APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1987, p. 2908–2913
0099-2240/87/122908-06$02.00/0

Vol. 53, No. 12

Biosynthesis of Indole-3-Acetic Acid by the Pine Ectomycorrhizal Fungus *Pisolithus tinctorius*

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Received 7 July 1987/Accepted 28 September 1987

Previous work has indicated that anatomical and morphological changes (stunting and dichotomy) in roots of various conifers may be influenced by plant-growth-regulating substances secreted by mycorrhizae. Indole-3-acetic acid (IAA) has been tentatively identified as a major auxin produced by some selected ectomycorrhizae. We report the isolation and detection of IAA as a secondary metabolite from *Pisolithus tinctorius* by thin-layer chromatography, high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent (monoclonal antibody) assay (ELISA), and unequivocal identification by gas chromatography-mass spectrometry (GC-MS). The thin-layer chromatography methods for auxin isolation described here are novel, with the use of heptane-acetone-glacial acetic acid as the migrating solvent and formaldehyde, H_2SO_4, and vanadate in detection. The acidic extract of the culture supernatant was methylated with ethereal diazomethane to detect IAA as methyl-3-IAA by HPLC, ELISA, and GC-MS. The quantitative amount of IAA detected ranged from 4 to 5 μmol liter^{-1} by HPLC and ELISA. Another unidentified metabolite was detected by GC-MS with a typical indole nucleus (m/z = 130), indicating that it could be an intermediate in auxin metabolism. Plant response (*Pseudotsuga menziesii*, Douglas fir) was monitored upon inoculation of *P. tinctorius* and L-tryptophan. There was a consistent increase in plant height and stem diameter as a result of the two treatments, with statistical differences in dry weights of the shoots and roots.

There has been much speculation that auxins might play a very important role in the symbiotic relationships between ectomycorrhizae and their host plants (for a review, see references 6 and 9). Various auxins often occur within ectomycorrhiza-infected roots of pine seedlings but not in all nonmycorrhizal roots (9, 11). Slankis (9) claimed that auxins in root cultures caused similar anatomical and morphological changes characteristic of ectomycorrhizae. These changes included radial elongation of outer cells, swollen roots with reduced length, and absence of root hairs. At high nitrogen levels, the synthesis of auxins by fungal symbionts was found to be inhibited (7). Meyer (6) reported that mycorrhizal production of auxins could influence carbohydrate metabolism (presence of soluble sugars and translocation) in the host roots. It has been proposed that hyperauxiny of mycorrhizal roots may be due to the inactivation of the host auxin oxidase (8, 12). Also, an exogenous supply of synthetic auxins at a supraoptimal concentration has been shown to enhance mycorrhizal formation within *Pinus virginiana* seedlings (J. G. Palmer, Ph.D. thesis, George Washington University, Washington, D.C., 1954).

The ability of ectomycorrhizae to produce indole auxins in pure culture has been demonstrated when L-tryptophan (TRP) is provided in the medium (7, 13). Tomaszewski and Wojciechowska (12) reported that culture filtrates of *Suillus variegatus* contained 200 μM indole-3-acetic acid (IAA) equivalent by the standard *Avena* coleoptile curvature test. Ho (2) recently demonstrated that several strains of *Pisolithus tinctorius* produced various phytohormones, including IAA, in a non-TRP-supplemented medium by putative identification with thin-layer chromatography. Other genera of ectomycorrhizae isolated from *Pinus silvestris* reported to produce auxins include *Amanita muscaria*, *Paxilus involutus*, *Suillus luteus*, *Suillus bovinus*, and *Rhizopogon luteolus*, on the basis of cochromatographed standards (10). Much of the evidence for IAA detection in these citations is based on thin-layer chromatography with chromogenic agents such as Salkowski or Ehrlich and auxin bioassays. However, Ek et al. (1) demonstrated that IAA was produced by over 16 mycorrhiza-forming fungi through gas chromatography-mass spectrometry analyses.

A fungal symbiont which shows considerable value in the field of forestry is the gasteromycete *P. tinctorius* (Pers.) Coker et Couch. The natural occurrence of *P. tinctorius* has been detected in 33 countries of the world and 38 states in the United States (3). It can form ectomycorrhizae with a broad range of hosts in diverse extreme environments with tolerance to high temperature, extreme soil pH, and high levels of SO_4^{2-}, Al^{3+}, and Mn^{2+} (4). The use of *P. tinctorius* has potential application in forestation programs throughout the world, but it is now evident that isolates differ in their effectiveness as ectomycorrhizal inocula. We report here a strain of *P. tinctorius* capable of producing IAA as detected by thin-layer chromatography, high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay, and gas chromatography-mass spectrometry which can promote Douglas fir growth when provided with TRP as the auxin precursor in the nanogram-to-microgram range per kilogram of soil. (Patent rights are pending on the use of TRP and its derivatives as agrochemicals influencing plant growth and development.)

MATERIALS AND METHODS

Culture. *P. tinctorius* 471 was obtained from the Corvallis Forest Service Laboratory, University of Oregon, after being isolated in Georgia with the host being a *Pinus* sp. The culture was maintained on potato dextrose agar in cold storage. To promote IAA production, the fungus was grown

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in 2 liters of liquid medium containing the following (in grams per liter): glucose, 10.0; TRP, 1.0 (sterile filtered, with a 0.45-μm-pore-size filter); and yeast extract, 0.1, as well as 10.0 ml each of phosphate buffer and trace element solutions. The phosphate buffer consisted of the following (in grams per liter): KH_2PO_4, 1.36; Na_2HPO_4, 2.13; and $MgSO_4 \cdot 7H_2O$, 0.2. The trace-element solution contained 1 ml of concentrated H_2SO_4 and the following (in milligrams per liter): $CaCl_2$, 530; $FeSO_4 \cdot 7H_2O$, 200; $MnSO_4 \cdot H_2O$, 20; $CuSO_4 \cdot 5H_2O$, 40; $ZnSO_4 \cdot 7H_2O$, 20; H_3BO_3, 3; $CoCl_2$, 4; and $Na_2MoO_4 \cdot 2H_2O$, 4. We were unable to detect IAA when TRP was replaced by NH_4NO_3 as a nitrogen source. The pH of the medium was adjusted to 7.0 with KOH before autoclaving. Glucose was autoclaved and added separately to the medium. The culture was continuously agitated at 200 rpm at room temperature.

Extraction. The culture was harvested by centrifugation at 9,000 × *g* for 20 min at 4°C. The supernatant was filtered through a 0.45-μm-pore-size filter, adjusted to pH 8.1 with Na_2CO_3, and partitioned three times against an equal volume of ethyl acetate. The aqueous phase was then adjusted to pH 2.8 with HCl and partitioned as described above with ethyl acetate (three times). The ethyl acetate (containing the acidic indoles) was evaporated to a dry residue and taken up in methanol.

Methylation. A methanolic solution of IAA (~1 mM) was treated with an excess of ethereal diazomethane. Diazomethane (~50 μmol) was generated with a Diazald Kit (Aldrich Chemical Co., Inc., Milwaukee, Wis.) by using anhydrous ether, diethylene glycol monoether, and 30% NaOH chilled in an ice-salt bath at 0°C with the addition of 0.025 mol of the precursor *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Thin-layer chromatography. The plates were Toxi-grams (4.2 by 11.5 cm) (American Scientific Products, Los Angeles, Calif.), in which blank B consisted of glass-fiber paper impregnated with silica gel and blank A was the same but treated with vanadate salts. The chromatograms were activated on a hot plate for 5 min before use. The acidic extract was concentrated to dryness and suspended in methanol and spotted in duplicate along with a IAA standard (20 μg of IAA μl of methanol^{-1}). The solvent system included heptane-acetone-glacial acetic acid (50:50:1, vol/vol/vol) and chloroform-ethyl acetate-formic acid (50:40:1, vol/vol/vol). Ascending chromatography was performed in a glass chromatographic chamber at room temperature. Detection consisted of one of the following: (i) exposure to ferric perchloric nitrate (FPN) solution composed of 5 ml of 5% ferric chloride, 45 ml of 20% (vol/vol) perchloric acid, and 50 ml of 50% (vol/vol) nitric acid; or (ii) exposure to formaldehyde vapors, followed by dipping the chromatogram in concentrated H_2SO_4, clearing it with deionized water, and viewing it with transmitted light of long-wave UV (366 nm).

HPLC. The liquid chromatographic separation was performed on a Beckman model 330 isocratic liquid chromatograph equipped with a model 110A pump, model 210 sample injector, and model 165 variable wavelength detector. The detector was operated at 280 nm. A 3390A integrator (Hewlett-Packard Co., Palo Alto, Calif.) was used to quantitate peaks. An Econosphere (C_{18}) 5-μm reverse-phase column (4.6 by 250 mm) (Alltech Associates, Inc., Los Altos, Calif.) was selected for the analysis. The packing material consisted of spherical silica with the following specifications: surface area, 200 m^2 g^{-1}; pore diameter, 8 nm; pore volume, 0.8 ml g^{-1}; and carbon load, 10%. An octadecylsilane (5-μm) guard column (30 by 4.6 mm) (Bio-Rad Laboratories, Rockville Centre, N.Y.) was used to protect the analytical column. Separation was optimized by using an isocratic system containing 45% methanol and 55% water, acidified to pH 2.5 with H_3PO_4 as described elsewhere (1a). The operating conditions for the detection of IAA were as follows: flow rate, 1.0 ml min^{-1}; chart speed, 0.5 cm min^{-1}; temperature, ambient; sample size, 20 μl.

IAA was tentatively identified by matching the elution time with a reference standard and was confirmed by UV spectrometry. The UV spectra of IAA and methyl-3-IAA (CH_3-IAA) were determined on a Beckman model 165 variable wavelength detector on line with the HPLC with the following specifications: selected wavelength range, 230 to 340 nm; spectral bandwidth, 7 nm; sample cell, 9 μl; path length, 10 mm; and accuracy, ±2 nm. Spectra of reference standards (10^{-4} M) were conducted in the previously described mobile phase at 25°C. Auto-background correction by internal normalization was provided during the peak scan.

Enzyme immunoassay. The IAA immunoassay was performed with a test kit purchased from Idetek (San Bruno, Calif.). The enzyme tracer consisted of IAA coupled to alkaline phosphatase. A stock solution of IAA methyl ester (Fluka, Ronkonkoma, N.Y.) was subjected to serial dilutions to give a final range of 1 to 500 pmol assay^{-1}. A 100-μl portion of the standard sample was then added to the monoclonal antibody along with 100 μl of diluted tracer. The wells were sealed and incubated at 4°C for 3 h. After incubation, the wells were washed with 25 mM Tris-saline buffer (0.85% NaCl), pH 7.5.

The alkaline phosphatase assay was conducted by adding 200 μl of freshly prepared *p*-nitrophenyl phosphate and incubating it at 37°C for 60 min. The optical intensity of the product, *p*-nitrophenol, was read at 405 nm. The quantitative determination of IAA by the immunoassay was based on the percent binding as follows: % binding = $[(OD_B - OD_{UNB})/(OD_{B_0} - OD_{UNB})] \times 100$, where OD_B is the optical density in the presence of IAA, OD_{UNB} is the absorbance in the presence of excess IAA (nonspecified binding), and OD_{B_0} is the absorbance in the absence of IAA (maximum enzyme binding to coat surface [100% binding]). Percent binding (% B/B_0) versus concentration of methyl-IAA (pmol) was plotted, giving a sigmoidal plot (Fig. 1). A linear standard curve was plotted by using a log-logit function: logit (% B/B_0) = $\ln [(B/B_0)/(100 - B/B_0)]$.

Gas chromatography-mass spectrometry. For unequivocal identification of IAA, the methyl ester was confirmed by gas chromatography-mass spectrometry. The gas chromatograph was a Hewlett-Packard 5790 equipped with a db-5 capillary column (30 m, 0.25-μm inside diameter) (J & W Scientific, Rancho Cordova, Calif.). The mass spectrometer was a VG Analytical 7070 EHF high-resolution mass spectrometer. The operating conditions were as follows: injector temperature, 220°C; detector temperature, 280°C; column temperature, 50°C for 1 min to 280°C at 10°C min^{-1}; mass spectrometry scan, 200- to 500-s^{-1} interval; resolution, 1,000; electron impact, 70 eV; and ionizing source temperature, 200°C.

Plant response. A glasshouse experiment was initiated to test the response of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) to the inoculation of *P. tinctorius* and the IAA precursor, TRP. Roots of small seedlings were submerged into the fungus culture grown to the late log phase. The seedlings were then transplanted into plastic pots (11 by 35 cm) containing 3 kg of Hanford sandy loam soil (coarse-loamy, mixed nonacid, thermic Typic Xerorthent). The

control consisted of untreated seedlings. After 2 weeks, the plants were treated with either 500 ml of deionized water or a TRP solution ranging from 10^{-3} to 10^{-8} M. Seedlings were continuously treated with Hoagland mineral nutrient solution (1/2 strength) on a monthly basis. The plants were monitored in growth by measuring plant height and stem diameter up to a 1-year period. At the end of the experiment, analyses were made on the dry weights of shoots and roots.

RESULTS AND DISCUSSION

Thin-layer chromatography. Two chromatographic methods were developed to separate and isolate IAA as a secondary metabolite. In the first procedure, 15 μl each of the acidic extract and standard IAA were spotted on blank B Toxi-grams, with the primary solvent being heptane-acetone-glacial acetic acid. IAA was located with FPN at R_f = 0.72. The zone corresponding to the marker spot from an unstained side was then punched out with a disk punch and run on another blank B with the secondary solvent, chloroform-ethyl acetate-formic acid. The chromatogram was dried, dipped in FPN, and confirmed to be IAA (R_f = 0.81).

To verify the presence of IAA in the culture supernatant, a second chromatographic procedure was developed. A 10-μl spot in duplicate with an IAA standard was applied to blank B. The primary solvent was heptane-acetone-glacial acetic acid, with detection by FPN. A nonstained punched disk was then placed in a blank A chromatogram. The secondary migration was carried out with the same solvent but with detection by the classical Marquis and Mondelins reaction (formaldehyde, H_2SO_4, and vanadate). The zone corresponding to standard IAA was viewed with transmitted light of long-wave UV (366 nm) and showed fluorescence, confirming the presence of IAA as a fungal metabolite.

HPLC. Previous work in this laboratory has established the operational conditions to separate indoles with an isocratic HPLC system (1a). The stationary phase and composition of the mobile phase were well suited for the separation and isolation of IAA. The low pH (2.5) suppressed dissociation of the hydrogen atoms (ion suppression) and allowed good resolution among the metabolites detected in the acidic extract. A prominent peak was observed with an elution time of 5.18 min, tentatively identified as IAA (5.3 μmol liter^{-1}). To verify its identification, the acidic extract was subjected to methylation with ethereal diazomethane. The methylated sample was then run under the same HPLC operational conditions as described above. CH_3-IAA was identified by cochromatography with an elution at 6.74 min (Fig. 2). For further confirmation, a UV spectrum of the solute was compared with the reference standard, CH_3-IAA. Regions of 240 to 250 nm and >300 nm had low levels of absorption with a maximum OD observed within the range of 270 to 280 nm. UV spectrometry confirmed the detection of CH_3-IAA.

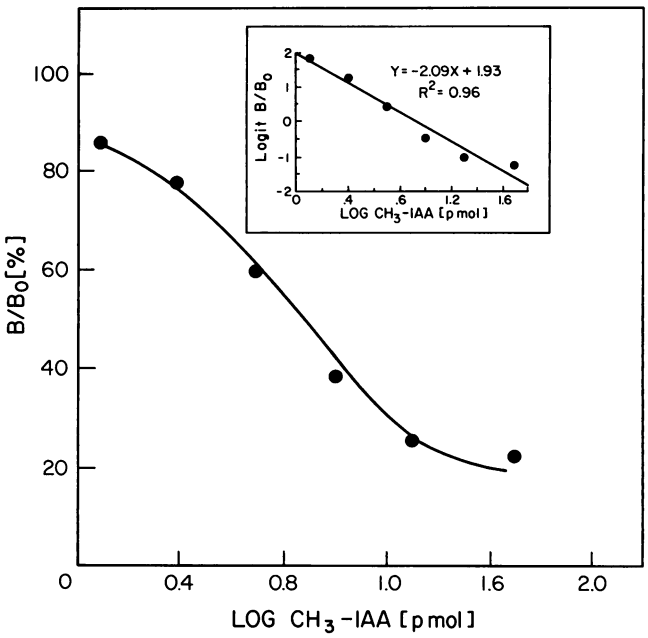

FIG. 1. Standard curve for IAA enzyme immunoassay. Tracer, alkaline phosphatase-IAA methyl ester; standard, IAA methyl ester; B, binding of tracer to monoclonal antibody in the presence of standard IAA; B_0, absence of IAA; logit $B/B_0 = \ln[(B/B_0)/100 - B/B_0)]$.

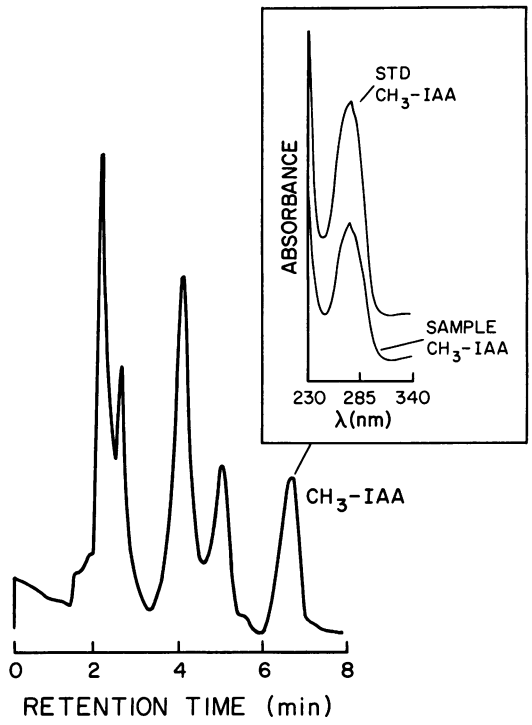

FIG. 2. HPLC chromatogram of a methylated acidic extract of *P. tinctorius*. Column, 5-μm Econosphere (C_{18}); flow rate, 1 ml min^{-1}; mobile phase, isocratic 45% methanol and 55% water acidified to pH 2.5 with H_3PO_4; sample injected, 20 μl; detection, 280 nm. STD, Standard.

Enzyme immunoassay. Enzyme-linked immunosorbent assay was used to quantitate the IAA present within the culture supernatant of *P. tinctorius*. The assay is based on the principle of competitive binding, in which IAA in the extract competes with the tracer (IAA labeled with alkaline phosphatase). The intensity of *p*-nitrophenol (enzyme product) was inversely proportional to the sample IAA concentration. A typical standard curve obtained with IAA methyl ester is shown in Fig. 1. The assay detection limit is 0.5 to 1 pmol with a reported measuring range of up to 500 pmol of IAA. This technique is highly specific for IAA. Among the auxins, only indole-3-acetylglycine is somewhat comparable in reactivity (58%). However, this compound is not known to occur as a microbial secondary metabolite in the metabolism of auxins. TRP does not cross-react at all in this assay

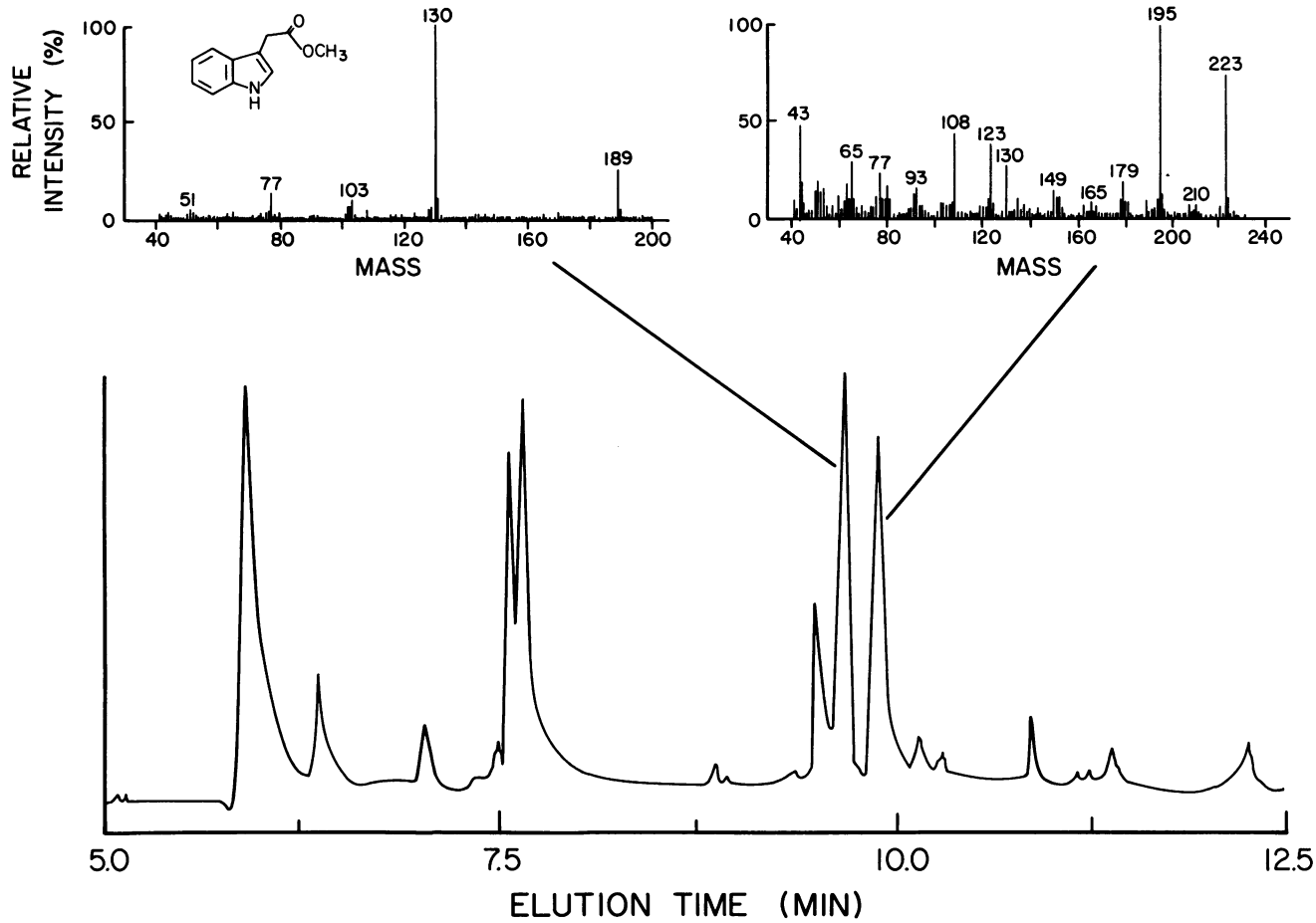

FIG. 3. Gas chromatogram of a methylated acidic extract of *P. tinctorius*. Column, db-5 capillary; injector temperature, 220°C; detector temperature, 280°C; column temperature, 50°C for 1 min to 280°C at 10°C min^{-1}; mass spectrometry scan, 200- to 500-s^{-1} interval; resolution, 1,000; electron impact, 70 eV; and ionizing source temperature, 200°C.

(0.04%). The immunoassay confirmed the detection of IAA (4.12 μmol $liter^{-1}$) in the culture supernatant of *P. tinctorius*.

Gas chromatography-mass spectrometry. Gas chromatography-mass spectrometry was used for the unequivocal identification of methylated IAA in the supernatant culture. The identity of IAA was determined on the basis of its molecular weight and characteristic fragmentation pattern in relation to a reference standard. Figure 3 shows the gas chromatogram of the methylated acidic extract. The identity of IAA was confirmed by the appearance of major ions as follows (*m/z* [relative intensity, %]): molecular ion, 189 (26); base ion, 130 (100); and others, 103 (11), 77 (13), and 51 (7). The base ion is thought to be a protonated methyl-3-indole or quinolinium cation (5).

Another unidentified metabolite of interest within the same extract had a typical indole nucleus (*m/z* = 130) (Fig. 3). The major ions within the mass spectrum were as follows (*m/z* [relative intensity, %]): molecular ion, 223 (75); base ion, 195 (100) and others, 210 (10), 179 (21), 165 (10), 149 (13), 130 (25), 123 (38), 108 (41), 93 (15), 77 (23), 65 (29), and 43 (45). On the basis of the exact molecular weight of 223.0837, the elemental composition best fit the formula $C_{11}H_{13}NO_4$ with the number of rings and double bonds being six. Analysis of the mass fragmentation data reveals that this metabolite could also possibly be an indole metabolite.

Plant response. After one growing season, *P. tinctorius* ectomycorrhizae stimulated the growth of potted seedlings of Douglas fir only when supplied with low concentrations of TRP. Analyses of variance for seedling height, stem diameter, shoot-root dry weight, and root/shoot ratio showed some significant differences among the treatments. Seedlings ranged from 15 to 33 cm in height, 0.22 to 0.88 cm in stem diameter, 2.91 to 5.14 g in shoot dry weight, 2.85 to 5.85 g in root dry weight, and 32.2 to 54.6 cm in root length. The cumulative increase in plant height and stem diameter was monitored up to 11 months (Fig. 4 and 5). There was basically no difference in growth between the inoculated and uninoculated treatments; however, the addition of a dilute solution of TRP with *P. tinctorius* promoted a dramatic increase. The more dilute treatments (10^{-6} to 10^{-8} M, 0.34 to 34 μg kg of $soil^{-1}$) resulted in the greatest response, indicating a physiological rather than nutritional effect. In regard to the biomass of shoots and roots, a similar trend was observed with enhanced yield among the 10^{-7} and 10^{-8} M TRP treatments (Fig. 6). Upon application of 10^{-8} M, the shoot and root dry weights were increased by 1.44- and 1.60-fold, respectively. The root/shoot ratio indicated that the ectomycorrhiza-infected seedlings subjected to TRP partitioned their assimilates to the root. This was particularly evident with the 10^{-6} to 10^{-8} M application. The higher concentration of TRP applied at 10^{-3} M inhibited the dry weights of the seedlings. The most dramatic influence among the interac-

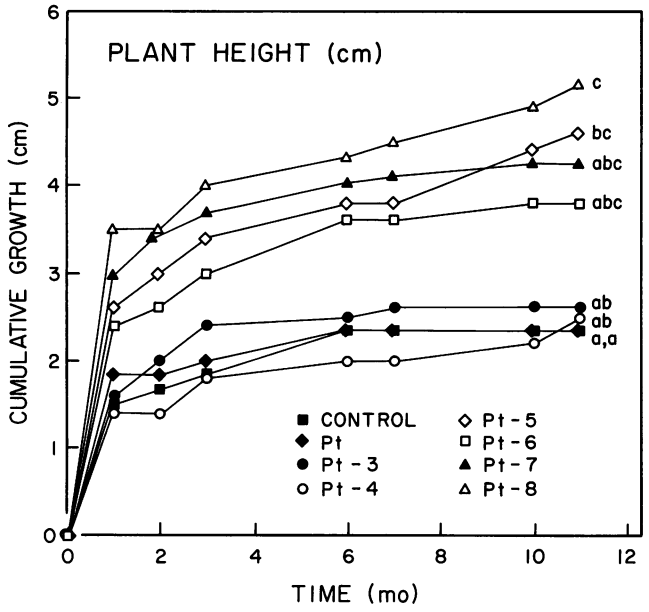

FIG. 4. Influence of *P. tinctorius* (Pt) and TRP (10^{-3} to 10^{-8} M) on plant height of Douglas fir. Treatments followed by the same letter are not significantly different at the 0.05 level according to the Duncan multiple range test.

tion of *P. tinctorius* and TRP was the seedling root length (Fig. 6). The root length reflected a phytohormone effect, with elongation being promoted by 1.24- to 1.28-fold upon 10^{-6} to 10^{-8} M TRP applications. Root examination revealed that the mycelial inoculum of *P. tinctorius* was highly effective in forming ectomycorrhizae on the seedlings. However, the effectiveness of this isolate on plant growth was not much different from that of the uninoculated stock seedlings unless TRP was provided in the nanogram-to-microgram range as a precursor of IAA (Fig. 7).

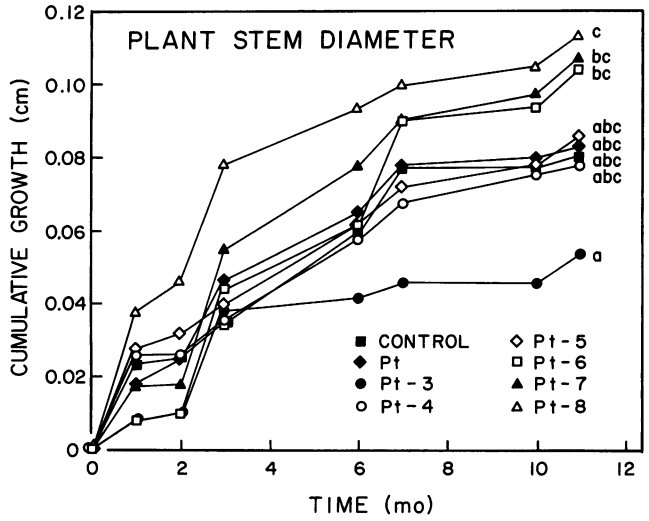

FIG. 5. Influence of *P. tinctorius* (Pt) and TRP (10^{-3} to 10^{-8} M) on the stem diameter of Douglas fir. Treatments followed by the same letter are not significantly different at the 0.05 level according to the Duncan multiple range test.

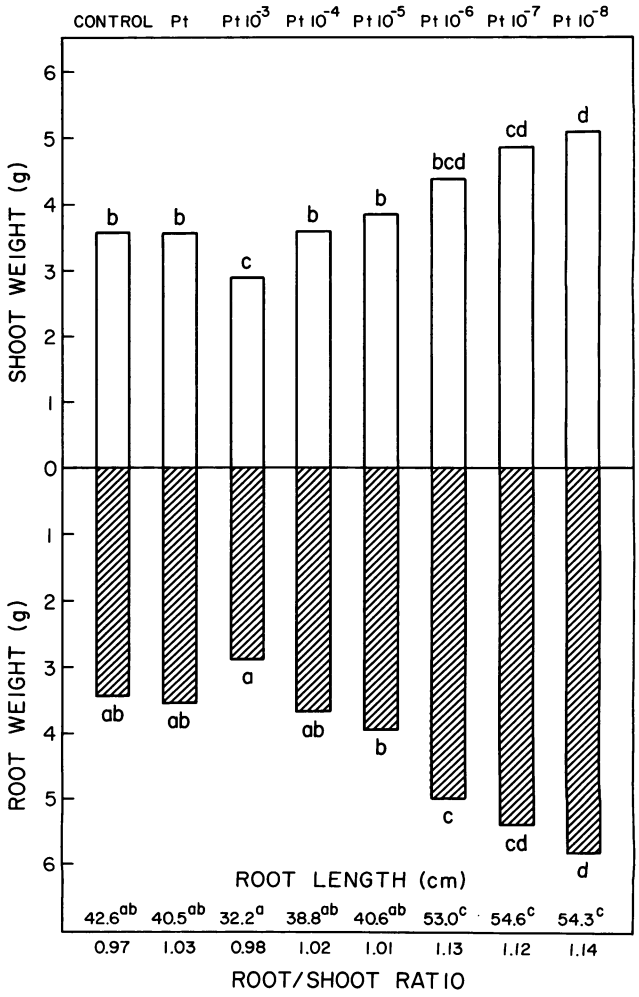

FIG. 6. Influence of *P. tinctorius* (Pt) and TRP (10^{-3} to 10^{-8} M) on the biomass and root length of Douglas fir. Treatments followed by the same letter are not significantly different at the 0.05 level according to the Duncan multiple range test.

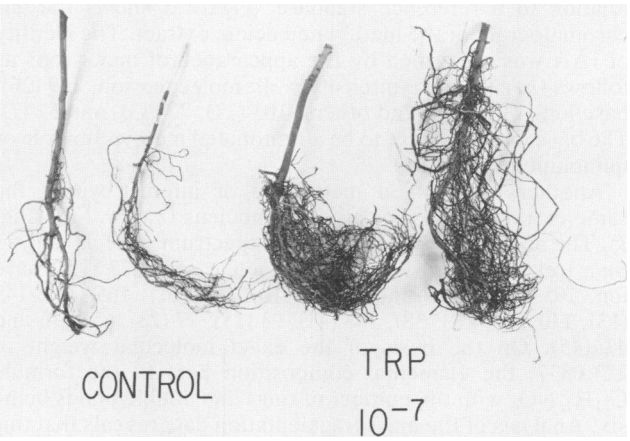

FIG. 7. Established ectomycorrhizae formed by *P. tinctorius* in the presence of 10^{-7} M TRP (3.4 μg kg of soil^{-1}).

ACKNOWLEDGMENTS

Cooperative research was supported by U.S. Department of Agriculture Forest Service grant PSW-86-0007CA.

We thank Susan Lim for her assistance in the laboratory, Ben Johnson for the glasshouse work, and Randy Molina for supplying us with the *P. tinctorius* culture.

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