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RESPONSE OF NURSERY SOIL MICROBIAL POPULATIONS TO VOLATILES PURGED FROM SOIL AROUND DOUGLAS-FIR ECTOMYCORRHIZAE

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Summary—Ectomycorrhizae produce biologically-active volatile compounds *in vitro*. Although the microbial populations in the soil surrounding ectomycorrhizae are known to differ quantitatively and qualitatively from those in the soil surrounding nonmycorrhizal roots, it is not known whether volatiles contribute to this difference. An apparatus which slowly purges volatiles from a "donor" test soil into a "receiver" soil was used to determined the effect of volatiles from pasteurized "donor" soils containing ectomycorrhizal Douglas-fir seedlings on *Fusarium* and other microbial populations of a "receiver" nursery soil. Seedlings which were ectomycorrhizal with *Laccaria laccata* or a *Hebeloma* sp., produced volatiles which significantly increased bacterial populations in receiver soils. The populations of actinomycetes, *Fusarium*, extracellular chitinase producers, facultative anaerobes and phosphate-solubilizing bacteria were not significantly influenced by volatiles from ectomycorrhizal seedlings compared to those from nonmycorrhizal seedlings. Factors which may influence the quantity and quality of volatiles produced by an ectomycorrhizal seedling and the consequent effect on microbial populations in the ectomycorrhizosphere are discussed.

INTRODUCTION

Fusarium oxysporum Schlecht. causes a seedling disease of the major conifers grown in the Pacific Northwest nurseries (Bloomberg, 1981). In contrast to nursery soils, coniferous soils covered with needle litter rarely harbor detectable populations of *Fusarium* (Schisler and Linderman, 1984). The exclusion of *Fusarium* from coniferous forest soils has been attributed to the lack of annual plants in coniferous forest soils (Toussiun, 1975) and to the germination and lysis effect of needle duff leachates (Hammerschlag and Linderman, 1975) and the forest soil microbiota (Schisler and Linderman, 1984) on *Fusarium* propagules.

Ectomycorrhizae may also contribute to the exclusion of *Fusarium* from coniferous forest soils. Ectomycorrhizae can improve plant nutrition, growth and drought stress tolerance and act as deterrents to pathogenic root infections (Marx, 1972). Inoculating fumigated nursery soil with spores of *Laccaria laccata*, a common ectomycorrhizal symbiont in nursery and forest soils, was shown to suppress *Fusarium* disease (Sinclair *et al.*, 1975). In greenhouse studies, *L. laccata* also decreased *Fusarium* disease expression and increased seedling growth (Sinclair *et al.*, 1982). This symbiont produces diffusible extracellular metabolites and induces primary host roots to produce phenolic compounds which may account for the protective effect of *L. laccata* against *Fusarium* (Sylvia, 1983; Sylvia and Sinclair, 1983a, b). The production of antifungal, antibacterial or antiviral compounds by over 100 species of ectomycorrhizal fungi (Marx, 1982) suggests that ectomycorrhizal fungi other than *L. laccata* could also influence *Fusarium* survival and disease.

Volatile compounds can stimulate or inhibit germination of fungal propagules and subsequent germ tube growth (Fries, 1973). Ectomycorrhizae produce solvent-extractable volatile compounds which can inhibit the *in vitro* growth of several root pathogens (Krupa and Fries, 1971; Krupa and Nylund, 1972; Krupa *et al.*, 1973). Furthermore, Graham and Linderman (1980) demonstrated, *in vivo* under aseptic conditions, that ectomycorrhizae could release significant amounts of ethylene into the mycorrhizosphere soil, and Smith (1976) has suggested that ethylene may play a role in soil fungistasis. The results from several studies indicate that volatile production by ectomycorrhizal fungal symbionts may influence *Fusarium* or microbial groups deleterious to *Fusarium* (Stack and Sinclair, 1975; Schisler and Linderman, 1989). It is documented that the soil microbial populations around ectomycorrhizae differ quantitatively and qualitatively from populations in soil around nonmycorrhizal roots. These differences may render a root resistant to pathogenic attack (Malajczuk and McComb, 1979; Strzelczyk and Pokojska-Burdziej, 1984). It is not known, however, whether volatile compounds from ectomycorrhizae can incite or maintain these microbial differences *in situ*. Using a device specifically constructed to purge volatile compounds from the soil around seedling roots and introduce them into a second soil (Schisler and Linderman, 1989), we conducted an experiment to determine whether (a) volatiles from ectomycorrhizae formed by inoculating Douglas-fir seedlings with Pacific Northwest isolates of the ectomycorrhizal genera *Hebeloma* and *Laccaria* significantly

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influence soil populations of *Fusarium* and selected soil microbial groups and (b) volatiles from ectomycorrhizae formed by different ectomycorrhizal fungal isolates differ in their influence on soil microbial group populations.

MATERIALS AND METHODS

Ectomycorrhizal fungi

Four ectomycorrhizal fungi common to Pacific Northwest forest and nursery soils (Trappe and Strand, 1969) were selected for inoculation of Douglas-fir seedlings. Sites where isolates were obtained are briefly described below, including the dominant conifer species present at the site.

- —*Hebeloma crustuliniforme* isolate 2 (Hecr2)*—Bald Mountain, Oregon, 1890 m, *Abies concolor–Pinus contorta* mix.
- —*Hebeloma* sp. isolate S260T (HeS260T)—Upper Battle Greek, Oregon, 1460 m, *P. contorta–Pseudotsuga menziesii* mix.
- —*L. laccata* isolate T813 (LlT813)—Southwest Washington nursery, 360 m, *P. menziesii.*
- —*L. laccata* isolate B101 (LlB101)—Barrier, British Columbia, 1300 m, *P. menziesii.*

Liquid shake cultures of ectomycorrhizal fungi were prepared in 125 ml flasks containing 60 ml of modified Melin–Norkrans (MMN) (Marx and Kenney, 1982) broth. Flasks were inoculated with 3–5 0.5 cm plugs taken from the periphery of 2–3 week-old ectomycorrhizal fungal colonies grown on MMN agar. Liquid cultures were grown at $22 \pm 2°C$ for 28 days before use. Vermiculite inoculum was prepared in 1 l. flasks containing 600 ml of a mixture of 29:1 (v/v) fine vermiculite and hypnum peat moistened with MMN broth and sterilized by autoclaving for 60 min at 121°C. Flasks were inoculated with 10–15, 0.5 cm dia MMN agar plugs colonized by each fungus and were held in darkness at $22 \pm 2°C$ for 8 weeks before use.

Seedling preparation and inoculation

A mixture of fine vermiculite and sand and loam soil (1:1:1 /v/v/v) was air–steam pasteurized (60°C for 30 min), placed in 45 ml tubes (Ray Leach Conetainers, Canby, OR 97002), and sown with Douglas-fir seeds which had been soaked in 30% H_2O_2 for 120 min and then rinsed with tap H_2O for 12 h. Trees were thinned to one per tube at 5 weeks and inoculated at 6 weeks by injecting hyphal suspensions of ectomycorrhizal fungi directly onto the roots, using a hypodermic syringe, without disturbing the soil or roots. Hyphal suspensions were prepared by macerating (Virtis blender, 2 s at high speed) 28 day-old liquid cultures of ectomycorrhizal fungi and washing hyphal fragments twice with sterile distilled H_2O. Hyphal suspensions were maintained at approx. 0°C throughout preparation and then 4.5 ml of suspension containing fresh mycelium (equal to approx. 5 mg dry weight) was used to inoculate each seedling. Seedlings were grown at $24 \pm 3°C$ under ambient conditions and supplemented light (high pressure sodium vapor lamps, average = 300 $\mu E\ m^{-2}\ s^{-1}$) and fertilized weekly to saturation with 1/4 strength phosphorus Long Ashtons nutrient solution (Hewitt, 1966) until transplanted into the volatile exchange system (VES).

*Isolate number used in Fig. 1 and Table 1.

Volatile exchange system and experimental treatments

The VES used was described by Schisler and Linderman (1989). Donor soil cups contained ectomycorrhizal seedling plugs transplanted into a 1:1:1 mixture of fine vermiculite ectomycorrhizal-fungus inoculum, pasteurized sand and pasteurized loam soil. Vermiculite inoculum was washed three times with sterile distilled water and squeezed gently through cheese cloth before incorporation into donor cup soil mixes. Seedlings were 10 weeks old when transplanted into donor soil cups. Controls consisted of similarly prepared though uninoculated donor soil mixes planted with a nonmycorrhizal seedling. A 3:2 (v/v) Kellogg Oregon nursery soil (Schisler and Linderman, 1989): sand mix was used as the receiver soil in all cases.

Lighting and temperatures for seedling growth were as described above. Seedlings were harvested after terminating the experiment and measurements of stem caliper, root and shoot dry weights, and number of buds and lateral branches were made, as well as the % of short roots that were ectomycorrhizal.

There were six donor-receiver cup pairs for each treatment which were placed in a completely randomized design. The moisture contents of donor soil mixes were adjusted to −0.01 MPa (1 bar = 0.1 MPa) and the receiver soil mix to −0.02 MPa (by misting the soils with sterile distilled water and measuring the soil matric potential with a tensiometer) before filling the cups. The soil moisture was approximately reestablished to these potentials every 4–5 days during the experiment by adding sdH_2O up to the initial soil cup weights. Volatiles were purged from donor cups from 8–10 a.m. and 8–10 p.m. every day with compressed air at a flow rate of 3–5 ml min^{-1} measured at the receiver cup.

Analysis of microbial profiles

Six receiver soils per treatment were assayed at 40 and 90 days to determine populations of the following taxonomic and functional microbial groups: bacteria, actinomycetes, *Fusarium*, extracellular chitinase producers, facultative anaerobes and phosphate-solubilizing bacteria. Bacteria and actinomycete populations were assayed to indicate the total potential biological activity of receiver soils. Materials and methods used for estimating populations of microbial groups were described for most groups by Schisler and Linderman (1989). Phosphorus-solubilizing bacteria were detected using modified Pikovskaya's medium (Sundara Rao and Sinha, 1962) [using 0.4 g $MgSO_4$ and 16 g l^{-1} "gelrite" (Merck & Co., San Diego, Calif.) solidifying agent]. Bilayer plates were prepared by pouring 6 ml of this hot medium into plates containing solidified "gelrite" medium. Bacterial colonies from soil dilutions which were able to solubilize tricalcium phosphate were identified by a cleared zone in the gel around the colony and counts were determined after 6 days at 22–25°C.

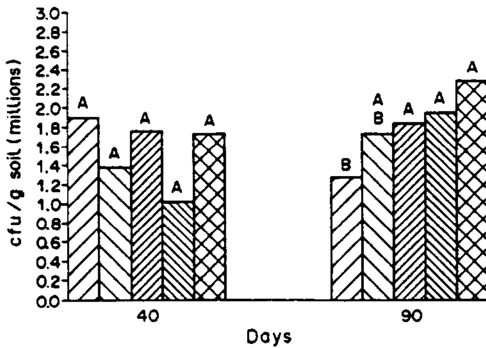

Fig. 1. Effect of volatiles from soils containing ectomycorrhizal Douglas-fir seedlings on the numbers of bacteria isolated from a receiver nursery soil. Values within an assay date not followed by the same letter are significantly different, $P < 0.05$ (Fisher's protected L.S.D. test). ▨ Control, ▧ Hecr2, ■ HeS260T, ▩ LT813, ▣ L1B101.

Data on microbial populations from receiver soil cups which were connected to donor cups containing the three most heavily ectomycorrhizal seedlings of each treatment were used for statistical analysis. Data were analyzed by one-way analysis of variance and means separated by Fishers protected L.S.D. test.

RESULTS

Volatiles from donor cups containing ectomycorrhizal seedlings frequently increased bacterial populations in receiver soils compared to controls ($P < 0.05$) at 90 but not 40 days (Fig. 1). The presence of ectomycorrhizal seedlings in donor cups did not significantly influence populations of actinomycetes, *Fusarium*, extracellular-chitinase-producers, facultative anaerobes or phosphate-solubilizing bacteria in receiver soils at either 40 or 90 days. Extreme variability in the numbers of extracellular-chitinase-producing organisms recovered from receiver soils at 90 days prohibited apparently large differences between treatment means from being statistically separated.

The technique of inoculating seedlings with ectomycorrhizal fungi by injecting hyphal suspensions directly onto intact root systems was largely successful in achieving colonization (Table 1), though the proportion of mycorrhizal short roots varied considerably (37–73%). Root systems of control seedlings did not become mycorrhizal. The shoot dry weights of seedlings colonized by *L. laccata* isolate T813 were significantly greater ($P < 0.05$) than controls. The root dry weights of seedlings colonized by *Hebeloma* S260T were significantly less than controls ($P < 0.05$).

DISCUSSION

Volatiles from ectomycorrhizae and the surrounding soil influenced the populations of the soil bacteria (Fig. 1). Volatiles may, therefore, be partially responsible for inducing the quantitative and qualitative differences in the microbial populations in soil around mycorrhizae (mycorrhizosphere) vs nonrhizosphere soils that were reported by Malajczuk and McComb (1979) and Strzelczyk and Pokojska-Burdziej (1984). Volatile-mediated increases in microbial populations of ectomycorrhizosphere soil may partially account for the resistance of some ectomycorrhizal root systems to fungal pathogen attack (Malajczuk and McComb, 1979; Sinclair *et al.*, 1982), since rhizosphere microorganisms could provide additional protection to the physical barrier of the ectomycorrhizal mantle around roots or by the production of antibiotics or siderophores.

Though bacterial populations increased in response to volatiles from ectomycorrhizal seedlings, populations of other microbial groups monitored did not change significantly. This could be due to other microbial groups being less effective competitors or utilizers of volatile carbon compounds. The quantity and quality of volatiles reaching the receiver soil cups may also be important. The length of time volatiles were purged in this investigation was shorter than that use in another study when more significant changes were observed, mediated by volatile compounds produced by microbial populations (Schisler and Linderman, 1989). The use of a pasteurized donor soil mix may also have lessened the quantity and quality of volatiles produced in donor soils since pasteurization may have eliminated ectomycorrhizosphere-competent microorganisms capable of producing volatiles or converting volatiles from ectomycorrhizae into biologically-active compounds.

The appearance of significance differences in bacterial populations of receiver soils after 90 days, but not 40 days, of exposure to volatiles from donor soils may be due to a number of factors. Since the morphology and physiology varies considerably as ectomycorrhizae mature (Piche and Peterson, 1984), this may delay the production of volatiles in sufficient quantities to affect microbial populations in receiver soil. Furthermore, sluggishness in the establishment of mycorrhizal short roots due to the potentially inhibitory effect of frequently purging donor

Table 1. Comparison of tree growth measurements and percent mycorrhizal short roots of seedlings inoculated with ectomycorrhizal fungi

Treatment	Top height (cm)	Stem caliper (mm)	Root dry weight (mg)	Shoot dry weight (mg)	Root shoot ratio	Buds (No.)	Lateral braches (No.)	Mycorrhizae (%)
1. Control	5.8a	1.2a	235a	152b	1.57a	3.7a	0.0a	0b
2. Hecr2	5.8a	1.2a	189a	144b	1.31a	4.3a	0.0a	37ab
3. HeS260T	5.7a	1.2a	127b	119b	1.07a	3.3a	0.0a	50a
4. L1T813	6.9a	1.3a	221a	221a	1.01a	3.0a	0.3a	53a
5. L1B101	6.8a	1.1a	203a	164ab	1.32a	3.0a	0.3a	73a

Values within a column not followed by the same letter are significantly different $P < 0.05$ (Fisher's protected L.S.D. test).

soil volatiles may be involved. Finally, soil absorption and adsorption, and microbial conversion of volatiles from ectomycorrhizae may have decreased concentrations of volatiles leaving donor soil cups and delayed receiver soil microbial population changes.

The importance of the size of the ectomycorrhizal root system and the proportion of infected short roots in influencing volatile-mediated changes in microbial populations is unknown. Although the average root dry weight of *Hebeloma* (HeS260T)-infected seedlings was less than controls or other ectomycorrhizae treatments (Table 1), volatiles from these ectomycorrhizal seedlings caused significant increases in bacterial populations in receiver soils (Fig. 1); and these increases were similar to those induced by volatiles from the other larger ectomycorrhizal root systems. Comparisons of the influence of heavily vs lightly *Laccaria*-infected seedlings showed that volatiles from heavily-infected seedlings consistently stimulated higher (though not statistically significant) bacterial populations in receiver soils (D. A. Schisler, unpublished results). Further studies on the influence of the degree of mycorrhizal infection on the quantity and quality of volatiles produced and their effect, *in vivo*, on soil microbial populations are needed.

The identity of the volatiles involved in this study remains unknown. Krupa *et al.* (1973) identified volatile terpenes present in solvent-extracted ectomycorrhizae; such compounds are possible candidates as the active components in this study. Graham and Linderman (1980) also demonstrated that a wide range of ectomycorrhizal fungi were capable of ethylene production *in vitro*, and both *Laccaria* and *Hebeloma* were among the best producers. Furthermore, they demonstrated that ethylene gas was released into the growth medium from ectomycorrhizae synthesized aseptically. Thus, ethylene is yet another candidate as an active volatile component for qualitative analysis of the purged volatiles in the VES and is a possible subject for study.

Inoculating seedlings by injecting hyphal suspensions of ectomycorrhizal fungi directly onto intact root systems, though labor-intensive, is very suitable for studies where the use of intact root systems is essential. Additionally, our preliminary trials have shown that vegetative inoculum of fungi, which previously have been ineffective in producing ectomycorrhizae, frequently formed ectomycorrhizae using this technique.

We have demonstrated that ectomycorrhizae are capable of producing biologically-active compounds which influence the microbial populations of ectomycorrhizosphere soil. The extent to which volatiles from ectomycorrhizae influence microbial populations in soil, however, remains unclear due to difficulties in delivering the same quantity and quality of volatiles to receiver soils as that produced *in situ* by ectomycorrhizae and ectomycorrhizosphere microorganisms.

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