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Effects of exogenous diamines on the interaction between ectomycorrhizal fungi and adventitious root formation in Scots pine in vitro

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Summary Production of free and conjugated polyamines by two ectomycorrhizal fungi, *Pisolithus tinctorius* (Pers.) Coker and Couch and *Paxillus involutus* (Batsch) Fr., was studied in vitro. Spermidine was the main polyamine in the mycelium of both fungi. *Paxillus involutus* also produced large amounts of the diamine putrescine, whereas *Pisolithus tinctorius* contained traces of the diamine cadaverine and released into the culture medium an unknown compound probably related to cadaverine or *N*-methylputrescine. Both fungi accelerated adventitious root formation and increased subsequent root growth of Scots pine (*Pinus sylvestris* L.) hypocotyl cuttings in vitro. Exogenous cadaverine enhanced rooting caused by *Pisolithus tinctorius* and also promoted mycorrhiza formation by the fungus. Putrescine and *Paxillus involutus* had a synergistic effect on root initiation, but not on subsequent root growth. We conclude that specific diamines may be involved in the interaction between ectomycorrhizal fungi and adventitious root formation in Scots pine, and that the effects of specific exogenous polyamines are dependent on the fungal strain and its ability to produce these compounds. The finding that *Paxillus involutus* enhanced rooting and root growth without mycorrhiza formation indicates that fungal-induced rooting is not necessarily related to visible mycorrhiza formation.

Keywords: cadaverine, Paxillus involutus, Pinus sylvestris, Pisolithus tinctorius, putrescine.

Introduction

A mycorrhiza is a symbiotic association between a fungus and the roots of a plant, in which the fungal partner provides nutrients and water to the host plant in exchange for photosynthates (Smith and Read 1997). During mycorrhiza formation, both symbiotic partners undergo several morphological changes. The mycelium starts to branch intensively and hyphal tips may fuse (Jacobs et al. 1989). In plant roots, root hair proliferation is inhibited and lateral root formation may be activated (Smith and Read 1997). For example, dichotomous branching of mycorrhizal short roots is characteristic of pine (*Pinus*) species (Duddridge and Read 1984*a*, 1984*b*, Smith and Read 1997).

Because mycorrhizal fungi have an important role in root growth, it might be possible to use them to promote formation of adventitious roots on difficult-to-root woody plants. Specific symbiotic fungi have been studied as rooting agents both in vitro and in vivo. Depending on the study, inoculation has resulted in higher rooting frequency or a greater number of adventitious and lateral roots or both (Linderman and Call 1977, Gay 1990, Supriyanto and Rohr 1994, Normand et al. 1996, Fortuna et al. 1998, Karabaghli et al. 1998, Niemi et al. 2000).

Fungal indole-3-acetic acid (IAA) is related to root enhancement in vitro (Gay 1990, Normand et al. 1996, Karabaghli et al. 1998). However, high fungal IAA production does not necessarily lead to the highest rooting frequency. In micropropagated shoots and hypocotyl cuttings of *Pinus pinaster* (Ait.) Sol., for example, the continuous release of small amounts of fungal IAA proved sufficient for root formation (Normand et al. 1996). In open culture systems with cuttings (Niemi et al. 2000) and intact seedlings (Nylund et al. 1994, Scagel and Linderman 1998*a*, 1998*b*), the role of fungal IAA has also proved complicated, indicating that root promotion by fungi is highly dependent on culture conditions and the characteristics of the fungal species or strains.

The polyamines, spermidine and spermine, and their precursor diamine putrescine, are widespread in living organisms and have been implicated in the regulation of growth and morphogenesis (reviewed by Galston and Flores 1991, Walters 2000, Tiburcio et al. 1997). In plants, fruit and root formation are examples of morphogenetic processes during which the concentrations of some polyamines change considerably (reviewed by Egea-Cortines and Mizrahi 1991, Galston and Flores 1991, Kevers et al. 1997). Polyamines are also involved in mycorrhizal symbiosis (El Ghachtouli et al. 1995, Kytöviita and Sarjala 1997, Fornalé et al. 1999, Sarjala 1999, Walters 2000). The formation of mycorrhizae can result in changes in both total polyamine concentration and in the ratios between individual polyamines in plants (Kytöviita and Sarjala 1997,

Goicoechea et al. 1998). Furthermore, a supply of exogenous polyamines may increase mycorrhiza frequency (El Ghachtouli et al. 1995).

To our knowledge, the involvement of polyamines in the interaction between an ectomycorrhizal fungus and a plant producing adventitious roots has not been studied. We investigated in vitro production of polyamines by two ectomycorrhizal fungi, *Pisolithus tinctorius* (Pers.) Coker and Couch and *Paxillus involutus* (Batsch) Fr. We also studied the effects of exogenous diamines produced by the fungi on the interaction between Scots pine (*Pinus sylvestris* L.) hypocotyl cuttings and the fungi during rooting. Rooting was carried out both with and without a seedling to determine whether mycorrhiza formation by a seedling affected rooting of the cutting.

Materials and methods

Biological materials

The ectomycorrhizal fungi of Scots pine used were *Pisolithus tinctorius*, originally obtained from the culture collection of the Swedish Agricultural University, Uppsala, Sweden (Strain 1984a), and *Paxillus involutus* (Strain H), which was kindly provided by Prof. V. Hintikka (University of Helsinki, Finland). These fungi were chosen because they differ in their ability to produce IAA (Niemi et al. 2000) and polyamines (data not shown). Both fungi were successfully maintained in the culture collection of the University of Kuopio, Finland by cultivating the mycelia on Melin-Norkrans (MMN1) agar medium (Marx 1969) according to modifications by Heinonen-Tanski and Holopainen (1991).

Open-pollinated Scots pine seeds originating from Konginkangas in central Finland (63° N, 26° E) were surface-sterilized for 20 min with 2% calcium hyphochlorite, rinsed in sterile water and germinated on 0.7% water agar in glass jars. The germinating seeds were incubated in a growth chamber at 22 ± 1 °C providing a 16-h photoperiod (130–140 μmol m^{-2} s^{-1}, cool white lights F25W/30"/133-T8, Sylvania, Germany). Hypocotyl cuttings for the rooting experiments were prepared from 17-day-old seedlings by cutting the stem about 5 mm above the root collar. The seedlings were germinated as described for the hypocotyl cuttings.

Radial growth of fungi on medium containing cadaverine and putrescine

A mycelial plug, 5 mm in diameter, was cut from the edge of a 1-month-old fungal colony and placed in the middle of a plate of MMN2 agar medium containing 3.7 mM KH_2PO_4, 1.9 mM $(NH_4)_2HPO_4$, 0.45 mM $CaCl_2$, 0.43 mM NaCl, 0.61 mM $MgSO_4 \cdot 7H_2O$, 0.2 μM thiamine HCl, 18.4 μM $FeCl_3 \cdot 6H_2O$ and 41.6 mM glucose, pH 5.7 (Marx 1969). The medium was supplemented with 0.1 or 0.5 mM diamine cadaverine (dihydrochloride, Sigma) or diamine putrescine (dihydrochloride, Sigma). Diamines as the sole nitrogen (N) source were studied by replacing NH_4-N in the medium with the same amount of N in the form of cadaverine or putrescine. Diamine solutions were filter-sterilized and added to the autoclaved medium. The fungi were cultivated for 3 weeks in the dark at 21 ± 1 °C, and growth of the mycelium from the edge of the agar plug was determined weekly. Each treatment contained four replicates.

Rooting of hypocotyl cuttings in the absence of a seedling

Petri dishes, 14 cm in diameter, contained about 65 ml of MMN2 medium (pH 5.7), which was supplemented with 0.5 mM cadaverine or putrescine and 1.1 mM glucose. The concentration of glucose was reduced to 1.1 mM to induce interaction between the plant and the fungus. The medium was solidified with 2.0% agar. The surface of the agar was covered with a sterile moist filter paper. Individual cuttings were placed horizontally on the filter paper and inoculated with two mycelial plugs (Figure 1a), which were 5 mm in diameter and cut from the edge of 4-week-old cultures of *Pisolithus tinctorius* or *Paxillus involutus*. One plug was placed close to the base of the hypocotyl and the other plug was placed about 1 cm below the base. In the control cultures, agar plugs were substituted for mycelial plugs. To prevent desiccation, the fungus and the base of the cutting were covered by a semicircular piece of moist filter paper (Figure 1b). The fungus and the base of the cutting were protected from direct illumination by a

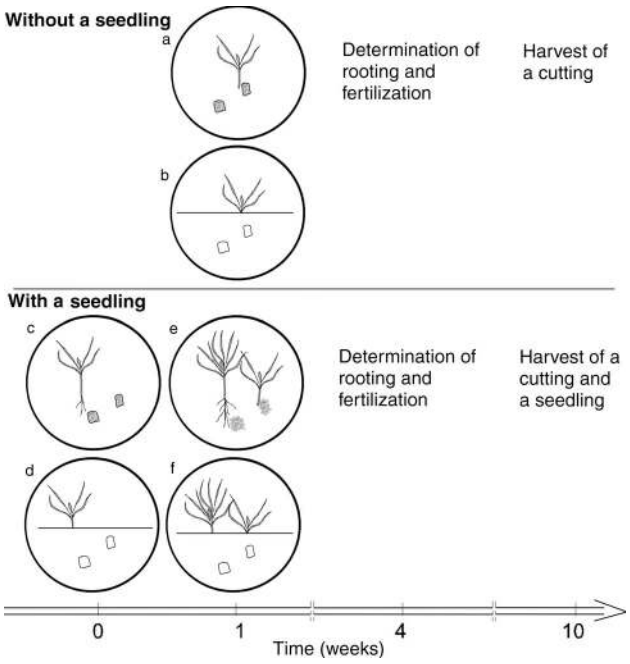

Figure 1. Rooting of Scots pine hypocotyl cuttings in the absence (a and b) and presence of a seedling (c–f) on MMN2 rooting medium covered by a moist filter paper. The hypocotyl cutting grown alone was inoculated with two mycelial plugs of *Pisolithus tinctorius* or *Paxillus involutus* (a) and the base of the cutting and the fungus were covered by a semicircular piece of moist filter paper (b). When the cutting was rooted with the seedling, the seedling was first inoculated with the fungus (c) and the root system and the fungus were covered by a semicircular piece of moist filter paper (d). One week later, the cutting was transferred to the agar next to the seedling and the mycelial plugs (e and f). The size of the cutting and seedling in relation to that of the petri dish is not to scale.

semicircle of brown paper placed on the lid of the petri dish. The petri dishes were slanted at 70° and incubated in the growth chamber under the same conditions as described for germination. There were nine to 12 replicates per treatment, and each treatment was repeated twice.

Rooting of hypocotyl cuttings in the presence of a seedling

A 17-day-old seedling was transferred from the germination medium and placed horizontally on MMN2 medium containing 0.1 or 0.5 mM cadaverine or putrescine and 1.1 mM glucose, covered by a moist filter paper and inoculated with two mycelial plugs of *Pisolithus tinctorius* or *Paxillus involutus* (Figure 1c). The root system and the mycelial plugs were covered by a semicircular piece of moist filter paper (Figure 1d). After 1 week, a 17-day-old cutting was placed next to the seedling so that the base of the cutting was about 1.5 cm away from the root collar of the seedling and at the same distance from the mycelial plugs as in the experiment without the seedling (Figures 1e and 1f). At this time the fungi showed only slight growth (Figure 1e). Rooting was carried out as in the absence of the seedling. There were nine to 12 replicates per treatment, and each treatment was repeated two or three times.

Measurements

The number of rooted hypocotyl cuttings was recorded 4 and 9 weeks after the hypocotyls were placed on the medium (Figure 1). Concomitant with the first determination of rooting, the cultures were fertilized by pouring 3 ml of liquid MMN2 medium without glucose over the root system. The cuttings and seedlings were harvested 9 and 10 weeks after being placed on the medium, respectively. We recorded the number of rooted cuttings and the number of adventitious roots per cutting. The length and fresh weight of the roots and the number of lateral roots, as well as shoot fresh weights, were also determined for both cuttings and seedlings. The number of mycorrhizal root tips was evaluated with the aid of a dissecting microscope and mycorrhizal structures were assessed with a scanning electron microscope.

Polyamine analyses of fungi and Scots pine needles

For polyamine analyses, the fungi were cultivated in 40 ml of MMN2 liquid medium containing 41.6 mM glucose (Marx 1969). Two mycelial plugs, each 5 mm in diameter, were cut from the margin of a 1-month-old fungal colony, placed in the medium and cultivated in the dark at 21 ± 1 °C. Three weeks later, the mycelium and culture medium were separated by filtration. The mycelium was washed with distilled water, carefully dried between filter paper at room temperature and then weighed. The culture filtrate was freeze-dried. Both the mycelia and culture filtrates were stored at –80 °C until analyzed.

At the end of the 9-week rooting period, free polyamines were analyzed in needles of cuttings that had rooted in the presence of a seedling. Fresh needle samples were weighed and stored at –80 °C until analyzed.

Both free and conjugated polyamines in the mycelium and culture filtrate, as well as free polyamines in the needles, were extracted in 5% (v/v) $HClO_4$ according to Fornalé et al. (1999) and Sarjala and Kaunisto (1993), respectively. Both soluble (hydrolyzed supernatant) and insoluble conjugated (hydrolyzed pellet) polyamines were determined from the mycelia, but only soluble conjugated forms were determined in the culture filtrates. Polyamines in the crude and hydrolyzed extracts were dansylated and then separated by high performance liquid chromatography (HPLC, Merck/Hitachi, Darmstadt, Germany) as described by Sarjala and Kaunisto (1993). The concentrations of polyamines in the mycelia and culture filtrates and those in the needles were expressed as nmol g^{-1} fresh weight of fungal mycelium and nmol g^{-1} fresh weight of needle, respectively.

Scanning electron microscopy

Root tips and longitudinal sections of tips covered by a hyphal mantle were fixed and dehydrated according to Honegger (1985), as described by Niemi et al. (2000). Dehydrated segments were subjected to a critical point dryer and coated with a 4:1 (w/w) mix of gold and palladium in a sputter coater (Polaron E5100, Milton Keynes, U.K.). Sections were photographed with a Jeol JSM 35 scanning electron microscope (Sundbyberg, Sweden) at 15 kV.

Statistical analyses

Radial growth data for *Paxillus involutus* for a 3-week culture period and for *Pisolithus tinctorius* for the first week of culture were log-transformed to correct for heterogeneity of variance. Where analysis of variance showed the effect of the medium to be significant ($P < 0.05$), pairwise comparisons of treatment means were performed by means of Tukey's honestly significant difference (HSD) test. Radial growth data for *Pisolithus tinctorius* for the second and third week of culture were analyzed with a non-parametric Kruskal-Wallis test. Differences in the seedling and hypocotyl growth parameters, except root/shoot ratios, were compared with a non-parametric Kruskal-Wallis test combined with the Mann-Whitney *U*-test with Bonferroni correction (Zar 1984, Altman 1991). Owing to conservatism of the Bonferroni correction, differences among both fungal treatments within the same medium and different media within the same fungal treatment were analyzed separately. The shoot/root ratio data were log-transformed and significant treatment effects as shown by analysis of variance were subject to pairwise comparison by means of Tukey's HSD test. Because the rooting experiments with and without a seedling were carried out separately, no statistical comparisons were performed.

Results

Production of polyamines by ectomycorrhizal fungi

Spermidine was the most abundant polyamine in the 3-week-old mycelia of both fungi (Table 1). It was predominantly in a free form but was also found in soluble and insoluble conjugated forms. The mycelium of *Paxillus involutus* contained large amounts of free putrescine but not cadaverine, whereas

Table 1. Concentrations of free and conjugated (soluble and insoluble) polyamines (nmol g^{-1} fresh weight of mycelium) in the mycelium and culture filtrate of *Pisolithus tinctorius* and *Paxillus involutus* after a 3-week culture in liquid MMN2 medium. Values are means (± SD) of three replicates. Symbols: * = polyamines were found in only one or two replicates; ** = the concentration of the unknown polyamine was not determined.

Polyamine	Mycelium		Culture filtrate	
	Pisolithus tinctorius	*Paxillus involutus*	*Pisolithus tinctorius*	*Paxillus involutus*
Putrescine				
Free	2.8 ± 4.8*	113.1 ± 35.0	0	25.4 ± 7.9
Soluble conjugated	16.6 ± 7.6	62.2 ± 62.2*	0	0
Insoluble conjugated	2.4 ± 0.7	0		
Spermidine				
Free	211.7 ± 124.8	355.3 ± 85.5	16.4 ± 9.6	3.6 ± 1.5
Soluble conjugated	24.5 ± 41.1*	123.7 ± 123.5*	0	0.5 ± 0.8*
Insoluble conjugated	5.1 ± 1.7	12.1 ± 10.7		
Spermine				
Free	4.0 ± 1.9	3.0 ± 1.6	0.4 ± 0.2	10.6 ± 5.8
Soluble conjugated	0.6 ± 0.7*	0.4 ± 0.6*	0	0
Insoluble conjugated	0.9 ± 0.8*	3.4 ± 2.8		
Cadaverine				
Free	4.3 ± 7.4*	0	**[1]	0[1]
Soluble conjugated	1.4 ± 2.4*	0	0[1]	0[1]
Insoluble conjugated	0	0		

[1] Unknown polyamine.

traces of both putrescine and cadaverine were found in the mycelium of *Pisolithus tinctorius*.

The culture filtrate of *Pisolithus tinctorius* contained no cadaverine, but an unknown compound, with a retention time (14.65 min) between that of cadaverine (14.09 min) and *N*-methylputrescine (15.40 min), was detected (Table 1). Cadaverine and *N*-methyputrescine have the same molecular weight, as well as the same number of carbon atoms and amino groups, suggesting that the unknown compound may be related to cadaverine or *N*-methylputrescine. Free putrescine was found in the culture filtrate of *Paxillus involutus*, but not that of *Pisolithus tinctorius* (Table 1). Free spermidine and spermine were found in the culture filtrates of both fungi, and in the case of *Paxillus involutus*, the spermine concentration was about three times higher in the culture filtrate than in the mycelium.

Radial growth of fungi on medium containing cadaverine or putrescine

The addition of cadaverine or putrescine to the MMN2 agar medium enhanced radial growth of *Pisolithus tinctorius* (Figure 2a). At the end of the first week in culture, the growth of the mycelium was significantly ($P < 0.05$) increased by 0.5 mM cadaverine, and by the end of the 3-week cultivation the mycelium had reached the edge of the petri dish. Although *Pisolithus tinctorius* was able to use both cadaverine and putrescine as the sole N source, replacement of NH_4-N by these diamines resulted in reduced radial growth (Figure 2a).

Paxillus involutus was able to grow on medium containing cadaverine or putrescine as the only N source (Figure 2b) and, after the first week of culture, the radial growth of the mycelium was significantly ($P < 0.05$) higher on these media than on the control MMN2. However, at the end of the experiment the mycelium grew most vigorously on the control MMN2 medium (Figure 2b).

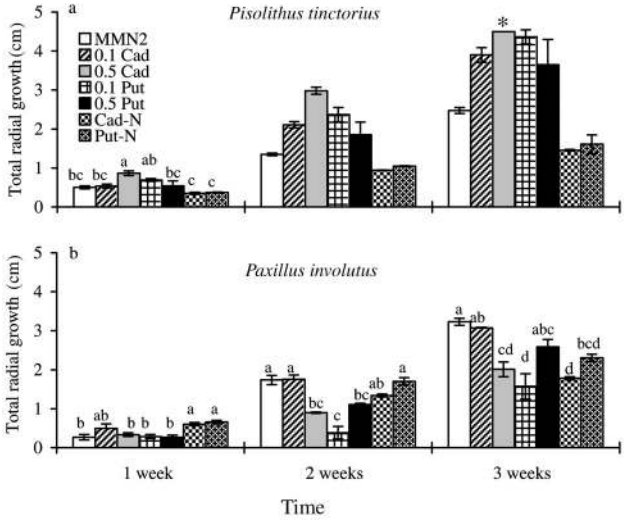

Figure 2. Total radial growth of *Pisolithus tinctorius* and *Paxillus involutus* on MMN2 agar medium supplemented with 41.6 mM glucose in the presence or absence of 0.1 or 0.5 mM cadaverine (Cad) or putrescine (Put). In the Cad-N and Put-N media, NH_4-N was replaced by the same amount of N in the form of cadaverine or putrescine. Values are means ± SE. Different letters above the columns denote significant ($P < 0.05$) differences between means (Tukey's honestly significant difference) within the experimental week. For the growth of *Pisolithus tinctorius* during the last 2 weeks, the Kruskal-Wallis test was used; 2 weeks, $P = 0.01$; 3 weeks, $P = 0.01$. An asterisk indicates that the mycelium had reached the edge of the petri dish.

Rooting of Scots pine hypocotyls in the absence of a seedling

Twenty percent of the non-inoculated hypocotyl cuttings, growing on the MMN2 rooting medium lacking polyamines, formed roots within the first 4 weeks (Figure 3a). The addition of 0.5 mM cadaverine or putrescine alone decreased root formation, whereas inoculation with the fungus increased the percentage of cuttings with roots (Figure 3a). Within the first month in culture, the presence of *Pisolithus tinctorius* and *Paxillus involutus* resulted in twofold higher rooting percentage compared with the non-inoculated cuttings. Addition of specific diamines to the medium further increased rooting percentage: more than 60% of the cuttings inoculated with *Pisolithus tinctorius* on the cadaverine-containing medium or inoculated with *Paxillus involutus* on the putrescine-containing medium formed roots within the first month (Figure 3a). At the end of the 9-week culture period, the percentage of cuttings with roots was high for both non-inoculated and inoculated cultures. The highest rooting percentage (94%) was on cuttings inoculated with *Pisolithus tinctorius* on the putrescine-containing medium, whereas the lowest percentage (78%) was on non-inoculated cuttings growing on the medium containing putrescine.

At harvest, root growth of the non-inoculated cuttings was unaffected by diamines (Figures 4a–d): the mean number of adventitious roots on the non-inoculated cuttings was about 1.5 on all media. Inoculation with *Pisolithus tinctorius* and *Paxillus involutus* resulted in a significant ($P < 0.05$) increase in adventitious root length (except on the control MMN2 medium without diamines, Figure 4a), number of lateral roots (Figure 4b) and fresh weight of the roots (Figure 4c). Furthermore, enhanced root growth and the presence of the fungal mycelium increased the root/shoot ratio (Figure 4d). However, the mean number of adventitious roots was unchanged as a result of fungal inoculation (data not shown). *Pisolithus tinctorius* and cadaverine had a synergistic effect on root growth. Both adventitious root length and number of lateral roots were significantly ($P < 0.05$) increased by the combined treatment compared with the fungal treatment alone. On the other hand, the combination of *Paxillus involutus* and putrescine, which enhanced root initiation, had no influence on subsequent root growth. Cuttings growing on the medium containing putrescine turned reddish, and thus seemed to suffer from the presence of *Paxillus involutus*.

Rooting of Scots pine hypocotyls in the presence of a seedling

In the presence of a seedling, the rooting percentage of the non-inoculated hypocotyl cuttings was increased by 0.5 mM, but not 0.1 mM, diamines within the first 4 weeks (Figure 3b). However, the inoculated cuttings rooted more rapidly than the non-inoculated cuttings. After a 4-week culture period, rooting of the inoculated cuttings on the MMN2 medium was about 50% and addition of 0.5 mM cadaverine further increased it (Figure 3b). The combined putrescine and *Paxillus involutus* treatment did not have the same positive effect on root initiation as it did in the absence of the seedling. At the end of the 9-week rooting period, *Pisolithus tinctorius* resulted in 100% rooting of cuttings on medium containing 0.1 mM cadaverine or putrescine and *Paxillus involutus* resulted in 100% rooting of cuttings on medium containing putrescine. Non-inoculated cuttings rooted best (85%) on the medium supplemented with 0.1 mM cadaverine and rooting was lowest (68%) on the control medium.

In general, the presence of a seedling on the rooting medium decreased root growth of the cuttings (Figure 4), although root growth of the cuttings in the presence of a seedling was significantly ($P < 0.05$) improved by the fungi (Figures 4e–h). In most cases, *Paxillus involutus* enhanced root growth more than *Pisolithus tinctorius*. *Pisolithus tinctorius* and cadaverine had a synergistic effect on root growth, and the same trend was also observed with *Paxillus involutus* and 0.5 mM cadaverine. In both cases, differences between media were insignificant.

Growth of Scots pine seedlings

Both fungi increased the fresh weight of the root system significantly ($P < 0.05$) (Table 2). Main root length and the number of lateral roots on the different media were also significantly ($P < 0.05$) increased by the fungi, except by *Pisolithus tinctorius* on the control MMN2 medium. Improved root growth together with the presence of the mycelium resulted in increased root/shoot ratios. Addition of diamines to the medium did not influence the interaction between fungus and seedling.

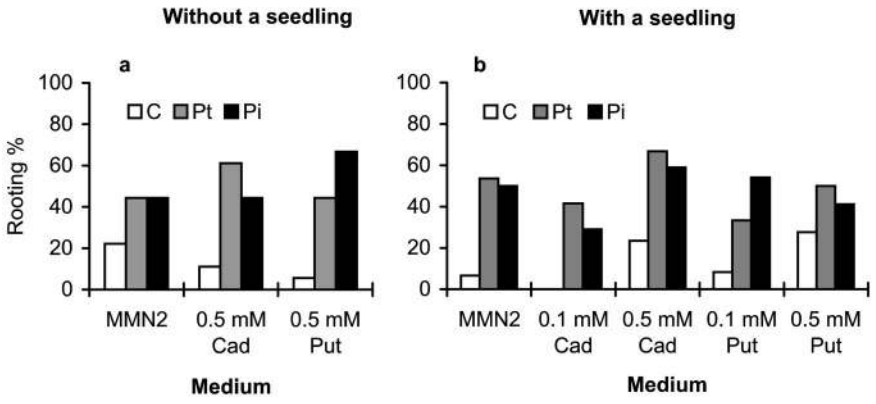

Figure 3. Effect of *Pisolithus tinctorius* (Pt) and *Paxillus involutus* (Pi), as well as cadaverine (Cad) and putrescine (Put), on the rooting of Scots pine hypocotyl cuttings after 4 weeks in culture. Rooting was carried out without (a) and with (b) a seedling.

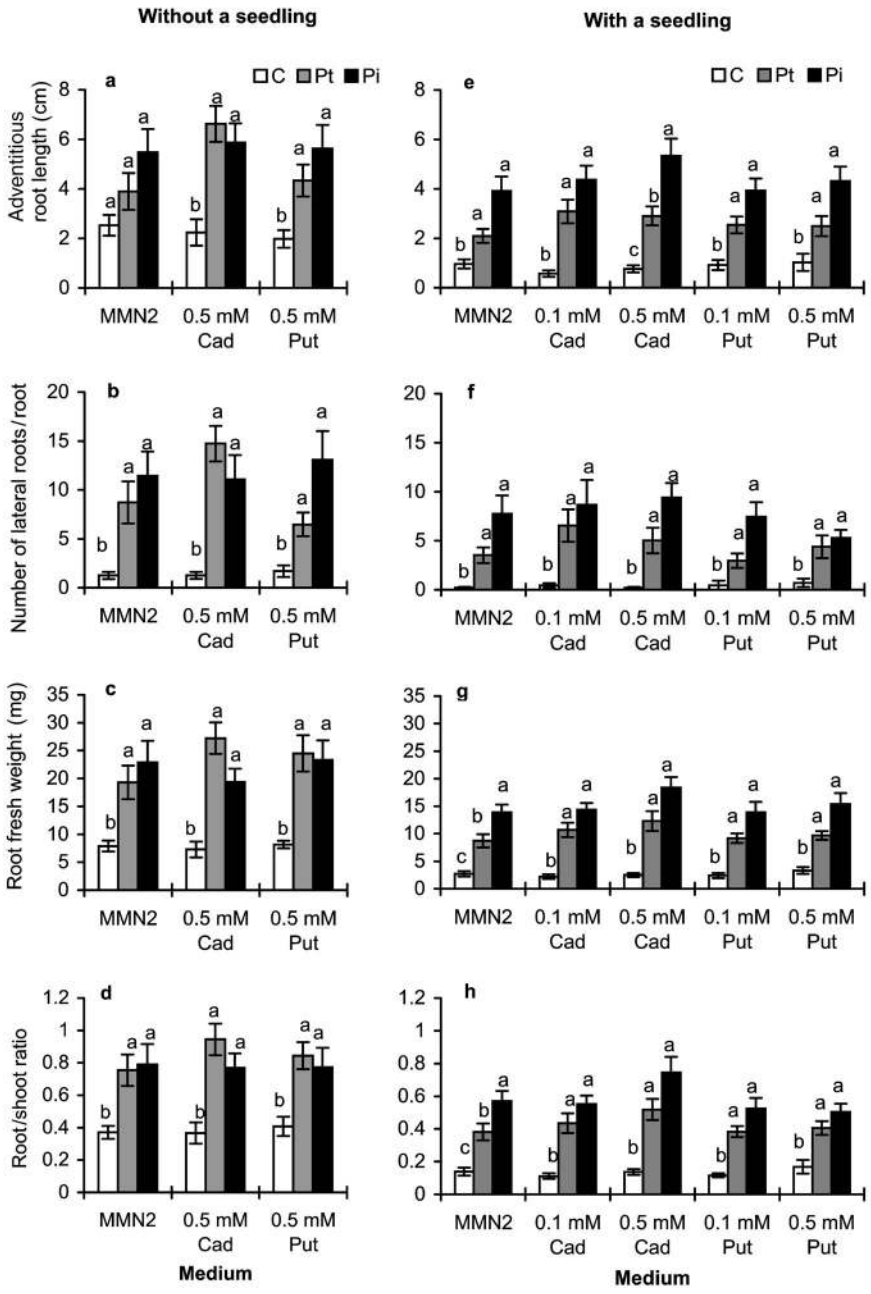

Figure 4. Effect of *Pisolithus tinctorius* (Pt) and *Paxillus involutus* (Pi), in the presence or absence of cadaverine (Cad) or putrescine (Put), on the root growth of Scots pine hypocotyl cuttings at the time of harvest, i.e., after 9 weeks in culture. Rooting was carried out without (a–d) and with a seedling (e–h). Values are means ± SE. Different letters above the columns denote significant ($P < 0.05$) differences between means (Kruskal-Wallis combined with Mann-Whitney *U*-test with Bonferroni correction, except for root/shoot ratios, for which ANOVA combined with Tukey's honestly significant difference test was used).

Mycorrhiza formation in roots of cuttings and seedlings

Pisolithus tinctorius formed a thick mantle over the lateral roots of both cuttings and seedlings, resulting in inhibition of root hair development (Figure 5a). However, Hartig net formation between the cortical cells was observed only in the seedling roots.

For cuttings grown without a seedling, addition of 0.5 mM cadaverine increased the number of root tips covered by the mycelium from 34 to 43%. For seedlings, the addition of 0.1 mM cadaverine increased the proportion of mycorrhizal root tips from 54 to 70%. Among treatments, the hyphal mantle was thickest in the presence of cadaverine. In cuttings rooted in the presence of a seedling, the number of lateral root tips was low and treatment differences were not observed.

Paxillus involutus did not form mycorrhizal structures in our experiments. In the absence of the seedling, the fungus grew aggressively, especially on medium containing putrescine. The mycelium grew over the root system, but it did not form a mycorrhizal mantle or inhibit root hair development (Figure 5b).

Free polyamines in hypocotyl needles

Putrescine was the major free polyamine in the needles of both non-inoculated and inoculated hypocotyl cuttings growing with a seedling. The addition of putrescine or cadaverine to the medium had no significant effect on the endogenous free poly-

Table 2. Growth of Scots pine seedlings inoculated with the ectomycorrhizal fungi *Pisolithus tinctorius* and *Paxillus involutus* on MMN2 agar medium in the presence or absence of cadaverine or putrescine. Values are means ± SD after a 10-week culture. Different letters following the values denote significant ($P < 0.05$) differences between means within the medium (Kruskal-Wallis combined with Mann-Whitney *U*-test with Bonferroni correction, except for root/shoot ratios for which ANOVA test combined with Tukey's highest significant difference test was used).

Medium	Main root length (cm)	Number of lateral roots	Root fresh weight (mg)	Root/shoot ratio
MMN2				
Control	3.8 ± 1.8 b	8.2 ± 3.1 b	8.2 ± 4.9 c	0.29 ± 0.14 c
Pisolithus tinctorius	4.9 ± 1.7 b	16.7 ± 14.9 ab	17.0 ± 7.1 b	0.45 ± 0.18 b
Paxillus involutus	7.6 ± 2.5 a	24.3 ± 10.3 a	24.4 ± 6.0 a	0.60 ± 0.13 a
0.1 mM Cadaverine				
Control	2.6 ± 1.1 b	6.2 ± 2.6 b	6.2 ± 2.3 b	0.24 ± 0.08 b
Pisolithus tinctorius	5.8 ± 2.0 a	15.1 ± 8.5 a	19.6 ± 5.7 a	0.60 ± 0.21 a
Paxillus involutus	6.9 ± 3.2 a	19.0 ± 11.0 a	22.5 ± 9.9 a	0.56 ± 0.28 a
0.5 mM Cadaverine				
Control	3.4 ± 1.8 b	6.7 ± 2.8 c	7.6 ± 4.3 c	0.34 ± 0.18 b
Pisolithus tinctorius	6.3 ± 2.7 a	15.4 ± 5.9 b	18.0 ± 7.3 b	0.54 ± 0.19 a
Paxillus involutus	8.3 ± 4.6 a	26.5 ± 9.7 a	24.1 ± 6.9 a	0.68 ± 0.24 a
0.1 mM Putrescine				
Control	3.2 ± 1.6 b	6.3 ± 2.7 b	8.2 ± 4.2 c	0.26 ± 0.09 b
Pisolithus tinctorius	5.7 ± 1.7 a	21.6 ± 12.0 a	18.1 ± 6.6 b	0.44 ± 0.19 a
Paxillus involutus	8.5 ± 2.7 a	22.4 ± 8.2 a	26.9 ± 7.0 a	0.56 ± 0.18 a
0.5 mM Putrescine				
Control	3.9 ± 2.2 c	6.4 ± 4.5 c	8.5 ± 7.3 c	0.30 ± 0.23 b
Pisolithus tinctorius	6.3 ± 3.1 b	17.1 ± 10.5 b	17.2 ± 5.5 b	0.47 ± 0.17 a
Paxillus involutus	9.6 ± 3.5 a	30.2 ± 14.0 a	26.8 ± 7.8 a	0.63 ± 0.27 a

amine pools at the end of the 9-week culture period. Similarly, the fungal inoculations had no significant effect on the endogenous free polyamine pools at the end of the 9-week culture period, although the endogenous spermidine and spermine pools were generally lower in inoculated cuttings than in control cuttings.

Discussion

Inoculation with *Pisolithus tinctorius* or *Paxillus involutus* accelerated root formation and increased the growth of roots formed on Scots pine hypocotyl cuttings in vitro. Similar results were obtained in earlier in vitro studies with hypocotyl

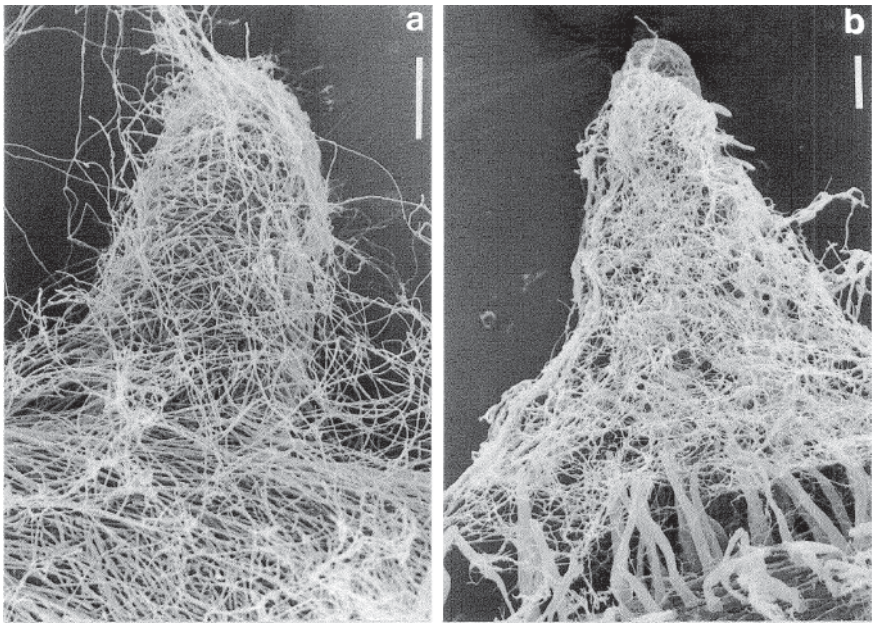

Figure 5. Scanning electron micrographs of the growth of fungal mycelium around the lateral root of a rooted Scots pine hypocotyl cutting. (a) The hyphae of *Pisolithus tinctorius* cover the root as a thick and dense mantle. Root hair development is inhibited. (b) The hyphae of *Paxillus involutus* grow as a mat over the root without forming a mantle. Root hair formation continued under the hyphae. The scale bars represent 100 μm.

cuttings (Gay 1990, Normand et al. 1996, Karabaghli et al. 1998) and micropropagated shoots (Supriyanto and Rohr 1994, Normand et al. 1996) of several coniferous species. The importance of fungal IAA for in vitro rooting has been well documented (Gay 1990, Normand et al. 1996, Karabaghli et al. 1998). However, rooting does not necessarily increase with increased IAA production (Normand et al. 1996). *Pisolithus tinctorius* can synthesize a high amount of IAA without exogenous tryptophan, whereas the IAA synthesis in *Paxillus involutus* is highly dependent on the presence of this precursor (Niemi et al. 2000). In the present study, *Paxillus involutus* enhanced rooting and root growth as much or even more than *Pisolithus tinctorius*, indicating either that only a very small amount of fungal IAA is needed for root formation in hypocotyl cuttings (Normand et al. 1996) or that a lack of IAA can be compensated for, at least in part, by other fungal compounds.

Fungal polyamines have been implicated in the interaction between ectomycorrhizal fungi and plant roots (Kytöviita and Sarjala 1997, Fornalé et al. 1999, Sarjala 1999). We found that spermidine was the major polyamine in the 3-week-old mycelium of both fungi, but that *Paxillus involutus* also produced large amounts of putrescine. Zarb and Walters (1994) reported on the ability of some ectomycorrhizal fungi to produce cadaverine and its homologs. In our study, *Pisolithus tinctorius* contained traces of cadaverine, and an unknown compound, probably a cadaverine-like free polyamine, was found in the culture filtrate. Although the culture filtrate of *Paxillus involutus* did not contain cadaverine, the fungus was able to use cadaverine as an N source (cf. Fornalé et al. 1999).

The effects of *Pisolithus tinctorius* on formation and growth of roots were enhanced by exogenous cadaverine, and the same tendency was observed for mycorrhiza formation. The reactions of the non-inoculated cultures to cadaverine, which was not found in the polyamine pool of Scots pine (e.g., Sarjala and Kaunisto 1993, Sarjala 1996, Sarjala et al. 1997), were variable and, in the absence of a seedling, a negative response was observed. These findings indicate that cadaverine affected the *Pisolithus tinctorius*–Scots pine interaction mainly through the fungus. The increase in radial growth of the mycelium in response to cadaverine suggests that cadaverine also improved the growth of *Pisolithus tinctorius* on the rooting medium, perhaps resulting in more rapid and extensive contact with the cutting. Because a close relationship between polyamines and IAA has been observed during root formation (reviewed by Galston and Flores 1991, Kevers et al. 1997), it is possible that there was an interaction between cadaverine and IAA released by *Pisolithus tinctorius* (Niemi et al. 2000).

Putrescine and *Paxillus involutus* had a synergistic effect on root initiation only when the cutting was grown in the absence of a seedling. The minor positive effect of exogenous putrescine may indicate that the amount of putrescine released by *Paxillus involutus* was sufficient for the fungus–cutting interaction or that putrescine was not associated with fungal-induced increases in root growth and lateral root formation.

Stimulation of roots by *Paxillus involutus* without mycorrhiza formation corroborates in vivo studies with cuttings (Linderman and Call 1977, Niemi et al. 2000). In the presence of a seedling, root growth increased more in response to *Paxillus involutus* than to *Pisolithus tinctorius*, suggesting that mycorrhiza formation by the seedling affected the cutting–fungus interaction. In general, the slow growth of roots of cuttings in the presence of a seedling was probably a result of nutrient utilization by the seedling.

To summarize, the enhanced formation and growth of Scots pine adventitious roots in response to *Pisolithus tinctorius* was further increased by exogenous cadaverine, a diamine produced by the fungus. Furthermore, exogenous cadaverine enhanced mycorrhiza formation by the fungus. It is unclear whether the role of cadaverine was to stimulate the growth of *Pisolithus tinctorius* or to interact with fungal growth regulators, such as IAA, or both. The effect of exogenous cadaverine or putrescine was generally weak or absent in cuttings grown in the presence of *Paxillus involutus*, which has a relatively high putrescine production capacity. We conclude that the effects of specific exogenous diamines are dependent on both the fungal strain and its ability to produce these compounds.

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