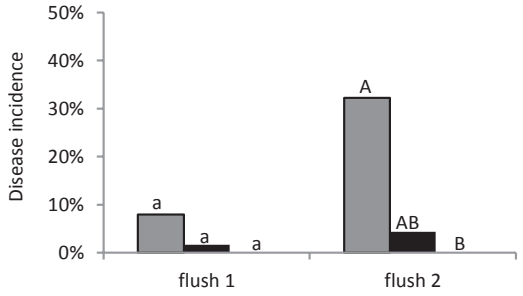


Figure 6b. The effect of 0 – 1.25 % 1-octen-3-ol in water on the average weight of healthy mushrooms per container. Mushroom cultures treated with water (grey bars), 0.125 % 1-octen-3-ol (black bars) or 1,25% 1-octen-3-ol (white bars). Bars represent an average of 6 replicates. Significant (α=0.05) differences were determined by non-parametric multiple comparison. Letters indicate significant differences in each flush.

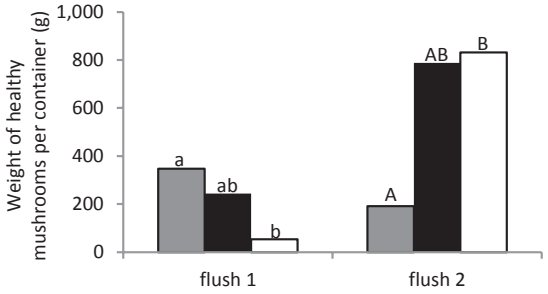
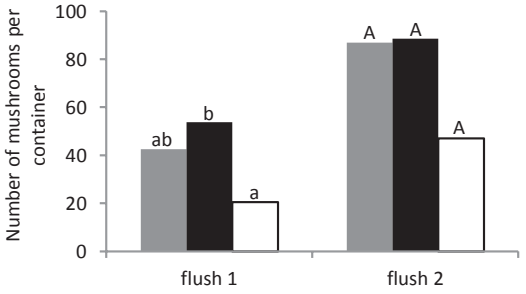


Figure 6c. The effect of 1-octen-3-ol in water on the average number of mushrooms (diseased and healthy) per containers. Mushroom cultures treated with water (grey bars), 0.125 % 1-octen-3-ol (black bars) or 1,25% 1-octen-3-ol (white bars). Bars represent an average of 6 replicates. Significant (α=0.05) differences were determined by non-parametric multiple comparison. Letters indicate significant differences in each flush.



EFFECT OF 1-OCTEN-3-OL ON DRY BUBBLE DISEASE IN A COMMERCIAL SETTING

The effect of 1-octen-3-ol on dry bubble disease was tested in a large climate-controlled chamber mimicking commercial mushroom cultivation. Treatment with 1.25 % 1-octen-3-ol in water reduced the number of mushrooms with symptoms of dry bubble disease after 3 flushes significantly (figure 7a). The reduction in disease incidence by the 1.25 % 1-octen-3-ol treatment was comparable to the disease reduction obtained by the standard treatment with Sporgon. Treatments with more diluted 1-octen-3-ol did not affect the number of diseased mushrooms significantly.

The weight of healthy mushrooms was significantly reduced after inoculation with *L. fungicola* (figure 7b). In the first flush, both Sporgon and treatment with 1.25 % 1-octen-3-ol reduced the weight of healthy mushrooms, however, this negative effect was compensated in the second and third flush. The total weight of healthy mushrooms after application of 1.25 % 1-octen-3-ol was not significantly different from the non-inoculated control. Application of 1-octen-3-ol after the first flush reduced the weight of healthy mushrooms that were produced in the second and third flush. Mushroom that had already partially formed at the time of 1-octen-3-ol application discolored within a day (not shown).

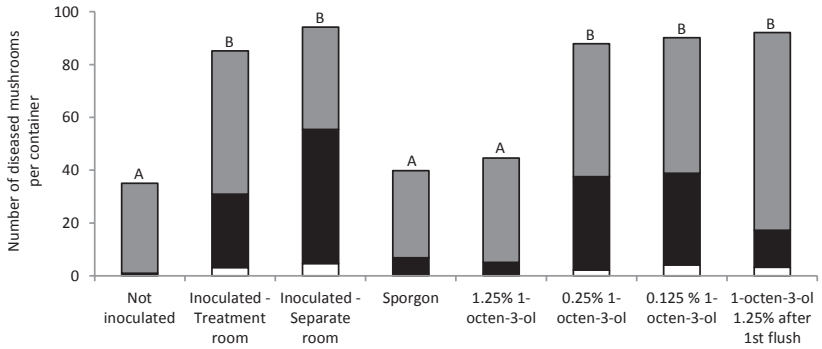


Figure 7a Effect of treatment with 0 – 1.25 % 1-octen-3-ol in water on the number of diseased mushrooms in a setting resembling commercial production of mushrooms. Mushroom cultures were inoculated with *L. fungicola* on day 1 and treated on day 3 or immediately after the first flush were harvested. Bars indicate average of 10 replicates. Stacks within bars differentiate flush 1(white), flush 2 (black) and flush 3 (grey). Capitals indicate significant (α=0.05) differences according to ANOVA.

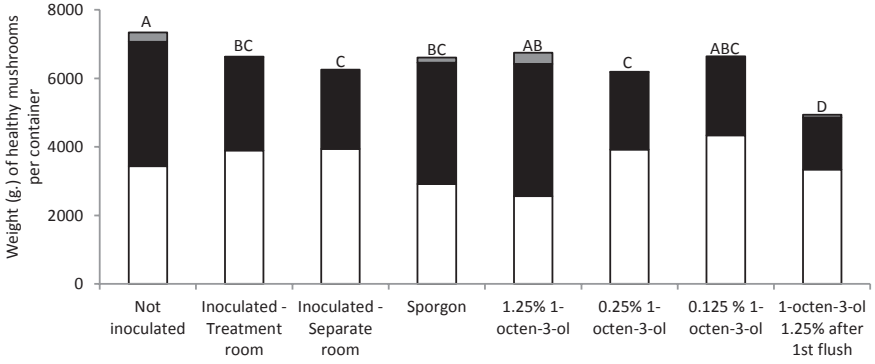


Figure 7b Effect of treatment with 0 – 1.25 % 1-octen-3-ol in water on the weight of healthy mushrooms in a setting resembling commercial production of mushrooms. Mushroom cultures were inoculated with *L. fungicola* on day 1 and treated on day 3 or immediately after the first flush were harvested. Bars indicate average of 10 replicates. Stacks within bars differentiate flush 1(white), flush 2 (black) and flush 3 (grey). Capitals indicate significant (α=0.05) differences according to ANOVA.

EFFECT OF 1-OCTEN-3-OL ON VEGETATIVE GROWTH OF *L. FUNGICOLA* AND *A. BISPORUS*

As colonization of the casing by *A. bisporus* was retarded in 1-octen-3-ol treated mushroom cultures, the effect of 1-octen-3-ol on vegetative growth was investigated. Vegetative growth of both *A. bisporus* and *L. fungicola* on PDA was negatively affected by 1-octen-3-ol (figure 8). Whereas 0.2 or 2 µmol 1-octen-3-ol did not affect fungal growth, 20 µmol 1-octen-3-ol reduced growth of both fungi and 200 µmol inhibited their growth completely.

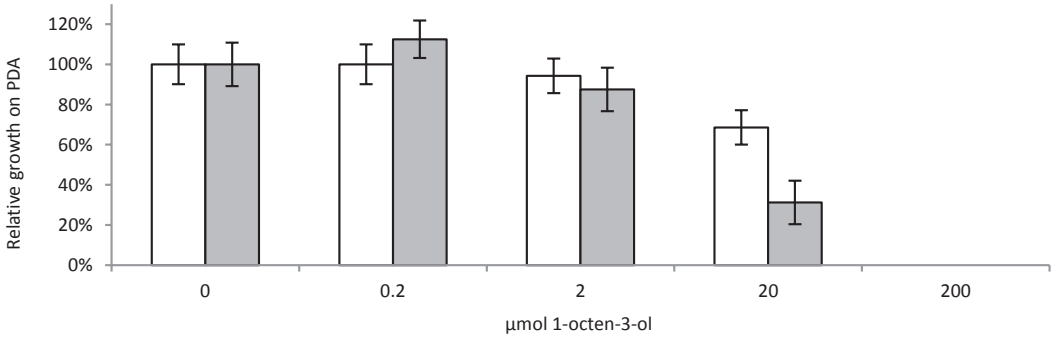


Figure 8. Effect of 1-octen-3-ol on vegetative growth of *L. fungicola* (white bars) and *A. bisporus* (grey bars). The average radius of fungal colonies relative to the control on PDA without 1-octen-3-ol are presented. Error bars represent standard deviation.

DISCUSSION

The volatile 1-octen-3-ol it is a potent inhibitor of fungal growth and spore germination. In *Penicillium paneum,* this volatile is a self-inhibitor that prevents spore germination at high spore densities (Chitarra et al., 2005). 1-octen-3-ol also inhibits radial growth of several fungi (Okull *et al.*, 2003; Chitarra *et al.*, 2005), suggesting that it has a broad antifungal effect.

In this study, it was found that colonies of *L. fungicola*, the causal agent of dry bubble disease, also produce volatile metabolites that inhibit the germination of *L. fungicola* spores (figure 1). However, static head-space analysis of growing *L. fungicola* colonies showed that *L. fungicola* does not produce 1-octen-3-ol (unpublished results). Nonetheless, 1-octen-3-ol is a major component of the volatiles that cause the typical smell of the white button mushroom (Combe*t et al.*, 2006; Noble *et al.*, 2009). As germination of spores of *L. fungicola* is strongly inhibited by 1-octen-3-ol (figure 2), we investigated if exogenous application of high concentrations of 1-octen-3-ol is effective in controlling dry bubble disease.

Exogenous application of volatile 1-octen-3-ol in mushroom cultures significantly reduced dry bubble disease. Both continuous exposure and exposure for only the first 5 days of mushroom cultivation were effective in controlling the disease (figure 3). Continuous exposure to 1-octen-3-ol negatively affected the formation of mushrooms. Noble *et al.* (2009) reported that high levels of 1-octen-3-ol can inhibit fruiting body formation in *A. bisporus.* When applied as a volatile, continuous exposure to 1-octen-3-ol reduced the weight of mushrooms in the first flush of mushroom cultures and the number of fruiting bodies was also lower. In the second flush, however, mushroom cultures continuously exposed to 1-octen-3-ol had equal number and higher weight of mushrooms than the non-exposed control treatment. It was observed that vegetative growth in the continuously exposed cultures was retarded (data not shown) and that radial growth of *A. bisporus* was inhibited by high levels of 1-octen-3-ol (figure 8). Inhibition of growth could alternatively explain the observed inhibition of fruiting body formation by 1-octen-3-ol found by Noble *et al.* (2009).

The negative impact of 1-octen-3-ol on mushroom yield was prevented by only exposing the mushroom cultures for the first five days of the experiment. Apparently, the casing layer acts as a buffer and protects the *A. bisporus* mycelium that it covers.

Treatment of infected mushroom cultures with a 1.25 % suspension of 1-octen-3-ol in water also significantly reduced the dry bubble disease in small scale mushroom incubators (figure 6). Under culturing conditions closely resembling commercial mushroom cultivation, 1.25 % 1-octen-3-ol was as effective as Sporgon, the fungicide that is generally used by the industry to control dry bubble disease (figure 7).

1-octen-3-ol can affect the growth and germination of *L. fungicola* directly. However, the effect of 1-octen-3-ol on dry bubble disease may also have been indirect by affecting microbial populations in the casing. Noble *et al.* (2009) demonstrated that 1-octen-3-ol exposure results in increased population densities of total bacteria and *Pseudomonas spp.* in the casing layer. Also in our study, exposure to 1-octen-3-ol, either continuous or temporary, caused an increase in population densities of total bacteria and *Pseudomonas spp.* (figure 5). Because spore germination of *L. fungicola* is inhibited by the casing's microbial population, a phenomenon known as fungistasis (Chapter 3), an increase in bacterial populations by 1-octen-3-ol could have consequences for soil fungistasis.

L. fungicola is not the only pathogenic fungus that is sensitive to 1-octen-3-ol. We observed that 1-octen-3-ol inhibits radial growth (on PDA) of the mushroom pathogens *Mycogone perniciosa* and *Trichoderma aggressivum* and the plant pathogens *Rhizoctonia solani, Fusarium oxysporum f. sp. raphani* and *Botrytis cinerea* (unpublished results). Thus 1-octen-3-ol may be used to control a broad range of fungal diseases both in mushroom and plant crops. Also, 1-octen-3-ol has been shown to trigger a defense response in *Arabidopsis thaliana (*Kishimoto *et al., 2007)*. Combined effects of induced defense responses and direct inhibition by 1–octen-3-ol could result in robust effectiveness in controlling fungal diseases in plants.

1-octen-3-ol could play an interesting role in the ecology of the interaction between *A. bisporus* and *L. fungicola*. The formation of fruiting bodies in *A. bisporus* is inhibited by 1-octen-3-ol, and this inhibition is lifted when 1-octen-3-ol concentrations are lowered (Noble *et al.*, 2009). Thus the fact that this fungal pathogen can only infect the fruiting bodies of *A. bisporus* (Bernardo *et al.*, 2004; Calonje *et al.*, 2000a) may coincide with 1-octen-3-ol levels in the mushroom cultures. Germination of *L. fungicola* is postponed until fruiting body formation when the levels of 1-octen-3-ol drop. Germination of *L. fungicola* spores is inhibited by 1-octen-3-ol (figure 2), however, on PDA or 10 mM glucose, the spores were less sensitive. Thus when more nutrients are available *L. fungicola*, is less affected by 1-octen-3-ol. Similarly, it was shown in Chapter 3 that nutrients leaching from *A. bisporus* hyphae lift inhibition of *L. fungicola* spore germination that is caused by bacterial activity in the casing. Spores of *L. fungicola* did not seem to be affected by the amounts of 1-octen-3-ol produced by *A. bisporus* in an incubator filled with actively growing cultures of mushrooms (figure 4). It is not likely that in commercial mushroom cultivation 1-octen-3-ol levels in the air exceed the levels in our incubators, as, in comparison to the average mushroom farm, there is little headspace in our system. However, levels of 1-octen-3-ol in the casing can locally still exceed the threshold for inhibition. In this respect, it should be noted that the amount of 1-octen-3-ol produced by *A. bisporus* can differ between strains of the mushroom (Cruz *et al.*, 1997). Selecting *A. bisporus*

strains that produce high levels of 1-octen-3-ol and that are not affected by these compounds might result in the discovery of more resistant mushroom varieties.
Recently, it was postulated that 3-octanone is the main volatile produced by whole fruiting bodies of *A. bisporus (*Combet *et al., 2009)*. Noble *et al.* (2009) found that, similar to 1-octen-3-ol, 3-octanone was produced by *A. bisporus* in compost and casing under axenic conditions and was also detected at lower concentrations in non-axenic cultures. In the same study, 3-octanone did not affect the formation of primordia. In a pilot study, 3-octanone was found to inhibit *L. fungicola in vitro*. Given the effectiveness of 1-octen-3-ol in this study it seems worthwhile to also study effects of 3-octanone on dry bubble disease *in vivo* and its effects on growth of *A. bisporus*.
This study demonstrated that 1-octen-3-ol effectively controls dry bubble disease. Although application of 1-octen-3-ol can also negatively affect the production of mushrooms, this can be prevented by applying 1-octen-3-ol before the casing is colonized by *A. bisporus*. We showed that 1-octen-3-ol and Sporgon are equally effective in controlling dry bubble disease. 1-octen-3-ol therefore has the potential to be used as an alternative by mushroom growers in treating dry bubble disease.

ACKNOWLEDGEMENT

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Summarizing discussion

INTRODUCTION

As with most other crops, the cultivation of the white button mushroom is affected by a wide range of pests and pathogens. Populations of most of these harmful organisms can be controlled through measures of hygiene that aim to exclude and/or contain them. Nonetheless, infections do occur that severely affect mushroom production. One of the most devastating pathogens of *Agaricus bisporus* is *Lecanicillium fungicola* that causes dry bubble disease. Symptoms caused by *L. fungicola* depend on the developmental stage of the mushroom culture (North and Wuest, 1993). When fully developed mushrooms are infected only small necrotic lesion will appear that may grow out wart-like. When infections occur earlier in the development they can cause severe deformations of the stipe (stipe blow-out) and can ultimately lead to totally amorphous masses of mushroom mycelium, the so called dry bubble (chapter 2: figure 1).
Control of dry bubble disease is difficult. Broad-spectrum fungicides can also severely affect the fungal host. Furthermore, fungicides that have been used in the past are no longer available or have a reduced effectiveness because the pathogen has developed resistance (Bollen and van Zaayen, 1975; Fletcher and Yarham, 1976; Gea, 1996; Wuest *et al.*, 1974). Currently, the mushroom industry relies on Sporgon (effective compound: prochloraz-manganese). In fact, in many countries, Sporgon is the only remaining chemical still permitted for the control of dry bubble disease. As *L. fungicola* is also becoming tolerant to Sporgon (Gea *et al.*, 2005; Grogan, 2008), innovative control measures for this pathogen are urgently needed. The research described in this thesis set out to develop alternatives to control dry bubble disease.

L. FUNGICOLA'S ARRIVAL IN THE MUSHROOM CULTURE

Dry bubble disease is a commonly occurring and widespread problem (Largeteau and Savoie, 2010). This indicates that commercial strains of *A. bisporus* are very susceptible to the pathogen. Over and above this, *L. fungicola* produces large amounts of spores after infection that easily spread when mushroom cultures are watered. As a result of this rapid dispersal, dry bubble disease can increase exponentially with every flush, leading to an epidemic (White, 1981). Therefore, to manage the disease it is of primary importance to prevent infection. Spread of *L. fungicola* occurs via dispersal of its spores. After a previous disease outbreak on a farm, spores of *L. fungicola* may still be present and thus serve as inoculum for following mushroom cultures. The spores of *L. fungicola* can survive for 7-8 months under dry conditions (Fekete, 1967) and even longer in soil (Cross and Jacobs, 1968). However, spores of *L. fungicola* die when exposed to temperatures higher than 42 ˚C (Fekete, 1967). Thus, steam treatment of growth facilities can be effective to eliminate spores of *L. fungicola.*

The primary source of *L. fungicola* infection can also be outside the farm. However, the mushroom substrates are an unlikely source of infective propagules. *L. fungicola* will not survive the high temperatures (70 to 80 ˚C) that occur during the composting process. Moreover, *L. fungicola* can not proliferate saprophytically in casing soil. This is caused by the microbial community in the black peat that is used as casing soil (Cross and Jacobs, 1968; Chapter 3). Therefore, introduction of *L. fungicola* to mushroom cultures likely takes place on the mushroom farm.

Spores of *L. fungicola* are produced in a mucilage and stick easily to all sorts of objects that can then serve as vectors of the disease (Ware, 1933). Personnel, equipment and insects have been identified as important vectors of *L. fungicola* spores (Fekete, 1967; Cross and Jacobs, 1968; Wong and Preece, 1987; Largeteau *et al.*, 2004a; Shamshad *et al.*, 2009a). Personnel and equipment can transport *L. fungicola* from other farms or other parts of the same farm were dry bubble disease is present. Thorough training of personnel to recognize dry bubble disease and regular cleaning of equipment is of evident importance for prevention. Insects can similarly transport Lecanicillium from farm to farm, but can potentially also bring in spores from infected mushrooms in the wild. Although the host range of *L. fungicola* is not clearly defined, it has been reported on mushroom species other than *A. bisporus* (Gea *et al.*, 2003; Marlowe, 1982; Zare and Gams, 2008). It is known that *Megaselia halterata* phorid flies are attracted by compost colonized by *A. bisporus* (Tibbles *et al.*, 2005). Indeed during the filling of mushroom production cells, large numbers of flies are present (personal communication J. Hooijmans, mushroom grower, Kerkdriel, the Netherlands). *L. fungicola* spores stuck to these flies may therefore be the primary inoculum source.
It is generally accepted that *L. fungicola* can only infect the fruiting bodies of *A. bisporus* and not the vegetative mycelium (Bernardo *et al.*, 2004; Calonje *et al.*, 1997; Calonje *et al.*, 2000a). When compost colonized by *A. bisporus* is added to a plate colonized by *L. fungicola*, *A. bisporus* even seems to overgrow *L. fungicola* (unpublished observations). As a consequence, infection of mushroom cultures by *L. fungicola* must take place in the casing. After *L. fungicola* spores have been introduced to the casing, spore germination is a crucial step in the infection process.

ECOLOGY OF *L. FUNGICOLA* IN THE CASING

L. fungicola spore germination in the casing is strongly inhibited until the casing is colonized by *A. bisporus* (Cross and Jacobs, 1968; Chapter 3). The inhibition of fungal spore germination is common to most soils and this phenomenon is known as soil fungistasis (Lockwood, 1977; Garbeva *et al.*, 2011a). As casing fungistasis is annulled by sterilization

of the casing (chapter 3), it is hypothesized to be caused by the casing microflora. As casing fungistasis is annulled by addition of glucose, nutrients leaching from *A. bisporus* hyphae have been assumed to lead to germination of *L. fungicola* in the casing (Cross and Jacobs, 1968). Indeed, it was demonstrated in chapter 3 that addition of *A. bisporus*-associated sugars annuls fungistasis of the casing resulting in germination of *L. fungicola* spores. This indicates that casing fungistasis is based on competition for nutrients. Yet, spores of *L. fungicola* can germinate independent of external nutrients. As the spore germination of other fungal species can be inhibited by the continuous discharge of spore exudates, it has been proposed that fungistasis is brought about by the casing microflora acting as a nutrient sink (Hsu and Lockwood, 1973). It was demonstrated that leaching of *Fusarium oxysporum* spore exudates indeed reduced spore germination for this plant pathogenic fungus (chapter 3). However, for *L. fungicola* such a mechanism could not be demonstrated. Apparently, spores of *L. fungicola* develop nutrient dependency only in the presence of the casing microflora, but not because the casing microflora drains nutrients from the spores. Possibly, antifungal compounds produced by the microflora cause this phenomenon. Indeed, a bacterium that is well studied for its production of secondary metabolites (*Pseudomonas fluorescens* CHA0) inhibited spore germination, whereas a knock-out mutant that no longer produces antibiotics did not. Germination inhibition by the antibiotics of CHA0 was annulled by addition of any carbon source utilizable by Lecanicillium, indicating that *L. fungicola* spores are less sensitive to the antibiotics of CHA0 if enough energy is available. The carbon sources might be used to activate energy-demanding processes to cope with the antibiotics. *Botrytis cinerea* defends itself against the antibiotic 2,4-diactylphloroglucinol (DAPG) through the activation of an ABC-transporter and the production of a laccase that is involved in the degradation of DAPG (Schoonbeek *et al.*, 2002; Schouten *et al.*, 2008). Similar processes might be involved in the casing Notably, sterilized casing that was allowed to be recolonized by CHA0, did not develop effective fungistasis. Also, *in vitro* antagonistic *Pseudomonas* spp. that was isolated from casing could not reinstate fungistasis in sterilized casing. When 1% of non-sterile casing was added to sterilized casing, fungistasis returned within a week. Thus casing fungistasis appears to be brought about by cohorts of microbes and not by single species. Antibiotics produced by different species might work synergistically and collectively impose fungistasis, but it has also been demonstrated that bacteria adjust the production of secondary metabolites to the presence of specific competitors (Garbeva and de Boer, 2009; Garbeva *et al.*, 2011b).

Soil fungistasis is considered beneficial for pathogenic fungi as it prevents them from wasting energy without a suitable food source (Lockwood, 1977; Garbeva *et al.*, 2011a; Termorshuizen and Jeger, 2008). *Cochliobolus sativus* strains of which spores were unaf-

fected by fungistasis disappeared more rapidly from soil than strains of which spore germination was inhibited (Chin and Tinline, 1963). Sensitivity of fungi to fungistasis differs between species, but especially pathogens seem to be sensitive (de Boer *et al.*, 1998). Also for the mycoparasitic *L. fungicola*, fungistasis must be considered advantageous. If *L. fungicola* spores would germinate immediately after introduction to the casing, they would be faced with the strong competition for the few available nutrients available in the casing prior to arrival of *A. bisporus*. Dormant *L. fungicola* spores can survive for more than a year (Cross and Jacobs, 1968). After the casing is colonized by *A. bisporus*, *L. fungicola* then uses its endogenously stored nutrients to grow towards its host.

Fungistasis can be manipulated to reduce disease incidence. Such manipulation aims to either boost the fungistasis or temporarily lift it (Garbeva *et al.*, 2011a). Both scenarios are not likely to be applicable for management of dry bubble disease. Temporarily alleviating fungistasis can be accomplished through addition of nutrients and serves the purpose of exposing an activated pathogen to saprophytic competition (Lockwood, 1977; Garbeva *et al.*, 2011a). As root exudates of non-host plants are assumed to annul fungistasis, it is believed to be the main mechanism through which crop rotation reduces pathogen inoculum (Garbeva *et al.*, 2011a). Germination of *L. fungicola* spores could indeed be stimulated through the addition of e.g. glucose. However, as primary infection of the casing by *L. fungicola* is expected to take place on the farm, pre-treatment of the casing would be pointless. Treatment of casing on the farm would allow for little time between induced germination and colonization of the casing by *A. bisporus* and would more likely aid *L. fungicola* than negatively affect it. Temporary removing fungistasis by steam treatment of the casing immediately before covering the compost, was shown to aggravate dry bubble disease (Wuest *et al.*, 1970).

Strengthening fungistasis would aim to increase nutrient withdrawal by the microflora, which would subsequently camouflage the nutrients released upon arrival by *A. bisporus* (Garbeva *et al.*, 2011a). For example, efficient consumption of linoleic acid from seed exudate by *Enterobacter cloacae* prevented the germination of Pythium spores, resulting in control of seed infection by Pythium (Van Dijk and Nelson, 2000). However, in chapter 3 it is shown that *L. fungicola* spore germination is not stimulated by a specific compound, but rather by any C-source associated with *A. bisporus*. Camouflage of such a generic stimulus would require very efficient use of all carbon-sources leaching from *A. bisporus* hyphae, and it is questionable if this is feasible. Nonetheless, casing fungistasis illustrates that *L. fungicola* spores are affected by microbial antagonism.

BIOLOGICAL CONTROL OF DRY BUBBLE DISEASE

After germination, fungi are more sensitive to competition by microorganisms (Chin and Tinline, 1963). For the control of fungal plant pathogens, antagonistic microorganisms have been effectively exploited, resulting in commercial products (Weller *et al.*, 2002; Fravel, 2005; Haas and Defago, 2005). Such biological control agents affect the pathogen directly through competition for nutrients (Duijff *et al.*, 1993; Van Dijk and Nelson, 2000), or antibiosis (Chin-A-Woeng *et al.*, 2003; Haas and Defago, 2005), or indirectly through induced systemic resistance (Bakker *et al.*, 2007; Van Wees *et al.*, 2008). A prerequisite for an effective control agent is that it can maintain sufficient numbers on the site of control (Bull *et al.*, 1991). Independent of the mechanism of control, 10^5 colony-forming units (cfu) per gram root seems to be the threshold level below which there is no effective biological control (Raaijmakers *et al.*, 1995; Raaijmakers *et al.*, 1999). A biological control agent of dry bubble disease should thus be well adjusted to living in casing colonized by *A. bisporus* and preferably closely associated to the mushroom. Therefore, bacteria were isolated from colonized casing and were tested *in vitro* for their ability to inhibit *L. fungicola*. With a few well-characterized biological control strains it was determined that, *in vitro, L. fungicola* is sensitive to siderophore-instigated competition for iron and to antibiosis. The *in vitro* antagonistic isolates were all identified as *Pseudomonas spp*. This is the most studied genus when it comes to biological control bacteria (Chin-A-Woeng *et al.*, 2003; Bakker *et al.*, 2007; Weller, 2007; Höfte and Altier, 2010). Furthermore, *Pseudomonas* spp. form a dominant group within the bacterial casing microflora and population densities of these Pseudomonads increase when the casing is colonized by *A. bisporus* (Doores *et al.*, 1986; Samson, 1986; Miller *et al.*, 1995; Fermor *et al.*, 2000; Pardo *et al.*, 2002). Survival of *P. putida*, WCS358r and *P. fluorescens* WCS417r and WCS374r, was monitored during colonization of the casing by *A. bisporus*. Two of these strains, WCS358r and WCS417r, maintained population sizes well above 10^5 cfu/ g casing. As the pseudomonads that were isolated from colonized casing are likely better adapted to survival in this environment, it was assumed that these isolates would grow equally well or better than these WCS-strains.

None of the isolates from casing significantly affected disease incidence (chapter 4). The insensitivity of *L. fungicola* to microbial antagonism *in vivo* might be explained by the fact that the selected isolates behaved differently in the casing, than in the *in vitro* experiments. Possibly, they produce smaller amounts of antibiotics or siderophores and the concentrations of the produced compounds might have been too low to affect *L. fungicola*. Furthermore, the desensitization of *L. fungicola* spores to inhibitory compounds by nutrients of *A. bisporus* might explain the insensitivity of the pathogen to the selected

isolates. Also in the biological control of plant pathogens, *in vitro* antagonism is not a guarantee of *in vivo* disease control (Reddy *et al.*, 1994).

The concept "pathozone" was introduced by Gilligan (Gilligan, 1985). The pathozone can be defined as the zone surrounding plant roots in which a propagule of a pathogen must reside to successfully infect its host. For a fungal propagule the probability of infection decreases with increasing distance from its host. Around plants, it overlaps and mostly exceeds the rhizophere and it is limited by the saprophytic capabilities of a pathogen in a soil. It is envisaged that direct antagonism reduces the pathozone by reducing the distance that a pathogen can overgrow. In this way, microbial antagonism increases the probability that pathogenic propagules are outside the pathozone. In this respect, it was observed that *A. bisporus* colonizes the casing totally and very densely. Contrasting to when plants and soil-borne pathogens interact, there are no large parts of the casing left uncolonized by the mushroom mycelium. The pathozone consequently covers all of the casing. It can therefore be argued that, although the antagonistic bacteria in our assay might reduce the distance that *L. fungicola* can grow saprophytically, a suitable host is always nearby and infection efficacy is not significantly affected.

Biological control can sometimes develop naturally in disease suppressive soils. In such soils, pathogens do hardly, if at all, establish. Specific disease suppressiveness is caused by one or a limited number of microbial species. Suppressiveness often develops after several years of monoculture of the same plant species and is often triggered by a severe disease outbreak. It is therefore believed that plants somehow selectively stimulate antagonistic microorganisms and use them as a first line of defense (Cook *et al.*, 1995; Weller *et al.*, 2002). It is interesting to speculate about similar functions of beneficial microbes in the defense of mushrooms. In this respect, mycorrhizal mushroom-forming fungi also seem to stimulate bacteria that are beneficial to them (Frey-Klett *et al.*, 2007). Nonetheless, disease suppressive casing have to our knowledge never been reported. Given the fact that casings are used once and only for a few weeks, disease suppressive casing might not be able to develop in such short time.

Biological control of plant pathogens by microorganism often not or not only results from direct antagonism. Many beneficial microorganisms trigger a systemic response in plants upon which the plants potentiate their defenses and become more resistant to attack (Bakker *et al.*, 2007; Van der Ent *et al.*, 2009; Van Loon *et al.*, 1998; Van Wees *et al.*, 2008). Properties of *A. bisporus* that contribute to disease resistance have not clearly be defined (chapter 2) and it has not been investigated if mushrooms can similarly express induced systemic resistance.

ABSENCE OF SYSTEMIC DEFENSE REPONSES IN *A. BISPORUS*

It was recognized early on that pathogens themselves trigger a response in plants. Upon a first attack plants can recognize pathogens and systemically boost their defensive capabilities to prevent further spread and or a second attack of the pathogen (Ross, 1961; Durrant and Dong, 2004). It is thought that in this way plants can save valuable resources and only invest in costly defenses when needed (Heil and Baldwin, 2002). Although the mechanisms are quite different, functionally similar responses exist in animals. Ultimately, both animals and plants are less vulnerable to pathogens when they have had a previous encounter. The presence of functionally similar systems of potentiated responses to secondary attack in both plants and animals indicate that there may be a strong adaptive advantage for such mechanisms. This raised the hypothesis that innate immune systems with inducible defenses have also evolved in other eukaryotes, such as the fungi. Uncovering such induced resistance in mushrooms would allow for the development of new disease management strategies.

Mushrooms of *A. bisporus* strain Sylvan A15 did not induce resistance upon infection by *L. fungicola*. However, commercially available mushroom strains like A15 have been selected for high production and marketability and not for resistance. Possibly, the absence of induced resistance in Sylvan A15 might be a result of an ineffective defense response. *A. bisporus* strain MES01497 was found to be more resistant to dry bubble disease than Sylvan A15. In cultures of MES01497 that were inoculated with *L. fungicola*, fewer diseased mushrooms appeared in the first flush and disease incidence increased slower in subsequent flushes. However, also in mushrooms of MES01497 defenses could not be manipulated by a primary infection of *L. fungicola*. It was concluded that *A. bisporus* does not acquire a systemic resistance against *L. fungicola*. In this light, it is interesting to note that Soanes and Talbot (2010) found that Pathogen Recognition Receptors (PRR's) with a leucine-rich repeat domain are largely absent in the fungal kingdom. PRR's seem to have convergently evolved in both plants and animals to detect pathogens and are required for induced defence responses. It was proposed that fungi might have evolved different classes of PRR's or that fungi have distinct ways of dealing with pathogens that is not based on pathogen-recognition. If mushrooms do not recognize pathogens as such, it is logical consequence that pathogenic attack does not induce a systemic resistance in mushrooms. A lack of pathogen recognition would imply that mushrooms rely on constitutive and wound- induced defenses. Indeed, mushrooms are known for a wide array of constitutively present chemical compounds that are toxic to animals and many of which have fungicidal activity. Although it has been demonstrated that opossums learn to avoid eating poisonous mushrooms (Camazine, 1983) for most toxic or fungicidal compounds, it has not been addressed whether they confer an adaptive advantage. Based

on their biocidal activity their functions have nonetheless been assumed to be defense related (Sherratt *et al.*, 2005; Spiteller, 2008).
Besides these putative constitutive chemical defenses, mushroom have for centuries been known to respond to damage by changing color, taste or odor. These damage-activated changes are based on the enzymatic transformation of an inactive precursor molecule to a biological active compound (Spiteller, 2008). In *A. bisporus,* such wound-activated defense could possibly be recognized in the formation of melanins upon damage or infection (Jolivet *et al.*, 1998; Soler-Rivas *et al.*, 2001).

CONTROL OF DRY BUBBLE DISEASE WITH 1-OCTEN-3-OL, A COMPOUND PRODUCED BY AGARICUS

Spiteller (2008) proposed that 1-octen-3-ol and 10-oxo-*trans*-8-decenoic acid (ODA) might be a wound-activated chemical defense of mushrooms. These compounds are formed in *A. bisporus* through the enzymatic cleavage of linoleic acid, the main fatty acid found in mushrooms of *A. bisporus* (Wurzenberger and Grosch, 1982; 1984). Both ODA and 1-octen-3-ol can inhibited mycelial growth of *Penicillium expansum* (Okull *et al.*, 2003). 1-Octen-3-ol has also been implied as a volatile self-inhibitor of *A. bisporus* fruiting body formation (Noble *et al.*, 2009), and spore germination of *Penicillium paneum* is inhibited by 1-octen-3-ol (Chitarra *et al.*, 2004). In chapter 6 it was demonstrated that germination of *L. fungicola* spores was also blocked by 1-octen-3-ol. Notably, regular addition of 1-octen-3-ol to a mushroom culture effectively reduced dry bubble disease. Whereas a continuous exposure to high levels of 1-octen-3-ol reduced the weight of mushroom in the first flush, this yield loss was compensated for in the second flush. Interestingly, exposure of mushroom cultures to increased 1-octen-3-ol levels for the first 5 days of culture did control dry bubble disease but did not affect mushroom growth. Also applying a 1.25% 1-octen-3-ol suspension directly to a mushroom culture effectively reduced dry bubble disease to a similar extent as Sporgon. Taken together, 1-octen-3-ol is an excellent alternative for Sporgon.
Preliminary results showed that high levels of 1-octen-3-ol also negatively affect other fungi (*Mycogone perniciosae* and *Trichoderma aggressivum)* that are problematic in the production of mushrooms. Furthermore, growth of the plant pathogenic fungi *Rhizoctonia solanii, Fusarium oxysporum f. sp. raphani* and *Botrytyis cinerea* were found to be strongly inhibited by 1-octen-3-ol. Thus, 1-octen-3-ol is a promising compound to control not only diseases in mushroom culture but also in cultivation of crop plants.

CONCLUDING REMARKS

The research described in this thesis set out to find alternatives for the control of dry bubble disease. The use of antagonistic bacteria was explored, but appeared ineffective. The use of 1-octen-3-ol for treatment of dry bubble was demonstrated to be promising. With Sporgon as the only chemical available to control dry bubble disease, increasing resistance of *L. fungicola* towards this chemical (Gea *et al.*, 2005; Grogan, 2008), will undoubtedly result in dry bubble disease becoming an increasing problem. Development of *A. bisporus* strains that are more resistant to *L. fungicola* would support the control of dry bubble disease. However, research on *A. bisporus* is hampered by the difficulties in transforming this fungus. *Schizophyllum commune* is used as a model system to study mushroom development. Molecular tools are available for its effective transformation and recently its genome has been sequenced (Ohm *et al.*, 2010). Production of large amounts of *S. commune* mushrooms can be achieved by cultivating it on Sorghum grains (Berendsen and Schrier, unpublished results). Use of a model system such as *Schizophyllum commune* might facilitate the investigation of mushroom defense against pathogens. However, *S. commune* does not seem susceptible to dry bubble disease. No symptoms were observed after inoculation with *L. fungicola* and the pathogen did not grow on caps of *S. commune* as determined by qPCR (unpublished results). The study of dry bubble disease therefore seems restricted to the white button mushroom. Development of molecular tools in the future and the availability of the genome sequence of *A. bisporus* (http://genome.jgi- psf.org) will be instrumental to study the interaction between *A. bisporus* and its microbial attackers. These studies should reveal new ways to control dry bubble disease.

Nederlandse samenvatting

Net als ieder ander gewas, wordt de commerciële teelt van champignons (*Agaricus bisporus*) bemoeilijkt door een scala aan plaagdieren en pathogenen. Normaliter worden schadelijke organismen in toom gehouden met behulp van strikte hygiëne maatregelen. Dergelijke maatregelen zorgen ervoor dat de meeste ziekteverwekkende organismen niet op een paddenstoelenkwekerij voorkomen en/of zich niet verder over de kwekerij kunnen verspreiden. Desalniettemin veroorzaken sommige organismen grote verliezen in de champignonteelt. De belangrijkste veroorzaker van opbrengstverliezen is *Lecanicillium fungicola*. Deze schimmel, een lid van de *ascomycota*, veroorzaakt de droge mollenziekte. Symptomen van de droge mollenziekte zijn afhankelijk van het moment waarop de champignon door Lecanicillium worden geinfecteerd. Wanneer de infectie plaats vindt op reeds gevormde champignons dan ontstaan er bruine vlekken die wratachtig kunnen uitgroeien. Vindt de infectie in een eerder stadium van ontwikkeling plaats, dan kan dat leiden tot sterke vervorming van de champignon of in het ergste geval een onherkenbare en amorfe massa paddenstoelenmycelium, de zogenaamde droge mol.
Het is moeilijk de droge mollenziekte te bestrijden. Omdat breedwerkende fungciden ook Agaricus kunnen remmen, is er maar een beperkt aantal chemicaliën geschikt voor gebruik in de champignonteelt. Veel van de fungiciden die in het verleden gebruikt werden voor de bestrijding van *L. fungicola* zijn nu echter niet meer beschikbaar, omdat ze verboden zijn of omdat *L. fungicola* resistent is geworden. De paddenstoelenindustrie is daardoor erg afhankelijk geworden van één bestrijdingsmiddel, Sporgon, met als werkzame stof Prochloraz-manganese. In veel landen is Sporgon zelfs het enige nog beschikbare middel op de markt. Nu *L. fungicola* ook resistentie ontwikkelt tegen Sporgon, is er een dringende behoefte aan alternatieven om dit pathogeen te bestrijden. Voor een effectieve beheersing van mollenziekte is kennis van het pathogeen onontbeerlijk. Hoofdstuk 2 van dit proefschrift geeft een overzicht van de beschikbare literatuur over *L. fungicola* . Hieruit bleek ondermeer dat het vegetatieve mycelium van de champignon niet wordt aangetast, maar dat infecties door *L. fungicola* beperkt blijven tot vruchtlichamen van de champignon. Champignons worden gekweekt op een gecomposteerde mix van paarden- en kippenmest met stro. Deze compost wordt volledig gekoloniseerd door het vegetatieve mycelium van de champignon, maar hierop worden nog geen vruchtlichamen gevormd. Die worden pas aangemaakt zodra de compost is afgedekt met een laag dekaarde. Infecties door *L. fungicola* vinden plaats in de dekaarde. De ziekteverwekker wordt voornamelijk verspreid door transport van sporen door onder meer insecten, besmette apparatuur en personeel. Omdat commercieel gebruikte champignonrassen niet erg resistent zijn tegen de droge mollenziekte, neemt na een eerste infectie de ziektedruk exponentieel toe. Om droge mollenziekte te bestrijden is het dan ook belangrijk om een eerste infectie te voorkomen. Nadat sporen in van *L. fungicola* zijn geland in de dekaarde, zullen zij moeten kiemen en naar hun gastheer

moeten toegroeien om te kunnen infecteren. Kieming van sporen in de dekaarde lijkt dus een cruciale stap in het infectie proces.

ECOLOGIE VAN *L. FUNGICOLA* IN DE DEKAARDE

Sporenkieming van *L. fungicola* wordt in de dekaarde sterk geremd totdat de dekaarde wordt gekoloniseerd door het champignonmycelium. Remming van sporenkieming is beschreven voor veel schimmels in een groot aantal bodems en staat bekend onder de naam fungistase. Omdat fungistase voor *L. fungicola* wordt opgeheven door sterilisatie van de dekaarde, wordt aangenomen dat de in de dekaarde aanwezige microflora de fungistase veroorzaakt. Fungistase in de dekaarde kan ook worden opgeheven door toevoeging van glucose. Kolonisatie door het champignonmycelium zou fungistase in de dekaarde op een zelfde manier kunnen opheffen door nutrienten afkonstig van het mycelium. Daarmee in overeenstemming werd in hoofdstuk 3 aangetoond dat toevoeging van verschillende suikers, die zijn geassocieerd met het champignonmycelium, inderdaad leidt tot kieming van *L. fungicola* sporen in de dekaarde. Dit duidt erop dat de fungistase in de dekaarde veroorzaakt wordt door een tekort aan nutriënten. Echter, sporen van *L. fungicola* kunnen kiemen op water, dat wil zeggen zonder externe nutrienten. De sporen van *L. fungicola* zijn echter wel afhankelijk van externe nutriënten in aanwezigheid van de microbiële gemeenschap van de dekaarde. Met behulp van verschillende Pseudomonas isolaten werd aangetoond dat sporenkieming van *L. fungicola* wordt geremd door bacteriële antibiotica. Deze remming wordt opgeheven door toevoeging van suikers die voor Lecanicillium als koolstofbron bruikbaar zijn. Dit duidt erop dat de sporen van *L. fungicola* minder gevoelig worden voor kiemingsremmende substanties zodra er genoeg energie voor handen is. De fungistase in de dekaarde kan op een zelfde manier werken, waarbij inhiberende stoffen geproduceerd door de microflora de kieming van *L. fungicola* sporen remmen totdat Agaricus de dekaarde koloniseert. Daarna lekken er voedingstoffen uit het champignonmycelium waardoor Lecanicillium minder gevoelig wordt voor de inhibitie en kan kiemen. Waarschijnlijk zijn cohorten van microben samen verantwoordelijk voor de fungistase in de dekaarde en antibiotica geproduceerd door verschillende organismen zouden synergistisch fungistase kunnen veroorzaken.

BIOLOGISCHE BESTRIJDING VAN DE DROGE MOLLENZIEKTE

Antagonistische micro-organismen kunnen effectief worden gebruikt ter bestrijding van schimmelziekten en er zijn verscheidene commerciële gewasbeschermingsproducten op de markt. De organismen in dergelijke biologische bestrijdingsmiddelen kunnen direct werken op het pathogeen door concurrentie om nutriënten of door antibiose, maar ook

doordat ze systemische resistentie induceren. Een voorwaarde voor effectieve biologische bestrijding is dat het werkzame organisme zich in voldoende aantallen kan handhaven. Een biologisch bedrijfsmiddel van de droge mollenziekte moet dus aangepast zijn aan de omstandigheden in de door de champignon gekoloniseerde dekaarde. Daarom werden in hoofdstuk 4 bacteriën geïsoleerd uit gekoloniseerde dekaarde. Deze zijn vervolgens *in vitro* geselecteerd op hun vermogen om *L. fungicola* te remmen. Met enkele goed gekarakteriseerde bacterie stammen is vervolgens aangetoond dat *L. fungicola in vitro* gevoelig is voor siderofoor-afhankelijke concurrentie om ijzer en voor antibiose. De uit de dekaarde geïsoleerde stammen zijn vervolgens geïdentificeerd als *Pseudomonas* spp. Dit is ook het meest bestudeerde genus wat betreft biologische bestrijding. Bovendien zijn pseudomonaden dominant binnen de microflora van de dekaarde en worden ze gestimuleerd door aanwezigheid van Agaricus. Echter, geen van de geselecteerde pseudomonaden was in staat de droge mollen ziekte significant te reduceren. De verminderde gevoeligheid van *L. fungicola* voor inhiberende stoffen als gevolg van nutriënten die worden uitgescheiden door *A. bisporus,* zoals gevonden in hoofdstuk 2, kan de *in vivo* ongevoeligheid van Lecanicillium deels verklaren. Biologische beheersing van plantpathogenen is vaak niet alleen een gevolg van direct antagonisme. Sommige micro-organismen zijn in staat de afweer van planten aan te schakelen, waardoor planten resistenter worden tegen microbiële aanvallen. Of schimmels, en *A. bisporus* in het bijzonder, op dezelfde manier een systemisch resistentie kunnen verwerven is echter niet bekend.

SYSTEMISCHE AFWEER IN *A. BISPORUS*?

Planten kunnen, zodra ze een pathogeen herkennen, systemische hun afweer versterken. Daarmee gaan ze verspreiding van het pathogeen tegen en zijn nog niet geïnfecteerde delen van de planten beter beschermd tegen een nieuwe aanval van het pathogeen. Gelijktijdig stelt dit systeem de plant in staat om zijn schaarse middelen alleen te investeren in kostbare afweerreacties als daar ook daadwerkelijk behoefte aan is. Alhoewel de mechanismen van elkaar verschillen hebben ook dieren soortgelijke reacties op aanvallen door pathogenen. Uiteindelijk zijn zowel plant als dier minder vatbaar voor een pathogeen als ze daarmee eerder in contact zijn geweest. Het bestaan van zulke verdedigingssystemen in zowel plant als dier wijst op adaptieve voordelen. Het lijkt dan ook niet onlogisch als ook schimmels dergelijk immuunsystemen met induceerbare afweer hebben ontwikkeld. De ontdekking van zulke induceerbare afweer zou kunnen leiden tot de ontwikkeling van nieuwe manieren om paddenstoelenziekten te bestrijden Paddenstoelen van het commercieel veel gebruikte *A. bisporus* ras Sylvan A15 werden niet resistenter na een eerste infectie met *L. fungicola* (hoofdstuk 5). Echter, de champignonrassen die worden gebruikt in de commerciële paddenstoelen zijn

geselecteerd vanwege hun hoge opbrengsten, maar niet op resistentie. Mogelijk is de afwezigheid van geïnduceerde resistentie in paddenstoelen van Sylvan A15 te wijten aan de gebrekkige afweer in dit ras. Het *A. bisporus* ras MES01497 is resistenter tegen droge mollen ziekte dan Sylvan A15. Echter, ook paddenstoelen van MES01497 werden niet minder vatbaar na een eerste infectie van *L. fungicola*. Het lijkt er dus op dat *A. bisporus* geen resistentie induceert tegen in ieder geval droge mollenziekte. Of andere schimmels wel induceerbare systemisch afweer hebben is vooralsnog onbekend. Het is goed mogelijk dat het immuunsysteem van schimmels daarin flink verschilt van dat van planten en dieren. Het zou bijvoorbeeld kunnen zijn dat afweer in schimmels voor een groter deel afhankelijk is van verwonding-geïnduceerde reacties.

BESTRIJDING VAN DE DROGE MOLLEN ZIEKTE MET 1-OCTEN-3-OL, EEN DOOR AGARICUS GEPRODUCEERDE STOF

Splitsing van linolzuur in 1-octen-3-ol en 10-oxo-*trans*-8-decenoic acid (ODA) lijkt een verwonding geinduceerde afweer in schimmels. 1-octen-3-ol is een belangrijke component van de typische champignongeur. De kieming van sporen van een aantal schimmels wordt geremd door 1-octen-3-ol. In hoofdstuk 6 bleek dat ook sporen van *L. fungicola* in hun kieming werden geremd door 1-octen-3-ol. Tevens bleek het mogelijk om de droge mollen ziekte in een geïnfecteerde champignoncultuur aanzienlijk te verminderen door regelmatig 1-octen-3-ol toe te voegen. Toediening van een oplossing met 1,25% 1-octen-3-ol was even effectief in het bestrijden van de droge mollen ziekte als Sporgon onder semi-commerciële kweekcondities. Het Agaricus eigen 1-octen-3-ol lijkt dus een uitstekend alternatief voor Sporgon in de bestrijding van *L. fungicola.*

AFSLUITENDE OPMERKINGEN

Het in dit proefschrift beschreven onderzoek toont aan dat de droge mollenziekte van de champignon een moeilijk te bestrijden probleem is. *L. fungicola* blijkt uitstekend aangepast aan het leven in de dekaarde en is daar biologisch erg moeilijk te bestrijden. Daarentegen lijkt 1-octen-3-ol bruikbaar om ook op commerciële schaal de droge mol te bestrijden. Daarnaast valt ook te denken aan de ontwikkeling van resistentere champignonrassen. Meer kennis van het afweersysteem van de champignon zou die ontwikkeling ondersteunen. Toekomstig onderzoek kan daarbij gebruik maken van de genoomsequentie van *A. bisporus* die sinds kort beschikbaar is (http://genome.jgi- psf. org). Onderzoekers zullen er daarbij rekening mee moeten houden dat de afweer van paddenstoelen weleens aanzienlijk anders geregeld kan zijn dan die van planten en dieren.

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1 De Dijk, 1983, Zoveel ik kan, op: *Nooit meer Tarzan.*

Curriculum vitae

Roeland Berendsen was born in 's-Hertogenbosch, the Netherlands, on June 3rd 1982. After his graduation at the Stedelijk Gymnasium 's-Hertogenbosch, he started studying Biology at Utrecht University in September 2000. In 2001, he switched to study Environmental Sciences at the same university. During his academic education, he performed a nine month internship at the Microbiology group of Prof. dr. H.A.B. Wösten and wrote a thesis entitled "The fungal highway". In September 2006, he obtained his Master's degree. After graduation, he was employed at the Plant-Microbe Interactions (PMI) group of Prof. dr. C.M.J. Pieterse. There he performed the research described in this thesis under supervision of dr. P.A.H.M. Bakker and in collaboration with the Wösten-group. Since march 2011, he is employed as a post-doctoral researcher at PMI and works on the impact of plant defenses on the rhizosphere microflora.