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EFFECTS OF *Pisolithus arhizus* AND OTHER ECTOMYCORRHIZAL FUNGI ON *in vitro* ROOTING OF *Pinus monticola* ADVENTITIOUS SHOOTS[1]*

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Micropropagation of conifers permits the production of large numbers of selected genotypes but in *Pinus monticola* as well as several other systems, this process is limited by the difficulty of obtaining roots. The potential of ectomycorrhizal fungi to induce and improve adventitious root formation on in vitro micropropagated shoots was investigated.

Preliminary trials on de-rooted seedlings showed 100% rooting with *Laccaria bicolor, Rhizopogon rubescens* and *Pisolithus arhizus* while rooting on controls was at 79 %; however only *P. arhizus* had a significant effect on the rooting of micropropagated shoots.

Co-cultivation with *P. arhizus* in a medium amended with 0.3 μM 1-naphthaleneacetic acid (NAA) gave the best rooting percentages (57%).

Thin layer chromatography, high-performance liquid chromatography, and gas chromatography/ mass spectrometric analysis of the broth from a *P. arhizus* culture in NAA amended media indicated the presence of hydroxylated NAA metabolites.

La multiplication végétative *in vitro* rend possible la production d'un vaste nombre de génotypes choisis. Cependant, pour le *Pinus monticola* et d'autres essences diverses, cette méthode de production se trouve limitée par la difficulté d'obtenir des racines. Les auteurs ont examiné le potentiel des champignons ectomycorhiziens à activer et à améliorer la formation de racines adventives sur des tiges micropropagagées.

Une étude exploratoire à partir de semis déracinés montre qu'il est possible d'obtenir un enracinement de 100% avec le *Laccaria bicolor*, le *Rhizopogon rubescens* et le *Pisolithus arhizus*, comparativement à un enracinement de 79% avec les échantillons-témoins. Toutefois, seul le *P. arhizus* exerce une forte influence sur l'enracinement de tiges micropropagagées. La coculture avec le *P. arhizus* dans un milieu modifié par de l'acide naphtalène acétique (ANA) à raison de 0,3 μ*M* a donné les meilleurs taux d'enracinement (57 %).

Les analyses par chromatographie sur couche mince, par chromatographie liquide haute performance et par couplage chromatographie en phase gazeuse-spectrométrie de masse, du bouillon de la culture de *P. arhizus* dans un milieu modifié avec de l'ANA révèlent la présence de métabolites d'ANA hydroxylées.

Introduction

Micropropagation of conifers permits tree breeders to produce large numbers of selected genotypes but in many conifer systems this process is limited by the difficulties in obtaining roots. A method for production of adventitious shoots from *Pinus monticola* Dougl. embryos and buds has been developed but various factors investigated, including auxin treatments, did not improve rooting over that which occurs spontaneously at about 20% (Lapp and Malinek 1988).

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* This paper is dedicated to the memory of K.A. Harrison.

Many ectomycorrhizal fungi release growth regulators such as: auxins, cytokinins, ethylene, gibberellins, vitamins and amino acids into the culture medium (Kraigher *et al.* 1991; Frankenberger and Poth 1987; Strzelczyk *et al.* 1986). The effects of ectomycorrhizal fungi on rooting of cuttings or derooted hypocotyls has been examined by several workers. Navratil and Rochon (1981) found that the addition of a *Pisolithus tinctorius* inoculum to the vermiculite-based medium enhanced root and shoot development of four poplar hybrids. *Laccaria laccata* added to the rooting medium stimulated root initiation in apple rootstock cuttings as compared with indole-3-butyric acid treated cuttings (Branzanti *et al.* 1984). Kim and Lee (1990) reported that inoculation with *P. tinctorius* enhanced rooting of *Quercus acutissima* Carr. cuttings taken from 2, 4 and 20-year-old trees. Gay (1990) observed increased rooting percentages with *Pinus halepensis* hypocotyls in the presence of *Hebeloma hiemale* and tryptophan or with culture filtrates from this same combination and suggested that these fungi may be useful to enhance *in vitro* rooting.

This study investigates the potential of the ectomycorrhizal fungi *Laccaria bicolor, Pisolithus arhizus, Rhizopogon rubescens* and *Wilcoxina mikolae* to increase adventitious root formation of *in vitro* micropropagated *Pinus monticola* shoots and examines the interaction between *P. arhizus* (Scop: Pers.) Rauschert (syn. *Pisolithus tinctorius* (Pers.)(Coker & Couch) and the synthetic auxin 1-naphthaleneacetic acid (NAA).

Materials and methods

Derooted hypocotyls After stratification in running water at 4°C for 1 week, seeds were surface sterilized for 25 min in 30% H_2O_2 with the addition of a drop of Tween 80 and placed on 1% w/v water agar for germination in darkness. Germinated seeds were transferred to 3% w/v sucrose solution solidified with 5% Gel-Gro (ICN Biochemicals, Cleveland, OH, 44128) in glass jars for 21 days in controlled environmental conditions of 16 h light : 8 h dark at 23°C. Light was supplied from both cool white flourescent and incandescent sources at a photon density of 120 $\mu Em^{-2}s^{-1}$.

Roots were excised from the seedlings 1.0 cm above the transition zone between root and hypocotyl. The base of each hypocotyl was wounded with a scalpel before being placed in a vermiculite:peat (9:1) substrate with 0.5% pine charcoal (Sigma Chemical Co., St. Louis, MO, Number C-4386) added. Three 7- mm diameter inoculum plugs were cut from the growing edge of a 14-day fungal culture and placed in the substrate near the base of the inserted seedling. Experiments were carried out in 25 x 150 mm glass tubes under the same controlled conditions as previously described.

Fungal Cultures Cultures were maintained at 20° C in darkness on modified Melin Norkrans medium (MMN) (Marx, 1969). The following cultures were tested in rooting bioassays: *Laccaria bicolor* (Maire) Orton FTK 862A*, *Pisolithus arhizus* (Scop: Pers.) Rausch FTK 820A, *Rhizopogon rubescens* (Tul.) Tulasne FTK 861A and *Wilcoxina mikolae* Yang & Korf (E-strain) FTK 849A.

Still liquid cultures of *P. arhizus* were prepared by placing ten 7- mm diameter inoculum plugs cut from the perimeter of an actively growing culture, in 50 mL of MMN in a 125 mL flask with or without the addition of NAA (50 mg L^{-1}), before autoclaving. Cultures were incubated for 21 days in darkness at 20°C.

Micropropagated Shoots Micropropagated *P. monticola* shoots, derived from

* Accession number to culture collection held at Forintek Canada Corp.

mature embryos (Lapp and Malinek 1988) were harvested 13 weeks after hormone treatment and elongated for an additional 4 weeks on half strength Litvay's medium (Litvay *et al.*, 1981) modified to increase the iron concentration two fold. After each shoot was freshly cut on the diagonal and wounded at the base with a scalpel, it was inserted into the rooting medium. Rooting was carried out on half strength Gresshoff Doy medium (GD) (Sigma Chemical Co., St. Louis, MO). The pH was adjusted to 5.5 before autoclaving or the addition of the gelling agent (5% w/v Gel Gro). Tryptophan or indole-3-acetic acid (IAA) were filter sterilized and added to the medium after autoclaving whereas NAA was autoclaved in the medium. A fungal inoculum plug was introduced into the tube at the base of the slant. Agar (MMN) plugs were introduced into tubes that served as controls. Black paper collars were placed around the base of each tube to protect the medium from excessive light exposure. After 10 weeks, shoots were examined for root formation and rooted shoots were transferred to a vermiculite: peat substrate in a greenhouse mistbed.

Experimental Design Statistical analyses were done using NWA Statpak (Northwest Analytical Inc., Portland, Oregon, Version 4.1). The Chi-square test for independence was used to determine significant differences among treatments in rooting studies. Differences in root numbers or root lengths were examined by analysis of variance followed by Duncan's multiple range test. A completely random experimental design was used for all experiments. All experiments were duplicated.

Chemical Analyses All solvents were high-performance liquid chromatography (HPLC) grade. Analytical thin layer chromatography (TLC) was carried out on Merck, Kieselgel 60 F_{254} (Merck, Darmsdat), aluminum sheets, 5 x 2 x 0.02 cm; compounds were visualized by exposure to ultraviolet light (254 nm) and by dipping the plates in a 5% aqueous (w/v) sulphuric acid, followed by heating at 200°C. HPLC analysis was performed with the following Spectra Physics instrument system: liquid chromatograph model SP 8100, equipped with a dual pump and autosampler, ultraviolet/visible detector (variable wavelength) model SP 4200, and computing integrator model SP 4200. A reverse phase RP-8 Spheri-5 column (5 µm particle size silica, 22 x 0.46 cm internal diameter, Brownlee Columns, Applied Biosystems Inc., San Jose, CA, USA) equipped with a NewGuard TM RP-8 guard column, was used with a gradient elution of methyl alcohol-water-acetic acid (100:99:1, 5 min: 40:9:1, 20 min), a flow rate of 2.0 mL min^{-1}, and detection at 270 nm. Under these conditions, the retention time for NAA was 4.0 min. Gas chromatography-electron impact mass spectrometry (GC-EIMS) analysis was performed using a Finnigan Model 4500 instrument, having the gas chromatograph equipped with an Ultra-2 fused silica capillary column (50 m x 0.32 mm; Hewlett Packard, Palo Alto, CA, USA), in splitless mode injection with splitter closed for 0.8 min, at 50-250°C; temperature gradient 4°C min^{-1}, and helium as the carrier gas (flow rate 40 cm sec^{-1}, measured at 150°C). Diazomethane (ethereal solution) was prepared from p-toluenesulfonylmethylnitrosoamide (de Boer and Backer, 1963). Methylation of the broth extracts, dissolved in ethyl ether (2mL), was carried out at room temperature.

Twenty one day old liquid cultures of *P. arhizus* were filtered through several layers of cheesecloth. The broth from NAA-amended and from non amended cultures was lyophilized and maintained at -20° C for further studies. The freeze dried broth was diluted with water to one tenth of the initial volume and extracted with ethyl ether. The ethereal extracts were dried (Na_2SO_4) and concentrated under vacuum at 35°C. The concentrated extracts were analyzed by TLC using the solvent system dichloromethane methanol (96:4, double elution), HPLC and GC-EIMS. Medium (MMN) containing NAA was also extracted and analyzed as described above.

Results and discussion

All of the de-rooted seedlings co-cultured with ectomycorrhizal fungi showed 100% rooting while 79% of controls rooted (Table I). Seedlings cultured in the presence of *P. arhizus* had a significantly greater mean number of roots formed per seedling than the other treatments. Total mean root length per seedling was greatest (9.2) where seedlings were cultured with *L. bicolor*, but did not differ significantly from that of *P. arhizus* co-cultured seedlings (7.0).

Contingency Chi-square analysis of rooting percentages of micropropagated *P. monticola* shoots showed that amending the media with NAA or the auxin precursor, tryptophan, did not affect rooting ($p<0.5$) (Table II). Fungal treatments did significantly increase rooting ($p<0.005$) with a total of 22% for all *P. arhizus* treated shoots while controls and shoots cultured with *L. bicolor* or *W. mikolae* had 9% rooting. The media x fungus interaction was significant ($p<0.05$) indicating that within each fungal treatment, differences could be attributed to media amendments. Little callus formation was observed on those shoots co-cultivated with fungi. Viable hyphae, which initiated new cultures when placed on MMN agar plates, were observed in the vicinity and often covering growing root tips (Fig 1). However, no true mantle or Hartig net structures were visible in squash mounts of short roots.

Shoots co-cultivated with *P. arhizus* but not amended with tryptophan and control shoots with (Fig 4) and without NAA (Fig 3) all rooted at 20% (Table III). Co-cultivation with *P. arhizus* and with 0.3 μM NAA in the medium was the best rooting treatment (56.6%; Fig 2). Rooting with IAA was low (6.0%) and associated with extensive callus formation (Fig 5). Co-cultivation with *P. arhizus* improved rooting to 13.0% but rooting remained less than that of controls. However, callus production associated with the IAA treatment was greatly reduced in the presence of *P. arhizus* (Fig 5). No rooting was observed with a combination of NAA (0.3 μM) and IAA (0.5 μM). Although IAA has been detected in pure culture filtrates of *P. arhizus* (Frankenberger and Poth, 1987), auxin alone does not appear to be responsible for the mycorrhizal-enhanced root induction. Shoots cultured with *P. arhizus* in a tryptophan-amended medium produced the highest mean number of roots per shoot (3.7) as compared to controls (1.0) or to those cultured in unamended medium in the presence of *P. arhizus* (2.0). This is the first report of *in vitro* root induction on adventitious conifer shoots using *P. arhizus*.

Rooting percentages of micropropagated pine shoots were significantly increased when rooting occurred in the presence of *P. arhizus* in a NAA- or tryptophan-amended medium. Kim and Lee (1990) similarly observed improved rooting on cuttings from 20-yr-old *Quercus* trees when a 3000 ppm indole-3-butyric acid dip was coupled with *P. tinctorius* inoculation.

Both TLC and HPLC analysis of the broth extracts of NAA-amended cultures of *P. arhizus* revealed the presence of three acidic compounds which were not present in non-amended cultures of *P. arhizus*. One of these was identified as NAA (<10% recovery) by comparison with an authentic sample (TLC, HPLC and GC-EIMS). The other two acids appeared to result from NAA oxidation. GC-EIMS analysis of the methylated broth extracts indicated the presence of two different hydroxy derivatives of methyl NAA (M^+ *m/z* 200, and base peak *m/z* 141 $(M\text{-}CO_2Me)^+$) which had M^+ *m/z* 216 (35%) and a base peak *m/z* 157 $(M\text{-}CO_2Me)^+$ for the two unknown components. Work is in progress to determine the chemical structures of these two compounds.

Co-culturing micropropagated shoots with ectomycorrhizal fungi has been reported to result in mycorrhizae formation (Tonkin *et al.* 1989, Grellier *et al.* 1984).

Table I Comparative effects of 3 ectomycorrhizal fungi on rooting of de-rooted *Pinus monticola* seedlings.

Treatment	Total number of seedlings	Rooting (%) P<0.005	Mean number roots per seedling P<0.05	Total mean root length per seedling P<0.005
Control	19	78.9b*	1.7b	6.5b
Laccaria bicolor	20	100.0a	2.2b	9.2a
Rhizopogon rubescens	19	100.0a	2.5b	4.7b
Pisolithus arhizus	20	100.0a	2.8a	7.0ab

* Means within a vertical column followed by different letters are significantly different by contingency Chi-square for rooting or by Duncan's multiple range test for number of roots and root lengths.

Table II Percent rooting of micropropagated *Pinus monticola* shoots co-cultivated with *Pisolithus arhizus, Laccaria bicolor* or *Wilcoxina mikolae* on half-strength Gresshoff-Doy medium containing 1-naphthaleneacetic acid or tryptophan. Contingency χ^2's are also shown.

	*Pisolithus arhizus**	*Laccaria bicolor*	*Wilcoxina mikolae*	*Control*	*Mean*
Control	20.0	0.0	26.6	20.0	16.7a**
1-Napthaleneacetic acid 0.3 μM	53.3	26.6	0.6	20.0	26.7
Tryptophan 0.1 mM	46.6	13.3	13.3	13.3	21.7a
Tryptophan 0.01 mM	26.0	20.0	13.3	0.6	16.7a
Mean	36.7a	15.0b	15.0b	15.0b	

* Values represent mean values of 15 shoots per treatment.
** Means within a row or column followed by different letters are significantly different at $p<0.05$ by contingency χ^2.

Contingency χ^2

Comparison:	df	χ^2	P
media X rooting	3	2.539	0.4683
fungus X rooting	3	13.001	0.0046
media (fungus) X rooting	15	26.643	0.0317

Table III Rooting percentages of micropropagated *Pinus monticola* shoots co-cultivated with (+) or without (-) *Pisolithus arhizus* in the presence of tryptophan, indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA) on half-strength Gresshoff Doy medium.

Medium amendment	*Piaolithus arhizus* (+/-)	Rooting (%) n = 15 χ^2 = 19.8350 p < 0.005	Mean number of roots per shoot p<0.001
None	-	20.0bcd	1.0b
None	+	20.0bcd	2.0b
TRP (0.1 mM)	+	43.3ab	3.7a
NAA (0.3 μM)	-	20.0bcd	1.7b
NAA (0.3 μM)	+	56.6a	2.5b
IAA (0.5 μM)	-	6.0cd	3.0b
IAA (0.5 μM)	+	13.3cd	1.0b
NAA (0.3 μM) and IAA (0.5 μM)	-	0.0d	-

* Means within a vertical column followed by different letters are significantly different by contingency Chi-square for rooting or by Duncan's multiple range test for number of roots.

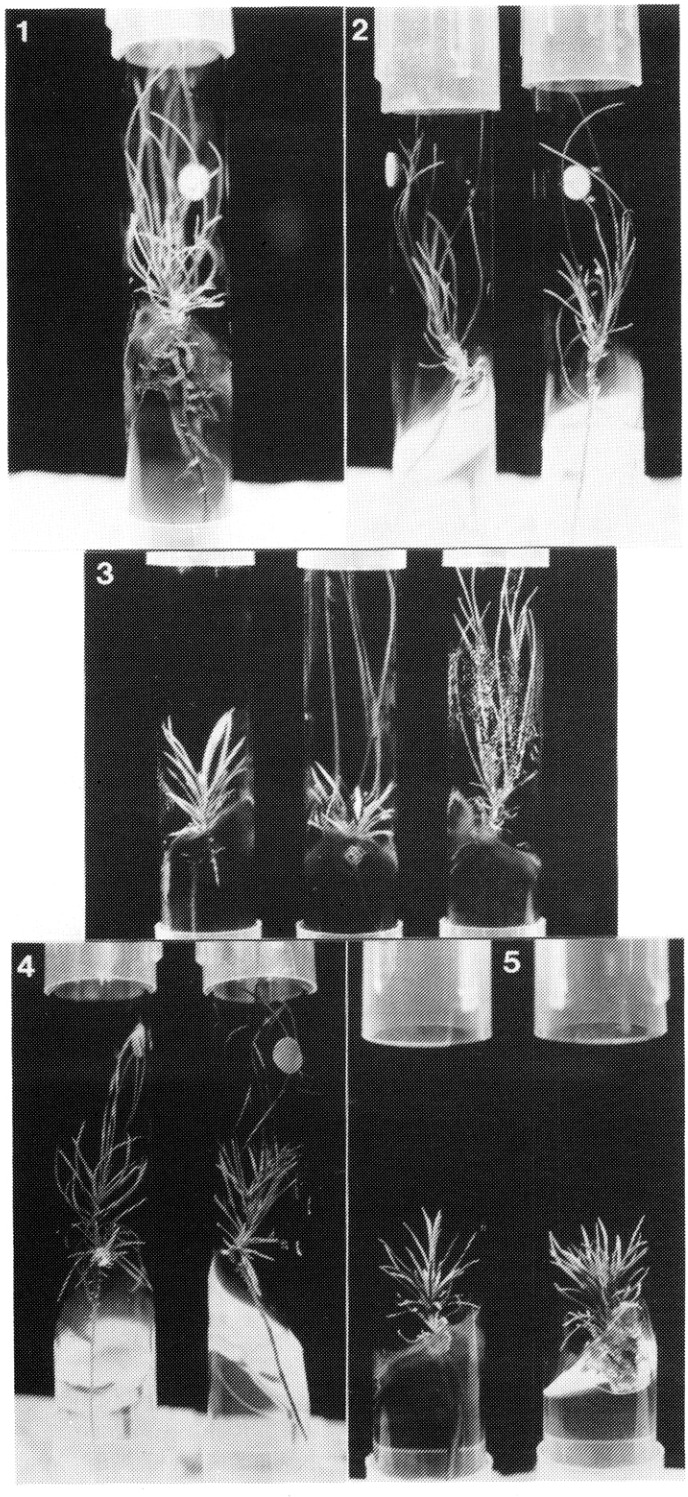
Fig 1 *Pinus monticola* shoot rooted in the presence of *P. arhizus* in Gresshoff Doy medium amended with tryptophan (0.1μM). (Diameter of the tube = 25 mm)

Fig 2 *Pinus monticola* adventitious shoots rooted in the presence of *P.arhizus* in Gresshoff Doy medium amended with NAA (0.3 μM). (Diameter of the tube = 25 mm)

Fig 3 *Pinus monticola* control shoots rooted in Gresshoff Doy medium. (Diameter of the tube = 25 mm)

Fig 4 *Pinus monticola* shoots rooted in Gresshoff Doy medium amended with NAA (0.3 μM). (Diameter of the tube = 25 mm)

Fig 5 Adventitious *Pinus monticola* shoots on Gresshoff Doy medium amended with IAA (0.5 μM) (right) and in the same medium plus *P. arhizus* (left) showing the absence of callus tissue in the presence of *P. arhizus*. (Diameter of the tube = 25 mm)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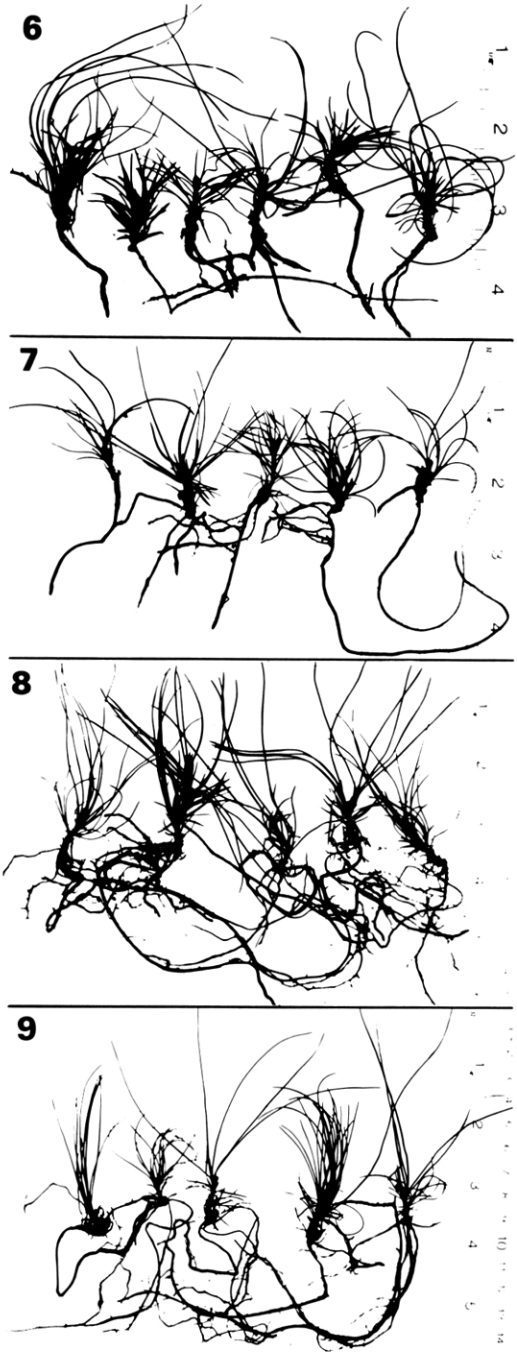

Figs 6-9 *Pinus monticola* adventitious shoots rooted in test tubes on Gresshoff Doy medium and transferred to a peat:vermiculite substrate in a greenhouse mistbed for 10 weeks: Fig 6 controls, Fig 7 NAA (0.3 μM) induced rooting, Fig 8 *Pisolithus arhizus* induced rooting, Fig 9 *P. arhizus* and NAA (0.3 μM) induced rooting.

However, Malajczuk and Hartney (1986) and Gay (1990) observed that ectomycorrhizae were initiated only on roots formed above the agar surface. The oxygen-poor agar substrate was probably detrimental to ectomycorrhizal formation in our experiment. Transferring these rooted shoots to a soil-like substrate *in vivo* did not result in ectomycorrhizal formation either. However, after two months in a greenhouse mistbed, the initial differences in root numbers per shoot, were still maintained (Figs 6-9).

Malajczuk and Hartney (1986) found that plantlets inoculated with ectomycorrhizal fungi survived transplantation from agar to peat substrate better than controls and Kim and Lee (1990) reported that the average survival rates of rooted *Quercus* cuttings were markedly improved when cuttings were inoculated with *P. tinctorius*. In liquid culture, *P. arhizus* produces extracellular metabolites which have been shown to inhibit a wide range of phytopathogenic fungi (Kope and Fortin 1990) and increased uptake of soil nutrients has been demonstrated with ectomycorrhizae (Harley and Smith 1983). Future studies should consider the effects of ectomycorrhizal inoculation treatments on root and shoot nutrition and the role of oxygenated NAA metabolites. Reinoculating with *P. arhizus* after root induction, or determining cultural conditions that are conducive to mycorhhizal formation as well as rooting may ultimately result in improved shoot as well as root quality and increased survival rates during transfer to field conditions, a critical stage in micropropagation.

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