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ORIGINAL PAPER

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Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture and in the rhizosphere of *Pinus sylvestris* grown in vitro

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Abstract Interactions between the ectomycorrhizal fungus *Laccaria laccata* and the soil fungus *Trichoderma virens* in co-culture and in the rhizosphere of *Pinus sylvestris* seedlings growing in vitro were investigated by light and scanning electron microscopy. The growth of *T. virens* was inhibited in co-culture. Shortened, more branched and sometimes deformed or injured hyphae of *T. virens* were observed in the zone of inhibition. Two-month-old mycorrhizae of *P. sylvestris*/*L. laccata* were inoculated with a conidial suspension of *T. virens* and examined at intervals of 7–24 h and 2, 3, and 6 days post-inoculation (p.i.). On non-mycorrhizal roots, conidia germination was high and long hyphae formed 3 days p.i. On mycorrhizal roots, short germ tubes were observed only sporadically. At 3 days p.i., the mantle hyphae of *L. laccata* grew towards the conidia and coiled around them. Extremely dense coils of hyphae were found around clusters of conidia. Deformation of conidia, breaks in conidial walls and their partial degradation were observed 6 days p.i.

Keywords *Laccaria laccata* · *Trichoderma virens* · *Pinus sylvestris* · Antagonism · Co-culture

Introduction

Few studies have shown only a benign relationship between ectomycorrhizal fungi and members of the soil hyphomycetes and zygomycetes (Moser 1963; Summerbell 1987). As a whole, the results of experiments on microbial competition for nutrients in the rhizosphere suggest that most filamentous fungi have a deleterious effect on the formation of mycorrhizae (Bowen and Theodorou 1979). The antagonism of *Trichoderma* spp. to mycorrhizal colonization throws into question their use as biological control agents against soil-borne pathogens in forest nurseries.

Trichoderma spp. and other soil saprobes may have particular significance for the formation of mycorrhiza by symbionts introduced into agricultural soils. In forest nurseries, mycorrhizal fungi are inoculated into fumigated soils which are usually heavily recolonized by species of *Trichoderma.* In such a soil environment, the mycorrhizal fungi can be easily eliminated before establishing itself in the root system.

Several species of *Trichoderma* are known to be mycoparasitic on soil saprobes and soil-borne plant pathogens (Benhamou and Chet 1996; Calistru et al. 1997; Dennis and Webster 1971c) and on mycorrhizal fungi (Rousseau et al. 1996). These fungi can produce both volatile and soluble antibiotics (Dennis and Webster 1971a, b) as well as fungal wall-degrading enzymes such as cellulases, chitinases and glucanases (Elad et al. 1982, 1983, 1984; Inbar and Chet 1994; Jacobs and Kamoen 1986). According to Summerbell (1987), *T. viride* Pers. and *T. polysporum* (Link ex Pers.) Rifai were antagonistic towards the mycorrhizal colonization of roots of *Picea mariana* (Mill.) B. S. P. seedlings by *Laccaria bicolor* (Maire) Orton in vitro. The presence of thin mycorrhizal mantles and apparently degraded *L. bicolor* hyphae in some *Trichoderma* treatments suggests mycoparasitic interactions.

In a study by Farquar and Peterson (1990), *Fusarium oxysporum* f. sp. *pini* Schlecht. Emend Snyd & Hans, when grown in co-culture with *Paxillus involutus* (Fr.), caused breaks in walls of the mycorrhizal fungus and the release of protoplasts. Such mycolytic activity is known to occur in fungi from all taxonomic groups (Hashiba and Yamada 1982; Peberdy 1976, 1979); however, information about mycoparasitism of mycorrhizal fungi on both pathogens and saprobes is very limited. In laboratory experiments by Lei et al. (1995), some ectomycorrhi-

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zal fungi were able to destroy vegetative mycelia of several soil-borne pathogens, inhibit the formation and germination of their conidia, sporangia and sclerotia, and even produce haustoria in hyphae of *Rhizoctonia solani* (Kühn).

Production of extracellular chitinase (Baek et al. 1999), biosynthesis of antibiotics and mycoparasitism are known also to be major mechanisms in the biocontrol activity of *T. virens* (Mill. Gidd. et Foster) von Arx against cotton seedling disease caused by *R. solani* (Howell et al. 2000) and cocoa black pod caused by *Phytophthora palmivora* (Butl.) (Krauss and Soberanis 2001).

Trichoderma virens originating from arable soils suppressed the growth in culture of *Heterobasidion annosum* (Fr.) Bref., the main pathogen of conifers on former agricultural lands (Werner and Zadworny 2002). However, in laboratory experiments the growth of the fungus was inhibited by *L. laccata* (Scop. ex Fr.) to a degree similar to other *Trichoderma* species, namely *T. harzianum* (Rifai), *T. koningii* (Oudem) and *T. viride* (Pers.) (Zadworny 2001).

The objectives of the present study were (1) to evaluate the antagonism of *L. laccata* to *T. virens* in a co-culture plate test; (2) to document the interaction between the two fungi in the rhizosphere of *Pinus sylvestris* grown in vitro; (3) to evaluate the individual and joint effects of the fungi on the dry weight growth of pine seedlings.

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 *Pinus sylvestris* in Poland. *Trichoderma virens* (Mill. Gidd. et Foster) von Arx, strain Z 49, originating from an agricultural soil near Poznań in Poland, was maintained on potato dextrose agar (Difco) at 24°C. *Pinus sylvestris* L. seedlings from the province 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 *T. virens* was carried out in Petri dishes according to the method described by Marx (1969). Petri dishes (10 cm in diameter) containing 10 ml of Pp nutrient agar were inoculated with discs (5 mm in diameter) of a 1-month-old mycelial mat of the mycorrhizal fungus. After establishing the mycelium, an inoculum of the soil fungus was placed at the opposite site of the plate. The cultures were incubated at 24°C in the dark. One week later, the morphology of the hyphae in the interaction zone was observed under light and phase-contrast microscopy.

Interaction between *L. laccata* and *T. virens* in the rhizosphere of pine seedlings

Pieces of *L. laccata* mycelium were transferred to 1-l Roux flasks containing 250 ml of liquid Pp medium at pH 5.5. After 2 weeks incubation, the medium was drained off and the mycelia were put into 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 into Petri dishes.

Pine seeds were soaked in water, surface sterilized with 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 aseptically transferred to Petri dishes containing the inoculum of *L. laccata*. The seedlings were incubated for 2 months in a growth room under fluorescent lights (Osram L36/W77 Flora) (100 µE m^{-2} s^{-1}) with a light period of 16 h and 60% RH at 24°C:20°C day:night temperatures.

Conidial suspensions of *T. virens* in sterile distilled water were obtained by scraping the surface of 2-week-old cultures using glass beads. Mycorrhizal roots were inoculated at a concentration of 4.75×10^6 conidia ml^{-1}. The control materials were non-mycorrhizal seedlings inoculated *T. virens* conidia at the same concentration.

For scanning electron microscopy (SEM), 15 roots of 2-month-old mycorrhizal seedlings and two roots of non-mycorrhizal plants and similar numbers each of mycorrhizal and non-mycorrhizal roots inoculated with *T. virens* were selected at intervals of 7–24 h and 2, 3, and 6 days post-inoculation (p.i.).

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 series of ethanols (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 using a Philips 515 scanning electron microscope at 15 keV.

Growth of pine seedlings in vitro

Ten sterile pine seeds were placed into 300 ml jars filled to half of their volume with an inoculum of *L. laccata* prepared as described above. After germination of the seeds, the jars were kept in a growth room under the described conditions. The following treatments were established: M – mycorrhizal plants, M + S – mycorrhizal plants plus *T. virens*, S – non-mycorrhizal plants plus *T. virens*, C – non-mycorrhizal plants without *T. virens* (control). Plants were inoculated with 1 ml of conidial suspension of *T. virens* at a concentration of 3.07×10^6 conidia ml^{-1}. The conidial suspension was injected into the rhizosphere of 1-month-old pine seedlings with the help of 20-cm-long needles.

Three months after inoculation with *T. virens*, the plants were gently removed from the jars. The roots were extracted from the substrate in tap water and rinsed twice with distilled water. The plants were divided into needles, trunks and roots, dried in the open air and subsequently in an oven at 60°C for 3 days and weighed. The experiment was replicated.

Statistical analysis

The data from each treatment were analyzed using Statistica PL (StatSoft Polska Inc.) software procedures. One-way analysis of variance (ANOVA) and Tukey's HSD test were applied.

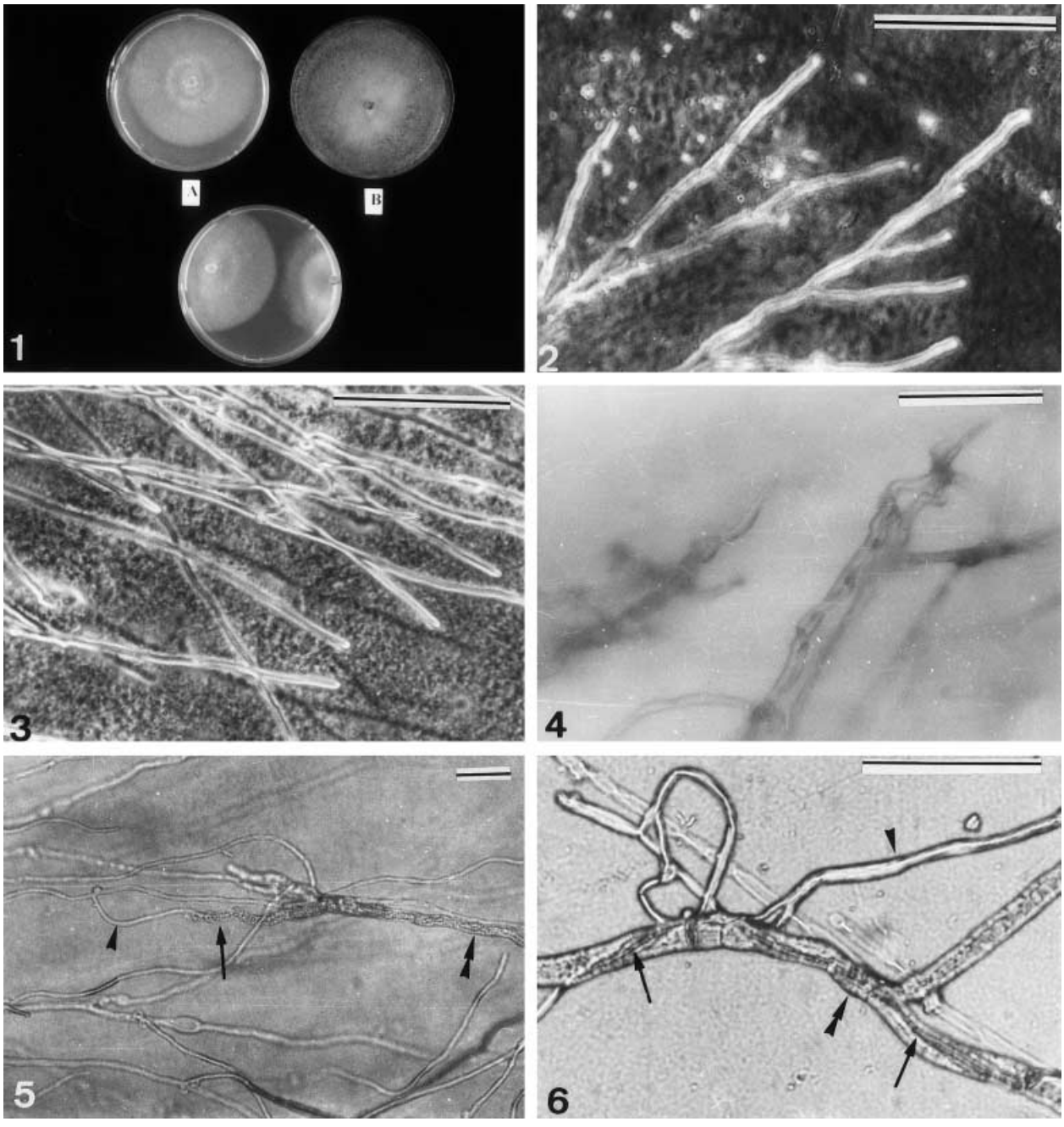


Fig. 1 Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture (*lower row*). Control cultures of *L. laccata* (**A**) and *T. virens* (**B**) (*upper row*)

Figs. 2–6 Light photomicrographs of the interactions between *L. laccata* and *T. virens* in the inhibition and contact zones in co-culture

Fig. 2 Abnormal, frequently branched hyphae of *T. virens* in the inhibition zone

Fig. 3 Hyphae of *L. laccata* in the zone of inhibition

Fig. 4 Mutual coiling of hyphae of the two fungi in the contact zone

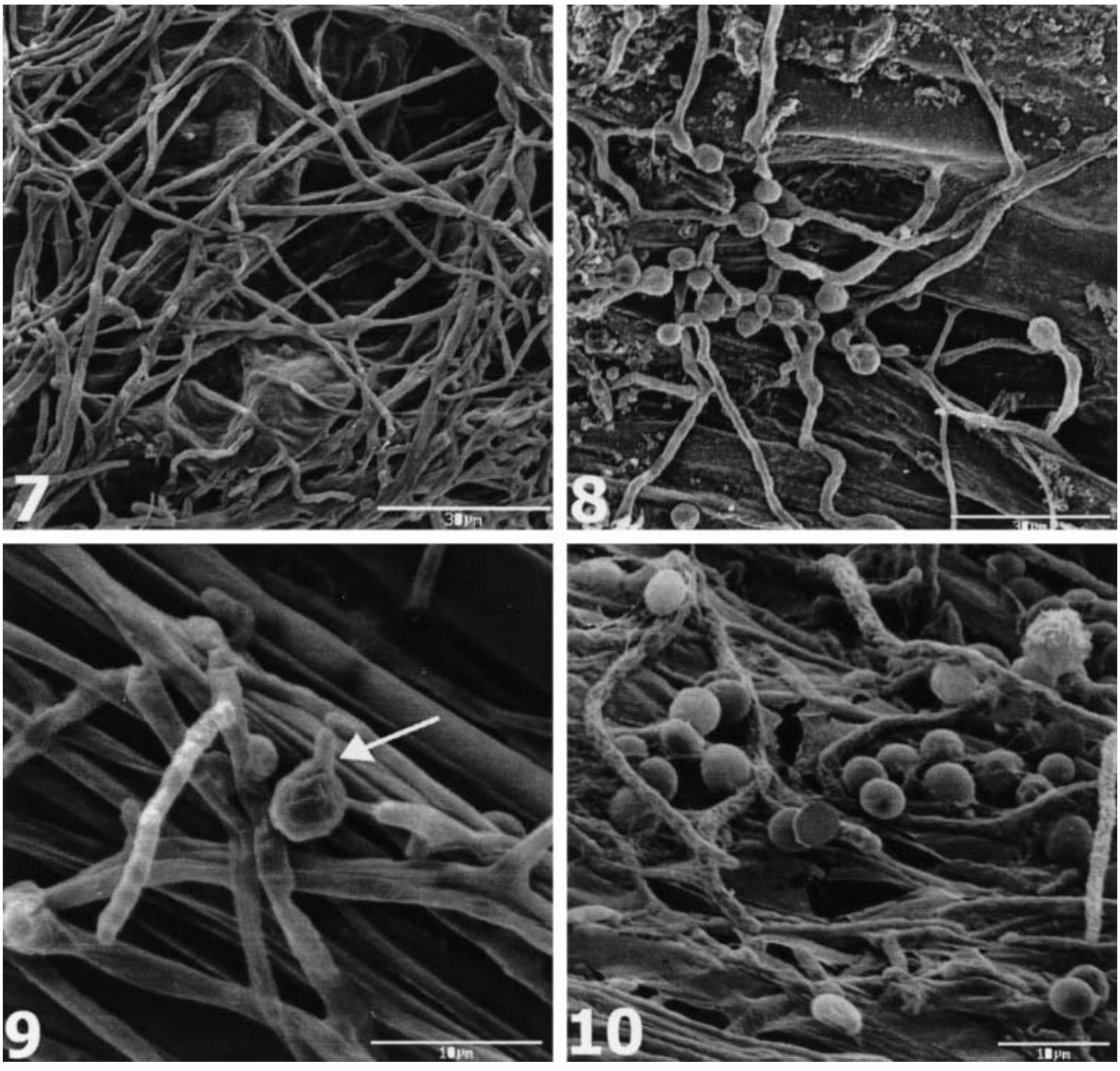
Fig. 5 Attachment of *L. laccata* hypha (*arrowhead*) to *T. virens* hypha (*double arrowhead*). A released, granular protoplast of *T. virens* is visible around hypha of *L. laccata* (*arrow*)

Fig. 6 Hypha of *L. laccata* (*arrowhead*) attached to a hypha of *T. virens* (*double arrowhead*). Note a thin hypha of *L. laccata* growing intracellularly (*arrows*); *bars* 50 µm

Results

Interactions in co-culture

In co-culture on Pp medium at pH 5.5, the outcome of the mycelial interaction may be categorized as contact inhibition or inhibition at a distance, with sparse hyphae of the two fungi interacting in the zone of inhibition. Although no changes in gross morphology of the fungal colonies were observed, *L. laccata* obviously negatively influenced the growth of *T. virens* mycelium (Fig. 1). In the zone of inhibition, the hyphae of *T. virens* were shortened and more branched (Fig. 2) or deformed. By contrast, the hyphae of *L. laccata* were unchanged



Figs. 7–14 Scanning electron micrographs of the interaction between *L. laccata* and *T. virens* in the rhizosphere of *Pinus sylvestris* seedlings

Fig. 7 Mantle hyphae of *L. laccata* in a patch of root poorly colonized by the fungus, 2 months after inoculation

Fig. 8 Germinated conidia and long hyphae of *T. virens* on the surface of a non-mycorrhizal root 3 days after inoculation

Fig. 9 A germinated conidium of *T. virens* with a short germ tube (*arrow*) on the mantle surface 2 days after inoculation

Fig. 10 Mantle hyphae of *L. laccata* growing circuitously around conidia of *T. virens* scattered on the mantle surface 2 days after inoculation

(Fig. 3.). Sporadic mutual coiling of hyphae was observed (Fig. 4), as well as breaks in walls of hyphae of *T. virens* with consequent protoplast release (Fig. 5). Moreover, some observations suggested penetration of *T. virens* hyphae by *L. laccata* (Fig. 6).

Mycoparasitism in the rhizosphere of pine seedlings

The mantles of 2-month-old mycorrhiza were white in color and consisted of at least two layers of hyphae. Clamp connections and anastomoses were evident. Mantles were thicker in the subapical region of roots. The surface mantle hyphae were still loosely organized. Embedding of the hyphae in mucilage resulted in a more compact mantle. In patches of roots that were less colonized, the hyphae were loosely organized but tightly adhered to the root surface. Frequent branches formed a network with scarce and thin hyphal strands (Fig. 7).

By 48 h p.i., *T. virens* conidia had germinated frequently on non-mycorrhizal pine roots. Long hyphae were observed on the root surfaces at 3 days p.i. (Fig. 8). In contrast, the conidia had not germinated on mycorrhizal roots by this time or had produced only sporadic short germ tubes (Fig. 9). The mantle hyphae of *L. laccata* grew circuitously around the conidia that were scattered on the mantle surface (Fig. 10). Dense coiling

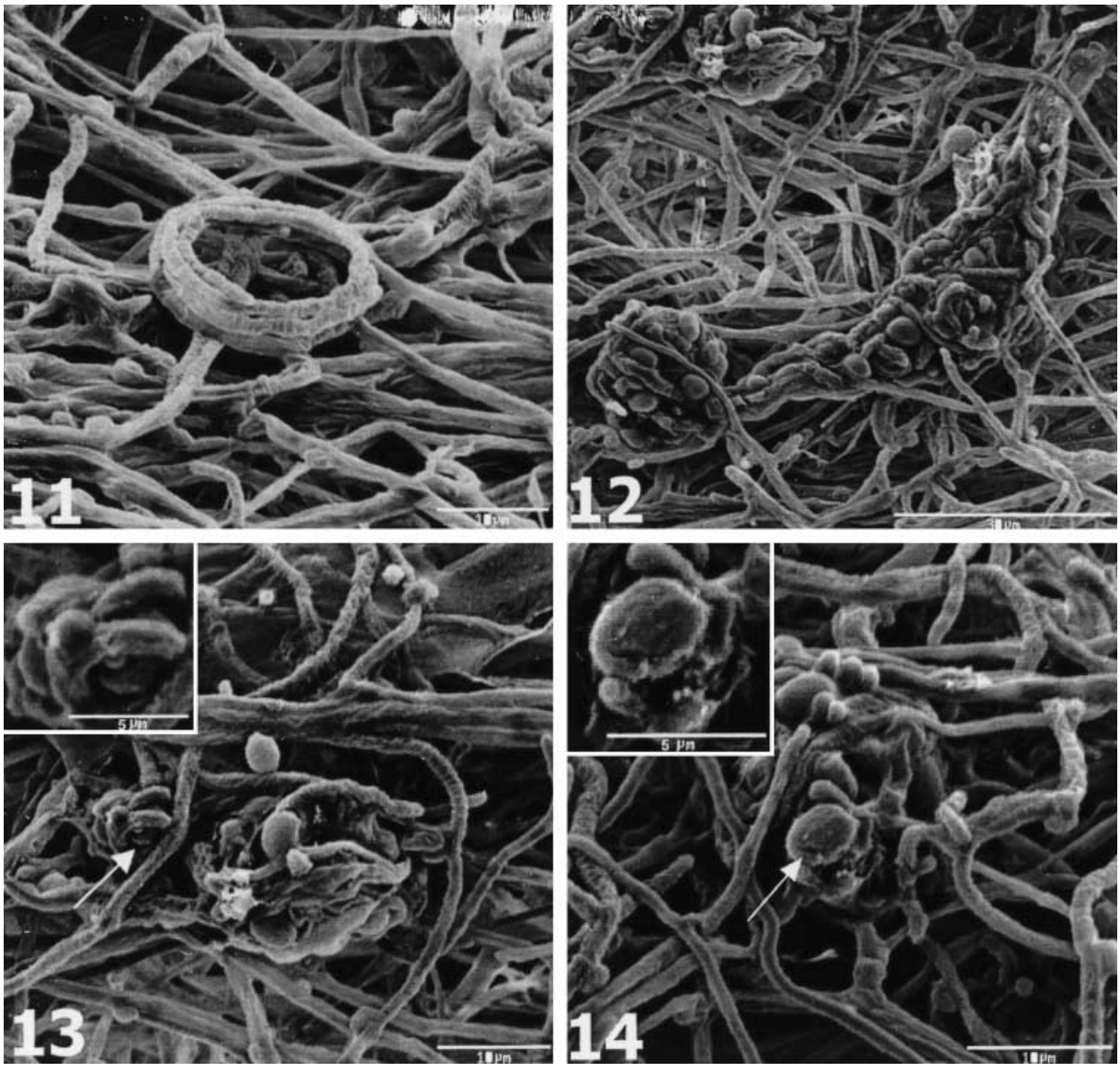


Fig. 11 A basket-like structure formed by the mantle hyphae of *L. laccata* 3 days after inoculation with conidia of *T. virens*

Fig. 12 Conidia concentrated into clusters and overgrown by the mantle hyphae 6 days after inoculation

Fig. 13 Collapsed and deformed conidia positioned inside a cluster. *Inset*: a conidium enveloped by short branches of a mantle hypha 6 days after inoculation

Fig. 14 Branched mantle hyphae coiling around deformed conidia. *Inset*: a crack in a conidial wall 6 days after inoculation

around them was observed as the process continued. In several cases, the mantle hyphae formed basket-like structures (Fig. 11). Unfortunately, they were usually empty, most probably due to application of a vacuum during the SEM fixation procedure. At 6 days p.i., the encirclement of individual conidia or groups resulted in their concentration into clusters (Fig. 12). The main mantle hyphae coiled around the clusters. Some hyphae formed short branches that tightly surrounded or "handcuffed" the particular conidia (Fig. 13). Most of the conidia inside the clusters were deformed. Some of those coiled heavily by short hyphae had totally collapsed or had small cracks in their walls (Fig. 14).

Growth of Scots pine seedlings in vitro

The influence of *L. laccata* and *T. virens* on the dry weight growth of pine seedlings varied significantly ($P<0.0001$). Both mycorrhizal plants and mycorrhizal plants inoculated with *T. virens* produced a significantly higher biomass of needles, trunks and roots and total biomass than non-mycorrhizal plants. However, there was no difference in the growth of mycorrhizal plants inoculated or uninoculated with *T. virens*. There was also no significant difference in the dry weight growth of non-mycorrhizal plants grown in the presence of *T. virens* and that of the control plants (Fig. 15).

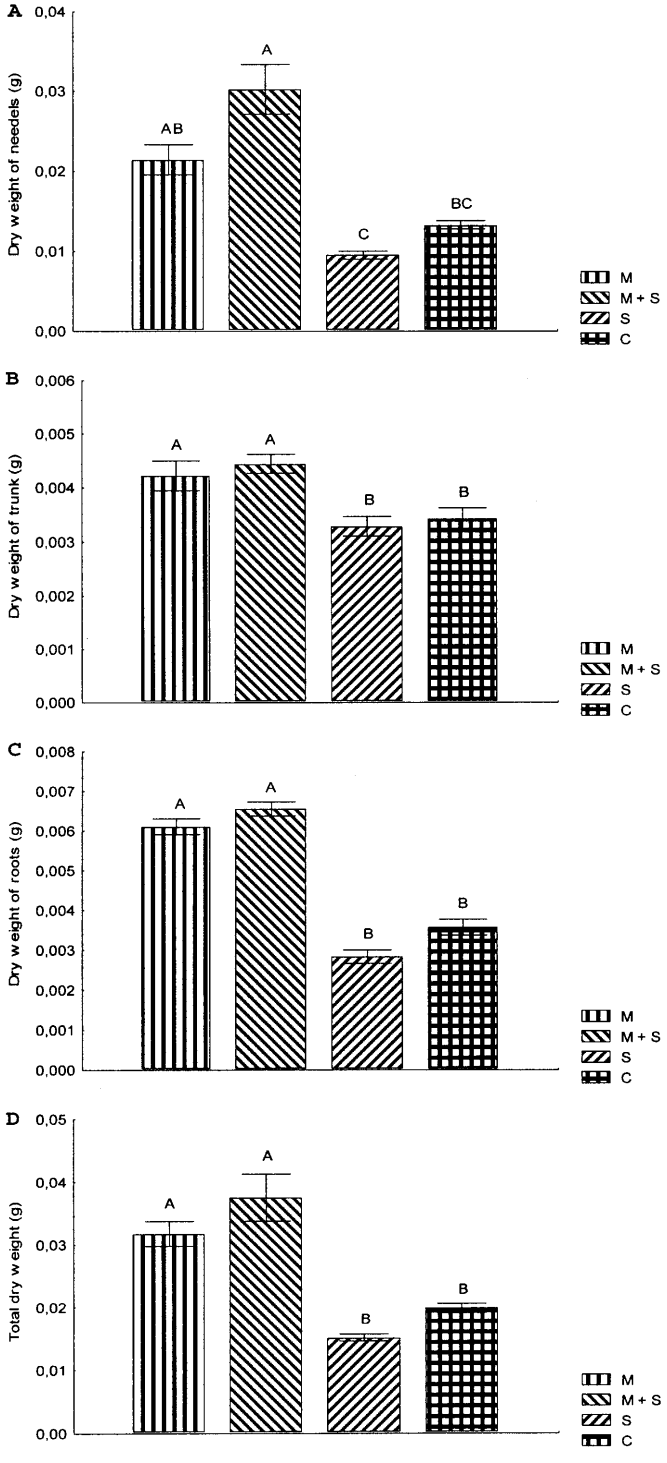


Fig. 15 Dry weight of needles (**A**), trunks (**B**), roots (**C**), and the total dry weight of Scots pine seedlings (**D**). Data are the means for each treatment. Means designated by the same letter do not differ significantly at the 5% level according to Tukey's HSD test; *bars* SE (n=20) (*C* control, *M* mycorrhizal, *M+S* mycorrhizal + *Trichoderma virens*, *S T. virens*)

Discussion

Few data exist on the inhibition of mycorrhiza formation by soil fungi in vivo. In pot experiments, different effects of *Trichoderma* species on the formation of mycorrhiza by two *Suillus* species were observed by Shemakhanova (1962). Sinclair et al. (1982) observed only a slight repression of *L. laccata* mycorrhiza formation on Douglas-fir by *Trichoderma*. According to Moser (1963), there was an adverse effect on mycorrhiza in nursery soils colonized by species of *Trichoderma* and *Botrytis*. By contrast, soil microfungi, particularly from the genera *Penicillium* and *Trichoderma*, inhibited formation of mycorrhiza in vitro (Vaartaja and Salisbury 1965), but specifically in experiments where all the studied fungi were inoculated together or where the soil fungi were added before establishment of mycorrhiza (Summerbell 1987).

In the present study, further development of *T. virens* was repressed when *L. laccata* was established in the root system. The success of *L. laccata* in inhibiting both the growth of hyphae of *T. virens* in co-culture and germination of its conidia in the rhizosphere may be related to the antibiotic activity of mycorrhizal fungus. However, the observed mycoparasitic activity of mantle hyphae on conidia of *T. virens* has not been reported.

Since mycorrhizal fungi require relatively rich artificial media that can modulate their reactions (Salzer et al. 1996) and, moreover, suppress the mycoparasitism of other fungi in vitro (Griffith and Barnett 1967), our results may differ from those in a soil environment. In artificial conditions, *L. laccata* may have used conidia of *T. virens* as a source of nutrients. In order to test this hypothesis, the dry weights of needles, trunks, roots and total biomass were measured for mycorrhizal plants uninoculated and inoculated with *T. virens*. All growth parameters were positively influenced by inoculation with *L. laccata* alone, with an apparent though statistically not significant further increase after additional inoculation with *T. virens*. Since the antagonism of *L. laccata* observed in the laboratory experiments may or may not be attenuated in natural habitats due to interactions with the another members of the microbial communities, the evidence for the antagonistic ability of the mycorrhizal fungus towards *Trichoderma* spp. must be confirmed in a non sterile environment. In co-culture, the strain of *L. laccata* studied was the most effective in limiting the growth of 23 isolates of other soil fungi common in agricultural soils (Zadworny 2001). Moreover, it easily formed mycorrhiza with Scots pine seedlings in a pot greenhouse experiment in which three agricultural soils and soils from two fallow areas were used as growth substrates (Werner and Zadworny 2001a, b).

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