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SHORT NOTE

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Behaviour of the hyphae of *Laccaria laccata* in the presence of *Trichoderma harzianum* in vitro

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Abstract The growth rate and the behaviour of *Laccaria laccata* and *Trichoderma harzianum* hyphae in co-culture and in the rhizosphere of 3-month-old *Pinus sylvestris* seedlings grown in vitro were investigated. In the interaction zone, hyphae of *L. laccata* became more pigmented and formed short branches growing towards the hyphae of the saprobic fungus, coiled around them and penetrated sporadically. Vacuolated hyphae of *T. harzianum* showed protoplasm granulation and breaks in walls followed by release of protoplasts. In the rhizosphere, the mantle hyphae of *L. laccata* showed a tendency to surround conidia of *T. harzianum*. No obvious penetration of the conidial walls by the hyphae of the mycorrhizal fungus was observed by scanning electron microscopy. Instead, in rare cases, the hyphae of *L. laccata* showed marked wrinkles, and a partial degradation of a mucilaginous material covering the mantle appeared to occur.

Keywords Co-culture · *Laccaria laccata* · Mycoparasitism · Rhizosphere · *Trichoderma harzianum*

Introduction

Very few data exist concerning mycoparasitism by ectomycorrhizal (ECM) fungi of soil saprobes. In previous studies (Werner et al. 2002; Werner and Zadworny 2003), we reported inhibition of germination of *Trichoderma virens* (Mill. Gidd. et Foster) von Arx conidia and spores of *Mucor hiemalis* (Wehmer) in the rhizosphere of *Pinus sylvestris* inoculated with *Laccaria laccata* (Scop. ex Fr.), and coiling around hyphae and spores of both saprobic fungi by mantle hyphae of the ECM fungus, which occasionally caused breaks in their walls.

Among fungi antagonistic to a wide range of root pathogens, species of *Trichoderma*, particularly *T. harzianum* (Rifai), have received most attention (Elad 2000; Whipps and Lumsden 2001). Nevertheless, little is yet known of their interaction with ECM fungi, and the mycoparasitic capabilities of *T. harzianum* in the rhizosphere have seldom been demonstrated (Lo et al. 1998). The purpose of this paper was to investigate interaction between *L. laccata* and *T. harzianum* in co-culture and in the rhizosphere of *P. sylvestris* seedlings grown in vitro.

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Materials and methods

Organisms and growth conditions

The source of *L. laccata* (Scop. ex Fr.) (strain 9-1) was 1-month-old mycelium grown on Pachlewski agar medium (Pachlewski 1983) at 24°C in the dark. *Trichoderma harzianum* (Rifai) (strain Mn 75), originating from an abandoned farmland soil at Mieczewo near Poznań in Poland was maintained on potato dextrose agar (Difco) at 24°C in the dark. *P. sylvestris* L. seedlings from the province of Bolewice (52°28N, 16°03E) were used.

Fungal interaction in co-culture

The fungal growth rate was assessed in 13.5 cm diameter Petri dishes containing 30 ml Pachlewski agar medium (Pachlewski 1983). The dishes were inoculated with discs (5 mm in diameter) of 1-month-old mycelial mat of *L. laccata* subcultured from stock culture and maintained on the same medium at 5°C. One week later, discs of *T. harzianum* mycelium of the same size were placed at the

opposite side of the plates at a distance of 5 cm. The fungi were also grown individually as controls. The cultures were incubated at 24°C in the dark. During 1 week, the mycelial growth was checked once a day and the growth rate of each fungus was calculated as described by Eckstein and Liese (1970). The experiment was repeated three times. Each replication consisted of ten plates. One way analysis of variance (ANOVA), based on individual data and comparisons of mean values using Tukey's HSD test at significance level $P<0.05$ were performed using the statistical analysis software Statistca PL 1997 (StatSoft Polska, Cary, N.C.). The morphology of the hyphae in the interaction zone was observed using light- and phase-contrast-microscopy (Axioskop; Zeiss, Jena, Germany).

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

Preparation of *L. laccata* inoculum, inoculation procedure and the growth conditions of *P. sylvestris* seedlings were as described by Werner et al. (2002).

Fig. 2 Light photomicrographs of the interaction between *L. laccata* and *T. harzianum* in co-culture (**A**–**E**) and scanning electron photomicrographs in the rhizosphere of *Pinus sylvestris* seedlings (**F**–**G**). **A** Vacuolated hypha of *T. harzianum* (*double arrowhead*) coiled by hypha of *L. laccata* (*arrowhead*) in the interaction contact zone. **B** Hyphae of *T. harzianum* in the interaction zone showing granular protoplasm and released protoplast (*arrow*). **C** Morphologically altered hyphae of *T. harzianum* (*double arrowhead*) in vicinity of *L. laccata* hyphae (*arrowhead*). *Inset* Higher magnification of hypha showing swollen short branches. **D** Hyphae of *L. laccata* (*arrowheads*) growing close to *T. harzianum* hyphae (*double arrowhead*). Note short branches of *L. laccata* (*arrow*) attached to hypha of *T. harzianum*. **E** Hypha of *L. laccata* firmly attached (*arrowhead*) to hypha of *T. harzianum* (*double arrowhead*). Note another hypha of *L. laccata* (*arrow*) growing intracellularly in thick hypha of *T. harzianum*. **F** Short branch of *L. laccata* mantle hypha attached to an individual conidium (*arrowhead*). Note degraded mucilaginous material (*) covering the mantle 3 days after inoculation and the presence of a clamp connection (*double arrowhead*). **G** Ramified short mantle hypha (*arrowhead*) showing a tendency to coil around *T. harzianum* conidium (*arrow*) 4 days after inoculation. *Bars* **A**, **B**, **D**, **E** 10 μm; **C** 50 μm (inset 5 μm); **F**, **G** 3 μm

Conidial suspension of *T. harzianum* in sterile distilled water was obtained by scraping the surface of 2-week-old cultures using glass beads. Mycorrhizal roots were

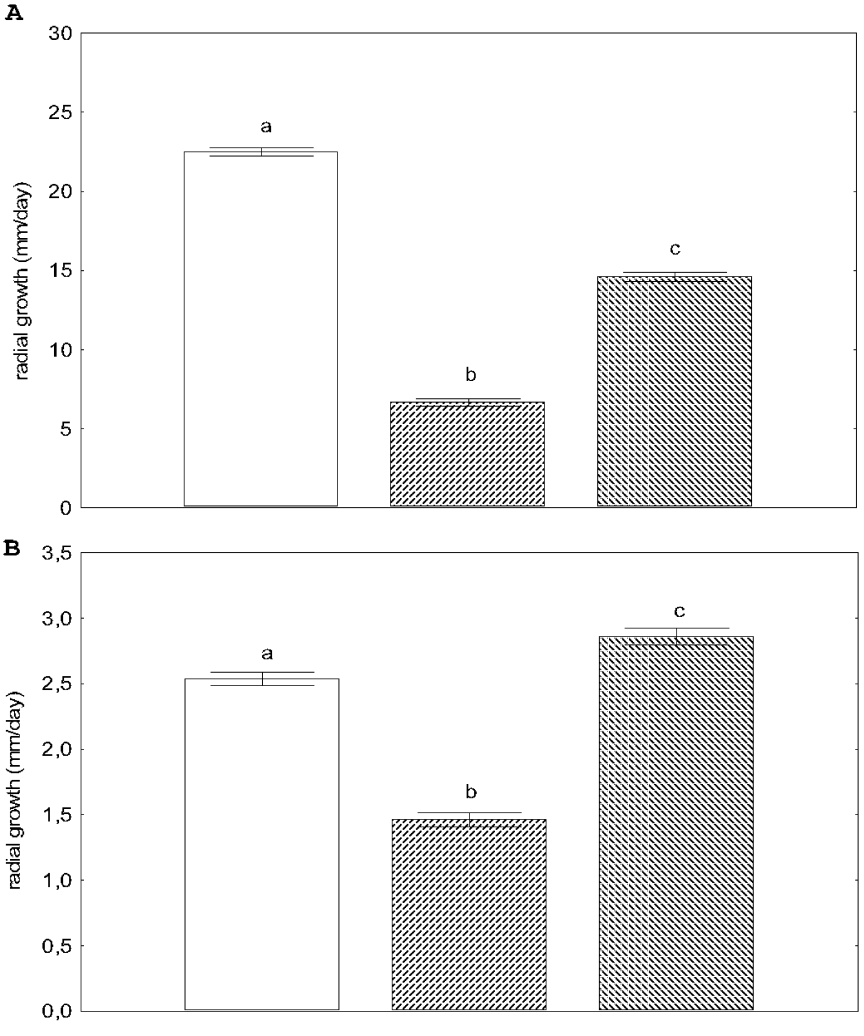
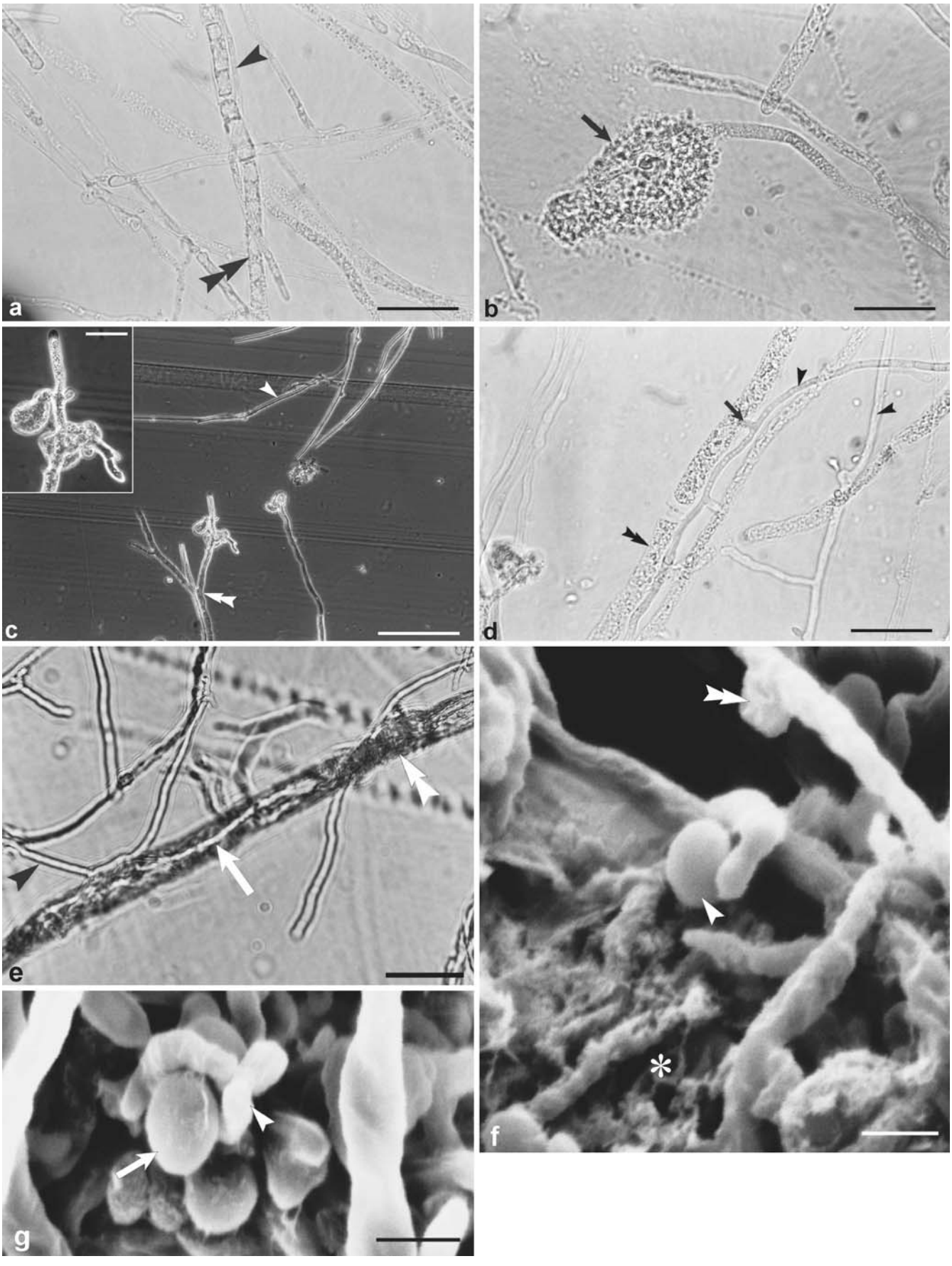


Fig. 1 Mean radial growth of *Trichoderma harzianum* (**A**) and *Laccaria laccata* (**B**) and in co-culture and in control plates. *Open bars* Radial growth in control, *upward hatched bars* growth towards the interacting fungus, *downward hatched bars* growth away from the interacting fungus. Means associated with different letters differ significantly at the 5% level using Tukey's HSD test. *Bars* Standard errors (n=30)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inoculated with a concentration of 3.45×10^6 conidia ml^{-1}. The control materials were nonmycorrhizal seedlings inoculated with the same concentration of *T. harzianum* conidia and mycorrhizal roots not inoculated with *T. harzianum*.

For scanning electron microscopy (SEM), 20 roots of 10 3-month-old mycorrhizal plants were selected at intervals of 3, 4, 6 and 8 days after inoculation with *T. harzianum*. Ten inoculated nonmycorrhizal roots, 5 nonmycorrhizal and a similar number of mycorrhizal plants were used as controls. The experiment was repeated three times. All samples were processed for SEM as described by Werner et al. (2002). The presence of clamp connections on the hyphae of *L. laccata* was the criterion used to distinguish between hyphae of the two fungi. Moreover, the hyphae of the latter are thinner and more ramified than those of *T. harzianum*.

Results

Interaction in co-culture

Expansion of mycelia of both colonies was slower in the direction of the other colony than it was when they were grown individually. In contrast to the lower growth rate of *T. harzianum* away from *L. laccata* (Fig. 1A), the growth rate of the latter fungus away from the former was significantly higher than in the control plates (Fig. 1B). Invasion and partial overgrowth of the *L. laccata* colony by *T. harzianum* and formation of a dense front of yellow-brown mycelium by *L. laccata* at the contact zone characterised the mycelial interaction.

In the interaction zone, some vacuolated hyphae of *T. harzianum* were coiled by hyphae of the mycorrhizal fungus (Fig. 2A). Moreover, they showed protoplasm granulation, breaks in walls followed by release of protoplasts (Fig. 2B), and swelling of short branches in the vicinity of *L. laccata* hyphae (Fig. 2C). Elongated hyphae of *L. laccata* produced short branches. Some of these obviously grew towards *T. harzianum* hyphae (Fig. 2D); however, their growth inside *T. harzianum* hyphae was observed only sporadically (Fig. 2E).

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

Three days after inoculation of nonmycorrhizal roots with *T. harzianum* conidia, there was an abundance of hyphae growing actively on the root surface (not shown). In contrast, germination of conidia on mycorrhizal roots was only seldom observed. The long mantle hyphae formed short branches, which attached to individual, ungerminated conidia (Fig. 2F). Four days after inoculation, the coiling of *L. laccata* hyphae around the conidia was observed frequently, but no breaks were seen in the conidial walls (Fig. 2G). At this stage of the interaction, the mucilaginous material covering the mantle had a granular, degraded appearance (Fig. 2F). Some wrinkled hyphae of *L. laccata* were occasionally observed (Fig. 2F). Such an alteration was never observed in the morphology of the short mantle hyphae attached to *T. harzianum* conidia (Figs. 2G) or in mantle hyphae of control roots. Similar observations were made 6 and 8 days after inoculation.

Discussion

Trichoderma spp. vary in their inhibitory effects towards other fungi (Hadar et al. 1979). The isolate of *T. harzianum* investigated here demonstrated antagonism towards *L. laccata*, resulting in overgrowing of the ECM fungal colony where its mycelium became more dense and pigmented. SEM micrographs also indicated some wrinkled or collapsed mantle hyphae of *L. laccata* on pine roots. These reactions did not appear when the mycorrhizal fungus was challenged with *T. virens* (Werner at al. 2002) or with *M. hiemalis* (Werner and Zadworny 2003). Induced pigment production in co-cultured fungi was described as a result of nutritional stress imposed by a more aggressive organism (Calistru et al. 1997; Murphy and Mitchell 2001), while collapse and loss of turgor of hyphae may suggest the influence of extracellular enzymes and/or volatiles produced by the aggressor (Calistru et al. 1997). Therefore, either nutritional stress or extracellular metabolites of *T. harzianum* could have caused the observed modification in *L. laccata*. Bursting of *T. harzianum* hyphae followed by protoplast release and their penetration may suggest an action of cell wall degrading enzymes. Although the parasitic ability of a number of isolates of *Trichoderma* spp. has been shown (Benhamou and Chet 1993; Elad et al. 1983; Doi et al. 1994), the present SEM investigation failed to indicate any parasitic activity of *T. harzianum* to *L. laccata*.

In the rhizosphere of *P. sylvestris* grown in vitro, *L. laccata* caused some cracks in the conidial walls of *T. virens* (Werner et al. 2002), while in the present study, mantle hyphae established close contact with conidia of *T. harzianum* within 3 days after inoculation but no alterations to the conidial walls were observed. Also, hyphal coiling by *L. laccata* around conidia of *T. harzianum* was never as dense as in the case of *T. virens*, whilst germination of *T. harzianum* conidia and formation of germ tubes were more often observed.

ECM fungi, which usually form dense mantles and forage large areas of soil, can greatly affect the amount of nutrients accessible for other microorganisms (Gadgil and Gadgil 1971, 1975; Pérez-Moreno and Read 2000). Since a large amount of nitrogen is necessary for germination of *Trichoderma* spp. conidia (Domsch et al. 1980), the inhibitory effect of the mantle hyphae of *L. laccata* on germination of *T. harzianum* conidia may be related to competition for resources that were in short supply for both fungi. Though the outcome of the mycelial interactions in vitro suggests different parasitic abilities of *L. laccata* towards soil fungi, to assess the contribution of

mycoparasitism of ECM fungi in nutrition of trees in environments poor in N and P, further studies on nutrient flux assessment to mycorrhizal mycelium are required.

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