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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 its 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. In addition, current knowledge on mushroom defence 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, called 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 causes significant losses in the commercial production of its main host, *Agaricus bisporus*. Annual costs for mushroom growers are estimated at 2–4% of 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: 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 because the host is also sensitive to fungicides. Notably, the 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*. On 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, so-called dry bubble. 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. *Lecanicillium fungicola* can be distinguished by erect distinct conidiophores with very unequally sized conidia aggregated in large, slimy globose heads. Conidiophores are verticillate with two to five whorls of three to seven phialides (Zare and Gams, 2008).

Although *L. fungicola* has been shown to infect other basidiomycetes (see below), *A. bisporus* is considered to be its main

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host. Worldwide, 40% of the commercially produced mushrooms belong to this species (http://www.isms.biz/edibles.htm). *Agaricus 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).

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).

GENETIC DIVERSITY

Dry bubble disease was first reported by Constantin and Dufour (1892), who described all bubble diseases then known, and referred to them as '*la môle*' disease. Derivations are still used to name dry bubble disease in French, Spanish, German and Dutch ('*môle sèche*', '*mole seca*', '*trockene Molle*' and 'd*roge 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 spore, a chlamydospore and big *Verticillium*-like conidia, and a second form bearing only small *Verticillium*-like conidia.

Later, Smith (1924) 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 that 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 has been concluded that, on the basis of the internal transcribed spacer (ITS) region and small subunit rDNA sequences, *V. fungicola* is 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, its optimum and maximum temperatures for growth, and, morphologically, in the 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 crops in Canada and the USA, whereas, 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 the morphology, virulence, fungicide resistance and random amplification of polymorphic DNA (RAPD) grouping of a large collection of *L. fungicola* var. *aleophilum* isolates. It was shown that, although initial isolates from the USA were genotypically and phenotypically diverse, more recent isolates were much more similar. On the basis of RAPD and amplified fragment length polymorphism (AFLP), it was concluded that European isolates of var. *fungicola* were also genetically homogeneous, although some polymorphisms existed and the population was less homogeneous than var. *aleophilum* (Largeteau *et al*., 2006). In this study, three French isolates appeared to be more polymorphous than a group of 15 isolates collected over a period of 27 years in the Netherlands, France and the UK, probably as a result of the diverging culture conditions of some French growers. Genetic homogeneity is most probably linked to common culture practices, including the casing material used (peat moss) and selection pressure caused by 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

Lecanicillium fungicola can infect mushrooms other than *A. bisporus* and *A. bitorquis*. The parasitic fungus has been isolated from *Pleurotus ostreatus*. On inoculation of healthy *P. ostreatus*, *A. bitorquis* and *A. bisporus*, these isolates caused disease and could be reisolated, thereby fulfilling Koch's postulates (Gea *et al*., 2003; Marlowe, 1982). *Lecanicillium fungicola* has also

been mentioned as a pathogen of *Coltricha perennis* and *Pleurotus sapidus* (Marlowe, 1982). In addition, *L. fungicola* has been isolated from the basidiomycetes *Marasmiellus ramealis*, *Thelephora terrestris*, *Henningsomyces candidus*, *Hypholoma capnoides* and *Laccaria laccata* (Gams *et al*., 2004; Zare and Gams, 2008). 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.

As *L. fungicola* is closely related to a variety of insect pathogens, it has been suggested that it is able to infect insects (Amey *et al*., 2007; Collopy *et al*., 2001; Yokoyama *et al*., 2006). 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, although 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 had observed that dry bubble disease was associated with the presence of insects (Ware, 1933). 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* was 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*., 2009b). Air collected on a mushroom farm contained 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 dispersal by employees and equipment has also been 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 with the initial population density of flies carrying *L. fungicola* spores, but the exponential spread of the disease in subsequent breaks was mainly a result of 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 infection is still under debate.

It is unlikely that *L. fungicola* is present in the compost delivered to farms, as the spores die at 40 °C and will thus not survive the composting process, where temperatures reach at least 70–80 °C (Van Griensven, 1988). Because of this, it was mentioned early on that the casing was more likely to be the 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 3 years. 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, 1967). Moreover, the anaerobic conditions and low pH are unfavourable for *L. fungicola*. In the UK in 1988, surface peat was used mainly (Visscher, 1988) and *L. fungicola* may survive on basidiomycete species that grow in and on such peat. Nowadays, in commercial farms, black peat is used mainly, 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 in *Verticillium* incidence in the mushroom industry in 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, 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 may 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* var. *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).

Fig. 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. Photographs J.A. van Pelt.

ECOLOGY OF *L. FUNGICOLA* IN THE CASING

Infection by *L. fungicola* most probably takes place in the casing, as it appears that *L. fungicola* cannot infect *A. bisporus* vegetative mycelium in the compost (Bernardo *et al*., 2004; Calonje *et al*., 2000a; Cross and Jacobs, 1968). 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 were visible. The phenomenon that the 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 the 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 *L. fungicola* spore germination. In agreement, Thapa and Jandaik (1987) demonstrated that, although spores of the pathogen can germinate in sterile water, germination 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 the 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 favour the proliferation of the pathogen.

MACROSCOPIC SYMPTOM DEVELOPMENT

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

- Necrotic lesions (Fig. 1B): brown, light-brown or grey discolorations on the cap or stipe, which can develop into warty outgrowths of the mushroom surface.
- Stipe blow-out (Fig. 1C): fruiting bodies are partially deformed. Deformation of the stipe is often accompanied by splitting or peeling of the stipe tissue.
- Dry bubble (Fig. 1D): undifferentiated amorphous mass of mushroom tissue, white, heterogeneously discoloured or homogeneously discoloured.

The time point of infection affects the type and severity of disease symptoms. Holmes (1971) harvested mushrooms from beds that were inoculated with *L. fungicola* at different time points after application of the casing layer. Disease incidence was lowest when the pathogen was inoculated during casing application and increased to a maximum after inoculation 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 *Agaricus* were deprived of nutrients by soil fungistasis, leading to reduced viability. Compared with inoculation at 14 days after casing, inoculation at 21 and 28 days after casing, when mature fruiting bodies were present, resulted in smaller 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). Indeed, *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, and necrotic

lesions developed 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 six 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, whereas, 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 lead to an amorphous mass of undifferentiated mushroom tissue, whereas infection at later stages, when tissue differentiation has begun, would lead to (partially) deformed mushrooms. Largeteau *et al*. (2007) also proposed that *L. fungicola* had 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. fungicola* var. *fungicola* on their host, and found that three isolates produced more diseased fruiting bodies than the others. Whereas these more aggressive isolates caused larger numbers of bubbles, other symptoms did not discriminate between 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 increased significantly the total numbers of mushrooms formed. These results suggest that infection with *L. fungicola* causes more primordia to develop. This could be caused by 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 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 to both the vegetative mycelium of *A. bisporus* and the mycelium of its developing fruiting bodies (Calonje *et al*., 1997, 2000a; Dragt *et al*., 1996; Shamshad *et al*., 2009a). 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*., 2009a) 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, as the medium was not specified.

The 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 surfaces of fruiting bodies of *A. bisporus* are lined with the hydrophobin ABH1, whereas the vegetative mycelium is covered by the hydrophobin ABH3 (Lugones *et al*., 1996, 1998). These hydrophobins render the surfaces of fruiting bodies and hyphae in contact with air or a hydrophobic surface hydrophobic. *Lecanicillium fungicola* also produces a hydrophobin, and the outer surface of the hyphae shows a rodlet structure typical for hydrophobins (Calonje *et al*., 2000b, 2002). Thus, it is likely that the pathogen and the host attach to each other by hydrophobic interactions between the hydrophobin layers. In *Magnaporthe 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* agglutinated sheep erythrocytes, and this haemagglutinating activity was inhibited in the presence of glucogalactomannan of *L. fungicola*. The authors concluded that the binding of the glucogalactomannan of *L. fungicola* to 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 cannot be excluded that hydrophobins play a role in this process. Previously, it has been shown that hydrophobins of *S. commune* have lectin-like activities (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 mitogen-activated protein kinase of *L. fungicola*, is up-regulated in cap lesions. Such mitogen-activated protein kinases play an important role in interactions between fungal pathogens and plants. However, *Lfpmk1* mutants did not show 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 haemagglutinating 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 intracellularly 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 (Calonje *et al*., 1997; Dragt *et al*., 1996). *Lecanicillium fungicola* produces a wide range of extracellular lytic enzymes. (Bidochka *et al*. 1999a, b; Calonje *et al*., 1997, 2000a; Juarez del Carmen *et al*., 2002; Kalberer, 1984; Mills *et al*., 2000; St Leger *et al*., 1997; Trigiano and Fergus, 1979). 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 smaller amounts when grown in minimal medium with simple carbon sources such as glucose, sucrose or fructose. 1,4-β-Glucanase was only produced in the presence of cell walls and not when grown on a simple carbon source medium. Electron microscopy showed that the 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, 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*.

Lecanicillium fungicola produces a chemotrypsin protease with narrow specificity and large amounts of broad-spectrum subtilisin-like proteases (Bidochka *et al*., 1999b; Kalberer, 1984; St Leger *et al*., 1997); however, their involvement in the infection process has not been studied to date. 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 lesions 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 the enzymatic oxidation of phenolic substrates into quinones, which subsequently autopolymerize into melanins. The formation of quinones is catalysed by polyphenol oxidases. Laccase and tyrosinase are the only polyphenol oxidases formed by *A. bisporus*, and 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).

The 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 is kept separated from its phenolic substrates through cellular compartmentalization. On 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 decompartmentalization, 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 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 with that after *P. tolaasii* infection. In both cases, infections resulted in a higher tyrosinase activity. In con-

trast, other pathogens caused a reduction in tyrosinase activity. It was proposed that the proteinases of both pathogens led to the degradation of tyrosinases in mushroom, and that active tyrosinase is an intermediate formed during the degradation of tyrosinases by proteinases (Soler-Rivas *et al*., 2000).

Laccase activity has been detected in the vegetative mycelium and primordia of *A. bisporus*. Laccase activity was also found in dry bubbles, but not in healthy mushrooms. The electrophoretic profile of laccases in dry bubbles differed from laccase found in vegetative mycelium of *A. bisporus*. This difference could be brought about by alteration of the host laccases by the pathogen or by *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 programme of the mushroom in the dry bubble. The difference in colour of *L. fungicola* lesions relative to lesions caused by other mushroom pathogens, which 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 defence against pathogens. In support of this, the 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 with healthy mushrooms pins. This suggests that *AbPPO2* is not involved in active defence of the mushroom against *L. fungicola* (Largeteau *et al*., 2010). In addition, 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 defence 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 whether 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. In addition, it has been found that cystidia have a defensive role against collembola in *Russula bella* and *Strobilurus ohsimae* fruiting bodies (Nakamori and Suzuki, 2007). It is tempting to speculate that mushroom species also invest in defence 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 favouring 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 have only revealed strains with partial resistance (Dragt *et al*., 1995; Largeteau *et al*., 2004b; Savoie and Largeteau, 2004; Savoie *et al*., 2004; Wuest and Harvey, 1978; Wuest and North, 1988). 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 in which cells in infected tissue die and encapsulate the infection (Greenberg *et al*., 1994). Like plants, toxic molecules also seem to take part in defence. Savoie and Largeteau (2004) tested 17 strains of *A. bisporus* and found a negative correlation between the 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 defence against *L. fungicola*, confirming the results presented by Thapa and Jandaik (1989). White rot fungi, such as *A. bisporus*, are relatively well able to cope with high levels of hydrogen peroxide, as they use extracellular peroxidases and oxidases to degrade lignin (Jansen *et al*., 2000). In addition to peroxide, antibiotics that are produced by *A. bisporus* may play a role in the defence against *L. fungicola* (Largeteau *et al*., 2006; Mamoun *et al*., 1995). The role of antibiotic production has been demonstrated in the interaction of *A. bisporus* with the green mould *Trichoderma harzianum*. An unidentified metabolite extracted from *A. bisporus* vegetative mycelium and fruiting bodies was able to inhibit the 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 the effects of antibiosis by *Agaricus* on the spore germination of *L. fungicola* (Largeteau *et al*., 2006; Mamoun *et al*., 1995). However, the inhibitory effect of *A. bisporus* strains in this test did not correlate with their dry bubble resistance (Mamoun *et al*., 1995).

In addition, there was no correlation of the susceptibility of different *A. bisporus* strains to *L. fungicola*, *P. tolaasii* and *Trichoderma aggressivum* (Largeteau *et al*., 2004b). This suggests that different pathogens are differentially recognized or affected

by different defence mechanisms. The different biochemical mechanisms of microbially induced diseases of *A. bisporus* have been reviewed recently by Largeteau and Savoie (2010). However (partial) resistance is not necessarily a result of active defence. Nondefence-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 with healthy tissue. A chitin deacetylase gene of *A. bisporus* was strongly up-regulated in infected tissue; however, RNAi hairpin-mediated gene silencing did not lead to increased susceptibility in infection trials. In addition, the 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 the identification of defence-related genes is probably difficult, as *L. fungicola* has been shown to affect the *A. bisporus* developmental programme in which a myriad of genes is involved. Largeteau *et al*. (2010) investigated the 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, *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 the 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 up-regulated in resistant strains and down-regulated in susceptible strains. This indicates that *hspA* is involved in the 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, the control of *L. fungicola* relies mainly on prevention and hygiene measures on mushroom farms. Active control of the disease is difficult because the chemicals used have become less effective as the pathogen has developed resistance and legislation is restricting their use. Therefore, dry bubble disease is likely to remain one of the most 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 knockouts. Future studies should focus on such a system, especially as the genome of *A. bisporus* will become available in the near future. This will facilitate the 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 prepare knockouts in *L. fungicola* are available, facilitating research into the pathogen side of the interaction (Amey *et al*., 2002, 2003)

In addition to new means to combat dry bubble disease, future research may also elucidate the way in which *L. fungicola* disrupts the developmental programme of *A. bisporus* and stops tissue differentiation. The elucidation of mechanisms through which the pathogen exerts this effect might also lead to the manipulation of mushroom development in a manner beneficial to the grower.

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