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Relationships Between Differential *In Vitro* Indole-Acetic Acid or Ethylene Production Capacity by Ectomycorrhizal Fungi and Conifer Seedling Responses in Symbiosis

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Abstract

To assess the relationship between *in vitro* plant growth regulator production by mycorrhizal fungi and plant growth responses in symbiosis, ectomycorrhizal fungi, characterized by their *in vitro* Indole Acetic Acid (IAA) and ethylene production capacity (quantified using HPLC and GC-MS methodology), were used to inoculate 8-week-old Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco.), Englemann Spruce (*Picea englemannii* Parry) and Lodgepole Pine (*Pinus contorta* Dougl.) seedlings maintained under glasshouse conditions. For six months after inoculation, seedlings were evaluated for changes in morphology and endogenous IAA content to determine any correlations between fungal IAA or ethylene production potential and plant responses. Morphological responses (seedling height, shoot weight, root collar diameter, number of lateral roots and root weight) varied with tree species and mycorrhizal fungus combination. *In vitro* fungal IAA production was significantly correlated to endogenous root IAA content and many morphological attributes of mycorrhizal Douglas-fir seedlings. *In vitro* fungal ethylene production was poorly correlated to Douglas-fir morphological responses, but positively correlated to root IAA for all three conifer species. These results suggest a relationship between auxin and ethylene production by ectomycorrhizal fungi and changes in endogenous IAA content of roots that could affect growth responses of conifer seedlings.

Keywords: Ectomycorrhizae, IAA, ethylene, conifers, roots

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Abbreviations

IAA = indole-acetic acid; HPLC = high performance liquid chromatography; GC = gas chromatography; PGR = plant growth regulating substance

1. Introduction

Microorganisms in the mycorrhizosphere have the potential to change the hormonal status of seedling roots (Tomaszewshi and Wojciechowska, 1973; Mitchell, 1984; Strzelczyk and Pokojska-Burdziej, 1984) by producing growth regulating substances like ethylene and auxins. Although mycorrhizal fungi are known to produce plant growth regulating (PGR) compounds *in vitro* (Ulrich, 1960; Graham and Linderman, 1981,1980; Radawska, 1982; Ek et al., 1983; Rouillon et al., 1985; Ho, 1986; Polojska and Strzelczyk, 1988; Gay et al., 1994), and ethylene and auxin can induce morphological changes in roots associated with mycorrhizal formations (Rupp et al., 1989a; Blake and Linderman, 1992; Alvarez and Linderman, 1983), there is no direct evidence of plant growth regulator (PGR) production by mycorrhizal fungi being responsible for morphological and physiological responses of a plant to mycorrhizal fungus colonization. One way to indirectly assess this question, however, is to test for differential plant biochemical and morphological responses to mycorrhizal fungi possessing reliable and stable differential capacities to produce IAA and ethylene *in vitro*. This paper presents the results of the examination of changes in endogenous IAA levels of conifer seedling roots and seedling morphology in response to inoculation with mycorrhizal fungi having differential *in vitro* IAA and ethylene production capability. Changes within the plant correlative to differential *in vitro* mycorrhizal fungal plant growth regulator production levels are considered indirect evidence of mycorrhizal fungal mediation of plant response.

2. Materials and Methods

Characterization of mycorrhizal fungal isolates for in vitro IAA production capacity

Gas chromatograph-mass spectrometry analyses were used to determine the levels of IAA in mycelia and culture filtrates of mycorrhizal fungi (Scagel,

1994). IAA concentration of fungal mycelia was determined by homogenizing the filtered mycelium in 80% cold methanol in a tissue grinder fitted with a Teflon pestle. The internal standard d_2-IAA was added and the homogenate allowed to stand at 5°C for 20 h for extraction. The sample was then filtered through glass fiber, and the mycelium dried overnight at 90°C for dry weight determinations. The methanol was evaporated and 50 ml 0.05 M K_2HPO_4 buffer (pH 8.0) was added to the residual water phase. The pH was lowered to 2.7 by addition of 2.5 M H_3PO_4 and the solution extracted (50 ml × 3) with ethyl ether. The ether fraction was dried over Na_2SO_4 and evaporated to dryness.

IAA concentration in the culture filtrates of the test fungi was determined by adding an internal standard of d_2-IAA to the culture filtrate and adjusting the pH to 2.7 with 2.5 M H_3PO_4. The culture filtrate was then extracted with ethyl ether, the ethereal fraction dried over Na_2SO_4 and evaporated to dryness.

Residues from ether extractions of mycelia and culture filtrates were dissolved in 0.1 ml pyridine and 0.1 ml N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA). After reacting at 50°C for 2 h, the IAA was quantified using GC-MS. Detection limit on the GC-MS was 10 pg per injection. Each sample was injected 3 times using a Carlo Erba (Fractovap MOD2900) GC-Finnigan MS (3200F) with an on-line computer system (6000). An OV11 glass WCOT (25 m × 0.28 mm ID) column was used. Injector temperature was 250°C and detector temperature was 300°C. The oven temperature was isothermal at 220°C. Carrier gas flow was 0.82 ml min^{-1} with a split ratio of 1:30. The mass spectrometer was operated in the multiple ion detection mode (MID). The mass spectra were all run with 70 KeV. The transfer line temperature was 300°C with an ion source temperature of 100°C. Molecular ions (319, 321) and the base peaks (202,204) were chosen for monitoring; the retention time was 3.45 min. Values are expressed in ng IAA mg^{-1} dry weight of mycelium.

The deuterated IAA was prepared according to Caruso et al. (1978) and used as an internal standard. Response factor was determined before each quantification series and was taken from a calibration curve. Values are expressed in ng IAA mg^{-1} dw of mycelium. Ethyl ether was freshly distilled prior to use to eliminate peroxidases.

Based on the extent and consistency of *in vitro* IAA production, mycorrhizal fungal isolates were categorized into groups with different *in vitro* IAA production capacities. Relative attributes of mycorrhizal fungal isolates used in this study are indicated in groups of high (HI), moderate (MI) and low (LI) *in vitro* IAA producers. Fungal species with representatives at all three levels of IAA production (HI, MI, LI) were used in inoculation experiments (Table 1).

Table 1. Experimental fungal isolates and their *in vitro* IAA and ethylene production characterisitcs (see Scagel, 1994)

Fungal isolate	Fungal species	PGR production designator*	Tree species**	Isolate number	Isolate origin
Ll-7	*Laccaria laccata*	LI	PSME, PICO, PIEN	S-22	Balco Inc.- Kamloops, BC, CA
Ll-11	*L. laccata*	HI	PSME, PICO, PIEN	B-101a	USDA-FS, Corvallis, OR
Ll-19	*L. laccata*	MI	PSME, PICO, PIEN	CS-23	CFS - Horse Mnt., BC, CA
Ll-2	*L. laccata*	ME	PIEN	Lala1	Univ. Wash. - Bald Mnt., OR
Ll-14	*L. laccata*	LE	PIEN	S-443	USDA-FS- Lost Prairie, OR
Ll-17	*L. laccata*	HE	PIEN	505	Balco Inc.-Kamloops, BC, CA
Rv-5	*Rhizopogon vinicolor*	LE	PSME	CS-31	Reisolation from USDA-FS #9428
Rv-7	*R. vinicolor*	HE	PSME	CS-33	Reisolation from USDA-FS #9444
Rv-8	*R. vinicolor*	ME	PSME	CS-34	Reisolation from USDA-FS R. vini
Sl-1	*Suillus luteus*	HE	PICO	CS-42	CFS - Vernon, BC, CA
Sl-2	*S. luteus*	LE	PICO	CS-41	CFS - Horse Mnt., BC, CA
Sl-3	*S. luteus*	ME	PICO	CS-36	CFS - Savona, BC, CA

*Inoculation treatments: C = control; HI = high IAA producer; HE = high ethylene producer; MI = moderate IAA producer; ME = moderate ethylene producer; LI = low IAA producer; LE = low ethylene producer. **Tree Species used in inoculations. PSME = *Psuedotsuga menziesii*, PICO = *Pinus contorta*, PIEN = *Picea englemanii*.

Characterization of mycorrhizal fungal isolates for in vitro ethylene production capacity

Gas chromatography was used to determine the levels of ethylene produced by mycorrhizal fungal cultures (Scagel, 1994). Cultures were grown at 20°C in the dark for 3 days and then placed for 60 min in a laminar flow hood without caps. After flushing the air space with sterile air, the tubes were sealed with rubber serum caps previously sterilized in 70% ethanol. After incubation in the dark at 20°C for another 96 h, a 1 cm^3 gas sample was withdrawn from each culture tube and analyzed for ethylene.

Test samples were injected into a Hewlett-Packard 5890A gas chromatograph equipped with a flame-ionization detector and a 1.8 m Poropak Q (80–100 mesh) stainless steel column. Column temperature was maintained at 70°C, detector and injector temperature at 150°C, and nitrogen carrier gas flowed at 30 ml min^{-1}. Ethylene was identified and quantified by cochromatography with a known amount of ethylene-in-air standard (6 µl l^{-1}).

Measurements were repeated weekly for 2 more weeks. Culture tubes were flushed with sterile air 96 h before sampling for ethylene. After the final ethylene measurement, hyphal fragments from fungal cultures were plated to check for viability and contamination. The remaining mycelium in each tube was filtered, washed, dried (70°C for 24 h) and weighed. Values are expressed in ng cm^{-3} mg^{-1} h^{-1}).

Based on *in vitro* ethylene production amounts and consistency over time, mycorrhizal fungal isolates were grouped with regard to their different *in vitro* ethylene production capacities. Relative attributes of mycorrhizal fungal isolates used in this study are indicated in groups of high (HE), moderate (ME) and low (LE) *in vitro* ethylene producers. Fungal species with representatives at all three levels of ethylene production (HE, ME, LE) were used in inoculation experiments (Table 1).

Mycorrhizal fungal isolates and inoculum production

The mycorrhizal fungi used in this study and their *in vitro* plant growth regulator production characteristics are given in Table 1. Characterization procedures for these isolates are given in Scagel (1994). Fungi were grown in flasks containing 2 l of semi-solid modified Melins-Norkrans (MMN) medium (Molina and Palmer, 1982). Cultures were stored in a dark incubator at 20°C and were agitated daily. After 5 weeks of growth, mycelium was harvested by filtration, fragmented using a Waring Blender (in 1 l sterile distilled water (dH_2O), and used to prepare a mycorrhizal inoculum mixture with equal volumes of fungal suspension, vermiculite and peat moss.

Seedling culture

Seeds of Interior Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (PSME), Englemann Spruce (*Picea englemanii* Parry) (PIEN), and Lodgepole Pine (*Pinus contorta* Dougl.) (PICO) were treated in 30% hydrogen peroxide for 2 h to substitute for cold stratification, washed in sterile dH_2O overnight and germinated on water agar at room temperature. Seedlings supporting growth of microbial contaminants were discarded. One week after germination, seedlings were transplanted into a mixture of steam sterilized vermiculite and peat moss. At 8 weeks, seedlings were transplanted into 21 × 4 cm diameter tubes containing a mixture of 3 parts air-steam pasteurized (70°C for 30 min.) soil (sandy loam with 0.8% organic matter, available P (Bray) 10 mg kg^{-1}, pH 6.5) and 1 part (v/v) mycorrhizal fungus inoculum.

Seedlings were maintained in a glass house for 4 months under a 16 h photoperiod of 10.5 klx (200 uE m^{-2} s^{-1} for 400–700 nm) and day/night temperatures of 23/18°C. Plants were watered as needed, although after fertilization at least 24 h elapsed before irrigation. Modified Hoagland's solution was applied to the growth medium (20 ml $cavity^{-1}$) every two weeks.

Seedling morphology

Seedling height and root collar diameter were measured two months after inoculation. At four months, seedlings were harvested, and final height and root collar diameter were recorded. Stems and roots not subsampled for IAA analysis were dried at 100°C for 72 h and their weight determined.

Growth was measured using the procedures described by Hunt (1982) and Ledig (1974). Relative growth rate (RGR) was determined by the equation: $RGR = (\ln X_n - \ln X_{n-1})/(T_n - T_{n-1})$, where X_n is the parameter at time T_n and X_{n-1}, is the parameter at time T_{n-1}. Thus, the calculated rate is the difference between the natural log of a parameter at (n) time and the natural log of the parameter at (n–1) time divided by the time period. The values are reported as percentage increases (%/time period).

Mycorrhizal colonization

Mycorrhiza formation at 4 months was determined by counting the number of primary laterals on a seedling and determining the percentage that were mycorrhizal. Random subsamples of half the root system were used to quantify the number of primary laterals per ml of root system. Representative mycorrhizae were examined for Hartig net development, and reisolations of the mycorrhizal fungi were performed.

Root IAA analysis

Samples for IAA analysis were taken from all 10 experimental seedlings in each conifer species-fungal isolate combination. Immediately after harvest, samples of root tissue were immersed in liquid nitrogen and stored at –20°C in the dark. All tissue was freeze-dried prior to extraction. Extraction was performed using modified methods of Cohen et al. (1987), Miller et al. (1990) and Miller and Roberts (1984) by grinding 6 mg of root tissue in 5 ml g^{-1} of 80% 2-propanol in 0.2 M imidazole buffer. $^{13}C_6$-(benzene ring) IAA (0.1–1 µg g^{-1} tissue) and 50,000 to 1,000,000 cpm of ^{3}H-IAA was added, and the extract was allowed to equilibrate for 1 h. The extract was centrifuged and supernatant pooled, then divided into volumes equivalent to 1 mg of tissue. Two replicates each were purified and quantified for the endogenous concentration of free-IAA and the two conjugated forms, ester- and amide-bound.

For purification of free-IAA, the volume of the 80% 2-propanol extract was increased with distilled water so that the organic solvent made up less than 20% of the total volume. This was then loaded into and passed through a preconditioned "Baker" amino column (3 ml) (preconditioned with one volume hexane followed with methanol, distilled water and 0.02 M imidazole buffer). The column was then rinsed with one column volume of ethyl acetate and acetonitrile, and then eluted with methanol with 2% acetic acid. The eluant was dried *in vacuo* and resuspended in 100 µl of 50% methanol. Further purification of the extract was obtained by HPLC on a C_{18} reverse-phase column (4.6 × 125 MM Whatman Partisil DS-3 column) with flow rate of 1 ml min^{-1} and 20% acetonitrile and 1% acetic acid as the mobile phase). Radioactive fractions were pooled, dried, resuspended in 100 µl of methanol and methylated with diazomethane. After methylation, the extract was evaporated under nitrogen and resuspended in 20 µl of ethyl acetate. HPLC analysis of extracts were performed by isocratic reverse phase on a Waters Associates Bondpak C_{18} Column (0.39 × 30 cm) equilibrated in water:acetonitrile:acetic acid (80:20:1) (v:v:v) at room temperature. Detection was at 280 nm and quantification was made by area integration through the Waters Data System. Values are given in ng g^{-1} root dry weight.

Hydrolysis and partial purification of IAA conjugates was carried out by hydrolysis with 1 N or 7 N NaOH for ester or amide conjugates, respectively. Prior to hydrolysis, the 2-propanol was removed *in vacuo*. For the 1 N hydrolysis, an equal volume of 2 N NaOH was added to the aqueous phase and hydrolysed at room temperature for 1 h. Appropriate amounts of NaOH were added directly to the plant extract for the 7 N hydrolysis. These samples were hydrolysed for 3 h at 100°C, after which the extracts were acidified with HCl to a pH of 2.5.

The hydrolysates were passed through a preconditioned (methanol and distilled water) Baker C_{18}-column. The column was rinsed with two volumes of distilled water and eluted with acetonitrile. An imidazole buffer (0.02 M, pH 6.9) was then added to the eluant to decrease the proportion of organic phase to 20% and neutralize the extract. The remaining purification steps were the same as described for the purification of free-IAA.

Experimental design and statistical analysis

For each of the three tree species (Douglas-fir, Englemann Spruce, Lodgepole Pine), six fungal isolates (and 1 control) and ten replicates of each treatment (7×10 = 70 seedlings/tree species) were used in a completely randomized design. Isolates were treated as distinct treatments and no nesting was done for fungal species. Means for each replication were analyzed for differences within species by one-way analysis of variance (ANOVA) using the SYSTAT (Wilkinson, 1988) statistical package. If differences were detected, means were compared using Fischer's Protected Least Significant Difference (FPLSD) test (p=0.05). Relationships between variables were assessed by Pearson Correlation Analysis using SYSTAT (Wilkinson, 1988) statistical package.

3. Results

Seedling growth responses to inoculation

Tree seedling roots colonized by mycorrhizal fungi that had been categorized by their capacity for *in vitro* production of IAA and ethylene, showed differential morphological responses to colonization 2 and 4 months after inoculation. All inoculated seedling heights were equal to or greater than non-inoculated controls. Seedling height 2 and 4 months after inoculation (Tables 2–4) showed differential responses to mycorrhizal colonization. Englemann Spruce (Table 3) and Lodgepole Pine (Table 2) showed the greatest response to inoculation with isolates characterized by their IAA production (Ll-7, Ll-11, Ll-19). There was very little variation in Douglas-fir height between different inoculation treatments (Table 4).

In general, mycorrhizal colonization had little effect on Lodgepole Pine and Englemann Spruce seedling root collar diameter when compared to non-inoculated control seedlings. Root collar diameter of Douglas-fir seedlings showed the greatest response to inoculation with isolates characterized by their IAA production (Ll-7, Ll-11, Ll-19). Seedling root collar diameter responses to inoculation were both conifer species and fungal isolate specific (Tables 2 and 4).

Table 2. Morphological characteristics of Lodgepole Pine seedlings 2 and 4 months after inoculation

Characteristic	Inoculation treatment*						
	C	HI	HE	MI	ME	LI	LE
Height growth							
2 month (cm)	2.43 ab***	2.64 bcd	2.87 d	2.63 bcd	2.49 abc	2.69 cd	2.34 a
4 month (cm)	8.64 bc	9.09 c	6.97 a	10.13 d	7.68 ab	9.78 cd	9.59 cd
Relative growth rate** (%)	63.50 c	61.20 c	44.40 a	67.80 cd	56.80 b	64.30 cd	70.60 d
Diameter growth							
2 month (mm)	1.34 b	1.29 b	0.87 a	1.43 b	0.98 a	0.78 a	1.28 b
4 month (mm)	1.54 abc	1.63 bc	1.23 ab	1.72 c	1.16 a	1.18 a	1.66 bc
Relative growth rate** (%)	6.90 a	11.70 c	17.30 d	9.20 b	8.40 b	20.10 d	12.90 c
Mycorrhizal colonization							
Lateral root colonization (#)	9.60 a	13.70 c	11.30 bc	12.40 bc	10.60 b	10.80 b	10.00 b
Lateral root colonization (%)	9.20 a	89.40 c	65.90 bc	88.30 c	53.20 b	65.20 bc	54.60

*Inoculation treatments: C = control; HI = high IAA producer (LI-11); HE = high ethylene producer (SI-3); MI = moderate IAA producer (LI-19); ME = moderate ethylene producer (SL-1); LI = low IAA producer (LI-7); LE = low ethylene producer (SI-2). **Relative growth rate = RGR = $(\ln X_n - \ln X_{n-1})/(T_n - T_{n-1})$, where X_n is the parameter at time T_n and X_{n-1}, is the parameter at time T_{n-1}. ***Means in the same row followed by the same letter are not significantly different ($p<0.05$; Fisher's Protected LSD)

Table 3. Morphological characteristics of Englemann Spruce seedlings 2 and 4 months after inoculation

Characteristic	Inoculation treatment* C	HI	HE	MI	ME	LI	LE
Height growth							
2 month (cm)	2.40 ab***	4.15 d	3.25 c	3.08 bc	2.77 bc	1.92 a	1.93 a
4 month (cm)	9.86 b	12.01 c	10.59 bc	13.86 d	7.35 a	9.29 b	10.09 b
Relative growth rate** (%)	70.7 b	53.1 a	59.6 a	75.3 b	48.5 a	73.7 b	79.8 b
Diameter growth							
2 month (mm)	0.79 a	1.05 bc	1.46 c	0.81 a	1.19 b	0.88 a	0.96 ab
4 month (mm)	1.24 a	1.48 a	2.24 b	1.39 a	1.47 a	1.31 a	1.27 a
Relative growth rate** (%)	22.9 c	17.2 b	21.4 bc	26.5 c	10.6 a	19.3 b	14.3 ab
Mycorrhizal colonization							
Lateral root colonization (#)	0.50 a	16.4 d	11.9 bc	14.1 cd	10.5 b	12.1 c	10.9 b
Lateral root colonization (%)	3.9 a	92.0 c	74.1 bc	81.3 bc	65.8 b	69.3 b	67.4 b

*Inoculation treatments: C = control; HI = high IAA producer (Ll-11); HE = high ethylene producer (Ll-17); MI = moderate IAA producer (Ll-19); ME = moderate ethylene producer (Ll-2); LI = low IAA producer (Ll-7); LE = low ethylene producer (Ll-14). **Relative growth rate = RGR = $(\ln X_n - \ln X_{n-1})/(T_n - T_{n-1})$, where X_n is the parameter at time T_n and X_{n-1}, is the parameter at time T_{n-1}. ***Means in the same row followed by the same letter are not significantly different ($p<0.05$; Fisher's Protected LSD)

Table 4. Morphological characteristics of Douglas-Fir seedlings 2 and 4 months after inoculation

Characteristic	Inoculation treatment*						
	C	HI	HE	MI	ME	LI	LE
Height growth							
2 month (cm)	2.35 b***	3.71 e	2.99 d	2.33 ab	2.64 c	2.65 c	2.25 a
4 month (cm)	7.64 a	11.86 c	7.14 a	9.89 b	7.34 a	7.65 a	7.33 a
Relative growth rate** (%)	57.2 bc	58.9 c	43.2 a	72.4 d	51.5 b	53.6 b	66.5 d
Diameter growth							
2 month (mm)	0.75 a	0.79 a	0.89 ab	0.96 ab	1.06 b	1.04 b	0.82 a
4 month (mm)	1.40 ab	1.65 ab	1.07 a	1.74 b	1.26 ab	1.83 b	1.31 ab
Relative growth rate** (%)	31.2 d	36.8 d	10.6 a	29.7 cd	8.7 a	19.1 b	23.4 b
Mycorrhizal colonization							
Lateral root colonization (#)	2.5 a	10.4 bc	11.9 c	12.5 c	15.0 d	11.2 bc	9.4 b
Lateral root colonization (%)	15.1 a	68.0 bc	73.1 c	76.7 c	77.1 c	63.4 bc	54.0 b

*Inoculation treatments: C = control; HI = high IAA producer (Ll-11); HE = high ethylene producer (Rv-7); MI = moderate IAA producer (Ll-19); ME = moderate ethylene producer (Rv-8); LI = low IAA producer (Ll-7); LE = low ethylene producer (Rv-5). **Relative growth rate = RGR = $(\ln X_n - \ln X_{n-1})/(T_n - T_{n-1})$, where X_n is the parameter at time T_n and X_{n-1}, is the parameter at time T_{n-1}. ***Means in the same row followed by the same letter are not significantly different ($p<0.05$; Fisher's Protected LSD).

Mycorrhizal inoculation increased shoot dry weight and root:shoot ratio of only some plants when compared to non-inoculated control seedlings (Fig. 1). Isolate HI (LI-11) and HE (SI-3) significantly increased both shoot dry weight and root:shoot ratio of inoculated Lodgepole Pine, and Englemann Spruce shoot dry weight and root:shoot ratio was increased by inoculation with only isolates HE (LI-17) and ME (LI-2). All inoculation treatments except LI (LI-19) increased the shoot dry weight and root:shoot ratio of Douglas-fir seedlings 4 months after inoculation.

Inoculation of Douglas-fir and Englemann Spruce seedlings with most isolates of mycorrhizal fungi resulted in significant increases in root dry weight over non-inoculated seedlings (Fig. 1). Root dry weight of Lodgepole Pine seedlings was significantly increased by isolates capable of high and low *in vitro* ethylene and IAA production (LI-11, LI-7) .

Number of primary lateral roots per ml was increased by inoculation with almost all isolates on Lodgepole Pine and Englemann Spruce, but only with some isolates on Douglas-fir (Fig. 2). Colonization was also isolate dependant (Table 2–4), but seedlings with the greatest colonization were usually those inoculated with isolates characterized by their *in vitro* IAA production.

Conifer root IAA responses to inoculation

Effects of inoculation on free and conjugate (ester) IAA of conifer roots varied with tree species (Fig. 3). Free IAA of roots was increased significantly by colonization with isolates HI (LI-11) and HE (Rv-7) on Douglas-fir but not Englemann Spruce or Lodgepole Pine. Only isolate HI (LI-11) significantly increased IAA conjugates (ester) in all three tree species.

Correlations between in vitro IAA production and seedling morphology

Correlations between *in vitro* IAA production by mycorrhizal fungi and seedling morphology were tree species specific (Table 5). No single morphological trait was significantly correlated to *in vitro* IAA production for all three tree species. Most morphological attributes not significantly correlated to *in vitro* IAA production capacity did show positive relationships to fungal IAA production capacity.

Correlations between in vitro ethylene production and seedling morphology

In vitro ethylene production by mycorrhizal fungi was positively correlated to relative height growth, primary lateral colonization and root dry weight for almost all three tree species (Table 5).

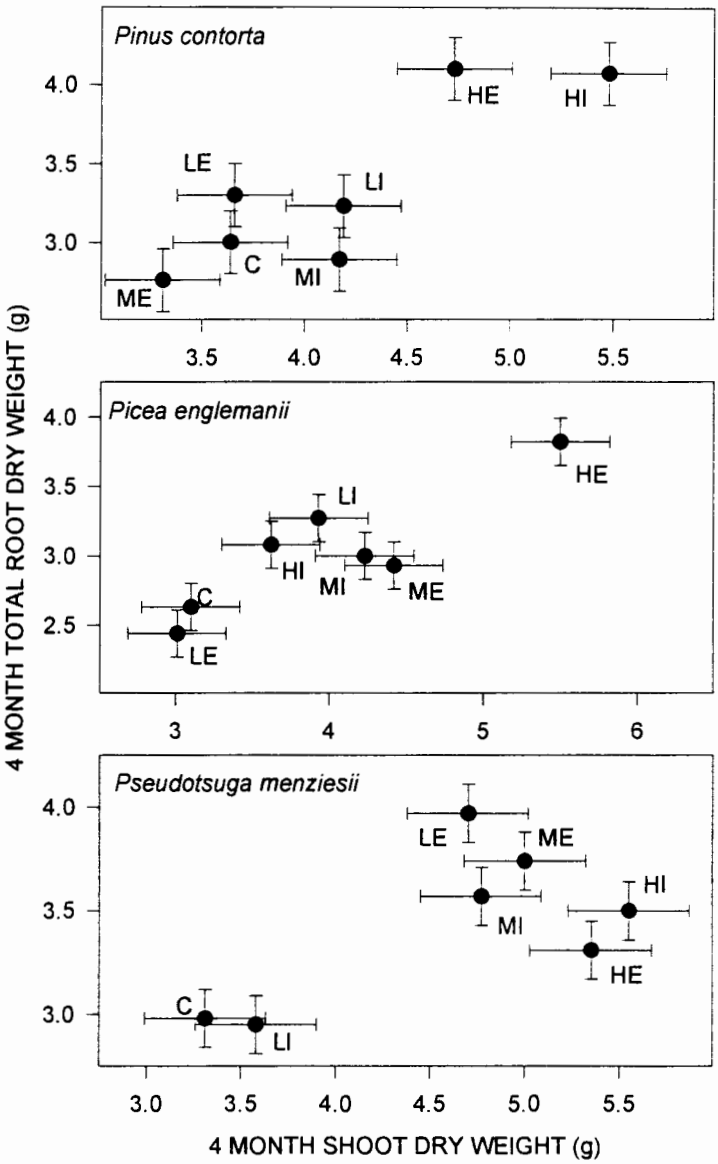

Figure 1. Relationship between above-ground dry weight and root dry weight of 4 month old *Pinus contorta, Picea englemanii* and *Pseudotsuga menziesii* seedlings inoculated with mycorrhizal fungal isolates capable of differential in vitro IAA and ethylene production. Inoculation treatments: C = control; HI = high IAA producer (Ll-11); HE = high ethylene producer (Rv-7); MI = moderate IAA producer (Ll-19); ME = moderate ethylene producer (Rv-8); LI = low IAA producer (Ll-7); LE = low ethylene producer (Rv-5). Bars represent Least Significant Differences ($p<0.05$; Fischer's Protected LSD).

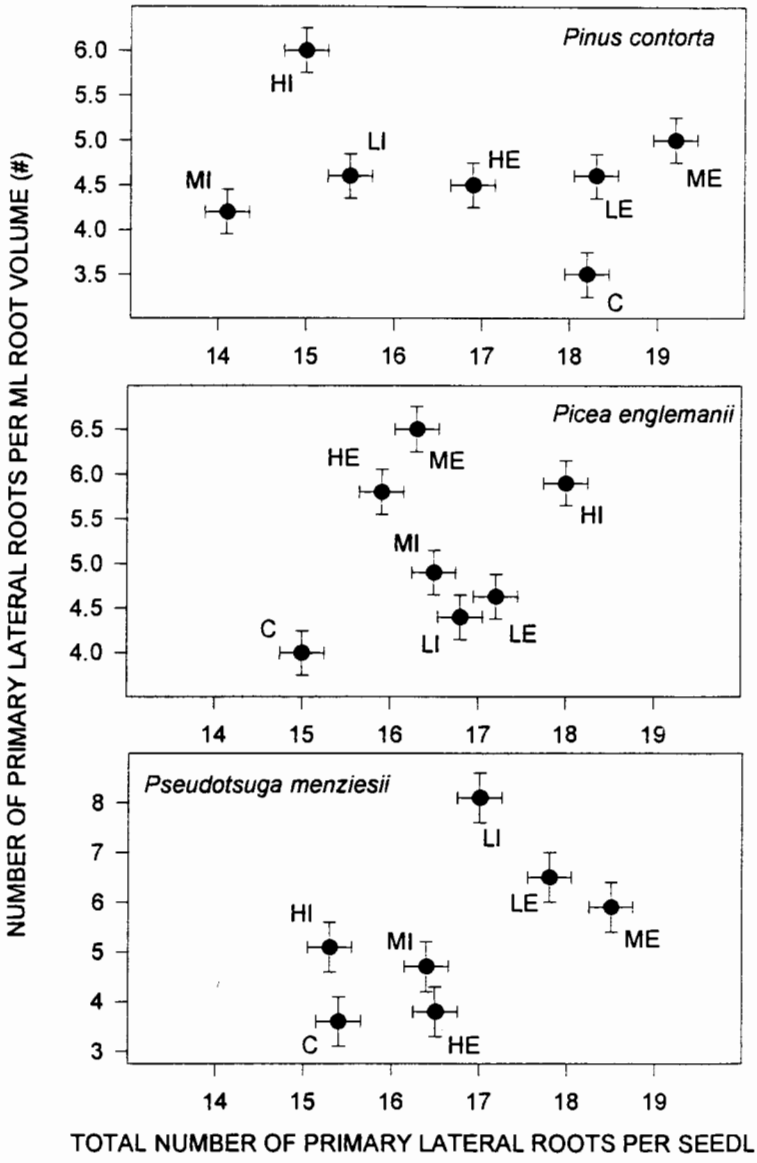

Figure 2. Relationship between number of primary later roots (per ml root volume) and total number of primary lateral roots (per seedling) of 4 month old *Pinus contorta, Picea englemannii* and *Pseudotsuga menziesii* seedlings inoculated with mycorrhizal fungal isolates capable of differential *in vitro* IAA and ethylene production. Inoculation treatments: C = control; HI = high IAA producer (Ll-11); HE = high ethylene producer (Rv-7); MI = moderate IAA producer (Ll-19); ME = moderate ethylene producer (Rv-8); LI = low IAA producer (Ll-7); LE = low ethylene producer (Rv-5). Bars represent Least Significant Differences ($p<0.05$; Fischer's Protected LSD).

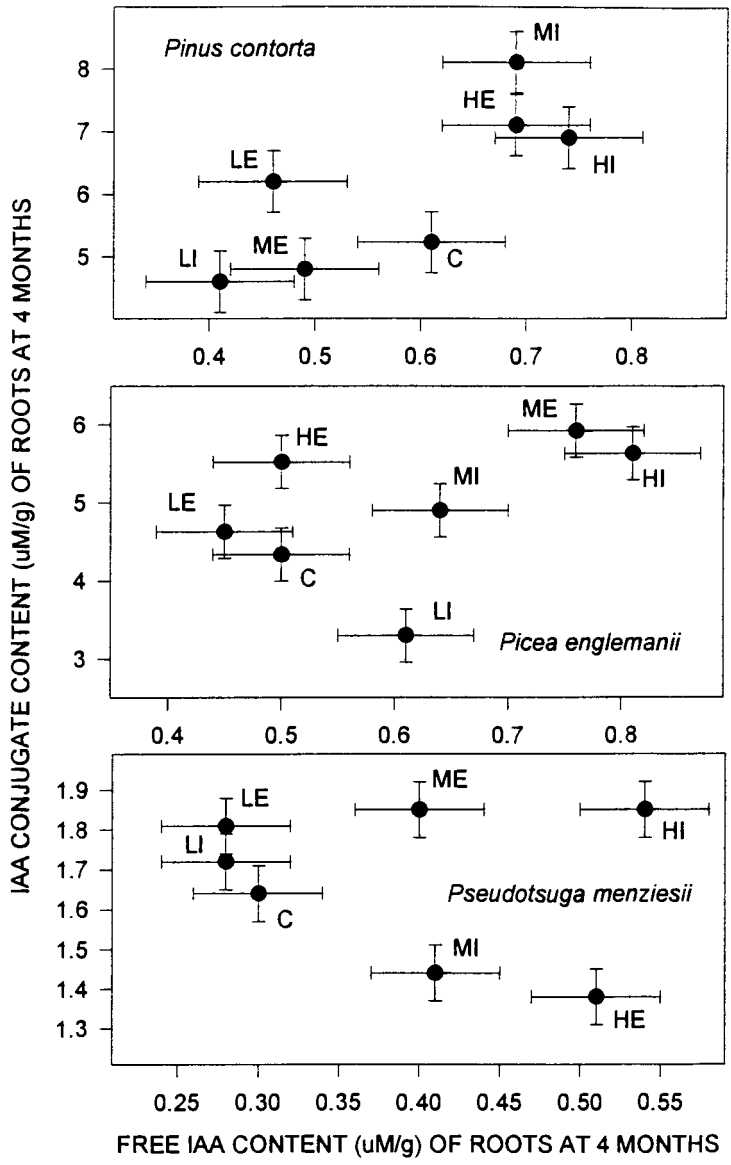

Figure 3. Relationship between IAA conjugate content of roots and free IAA content of roots of 4 month old *Pinus contorta, Picea englemannii* and *Pseudotsuga menziesii* seedlings inoculated with mycorrhizal fungal isolates capable of differential in vitro IAA and ethylene production. Inoculation treatments: C = control; HI = high IAA producer (LI-11); HE = high ethylene producer (Rv-7); MI = moderate IAA producer (LI-19); ME = moderate ethylene producer (Rv-8); LI = low IAA producer (LI-7); LE = low ethylene producer (Rv-5). Bars represent Least Significant Differences ($p<0.05$; Fischer's Protected LSD).

Correlations between in vitro PGR production, seedling morphology, and root IAA content

Endogenous root IAA and *in vitro* fungal IAA production capacity were significantly correlated for Lodgepole Pine and Englemann Spruce (Table 5). Endogenous root IAA and *in vitro* fungal ethylene production capacities were well correlated for all tree species. Douglas-fir endogenous root IAA was negatively correlated with *in vitro* IAA production capacity (Table 5).

Correlations between root IAA content and seedling morphology

Correlations between root IAA content and seedling morphology were tree species specific (Table 6). No single morphological trait was significantly correlated to root IAA content for all three tree species. Most morphological attributes not significantly correlated to root IAA content did show positive relationships to root IAA content.

4. Discussion

Root growth, as with other plant physiological processes, is regulated by phytohormones. Plant growth regulator production, metabolism and distribution within the plant is influenced by both physical and biological environmental factors. Plant growth regulator activity is influenced by concentration in the tissue, both absolute and in relation to other plant growth regulators, and tissue sensitivity. In this study, we sought to evaluate the potential for mycorrhizal fungi to influence the endogenous concentration of the major root-promoting plant growth regulators, IAA and ethylene. The overall hypothesis was that ectomycorrhizal fungi vary in their capacity to produce IAA and ethylene *in vitro*, and that high producers would have a greater influence on root growth of conifer seedlings than low producers, resulting in greater plant and root growth and thus increased potential for survival.

Previous *in vitro* production studies (Scagel, 1994) showed that there is considerable variation in IAA and ethylene production capacity, both within and between species of mycorrhizal fungi, allowing for the fungi to be clustered into groups based on their high, medium or low capacity to produce plant growth regulators. Selected isolates from these groups were evaluated in this study for their differential effects on conifer morphology and physiology to test whether high producers of IAA or ethylene would influence the level of endogenous IAA in roots more than low producers. A significant correlation between production capacity and increased IAA level in mycorrhizal fungus-

Table 5. Pearson Correlation Coefficients (p=0.05) between *in vitro* IAA or ethylene production by mycorrhizal fungi (6 isolates/tree species) and inoculation responses to colonization on three conifer species four months after inoculation

Characteristic	*In vitro* IAA production			*In vitro* ethylene production		
	PSME*	PICO	PIEN	PSME	PICO	PIEN
Height growth						
2 months	ns	0.68	0.75	0.43	ns	0.73
4 months	0.51	ns	0.61	ns	0.51	ns
Relative growth rate	0.57	ns	ns	0.52	0.69	0.66
Diameter growth						
4 months	ns	9.67	ns	ns	ns	0.70
Relative growth rate	0.54	ns	ns	0.42	ns	ns
Dry weight						
Shoot dry weight	ns	ns	ns	ns	0.49	ns
Root dry weight	ns	0.44	ns	ns	0.49	0.81
Root growth and root IAA content						
Number primary lateral roots	0.81	ns	0.76	ns	ns	ns
Lateral roots per ml	0.79	0.56	ns	0.79	ns	ns
Primary laterals colonized	ns	0.58	ns	0.54	0.59	0.52
Root IAA content (conjugate)	ns	0.41	0.75	0.49	0.62	0.79

*Tree species inoculated PSME = *Psuedotsuga menziesii*, PICO = *Pinus contorta*, PIEN = *Picea englemanii*. ns = No significant correlation between variables ($p<0.05$).

inoculated roots would be indirect evidence of mycorrhiza-mediated IAA production in conifer roots.

The data from this study indicate that the capacity for IAA or ethylene production by test fungi was not always correlated with elevated IAA concentrations in roots of all three tree species. The varied response was apparently influenced by tree species-fungal symbiont interactions. Only one high IAA producing fungus, *Laccaria laccata* (Ll-11) consistently increased endogenous root IAA above control roots for all three tree species tested. However, there was a strong correlation between ethylene production capacity and endogenous IAA content of roots of all three tree species. This is clear indirect evidence that ethylene production by the fungal symbiont stimulates IAA production in the roots. Even though others have shown ethylene stimulation of root growth in conifers (Graham and Linderman, 1981; Alvarez

Table 6. Pearson Correlation Coefficients (p=0.05) between root IAA content (free IAA and IAA conjugates) of three conifer species and inoculation responses to colonization by mycorrhizal fungi (6 isolates/tree species) four months after inoculation

Characteristic	Free IAA content of roots			IAA conjugate content of roots		
	PSME*	PICO	PIEN	PSME	PICO	PIEN
Height growth						
2 months	0.80	0.80	0.62	ns	0.72	0.73
2 months	0.57	ns	ns	ns	ns	ns
Relative growth rate	ns	ns	–0.70	ns	ns	–0.76
Diameter growth						
2 months	ns	ns	ns	ns	0.50	0.64
4 months	ns	0.45	ns	ns	0.61	0.49
Dry weight						
Shoot dry weight (4 months)	0.55	0.67	ns	ns	0.53	ns
Root dry weight	ns	0.51	ns	0.70	ns	ns
Root growth and root IAA content						
Number primary lateral roots	ns	0.47	0.46	ns	0.59	0.68
Lateral roots per ml	0.48	0.21	0.68	0.54	ns	0.84
Primary laterals colonized	0.54	ns	0.48	ns	0.61	ns
Root IAA content	0.75	0.41	ns	0.49	0.62	0.79

*Tree species inoculated PSME = *Psuedotsuga menziesii*, PICO = *Pinus contorta*, PIEN = *Picea englemanii*. ns = No significant correlation between variables ($p<0.05$).

and Linderman, 1986; Rupp et al., 1989a,b), it had not been previously shown that mycorrhiza-produced ethylene could increase IAA production in colonized roots. It has been shown, however, that flooding stress-induced ethylene could induce elevated IAA in tomato plants (Wample and Reid, 1979).

The last portion of the hypothesis tested here was that IAA or ethylene production by mycorrhizal fungi could affect root growth as mediated by increased IAA production. Alvarez and Linderman (1986) and Blake and Linderman (1992) showed that increased ambient ethylene in cold-stored conifer seedlings could stimulate root growth capacity at outplanting, but the mechanism was not determined. Furthermore, there was considerable variation in tree species response, both in terms of increased root growth

capacity and seedling growth and survival. A significant correlation between levels of IAA in the root and plant growth responses would be indirect evidence of IAA mediation of plant growth by mycorrhizal fungi.

In this study, levels of endogenous IAA, as potentially affected by different mycorrhizal fungi, was not consistently correlated with increased seedling growth for the parameters measured, i.e. height, stem diameter, root and shoot mass, and number of primary lateral roots per volume of the root system. There was, however, clearly mycorrhizal symbiont-tree species variation. For example, Douglas-fir height and stem dry weight were significantly correlated to the free IAA content of the roots, while the correlations were not as strong for Lodgepole Pine or Englemann Spruce. Although some morphological features showed significant responses to inoculation, responses could not be consistently correlated to endogenous root IAA contents for all three tree species.

There can be many factors that contribute to the inconsistent response or lack of correlation of IAA or ethylene producing capacity by ectomycorrhizal fungi and induced plant and root growth as mediated by changes in endogenous IAA levels. As Trewavas (1981) suggested, there is no evidence of plant growth regulators acting via changes in the concentration, and that all change in response must be attributed to changes in tissue sensitivity. This is the reason frequently given for lack of correlation between hormone concentration measured in a tissue and response. Changes in endogenous concentrations of IAA in tissues are often much smaller than would be expected relative to the magnitude of the changes in growth or development observed. It could be that response is the result of changes in the number of receptors (receptivity), receptor affinity, or a change in the subsequent chain of events (response capacity) (Firn, 1986). Furthermore, plant responses may not be in relation to actual levels of endogenous IAA, but rather to changes in the ratio of IAA to other plant regulators (Davies, 1986).

In this study, many environmental or cultural conditions could have influenced the capacity of selected ectomycorrhizal fungi to influence plant growth via induced changes in endogenous IAA levels in roots. A given set of plant growth conditions could favour the response by one tree species but not another. In that regard, the general hypothesis that any mycorrhizal fungus with high *in vitro* IAA or ethylene producing capacity would have greater capacity to increase IAA production in roots, is not true. Too many other variables influence the symbiotic association that cannot be known from *in vitro* tests. High IAA production by the fungus *in vitro* does not necessarily mean high IAA production at any selected time in the symbiosis. Furthermore, different tree species may have different capacity to produce IAA or ethylene in the absence of mycorrhizae, and that capacity can vary with developmental

stage of the plant and sensitivity to these growth regulators. For these reasons, and probably many more, it appears to be impossible to predict that high hormone-producing ectomycorrhizal fungi would stimulate root growth.

While the results presented here do not consistently confirm the hypothesis that high capacity for *in vitro* IAA and/or ethylene production by ectomycorrhizal fungi will induce increased endogenous IAA in root tissue and subsequent root growth, the data suggest that under specific conditions the hypothesis could be true. The mycorrhizal fungus-tree species combination is highly specific, so that it is not surprising that the sensitivity range of each conifer species to different growth regulators could be different (Zaerr and Lavender, 1980; Lavender, 1989). Nevertheless, in a dynamic plant growth system, the interaction could be highly effective and plant response could be clearly positive. The results presented here clearly support the contention that there is a relationship between fungus-produced IAA and endogenous IAA under some conditions, and furthermore, that there is a fungus-produced ethylene relationship to elevated endogenous IAA concentration in roots. These relationships could ultimately affect growth responses and survival of conifer seedlings outplanted in reforestation efforts.

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