Phenolic Compounds and Resistance to Fungal Pathogens Induced in Primary Roots of Douglas-Fir Seedlings by the Ectomycorrhizal Fungus *Laccaria laccata*

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ABSTRACT

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A mycorrhizal strain of *Laccaria laccata*, which can protect primary roots of seedlings of Douglas-fir (*Pseudotsuga menziesii*) from lethal root rot by *Fusarium oxysporum*, induced an accumulation of osmiophilic materials in cortical cells of primary roots. Histochemical tests demonstrated that the osmiophilic materials were primarily phenolic. One-month-old primary roots, incubated with *L. laccata*, its cell-free metabolites, or killed inoculum, were challenged with *F. oxysporum* or *Pythium irregulare*. Rate of advance of hyphae toward roots, extent of surface colonization and cortical infection by the pathogens, and amount of phenolic material in the cortex were estimated by optical techniques. Cortical infection by each pathogen was significantly less in seedlings affected by *L. laccata* or its metabolites than in controls. Hyphal frequency was inversely proportional to the concentration of osmiophilic materials. Rates of growth of the pathogens toward the root and intensity of rhizoplane colonization were not influenced by *L. laccata*. Thus, we judged antibiosis unimportant in root protection although *L. laccata* had been shown capable of inhibiting *F. oxysporum* in vitro. Phenolics induced by *L. laccata* in the primary root may be the basis for root protection.

Additional key words: biological control.

A mycorrhizal strain of *Laccaria laccata* (Scop.: Fr.) Berk. & Br. protected nonmycorrhizal seedlings of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) from root rot by *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans. in field experiments (34) and in controlled environments (35,37). The nature of this protection was not explained. Of the mechanisms proposed by Marx (26), *L. laccata* could protect roots by antibiosis, induced resistance, or alteration of rhizosphere microflora.

Most lethal infections by *F. oxysporum* begin in primary roots before seedlings are 3 wk old (5). *L. laccata*, like *F. oxysporum*, can invade radicles of Douglas-fir within days after germination (6). It thus gains a position where it may affect pathogenesis. In culture, *L. laccata* produced a diffusible substance that inhibited mycelial growth and delayed spore germination of *F. oxysporum* (39). This suggested antibiosis is a possible mechanism for root protection.

The response of primary roots to colonization and infection by mycorrhizal fungi had not been investigated. We hypothesized that *L. laccata* might induce root resistance to pathogens by inducing accumulations of phenolic materials. Phenolics accumulate rapidly during other host-parasite interactions (9,15,20) and can mediate disease suppression through inactivation of fungal enzymes or strengthening of plant structural components (22).

Our objectives were to characterize, anatomically and histochemically, the reaction of primary roots of Douglas-fir to *L. laccata* and determine the significance of antibiosis and induced host response in root protection by the mycorrhizal fungus.

MATERIALS AND METHODS

Systems for culture of seedlings and microorganisms. The responses of primary roots to colonization by *L. laccata* and other soil microorganisms were investigated in three gnotobiotic systems (sensu Kreutzer and Baker [21]) and in pasteurized soil.

A wick-culture system (39) allowed observation of primary roots and minimized their disruption at harvest. In that system, roots of intact seedlings of Douglas-fir grew gnotobiotically with microorganisms in 200 × 32-mm culture tubes while the shoots were exposed to ambient conditions of the growth chamber. The seedlings were transferred to tubes after surface sterilization of seed in 30% H_2O_2 for 2 hr and germination on moist, sterile, filter paper. In a modification of the wick culture system, *L. laccata* was separated from the root by a dialysis membrane (12,000–14,000 molecular weight cut off) to allow observations of effects of extracellular metabolites. The third system was a soil culture tube previously used for studies of root colonization by *L. laccata* (6). Seedlings in either wick-culture or soil-culture tubes grew for up to 6 wk in the greenhouse within an air-conditioned chamber containing a water bath that maintained roots at 22 C while shoots were subject to diurnal temperatures (18–27 C). Natural light was supplemented by mercury metal halide lamps to provide 300–450 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at seedling level for at least 14 hr/day.

For an experiment in pasteurized soil, Douglas-fir seedlings were grown in pots of nursery soil injected with *L. laccata* or steam-killed inoculum as previously described (35). Pots were held in an air-filtered chamber with the following conditions at plant level: 350 $\mu E \cdot m^{-2} \cdot sec^{-1}$ provided by fluorescent plus incandescent lamps for 16 hr daily; 24 and 16 C during light and darkness, respectively; and 60% relative humidity. The air filters (Micro Filter Sheet 05-600-32, Air Control, Huntingdon Valley, PA 19006) had prevented fortuitous ectomycorrhizal formation in previous experiments. Pots were rerandomized weekly and watered when soil surfaces became dry.

Microorganisms introduced to tubes or pots with roots of intact seedlings represented an array of ecological capabilities: mycorrhizal symbionts, pathogens, and saprobic biocontrol agents (Table 1). Stock cultures of *L. laccata* and *Cenococcum geophilum* Fr. were maintained on modified Melin-Norkrans agar medium (MMN) (25) amended with 50 ppm biotin and stored as plugs from plate cultures in sterile distilled water (SDW) at 2 C (27).

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Trichoderma harzianum Fifai, *Epicoccum purpurascens* Ehrenb.: Schlecht. (= *E. nigrum* Link), and *F. oxysporum* were maintained on potato-dextrose agar and stored as above. Plate cultures were started by comminuting mycelium plus agar for 5 sec in a small blender with sufficient SDW to yield a slurry and then dispersing the slurry over surfaces of fresh plates of MMN. *Pseudomonas cepacia* Burkh. was maintained on cornmeal agar and stored as a suspension in SDW at 22 C. Inoculum was produced by dispersing slurries of 2-wk-old plate cultures on MMN plates and incubating fast-growing organisms for 1 wk (*T. harzianum*, *E. purpurascens*, *F. oxysporum*, and *P. cepacia*) and slow-growing ones 2 wk (*C. geophilum* and *L. laccata*).

For each organism two plate cultures were leached twice for at least 12 hr in 400 ml SDW and a slurry was prepared from them. This provided inoculum nearly free from nutrients. A Cornwall syringe fitted with a 1.63-mm-diameter (14 gauge) cannula was used to inject 1.0 ml of slurry into each culture tube. A slurry of leached MMN or leached MMN plus steam-killed inoculum served as the control. Microorganisms were injected into tubes 2–3 days before the radicles (30–40 mm long) of intact seedlings were aseptically introduced. Experiments ran for 6 wk unless otherwise indicated.

Histochemistry. At the conclusion of each experiment, at least three primary roots per treatment were removed from the wick or soil system. Soil was gently washed from roots and adhering particles were removed with a camel's hair brush. Roots were observed with light microscopy (LM) when fresh or after embedment in epoxy resin. Transverse sections, 50 to 100 μm thick, of fresh roots were cut with a razor and immediately placed in SDW or a histochemical reagent. Reagents used to localize phenolics were nitrous acid (10) or saturated alcoholic vanillin-concentrated HCl (13). The reagent for lipids was 0.3% Sudan black B in 70% EtOH (2). Lignin was localized with 2% phloroglucinol in 95% EtOH-35% HCl (19). Reagents for proteins were 1% naphthol blue-black in 7% acetic acid (12) or 0.005% acid fuchsin in 1% acetic acid (11). Sections were soaked in reagents for 5–10 min and excess reagent removed with appropriate solvent. Autofluorescence of fresh, unstained sections in SDW or 0.1 M tris (hydroxymethyl)aminomethane buffer at pH 8.5 was observed with incident light fluorescence microscopy using a Zeiss Photomicroscope II with an HBO 200 W super-pressure mercury lamp, UG-5 exciter filter and No. 50 barrier filter.

Roots to be embedded in plastic were first fixed 12 hr at 22 C in 1% glutaraldehyde plus 0.5% caffeine (29) in 0.05 M sodium cacodylate buffer, pH 6.8. Specimens were washed with buffer plus 0.5% caffeine, followed by buffer alone, postfixed with 2% osmium tetroxide, dehydrated in an acetone series, and embedded in Spurr's low viscosity epoxy resin (36). Embedded specimens were sectioned at 20 μm with a sliding microtome. Sections were observed with Nomarski differential interference contrast optics and photographed with Kodak Ektachrome 160 professional film 5037.

Observations of pathogenesis. Two experiments were done. In the first, primary roots of Douglas-fir seedlings in soil culture tubes were inoculated with living or killed *F. oxysporum* 2 wk after receiving living or killed *L. laccata*. The mycorrhizal fungus was added to soil at seeding. Seedlings were inoculated with *F. oxysporum* by placing an infested Douglas-fir seed in a hole adjacent to the primary root at a depth of 3 cm. The hole was made with a sterile glass rod and was filled with soil after inoculation. At least three replicates per treatment were harvested 3, 8, 11, and 19 days after inoculation with *F. oxysporum*. Roots were observed with a dissecting microscope, and segments that were colonized by *L. laccata* and/or *F. oxysporum* were embedded in plastic for LM or prepared for scanning electron microscopy (SEM). Segments for SEM were fixed and dehydrated as described for LM, subjected to critical-point drying, mounted on aluminum stubs, coated with Au-Pd, and viewed at 10 kV with a AMC model 1000 scanning electron microscope.

The second experiment involved challenge of primary roots with *F. oxysporum* or *Pythium irregulare* Buism. after exposure to *L. laccata* or its metabolites or to killed inoculum. Seedlings were grown in wick-culture tubes where inoculum of the mycorrhizal fungus was injected onto the root or adjacent to it but separated from it by a dialysis barrier. After 4 wk, intact seedlings were aseptically transferred to microscope slides coated with 2% water agar. On each slide, one seedling was oriented lengthwise with its root in contact with the agar and its top extending beyond the slide. A surface-sterilized seed of Douglas-fir or oat internally infested with *F. oxysporum* or *P. irregulare*, respectively, was placed on the agar 5 mm from the root at a point 5 mm proximal from its tip. *P. irregulare* was grown in oat seeds as this fungus failed to infest seeds of Douglas-fir. The slides were incubated in the dark in moist chambers at 15 C. Growth of hyphae toward the root was recorded daily, and after 10 days the distal 10 mm of the root was excised and prepared for LM and SEM.

Colonization of the root surface by *F. oxysporum* was quantitatively estimated for three roots in each treatment that received this fungus. The estimate was obtained from scanning electron micrographs (×1,060) made at three random locations along the surface of the distal root segment. A grid of dots 5 mm apart was placed on each micrograph, and the number of dots on hyphal images was expressed as a percentage of total dots over hyphae plus root surface. Only hyphae that could not be identified as *L. laccata* were counted. *L. laccata*, identified by clamp connections, was not quantified.

A cortical infection index was determined for three roots per treatment by viewing longitudinal sections at ×1,250 with LM through a net reticle. The sections, 20 μm thick, were cut from three random locations in the distal segment. The number of intersections of the grid under which hyphae were seen was expressed as a proportion of the total number of intersections over cortical tissue.

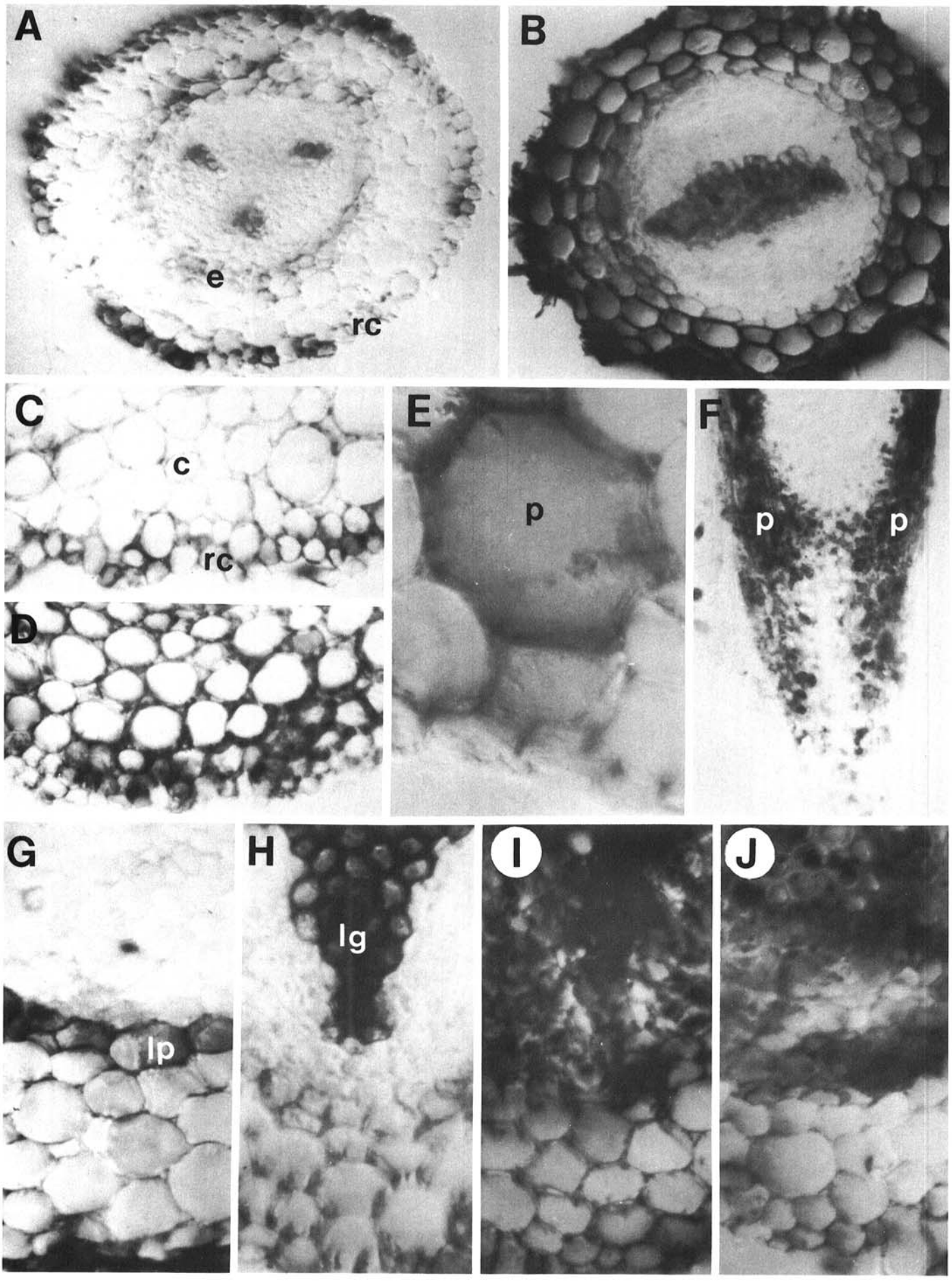
The relationship between osmiophilic compounds and fungal presence in the cortex was quantified by determining, in conjunction with the assessment of internal infection, the absorbance of light by sections stained with osmium tetroxide. Absorbance was measured with a Photovolt multiplier photometer (model 520-M) using an 80-μm spot size.

Living cultures of *L. laccata* often inhibited growth of seedlings in wick culture (39). To distinguish effects of *L. laccata* from those of extraneous factors, which might also cause slow root elongation, seedlings to be challenged with *F. oxysporum* were first segregated into two categories based on growth rate of roots in wick culture: rapidly growing (>60 mm at 4 wk) and slowly growing (<60 mm at 4 wk). Roots were not segregated before observations of surface colonization or before challenge with *P. irregulare*.

The experiment had nine treatments: *F. oxysporum* challenge to

TABLE 1. Characteristics of fungi and bacteria allowed to interact with primary roots of Douglas-fir

Organism and characteristics	Designation and origin of isolate
Laccaria laccata, slow-growing, lavender-pigmented isolate, mycorrhizal with Douglas-fir and red pine in laboratory tests (6,35).	T813, Washington, sporocarp associated with Douglas-fir seedlings.
Cenococcum geophilum, ecto-mycorrhizal symbiont with many tree species (40).	T1068, Georgia, ectomy-corrhiza of loblolly pine.
Pseudomonas cepacia, pathogen of onions, and biocontrol agent against Pythium and Fusarium damping-off of onion (7).	T1071, New York, organic soil.
Trichoderma harzianum, saprobe, widely employed as experimental biocontrol agent (8).	T1066, Georgia.
Epicoccum purpurascens, saprobe with biocontrol capability (4).	T1070, New York, soil.
Fusarium oxysporum, root-rotting strain pathogenic to Douglas-fir seedlings (5).	T1062, British Columbia, root of Douglas-fir seedling.
Pythium irregulare, pathogen that causes damping-off of many plant species (18).	T1065, New York, onion.


A
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(i) rapidly or (ii) slowly growing control roots, to (iii) rapidly or (iv) slowly growing roots colonized by *L. laccata*, to (v) rapidly or (vi) slowly growing roots exposed only to metabolites of *L. laccata*; *P. irregulare* challenge to (vii) control roots, to (viii) roots colonized by *L. laccata*, or to (ix) roots exposed to metabolites only. Seedlings treated with killed inoculum with or without a dialysis barrier were used interchangeably as controls because the barrier did not influence their development. The portion of the experiment involving challenge with *F. oxysporum* was done twice with similar results.

RESULTS

Histochemical changes induced by *L. laccata*. Transverse sections of 10-day-old tissue of roots grown gnotobiotically with or without *L. laccata* were compared for intensity and location of staining by reagents that react with phenolic compounds. Control roots stained with vanillin-HCl gave a positive (brown) reaction in the endodermis and occasionally in persistent root cap cells (Fig. 1A). Root tissue of the same age exposed to *L. laccata* gave a positive reaction with this reagent not only in the endodermis and persistent root cap cells but also throughout the cortex (Fig. 1B). Nitroso reagent gave similar results; phenolics stained orange-brown (Fig. 1C and D). Phenolics also accumulated in the cortex of roots separated from *L. laccata* by a dialysis barrier, indicating that an extracellular fungal metabolite with molecular weight $<14{,}000$ induced this response. As determined by microscopic observation, the dialysis barrier was not breached by this isolate of *L. laccata*. Roots that accumulated phenolics grew slowly in comparison to controls, but controls that in some cases grew slowly did not accumulate phenolics. Ten percent of the control roots grew as slowly as those inoculated with *L. laccata*.

Cortical cells of Douglas-fir roots grown in pasteurized soil with *L. laccata* also contained phenolic materials, but their distribution was less uniform than that found in gnotobiotic tests (Fig. 1E). Control roots from pasteurized soil gave a mixed reaction; some lacked phenolics while others had phenolic-containing cells scattered in the cortex. Phenolic materials stained in fresh sections generally diffused rapidly through the cytoplasm, cell walls, and intercellular spaces.

Root cap cells in all treatments were stained intensely by either vanillin-HCl or nitroso reagent (Fig. 1F).

Lipids, stained blue with Sudan black B, were concentrated in the endodermis (Fig. 1G), and their distribution was unaffected by fungal treatment. Lignified tissue was identified with phloroglucinol-HCl, which produced a dark red color (Fig. 1H). This reaction was limited to the xylem in all treatments. Staining of proteins by naphthol blue-black or acid fuchsin was strongest within the stele (Fig. 1I and J). Protein staining in the cortex ranged from diffuse to localized in scattered cells and was not related to colonization by *L. laccata*.

There was no difference in autofluorescence between controls and roots colonized by *L. laccata*. Autofluorescence, in water or buffer (pH 8.5), occurred only in xylem elements and endodermal cells (Fig. 2).

To investigate the specificity of the phenolic induction, seedling roots of Douglas-fir were cultured gnotobiotically with several common soil microorganisms. After 6 wk in wick culture all microorganisms were reisolated readily from the root surface. Root segments contained phenolics, which after treatment with OsO_4, appeared as black amorphous material, discrete globular bodies or granular structures within cortical cells (29). *Pseudomonas cepacia* and *Trichoderma harzianum* induced heavy accumulations of phenolics throughout the root cortex (Fig. 3A and B). Such accumulation precluded observation of cellular detail in thick sections. Response to *Cenococcum geophilum* varied within a root: phenolics were abundant in some cortical cells but absent from others (Fig. 3C). Some phenolic material accumulated in response to *Epicoccum purpurascens*, but this stain reaction was diffuse and weak (Fig. 3D). Stained phenolics were absent from cortical cells of roots colonized and infected by *F. oxysporum* (Fig. 3E). Response to *P. irregulare* was not tested.

Observations of pathogenesis. Roots of control seedlings in soil culture tubes were colonized and infected by *F. oxysporum* within 8 days after inoculation. The pathogen entered surface cells directly without forming specialized penetration structures. Within the root, the fungus ramified through cortical and stelar tissue and caused loss of host cell integrity (Fig. 4A). Surfaces of roots that had been inoculated with *L. laccata* 2 wk prior to inoculation with *F. oxysporum* were colonized by both fungi within 8 days after inoculation with the latter. The two organisms grew in close proximity to one another and there was no evidence for antagonism. Roots exposed to *L. laccata*, however, accumulated osmiophilic materials in cortical cells (Fig. 4B) and cortical cells were not infected by *F. oxysporum* during the 19 days of

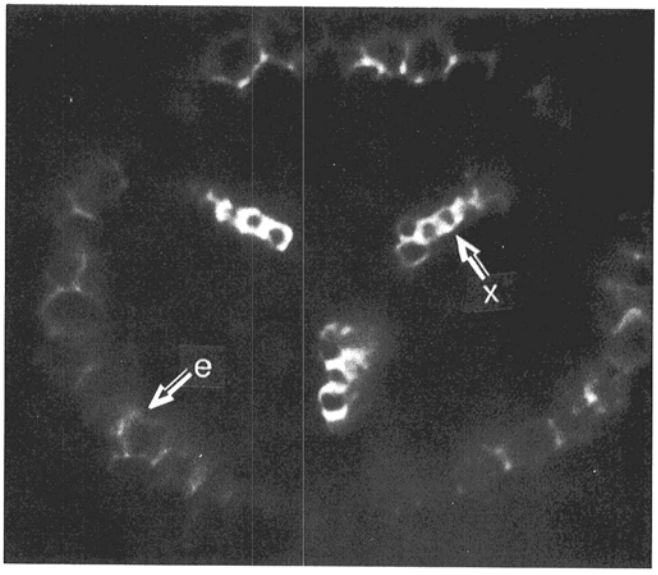

Fig. 2. Autofluorescence in xylem (x) and endodermis (e) of 10-day-old tissue from the primary root of a Douglas-fir seedling grown in a wick-culture tube with *Laccaria laccata*. The section was mounted in 0.1 M tris buffer and visualized with incident-light fluorescence photomicroscopy (×330).

Fig. 1. Reaction of fresh sections of primary roots of Douglas-fir with histochemical reagents. All figures except E and F represent transverse sections of 10-day-old tissue from seedlings grown in wick-culture tubes with or without isolate T813 of *Laccaria laccata*. **A,** Control root stained with vanillin-HCl. Positive reaction for phenolics occurred in persistent root cap cells (rc) and in the region of the endodermis (e) (×210). **B,** Root colonized by *L. laccata* and stained with vanillin-HCl. Phenolic materials accumulated uniformly throughout the cortex (×250). **C,** Control root stained with nitroso reagent. Phenolics are present in root cap cells (rc) but absent from cortex (c) (×360). **D,** Root colonized by *L. laccata* and stained with nitroso reagent. Positive reaction for phenolics throughout cortex (×380). **E,** Portion of transverse section of 10-day-old tissue from primary root of Douglas-fir seedling grown with *L. laccata* in pasteurized soil and stained with nitroso reagent. Phenolic-containing cell (p) is surrounded by cortical cells lacking stained material (×1,150). **F,** Longitudinal section of the primary root tip of a Douglas-fir seedling grown in soil culture tube with killed inoculum of *L. laccata* and stained with nitroso reagent. Phenolics (p) are localized in root cap and in persistent root cap cells along shaft of the root (×180). **G,** Section stained for lipids with Sudan black B. Lipids (lp) were concentrated in the endodermis (×510). **H,** Section stained for lignin with phloroglucinol-HCl. Lignin (lg) was restricted to xylem (×400). **I, J,** Sections stained with naphthol blue black and acid fuchsin, respectively, for localization of proteins. Staining was strongest within the stele and ranged from diffuse to localized in scattered cells in the cortex (×415).

observation. Infection status was evaluated by extensive microscopic observations.

Prior colonization of roots by *L. laccata* or exposure to its metabolites in wick-culture tubes did not reduce either the rate at which *F. oxysporum* approached the root (Table 2) or the extent of root surface colonization on agar-coated slides. *F. oxysporum* colonized 18% of the surface of control roots compared to 43 and 25%, respectively, for roots exposed to *L. laccata* or its metabolites. Where *F. oxysporum* and *L. laccata* grew together on the root surface (Fig. 4C), the two fungi were distinguished by the clamp connections of *L. laccata*. A hypha that had no clamp within the field of view was counted as *F. oxysporum*. The apparent stimulation of *F. oxysporum* on root surfaces in the presence of *L. laccata* was considered an artifact of this identification scheme. *P. irregulare* grew so rapidly (more than five times the rate of *F. oxysporum*) that reliable data for surface colonization could not be obtained.

Cortical invasion of roots by either pathogen on agar-coated slides was significantly reduced by prior exposure to *L. laccata* or its metabolites in wick culture with the exception noted below (Table 2). As with seedlings grown in soil, the majority of sections from roots affected by *L. laccata* had osmiophilic materials distributed throughout the cortex and lacked hyphae of pathogens (Fig. 4D), while roots of controls had abundant hyphae in the

TABLE 2. Effect of *Laccaria laccata* or its extracellular metabolites on growth of *Fusarium oxysporum* and *Pythium irregulare* from infested seed toward Douglas-fir roots, and on intensity of cortical infection[x]

Challenge to roots by:	Growth category and treatment of root in wick culture	Root length before challenge (mm)[y]	Growth of pathogen toward root (mm/day)	Cortical infection index[z]
F. oxysporum	Slow			
	Control	8 b	1.1 b	23 ab
	L. laccata	24 b	0.8 b	0 d
	Metabolites	7 b	0.7 b	5 cd
	Rapid			
	Control	67 a	0.6 b	25 ab
	L. laccata	94 a	0.6 b	26 a
	Metabolites	76 a	0.6 b	11 c
P. irregulare	Control	87 a	>5.3 a	27 a
	L. laccata	23 b	>5.3 a	8 cd
	Metabolites	14 b	>5.3 a	13 bc

[x] Seedlings were grown in wick-culture tubes for 4 wk with killed inoculum (control) or with inoculum of *L. laccata* on the primary root or separated from it by a dialysis barrier (metabolites). Seedlings were then aseptically transferred to glass slides coated with water agar and challenged by pathogens. Values for root length and growth rate toward root represent means of at least six replicates. Values followed by the same letter do not differ significantly according to Duncan's multiple range test ($P = 0.05$).

[y] Total length minus length of radicle when introduced to culture tube.

[z] Cortical infection index equals the number of intersections of an ocular grid under which hyphae of challenge pathogens were seen in longitudinal sections, as a proportion of the total number of intersections over cortex × 100. Values are means from three random sections from each of three roots. Values followed by the same letter do not differ significantly according to Duncan's multiple range test ($P = 0.05$).

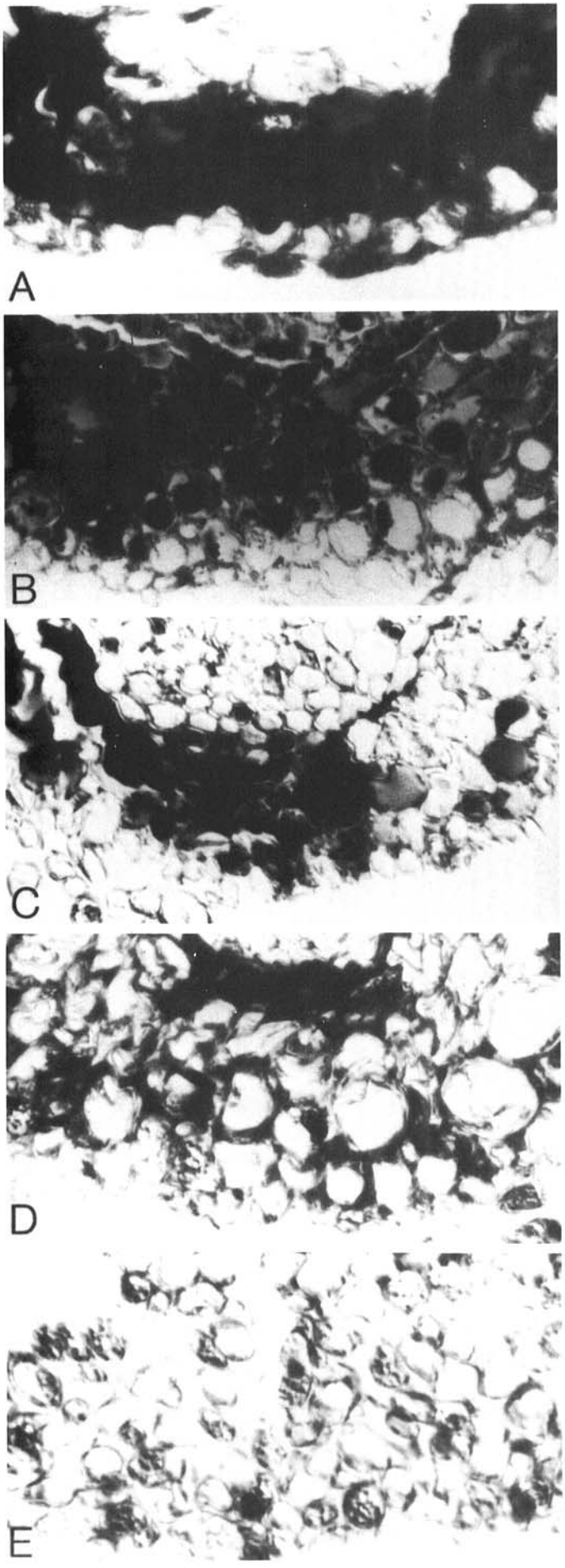

Fig. 3. Reaction of cortical tissue of Douglas-fir primary roots to soil microorganisms. Seedlings were grown in wick-culture tubes with the organisms for 6 wk, fixed in glutaraldehyde plus caffeine, postfixed in osmium tetroxide, embedded in plastic, and sectioned at 20 μm. Darkly stained osmiophilic materials accumulated in decreasing amounts in response to **A,** *Pseudomonas cepacia* (×380); **B,** *Trichoderma harzianum* (×305); **C,** *Cenococcum geophilum* (×230); and **D,** *Epicoccum purpurascens* (×370). Dense accumulation of osmiophilic materials precluded observation of cellular detail. **E,** Osmiophilic materials were absent and integrity of cortical cells was disrupted in sections infected with *Fusarium oxysporum* (×370).

cortex but no osmiophilic materials there (Fig. 4E). Some roots (~25%) grew rapidly in wick culture even when inoculated with *L. laccata*, and these were not protected from *F. oxysporum* (Table 2). Apparently these roots escaped the effect of the mycorrhizal fungus or the inoculum was ineffectual. Phenolic materials did not accumulate in their cortical cells and they resembled controls in the extent of infection by *F. oxysporum*.

A significant ($P < 0.01$), inverse relationship was found between the hyphal frequency of *F. oxysporum* and the absorbance of light by cortical tissue fixed in glutaraldehyde plus caffeine and stained with osmium tetroxide (Fig. 5).

DISCUSSION

L. laccata, its metabolites, and other mycorrhizal and nonmycorrhizal organisms induced an accumulation of osmiophilic materials in cortical cells of primary roots of Douglas-fir seedlings. When roots were challenged with fungal pathogens following incubation with *L. laccata*, invading hyphae were least abundant and often absent in tissue with the greatest accumulation of osmiophilic materials. Histochemistry of fresh sections showed that the induced materials were primarily phenolic.

Osmium tetroxide combines chemically with lipids, proteins,

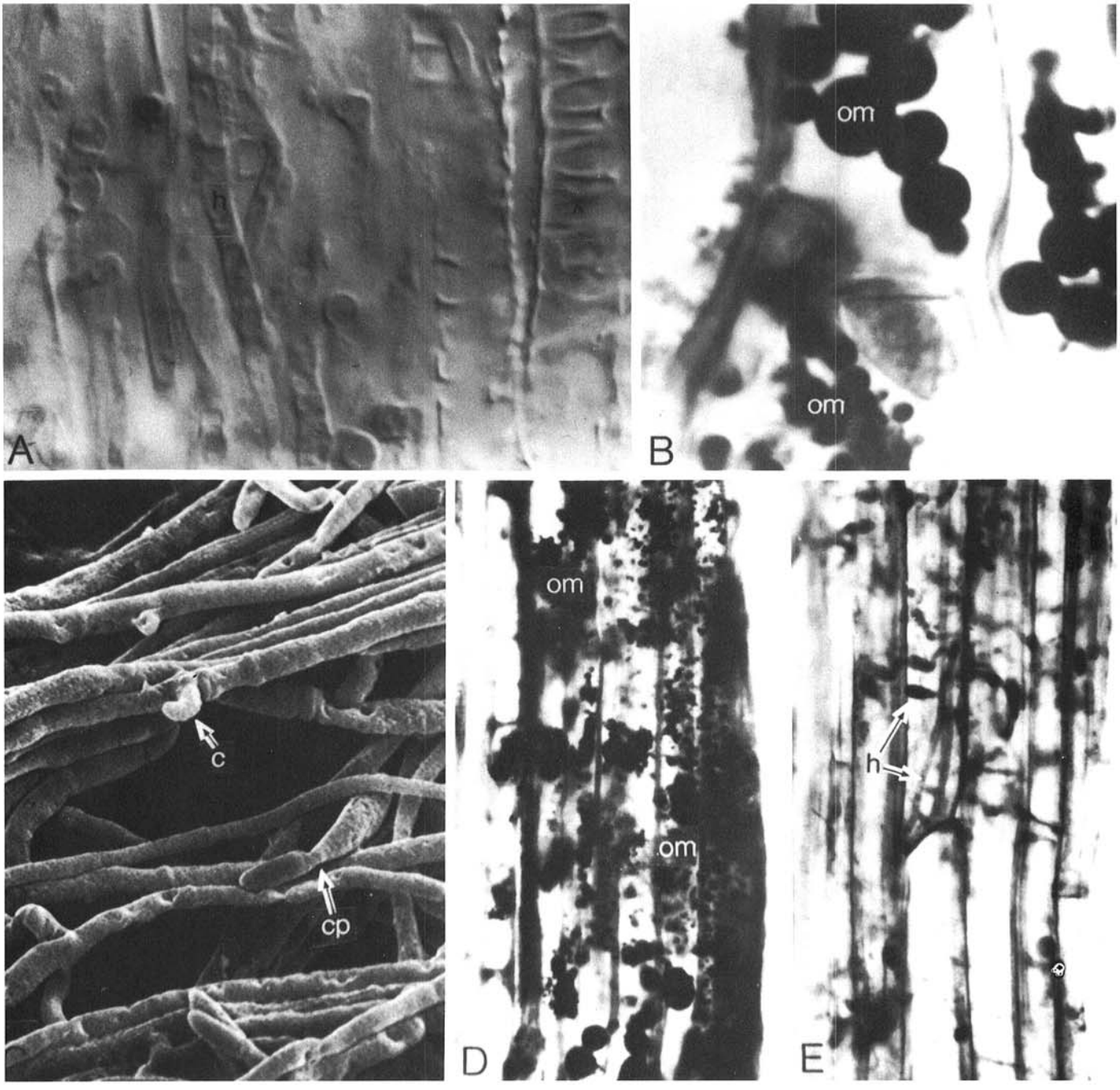

Fig. 4. Interaction of primary roots of Douglas-fir with *Laccaria laccata* and root pathogens. All figures except **C** represent longitudinal sections of root segments fixed in glutaraldehyde plus caffeine, postfixed in osmium tetroxide, embedded in Spurr's plastic, and sectioned 20 μm thick. **A, B,** Seedlings were grown in soil culture tubes with or without *L. laccata*, challenged with *Fusarium oxysporum* and observed 19 days later. **A,** Control showing hyphae (h) of *F. oxysporum* in macerated cortex, and secondary thickenings of xylem elements (x) (×1,580). **B,** Accumulation of osmiophilic material (om) in cortical cells of root exposed to *L. laccata* (×1,800). **C,** Scanning electron micrograph of surface of a root grown in a wick-culture tube with *L. laccata*, and subsequently challenged with *F. oxysporum*. Clamp connection (c) of *L. laccata* and conidium and phialide (cp) of *F. oxysporum* identify the fungi (×2,070). **D, E,** Cortex of seedlings grown in wick-culture tubes and challenged with *Pythium irregulare*. **D,** Root exposed to *L. laccata* shows accumulation of osmiophilic material (om) in cortical cells and absence of hyphae (×290). **E,** Control root has hyphae (h) of *Pythium irregulare* within cortex (×350).

and phenolics producing darkly stained structures (28,30). Differential staining with histochemical reagents is necessary for separation of these classes of compounds. Cortical cells of Douglas-fir exposed to *L. laccata* stained strongly with nitrous acid reagent, which reacts with *o*-dihydroxy phenols (33). Intense staining by vanillin indicated presence of catechins and condensed tannins (38). Primary roots of *Pinus resinosa* colonized by either the mycorrhizal strain of *L. laccata* or a nonmycorrhizal strain of this fungus, and Douglas-fir roots colonized by the nonmycorrhizal strain also accumulated phenolic material in cortical tissue (*unpublished*). Materials that accumulated in response to *L. laccata* did not stain with the lipid reagent, Sudan black B, and only inconsistently with indicators for protein. Phloroglucinol likewise failed to stain within cortical cells, indicating that lignification was not involved in cortical response to *L. laccata*.

Induced materials did not fluoresce under ultraviolet radiation. Radley and Grant (38) noted that most natural tanning agents have negligible autofluorescence. Swain (38) defined tannins as naturally occurring compounds containing sufficiently numerous phenolic hydroxyl groups to enable effective cross-links between proteins and other macromolecules. Tannins are thus capable of inhibiting enzymes and forming structural barriers to penetration. Ling-Lee et al (23) found that phenolic-containing cells in mycorrhizal roots of *Eucalyptus fastigata* had characteristic fluorescence ranging from deep orange to weak yellowish green. Fluorescence in their material, but not in ours, suggests that the phenolic materials observed were different.

All primary roots, whether exposed to *L. laccata*, other fungi, bacteria, or no microorganisms, contained phenolics in the root cap, endodermis, and stele. Accumulation of phenolics in cortical cells, however, was a nonspecific response to organisms in gnotobiotic systems and in pasteurized soil. Nonspecific induction may have been the reason why Piché et al (31) saw no difference in the phenolic content of ectomycorrhizal and noninoculated short roots of *Pinus strobus* grown in a nonsterile system.

The intensity of phenolic accumulation in Douglas-fir roots varied with identity of the inducing organism and with test conditions. Seedlings grown gnotobiotically with *P. cepacia* or *T. harzianum* had osmiophilic materials comparable in density to those induced by *L. laccata*. *Epicoccum purpurascens*, a common soil saprobe, induced only a weak and diffuse accumulation. Both of the former organisms have been used as biological control agents, and their protective influences have been attributed to antibiosis (7), hyperparasitism (17), or competition (24). Our observations suggest that they may induce resistance. Baker et al (1) found that resistance of carnation plants to Fusarium stem rot was induced by several microorganisms.

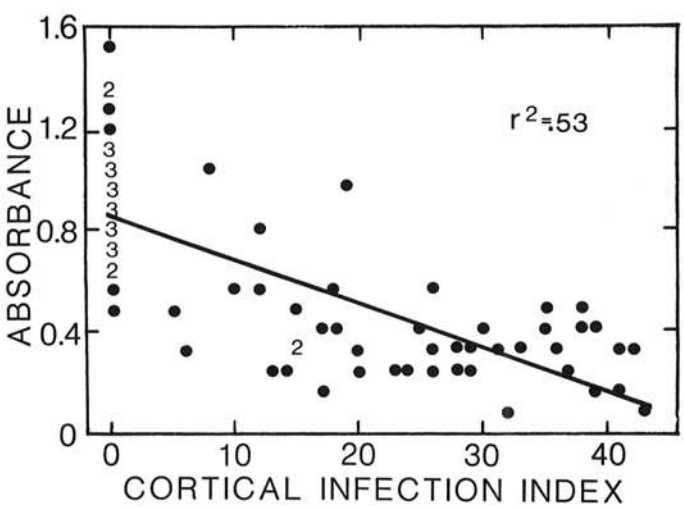

Fig. 5. Relationship between light absorbance of root cortical sections (20-μm thick, previously fixed in 1% glutaraldehyde plus 0.5% caffeine) and the frequency of hyphae of *Fusarium oxysporum* in the sections. Cortical infection index equals the number of intersections of a grid under which hyphae were seen as a proportion of the total number of intersections over cortex ×100. Numbers in graph represent multiple data points.

Gäumann and co-workers (14,15) were the first to demonstrate nonspecific induction of phenolics and disease resistance in plants by mycorrhizal fungi. Orchinol and other compounds capable of inhibiting many organisms were induced by mycorrhiza-forming *Rhizoctonia* species and by nonmycorrhizal organisms. Our findings were similar and extend the demonstration of induced resistance to the association of tree seedlings with ectomycorrhizal fungi. Although an array of microorganisms may elicit resistance, mycorrhizal fungi also have the potential for entering a symbiotic association with the host, stimulating growth, and enhancing survival of young plants. As evidenced by the difference between *L. laccata* and *C. geophilum* in phenolic induction, mycorrhizal fungi may differ in ability to elicit resistance. Such ability should be considered in the selection of symbionts for inoculation in nurseries.

Phenolics were absent from roots colonized and infected by *F. oxysporum*. The pathogen may have mechanisms that inhibit or avoid eliciting synthesis of fungistatic substances (3,16) and thereby allow it to invade the suscept.

Rates of growth of pathogens toward the root and intensity of rhizoplane colonization were not influenced by *L. laccata*. Furthermore, *L. laccata* and *F. oxysporum* grew together on the root surface with no observable suppression of one another. Thus, we judged antibiosis to be unimportant in root protection although *L. laccata* had been shown capable of inhibiting *F. oxysporum* in vitro (39).

We have presented direct evidence that a mycorrhizal strain of *L. laccata* can protect seedlings of Douglas-fir from root pathogens in the absence of other organisms. The potential role of other soil organisms in modifying root protection, however, appears large and deserves further study. Levels of root protection by *L. laccata* previously demonstrated in nursery beds were modest (34), and protection was discerned only in fumigated soil. Strains of mycorrhizal fungi, which colonize the primary root rapidly, compete aggressively with other soil organisms, and induce a consistent deposition of phenolics in roots may improve efficacy of biocontrol. Nonmycorrhizal organisms that colonize the radicle and induce resistance should also be investigated, not only for biocontrol ability but also for effects on mycorrhizal formation.

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