Mycorrhiza (2003) 13:41–47
DOI 10.1007/s00572-002-0195-4

ORIGINAL PAPER

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In vitro evidence of mycoparasitism of the ectomycorrhizal fungus *Laccaria laccata* against *Mucor hiemalis* in the rhizosphere of *Pinus sylvestris*

Received: 5 March 2002 / Accepted: 5 July 2002 / Published online: 9 August 2002

Abstract Interactions between the ectomycorrhizal fungus *Laccaria laccata* and the soil fungus *Mucor hiemalis* f. *hiemalis* in co-culture, and in the rhizosphere of in vitro-grown *Pinus sylvestris* seedlings were investigated by light- and scanning electron-microscopy. In co-culture, mycelial growth away from the *L. laccata* colony reduced the number of aerial hyphae at the contact zone and increased the density and compactness of the mycelium-characterized gross morphology of the saprobic fungus. Although the growth of *M. hiemalis* was suppressed, no penetration of *M. hiemalis* hyphae after the colony was entered by *L. laccata* was observed. Instead, dense coiling of *L. laccata* hyphae around sporangiophores, overpowering them and causing them to disappear, was quite common. On nonmycorrhizal roots, sporangiospores germinated heavily and formed long hyphae for 2 days post inoculation, whereas their germination was totally inhibited on mycorrhizal roots. At 3 days after inoculation, only sporangia were seen with mycelial mats firmly attached to the roots by the mantle hyphae, whereas some remnants of sporangiophores, ruptured sporangial walls and degraded hyphae of *M. hiemalis* were overgrown by the mantle hyphae. During the next 3 days, the mantle-hyphae-invading sporangia formed short, thin branches that grew directly towards individual spores, tapering off upon contact.

Keywords *Laccaria laccata* · *Mucor hiemalis* · Mycoparasitism · *Pinus sylvestris* · Rhizosphere

Introduction

Interactions between mycorrhizal fungi and other soil microorganisms are crucial in the understanding of the dynamics of rhizosphere populations affecting plant growth and health. Soil microorganisms are known to influence mycorrhizae development and function (Garbaye and Bowen 1989; McAfee and Fortin 1988; Summerbell 1987). Mycorrhizal fungi are also key components of soil microbiota, regulating the composition and population sizes of rhizosphere microorganisms. The activity of soil heterotrophs depends on their ability to decompose organic substrates and to compete for limited sources of carbon and nitrogen in the rhizosphere. At early developmental stages, mycorrhizal fungi with low saprobic capabilities do not compete well for nutrients with such low-specialized microorganisms, and are sensitive to their antagonistic or beneficial impacts (Azcón-Aguilar and Barea 1992). However, in general, the results of experiments on the dynamics of rhizosphere populations and microbial competition for nutrients suggest that most filamentous fungi have a negative influence on ectomycorrhiza formation, particularly when symbionts are introduced into fumigated soils of forest nurseries (Lamb and Richards 1978; Sinclair et al. 1975). Some interactions between mycorrhizal fungi and other soil microorganisms involving nutrient cycling and/or biological control benefit plant growth and health. This suggests that multipartite associations benefit the plant more than a single mycorrhizal symbiont (Paulitz and Linderman 1991).

Mycorrhizal fungi may influence plant growth and health directly by excreting growth-promoting and antimicrobial substances (Duchesne et al. 1987; Marx 1972; Mitchell et al. 1986; Strzelczyk and Pokojska-Burdziel 1984; Strzelczyk et al. 1985; Sylvia and Sinclair 1983) or indirectly by altering the root physiology and pattern of exudation into the mycorrhizosphere, which in turn can affect the composition of beneficial microbial populations. Many authors have shown that some bacteria and actinomycetes associated with the mycorrhizosphere are involved in this indirect effect of mycorrhizal fungi on plant growth (Leyval and Berthelin 1993) and biocontrol (Malajczuk and McComb 1979; Napierała-Filipiak et al. 2001). Conversely, only a limited amount of research has been reported on the direct interaction of mycorrhizal fungi with soil-borne fungal pathogens and saprobes, and

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the mycoparasitic capabilities of mycorrhizal fungi have been suggested in only a few cases (Lei et al. 1995; Werner et al. 2002; Zhao and Kuo 1988).

Once established, ectomycorrhizae are well supplied with nutrients and are protected due to the physical effect of the mantle and/or biosynthesis of antimicrobial substances, but the extramatrical mycelia still interact with fungal pathogens and saprobes. To date, little is known about the direct effect of mycorrhizal fungi on bulk-soil saprobic fungi. The existence of a benign relationship between ectomycorrhizal fungi and soil *Penicillia* and *Mucorales* was suggested by Moser (1963). The composition of fungi is controlled by the amount and form of carbon in the soil, depending on the conditions and preferences for decayed substrates. *Penicillia*, in general, degrade cellulose and xylan well, which makes them common soil inhabitants (Bääth and Söderström 1980), while the frequency of *Mucorales*, which decompose proteins, increases with the intensification of the decomposition and mineralization processes (Sierota and Kwaśna 1998).

Mucor hiemalis f. *hiemalis*, due to its rapid growth, is one of the commonest soil fungi and is the most frequent representative of *Mucorales* (*Zygomycotina*). It has a worldwide distribution and occurs in various microhabitats including grassland, arable soils, and forest litter. Its high mycelial extension rates allows the fungus to colonize fresh substrates rapidly. This applies particularly to the colonization of roots at early stages of pine and spruce development (Kuhlman 1969; Mańka and Truszkowska 1958). In an agricultural soil, *M. hiemalis* out-competed *Trichoderma harzianum* (Rifai), whereas in a forest soil *T. polysporum* (Link ex Pers. Rifai) maintained a competitive advantage over *M. hiemalis* (Wardle et al. 1993). Increased frequencies have been reported after mineral fertilization. *M. hiemalis* f. *hiemalis* is one of the least substrate-specific coprophilous *Mucoraceae*. The long list of dead substrates that it can colonize indicates its lack of any particular preference. As with most *Mucoraceae* it occurs predominantly in the uppermost soil layer, and over a very wide pH range, but particularly in neutral or slightly alkaline soils (Domsch et al. 1980). This fungus has antimicrobial and antifungal activities (Codignola and Gallino 1974/1975; Jeziorska 1974) and has been found amongst the hyperparasites of sclerotia of *Sclerotinia* spp. and *Claviceps purpurea* (Fries) Tulasne (Karhuvaara 1960; Makkonen and Pohjakalljo 1960).

The objectives of this study were: (1) to examine and characterize the in vitro interaction between the ectomycorrhizal fungus *Laccaria laccata* and the soil saprobic fungus *M. hiemalis* f. *hiemalis*, selected on the basis of its predominance over another members of *Mucorales* in a fertile arable soil, both in dual culture and in the rhizosphere of *Pinus sylvestris*; and (2) to gather more evidence about the role of the potential mycoparasitic capabilities of *L. laccata* in the growth suppression of soil saprobic fungi.

Materials and methods

Organisms and growth conditions

The standard source of *L. laccata* (Scop. ex Fr.), strain (9-1) was 1-month-old mycelium growing on Pp agar medium (Pachlewski 1983) at 24°C in the dark. The strain was isolated from a basidiocarp under *P. sylvestris* (L.). *M. hiemalis* f. *hiemalis* (Wehmer), strain (Bu 10) – originating from an agricultural soil at Bukowiec in Poland (Werner et al. 2001) – was maintained on potato dextrose agar (Difco) at 22°C. *P. sylvestris* seedlings from the provenance of Bolewice (52° 28′ N and 16 03′ E) were used in the study.

Fungal interaction in co-culture

Dual culture of *L. laccata* and *M. hiemalis* was carried out in Petri dishes (10 cm diameter). Plates containing 10 ml Pp agar medium at pH 5.5 were inoculated with discs (5 mm diameter) of 1-month-old mycelial mat of the mycorrhizal fungus. After establishing the mycelium, an inoculum of the soil fungus was placed at the opposite side of the plate. The cultures were incubated at 24°C in the dark. Each fungus grown individually served as controls. The outcome of the interaction in the Petri dishes was analyzed over the following 2 weeks.

Microscope slide cultures

Sterilized glass microscope slides were dipped in Pp medium to obtain a thin layer of agar and inoculated with hyphal tips of the fungi. The cultures were incubated in sterile Petri dishes as described by Stahl and Christensen (1992) at 24°C in the dark. After 3–5 days, the morphology of the hyphae and their behavior in the contact zone were observed under a light microscope at magnifications of up to 400×.

Interaction between *L. laccata* and *M. hiemalis* in the rhizosphere of pine seedlings

Pieces of *L. laccata* mycelium were transferred to 1 l Roux flasks containing 250 ml liquid Pp medium at pH 5.5. After 2 weeks of incubation, the medium was drained off and the mycelia were transferred to 300 ml jars (Sigma) containing a sterile mixture of peat and perlite (1:3 v/v) moistened with liquid Pp medium. Subsequently, the jars were shaken by hand twice weekly and incubated until all the mixtures were overgrown by the mycelia. The inocula were then transferred to Petri dishes.

Pine seeds were soaked in water, surface-sterilized with 0.2% $HgCl_2$ for 4 min and washed three times (15 min each) in sterile distilled water. Seeds were germinated on 0.6% agar (w/v) medium, in the dark at 24°C. Subsequently, they were transferred aseptically to Petri dishes containing the inoculum of *L. laccata* and incubated for 2 months in a growth room under fluorescent tubes (Osram L36/W77 Flora) (100 $\mu Em^{-2}\ s^{-1}$) with light 16 h/day, 60% relative humidity at 24:20°C day:night temperatures.

Roots of mycorrhizal and nonmycorrhizal (control) plants were inoculated either with a suspension of sporangiospores or mycelial mats of *M. hiemalis*. The sporangiospores were obtained by scraping the surface of 2-week-old cultures using glass beads and resuspending in sterile distilled water to a final concentration of 2.2×10^6 sporangiospores ml^{-1}. For inoculation of roots with mycelium, small discs (5 mm) cut from the margins of vigorously growing cultures were placed close to the roots.

For scanning electron microscopy (SEM), mycorrhizal and nonmycorrhizal roots (15 of each) inoculated with sporangiospores and a similar number of roots inoculated with mycelial mats of *M. hiemalis* as well as several mycorrhizal and nonmycorrhizal roots uninoculated with the soil fungus were selected at intervals of 7–24 h, and 2, 3, and 6 days post inoculation.

Preparation of roots for SEM

Small pieces (3–5 mm) of roots were fixed in 2.5% glutaraldehyde in 0.5 M cacodylate buffer at pH 7.2 for 24 h and postfixed in 2% OsO_4 in 0.5 M cacodylate buffer for 2 h at 4°C. The specimens were then washed in distilled water, dehydrated in an ascending ethanol series (10% steps, 15 min each) and critical point dried in a Balzers CPD-030 unit using CO_2 as a transition fluid. Specimens were then mounted on aluminum stubs and coated with gold (12–15 nm thick) using a Balzers SPD-050 sputter coater. Finally, the roots were observed in a Philips 515 scanning electron microscope at 15 keV.

Results

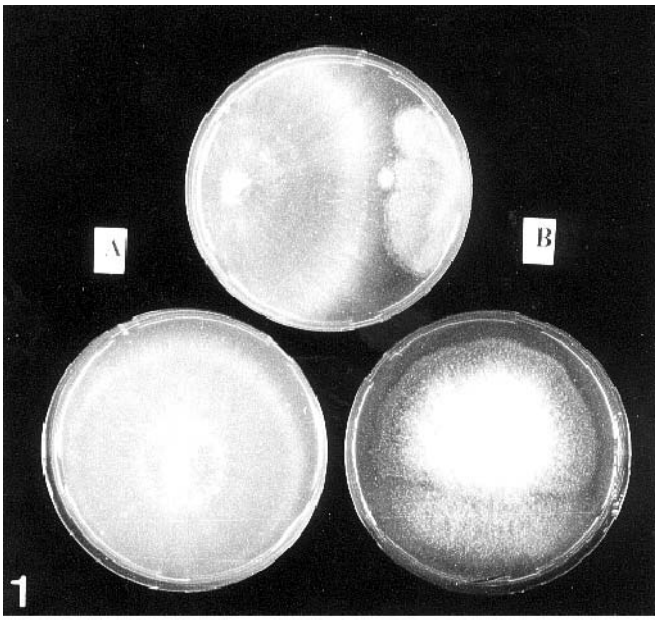
Interaction in co-culture

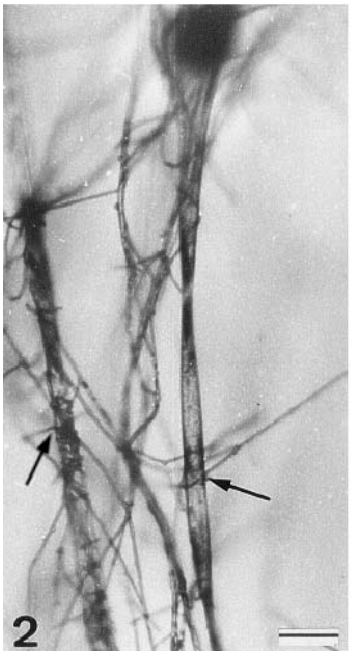
In paired cultures, *L. laccata* suppressed growth of *M. hiemalis* long before contact between hyphae of their colonies occurred, often when the two fungi were 3–4 cm apart. The growth rate of *L. laccata*, as indicated by the radius of its colony, was the same in co-culture and when grown individually, whereas the growth of *M. hiemalis* was evidently inhibited and there was no expansion of its mycelium in the direction of the colony of *L. laccata*. A common response of the saprobic fungus was a reduced number of aerial and substrate quick-growing hyphae at the proximal edges of the colony. Consequently, its mycelium was much more dense and compact in the presence of *L. laccata* than when grown alone (Fig. 1). The outcome of the mycelial interaction may be categorized as invasion-overgrowth. Observations made using slide cultures showed the ability of *L. laccata* hyphae to grow into colonies of the saprobic fungus along its hyphae. Although the hyphal growth of the latter was apparently inhibited, there was no evidence for penetration of its hyphae by *L. laccata*.

One day after the first contact between the two fungi, the hyphae of the mycorrhizal fungus coiled around sporangiophores of *M. hiemalis*. Aerial hyphae were seldom coiled and no coiling around sub-surface hyphae was observed. At the beginning, the main hyphae of *L. laccata* formed numerous short branches, usually at right angles (Fig. 2). Then, each branch coiled tightly around the sporangiophore. Abundant multiplication of *L. laccata* hyphae and dense coiling around the sporangiophore were observed within a day. Some hyphae grew in a wavy manner along the surface of the sporangiophore, while still others showed a tendency to coil around, creating a structure resembling a cocoon (Fig. 3). This dense plexus made it impossible to trace the growth of individual hyphae and their possible entry into the sporangiophore. However, in areas of colonies overgrown by *L. laccata* most sporangiophores were overpowered, became flaccid and disappeared with time.

Interactions in the rhizosphere of pine seedlings

In Petri dishes, *P. sylvestris* seedlings produced a heterorrhizic root system with a few short monopodial and dichotomous roots. The mantles of 2-month-old mycorrhiza were white and comprised two or three layers of hyphae forming a loose weft in the apical part of these roots (Fig. 4). In older parts of the roots, the mycelium was thicker, more compact and, in many root patches, embedded in a mucilaginous material.





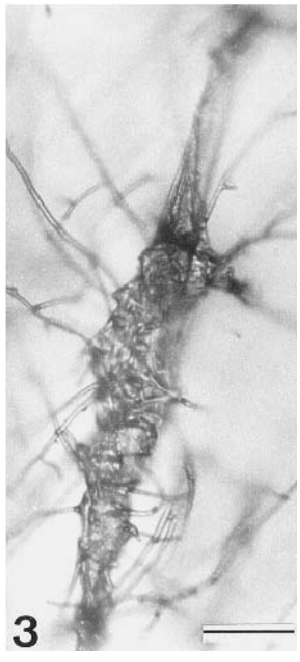
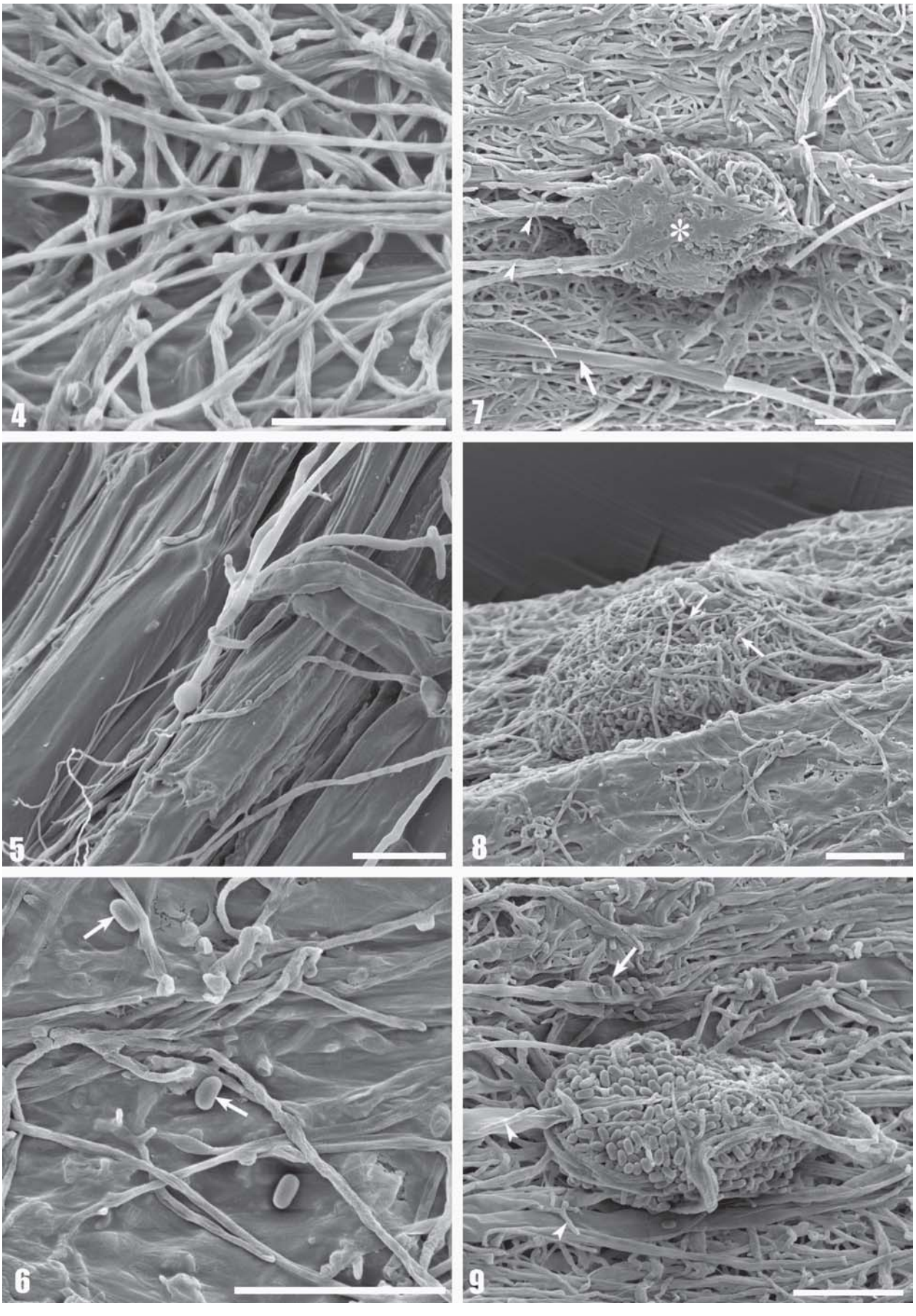


Fig. 1A, B Interaction between *Laccaria laccata* and *Mucor hiemalis* in co-culture (upper plate). Control cultures (lower plates) of *L. laccata* (left) and *M. hiemalis* (right)

Fig. 2 Interaction between *L. laccata* and *M. hiemalis* at the contact zone. Note elongated hyphae of *L. laccata* growing along sporangiophores of *M. hiemalis*, branching frequently at right angles and coiling around them (*arrows*). *Bar* 50 μm

Fig. 3 An advanced stage of high-density coiling around sporangiophore of *M. hiemalis* by hyphae of *L. laccata*. *Bar* 50 μm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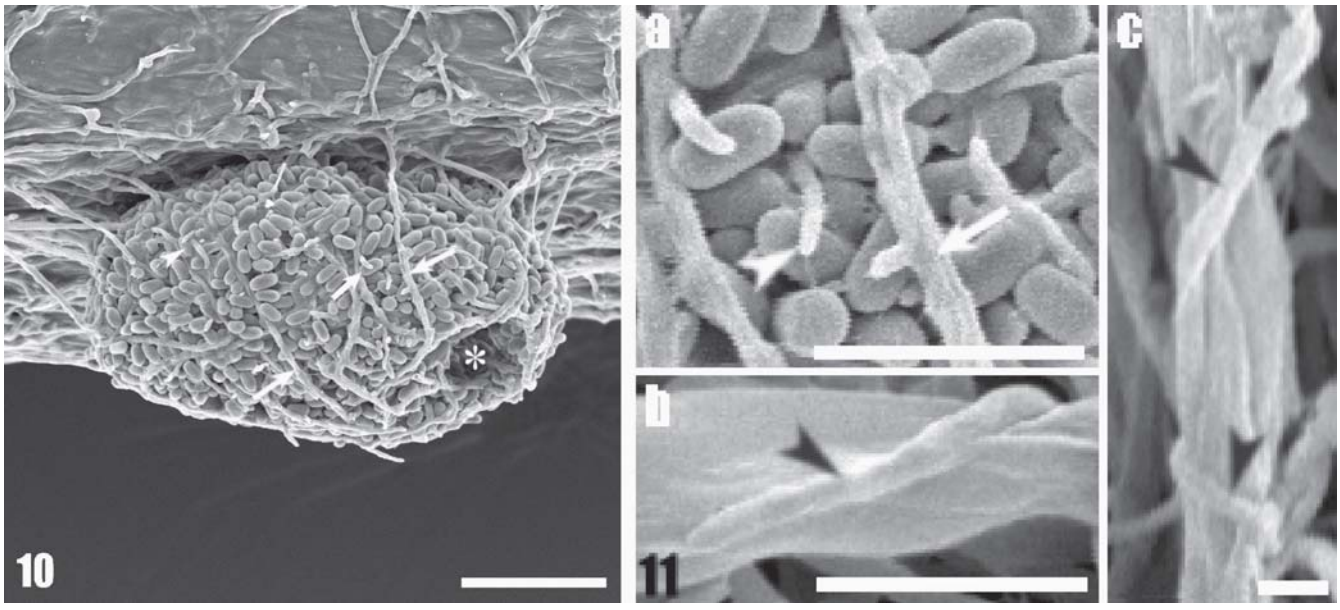

Fig. 10 Sporangium firmly attached to the root surface by the mantle hyphae (*arrows*) 6 days after inoculation. Note a hole (*) in the point of the degraded columnella and the close contact of short hyphae with sporangiospores (*arrowheads*). *Bar* 30 µm

Fig. 11a–c Enlarged areas of Figs. 7, 9 and 10, showing narrowed tips of short branches of the mantle hyphae of *L. laccata* (*arrow*) attached to **a** sporangiospores (*arrowhead*), **b** mantle hyphae (*arrowhead*) coiling a remnant of sporangial wall and **c** hyphae of *M. hiemalis*. *Bars* 10 µm

By 48 h after inoculation of nonmycorrhizal pine roots with suspension of sporangiospores, most of them germinated. One day later, a great number of elongated hyphae differing in size and structure (characteristic for *Mucor*) began to colonize the root surfaces (Fig. 5). Germination of sporangiospores was totally inhibited on mycorrhizal roots after inoculation with either the suspension (Fig. 6) or mycelial mats (Figs. 7, 8 and 9). During the 2–3 days after inoculation with the mycelial mat, mycelium of *M. hiemalis* adjacent to the mantle was totally colonized by the hyphae of *L. laccata* (Fig. 7). Three days after inoculation, there were no sporangiophores on the surfaces of mycorrhizal roots (Figs. 8, 9 and 10). Instead, many-spored sporangia, firmly attached to the root by the mantle hyphae, and occasional remnants of sporangiospores, ruptured sporangial walls and thick and collapsed hyphae of *M. hiemalis* could be seen 6 days post inoculation (Fig. 9). The mantle hyphae coiled around the remnants and invaded sporangia. Some of the hyphae formed short and thin branches, whose growth was obviously directed towards particular spores. The tips of the thin hyphae additionally tapered off at the point of contact with spores (Figs. 10 and 11).

◀ **Figs. 4–9** Scanning electron micrographs of interaction between *L. laccata* and *M. hiemalis* in the rhizosphere of *Pinus sylvestris*

Fig. 4 Hyphae of *L. laccata* forming a loose mantle on root surface 2 months after inoculation. *Bar* 30 µm

Fig. 5 Hyphae of *M. hiemalis* growing on the surface of a nonmycorrhizal root 3 days after inoculation with a suspension of sporangiospores. *Bar* 30 µm

Fig. 6 Ungerminated sporangiospores (*arrows*) scattered on the mantle surface 3 days after inoculation. Note abundant mucilaginous material covering the proximal part of the root and coating the mantle hyphae. *Bar* 30 µm

Fig. 7 A part of mycelium of *M. hiemalis* invaded by *L. laccata* showing intermingled hyphae of both fungi and the mantle hyphae growing on and coiling around hyphae of the former fungus (*arrowheads*), flattened sporangiophores and/or thick hyphae of *M. hiemalis* (*arrows*), 3 days after inoculation with mycelial mat. Note particularly dense and compact mycelium of *L. laccata* covering sporangium (*). *Bar* 30 µm

Fig. 8 Sporangium of *M. hiemalis* overgrown by the mantle hyphae (*arrows*) 3 days after inoculation. *Bar* 30 µm

Fig. 9 Sporangium and sparse sporangiospores (*arrows*) on the surface of the mycelial mat adjacent to a mycorrhizal root 6 days after inoculation. Note lack of sporangiophore and flattened thick hyphae of *M. hiemalis* and remnants of sporangial wall coiled by the mantle hyphae (*arrowheads*). *Bar* 30 µm

Discussion

In studies on the direct effect of ectomycorrhizal fungi on soil microbiota, mycoparasitism on soil-borne fungal pathogens and saprobes has seldom been taken into account. The mycoparasitic capacity of ectomycorrhizal fungi against *Rhizoctonia solani* (Kühn) was suggested by Zhao and Kuo (1980). A recent report by Lei et al. (1995) confirmed this assumption. Malajczuk (1988) described formation of a constriction ring and the ability to "capture" the germ tubes of *Phytophthora cinnamomi* (Rands.) by hyphae of fungi associated with the white type of ectomycorrhiza of *Eucalyptus marginata* (Donn ex Sm.). However, the author suggested that the primary agents reducing the activity of the pathogen were bacteria and actinomycetes.

The obvious lack of hyphal coiling around sporangiospores of *M. hiemalis* scattered on the mantles of *L. lac-*

cata, as compared to that described by us around conidia of *Trichoderma virens* (Mill. Gidd. et Foster) von Arx (Werner et al. 2002), may be explained in terms of a weak stimulus due to the low concentration of spores used in this study to exclude the effect of self-inhibitors. Also, the lack of uncontrovertible evidence of degradation of sporangiospores may be related to their high tolerance of the action of wall-degrading enzymes. Considering the ability of sporangiospores of true coprophilous fungi to survive in the intestinal passages of animals (Harper and Webster 1964; Mehrotra et al. 1965), a period of 6 days could be too short to allow degradation of the sporangiospores of *M. hiemalis* by *L. laccata*, but long enough to decompose and/or consume its hyphae and sporangiophores. As in all the *Mucorales*, the cell walls of *M. hiemalis* consist mainly of chitin. In nature, chitin is decomposed by chitinolytic soil actinomycetes and bacteria. Certain soil *Hyphomycetes* (species of *Aspergillus*, *Fusarium*, *Gliocladium*, *Trichoderma* and *Penicillium*) and soil *Zygomycetes* (species of *Absidia*, *Mortierella*, *Mucor*) are known to have chitinolytic activity, whereas only a few members of the *Basidiomycotina* are chitinolytic. Knowledge about the mycolytic activity of mycorrhizal fungi is still insufficient.

The results presented here, and our previous investigations (Werner et al. 2002), may suggest that the mycoparasitic capabilities of *L. laccata* expressed in artificial, in vitro, conditions may be related to nutrient deficiency that would be uncommon in the soil environment. Under natural conditions, increased nutrient availability for plants results from changes induced in the physicochemical properties of the microhabitats. The rhizosphere and mycorrhizosphere microorganisms, such as phosphate-solubilizing microorganisms, nitrogen fixers, siderophore producers, plant growth-promoting Rhizobacteria and mycorrhizal helper bacteria are known to improve plant nutrition (Azcón-Aguilar and Barea 1992; Garbaye 1994; Szaniszlo and Powell 1981).

According to De Boer et al. (1998, 2001), the production of chitinase, β-1,3-glucanase and protease by dune soil chitinolytic bacteria seems to be only a part of a lytic system that uses living hyphae of saprobic fungi, including *M. hiemalis*, rather than chitin as a source of nutrients. The results of the studies cited above suggest that, even in nutrient-poor sand dune soil, the inhibition of fungal growth was not accompanied by bacterial chitinase, and that antibiotics or other mechanisms were involved in the antagonism of the bacteria towards the target fungi. However, since our experiments were performed under sterile conditions, they would not reflect the natural conditions where individual microorganisms compete and interact with one another. Nevertheless, it would be worth studying the potential mycoparasitic capabilities of ectomycorrhizal fungi to improve plant growth and to suppress root diseases, particularly when trying to establish beneficial microbial populations on the roots of axenically propagated plants. Since the outcome of interactions with general soil microorganisms determine whether introduced ectomycorrhizal fungi can persist other than in a forest environment, mycoparasitic capabilities of fungi are of great importance.

Acknowledgements This study was financially supported by the Polish Academy of Sciences and Polish Committee for Scientific Research, grant No 6 PO4F 003 12. Special credit is due to Dr. Krystyna Idzikowska for providing assistance with scanning electron microscopy.

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