The effect of *Hypomyces perniciosus* on the mycelia and basidiomes of *Agaricus bisporus*  

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**Abstract**

*Hypomyces perniciosus* has been reported as a destructive pathogen of *Agaricus bisporus*. Previous research suggested that the pathogenesis may not only be perpetuated by *H. perniciosus*, but also by bacteria. Clarification of the interaction between *A. bisporus* and *H. perniciosus* is a prerequisite for the development of effective control measures against wet bubble disease. Here, the effects of *H. perniciosus* on *A. bisporus* mycelia are examined in dual culture on agar media and in open-ended test tubes. During disease development, the putative causal agents and cytology of wet bubble-diseased mushrooms were followed microscopically. The interaction between *H. perniciosus* and the basidiome of *A. bisporus* was also studied using dual-cultured *H. perniciosus* and basidiome tissues. Dual-cultured mycelia from both fungi showed that growth continued even after contact was made, without any observable antagonistic lines or cytoplasmic changes of *A. bisporus* mycelia. *Hypomyces perniciosus* could be isolated from diseased basidiomes any time after inoculation, but bacteria were only recovered after the basidiomes of *A. bisporus* had been killed by *H. perniciosus*. Dual culture of the basidiome tissue of *A. bisporus* and *H. perniciosus* on agar media established that *H. perniciosus* can independently and rapidly degrade the basidiomes of *A. bisporus*. We conclude that *H. perniciosus* has no pathogenic activity on the mycelial stage of *A. bisporus*, but it can destroy *A. bisporus* basidiomes in the absence of bacteria. Wet bubble disease is evidently not caused by bacteria, but by the fungus, although bacteria likely participate in the disease after invasion by the fungus.

**INTRODUCTION**

*Hypomyces perniciosus* Magnus, syn. *Mycogone perniciosa* (Magnus) Delacr. has been reported as the causal agent of wet bubble disease (WBD) in *Agaricus bisporus* (Lange) Imbach, the most widely cultivated mushroom in the world. The disease has been reported wherever *A. bisporus* is cultivated and results in drastic reductions in mushroom yield due to malformation and deterioration of the basidiomes [1]. In commercial mushroom farms, the disease is controlled by either selective fungicides or soil sterilization [2, 3]. However, in China, even with control measures in place, an estimated loss of 30% in mushroom production was reported [4].

Umar et al. [5] and Pieterse [6] suggested that bacteria may be the causal agent of the disease rather than *H. perniciosus* [6, 7]. This issue requires clarification to enable the development of scientifically based effective control methods, as bacteria and fungi can respond differently to particular fungicides. Han et al. [7] reported that there was no parasitic relationship between *H. perniciosus* and the mycelia of *A. bisporus* [8]. However, Pieterse [6] and Huang et al. [8] found that the mycelial growth of *H. perniciosus* increased in paired cultures with *A. bisporus* on agar media, resulting in the inhibition of the mycelial growth of *A. bisporus* [7, 8]. Pieterse [6] and Huang et al. [8] suspected that these interactions were caused by metabolites, possibly volatile compounds produced by the *Hypomyces*, because effects were observed before physical contact was made with the putative pathogen [7, 8]. However, previous studies have not examined any possible cytological changes or mycelial activity of *A. bisporus* caused by *H. perniciosus*. As *A. bisporus* is cultivated using composts selected specifically for the nutrient requirements of large-scale mushroom production, the growth conditions on composts in farms differ from those on agar media. We therefore also used compost as a substrate on which to study the mycelial interactions, as used in studies on diseases in *Lentinula edodes* (Berk.) Pegler by Cao et al. [9].

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**Keywords:** wet bubble disease; pathogenicity; mycelial activity; cytology; host-parasite interaction; bacteria.  
**Abbreviations:** CMA, corn meal agar; LM, light microscope; LSM, laser scanning microscope; MEA, malt extract agar; PDA, potato dextrose agar; SEM, scanning electron microscope; WBD, wet bubble disease; YPD, yeast peptone agar.
In order to clarify the host–parasite interactions in WBD, we isolated the putative pathogen and observed the histological and morphological changes of *A. bisporus* basidiomes using different types of microscopy. The effects on *A. bisporus* mycelia were examined in dual cultures on media using a light microscope (LM) and a laser scanning microscope (LSM) after the hyphae were stained with viability reagents. Furthermore, we examined the mycelial interactions between *H. perniciosus* and the mycelia of *A. bisporus* using the dual-culture method reported by Nitta and Miyazaki [10].

**METHODS**

**Strains of *H. perniciosus* and *A. bisporus***

The strain CCMJ1131 of *H. perniciosus* (a voucher strain was deposited in the Engineering Research Centre of the Chinese Ministry of Education for Edible and Medicinal Fungi, Jilin Agricultural University under accession no. CCMJ1131) was isolated from the basidiomes of *A. bisporus* (‘As2796’) that were naturally infected with the fungus in mushroom farms in Wuhan, China. The strain was confirmed as *H. perniciosus* by previously described procedures [11]. Mycelial cultures were obtained from the tissues of healthy basidiomes of *A. bisporus* (‘Sylvan A15’) collected from a mushroom farm in Shanghai.

**Compost and casing soil**

The compost for cultivation of *A. bisporus* was obtained from Da Shan He Farm, Shandong, China. It consisted of wheat straw, chicken manure and gypsum (10 : 6 : 0 : 5 w/w), and was prepared according to the protocol used on that farm. The casing soil, consisting of a 10 : 1 mixture of black peat and lime chalk, was obtained from CNC Grondstoffen BV (the Netherlands).

**Media**

The media for fungal cultures were prepared as follows: (1) potato dextrose agar (PDA; peeled and sliced potatoes 200 g, dextrose 20 g, agar 20 g and deionized water 1000 ml); (2) corn agar (CMA; corn flour 300 g, agar 17 g and deionized water 1000 ml); (3) malt extract agar (MEA; malt extract 25 g, agar 17 g and deionized water 1000 ml); (4) improved Fries agar (ammonium tartrate 5 g, sucrose 30 g, NH\(_4\)NO\(_3\) 1 g, KH\(_2\)PO\(_4\) 1 g, MgSO\(_4\) 7H\(_2\)O 0.5 g, NaCl 0.1 g, CaCl\(_2\) 2H\(_2\)O 0.1 g, yeast extract 0.5 g and deionized water 1000 ml). Bacteria were cultured on yeast peptone agar (YPD; yeast extract 5 g, peptone 5 g, NaCl 5 g, agar 15 g and deionized water 1000 ml).

**Cultivation of *A. bisporus***

Basidiomes of *A. bisporus* (‘Sylvan A15’), were produced as described by Daniel et al. [12] on mushroom beds under

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**Table 1.** Pathogenicity grades of *Hypomyces perniciosus* on *Agaricus bisporus* [10]

<table>
<thead>
<tr>
<th>Grade of pathogenicity</th>
<th>Observation of mycelial growth of <em>Agaricus bisporus</em> (A) and <em>Hypomyces perniciosus</em> (B) after their contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>5  Very strong</td>
<td>B observed in all or most parts of A (–)</td>
</tr>
<tr>
<td>4  Strong</td>
<td>Growth albeit limited to grade 5, was noted in area A.</td>
</tr>
<tr>
<td>3  Normal</td>
<td>Growth of A and B is inhibited at the centre, or if A enters the area of B (+), but with a low growth rate</td>
</tr>
<tr>
<td>2  Weak</td>
<td>A is observed in the area of B (+), but its growth rate is restricted</td>
</tr>
<tr>
<td>1  Very weak</td>
<td>A enters the area of B (+) and its growth rate is increased</td>
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</table>
controlled environmental conditions whose temperature and humidity were those used for commercial production [12].

Inoculation of A. bisporus basidiomes with H. perniciosus

When the first primordia appeared in the casing soil and the primordial cap reached 0.5 cm in diameter, one droplet (about 5 µl) of spore suspension of H. perniciosus at 1 x 10^5 ml^-1 was inoculated onto its surface. Control samples were prepared by inoculation with deionized water. Changes in disease symptoms were observed and recorded for approximately 20 days. Fungi and bacteria were isolated from WBD mushrooms at different disease progression stages (every 2 days until 20 days after inoculation). For isolation, five diseased mushroom basidiomes were selected randomly at the different stages and experiments were replicated twice. Fungal and bacterial isolations were conducted using the methods described by Osato [13] and Shi [14], respectively [13, 14].

The morphological characteristics of the isolates were observed by LM (Leica, Tokyo, Japan) as described by Umar and Van [15] and Shamshad et al. [17]. The method employed for LSM is described below: 1 µl of NUCLEAR-ID Blue/Red cell viability reagent (product number, ENZ-53005–C100) was mixed in 1 ml of PBS buffer (pH 6.8). This dye was a mixture of a blue fluorescent cell-permeable nucleic acid dye and a red fluorescent cell-permeable nucleic acid dye that is suitable for staining dead nuclei. The dye-stained nuclei were blue in living cells and fluoresced red in dead cells. This dye does not stain the cytoplasm and reacts with the nucleus. Tissue sections were placed directly onto glass microscope slides. The staining solution (3–5 µl) was dispensed in a volume sufficient to cover the cell monolayer. Samples extracted for LSM were protected from light using aluminium foil to cover the slides and incubated for 30 min at 37 °C. Any excess staining solution was removed, and a few drops of buffer were added to prevent the cells from drying out. The mycelium was then covered with a glass cover slip and observed under LSM with a dual filter set for 4’, 6-diamidino-2–phenylindole (DAPI) (Ex/Em: 350/470 nm) and Texas Red (Ex/Em: 540/605 nm). The presence of bacteria in the samples was checked with LM as described by Lu et al. [16].

**Inoculation of A. bisporus basidiome tissue with H. perniciosus on agar media**

Healthy A. bisporus basidiomes with cap diameters of about 3–4 cm and without evident disease symptoms were surface-sterilized with 75% ethanol for 30 s. About 5 mm² from the central part of the cap was cut out, and transferred to PDA in 90 mm-diameter Petri plates. Five millimeter-diameter plugs from the edge of actively growing H. perniciosus colonies on PDA were inoculated opposite to the sample from the A. bisporus basidiome on 90 mm-diameter Petri plates with PDA, leaving 2.5 cm between the two inocula. The control Petri dishes were inoculated solely with A. bisporus basidiome tissues. All Petri plates were incubated at 25 °C in darkness for 15 days. The ten Petri dishes used in each experiment were replicated twice. Any morphological changes of A. bisporus basidiome tissue were checked for and recorded by LM.

**Hyphal appearance and viability in dual cultures of A. bisporus mycelium and H. perniciosus**

Five millimeter-diameter plugs from the edge of actively growing A. bisporus and H. perniciosus colonies on PDA were inoculated at opposite sides of 90 mm-diameter Petri plates with four test media (see above). The controls consisted of plates with plugs of either H. perniciosus or A. bisporus at opposite sides of the Petri plates. Due to the different growth rates of the two fungi, A. bisporus was inoculated 7 days before H. perniciosus. All of the Petri plates were incubated at 25 °C in the dark over 20 days. Six Petri dishes were used per experiment and they were replicated three times. The appearance of the A. bisporus mycelium was observed using LM and LSM. Mycelial samples were taken from the basidiome at different developmental stages. These tissue samples were processed for LM and SEM as described by Umar and Van [15] and Shamshad et al. [17]. The method employed for LSM is described below: 1 µl of NUCLEAR-ID Blue/Red cell viability reagent (product number, ENZ-53005–C100) was mixed in 1 ml of PBS buffer (pH 6.8). This dye was a mixture of a blue fluorescent cell-permeable nucleic acid dye and a red fluorescent cell-permeable nucleic acid dye that is suitable for staining dead nuclei. The dye-stained nuclei were blue in living cells and fluoresced red in dead cells. This dye does not stain the cytoplasm and reacts with the nucleus. Tissue sections were placed directly onto glass microscope slides. The staining solution (3–5 µl) was dispensed in a volume sufficient to cover the cell monolayer. Samples extracted for LSM were protected from light using aluminium foil to cover the slides and incubated for 30 min at 37 °C. Any excess staining solution was removed, and a few drops of buffer were added to prevent the cells from drying out. The mycelium was then covered with a glass cover slip and observed under LSM with a dual filter set for 4’, 6-diamidino-2–phenylindole (DAPI) (Ex/Em: 350/470 nm) and Texas Red (Ex/Em: 540/605 nm). The presence of bacteria in the samples was checked with LM as described by Lu et al. [16].
Table 2. Results of the infection progression of *Hypomyces perniciosus* (H) on the *Agaricus bisporus* (A) basidiome

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Disease symptoms</th>
<th>Observation by LM</th>
<th>Observation by SEM</th>
<th>Observation by LSM</th>
<th>Isolation of</th>
<th>H</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>Conidia and clamydospores germinated, mycelial structure of A did not change</td>
<td>Same as for LM</td>
<td>Mycelia of A basidiome was alive</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Necrotic brown lesion</td>
<td>At the inoculation site, mycelia of A was brown and plasmolyzed</td>
<td>Chlamydospores germinated and attached on the surface of A. The mycelia of A was normal</td>
<td>Mycelia of A basidiome was alive</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>A appeared to be larger in size and on its surface H developed a small colony nearly 1 cm in diameter</td>
<td>Some mycelia of A showed deformation; H infected the basidiome</td>
<td>The infected parts of mycelia showed hydropic degeneration</td>
<td>Near the inoculation site the mycelia of A were dead</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>The cap of A continued to grow, while A turned brown and was covered with H; some droplets were produced on the surface of A</td>
<td>On the surface, H produced abundant conidia and clamydospores. Bacteria were observed in droplets, but none were observed in the centre of A</td>
<td>The mycelia of A continued to show hydropic degeneration and cytoplasmolysis</td>
<td>The mycelia of A were dead</td>
<td>+</td>
<td>Bacteria were visible in the droplets, but no bacteria were isolated from the centre of A</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>A became soft and stopped growing; the droplets were dry</td>
<td>The mycelia of A were scarce and barely observed. Large quantity of H in the basidiome. Bacteria were observed within or on the surface of A</td>
<td>Most mycelia of A showed hydropic degeneration and cytoplasmolysis, and the contents of the mycelium were empty, i.e. without organelles</td>
<td>Some mycelia of A dead</td>
<td>+</td>
<td>Bacteria were isolated from the inner parts or surface of A</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Loss of structural integrity observed, followed by collapse and eventual rotting</td>
<td>Mycelia of A few and barely observed; bacteria and H observed within or on the surface of A</td>
<td>Most mycelia of A were degraded and broken into pieces</td>
<td>Most of the mycelia of A were dead</td>
<td>+</td>
<td>Bacteria were isolated from the inner parts or surface of A</td>
<td></td>
</tr>
</tbody>
</table>

zone where the *A. bisporus* and *H. perniciosus* hyphae intermingled on the media. The appearance of the mycelium was observed at different stages (Fig. 1a): (1) the mycelium of *A. bisporus* prior to contact with mycelium of *H. perniciosus*; (2) the mycelium of *A. bisporus* when contact was established with the mycelium of *H. perniciosus*; and (3) the mycelium of *A. bisporus* and *H. perniciosus* when intermingled. The method of observation applied above was repeated for visualization of the basidiomata.

**Dual cultures in open-ended test tubes**

Open-ended test tubes (length 25 cm, diameter 2.2 cm) were filled with compost at the central part of the tubes for a length of 9 cm, and both ends were sealed with cotton plugs, as illustrated in Fig. 1(b). The tubes with the compost were then sterilized at 121 °C for 60 min. Ten millimetre-diameter mycelial agar discs from actively growing *A. bisporus* or *H. perniciosus* colonies cultured on PDA were inoculated at one side of the compost in tubes and incubated at 25 °C in darkness at a relative humidity of 85 % [7]. After inoculation, the time taken for the mycelia to reach the centre of the compost was noted and the growth speed was calculated. For dual cultures, each fungus was inoculated at opposite ends of the compost in tubes using the method described above. The inoculation time used for each fungus was adjusted so that both would make contact at the centre of the compost plugs. The growth of both *A. bisporus* and *H. perniciosus* was measured every 2 d. After contact between the two fungi occurred at the centre of the compost, microscopic observations were made to study the changes in the mycelia of both fungi. Observations were continued for 16 days after contact. If *A. bisporus* grew towards the growth zone of *H. perniciosus* after the contact, then its growth was recorded as ‘+’, while if the fungal growth area was occupied by *H. perniciosus* it could not grow further after contact, then it was recorded as ‘–’ [10]. The pathogenicity of *H. perniciosus* was evaluated in five grades (Table 1) as previously described by Nitta and Miyazaki [10]. Five tubes were used for each test, and the experiments were replicated three times.

**RESULTS**

**Basidiomes of *A. bisporus* infected with *H. perniciosus***

The hyphae of the mycelium in healthy *A. bisporus* basidiome were turgid, while the organelles were clearly visible and the nuclei were viable (Fig. 2a–d). The results of disease progression are shown in Table 2. Necrotic brown lesions develop on the basidiomes during the early stages of the disease, which is due to the germination of conidia and the
growth of the hyphae of the invading fungus (Fig. 3a). The spores of \textit{H. perniciosus} first attach to the surface of the \textit{A. bisporus} basidiomes, and then germinate and penetrate the \textit{A. bisporus} basidiome directly without the formation of appressorium-like structures (Fig. 3b). The cell contents of the hyphae of the \textit{A. bisporus} basidiomes in the necrotic brown lesion plasmolysed as seen in LM (Fig. 3c), but were still alive (Fig. 3d). A sample was taken from the brown necrotic regions and \textit{H. perniciosus} was positively isolated, but no bacteria were detected in the centre of the mushroom or on its surface using LM; only \textit{H. perniciosus} mycelia were observed.

The necrotic brown lesion became larger day by day. Six days after inoculation with \textit{H. perniciosus}, the mycelium of the inoculated area of the \textit{A. bisporus} basidiomes appeared to be dead when checked by LSM (Fig. 4a). The area was observed in LM and SEM, and a sample of the diseased area was removed and tested, revealing the presence of \textit{H. perniciosus} but not of any bacteria (Fig. 4b). Twelve days after inoculation with \textit{H. perniciosus}, the size of the diseased \textit{A. bisporus} basidiomes was smaller than that of the healthy uninfected ones. Brown droplets containing bacteria appeared on the infected basidiomes (Fig. 5a), and their surface was covered with \textit{H. perniciosus} (Fig. 5b). The centres of the basidiomes were also discoloured (Fig. 5c), and some mycelia of \textit{A. bisporus} showed degeneration and cytoplasmolysis (Fig. 5d). Bacteria could only be observed and isolated from the brown droplets. At the centre of the infected basidiomes, only \textit{H. perniciosus} was observed and isolated (Fig. 5e). Twenty days after inoculation, the basidiomes of \textit{A. bisporus} were altered and appeared to be softer, lacking structural integrity. Furthermore, when observed in LM, numerous bacteria were present, regardless of the depth of focus (i.e. the surface or the centre). The bacterial cells were of different sizes and shapes, and most were Gram-negative (Fig. 5f). Most of the mycelium of \textit{A. bisporus} had undergone degeneration and cytoplasmolysis by this time (data not shown).

Disease development in \textit{A. bisporus} basidiome tissue on culture media inoculated with \textit{H. perniciosus}

Three days after inoculation, both fungi made contact, and the necrotic brown lesions developed at the contact zone of \textit{A. bisporus} basidiomes tissue. Seven days later, the basidiomes piece were covered by fluffy white mycelium \textit{H. perniciosus} (Fig. 6a). In contrast, the control basidiomes had already sporulated (Fig. 6b). Fifty days later, the basidiomes

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**Fig. 3.** Inoculating \textit{Hypomyces perniciosus} on \textit{Agaricus bisporus} basidiome at 3 days. (a) The inoculated part of the \textit{A. bisporus} basidiome developed a necrotic brown lesion. (b) A germinated chlamydospore of \textit{H. perniciosus}. (c) Light microscopy features of the cap surface mycelium of an \textit{A. bisporus} basidiome necrotic brown lesion. (d) Laser scanning image of the cap-surface mycelium of an \textit{A. bisporus} basidiome’s necrotic brown lesion portion. Di, brown colour; Cl, clamydospore; N, nuclei.

**Fig. 4.** Six days after inoculating \textit{Hypomyces perniciosus} on an \textit{Agaricus bisporus} basidiome. (a) Laser scanning microscope features of the mycelium of the \textit{A. bisporus} basidiome, necrotic brown lesion portion. (b) Light microscopy features of the mycelium of the \textit{A. bisporus} basidiome, necrotic brown lesion portion. H, \textit{H. perniciosus}; N, nucleus.
turned yellow brown, with the production of yellow *H. perniciosus* chlamydospores on the surface, and the shape of the infected *A. bisporus* basidiomes tissue reduced in overall size (Fig. 6c). However, during the whole recorded time we did not observe any production of brown droplets. Observations by LM showed that most of the mycelia of *A. bisporus* had undergone degeneration and breakage. The structure of the mycelium became ill-defined and the hyphae of *H. perniciosus* filled the basidiomes, but not of any bacteria *(Fig. 6d)*. The disease symptoms mostly appeared to be the same as those with *in vitro* inoculations of *H. perniciosus* onto *A. bisporus* basidiomes.

**Cytology of mycelial growth in dual culture on media**

The growth of *A. bisporus* was expedited more when it was inoculated on PDA compared to the other tested media (Fig. 7a). On PDA and CMA, the mycelial growth of *A. bisporus* appeared to be denser than that on MEA (Fig. 7a–c), and in addition the mycelial growth on improved Fries medium was sparse (Fig. 7d).

In the dual cultures, before observable contact between the mycelia of the two fungi was made, the growth rate of *A. bisporus* was not inhibited. When contact was established, growth was still not inhibited for either mycelia, pigments were not produced and no zones of lysis were seen. Morphological changes of *A. bisporus* and *H. perniciosus*, such as ‘barrages’ of mycelium resistant to invasion, invasive mycelia fans, and aggregated mycelia structures, cords and rhizomorphs were also not observed. After contact, the reactions still did not differ significantly on the different media used. To the unaided eye, *A. bisporus* continued to proliferate and spread into the growth area of *H. perniciosus*, although the rate was inhibited. *Hypomyces perniciosus* also entered the growth area of *A. bisporus*, *(Fig. 6d)*.
but its proliferation was also restricted, especially on PDA (Fig. 7a). Both continued to grow. No observable pigments were produced and no zones of lysis were seen (Fig. 7a–d). Further, no morphological changes were evident in the A. bisporus mycelia.

The observations made by LM concentrated on the tips of the mycelial hyphae of A. bisporus that first made before contact with H. perniciosus. They showed that these were normal, without any apparent degradation or malformation, while the cytoplasm also remained organized, with no cytoplasmolysis or plasmolysis detected, and the vacuoles also remained visible (Fig. 8a). Observations by LSM using cell viability-testing reagents, showed that the nuclei of A. bisporus, particularly those in the upper parts of the mycelium, stained in blue and so had not undergone karyolysis (Fig. 8b). Observations by LM just after contact showed that the tip of the mycelia hyphae of A. bisporus remained unchanged and, as before, contact was made (Fig. 8c). The physical state of the nuclei, as observed in LSM, was also unchanged just after contact (Fig. 8d). Observations of the mycelium of A. bisporus and H. perniciosus by LM when they were intermingled showed that the mycelia hyphae of A. bisporus remained unchanged and as contact was just made (Fig. 8e). The physical state of the nuclei, as observed in LSM, was also unchanged after contact (Fig. 8f).

During the dual culture experiments we also observed some fractured hyphae of A. bisporus in both the control and the dual cultures on all of the media.

**Interactions in dual cultures in compost**

In the compost cultures, the mycelium of H. perniciosus reached the centre (4.5 cm) of the compost 11 d after inoculation, whereas A. bisporus required 21 d.

H. perniciosus and A. bisporus mycelia made contact 11 d after inoculations at the centre of the compost in the tubes. After contact was made, A. bisporus continued to grow into the area occupied by H. perniciosus [Fig. 9 (+)], although the speed was reduced. H. perniciosus barely entered the area occupied by A. bisporus [Fig. 9 (−)]. There were no observable antagonistic lines where the hyphae intermingled (Fig. 10). The pathogenicity of H. perniciosus to A. bisporus mycelia, based on Table 1, was evaluated as weak [10].

**DISCUSSION**

Our results on the progression of the development of WBD in A. bisporus basidiome, and on the effect of H. perniciosus on basidiome pieces in culture indicated that H. perniciosus can kill and cause cytological changes in A. bisporus basidiomes, confirming it as a necrotroph. Bacteria were not seen to be involved in the onset of the disease. We therefore hypothesize that the increase in the number of bacteria seen in the later stages of WBD progression is most probably attributable to them being introduced into the casing mixture used in mushroom cultivation. Generally, the standard casing soil used in commercial mushroom cultivation contains bacteria, as certain bacteria are indispensable in the fructification process [18, 19]. When using sterilized casing soil, only mycelial growth occurs without fructification.
In a study that explained how certain bacteria induced the fructification of *A. bisporus*, Hayes [18] revealed that these bacteria are stimulated by various volatile metabolic products of the *A. bisporus* mycelium, and that the bacteria remove an excess of those products, which would inhibit fructification [18]. However, the bacteria in the commonly used casing soil may also contain some saprotrophic bacteria or parasitic organisms [20]. In common culture conditions they are inhibited by the beneficial microbes in the casing soil, or the culture conditions restrict their dissemination [7]. Therefore, they remain balanced with the beneficial microbes and cannot influence the yield of *A. bisporus*. However, when the mushrooms are infected by some other parasitic organisms, these saprotrophic or parasitic bacteria can invade and reproduce on the diseased *A. bisporus* basidiomes. Consequently, in this paper we concluded that during WBD progression, the *A. bisporus* basidiomes are first invaded by *H. perniciosus*, and only later do these opportunistic bacteria become established, when they may also participate in causing degradation of the diseased basidiomes.

Observations made using dual cultures of *H. perniciosus* and mycelia of *A. bisporus* on different media and compost showed that *H. perniciosus* has almost no pathogenic activity against the mycelial stage of *A. bisporus*. In contrast, Pieterse [6] and Huang et al. [8] reported that growth of *H. perniciosus* was stimulated, and that of *A. bisporus* inhibited, before their contact [6, 8]. They suggested that these interactions were caused by metabolites or volatile compounds from *H. perniciosus*. Our results, however, showed that there was only limited inhibition of the mycelial growth of *A. bisporus* by *H. perniciosus*. This growth restriction after contact may not be caused solely by chemical products from *H. perniciosus*, but also by nutritional requirements. Because, after contact was made by both fungi, there was competition for the same source of nutrition i.e. that portion of media, therefore inhibition of growth was noted in both organisms.

Nevertheless, as *H. perniciosus* can cause significant morphological changes in *A. bisporus* basidiomes, we suggest that their interactions in the mycelial stages and in

Fig. 8. Light and laser scanning microscopy of the mycelium of *Agaricus bisporus*. (a) Hyphae of *A. bisporus* before making contact with *H. perniciosus* on CMA at 2 d. (b) Actively growing hyphae of *A. bisporus* before making contact with *H. perniciosus* on CMA at 2 d. (c) Hyphae of *A. bisporus* and *H. perniciosus* just before making contact on CMA at 4 d. (d) Actively growing hyphae of *A. bisporus* just after making contact with *H. perniciosus* on CMA at 4 d. (e) Hyphae of *A. bisporus* and *H. perniciosus* after making contact with each other on MA at 13 d. (f) Actively growing hyphae of *A. bisporus* after making contact with *H. perniciosus* on MA at 13 d. A, *A. bisporus*; H, *H. perniciosus*; N, nuclei; bar, 10 µm.

Fig. 9. The growth of *Hypomyces perniciosus* and *Agaricus bisporus* from the first contact point.
basidiomes may occur via different mechanisms. Different chemical compounds from the basidiome and mycelial surfaces of A. bisporus may be the only reason why the A. bisporus basidiome becomes infected by H. perniciosus [21–23]. Furthermore, it seems probable that the release of pathogenic substances (toxins, enzymes, and antifungal metabolites) from H. perniciosus has to be induced by the basidiomes of A. bisporus. The attachment of H. perniciosus to the basidiome of A. bisporus could be initiated through a specific interaction between the surface molecules of both fungi.

Fig. 10. Mycelial interactions of Hypomyces perniciosus and Agaricus bisporus in compost in the two-sided open test tubes. (a) Two days after contact. A. bisporus did not grow within the area occupied by H. perniciosus. (b) Eight days after contact. A. bisporus grew towards the area occupied by H. perniciosus. (c) Sixty days after contact. A. bisporus grew within most of the area occupied by H. perniciosus. A, A. bisporus; H, H. perniciosus; C, contact point.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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