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ORIGINAL PAPER

Absence of induced resistance in *Agaricus bisporus* against *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*.

Keywords *Lecanicillium fungicola* · *Agaricus bisporus* · Induced resistance · Dry bubble disease · Systemic acquired resistance

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Introduction

Dry bubble disease, caused by the ascomycete *Lecanicillium fungicola* [synonyms: *Verticillium fungicola* (Preuss) Hassebrauk, *Verticillium malthousei* (Preuss) Ware], is a serious problem in the 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 (North and Wuest 1993; Bernardo et al. 2004). Whereas infections of matured mushrooms will only cause necrotic lesions, infections in developing mushrooms or primordia will lead to deformed mushrooms or even totally amorphous masses of mushroom tissue, called dry bubble. 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 (Berendsen et al. 2010). In contrast, a wealth of information is available on interactions between plants and 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 have developed that could overcome this defense. In response, both plants and animals have evolved an innate immune system that is activated upon recognition of a pathogen (Jones and Dangl 2006; Martinon et al. 2009; Spoel and Dong 2012). 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 Savoie and 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, RNAi hairpin-mediated suppression of some of these genes did not lead to increased susceptibility towards *L. fungicola.* This demonstrates that identification of defense-related genes is difficult, likely because *L. fungicola* affects the developmental programming of *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, 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 multicellular 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.

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 (110 × 100 × 80 mm; Eco2box, Ophasselt, Belgium). Phase 2 compost (CNC, Milsbeek, The Netherlands) was mixed with 1 % (w/w) fully colonized sorghum granules and incubated at 24 °C and 95 % humidity for 2 weeks.

Cultivation of *A. bisporus*

Mushrooms were cultured in controlled-climate incubators (Snijders scientific, Tilburg, the Netherlands, dimensions 99 × 63 × 134 cm). Plastic containers (17.5 × 27.5 × 22.5 cm) 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 5×10^3, 5×10^4, 5×10^5, 5×10^6 or 5×10^7 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 h 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 below.

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 (10^7 spores/ml) and incubated for 72 h 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 (Sylvan spawn, Horst, the Netherlands)
A. bisporus MES01497	Wild type from Dutch PRI collection selected for resistance against *L. fungicola* (PRI, Wageningen, The Netherlands)

Subsequently, material was collected by cutting 4 mm deep, 4 × 8 mm pieces from infected mushroom caps, starting from the center of the lesion). 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.

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 (1×10^7 spores/ml) or 2 µl sterile demineralized water on one side of the cap. After 24 h, the other side of all mushroom caps was inoculated with 2 µl of a fresh *L. fungicola* suspension (1×10^7 spores/ml). Mushrooms were cultivated for another 72 h before 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, five symptomless mushrooms were inoculated with 2 µl *L. fungicola* spore suspension (1×10^7 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 four 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 ten small mushrooms (1–1.5 cm cap diameter) still attached to the mycelium or ten 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 h.

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). Five freshly harvested mushrooms were placed in 10 ml 0.1 or 1 mM methyl jasmonate (MeJA) in 1 % (v/v) ethanol, 1 or 10 mM SA in 1 % (v/v) ethanol or in 1 % (v/v) ethanol (control treatment). Mushrooms were incubated at room temperature for 24 h and subsequently point inoculated with *L. fungicola* (see above). Lesion diameters were measured after 72 h.

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 1,500 shakes/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 °C for 30 min with gently mixing every 10 min. After adding 700 µl phenol:chloroform:isoamylalcohol (24:24:1), samples were homogenized by vortexing and subsequently centrifuged (5 min, 11,700×*g*). The aqueous layer was transferred to a clean tube. An equal volume of chloroform:isoamylalcohol (24:1) was added. Samples were homogenized and then 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 °C.

Development of qPCR test to detect *L. fungicola*

Previously determined rDNA sequences of *L. fungicola var. aleophilum* (GenBank Acc.No AF324876, AB111494), *L. fungicola var. fungicola* (GenBank Acc.No. AB107135, AF324874) and of *Lecanicillium flavidum* (GenBank Acc.No. AB112030, AF324877) were compared to design specific real-time PCR primers and probe which determine both *L. fungicola var. aleophilum* and *L. fungicola var. fungicola* and not *L. flavidum*. The alignment of these rDNA sequences was performed using the Megalign module of the Lasergene software (DNASTAR Inc., Madison, WI, USA).

Different primers/probe combinations were designed with the Primer Express version 1.5 Software (Applied Biosystems, Foster City, CA, USA). The primers were manufactured by Sigma-Genosys (Haverkill, UK). The TaqMan probe, labelled with 5′-FAM as the reporter dye and with a non-fluorescent 3′-quencher (NFQ), was manufactured by Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). The potentially best discriminating combination was selected: primers f.vff.vfa® (5′-CGTCCG GGTGGCCTT-3′) and r.vff.vfa® (5′-TGCCTTG CGGCGGAT-3′) and probe P.vff.vfa® (5′-6FAM-TG TATTACCAGTATCTTCTG-3′NFQ). To minimize the likelihood of non-specific detection, the primers and probe were compared with all available relevant sequences by using the BLAST database search program (Altschul et al. 1997) (http://www.ncbi.nlm.nih.gov/BLAST). No marked sequence homology between the probe or primer sequences with organisms in the database were found.

Quantification of *L. fungicola* DNA by quantitative polymerase chain reaction (qPCR)

The Eurogentec qPCR core kit (Eurogentec, The Netherlands) or the PCR-2×-mastermix of TaKaRa (Takara Premix Ex Taq™,Otsu, Shiga, 520-2193, Japan) was used according to the instructions of the manufacturers. The amplification mixture further consisted of 300 nM of probe, 300 nM forward and reverse primer and 2 µl of DNA in a final volume of 30 µl. Primer and probe concentration had been optimized (concentrations varying from 50 to 900 nM have been assayed) as well as annealing temperature. Real-time PCR amplifications were performed with a 7900HT Fast Real-Time PCR System (Applied Biosystems, The Netherlands) or an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA, USA). Cycling conditions were 50 °C for 2 min to degrade uracil-containing DNA and 95 °C for 10 min to inactivate uracil-N-glycosylase, followed by 40 cycles containing a denaturation step at 95 °C for 15 s and an annealing step at 60 °C for 1 min. The emission was measured at the annealing-extension step.

Data were analyzed with SDS 2.2.2 software or with ABI PRISM sequence detection system software (version 1.9.1). C_t values of samples were converted to *L. fungicola* 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.

Using the Wizard Magnetic DNA Purification System for Food (Promega,Madison, WI53711 USA), DNA was isolated from different pure fungal cultures, pathogenic for *A. bisporus*, listed in supplementary Table 1 to test the specificity of the qPCR test. The DNA extract was diluted to 1 ng/µl before use.

Statistics

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

Results

Specificity of qPCR test

FAM fluorescence could only be measured when the assay contained DNA from *L. fungicola var. fungicola* or *L. fungicola var. aleophilum*. No fluorescence was measured when DNA of *L. flavidum, Scytalidium thermophilum, Verticillium biguttatum, Cladobotyrum dendroides, Trichoderma agressivum var. europeanum, Trichoderma harzianum, Trichoderma koningii, Mycogone perniciosa, Mycogone cervina, Mycogone calospora, Mycogone rosea, Pseudomonas tolaasii, Pseudomonas reactans, Pseudomonas fluorescens complex, Pseudomonas putida, Pseudomonas gingeri* or *Pseudomonas agarici* was used as template DNA .

Detection of *L. fungicola* on *A. bisporus* 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 (Fig. 1a). The amount of *L. fungicola* DNA on mushrooms that were inoculated with $\leq 10^4$ 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 h, *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. The amount of *L. fungicola* DNA detected was least variable after inoculation with the highest density spores. Therefore, mushroom caps were inoculated with 10^5 spores in subsequent experiments. Most *L. fungicola* DNA was detected within the lesion area on the mushroom cap, as investigated for inoculation with 10^4 spores (Fig. 1b). At 72 h after inoculation, *L. fungicola* DNA was detected in cap tissue directly adjacent to the lesion but it was a 1,000 fold lower than inside the discolored tissue.

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 h 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

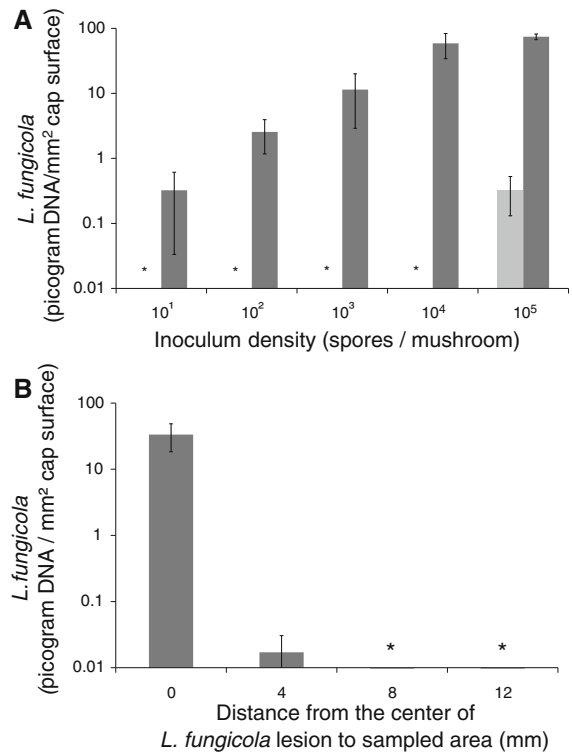

Fig. 1 **a** Quantification of growth of *L. fungicola* on a harvested mushroom cap. *L. fungicola* DNA from necrotic lesions was quantified immediately after inoculation (*light grey bars*) or after 72 h incubation (*dark grey bars*). *Bars* represent average *L. fungicola* DNA (pg/mm^2 cap surface) of three samples as determined by qPCR. *Error bars* represent standard deviation. *Asterisks* below detection limit. **b** Quantification of growth of *L. fungicola* on a harvested mushroom cap. DNA was isolated from small blocks cut from concentric rings around a necrotic lesions on mushroom caps 72 h after inoculation). *Bars* represent average *L. fungicola* DNA (pg/mm^2 cap surface) of three samples as determined by qPCR. *Error bars* represent standard deviation

differences in lesion size were obtained between the samples (Fig. 2a). 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 1 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 symptomless mushrooms were point inoculated with 2 µl of *L. fungicola* spore suspension in all boxes. Lesion size was determined 4 days after the fruiting bodies were inoculated. No significant differences in lesion size on mushrooms in healthy and diseased cultures were detected (Fig. 2b). qPCR analysis confirmed these results (data not shown).

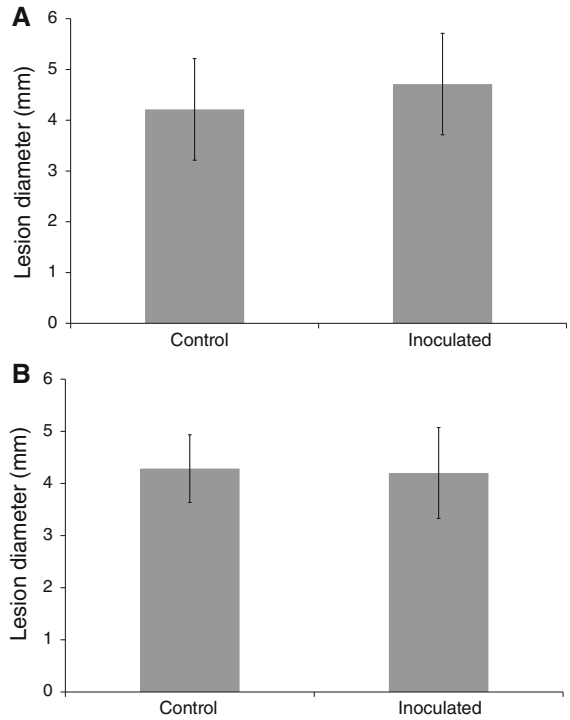

Fig. 2 **a** Absence of induced resistance upon secondary infection of caps of *A. bisporus* strain Sylvan A15 cultures in *boxes*. Development of lesions on mushroom caps of *A. bisporus* strain A15 that had or that had not been previously inoculated with *L. fungicola*. *Bars* represent average lesion diameter of eight mushrooms. *Error bars* denote standard deviation. **b** 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 induces 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 and 0.1 mM) and of SA (1 or 10 mM) for 24 h and were subsequently point inoculated with 2 µl of a *L. fungicola* spore suspension. The size of lesions that developed in 72 h following infection by *L. fungicola* was not significantly affected by pre-treatment with either MeJA or SA (Fig. 3).

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 (Fig. 4a). In the first flush around 20 % of the mushrooms of strain A15 showed symptoms of disease whereas MES01497 was completely healthy.

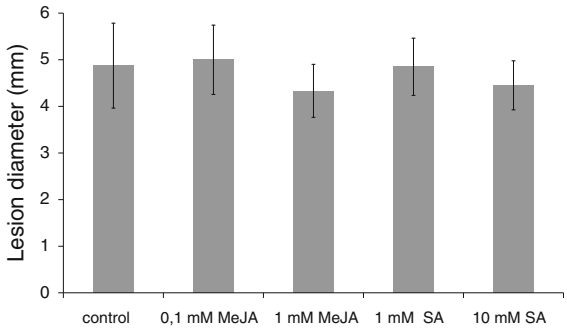

Fig. 3 Effect of SA and MEJA application on *L. fungicola* lesion development. A spore suspension of the pathogen was point inoculated on harvested mushrooms. *Bars* represent average lesion diameter on five mushrooms. *Error bars* represent standard deviation

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 (Fig. 4b). 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.

It was also investigated if after point inoculation on the caps, *L. fungicola* lesions would develop less on MES01497 than on Sylvan A15. Mushrooms of both strains were inoculated immediately after being harvested or while still growing in culture. After 4 days, *L. fungicola* lesion diameters were determined (Fig. 5). 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.

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

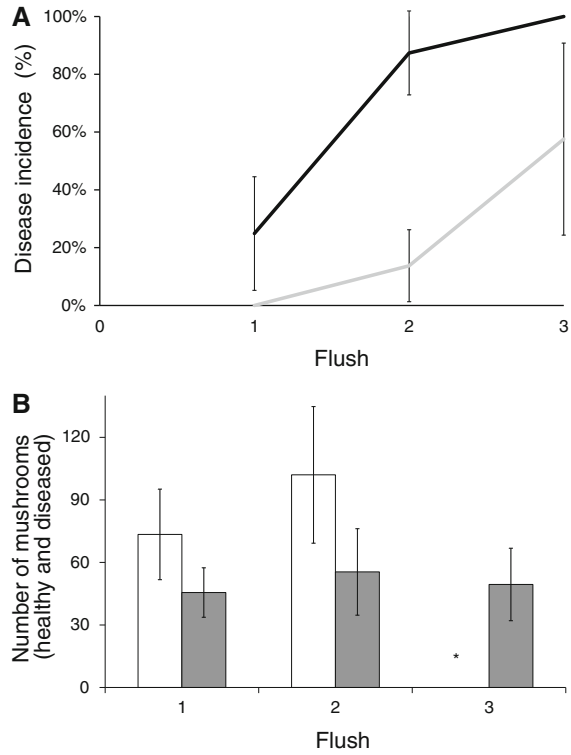

Fig. 4 **a** Dry bubble disease incidence in mushrooms of *A. bisporus* strains A15 (*black line*) and MES01497 (*grey line*). The average number of diseased mushrooms as a percentage of total mushrooms is depicted for each flush. *Bars* represent the average of nine containers. *Error bars* represent standard deviation **b** Number of mushrooms produced by *A. bisporus* strains A15 (*white bars*) and MES01497 (*dark grey*). *Bars* represent the average of nine containers. *Error bars* represent standard deviation. *Asterisks* could not be determined

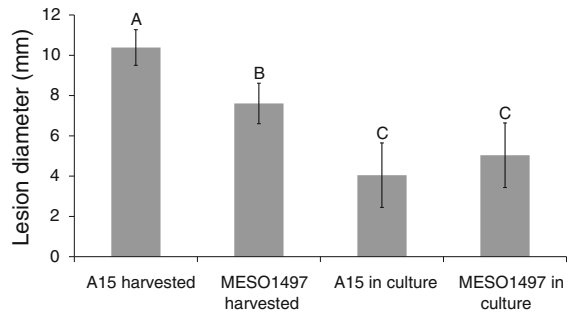

Fig. 5 Lesion diameter on caps of harvested mushrooms and mushrooms in culture of *A. bisporus* strains MES01497 and Sylvan A15 72 h after inoculation with *L. fungicola*. *Different letters* indicate significant differences as determined by ANOVA ($\alpha = 0.05$)

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 (Fig. 6a), nor development of dry bubble in neighboring fruiting bodies (Fig. 6b) could inhibit lesion development upon challenge inoculation with *L. fungicola* on mushroom caps.

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 (Berendsen et al. 2010).

The possibility of increasing the resistance of white button mushroom to *L. fungicola* infection was investigated by observation of lesion sizes and by determination of *L. fungicola* DNA using a newly developed TaqMan qPCR test specific to *L. fungicola*. 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, 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 (Fig. 1). 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 were only 24 h 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. Furthermore, initial inoculation of mushroom caps with *P. tolaasii* did not affect infection by *L. fungicola*

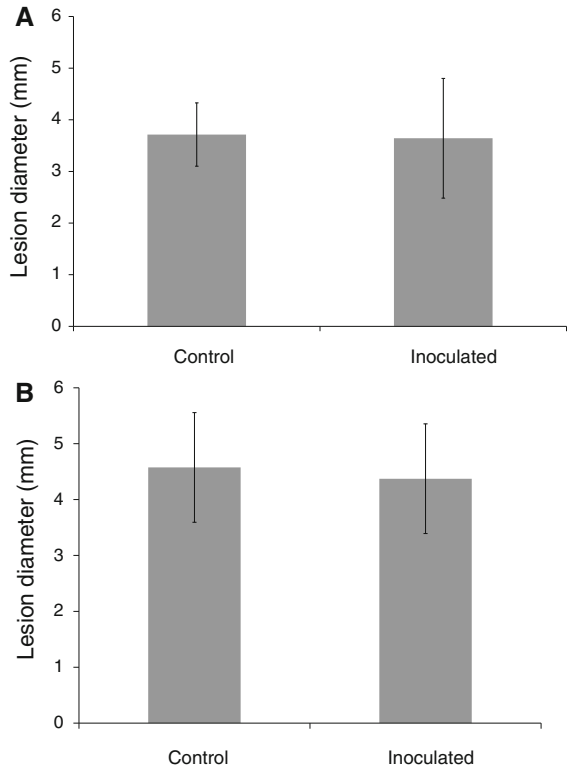

Fig. 6 **a** Cap assay for induced defense. Development of lesions on mushroom caps of *A. bisporus* strain MES01497 cultures in *boxes* that had either (*inoculated*) or not (*control*) been previously infected. *Bars* show average lesion diameter of ten mushrooms. **b** Infection of healthy mushrooms 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

(data not shown). Apparently, pathogenic 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 neighbouring 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 neighbouring 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 (Fig. 3). 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 efficiently and less consistently 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 (Fig. 4), 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. This seems to concur with findings of Foulongne-Oriol et al. (2011). They 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. More recently, Foulongne-Oriol et al. (2012) found that quantitative trait loci (QTL) determining resistance against the dry bubble symptom are on distinct genomic regions from resistance QTL to necrotic lesions. Nonetheless, 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 (Fig. 5). 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. For example, 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. 2004; Foulongne-Oriol et al. 2011, 2012). 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. fungicola* did not affect development of secondary infections in any of our experimental approaches (Fig. 6).

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 odour, 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.

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