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Title: Microbial Analysis of Coniferous Forest and Nursery Soils:

Effects of Ectomycorrhizae, Volatiles, and Humic-rich Organic

Substrates.

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Dr. Robert G. Linderman

Fusarium spp. frequent conifer nursery soils yet are rarely
recovered from coniferous forest soils. Experiments were performed
to determine what influence humic-rich organic amendments and
volatiles from soil, ectomycorrhizal fungi and ectomycorrhizae have
on the populations of several physiologic and taxonomic microbial
groups potentially related to this phenomenon.

A comparative microbial population analysis showed that
coniferous forest soils contained lower numbers of bacteria, fusaria,
facultative anaerobes, and putative nitrogen-fixing bacteria than
nursery soils, while the populations of actinomycetes, extracellular
chitinase producers, fluorescent pseudomonads, and phosphate
solubilizers did not differ. Higher concentrations of NH_4-N which in
the gaseous ammonia form can deleteriously affect Fusarium, were
found in forest soils.

The effects of volatiles purged from forest soils differed from
that of nursery soils on the populations of several microbial groups

in a nursery soil. Volatiles from a forest soil planted with a Douglas-fir seedling had a different effect on soil microbial populations than volatiles from the soil without a seedling. The importance of volatile-mediated microbial group population changes in relation to Fusarium exclusion from coniferous forest soils was not readily determinable. Volatiles from the soil surrounding ectomycorrhizal Douglas-fir seedlings significantly increased the bacterial population of a soil, suggesting that volatiles from ectomycorrhizae are involved in maintaining microbial populations in the ectomycorrhizosphere.

Volatiles from ectomycorrhizal fungi frequently stimulated Fusarium oxysporum colony expansion in vitro. Volatiles from forest or nursery soils had no differential effect on F. oxysporum colony expansion.

Amending three coniferous nursery soils with three different organic materials with a high content of humic substances frequently increased, but sometimes decreased, seedling growth. The degree of seedling growth stimulation or inhibition varied with the amendment, soil, and amount of amendment. Composted grape pomace stimulated tree growth in all soils and also increased the numbers of several soil microbial groups potentially deleterious to Fusarium. All amendments reduced Fusarium-induced damping-off in one of the soils. Ectomycorrhizae were increased by only one amendment and then in only one soil.

MICROBIAL ANALYSIS OF CONIFEROUS FOREST AND NURSERY SOILS:

EFFECTS OF ECTOMYCORRHIZAE, VOLATILES, AND HUMIC-RICH

ORGANIC SUBSTRATES

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I dedicate this work to my family whose love continues to strengthen me in all walks of life.

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INTRODUCTION

Fusarium spp. incite diseases of economic importance in Pacific
Northwest conifer nurseries (Bloomberg, 1981). Host symptoms induced
by pathogen infection include seed rot, pre- and post-emergence
damping-off, cotyledon blight, root rot (Bloomberg and Lock, 1972),
and hypocotyl rot (Brownell and Schneider, 1983). Though high
pathogen populations are commonly found in conifer nursery soils,
Fusarium is only rarely recovered from coniferous forest soils
covered with needle litter (Morrow, 1932; Thornton, 1960; Park, 1963;
Schisler and Linderman, 1984) and has not been reported as a seedling
pathogen in these soils. Investigations of this phenomenon have
suggested that needle litter leachates (Menzinger, 1969; Toussoun et
al., 1969; Hammerschlag and Linderman, 1975), the lack of annual
plants in coniferous forest soils (Toussan, 1975) and the forest soil
microbiota (Schisler and Linderman, 1984) contribute to the demise of
Fusarium when it is introduced into coniferous forest soils (Smith,
1967).

Several taxonomic or physiological groups of microorganisms may
be involved in the exclusion of Fusarium from coniferous forest
soils. Bacteria and actinomycetes can contribute to the overall
suppressiveness of a soil toward fungal pathogens (Baker and Cook,

1974; Cook and Baker, 1983). Fluorescent pseudomonads,
extracellular-chitinase-producing organisms, and facultative
anaerobes produce metabolites which can directly inhibit the survival
or activity of pathogenic fungi (Smith, 1976; Elad et al., 1982,
1983; Weller, 1985), while the activity of phosphate solubilizing
bacteria in soil may increase plant phosphorus nutrition (Goldstein,
1986) which can result in decreased conifer seedling succulence and
damping-off (Bloomberg, 1981).

The influence of soil volatiles and humic-rich organic
substances on the populations of microbial groups and Fusarium in
soils, though unknown, may be considerable. Volatiles contribute to
soil fungistasis (Lockwood, 1977), can deleteriously affect hyphal
growth and survival of Fusarium (Moore-Landecker and Stotzky, 1973;
Okazaki, 1985), and can influence populations of soil microorganisms
when diffused through soil (Gilbert et al., 1969). Furthermore,
volatiles inhibitory to root pathogenic fungi are produced by
ectomycorrhizae (Krupa and Nylund, 1972; Krupa et al., 1973; Graham
and Linderman, 1980) and ectomycorrhizal fungi (Krupa and Fries,
1971; Graham and Linderman, 1980). Biologically active volatile
compounds produced by soil microbes, ectomycorrhizae and/or released
during organic matter decay can include, but are not limited to,
ethylene, ammonia, low molecular weight aldehydes and alcohols, and
terpenoid compounds (Krupa et al., 1973; Linderman and Gilbert, 1975;
Stotzky and Schenck, 1976; Schippers et al., 1982). The content of
humic substances is depleted in soils under extended cultivation
(Parsons, 1985) but is high in soils supplied with organic matter

(Millar, 1974); organic matter being supplied in the form of needle litter in the case of coniferous forest soils. Humic substances can also influence the microbial balance of a soil (Visser, 1985a).

Determining whether soil volatiles and humic substances influence _Fusarium_ populations and/or the quantity and quality of microbes present in a soil would greatly contribute to our understanding of the nature of _Fusarium_ exclusion from coniferous forest soils and factors involved in the maintenance of soil microbial balances in general. Additionally, it would help one determine the feasibility of transferring biocontrol systems from forest to nursery soils. Accordingly, the objective of these investigations was to assess the effects of humic-rich organic amendments and volatiles from forest and nursery soils, ectomycorrhizae and ectomycorrhizal fungi on _Fusarium_ and the populations of microbial groups potentially deleterious to _Fusarium_.

CHAPTER 1

COMPARATIVE POPULATION ANALYSIS OF SELECTED MICROBIAL GROUPS
IN PACIFIC NORTHWEST CONIFEROUS FOREST AND NURSERY SOILS.

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SUMMARY

In order to determine the role of the microbiota in the
exclusion of _Fusarium_ from coniferous forest soils, a comparative
population analysis of taxonomic and physiologic microbial groups in
forest and nursery soils was undertaken. The average numbers of
bacteria, fusaria, facultative anaerobes, and putative nitrogen
fixing bacteria were less in forest than nursery soils, while the
populations of actinomycetes, extracellular chitinase producers,
fluorescent pseudomonads, and phosphate solubilizers did not
significantly differ between the two soil types. Higher soil
fertility and/or recent organic amendments to nursery soils was
frequently associated with higher microbial counts. Higher
concentrations of NH_4-N, which in the gaseous ammonia form can

deleteriously affect Fusarium, were found in forest soils than in
nursery soils. The possibility that ammonia gas and in situ
microbial activity in forest soils influences Fusarium survival is
discussed.

INTRODUCTION

It has been known for some time that coniferous forest soils
generally do not harbor detectable populations of Fusarium (Morrow,
1932; Thornton, 1960; Park, 1963; Schisler and Linderman, 1984).
However, Fusarium spp., which cause a number of economically
important conifer seedling root diseases (Bloomberg and Lock, 1972;
Brownell and Schneider, 1983), commonly are found in high populations
in nursery soil (Bloomberg, 1981). In 1967, Smith reported that
Fusarium oxysporum Schlecht. disappeared from the root systems of
infected sugar pine (Pinus lambertiana) transplanted into native
forest soil, and that Fusarium was no longer recoverable from roots
after 3 years. The inability of Fusarium to survive in coniferous
forest soils has subsequently been attributed to the deleterious
effects of needle litter leachates (Menzinger, 1969; Toussoun et al.,
1969; Hammerschlag and Linderman, 1983), the lack of annual plants in
coniferous forest soils (Toussan, 1975) and on the effects of the
forest soil microbiota (Schisler and Linderman, 1984) on Fusarium
macroconidia, hyphae and chlamydospores.

Though numerous studies have been reported on the microbial
populations of coniferous forest or agricultural soils (Clark and
Paul, 1970; Nioh, 1977; Schnurer et al., 1986), few have compared the

microbial populations of soils from these two ecosystems (Theodorou, 1984). Moreover, little is known regarding the relative populations of physiological or functional groups (Katznelson and Rouatt, 1957) of microorganisms in forest and nursery soils. The lower fertility of forest soils compared to nursery soils, and the selective influence of humic substances on microbial growth and activity (Martin et al., 1976; Nioh, 1976; Visser, 1985) in forest soils, suggests that populations of physiologically distinct microbial groups may differ between forest and nursery soils. Furthermore, needle duff leachates contain chemical compounds that can inhibit nitrifying organisms which consequently leads to NH_4 accumulation in forest soils (Lodhi and Killingbeck, 1980; White, 1986). High concentrations of NH_3 gas can increase the lysis of macroconidia of several Fusarium species in soil and decrease chlamydospore formation (Loffler and Schippers, 1985; Loffler et al., 1986) and thus could partially account for the exclusion of Fusarium from coniferous forest soils.

This study was undertaken to determine if 1) the populations of several taxonomic and physiological groups of microorganisms in selected Pacific Northwest coniferous forest and nursery soils differed, 2) microbial group population differences between forest and nursery soils could relate to the observed exclusion of Fusarium from coniferous forest soils, and 3) NH_4-N levels are higher in selected coniferous forest soils than nursery soils.

MATERIALS AND METHODS

Soil sites and preparation

Soils from 12 coniferous forest and 12 coniferous nursery sites
were sampled in early summer after seasonal rains had ceased for most
of the Pacific Northwest. Forest sites were located from Yakima
County, Washington, to Jackson County, Oregon. Nursery sites were
located in the region from Skamania County, Washington, to Klamath
County, Oregon. Sites were distributed over a wide range of soil
types, slopes and elevations. Whenever possible, forest soil sites
were selected which were in close proximity to nursery soil sites.
The dominant conifer species present at the forest sites included one
or more of the following: Douglas-fir (Pseudotsuga menziesii), noble
fir (Abies procera), grand fir (Abies grandis), silver fir (Abies
amabilis), ponderosa pine (Pinus ponderosa), lodgepole pine (Pinus
contorta), and western hemlock (Tsuga heterophylla). Herbaceous
understory vegetation was negligible due to preferential selection of
sites with closed conifer canopies and well developed needle duff
layers. Nursery soil sites were fallow, seeded with Douglas-fir, or
cropped with one of several cover crops, Douglas-fir, or lodgepole
pine.

At each site, four or five samples of approximately 3 L were
collected from the top 10 cm of mineral soil, pooled, and
refrigerated for a maximum of 60 days at 5°C until assayed for
microbial group populations. Sampling locations at each site were
selected at random within a 25 m by 25 m sampling area. Prior to

assaying soils for microbial populations, pooled samples were sieved
through a 2 mm mesh screen.

Five forest and five nursery soils were chosen for nutritional
and chemical property analysis (Harris Laboratories, Inc., Lincoln,
NE) on the basis of the proximity of a nursery and forest soil site
or the uniqueness of the dominant plant cover at a site and are
described below.

Nursery

-- Elkton, Oregon (soil N2)[1] - Elevation, 60 m, silt loam,
   Pseudotsuga menziesii

-- Bonanza, Oregon (soil N5) - Elevation, 1280 m, coarse sandy
   loam, Avena sativa, Pisum sativum rotation for 6 years.

-- Bonanza, Oregon (soil N6) - Elevation, 1280 m, coarse sandy
   loam, Pinus contorta

-- Mt. Hood, Oregon (soil N9) - Elevation, 590 m, coarse sandy
   loam, Avena sativa for 1 year

-- Wind River, Washington (soil N10) - Elevation, 360 m, sandy
   loam, Pseudotsuga menziesii

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[1] Soil number used in Tables 1-2, 1-3

Forest

 -- Mt. McLoughlin, Oregon (soil F1) - Elevation, 1370 m,

  gravelly loamy sand, <u>Abies</u> <u>amabilis</u>

 -- Mt. Hood, Oregon (soil F8) - Elevation, 600 m, coarse sandy

  loam, <u>Pseudotsuga</u> <u>menziesii</u>

 -- Mary's Peak, Oregon (soil F9) - Elevation, 1200 m, clay

  loam, <u>Pseudotsuga</u> <u>menziesii</u>, <u>Abies</u> <u>amabilis</u>

 -- Cascadia, Oregon (soil F10) - Elevation, 260 m, clay loam,

  <u>Pseudotsuga</u> <u>menziesii</u>

 -- Wind River, Washington (soil F12) - Elevation, 360 m, sandy

  loam, <u>Pseudotsuga</u> <u>menziesii</u>

<u>Analysis of microbial profiles</u>

 Three sub-samples from each site's pooled soil sample were used
for dilution plate analysis of soil microbial populations of the
following taxonomic and functional microbial groups: bacteria,
actinomycetes, extracellular chitinase producers, <u>Fusarium</u>,
fluorescent pseudomonads, facultative anaerobes, phosphate-
solubilizing bacteria and putative nitrogen fixers. Bacteria and
actinomycete populations were assayed to indicate the total potential
biological activity of the soils. Microorganisms capable of
producing extracellular chitinase have been implicated in the lysis
of hyphae of some plant pathogenic fungi (Mitchell and Alexander,
1963; Tominaga and Tsujisaka, 1976; Elad <u>et</u> <u>al</u>., 1982, 1983), while
many fluorescent pseudomonads are potent biological control agents

(Weller, 1985). Facultative anaerobes frequently are producers of ethylene, a volatile compound which may be involved in soil fungistasis (Smith, 1976). Phosphate-solubilizing bacteria in plant rhizosphere soil could potentially increase plant phosphorus nutrition and growth (Goldstein, 1986). Decreased seedling succulence and _Fusarium_-induced damping-off has been observed with increased phosphorus nutrition of coniferous seedlings (Bloomberg, 1981). The population of putative nitrogen-fixing organisms was estimated as an indicator of the potential of a soil to fix N_2 gas. Materials and methods used for estimating populations of microbial groups are as described elsewhere for most groups (Schisler, Chapters 2 and 3). The number of putative nitrogen-fixing organisms was estimated by dilution plating of soil 0.1% H_2O agar suspensions onto Rennie's combined carbon medium (Rennie, 1981). Counts were determined after plates were incubated for 6 days in "Gas-Pak" (BBL Microbiology Systems, Cockeysville, MD) anaerobic chambers at 22-25°C. The data were analyzed using a one-way analysis of variance and means separated using Fisher's protected L.S.D. test.

RESULTS

The number of microorganisms recovered from forest and nursery soils differed significantly for several physiologic and taxonomic microbial groups. The average population of _Fusarium_ in forest soils was significantly less ($P < 0.01$) than for nursery soils, (Table 1-1), and _Fusarium_ was recovered from only 1 forest soil but from all 12 nursery soils (Table 1-2). The average number of bacteria,

facultative anaerobes, and putative nitrogen-fixing bacteria recovered from forest soils was also less (P <0.05) than for nursery soils. The populations of actinomycetes, extracellular-chitinase-producing organisms, fluorescent pseudomonads, and phosphate-solubilizing bacteria did not differ between the two soil types (Table 1-1).

Higher soil fertility and/or the recent incorporation of crop residues into nursery soils was associated with higher numbers of microorganisms being recovered from such soils. Nursery soils N2 and N6 generally were high in fertility (Table 1-3) and contained high populations of bacteria and phosphate-solubilizing bacteria compared to most other forest and nursery soils (Table 1-2). Furthermore, nursery soil N3, amended with mint refuse and sawdust the previous fall, contained the highest number of bacteria and facultative anaerobes of any soil. Interestingly, forest soils F4 and F6 contained significantly higher numbers of extracellular chitinase-producing organisms than did any other soils (P < 0.05, Table 1-2).

The nursery soil from Mt. Hood, OR (N9) contained significantly greater numbers of actinomycetes (P< 0.01) and lower numbers of fusaria (P < 0.01) than did the forest soil near it (F5) (Table 1-2). Conversely, the nursery soil from Wind River, WA (N10) harbored fewer actinomycetes (P < 0.05) than did the forest soil near it (F12).

Forest soils tended to contain greater quantities of NH_4-N than did the nursery soils analyzed (Table 1-3).

DISCUSSION

Not unexpectedly, Fusarium was found to occur in high populations in all nursery soils, while forest soils contained no recoverable Fusarium populations except for one soil. This finding supports other reports of Fusarium exclusion from coniferous forest soils (Morrow, 1932; Thornton, 1960; Park, 1963; Toussoun et al., 1969) and our earlier report in which Fusarium was detected at low levels in a forest soil (Schisler and Linderman, 1984). The source of these infrequent pockets of Fusarium in forest soils is unknown as is whether they are an inoculum source for Fusarium infestation of conifer seed and seed cones (Graham and Linderman, 1983).

Forest soils, which tended to be lower in fertility than the nursery soils analyzed (Table 1-3), also supported a lower average population of several taxonomic and physiologic microbial groups (Table 1-1). Nitrogen and, to some extent, phosphorus can be immobilized in needles during litter decomposition (Edmonds, 1984) which may partially account for the lower microbial populations found in the forest soils. Compounds deleterious to the growth and survival of some soil microbes are present in forest floor litter extracts (Lodhi and Killingbeck, 1980; White 1986). Further, the microbial population estimates obtained for soils is based on that fraction of the soil microbiota able to grow on laboratory media. The actual microbial population present in forest soils is likely greater than that which was recovered, since humic substances appear essential to the growth of some soil microbes (Visser, 1985). The high pH used in the selective media may also have reduced forest soil

microbial population estimates by excluding organisms adapted
(Williams et al., 1971) to the low pH commonly found in forest soils
(Table 1-3). Finally, the populations of taxonomic and microbial
groups in forest soils are subject to seasonal variation (Nioh, 1977;
Soderstrom, 1979; Bathe, 1982) and, therefore, may be more closely
aligned to that of nursery soils during other times of the year.

The association of high soil fertility and high microbial
populations (soil N2 and N6) has been noted in previous studies
(Theodorou, 1984; Schnurer, 1986). Interestingly, soils high in
soluble phosphate (soils N2 and N6, Table 1-3) also contained high
populations of phosphate-solubilizing bacteria (Table 1-2). Organic
matter additions frequently cause an increase in soil microbial
activity and populations (Bell, 1974) as was seen in the nursery soil
which had been previously amended with mint refuse and sawdust (N3,
Table 1-2).

Trends in the differences between the average populations of
taxonomic or physiological microbial groups from nursery and forest
soils (Table 1-1) or nursery and forest soils of similar parent
material (Soils N9 vs. F5, N10 vs. F12; Table 1-2) were not readily
apparent. Comparative population analyses of forest and nursery soil
microbial groups, therefore, do not convincingly suggest any specific
microbial group as contributing to the exclusion of Fusarium from
coniferous forest soils. However, the microbial population numbers
in forest soils is not necessarily an indicator of how active these
microbes are in situ. If the high populations of extracellular-
chitinase-producing organisms in forest soils (Table 1-1) actively

produce enzymes in situ, they would likely contribute to the deleterious nature of forest soils toward Fusarium. NH_4-N, which was found to occur in high concentrations in forest soils (Table 1-3), may in the gaseous ammonia form, also contribute to the demise of Fusarium in forest soils by stimulating the lysis of Fusarium macroconidia and decreasing Fusarium chlamydospore formation (Loffler and Schippers, 1985; Loffler et al., 1986).

Table 1-1. Average number of colony-forming units of bacteria and actinomycetes recovered per gram of nursery or coniferous forest soil.

Soil	Bacteria	Actino	Chitin	Fusarium	Fluor. pseudo.	Facult. anaerobes	Phos. sol.	Putative N-fixers
Nursery	7.68E+07[a]	1.72E+06	1.02E+06	3.96E+03	1.85E+06	6.50E+06	9.13E+06	1.50E+06
Forest	5.88E+06*	1.12E+06	1.97E+06	5.55E+00**	3.21E+05	4.99E+05*	9.30E+05	5.21E+05*

Forest soil values within a column followed by a "*" or "**" are significantly different from the nursery soil value, $P < 0.05$, $P < 0.01$, respectively (Fisher's protected L.S.D. test).

[a] Table values are in scientific notation, ie, 7.68E+07 = 7.68×10^7

Table 1-2. Number of colony-forming units of bacteria and
actinomycetes recovered (per gram soil) from a variety of nursery and
forest soils (nr = none recovered).

Soil[a]	Bacteria	Actino	Chitin	Fusarium	Fluor. pseudo.	Facult. anaerobes	Phos. sol.	Putative N-fixers
N1	4.9E+07[b]	2.7E+03	1.1E+05	1.4E+02	7.5E+06	9.8E+05	8.6E+06	4.6E+05
N2	2.1E+08	1.1E+06	2.8E+06	2.4E+03	7.2E+06	8.7E+05	7.1E+07	7.7E+05
N3	2.8E+08	3.2E+06	3.2E+06	1.6E+03	7.2E+06	8.3E+06	9.4E+06	2.4E+06
N4	2.7E+07	3.9E+05	3.4E+05	1.8E+02	nr	7.5E+05	2.9E+06	5.5E+05
N5	1.1E+07	3.2E+06	1.3E+06	3.2E+03	8.3E+04	2.3E+06	1.1E+06	1.5E+06
N6	2.2E+08	1.1E+06	7.5E+05	1.1E+04	nr	1.1E+06	1.0E+07	7.2E+05
N7	6.5E+07	6.3E+05	2.0E+05	3.8E+03	1.1E+05	2.3E+07	2.4E+06	2.4E+06
N8	1.3E+07	1.1E+06	3.7E+05	4.6E+03	9.8E+04	2.9E+07	9.9E+05	3.3E+06
N9	1.2E+07	2.2E+06	3.8E+05	4.1E+03	1.6E+04	1.6E+06	7.5E+05	1.7E+06
N10	9.3E+06	8.3E+05	1.5E+06	6.0E+02	1.6E+04	4.3E+06	1.1E+06	9.3E+05
N11	1.7E+07	4.2E+06	8.6E+05	7.8E+03	4.2E+03	4.3E+06	8.9E+05	1.8E+06
N12	5.8E+06	2.6E+06	4.6E+05	8.2E+03	7.6E+03	1.1E+06	2.4E+05	1.3E+06
F1	7.7E+06	2.2E+06	1.3E+06	nr	4.2E+05	3.5E+06	1.1E+06	5.1E+04
F2	3.4E+06	9.1E+05	6.8E+05	nr	2.2E+06	5.2E+04	5.2E+05	1.1E+04
F3	4.2E+05	2.3E+06	1.2E+06	nr	4.2E+04	3.3E+05	2.6E+05	1.3E+05
F4	6.4E+06	2.7E+05	6.7E+06	6.7E+01	6.2E+05	1.8E+04	1.6E+06	4.0E+04
F5	2.7E+06	3.6E+04	1.1E+06	nr	5.4E+03	4.5E+04	1.0E+06	2.0E+04
F6	1.0E+07	5.5E+05	5.6E+06	nr	1.9E+05	1.9E+04	1.2E+06	4.0E+04
F7	3.3E+06	3.1E+05	3.3E+05	nr	1.4E+03	2.1E+04	3.1E+05	1.4E+05
F8	5.6E+06	9.3E+05	5.6E+04	nr	6.5E+04	5.1E+05	1.2E+06	4.4E+06
F9	1.3E+07	6.2E+05	2.9E+06	nr	1.9E+04	6.5E+05	1.7E+06	2.0E+05
F10	8.0E+06	2.6E+06	1.9E+06	nr	2.6E+04	5.0E+05	1.2E+06	1.1E+06
F11	1.1E+06	1.1E+05	2.2E+05	nr	7.2E+03	1.6E+04	1.2E+05	1.3E+04
F12	8.7E+06	2.6E+06	1.9E+06	nr	2.7E+05	2.8E+05	8.9E+05	1.3E+05
α = 0.05	4.07E+07	5.91E+05	1.97E+06	1.79E+03	2.12E+06	6.24E+06	1.66E+06	2.27E+06
α = 0.01	5.43E+07	7.89E+05	2.64E+06	2.39E+06	2.84E+06	8.33E+06	2.22E+06	3.03E+06

[a] Nursery soils = N1-N12; forest soils = F1-F12.   [b] Table values are in scientific notation.

Table 1-3. Chemical and nutritional properties
of forest and nursery soils.

Soil	pH	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	% OM	CEC	NH_4 (ppm)	NO_3 (ppm)
N2	5.1	114	261	1299	11.0	3.1	21.2	1	22
N5	6.1	43	273	1014	15.8	2.4	10.5	1	1
N6	6.5	104	402	1181	9.6	2.0	8.9	6	8
N9	5.7	46	109	813	6.3	4.2	11.7	6	19
N10	5.9	117	101	710	2.9	4.5	10.1	1	9
F1	6.4	27	239	1537	15.3	8.6	14.3	11	6
F8	6.1	22	68	458	5.1	4.5	9.8	23	2
F9	4.3	33	94	137	5.8	25.9	20.2	3	50
F10	5.7	88	419	2003	5.9	5.7	22.6	12	16
F12	5.5	53	169	1181	14.7	7.2	16.2	29	10

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CHAPTER 2

SELECTIVE INFLUENCE OF VOLATILES PURGED FROM CONIFEROUS FOREST

AND NURSERY SOILS ON MICROBES OF A NURSERY SOIL.

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SUMMARY

Numerous genera of ectomycorrhizal fungi and other soil microbes

produce volatile compounds in vitro. The consequence of in vivo

volatile production by soil microorganisms is unknown largely due to

difficulties in separating volatile effects from other microbially

mediated effects. An apparatus which slowly purges volatiles from a

"donor" test soil into a "receiver" soil was used to determine

differential effects of volatiles from "donor" forest or nursery

soils on Fusarium and other microbial populations of a "receiver"

nursery soil. "Donor" soils were either treated or not with methyl

bromide and aerated-steam, and were planted or not with Douglas-fir

seedlings. "Donor" soil origin and tree presence significantly

altered populations of several taxonomic and functional microbial

groups in "receiver" nursery soils, demonstrating for the first time that soil volatiles selectively influence soil microbe populations in vivo. Though functional microbial group populations were differentially altered by volatiles from forest and nursery soils, the importance of this phenomenon in relation to Fusarium exclusion from coniferous forest soils is not readily determinable.

INTRODUCTION

Fusarium oxysporum Schlecht. can induce seed rot, pre- and post-emergence damping-off, cotyledon blight, hypocotyl rot and root rot of conifer seedlings in Pacific Northwest nurseries (Bloomberg and Lock, 1972; Brownell and Schneider, 1983). Fusarium, however, has not been reported as a seedling pathogen in coniferous forest soils covered with needle litter and rarely reaches detectable population levels in these soils (Thornton, 1960; Park, 1963; Schisler and Linderman, 1984; Schisler, Chapter 1). In 1967, Smith reported that Fusarium oxysporum could not be recovered from roots of previously infected sugar pine (Pinus lambertiana Dougl.) seedlings transplanted into native forest soils three years earlier. Further studies demonstrated that forest soils stimulate Fusarium oxysporum chlamydospore and macroconidia germination followed by germ-tube lysis owing to the influence of needle duff leachates and the forest soil microbiota (Menzinger, 1969; Toussoun et al., 1969; Hammerschlag and Linderman, 1975; Schisler and Linderman, 1984).

The importance of volatiles in the survival of _Fusarium_ in forest soils is unknown, although they could conceivably exert considerable influence. Ectomycorrhizae produce numerous volatile terpenoid compounds which can inhibit the growth of various root pathogens _in vitro_ (Krupa and Fries, 1971; Krupa and Nylund, 1972; Krupa _et al._, 1973). Furthermore, volatiles from the spores of ectomycorrhizal fungi or microbes associated with these spores may have protected the roots of conifers from _Fusarium_ infection in glasshouse trials (Stack and Sinclair, 1975). Forest soils also are high in organic matter which, when decomposing, can release volatiles inhibitory to fungi (Gramss, 1986a, b). Biologically active volatile plant residue decomposition products, which include low molecular weight aldehydes and alcohols, can also contribute to decreased survival of pathogenic fungal resting structures, presumably directly, or indirectly via influence on associated antagonistic microorganisms (Linderman and Gilbert, 1969, 1975; Gilbert and Linderman, 1971). Volatiles can cause morphological abnormalities in _Fusarium_ and other fungi (Moore-Landecker and Stotzky, 1973) which may partially account for the abnormal germ tubes and replacement chlamydospores which result when _Fusarium_ chlamydospores are germinated in forest soils.

Volatiles are reported to affect fungi in numerous ways including: the stimulation of propagule germination (King and Coley-Smith, 1968; Linderman and Gilbert, 1969; Punja _et al._, 1984), the orientation of hyphal growth (Fries, 1973; Koske, 1982), and the inhibition of hyphal growth and propagule germination (Robinson and

Park, 1966; Hora and Baker, 1972; Stotzky and Schenck, 1976; Gupta
and Tandon, 1977; Lockwood, 1977; Schippers and Pruis, 1978; Pavlica
et al., 1978; Schippers et al., 1982; Herrington et al., 1985).
Definitive information on how volatiles influence microbial balances
in soils is more limited. Muller (1965) demonstrated that volatile
terpenes from leaves of Salvia leucophylla decreased the number of
bacteria recovered from soil. Hayes and co-workers (1969) showed
that Agaricus bisporus emanates volatile compounds which cause
populations of Pseudomonas putida to increase, and that this
pseudomonad produces volatiles which stimulate sporophore formation
in A. bisporus. Volatiles from alfalfa, when diffused through soil,
can increase or decrease populations of soil bacteria, fungi, and
actinomycetes (Gilbert et al., 1969; Owens et al., 1969). Though
literature reporting the effects of volatiles on fungi and soil
microbes in vitro is voluminous, studies on the influence of
naturally occurring levels of soil volatiles on the microbial ecology
of a soil have not been attempted. The difficulty in experimentally
assaying the influence of volatiles from a given soil on the
microbial population of a second soil undoubtedly accounts for this
void. Using a device specifically constructed for such experiments,
our objectives in this study were to determine if a) volatiles from
forest and nursery soils differ in their effect on the microbial
profile of a test soil, b) volatiles from forest soils, forest soil
microbes and/or mycorrhizae directly decrease Fusarium populations or
increase populations of soil microorganisms potentially deleterious

to _Fusarium_, and c) <u>in vivo</u> studies on the influence of soil
volatiles on microbial populations are feasible.

MATERIALS AND METHODS

Soil sites and preparation

Soils from two coniferous forest sites and three nursery sites
were sampled in early summer after seasonal rains had ceased in the
Pacific Northwest. Each soil site is briefly described below,
including the predominant conifer species present at the site.

Forest-

-- Mary's Peak, Oregon (soil F1)[1] - Elevation, 1200 m, clay
   loam, <u>Pseudotsuga</u> <u>menziesii</u>, <u>Abies</u> <u>amabilis</u>

-- Cascadia, Oregon (soil F2) - Elevation, 60 m, silt loam,
   <u>Pseudotsuga</u> <u>menziesii</u>

Nursery

-- Elkton, Oregon (soil N1) - Elevation, 60 m, silt loam,
   <u>Pseudotsuga</u> <u>menziesii</u>

-- Kellogg, Oregon (soil N2) - Elevation, 60 m, silt loam,
   <u>Pseudotsuga</u> <u>menziesii</u>

-- Mt. Hood, Oregon (control) - Elevation, 590 m, coarse sandy
   loam, <u>Pseudotsuga</u> <u>menziesii</u>

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[1] Soil number used in Figures 2-3 through 2-16.

For forest sites, herbaceous understory vegetation was minimal due to preferential selection of sites with closed canopies and well developed needle litter layers. At each site, four or five samples of approximately 3 L were collected from the top 0-10 cm of mineral soil, pooled, and refrigerated at 5°C until needed. Sampling locations at each site were randomly selected within a 25 m by 25 m sampling area. Prior to experimental use, pooled samples were sieved through a 2 mm mesh and mixed with pasteurized (60°C aerated steam for 30 min) river sand (60 soil:40 sand).

Volatile exchange apparatus (Figs. 2-1 and 2-2)

A volatile exchange system was designed in which ambient greenhouse air passes from an air compressor through dust and particulate oil filters into the main manifold (9.5 cm I.D. PVC pipe) (Figs. 2-1, 2-2, a) of the volatile exchange system (VES) and subsequently into ten submanifolds (5.1 cm I.D. PVC pipe) (Fig. 2-2, b). Tygon tubing (0.635 cm I.D.) (c) was used to connect 10 ports on each submanifold to the tops of donor soil cups (plastic, 210 ml capacity) (d). Air flow was at 3-5 ml/min through tygon tubing to the top of a sealed donor cup, through the soil column (purging donor soil volatiles), through holes in the bottom of the donor soil cup (e) and into a plastic sleeve below (f). The displaced donor soil atmosphere then passes through tubing (g), into the top of a sealed receiver soil cup (h) and flows through the receiver soil column (exposing receiver soil microflora to donor soil volatiles). Flow continues out holes in the bottom of the receiver soil cup (i), into

a plastic sleeve below (j), and finally out of the system through a

hole in the sleeve (k). A bubble flow meter was connected to this

hole to measure air flow rates. Flow rate for individual cups was

altered by using adjustable clamps (l). Quick release tubing

connectors (m) provided access to cups for watering with a hypodermic

syringe. A fiberglass plug (n) between donor and receiver cups was

used to minimize the chance of microbial propagule transfer between

cups to assure that changes in receiver soil microbial profiles were

due to volatile effects alone. A 1 cm thick layer of chicken grit

(gage #2) was placed on the bottom of cups and on the top of soil

columns to assure air passage ports did not become clogged with

soil. Lids of cups, with a 0.5 cm hole in the center and slit from

the center to one edge, allowed seedlings to be planted in cups with

minimal damage to tree shoots and roots. All lids were sealed onto

cups using adhesive clay (Permagum, Virginia Chemicals, Portsmouth,

VA).

Experimental treatments

Mary's Peak, Cascadia (forest soils), Elkton, and Kellogg

(nursery soils) soil mixes were used as "donors" of soil volatiles,

while the Mt. Hood mix (nursery soil) was used as a "receiver" soil

in all cases. Sand was added to natural soils to provide additional

soil porosity needed to facilitate uniform flow rates of volatiles

from donor to receiver soils. "Donor" soils were pasteurized (60°C

aerated steam for 30 min) and fumigated (P-F) (0.454 kg of a 95%

methyl bromide, 5% chloropicrin mixture and 12 L soil spread 2 cm

thick in a 2.5 m^3 sterilizing chamber for 24 h) or were left untreated. Differential effects on the microbial profile of the receiver soil due to volatiles from P-F vs. fresh donor soils would imply that volatiles from the soil microbiota influence microbial populations.

Cups containing fresh and P-F Mary's Peak (forest) and Elkton (nursery) donor soils were planted or not with a 30-day-old Pseudotsuga menziesii seedling at day 30 of the experiment. Seedlings were grown from seed in flats in greenhouses at 24 ± 3°C under ambient and supplemented (high pressure sodium vapor lamps) light (average = 300 microeinsteins/m^2/sec) and fertilized biweekly to saturation with 1/4 strength phosphorus Long Ashtons solution (Hewitt, 1966) prior to transplanting into the VES. Because of the potential to accumulate soluble salts when fertilizing closed soil systems, trees were not fertilized after being transplanted. Seedlings were harvested after terminating the experiment and scored to determine the % of short roots that were ectomycorrhizal.

There were 8 donor-receiver cup pairs for each treatment which were placed in a completely randomized design. Controls were the Mt. Hood receiver soil mix used as a donor soil. All donor and receiver soil mixes were adjusted to -0.15 bars (by misting soils with sterile distilled H$_2$O and measuring soil matric potential with a tensiometer) prior to filling cups. Soil moisture was approximately reestablished at this potential every 4-5 days during the experiment by adding sterile distilled water to the initial soil cup weights. Volatiles were purged from donor cups from 8 p.m. till 8 a.m. each day.

Analysis of microbial profiles

Receiver soils were assayed at 0, 30, 60, and 120 days to determine populations of the following taxonomic and functional microbial groups: bacteria, actionomycetes, Fusarium, extracellular chitinase producers, fluorescent pseudomonads, and facultative anaerobes. Organisms which produce extracellular chitinase have been implicated in the lysis of hyphae of some plant pathogenic fungi (Mitchell and Alexander, 1963; Tominaga and Tsujisaka, 1976; Elad et al., 1982, 1983). Fluorescent pseudomonads can be potent biological control agents (Weller, 1985) while facultative anaerobes often are producers of ethylene, a volatile compound which may be involved in soil fungistasis (Smith, 1976). Estimates of populations of microbial groups in receiver soils were made by serially diluting 0.75 g dry weight equivalent of fresh receiver soil with 0.1% water agar and plating aliquots of appropriate dilutions on tryptic soy agar + 100 ppm cycloheximide (Difco), colloidal chitin agar, peptone PCNB agar (PPA) (Nash and Snyder, 1962), colloidal chitin agar, modified Kings B agar (Sands and Rovira, 1970), and tryptic soy agar + 1 ppm resazurin (redox potential indicator dye) + 275 ppm L-cysteine-HCL$\cdot$H$_2$O (reducing agent) to enumerate bacteria, actinomycetes, fusaria, extracellular chitinase producers, fluorescent pseudomonads, and facultative anaerobes respectively. All media except PPA were adjusted to pH 7.1. Colloidal chitin media consists of: 980 ml H$_2$O, 3.5 g colloidal chitin (Hsu and Lockwood, 1975), 16.0 g "gelrite" (Merck & Co., San Diego, CA), 7.2 g K$_2$HPO$_4$$\cdot$3H$_2$O, 2.8 g KH$_2HPO_4$$\cdot$3H$_2$O, 0.5 g MgSO$_4$$\cdot$7H$_2$O, 0.01 g CaCl$_2$,

0.005 g $FeSO_4 \cdot 7H_2O$, and 2.0 g $(NH_4)SO_4$ added from 10.0 g/100 ml dH_2O

stock solution autoclaved separately. Six ml of this preparation

were poured, while hot, into plates containing solidified "gelrite"

medium (8.0 g "gelrite" + 0.75 g $MgSO_4 \cdot 7H_2O/L \, dH_2O$). Colloidal

chitin degradation by extracellular chitinase was readily detected by

cleared zones around chitinase producing colonies on this medium.

Plates were incubated at 22-25°C for 3 (bacteria), 4 (_Fusarium_), 6

(actinomycetes), or 7 (facultative anaerobes) days. Anaerobic

incubation was carried out in a "Gas Pak" (BBL Microbiology Systems,

Cockeysville, MD) anaerobic chamber. The data were analyzed by one-

way analysis of variance and means separated by Fishers protected

L.S.D. test.

RESULTS

Donor soil volatiles frequently influenced receiver soil

microbial profiles ($P < 0.05$), although the specific groups

influenced varied over the course of the experiment. Volatiles from

forest soils often differed from nursery soils in their influence on

the microbial profiles of the receiver soil. After a decline from

initial populations, bacteria counts were not significantly

influenced by fresh or P-F forest and nursery soil volatiles after 30

or 60 days (Figs. 2-3, 2-4). At 120 days, fresh Mary's Peak soil

volatiles had increased bacterial populations in the receiver soil

above those of the control, while volatiles from both fresh nursery

soils had significantly decreased bacterial populations (Fig. 2-3).

Also at 120 days, P-F Kellogg nursery soil volatiles had decreased

bacterial populations in the receiver soil (Fig. 2-4). Fresh and P-F
forest and nursery soil volatiles caused a variety of changes in the
populations of chitinase-producing organisms in the receiver soils
(Figs. 2-5, 2-6). For fresh soils, initial volatile-mediate declines
in the populations of chitinase-producing organisms at 30 days, were
no longer apparent at 60 days, and populations were stimulated by
fresh Mary's Peak forest soil volatiles after 120 days (Fig. 2-5).
Similar trends were seen for the P-F forest and nursery soils (Fig.
2-6). Though treatments occasionally differed significantly from
controls, trends in the influence of volatiles from fresh or P-F
forest and nursery soil on populations of Fusarium in the receiver
soil were not readily apparent (Figs. 2-7, 2-8).

The effects of volatiles from soil microbes, a conifer seedling,
or both on receiver soil microbial profiles varied depending on the
donor soil (forest vs. nursery) and the specific microbial group
assayed. Tree presence or P-F of Mary's Peak forest or Elkton
nursery donor soils did not significantly influence receiver soil
populations of actinomycetes at any assay date (Figs. 2-9, 2-10).
Populations of extracellular chitinase-producing organisms were
significantly decreased ($P < 0.05$) by day 120 in soils receiving
volatiles from P-F, seedling-planted Mary's Peak soil compared to
fresh Mary's Peak soil not containing a seedling (Fig. 2-11).
Identical treatments of the Elkton nursery soil resulted in no
differences in volatile-mediate populations of chitinase-producing
organisms (Fig. 2-12). Facultative anaerobe populations in receiver
soils were reduced by volatiles from the P-F and/or seedling

treatments of the Mary's Peak forest donor soil at the day 60 assay
(Fig. 2-13). This difference was no longer seen by day 120.
Similarly, an initial difference in the receiver soil population of
facultative anaerobes exposed to volatiles from fresh vs. P-F Elkton
nursery donor soil was only apparent during the first monitoring
(Fig. 2-14). Neither the P-F nor the seedling treatments of Mary's
Peak donor soil resulted in volatile-mediated differences in the
population of Fusarium in receiver soils (Fig. 2-15). Conversely,
volatiles from fresh Elkton nursery donor soil planted with a
Douglas-fir seedling inhibited Fusarium populations in receiver soils
compared to the same soil alone at day 60 (30 days since seedling
transplant) (Fig. 2-16), though this effect was not seen at day
120. In no cases did treatments of donor soils result in significant
differences in receiver soil populations of fluorescent
pseudomonads. All seedlings planted in fresh Mary's Peak forest soil
were mycorrhizal (average % short roots mycorrhizal = 33 ± 24) when
examined at the end of the experiment. No mycorrhizae were present
for seedlings planted in any other soils.

DISCUSSION

Results from this study indicate for the first time that
naturally occurring levels of soil volatiles influence the
populations of soil microorganisms. Volatiles from forest and
nursery soils often differed in their influence on the populations of
receiver soil microorganisms (Figs. 2-3 - 2-6). The higher organic
matter content in forest soils may account for this differential

influence since organic amendments to soil can decrease the disease potential of a soil (Sun and Huang, 1985) and stimulate the production of volatiles which can lyse Fusarium chlamydospores (Okazaki, 1985). Furthermore, organic matter content has been closely correlated with the production of ethylene and other C3 and C4 compounds in soils (Goodlass and Smith, 1978a, b). NH_4 released during organic matter degradation will tend to persist in forest soils (White, 1986), and is a potent inhibitor of fungi including Fusarium when in the gaseous ammonia (NH_3) form (Schippers and Palm, 1973; Bacon, 1986). Humic substances, more prevalent in forest than nursery soils, can also influence the type and amount of volatiles produced by a soil (Stevenson et al., 1970).

Though volatiles differentially stimulated or inhibited receiver soil microbial groups, these differences often did not persist over time or were difficult to correlate with Fusarium exclusion from native coniferous forest soils. Populations of Fusarium fluctuated considerably during the investigation (Figs. 2-7, 2-8), and fresh or P-F forest soil volatiles never decreased and sometimes increased receiver soil Fusarium populations compared to the control. This may have resulted from chemical, physical and biological factors in donor soil cups removing or altering volatiles before these volatiles could be transferred to receiver cups. Volatiles can be removed from the soil atmosphere by adsorption, absorption, chemical conversion, and microbiological transformation (Stotzky and Schenck, 1976). Interestingly, the capacity of a soil to degrade ethylene has been correlated with the amount of ethylene present in the soil (Sawada et

al., 1985). A high degradation rate, in donor soils, of compounds capable of directly or indirectly influencing Fusarium populations would account for the observed variable effect of forest and nursery soil volatiles. The quantity and quality of volatiles produced in a soil can vary with time as soil substrates are utilized by microorganisms (Adamson et al., 1975; Francis et al., 1975). This could partially account for receiver soil microbial population differences not persisting over time, since forest soil litter would be continually decomposing during the experiment. Changes in the numbers of anaerobic microsites in donor soils during the experiment may also be involved since single substrates can give rise to a variety of volatiles depending on whether these substrates are aerobically or anaerobically utilized (Stotzky and Schenck, 1976).

Numerous soil microorganisms produce volatile compounds which are biologically active (Hutchinson, 1971, 1973; Stotzky and Schenck, 1976). Rarely in this investigation, however, did volatiles from P-F donor soils differ from fresh soils in their influence on the receiver soil microbiota (Fig. 2-9 - 2-16). Possibly the destruction of soil aggregates in preparing donor soil mixes deleteriously influenced the microbial balance and associated volatile-producing character of native fresh soils (Stotzky, 1986). The apparent unimportance of the soil microbiota as a volatile producer could also result from the rapid recovery of initially low microbial populations in P-F donor soils to population levels near that of fresh soil. This population recovery can take place as quickly as 11 days after fumigation (Ridge and Theodorou, 1972) and bacterial counts

approximately 1/10th that of fresh soil were measured only 5 days

after P-F treatment (Schisler, unpublished results). Our results,

therefore, likely indicate only that the influence, on microbial

populations, of volatiles from a qualitatively changed donor soil

microbiota usually did not differ from the influence of volatiles

from fresh soils. The importance of volatiles from the forest and

nursery soil microbiota in influencing soil populations of

microorganisms and Fusarium, we feel, remains undetermined.

Volatiles from soil planted with a Douglas-fir seedling

occasionally had a significant influence on receiver soil microbial

populations (Fig. 2-11, 2-13, 2-16). Though in vitro studies by

Krupa and co-workers (1971, 1972, 1973) indicated volatiles from

ectomycorrhizae were inhibitory to several root pathogenic fungi, in

only one instance did our treatment containing ectomycorrhizal

Douglas-fir seedlings (fresh Mary's Peak forest donor soil)

significantly influence receiver soil microbial populations (Fig. 2-

13, day 60), and Fusarium populations were never significantly

altered. Perhaps our experimental conditions selected for

ectomycorrhizal fungi which are not representative of those active in

native forest soils. Further, it seems probable that more time was

needed for volatile-mediated decreases in soil Fusarium populations

to occur, since it took two years for Fusarium populations in

coniferous forest soils to decline in Smith's original study

(1967). It is also possible that concentrations of volatiles

deleterious to <u>Fusarium</u> that were purged from donor cups were insufficient to have any effect.

It is not surprising that seedling presence in soils resulted in volatiles which influenced receiver soil microbial populations. Conifer seedling roots can produce a variety of volatile compounds (Nordlander, 1986). Fluctuations in the effects of volatiles from seedling-planted donor soils on receiver soil microbial populations could have resulted from changes in the seedling volatile exudation pattern over time. Plant age, fertility and size can influence the quantity and quality of volatile and non-volatile exudates released from plant roots (Rovira, 1985; Curl and Truelove, 1986).

The VES has proved a valuable tool in studying the effects of naturally occurring levels of soil volatiles <u>in vivo</u>. However, difficulties in possible degradation, absorption, or adsorption of volatiles before being purged from donor soil cups must be considered when interpreting any experimental results. Volatiles from coniferous forest soils tended to differ from those of nursery soils in their stimulatory or inhibitory effect on specific microbial group populations. The importance of this phenomenon in relation to <u>Fusarium</u> exclusion from coniferous forest soils is not readily determinable. <u>In vivo</u> studies using the VES and comparing the influence of volatiles from several Douglas-fir ectomycorrhizal fungi on populations of <u>Fusarium</u> and selected microbial groups are reported in the following chapter.

The design of the VES described in this paper lends itself to many other potentially fruitful studies on the influence of soil

volatiles on various biological processes in soil. Plant roots, for instance, could act as targets or producers of volatiles in studies on allelopathy (Putnam and Duke, 1978). Furthermore, donor and receiver cups can be easily introduced or removed from experiments, allowing great flexibility in selecting and/or changing volatile donor and receiver combinations during an experiment. Finally, identification of specific volatiles being purged from donor soils could be determined by gas chromatographic analysis of samples removed from the airspace within the donor cup sleeve.
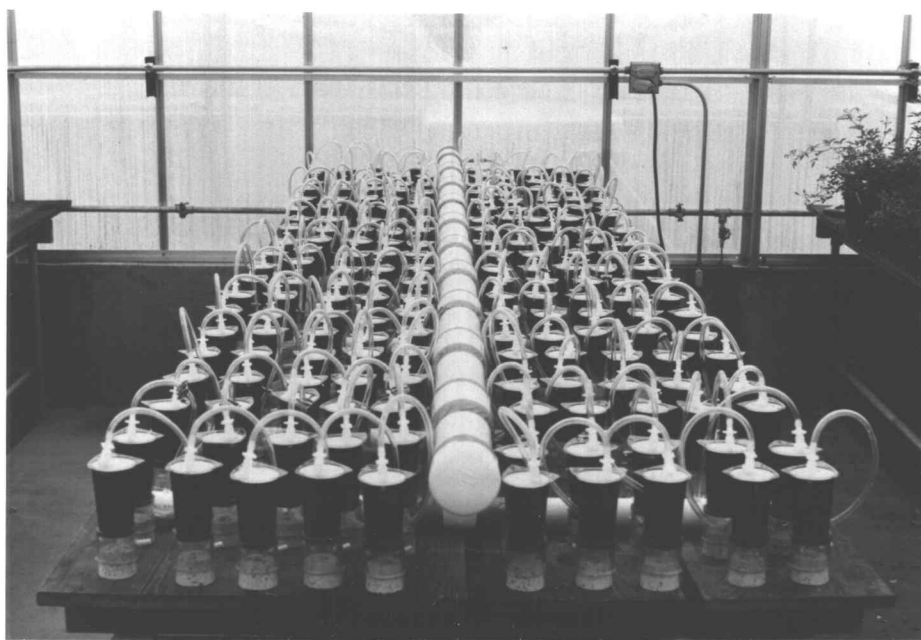
Figure 2-1. Volatile exchange system (VES) for purging the atmosphere from donor soils into receiver soils.
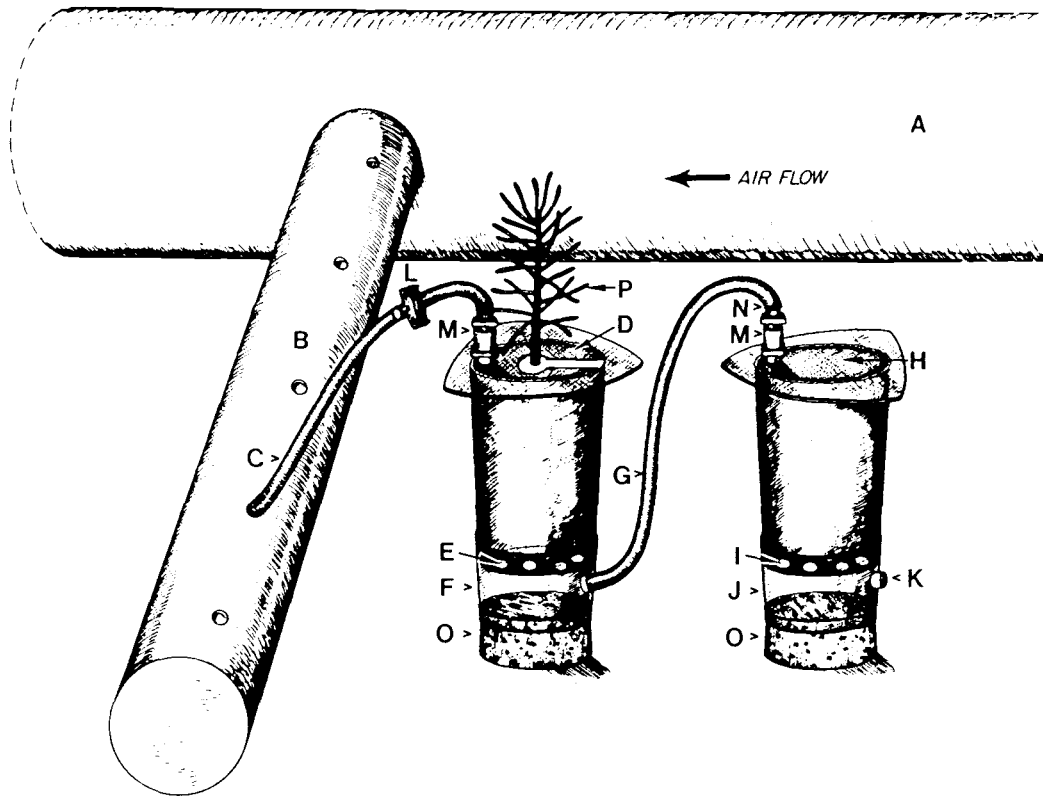
Figure 2-2. Schematic diagram of a donor and receiver cup of the
volatile exchange system (VES). (a) main manifold, (b) submanifold,
(c) tygon tubing, (d) top of donor soil cup, (e) holes in the bottom
of donor soil cup, (f) plastic sleeve, (g) tygon tubing, (h) top of
receiver soil cup, (i) holes in the bottom of receiver soil cup, (j)
plastic sleeve, (k) hole in plastic sleeve, (l) adjustable clamp, (m)
quick release tubing connectors, (n) fiberglass plug, (o) cork
stopper, (p) conifer seedling.
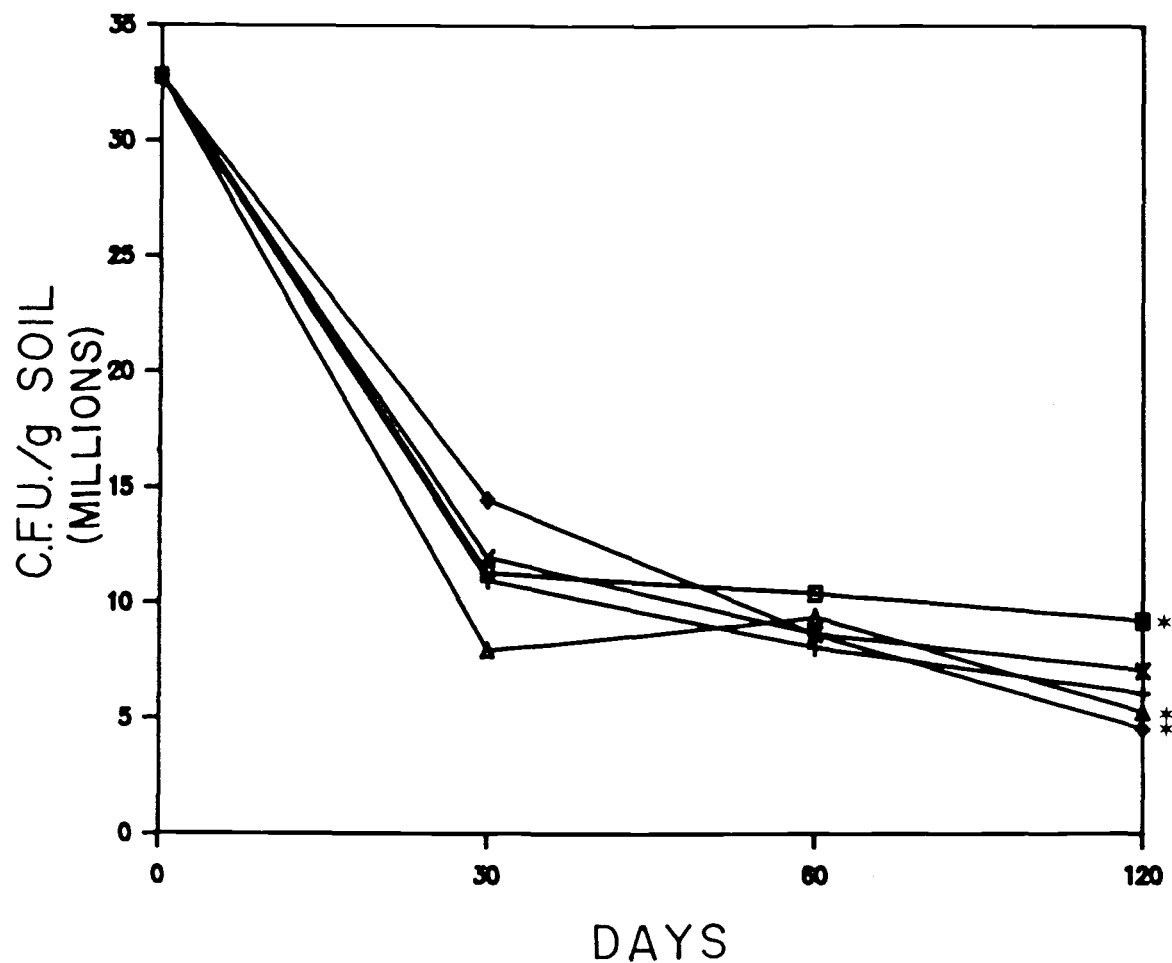
Figure 2-3. Effect of volatiles from fresh forest and nursery soils on the numbers of bacteria isolated from a receiver nursery soil. Values within an assay date followed by a " * " are significantly different from the control, P < 0.05 (Fisher's protected L.S.D. test). —□— F1, —+— F2, —◇— N1, —△— N2, —✕— control.

Figure 2-4. Effect of volatiles from pasteurized and fumigated forest and nursery soils on the numbers of bacteria isolated from a receiver nursery soil. Values within an assay date followed by a " * " are significantly different from the control, $P < 0.05$ (Fisher's protected L.S.D. test).

— □ — F1, — + — F2, — ◇ — N1, — △ — N2, — × — control.
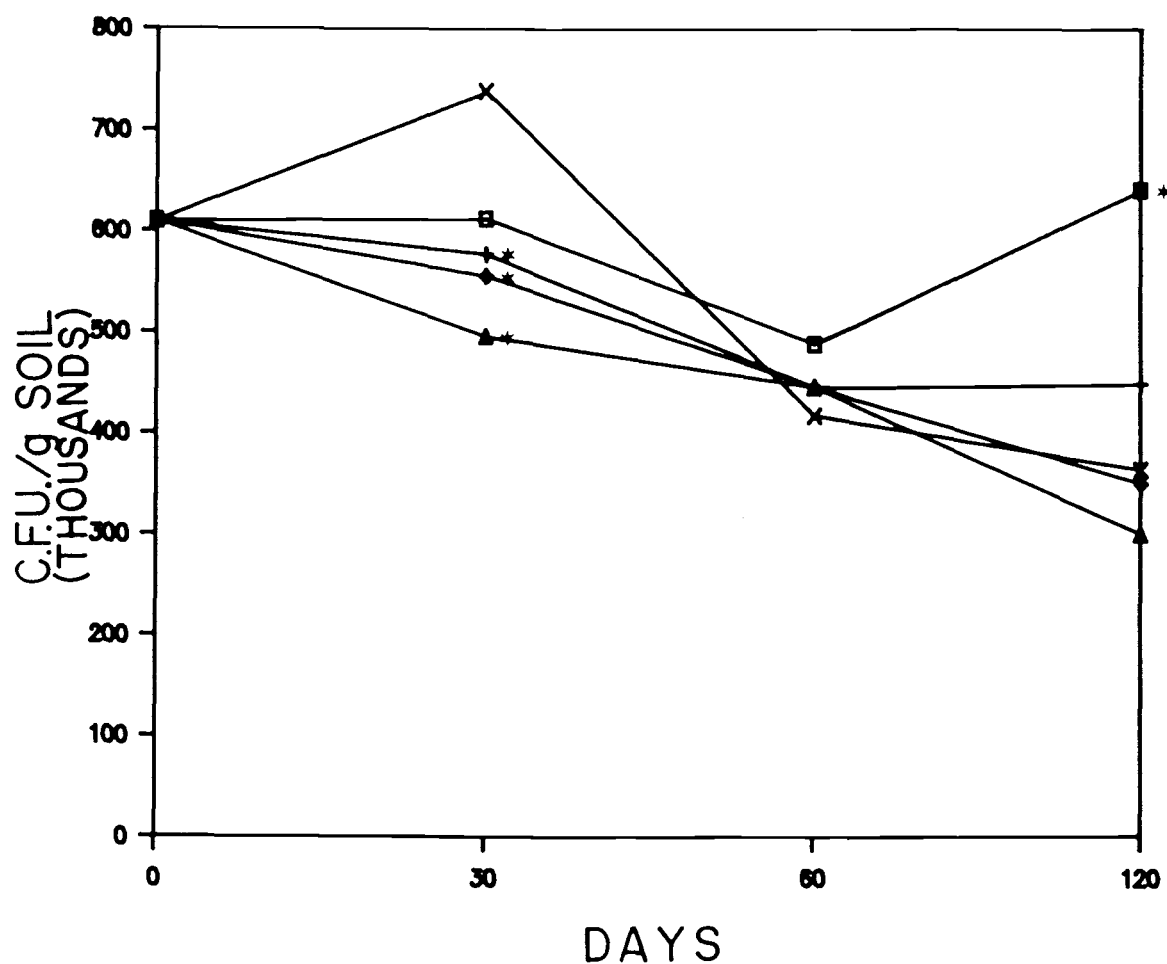
Figure 2-5. Effect of volatiles from fresh forest and nursery soils on the numbers of extracellular-chitinase-producing microorganisms isolated from a receiver nursery soil. Values within an assay date followed by a " * " are significantly different from the control, P < 0.05 (Fisher's protected L.S.D. test).

— □ — F1, — + — F2, — ◇ — N1, — △ — N2, — × — control.

Figure 2-6. Effect of volatiles from pasteurized and fumigated forest and nursery soils on the numbers of extracellular-chitinase-producing microorganisms isolated from a receiver nursery soil. Values within an assay date followed by a " * " are significantly different from the control, $P < 0.05$ (Fisher's protected L.S.D. test). — □ — F1, — + — F2, — ◇ — N1, — △ — N2, — x — control.
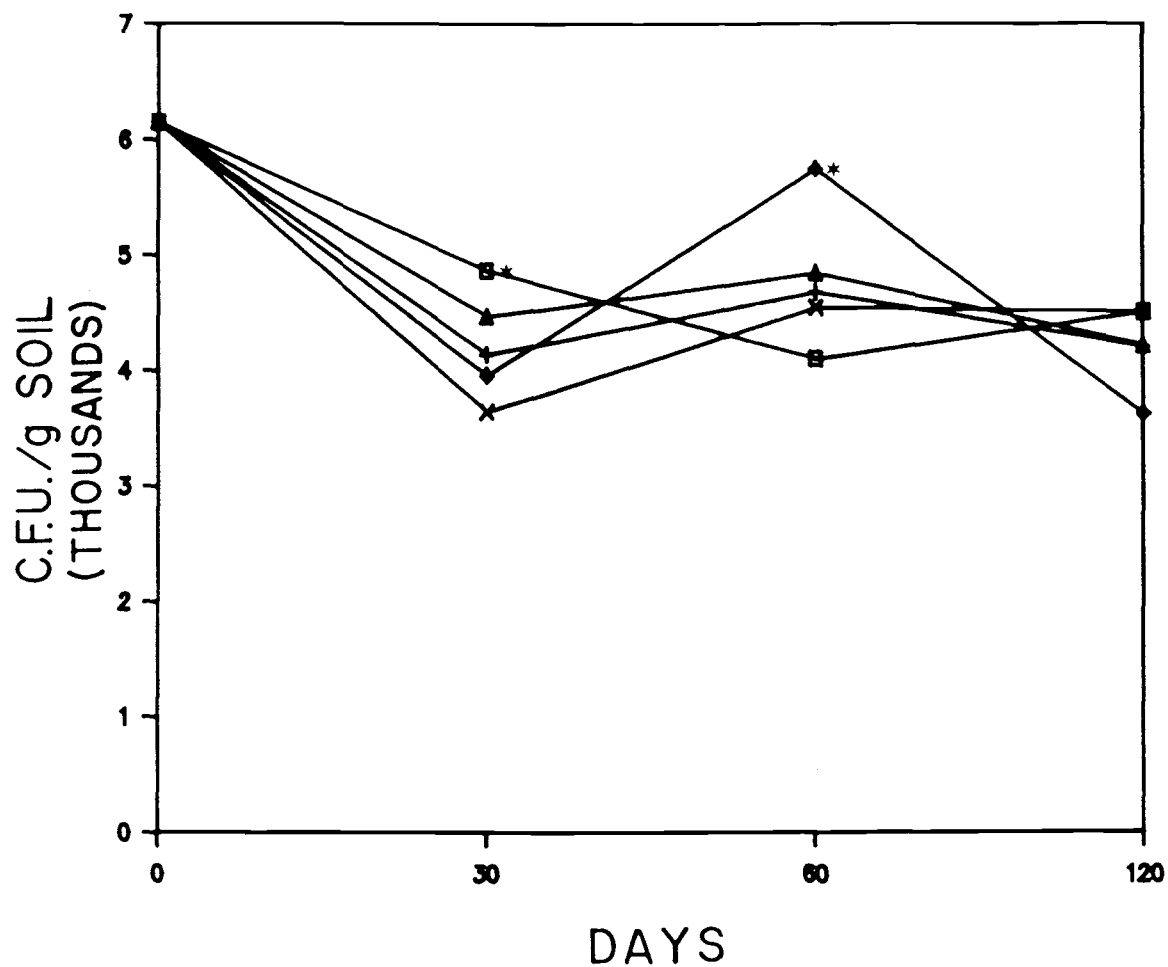
Figure 2-7. Effect of volatiles from fresh forest and nursery soils on the number of _Fusarium_ propagules recovered from a receiver nursery soil. Values within an assay date followed by a " * " are significantly different from the control, $P < 0.05$ (Fisher's protected L.S.D. test).

—□— F1, —+—F2, —◇— N1, —△—N2, —✕—control.
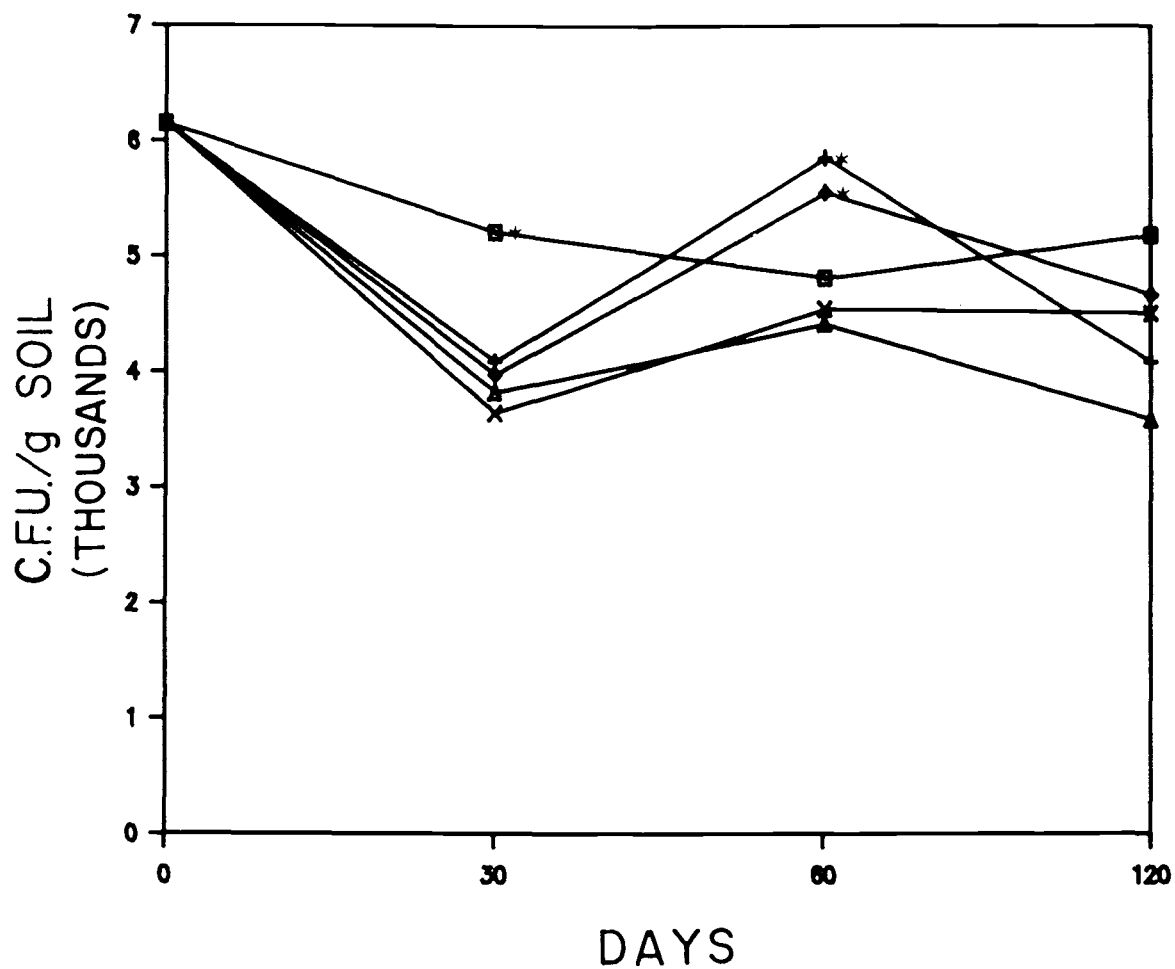
Figure 2-8. Effect of volatiles from pasteurized and fumigated forest and nursery soils on the number of _Fusarium_ propagules recovered from a receiver nursery soil. Values within an assay date followed by a " * " are significantly different from the control, P < 0.05 (Fisher's protected L.S.D. test).

—□— F1, —+— F2, —◇— N1, —△— N2, —✕— control.

Figure 2-9. Effect of volatiles from a fresh or pasteurized and fumigated forest soil, planted or not with a Douglas-fir seedling, on the numbers of actinomycetes 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). ☐ pre-experiment count, ▨ fresh F1, ▨ fresh F1 with seedling, ▨ P-F F1, ▨ P-F F1 with seedling.

Figure 2-10. Effect of volatiles from a fresh or pasteurized and fumigated nursery soil, planted or not with a Douglas-fir seedling, on the numbers of actinomycetes 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). ☐ pre-experiment count, ▨ fresh Nl, ▨ fresh Nl with seedling, ▨ P-F Nl, ▨ P-F Nl with seedling.

Figure 2-11. Effect of volatiles from a fresh or pasteurized and fumigated forest soil, planted or not with a Douglas-fir seedling, on the numbers of extracellular-chitinase-producing microorganisms 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). ☐ pre-experiment count, ⧄ fresh Fl, ⧅ fresh Fl with seedling, ⧄ P-F Fl, ⧅ P-F Fl with seedling.

Figure 2-12. Effect of volatiles from a fresh or pasteurized and fumigated nursery soil, planted or not with a Douglas-fir seedling, on the numbers of extracellular-chitinase-producing microorganisms 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). ☐ pre-experiment count, ▨ fresh Nl, ◩ fresh Nl with seedling, ▨ P-F Nl, ▨ P-F Nl with seedling.

Figure 2-13. Effect of volatiles from a fresh or pasteurized and fumigated forest soil, planted or not with a Douglas-fir seedling, on the numbers of facultative anaerobes 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). ☐ pre-experiment count, ▨ fresh Fl, ▧ fresh Fl with seedling, ▨ P-F Fl, ▨ P-F Fl with seedling.
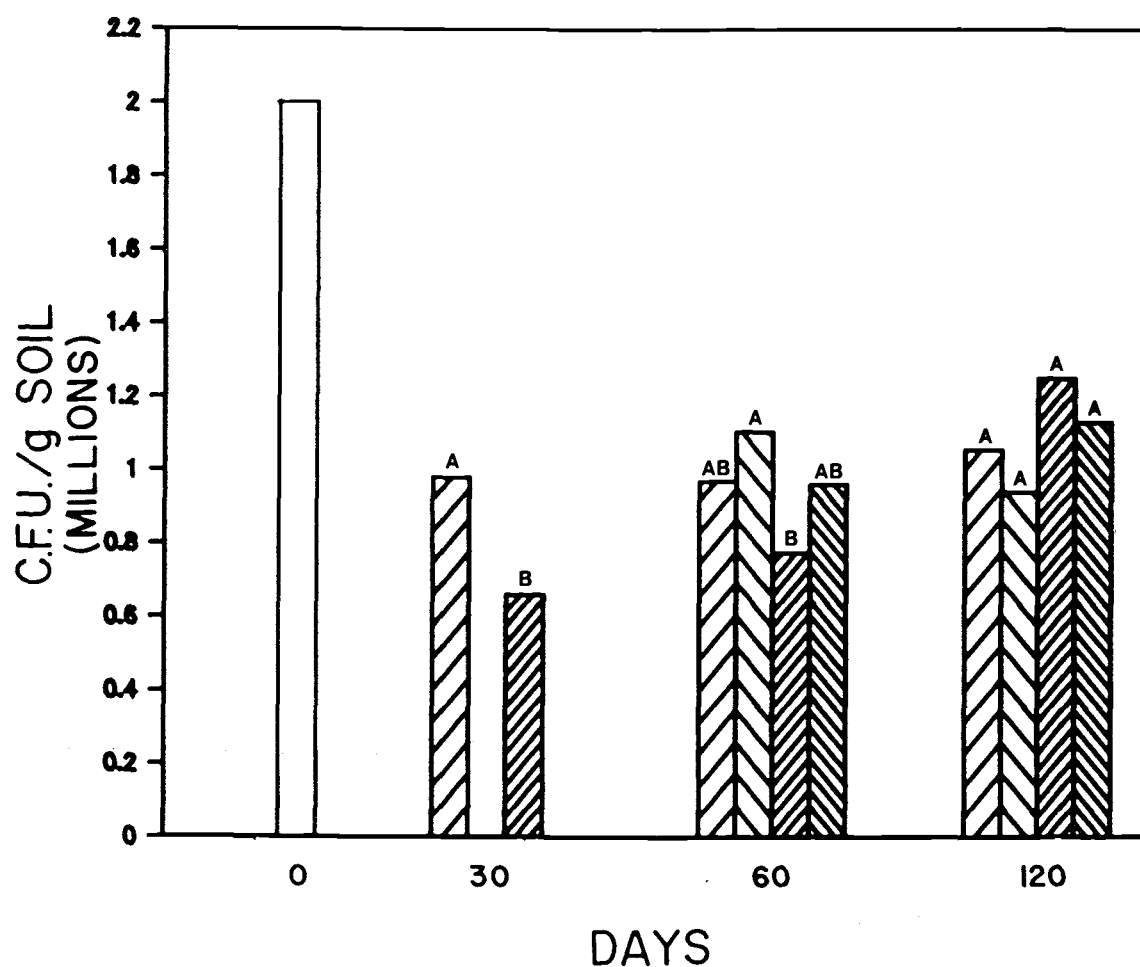
Figure 2-14. Effect of volatiles from a fresh or pasteurized and fumigated forest soil, planted or not with a Douglas-fir seedling, on the numbers of facultative anaerobes isolated from a receiver nursery soil. Values within an assay date not followed by the same letter are significantly different, P < 0.05 (Fisher's protected L.S.D. test). ☐ pre-experiment count, ▨ fresh Nl, ◪ fresh Nl with seedling, ▨ P-F Nl, ▨ P-F Nl with seedling.

Figure 2-15. Effect of volatiles from a fresh or pasteurized and fumigated forest soil, planted or not with a Douglas-fir seedling, on the number of Fusarium propagules recovered 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). ☐ pre-experiment count, ▨ fresh F1, ▨ fresh F1 with seedling, ▨ P-F F1, ▨ P-F F1 with seedling.
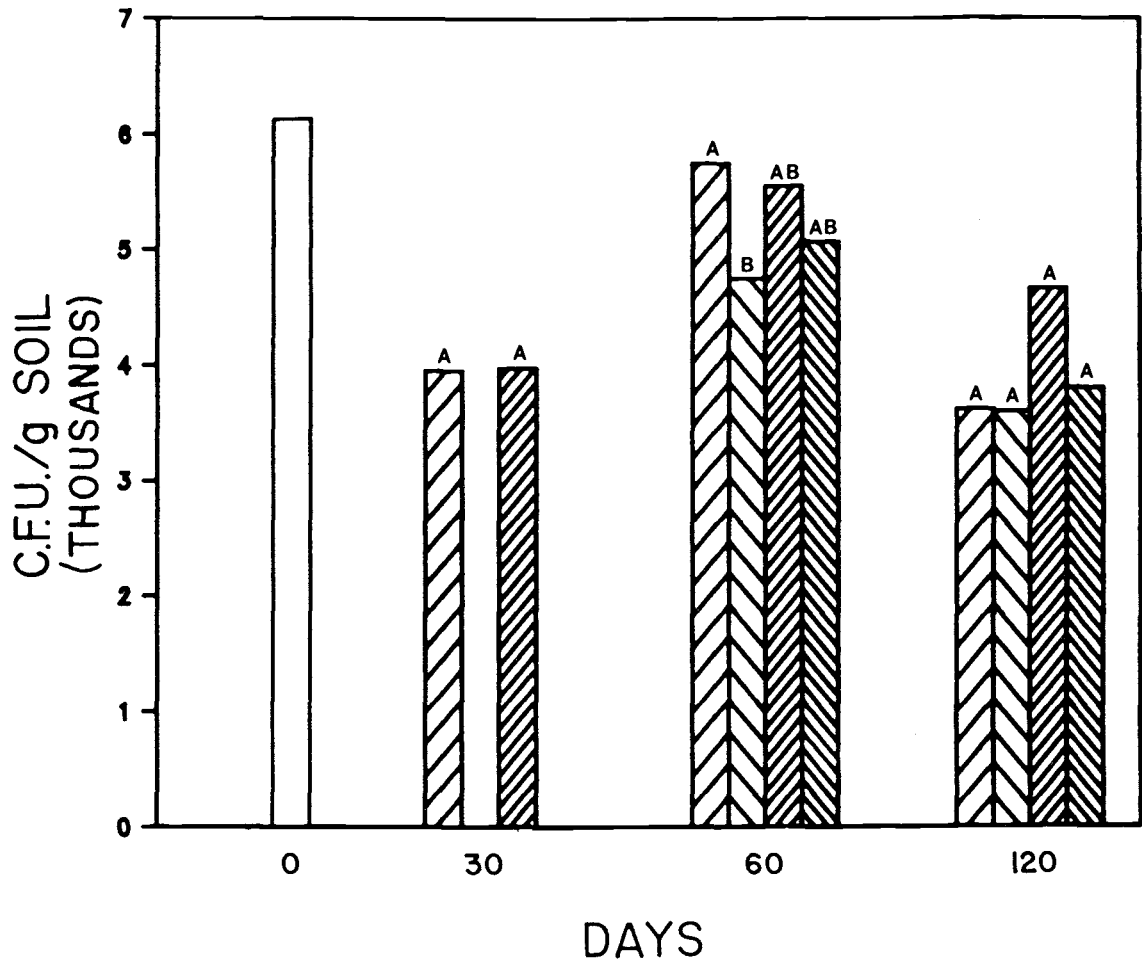
Figure 2-16. Effect of volatiles from a fresh or pasteurized and fumigated nursery soil, planted or not with a Douglas-fir seedling, on the number of _Fusarium_ propagules recovered from a receiver nursery soil. Values within an assay date not followed by the same letter are significantly different, P < 0.05 (Fisher's protected L.S.D. test).   □ pre-experiment count, ▨ fresh N1, ▨ fresh N1 with seedling, ▨ P-F N1, ▨ P-F N1 with seedling.

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CHAPTER 3

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 determine 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. 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 Pacific Northwest nurseries (Bloomberg and
Lock, 1972; Bloomberg, 1981). In contrast to nursery soils,
coniferous soils covered with needle litter rarely harbor detectable
population levels of Fusarium (Thornton, 1960; Park, 1963; Schisler
and Linderman, 1984; Schisler, Chapter 1). The exclusion of Fusarium
from coniferous forest soils (Smith, 1967) has been attributed to the
lack of annual plants in coniferous forest soils (Toussan, 1975) and
to the lytic effect of needle duff leachates (Menzinger, 1969;
Toussoun et al., 1969; 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; Harley and Smith, 1983).
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, Laccaria 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 Laccaria 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) indicates ectomycorrhizal fungi other than Laccaria laccata could also influence Fusarium survival and disease.

Volatile compounds can stimulate as well as inhibit fungal propagule germination and subsequent germ tube growth (Fries, 1973; Hutchinson, 1971, 1973; Stotzky and Schenck, 1976). Ectomycorrhizae produce 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 vitro, that ectomycorrhizae could release significant amounts of ethylene into the mycorrhizosphere soil, and Smith (1976) has suggested that ethylene may play a significant 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, Chapter 2). It is well 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 (Katznelson et al., 1962; Neal et al., 1964; Neal et al., 1968; Malajczuk, 1979; Strzelczyk and Pokojska-Burdziej, 1984). It is not known, however, if natural levels of volatile compounds from ectomycorrhizae can incite or maintain these microbial difference in vivo. Using a device specifically constructed to purge volatiles from a soil and introduce these volatiles into a second soil, we conducted an experiment to determine if (a) volatiles from ectomycorrhizae formed by inoculating Douglas-fir seedlings with Pacific Northwest isolates of the ectomycorrhizal genera Hebeloma and Laccaria significantly 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 predominant conifer species present at the site.

-- <u>Hebeloma</u> <u>crustuliniforme</u> isolate #2 (Hecr2)[1] - Bald

Mountain, Oregon, 1890 m, <u>Abies</u> <u>concolor</u>-<u>Pinus</u> <u>contorta</u> mix.

-- <u>Hebeloma</u> sp. isolate #S260T (HeS260T) - Upper Battle Creek,

Oregon, 1460 m, <u>Pinus</u> <u>contorta</u>-<u>Pseudotsuga</u> <u>menziesii</u> mix.

-- <u>Laccaria</u> <u>laccata</u> isolate #T813 (L1T813) - Southwest

Washington nursery, 360 m, <u>Pseudotsuga</u> <u>menziesii</u>.

-- <u>Laccaria</u> <u>laccata</u> isolate #B101 (L1B101) - Barrier, British

Columbia, 1300 m, <u>Pseudotsuga</u> <u>menziesii</u>.

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 three to five
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
$22\pm2°C$ for 28 days prior to use. Vermiculite inoculum was prepared
in 1 L flasks containing 600 ml of a mixture of 29:1 fine vermiculite
and hypnum peat moistened with MMN broth and sterilized. Flasks were
inoculated with 10-15, 0.5 cm diameter MMN agar plugs colonized by
each fungus and incubated in darkness at $22\pm2°C$ for 8 weeks prior to
use.

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[1] Isolate number used in Figures 3-1 through 3-6.

Seedling preparation and inoculation

A mixture of fine vermiculite, sand and loam soil (1:1:1 vol/vol/vol) was air-steam pasteurized (60°C/30 min), placed in 45 cc tubes (Ray Leach Cone-tainers, Canby, Oregon), and then sown with Douglas-fir seeds which had been soaked in hydrogen peroxide (30% H_2O_2, 120 min) and then rinsed with tap H_2O for 12 h. Trees were thinned to 1/tube at 5 weeks and inoculated at 6 weeks by injecting hyphal suspensions of ectomycorrhizal fungi directly onto intact soil root systems using a hypodermic syringe. 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 sd H_2O. Hyphal suspensions were maintained at approximately 0°C throughout preparation and then 4.5 ml of suspension containing approximately 5 mg of dry weight equivalent fresh mycelium was used to inoculate each seedling. Seedlings were grown at 24 ± 3°C under ambient and supplemented (high pressure sodium vapor lamps) light (average = 300 microeinsteins/m^2/sec) and fertilized weekly to saturation with 1/4 strength phosphorus Long Ashtons solution (Hewitt, 1966) until transplant into the volatile exchange system (VES).

Volatile exchange system and experimental treatments

The VES used in this study was as described previously (Chapter 2). 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 prior to 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 non-mycorrhizal seedling. A 60:40 Kellogg Oregon nursery soil (Schisler, Chapter 2):sand mix was used as the receiver soil in all cases.

Lighting and temperatures for seedling growth were as described above. Because of the potential to accumulate soluble salts when fertilizing closed soil systems, trees were not fertilized after being transplanted into donor cups. 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 6 donor-receiver cup pairs for each treatment which were placed in a completely randomized design. The moisture level of donor soil mixes was adjusted to -0.1 bars and the receiver soil mix was adjusted to -0.2 bars (by misting the soils with sterile distilled water and measuring the soil matric potential with a tensiometer) prior to filling 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 cc/min 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.
Microorganisms which produce extracellular chitinase have been
implicated in biological control of plant pathogenic fungi (Mitchell
and Alexander, 1963; Tominaga and Tsujisaka, 1976; Elad et al., 1982,
1983), while facultative anaerobes frequently are capable of ethylene
production, which could contribute to soil fungistasis (Smith,
1976). Phosphate-solubilizing bacteria in plant rhizosphere soil
could potentially increase plant phosphorus nutrition and growth
(Goldstein, 1986). Decreased seedling succulence and damping-off has
been observed with increased phosphorus nutrition (Bloomberg,
1981). Materials and methods used for estimating populations of
microbial groups were as described elsewhere for most groups
(Schisler, Chapter 2). 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, CA) 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 incubation at 22-25°C.

Microbial population data from receiver soil cups which were connected to donor cups containing the three most heavily ectomycorrhizal seedlings of each treatment was used for statistical analysis. Data was 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 receiver soil bacterial populations compared to controls (P < 0.05) at 90 but not 40 days (Fig. 3-1). The presence of ectomycorrhizal seedlings in donor cups did not significantly influence populations of actinomycetes (Fig. 3-2), Fusarium (Fig. 3-3), extracellular-chitinase-producers (Fig. 3-4), facultative anaerobes (Fig. 3-5), or phosphate-solubilizing bacteria (Fig. 3-6) 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 3-1), though the percent 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 Laccaria 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

Results from this investigation indicate that volatiles from ectomycorrhizae and the surrounding soil influence the populations of the soil bacteria (Fig. 3-1). Volatiles, therefore, apparently could be at least partially responsible for inducing the quantitative and qualitative differences in the microbial populations of mycorrhizosphere vs. nonmycorrhizosphere soils in earlier reports (Katznelson et al., 1962; Neal et al., 1964; Neal et al., 1968; Malajczuk, 1979; 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, 1979; Sinclair et al., 1982), since rhizosphere microorganisms could impede fungal pathogen attack by providing additional protection to the physical barrier of the ectomycorrhizal mantle around roots or by the production of antibiotics and/or siderophores (Baker and Cook, 1974; Cook and Baker, 1983).

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 receiver soil cups likely also is important. The length of time volatiles were purged in this investigation was shorter than that used in another study when more significant volatile mediated microbial population changes were observed (Chapter 3). 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 ectomycorrhizae volatiles into biologically active compounds.

The appearance of significant 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. The morphology and physiology of ectomycorrhizae varies considerably as ectomycorrhizae mature (Piche and Peterson, 1984), which may have delayed the production of volatiles in sufficient quantities to affect receiver soil microbial populations. Furthermore, sluggishness in the establishment of mycorrhizal short roots due to the potentially inhibitory effect of frequently purging donor 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 percentage of infected short roots in influencing volatile-

mediated changes in microbial populations is unknown. Though the average root dry weight of He S260T-infected seedlings was less than controls or other ectomycorrhizae treatments (Table 3-1), volatiles from these ectomycorrhizal seedlings caused significant increases in receiver soil bacterial populations (Fig. 3-1) 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 (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 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 in the present study. Qualitative analysis of the purged volatiles in the VES is possible, and is the subject of a future 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 being important producers of 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.

Table 3-1. Comparison of tree growth parameters and percent mycorrhizae of seedlings inoculated with ectomycorrhizal fungi or non-mycorrhizal

Treatment	Top height (cm)	Stem caliper (mm)	Root dry weight (g)	Shoot dry weight (g)	Root/ shoot	# buds	Lateral branches	% mycor- rhizae
1 control	5.8a	1.2a	0.235a	0.152b	1.57a	3.7a	0.0a	0b
2 Hecr2	5.8a	1.2a	0.189a	0.144b	1.31a	4.3a	0.0a	37ab
3 HeS260T	5.7a	1.2a	0.127b	0.119b	1.07a	3.3a	0.0a	50a
4 L1T813	6.9a	1.3a	0.221a	0.221a	1.01a	3.0a	0.3a	53a
5 L1B101	6.8a	1.1a	0.203a	0.164ab	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).
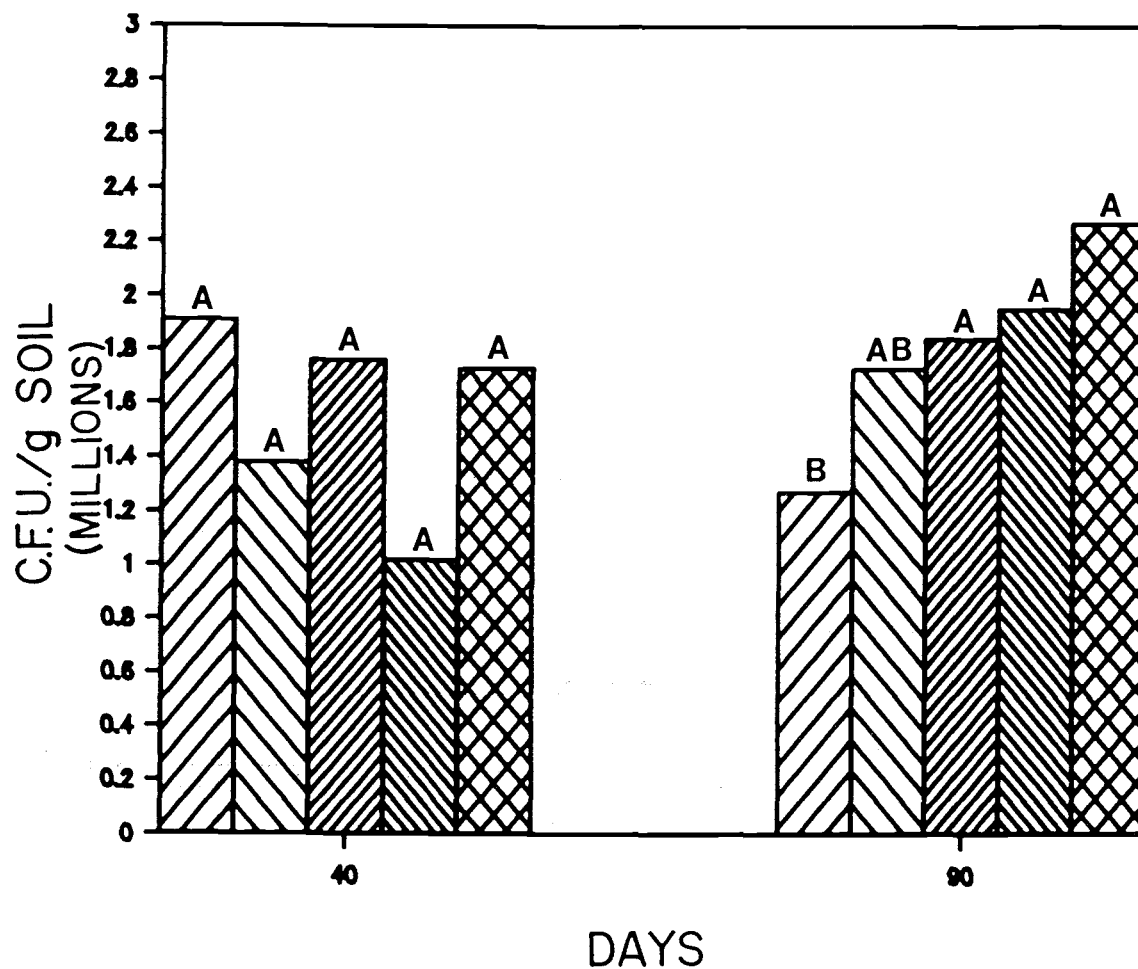
Figure 3-1. Effect of volatiles from soils containing ectomycorrhizal Douglas-fir seedlings on the numbers of bacteria isolated from a receiver nursery soil. Values within an assay date not followed by the same letter are significantly different, $P < 0.05$ (Fisher's protected L.S.D. test). ▨ Control, ◹◸ Hecr2, ▨ HeS260T, ▨ L1T813, ▨ L1B101.
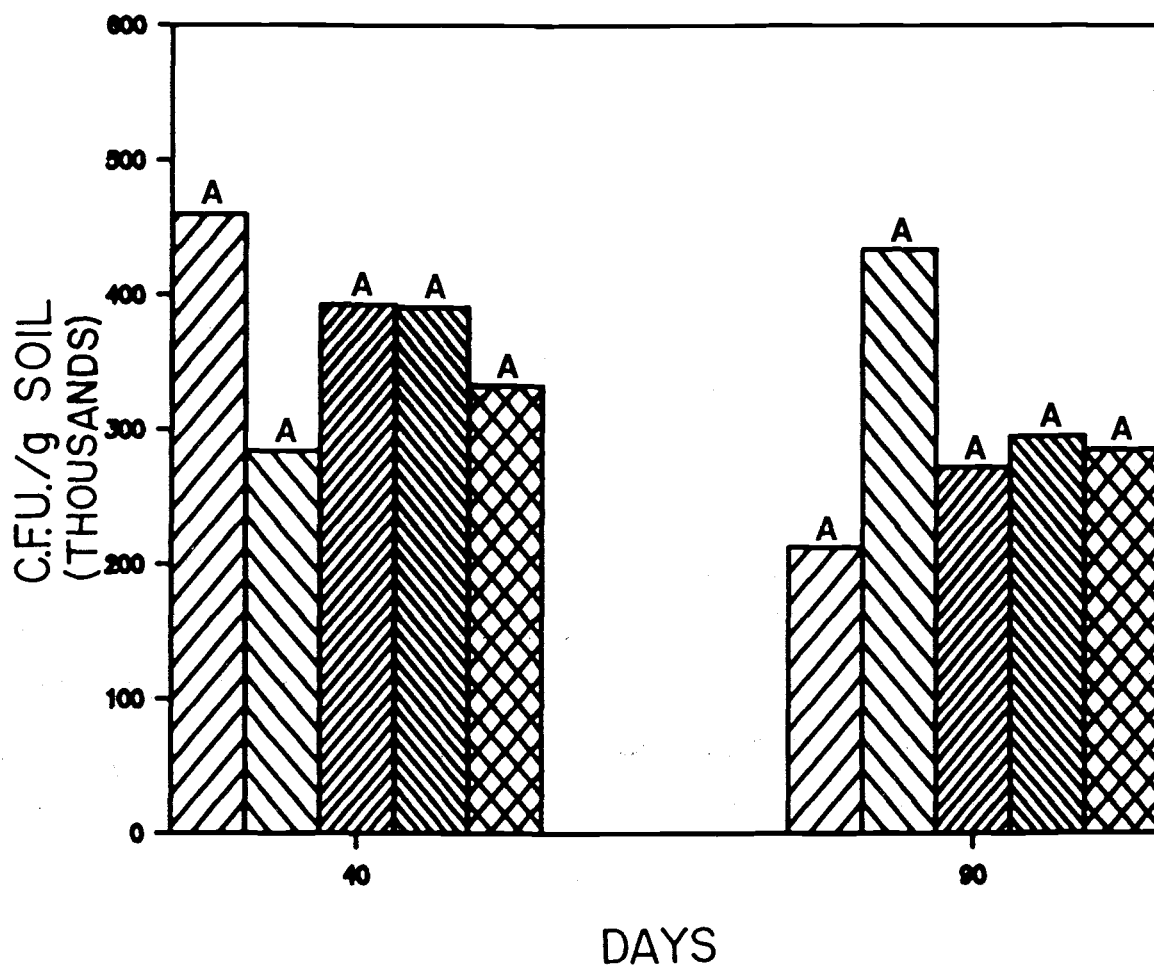
Figure 3-2. Effect of volatiles from soils containing ectomycorrhizal Douglas-fir seedlings on the numbers of actinomycetes isolated from a receiver nursery soil. Values within an assay date not followed by the same letter are significantly different, $P < 0.05$ (Fisher's protected L.S.D. test). ▨ Control, ▧ Hecr2, ▨ HeS260T, ▨ L1T813, ▨ L1B101.

Figure 3-3. Effect of volatiles from soils containing ectomycorrhizal Douglas-fir seedlings on the number of Fusarium propagules recovered 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, LIT813, LIB101.
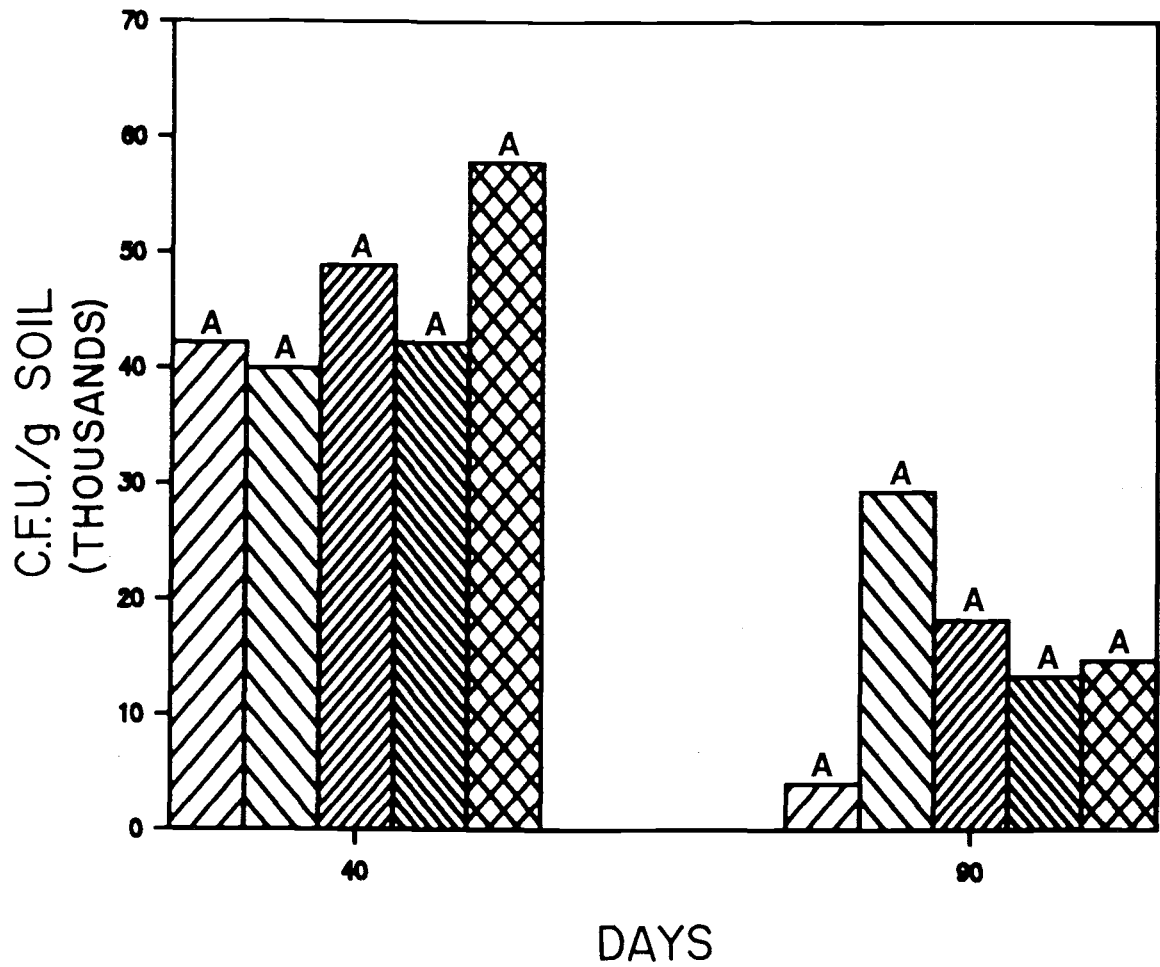
Figure 3-4. Effect of volatiles from soils containing
ectomycorrhizal Douglas-fir seedlings on the numbers of
extracellular-chitinase-producing microorganisms isolated from a
receiver nursery soil. Values within an assay date not followed by
the same letter are significantly different, P < 0.05 (Fisher's
protected L.S.D. test). ▨ Control, ▧ Hecr2,
▨ HeS260T, ▨ L1T813, ▨ L1B101.
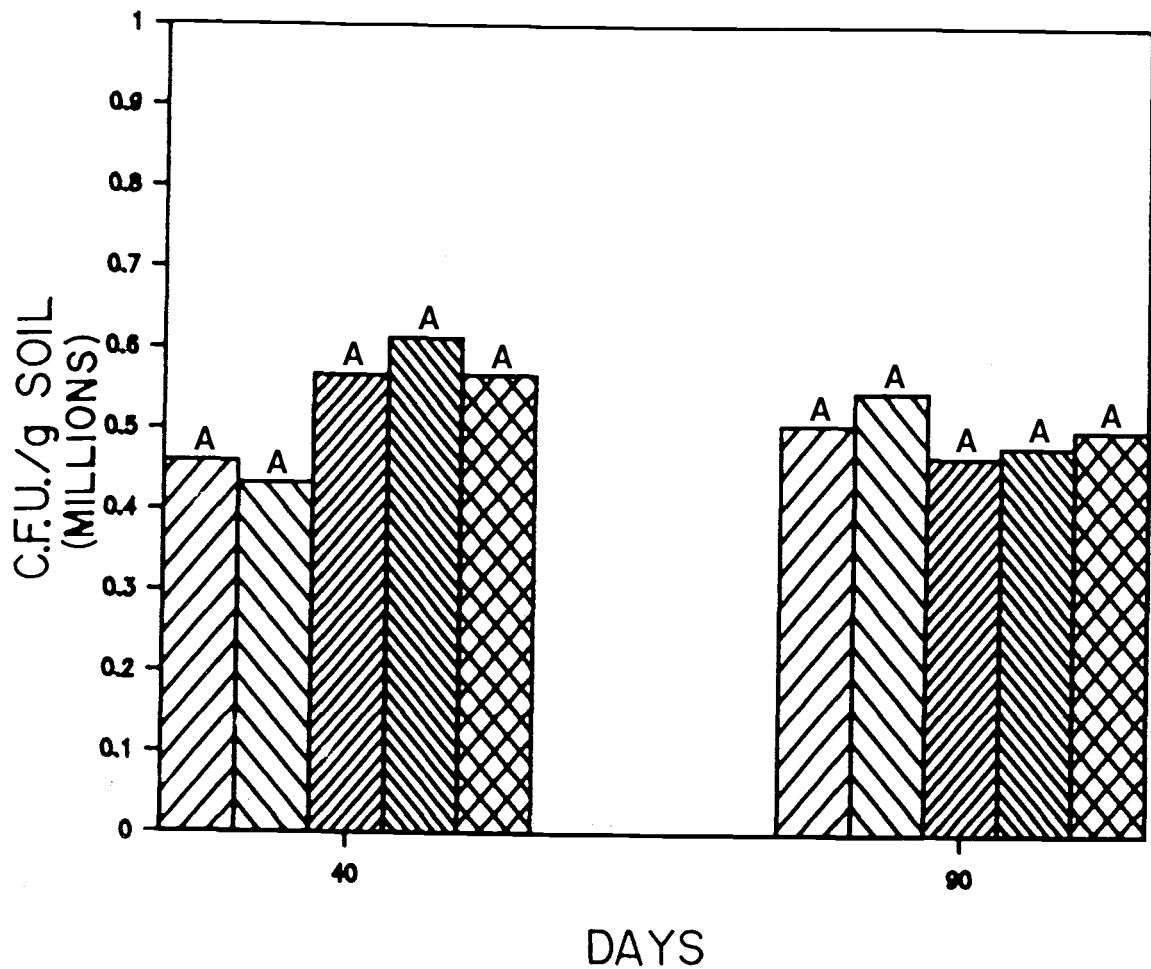
Figure 3-5. Effect of volatiles from soils containing

ectomycorrhizal Douglas-fir seedlings on the numbers of facultative

anaerobes recovered 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, LlT813,

LlB101.

Figure 3-6. Effect of volatiles from soils containing ectomycorrhizal Douglas-fir seedlings on the numbers of phosphate-solubilizing 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, ▨ L1T813, ▨ L1B101.

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CHAPTER 4

THE INFLUENCE OF HUMIC-RICH ORGANIC AMENDMENTS TO CONIFEROUS

NURSERY SOILS ON DOUGLAS-FIR GROWTH, DAMPING-OFF,

AND ASSOCIATED SOIL MICROORGANISMS.

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SUMMARY

Fusarium spp. are conspicuously absent from coniferous forest

soils, yet frequent conifer nursery soils. To test the hypothesis

that loss of humus from nursery soils may affect _Fusarium_ spp.

survival, 3 nursery soils were amended with 4 levels of 3 organic

materials high in humic content. Amendment-induced increases and

occasional decreases in tree growth varied with soil origin. A humic

amendment that stimulated tree growth in all soils also increased the

numbers of several soil microbial groups (total bacteria,

actinomycetes, extracellular-chitinase producers, and facultative

anaerobes). Fusarium-induced damping-off declined in 1 of 3 soils

for all amendments. Ectomycorrhizae were increased by only 1

amendment and then in only one soil. The complex nature of soil-humic interactions and the physiological action of these substances on roots and microbial cells complicates predicting the efficacy of humic amendments to nursery soils.

INTRODUCTION

In 1967, Smith reported that Fusarium oxysporum (Schlecht.), a pathogen of major economic importance on nursery-grown, first-year coniferous seedlings, did not persist on the roots of infected sugar pine (Pinus lambertiana) seedlings transplanted into a native pine forest. Fusarium, in fact, has not been reported as a pathogen of coniferous seedlings in forest soils and rarely reaches detectable populations in soils covered with a thick needle litter layer (Morrow, 1932; Thornton, 1960; Park, 1963; Schisler and Linderman, 1984; Schisler, Chapter 1). The inability of Fusarium to establish in forest soils has been attributed to the lack of annual plants in coniferous forest soils (Toussoun, 1975) and to the lytic effect of needle duff leachates (Menzinger, 1969; Toussoun et al., 1969; Hammerschlag and Linderman, 1975) and the forest soil microbiota (Schisler and Linderman, 1984) on Fusarium macroconidia, chlamydospores and hyphae.

Coniferous forest soils frequently are higher in humic-rich organic matter than nursery soils due to humus additions to forest soils from needle litter decomposition (Millar, 1974; Haider et al., 1975; Kuiters and Sarink, 1986) and the loss of humus from nursery soils due to cultivation (Browning, 1983; Parsons, 1985; Maciak and

Harms, 1986). Humic substances are involved in biochemical and physiological processes in plants and soil microbes which could indirectly influence the survival of Fusarium in forest soils. Humic and fulvic acids can complex with plant nutrients in the soil solution, which keeps nutrients in solution (Vaughan and Malcolm, 1985) and often results in increased plant root and shoot growth and nutrition (Lee and Bartlett, 1976; Mylonas and McCants, 1980b; Malik and Azam, 1985). Humic substances are also reported to improve soil structure (Chaney and Swift, 1986) and detoxify soil by adsorbing metals deleterious to plant growth (Schnitzer, 1986; Tan and Binger, 1986), processes which can improve plant health and resistance to pathogen attack.

Humic substances are known to increase microbial growth and activity (Martin et al., 1976; Nioh, 1976). Visser (1985a) found that higher numbers of a wide range of taxonomic and functional groups of bacteria were recovered from soils if selective media contained humic acids extracted from soil. Humic substances apparently modify cellular activity and growth due to their influence as growth factors (Visser, 1985a), their nutritive value (Mathur and Paul, 1966; Clark and Paul, 1970) and/or their influence on cell membrane permeability (Vaughan and MacDonald, 1971, 1976; Visser, 1985b). Microbial populations which increase due to humic substance amendments to soils include physiologic groups potentially deleterious to Fusarium survival (Visser, 1985a).

Humic substances in forest soils may also contribute to the exclusion of Fusarium from coniferous forest soils due to their

tendency to complex with soil enzymes. Purified soil enzymes are often easily degraded in laboratory studies, yet are extremely resistant to degradation when complexed with humic substances (Skujins, 1976). Substrates of many of these enzyme-humic complexes are components of fungal cell walls (Ladd, 1972; Rowell et al., 1973; Rodriguez-Kabana et al., 1983; Sarker, 1986).

The feasibility of adding humic substances to nursery soils in order to restore a microbially-mediated Fusarium suppressiveness similar to that of forest soils is unknown. Furthermore, the effect of humic substances on ectomycorrhizae development is not well studied. We proposed to determine if amendments of humic-rich organic compounds to nursery soils would affect: a) soil suppressiveness to Fusarium, b) populations of several microbial groups with potential for biocontrol, c) ectomycorrhizae, and d) Douglas-fir seedling growth.

MATERIALS AND METHODS

Soil sites and preparation

Soils from three Pacific Northwest bareroot conifer nurseries were sampled in early summer after seasonal rains had ceased. Each soil site is briefly described below, including the predominant conifer seedling species present at the site.

-- Brownsville, Oregon - Elevation, 110 m, clay loam,
    Pseudotsuga menziesii

-- Mt. Hood, Oregon - Elevation, 590 m, coarse sandy loam,
    Pseudotsuga menziesii

      -- Kellogg, Oregon - Elevation, 60 m, silt loam, <u>Pseudotsuga</u>

        <u>menziesii</u>

At each site, four or five samples of approximately 3 L were

collected from the top 10 cm of soil, pooled, and refrigerated at 5°C

until needed. Sampling locations at each site were selected at

random within a 25 m by 25 m sampling area. Prior to experimental

use, pooled samples were sieved through a 2 mm mesh and mixed with

pasteurized (60°C aerated steam for 30 min) river sand (60 soil:40

sand).

Humic-rich organic amendments

Three products known to be high in humic substances were

selected for experimental use: a) composted grape pomace (CGP)

(ET100, Ortek, Belleveue, WA, 98006); b) Hypnum peat (HP), a high-

humic-content hypnum (vs. sphagnum) based peat (The Bonaparte

Company, Renton, WA); and c) powdered oxidized lignite coal

(leonardite) (L) (Moms, Intertec Inc., Portland, OR, 97217). Organic

amendments were analyzed at the Oregon State University Soil Testing

Laboratory for chemical and nutrition properties., and specific humic

acid fractions prepared using the techniques of Stevenson (1965).

Experimental treatments

Nursery soils were amended with 1, 2, 5, and 10% (by volume)

CGP, HP, and L. Controls consisted of each nursery soil without

amendments. Amended soil mixes were then sown with 4 surface-

sterilized Douglas-fir seeds per 165 ml "supercell" container (Ray

Leach Cone-tainers, Inc.). Tubes were top-dressed with 0.6 g of 18-

6-12 Osmocote fertilizer and #2 gage chicken grit to slow moisture

loss from tubes during seed germination, and placed in a completely

randomized factorial design. Seedlings were grown at glasshouse

temperature (23 $\pm$ 4°C) under ambient and supplemented (high pressure

sodium vapor lamps) light (average = 350 microeinsteins/m^2/sec).

The number of damped-off and healthy seedlings was recorded for

six replicates of 28 trees/rep until 6 weeks after seeding. The root

systems of 15-25 damped-off seedlings from each soil were surface-

sterilized and plated on peptone, PCNB agar (PPA, Nash and Snyder,

1962) and selective V-8 agar (SV-8, Schmithenner, 1973) to determine

if Fusarium or Phytophthora and Pythium, respectively, were

associated with seedling damping-off. Seedlings were then thinned to

1 per tube.

Twenty-six weeks after seeding, 15 seedlings per treatment were

selected at random and the percent of short roots with mycorrhizae,

top height, stem caliper, root dry weight, shoot dry weight,

root/shoot ratio, number of buds, and number of lateral branches

determined for each seedling. Analysis of variance was performed on

a 3x3x4 factorial data set which resulted from the removal of the

data from each control soil. Controls were then reinstated and data

for each soil analyzed separately using a (3x4)+1 analysis of

variance. Means within each soil type were separated from their

respective controls using Fishers protected L.S.D test. After

seedlings were harvested, samples of representative amended soil

mixes were analyzed for chemical and nutrition properties at the Oregon State University Soil Testing Laboratory.

Analysis of microbial profiles

After seedling harvest, 4 tubes per treatment were selected at random for dilution plate analysis of soil microbial populations of the following taxonomic and functional microbial groups: bacteria, actinomycetes, extracellular chitinase producers, Fusarium, fluorescent pseudomonads, and facultative anaerobes. Bacteria and actinomycete populations were assayed to indicate the total potential biological activity of the soils. Microorganisms capable of producing extracellular chitinase have been implicated in the lysis of hyphae of some plant pathogenic fungi (Mitchell and Alexander, 1963; Tominaga and Tsujisaka, 1976; Elad et al., 1982, 1983), while many fluorescent pseudomonads are potent biological control agents (Weller, 1985). Facultative anaerobes frequently are capable of producing ethylene, a volatile compound which could contribute to soil fungistasis (Smith, 1976). Estimates of populations of soil microbial groups were made by dilution platings on selective media as described previously (Schisler, Chapter 2). Estimates of the populations of these same microbial groups were also made for the humic-rich organic amendments alone.

Extracts from each of the 4 soils per treatment were also prepared to determine the comparative suppressiveness of amended soils towards sporangia and zoospore production by Phytophthora cinnamomi, a sensitive indicator of general soil suppressiveness

(Broadbent and Baker, 1974). Extracts were prepared by flooding 0.75 g dry weight equivalent of fresh soil in a 125 ml flask with 75 ml sd H_2O, and decanting the supernatant after 4 days incubation at 23 ± 2°C. Five ml of each extract were then used to flood three 5 mm-diameter by 1 mm-thick disks of V-8 juice agar taken from the periphery of 2-day-old colonies of P. cinnamomi (Ribeiro, 1978) in 5 cm-diameter petri dishes. After 48 h, sporangia that had grown out from the disks were cold-shocked at 5°C for 40 min, warmed to room temperature, and appropriate serial dilutions of the extract containing released zoospores were plated on SV-8 agar. The number of viable P. cinnamomi zoospores/ml of extract were indicated by the number of colonies formed after dark incubation at room temperature for two days. Data for each soil type was analyzed using a (3x4)+1 analysis of variance, and means of all microbial counts within each soil type were separated from their respective controls using Fishers protected L.S.D. test.

RESULTS

Seedling growth and soil-amendment analysis

Humic amendments frequently increased and sometimes decreased seedling growth for those parameters measured (Table 4-1). Significant soil x amendment interactions precluded pooling of the soil data to obtain overall humic amendment effects on tree growth. The CGP amendment often increased (P < 0.01, 0.05) seedling top heights, stem calipers, shoot weights and the number of buds and

lateral branches per seedling at the 5 and 10% amendment rate. The
HP amendment significantly (P < 0.05) increased seedling top height
and number of buds for seedlings grown in the Brownsville soil (Table
4-1), but otherwise did not stimulate tree growth in the soils
tested. The L amendment did not increase, and at some amendment
rates, decreased seedling growth (Table 4-1). The chemical and
nutritional analysis of the amendments used and selected amended
soils at the end of the experiment showed CGP to be higher in
phosphorus and potassium than the other amendments (Tables 4-2, 4-
3). Of the three organic amendments, HP and L had the highest
proportion of humic acid and fulvic acid, respectively (Table 4-2).

Ectomycorrhizae and seedling health/damping off

Soil origin was criticial in determining the effect of
amendments on mycorrhiza development, seedling damping-off and
seedling health. Each amendment at every rate significantly (P <
0.01) decreased seedling damping-off and increased the number of
healthy seedlings (P < 0.01) in the Brownsville soil (Table 4-1), but
had no effect in the other two soils. Interestingly, Fusarium
populations in non-amended soils were highest in the Brownsville soil
(Table 4-4). Fusarium was the only pathogen isolated from the roots
of damped-off seedlings grown in the Brownsville and Kellogg soils,
while Fusarium and Pythium were isolated with equal frequency from
the roots of damped-off seedlings grown in the Mt. Hood soil.
Ectomycorrhizae increased in Kellogg soil amended with 1 or 5% CGP

(Table 4-1), but amendments were otherwise ineffective in influencing ectomycorrhiza formation.

Microbial analysis, soil suppressiveness

Humic-rich organic amendments sometimes significantly increased and sometimes decreased the number of bacteria, actinomycetes, extracellular-chitinase-producing organisms, fusaria and facultative anaerobes recovered from amended soils (Table 4-4), while amendments had no effect on the numbers of fluorescent pseudomonads recovered from any soil. Although amendment effects on microbial populations varied, depending on the soil, CGP generally increased populations of bacteria, actinomycetes, extracellular chitinase producers and facultative anaerobes, especially at the highest amendment rates. Surprisingly, general soil suppressiveness to Phytophthora was decreased in 2 and 10% CGP-amended Brownsville soil, as measured by an increased in viable zoospores produced in soil extracts (Table 4-4).

Several amendment levels of HP increased (P < 0.05) populations of extracellular chitinase-producing organisms in the Mt. Hood soil. In other soils, the highest levels of HP amendment increased (P < 0.05) the recoverable Fusarium population and decreased soil suppressiveness to Phytophthora (Table 4-4). Several amendment levels of HP decreased the population of facultative anaerobes in the Mt. Hood soil (Table 4-4).

Several levels of the L amendment increased the recoverable bacteria and chitinase producer populations in the Mt. Hood soil, but

decreased the number of chitinase producers in the Brownsville soil and the number of facultative anaerobes in the Mt. Hood soil (Table 4-4).

Total bacterial and actinomycete populations as well as extracellular chitinase producers and facultative anaerobes at levels considerably higher than those of control soils were recovered from the CGP amendment alone (Table 4-5). Populations of these groups were similar or less than those of control soils for the HP and L amendments, respectively. Detectable populations of Fusarium were not present in any of the amendments used.

DISCUSSION

Humic amendments to nursery soils varied in their influence on soil microbial populations, depending on the specific amendment and soil considered. High background microbial counts for the CGP amendment (Table 4-5) would appear to at least partially account for the increased microbial populations in soil amended with 5 and 10% CGP, although microbial group populations in the HP and L amendments are too low to explain the microbial population increases occasionally seen in soils amended with these substances. Microbial population increases likely also resulted from the nutritive value of the amendments (Mathur and Paul, 1966; Clark and Paul, 1970) and perhaps from humic-mediated increases in microbial cell membrane permeability to nutrients (Visser, 1985b). Interestingly, high concentrations of the HP and L amendments occasionally decreased the populations of several microbial groups in amended soils (chitinase

producers in Brownsville soil; bacteria, chitinase producers and facultative anaerobes in Mt. Hood soil, Table 4-4). The concentration-dependent nature of the effects of humic substances on soil microbes has been noted previously (Visser, 1985a).

The seedling growth enhancement frequently observed in humic-amended soils appears to be at least partially due to increased soil fertility, especially in CGP-amended soils (Tables 4-2, 4-3). Humic substances are also known to improve soil structure (Flaig, 1975), prevent the leaching of nutrients from soils (Vaughan and Ord, 1985) and increase plant cell permeability to nutrients (Vaughan and MacDonald, 1976; Vaughan and Malcolm, 1985), processes which also could contribute to increased seedling growth. Auxin-like qualities have also been attributed to humic substances (O'Donnell, 1973). The inhibitory effect of high concentrations of the L amendment on some plant growth parameters parallels reported observations that humic substances can inhibit plant growth at high concentrations (Elgala et al., 1978; Mylonas and McCants, 1980a).

Several soil chemical, physical and microbiological factors may be involved in the variable influence of humic substances on ectomycorrhiza formation and seedling damping-off (Table 4-1). Clay colloids can absorb humic substances, thus modifying humic substance availability, depending on the quantity and quality of clays present in a soil (Schnitzer, 1986). The growth and development of ectomycorrhizal fungi could, in turn, be affected (Tan and Nopamornbodi, 1979). Resident populations of root pathogenic fungi may have differed between soils, with differential responses of these

fungi to humic substances accounting for differential damping-off responses between soils. Nutrient differences between soils (Table 4-3) likely altered seedling nutrition which can affect ectomycorrhiza formation (Harley and Smith, 1983) and seedling damping-off (Bloomberg, 1981). Lastly, the amount and relative proportions of inorganic ions in a soil can influence the quantity and quality of stable enzyme-humic complexes formed (Mayaudon, 1968; Maignan, 1982) which could affect the lytic nature of a soil.

Results from this study point to the difficulty in predicting the efficacy of adding high humic-content organic materials to nursery soils to control seedling damping-off and increase seedling growth and ectomycorrhizae. Reconstruction of a forest-like Fusarium suppressiveness in nursery soil via humic amendment is complicated by the fact that commercially available humic products vary greatly from each other and undoubtedly from forest soil humus in type, content and availability of humic substances. The contribution of phenolic substances present in the leachates of coniferous needle litter (Blaschke, 1979) to Fusarium exclusion from forest soils is another factor difficult to establish in nursery soils by the addition of humic-rich substances alone. Lastly, the high soil fertility of nursery soils and the resultant change in soil microbial population profiles (Schisler, Chapter 1) defies reconstruction of a forest soil-like Fusarium suppressiveness in nursery soils by the simple addition of humic-rich organic amendments.

ACKNOWLEDGMENTS

Table 4-1. Comparison of growth parameters, percent mycorrhizae and damping-off of seedlings grown in three nursery soils amended with high humic content organic materials.

Soil/ blend	Top height (cm)	Stem caliper (mm)	Root dry weight (g)	Shoot dry weight (g)	Root/ shoot	# buds	Lateral branches	% mycor- rhizae	Damping- off %	Germ. healthy %
				BROWNSVILLE						
CGP 1%	6.4	1.51	0.255	0.216	1.31	5.9	0.9	2.3	13**	63**
CGP 2%	6.3	1.42	0.268	0.195	1.43	7.5	1.1	3.7	13**	62**
CGP 5%	6.6*	1.59	0.329	0.247	1.38	9.8**	2.6**	4.3	10**	68**
CGP 10%	6.9**	1.66*	0.308	0.269	1.19	8.2	2.3**	0.5	7**	69**
HP 1%	6.2	1.32	0.284	0.174	1.74	7.1	0.7	1.3	8**	74**
HP 2%	5.9	1.39	0.265	0.211	1.53	5.9	0.7	6.1	8**	56**
HP 5%	6.4	1.47	0.278	0.191	1.56	6.9	0.6	2.1	14**	61**
HP 10%	6.7*	1.47	0.322	0.218	1.52	8.7*	1.7	4.8	11**	60**
L 1%	5.6	1.38	0.249	0.173	1.59	6.4	0.3	2.2	11**	63**
L 2%	6.4	1.45	0.270	0.193	1.49	8.0	0.7	4.1	5**	71**
L 5%	5.9	1.42	0.259	0.187	1.54	6.2	0.7	1.5	2**	76**
L 10%	5.4	1.35	0.226**	0.178	1.64	5.5	0.2	3.1	7**	73**
control	5.7	1.45	0.307	0.186	1.69	6.7	0.9	6.5	33	38
				MT. HOOD						
CGP 1%	5.2	1.21	0.211	0.152*	1.57	4.7	1.0	4.1	26	25
CGP 2%	5.0	1.21	0.213	0.125	1.83	4.3	0.9	2.2	19.	19
CGP 5%	6.7**	1.25	0.217	0.182**	1.27	4.1	0.5	2.7	17	28
CGP 10%	5.9**	1.25	0.225	0.185**	1.21	4.3	1.0	0.2	27	24
HP 1%	4.5	1.11	0.150	0.112	1.53	3.3	0.7	0.7	33	17
HP 2%	4.8	1.18	0.176	0.111	1.75	3.6	0.7	1.1	20	19
HP 5%	5.0	1.12	0.168	0.117	1.60	3.4	0.7	1.1	29	20
HP 10%	5.1	1.14	0.185	0.138	1.48	4.8	0.8	0.4	16	33
L 1%	4.2	1.08	0.140*	0.102	1.49	2.9	1.0	2.3	26	35
L 2%	4.7	1.22	0.194	0.134	1.63	4.1	0.9	0.1	22	37
L 5%	4.6	1.10	0.163	0.127	1.43	3.3	0.7	0.1	28	36
L 10%	4.6	1.24	0.209	0.143	1.58	4.2	1.3	2.7	23	33
control	4.3	1.13	0.190	0.104	1.91	4.0	0.4	0.5	24	26
				KELLOGG						
CGP 1%	5.3	1.08	0.162	0.110	1.52	4.7	0.9	12.7**	4	72
CGP 2%	5.6	1.08	0.179	0.120	1.59	4.7	0.5*	3.7	5	69
CGP 5%	6.0*	1.34**	0.292**	0.166*	1.82	7.7**	0.9	8.0*	4	68
CGP 10%	7.7**	1.50**	0.431**	0.262**	1.75	9.0**	2.9**	0.0	8	62
HP 1%	4.9	1.16	0.183	0.099	1.87	4.5	0.4*	0.0	6	77
HP 2%	5.0	1.10	0.164	0.108	1.53	4.5	0.6	1.1	13	65
HP 5%	5.2	1.13	0.182	0.116	1.65	4.1	0.7	1.1	5	65
HP 10%	5.0	1.17	0.159	0.114	1.52	3.9	0.5*	3.1	5	73
L 1%	4.9	1.09	0.186	0.101	1.88	5.1	0.1**	0.9	5	69
L 2%	5.3	1.16	0.177	0.116	1.69	4.1	0.1**	0.7	6	67
L 5%	4.8	1.06	0.162	0.089*	1.85	4.3	0.3*	0.8	4	64
L 10%	4.9	1.10	0.175	0.100	1.80	3.8	0.3*	1.1	9	63
control	5.2	1.14	0.148	0.125	1.36	3.8	1.4	2.0	7	61

Values within the same column of a soil followed by a "*", or "**" are significantly different from their associated control. P < 0.05. P < 0.01. respectively (Fisher's protected L.S.D. test).

Table 4-2. Chemical and nutritional properties of humic-rich organic amendments.

Amendment	pH	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	% OM	CEC	NH_4 (ppm)	NO_3 (ppm)	% Humin	% Humic acid	% Fulvic acid
CGP	7.2	334	15990	4060	1560	41.4	69.5	254.1	10.9	91.0	1.5	7.5
HP	5.2	8	55	10200	672	53.8	78.1	15.4	372.4	79.0	12.0	9.0
L	4.8	9	129	3980	492	14.4	32.0	79.8	1.0	85.5	4.5	10.0

Table 4-3. Chemical and nutritional properties of nursery soils
amended with humic-rich organic materials.

Soil/amend	pH	P (ppm)	K (ppm)	Mg (ppm)	% OM	CEC	NH$_4$ (ppm)	NO$_3$ (ppm)
			Brownsville					
CGP 5%	5.7	29	417	372	1.95	16.4	2.8	5.8
HP 5%	5.5	24	179	360	2.33	18.4	2.4	5.6
L 5%	5.6	20	140	360	2.06	15.0	2.8	2.3
control	5.4	26	152	348	1.08	16.8	2.8	11.7
			Mt. Hood					
CGP 5%	6.1	20	406	156	2.93	9.3	4.7	16.4
HP 5%	6.1	10	129	132	2.47	9.8	2.8	3.4
L 5%	5.9	10	129	132	2.58	9.3	2.4	6.7
control	6.0	10	144	132	1.52	7.3	4.8	13.9
			Kellogg					
CGP 5%	5.5	28	359	180	1.48	10.7	2.4	9.7
HP 5%	5.5	17	160	204	1.75	10.0	2.8	7.4
L 5%	5.4	18	179	216	1.70	10.5	2.8	4.5
control	5.2	20	172	168	0.88	10.0	28.6	10.2

Table 4-4. Effect of humic-rich organic amendments of nursery soils on
Phytophthora suppressiveness and on the numbers of bacteria, actinomycetes,
and Fusarium propagules isolated.

Soil/ blend	Bacteria	Actino	Chitin	Fusarium	Fluor. pseudo.	Facult. anaerobes	Phytoph.
BROWNSVILLE							
CGP 1%	3.8E+06[a]	1.9E+06	3.8E+05	2.6E+03	6.6E+03	5.6E+05*	34
CGP 2%	3.9E+06	2.3E+06	4.2E+05	3.2E+03	3.8E+03	1.2E+06*	103*
CGP 5%	3.9E+06	2.1E+06	2.1E+05	3.3E+03	5.8E+03	1.3E+06**	67
CGP 10%	5.9E+06**	2.6E+06**	5.0E+05	3.9E+03	2.9E+03	1.5E+06**	95*
HP 1%	3.6E+06	1.8E+06	4.2E+05	5.7E+03	8.9E+03	1.1E+06	52
HP 2%	2.4E+06	1.8E+06	2.8E+05	4.0E+03	8.7E+03	1.1E+06	77
HP 5%	3.1E+06	1.9E+06	4.4E+05	4.0E+03	6.1E+03	1.1E+06	131**
HP 10%	4.1E+06	2.0E+06	3.0E+05	4.2E+03	8.0E+03	1.0E+06	120**
L 1%	3.6E+06	1.6E+06	2.2E+05	2.6E+03	2.6E+03	1.0E+06	29
L 2%	3.1E+06	1.8E+06	1.6E+05*	3.3E+03	5.1E+03	8.5E+05	70
L 5%	2.9E+06	1.6E+06	1.5E+05*	5.0E+03	5.7E+03	6.5E+05	58
L 10%	2.4E+06	1.8E+06	1.8E+05	4.5E+03	2.7E+03	8.7E+05	43
control	3.3E+06	1.9E+06	4.0E+05	4.0E+03	9.4E+03	8.9E+05	28
MT. HOOD							
CGP 1%	2.8E+06	2.5E+06	5.3E+05**	1.5E+03	1.8E+03	1.8E+06	4
CGP 2%	3.2E+06	2.5E+06	4.0E+05*	1.4E+03	1.9E+03	2.0E+06	6
CGP 5%	5.2E+06**	2.9E+06	4.1E+05*	1.8E+03	1.1E+03	2.6E+06	6
CGP 10%	4.4E+06*	2.6E+06	3.4E+05	1.2E+03	9.8E+02	2.6E+06	3
HP 1%	3.7E+06	2.9E+06	4.2E+05*	9.8E+02	1.1E+03	1.7E+06*	9
HP 2%	4.1E+06	2.9E+06	2.2E+05	1.1E+03	8.5E+02	2.2E+06	1
HP 5%	3.8E+06	2.5E+06	4.2E+05*	8.7E+02	4.3E+02	1.9E+06	4
HP 10%	2.9E+06	2.4E+06	2.5E+05	5.3E+02	5.3E+02	1.5E+06*	12
L 1%	3.4E+06	2.3E+06	2.8E+05	1.1E+03	3.7E+02	1.7E+06*	12
L 2%	3.7E+06	2.9E+06	2.9E+05	2.1E+03	4.2E+02	1.3E+06*	1
L 5%	4.6E+06**	3.2E+06	5.1E+05**	2.2E+03	1.3E+03	2.3E+06	15
L 10%	4.3E+06*	3.2E+06	3.9E+05*	1.6E+03	6.7E+01	1.9E+06	2
control	2.8E+06	2.2E+06	2.3E+05	2.3E+03	1.8E+02	2.4E+06	7
KELLOGG							
CGP 1%	2.5E+06	5.5E+05	6.3E+04	1.7E+03	0.0E+00	4.0E+05	204
CGP 2%	2.7E+06	1.1E+06*	1.8E+05	2.1E+03	0.0E+00	5.3E+05	149
CGP 5%	2.8E+06	1.4E+06**	2.8E+05*	3.9E+03	0.0E+00	5.1E+05	222
CGP 10%	5.2E+06**	2.0E+06**	3.4E+05**	6.6E+03*	4.3E+03	9.8E+05**	290
HP 1%	2.1E+06	7.3E+05	1.1E+05	2.5E+03	1.7E+01	3.7E+05	69
HP 2%	3.0E+06	7.4E+05	2.0E+05	2.4E+03	3.5E+02	3.5E+05	20
HP 5%	3.3E+06	8.1E+05	1.2E+05	6.3E+03*	1.7E+02	3.6E+05	158
HP 10%	1.9E+06	7.8E+05	1.5E+05	3.3E+03	3.3E+01	7.6E+05	158
L 1%	2.4E+06	8.4E+05	1.2E+05	4.4E+03	1.6E+03	5.0E+05	16
L 2%	3.5E+06	7.5E+05	1.3E+05	4.9E+03	3.5E+02	3.6E+05	73
L 5%	2.0E+06	6.5E+05	5.3E+04	2.7E+03	8.3E+01	6.0E+05	80
L 10%	1.7E+06	7.5E+05	9.8E+04	4.7E+03	3.3E+02	6.2E+05	22
control	2.3E+06	7.5E+05	1.3E+05	2.7E+03	4.0E+02	5.8E+05	135

[a] Values within the same column of a soil followed by a "*", or "**" are significantly
different from their associated control, P < 0.05, P < 0.01, respectively (Fisher's
protected L.S.D. test). Table values are in scientific notation. i.e., 3.8E+06 = 3.8 x
10^6.

Table 4-5. Number of colony-forming units of bacteria
and actinomycetes recovered (per gram material) from humic-rich
organic amendments.

Amendment	Bacteria	Actino	Chitin	Fusarium	Fluor. pseudo.	Facult. anaerobes
CGP						
Average	1.6E+08[a]	6.9E+07	5.3E+05	0.0E+00	5.8E+04	1.2E+06
Std dev.	2.1E+07	1.0E+07	7.3E+04	0.0E+00	2.9E+04	2.5E+05
HP						
Average	1.6E+07	5.0E+05	6.9E+04	0.0E+00	7.3E+03	1.6E+05
Std dev.	3.2E+06	6.1E+04	1.3E+04	0.0E+00	2.5E+03	1.3E+04
L						
Average	1.2E+04	1.0E+04	5.0E+02	0.0E+00	0.0E+00	2.5E+02
Std dev.	3.2E+03	5.5E+03	5.5E+02	0.0E+00	0.0E+00	8.7E+01

[a] Table values are in scientific notation, i.e., 3.8E+06 = 3.8×10^6.

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SUMMARY AND CONCLUSIONS

Results from these studies confirmed that Fusarium only rarely exists in coniferous forest soils, but commonly occurs in nursery soils. Furthermore, nursery soils frequently harbor higher populations of taxonomic and physiologic microbial groups than do forest soils, likely due, at least partially, to higher soil fertility in nursery soil and the deleterious effect of compounds present in forest floor litter extracts on the populations of forest soil microbes. Accumulation of higher concentrations of NH_4-N (which as gaseous ammonia can be deleterious to Fusarium survival) in forest soils than nursery soils may contribute to the exclusion of Fusarium from coniferous forest soils.

Volatiles from forest soils, ectomycorrhizae, and ectomycorrhizal fungi influenced the populations of microbial groups potentially deleterious to Fusarium and Fusarium mycelial growth. The potential relationship of these effects to the demise of Fusarium in coniferous forest soils was not always clear, but results did indicate, for the first time, that naturally occurring concentrations of soil volatiles influence the microbial populations of a soil. In addition to mucilages, exudates and secretions from ectomycorrhizae, volatile compounds must be considered as selectively influencing microbial populations in the ectomycorrhizosphere.

Amending nursery soils with organic materials high in humic content frequently increased, but sometimes decreased, soil microbial populations. Amendments decreased Fusarium-induced damping-off in

one soil, but were not effective in two other soils, while seedling growth was enhanced by amendments in all soils. The complex nature of interactions between soil, humic substances, plant roots and microorganisms complicates our ability to accurately predict the efficacy of amending nursery soils with humic substances as a prophylactic measure against Fusarium-induced seedling disease.

The information gained from these and former studies indicate that the following components of coniferous forest soils contribute, or may contribute, to the exclusion of Fusarium from these soils:

1. Soil volatiles - Directly, or indirectly via selective influences on soil microbes, deleterious to Fusarium survival in forest soils. A diversity of compounds may be involved including ammonia, ethylene, terpenes, and low molecular weight alcohols and aldehydes.

2. Needle litter leachates - Contain compounds which inhibit the growth of annual plants that otherwise would serve as source of saprophytic inoculum increase. Also affects Fusarium survival by stimulating propagule germination and lysis in forest soils.

3. Humic substances - Possibly renders soil enzymes deleterious to Fusarium survival less vulnerable to degradation in soil. Selectively increase the populations, and possibly

the activities, of forest soil microbes deleterious to
Fusarium.

4. Deleterious soil microbiota - Involved in the process of
Fusarium propagule germination and lysis in forest soils.
Ectomycorrhizal fungi and ectomycorrhizae produce several
volatile compounds deleterious to Fusarium (including ethylene,
terpenoids, and low molecular weight alcohols) and
ectomycorrhizae exhibit increased resistance to root pathogen
attack.

Studies, such as those presented in this thesis, which
investigate the effect of any single edaphic factor on the microbial
community present in an intact soil are more likely to produce
results which accurately reflect the in situ effect of that factor
than studies which examine the effect of that factor outside the
natural soil environment. Once the effect of a given edaphic factor
is established in studies employing whole soils, research which
isolates individual components of a soil and its environment can be
justified. It is hoped that these investigations will further
stimulate research in the area of the role of soil volatiles and
humic substances in soil microbial ecology.

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APPENDICES

APPENDIX

IN VITRO RESPONSE OF FUSARIUM OXYSPORUM TO VOLATILES

FROM ECTOMYCORRHIZAL FUNGI AND CONIFEROUS FOREST AND

NURSERY SOILS

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SUMMARY

The influence of volatiles from ectomycorrhizal fungi and
coniferous forest and nursery soil on Fusarium oxysporum mycelial
growth was determined using a petri plate assay. Volatiles from 7 of
9 ectomycorrhizal fungi tested stimulated the vegetative growth of F.
oxysporum. Volatiles from some isolates either did not affect or
stimulated F. oxysporum growth depending on the medium used to
culture the ectomycorrhizal fungus. F. oxysporum colony growth was
not differentially influenced by volatiles from coniferous forest and
nursery soils.

INTRODUCTION

Needle duff leachates and the forest soil microbiota are apparently partially responsible for the exclusion of Fusarium from coniferous forest, but not nursery, soils (Toussoun et al., 1969; Hammerschlag and Linderman, 1975; Schisler and Linderman, 1984). Ectomycorrhizae may also be involved in this phenomenon. Ectomycorrhizae are more resistant to root pathogen attack than are nonmycorrhizal roots (Marx, 1972). One possible reason for this resistance is that ectomycorrhizal fungi and mycorrhizae produce biologically active volatile compounds. Ectomycorrhizal fungi produce volatile compounds which possess fungistatic activity (Krupa and Fries, 1971; Graham and Linderman, 1980). In addition, the infection of roots with ectomycorrhizal fungi can cause the accumulation of volatile compounds (Krupa and Fries, 1971; Graham and Linderman, 1980) inhibitory to root pathogenic fungi (Krupa and Nylund, 1972; Krupa et al., 1973; Smith, 1976) and the soil microbiota can be influenced by volatiles from ectomycorrhizae (Schisler, Chapter 3).

Forest soil volatiles from sources other than ectomycorrhizae may also influence Fusarium survival. Volatiles from soil can influence the activities of soil fungi, including Fusarium (Hora and Baker, 1972; Pavlica et al., 1978; Okazaki, 1985) and the microbial population of a soil (Gilbert et al., 1969; Schisler, Chapter 2). The current study was designed to determine (a) if soil volatiles and volatiles from ectomycorrhizal fungi influence the vegetative growth of Fusarium oxysporum in vitro and (b) if volatiles from forest soils

differ from nursery soils in their effects on the vegetative growth
of Fusarium oxysporum in vitro.

MATERIALS AND METHODS

Ectomycorrhizal fungi

Nine ectomycorrhizal fungi, representing five genera common to
Pacific Northwest forest soils, were selected for use in this
study. Sites where isolates were obtained are briefly described
below, including the predominant conifer species present at the site:

-- Laccaria laccata isolate #S-446 (LL S446)[1] - Mary's Peak,
Oregon, 310 m, Pseudotsuga menziesii.

-- Laccaria laccata isolate #S-467 (LL S467) - Black Prince
Coal Mine, Centralia, Washington, elevation unknown,
Pseudotsuga menziesii.

-- Hebeloma sp. isolate #S-260T (He S260T) - Upper Battle
Creek, Oregon, 1460 m, Pinus contorta-Pseudotsuga
menziesii mix.

[1] Isolate abbreviation used in tables.

-- <u>Hebeloma</u> <u>crustuliniforme</u> isolate #2 (Hecr 2) - Bald Mountain, Oregon, 1890 m, <u>Abies</u> <u>concolor</u>-<u>Pinus</u> <u>contorta</u> mix.

-- <u>Cenococcum</u> <u>geophilum</u> isolate #6 (Cege 6) - Oklahoma Gulch Pass, Washington, 900 m, <u>Pinus</u> <u>ponderosa</u>.

-- <u>Cenococcum</u> <u>geophilum</u> isolate #7 (Cege 7) - Wenatchee, Washington, 1250 m, <u>Pinus</u> <u>ponderosa</u>, <u>Pseudotsuga</u> <u>menziesii</u>.

-- <u>Suillus</u> <u>luteus</u> isolate #1 (Sulu 1) - Bald Mountain, Oregon, 1890 m, <u>Abies</u> <u>concolor</u>-<u>Pinus</u> <u>contorta</u> mix.

-- <u>Suillus</u> <u>lakei</u> isolate #1 (Sula 1) - Wenatchee river drainage, Washington, 760 m, <u>Pseudotsuga</u> <u>menziesii</u>-<u>Tsuga</u> <u>heterophylla</u>-<u>Picea</u> <u>mariana</u> mix.

-- <u>Rhizopogon</u> sp. isolate #15 (Rhizo 15) - Buck Mountain, Oregon, 460 m, <u>Pseudotsuga</u> <u>menziesii</u>.

<u>Soil sites and preparation</u>

Five forest and five nursery soils were used in this study. Soils were obtained and prepared for experimental use as described previously (Schisler, Chapter 1). Soil sites varied in dominant vegetation, elevation, soil type and location and the chemical and nutritional characteristics of these soils varied considerably (Schisler, Chapter 1).

Assay of the effects of volatiles on Fusarium vegetative growth

One mm^{+3} blocks were cut from the periphery of 3-4-day-old

Fusarium oxysporum Schlect. (isolate F-35) colonies growing on water

agar. Two blocks were placed approximately 3 mm apart on 6x6 cm

pieces of sterile dialysis or cellophane membranes in 8 cm diameter

water agar plates. Plates were incubated in the dark at 22±1°C for

12-15 h prior to exposing Fusarium colonies to volatiles from either

soil or ectomycorrhizal fungi. The influence of soil volatiles was

assayed by lightly packing 15 g dry weight equivalent of fresh soil

into the bottom of sterile petri plates. The bottom half of water

agar plates containing 12-15 h old F. oxysporum colonies on membranes

were then inverted, placed on top of the plates containing soil and

sealed in place using parafilm. Controls consisted of water agar

plates inverted over plates containing sterile moistened

vermiculite. Volatile-mediated differences in Fusarium colony

expansion were then measured after 55-60 h incubation in darkness at

22±1°C. There were four replicate plates for each soil and the

average diameter of the two colonies present for each replicate was

used in the statistical analysis. Data was analyzed using a one-way

analysis of variance and treatment means separated from controls

using Fisher's protected L.S.D. test.

The influence of volatiles from ectomycorrhizal fungi was

assayed in a manner similar to that described above except

ectomycorrhizal fungi grown for 2-4 weeks in the dark at 22±3°C on

MMN agar (Marx and Kenny, 1982), Malt agar (Palmer, 1971), and PDA

(Difco) were used instead of plates containing soils. Controls

consisted of uninoculated MMN agar, Malt agar, or PDA plates. Four, or occasionally five (Cege 6, Cege 7), 0.5 cm diameter plugs taken from the periphery of ectomycorrhizal fungal colonies were used to inoculate the plates used in this study. Data obtained from four replicate plates of each fungal isolate were analyzed as described above.

RESULTS

Volatiles from ectomycorrhizal fungi increased, or sometimes did not affect, F-35 colony expansion compared to controls; depending on the ectomycorrhizal fungus isolate and the medium on which it was grown (Table A-1). The two isolates of Hebeloma (He S260T, Hecr 2) consistently stimulated F-35 colony expansion. Conversely, the Suillus isolates (Sula 1, Sulu 1) did not increase F-35 mycelial growth regardless of the medium these isolates were grown on. Both isolates of Cenococcum (Cege 6, Cege 7), when grown on MMN, produced volatiles which stimulated F-35 colony expansion, but neither were successfully grown on Malt agar or PDA.

Volatiles from soils did not significantly influence F-35 colony expansion as compared to controls (Table A-2). The effect of volatiles from nursery soils on F-35 colony expansion did not differ from that of forest soils (Table A-2).

DISCUSSION

The stimulation, rather than inhibition, of F-35 colony growth which resulted when colonies were exposed to volatiles from

ectomycorrhizal fungi (Table A-1) is likely due to a combination of factors. Krupa and Nylund (1972) demonstrated that Boletus variegatus produces ethanol when grown vegetatively, and that low concentrations of this volatile compound stimulate the growth of root pathogenic fungi in vitro, whereas higher concentrations are inhibitory. A similar volatile concentration-dependent response by the root pathogenic fungus Sclerotium rolfsii has been demonstrated by Linderman and Gilbert (1969). Perhaps, therefore, deleterious volatile concentrations were not achieved using this assay system due to volatile absorption by the agar or volatile escape from the assay chambers through the parafilm seals. Furthermore, nutrition is known to be important in determining whether microorganisms produce compounds stimulatory, deleterious, or of no consequence to the growth of pathogenic fungi (Gilbert and Linderman, 1971). Growing ectomycorrhizal fungi on a growth medium based on a different quantity and quality of nutrients may result in these fungi producing volatile compounds inhibitory to F-35 colony growth. Finally, ectomycorrhizae produce volatile compounds distinctly different from those produced by ectomycorrhizal fungi alone (Krupa and Nylund, 1972; Krupa et al., 1973) and thus the effect of volatiles produced by ectomycorrhizal fungi on Fusarium may not accurately reflect the influence of volatiles produced by ectomycorrhizae in forest soil ecosystems. Alternatively, the assay system used in this study may accurately reflect the influence of volatiles from ectomycorrhizal fungi on Fusarium mycelial growth. If volatiles from ectomycorrhizal fungi stimulated Fusarium mycelial growth in forest soils to the

exclusion of chlamydospore formation, even during periods of
unfavorable nutrition for growth, lysis of Fusarium hypahe would
likely result.

Though soil volatiles are known to influence soil microbial
populations (Gilbert et al., 1969; Schisler, Chapter 2), F-35 colony
growth was not effected by volatiles from nursery or forest soils
(Table A-2). Pavlica and coworkers (1978) found that concentrations
of volatiles considerably above those detected in soil were necessary
to inhibit fungal spore germination. Thus the dilution of soil
volatiles which would take place during diffusion of these substances
into the airspace separating the F-35 colonies from the soil may
account for the lack of any observed influence on F-35 colony
growth. Additionally, volatiles may have been more appropriately
tested for their effect on Fusarium macroconidial germination instead
of colony expansion since volatiles are thought to be important in
fungistasis (Hora and Baker, 1972; Lockwood, 1977). Adequate
nitrogen nutrition can nullify factors contributing to fungistasis
(Lockwood, 1977), a factor which may also have kept potential
volatile-mediated effects on F-35 colony expansion from being seen
due to the nutrient content present in unwashed water agar. Lastly,
a concentration of volatiles sufficiently high enough to influence F-
35 colony expansion may have been achieved if soils had been amended
with organic or humic substances in order to create a soil
environment analogous to a forest soil covered with a needle litter
layer. The demonstration by Okazaki (1985) that glucose amendment of
soils resulted in volatiles being produced which lysed Fusarium

chlamydospores lends credence to the possibility that concentrations of volatiles sufficient to influence F-35 colony growth _in vitro_ could be achieved by amending test soils with organic or humic substances.

Table A-1. Effect, _in vitro_, of volatiles from ectomycorrhizal fungi grown on several different media on the mycelial growth of _Fusarium oxysporum_.

Fungal isolate	F-35 colony diameter (mm)		
	MMN	MA	PDA
LL S446	25.1	23.2	23.4**
LL S467	26.6	24.3*	23.1**
He S260T	28.1*	24.1*	24.1**
Hecr 2	27.6*	24.9**	24.3**
Cege 6	28.8**	-	-
Cege 7	27.9*	-	-
Sulu 1	26.9	23.6	20.6
Sula 1	27.5	21.8	19.9
Rhizo 15	26.4	24.3*	21.4**
Control	25.6	22.2	19.2
$\alpha = 0.05$	1.97	1.60	1.42
$\alpha = 0.01$	2.66	2.17	1.93

Values within the same column followed by a "*", or "**" are significantly different from their associated control, $P < 0.05$, $P < 0.01$ respectively (Fisher's protected L.S.D. test).

Table A-2. Effect, _in vitro_, of volatiles from nursery and
coniferous forest soils on the mycelial growth of
Fusarium oxysporum.

Donor soil[a][b]	F-35 colony diameter (mm)
N2	25.3
N5	25.7
N6	25.8
N9	25.6
N10	24.8
F1	25.5
F8	26.1
F9	25.8
F10	25.6
F12	25.9
control	25.4
Average nursery	25.4
Average forest	25.8

[a] Nursery soil = N2, N5, N6, N9, N10; forest soils = F1, F8, F9, F10, F12.

[b] Soil sites are described in Chapter 1.

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