

## 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 significant diseases of conifer seedlings in nurseries (Bloomberg and Lock, 1972; Brownell and Schneider, 1983). *Fusarium*, apparently does not affect seedlings in coniferous forest soils covered with needle litter, however, and rarely can even be detected in such soils (Thornton, 1960; Park, 1963; Schisler and Linderman, 1984). Smith (1967) reported that *F. oxysporum* could not be recovered from roots of previously infected sugar pine (*Pinus lambertiana* Dougl.) seedlings transplanted into native forest soils 3 years earlier. Further studies demonstrated that forest soil needle litter extracts stimulate germination of chlamydo-spore and macroconidia of *F. oxysporum* followed by germ-tube lysis (Menzinger, 1969; Toussoun *et al.*, 1969; Hammerschlag and Linderman, 1975; Schisler and Linderman, 1984).

Forest soils generally contain a large fungal biomass, including ectomycorrhizal fungi, which generates significant amounts of volatile compounds (Krupa and Fries, 1971; Krupa *et al.*, 1973). Furthermore, the high organic content of such soils supports significant activity of other microbes which could also generate volatile compounds. Hypothetically, such volatiles could affect the survival of fusaria (Krupa and Nylund, 1972; Moore-Landecker and Stotzky, 1973; Stack and Sinclair, 1975), possibly by stimulation of propagule germination; affecting the orientation of hyphal growth; or inhibition of hyphal growth and propagule germination (Linderman and Gilbert, 1975).

Though literature reporting the effects of volatiles on fungi and soil microbes *in vitro* is voluminous, studies on the influence of naturally occurring amounts of soil volatiles on the microbial ecology of a soil have not been attempted. The difficulty in experimentally exposing soil microbes to soil-produced volatiles without interference from other biologically-active compounds produced in soil undoubtedly accounts, in part, for this void. Using a device specifically constructed to transfer volatiles from one soil to another which could then be assayed for microbial population shifts, we proposed to determine whether: (a) *in vivo* studies on the influence of soil volatiles on microbial populations are feasible; (b) volatiles from forest and nursery soils differed in their effect on the microbial profile of a test soil; and (c) volatiles from forest soils, forest soil microbes or mycorrhizae directly decrease *Fusarium* populations or increase populations of soil microorganisms potentially deleterious to *Fusarium*.

### MATERIALS AND METHODS

#### *Soil sites and preparation*

Soils from two coniferous forest sites, Mary's Peak (F1) and Cascadia (F2) dominated primarily by Douglas-fir (*Pseudotsuga menziesii*) and three nursery sites, Elkton (N1), Kellogg (N2) and Mt Hood (control) all planted to Douglas-fir, were sampled in early summer after seasonal rains had ceased in the Pacific Northwest. 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 approx. 3l. each were collected from the top 0-10 cm of mineral soil, pooled and refrigerated at 5°C until needed. Sampling locations at

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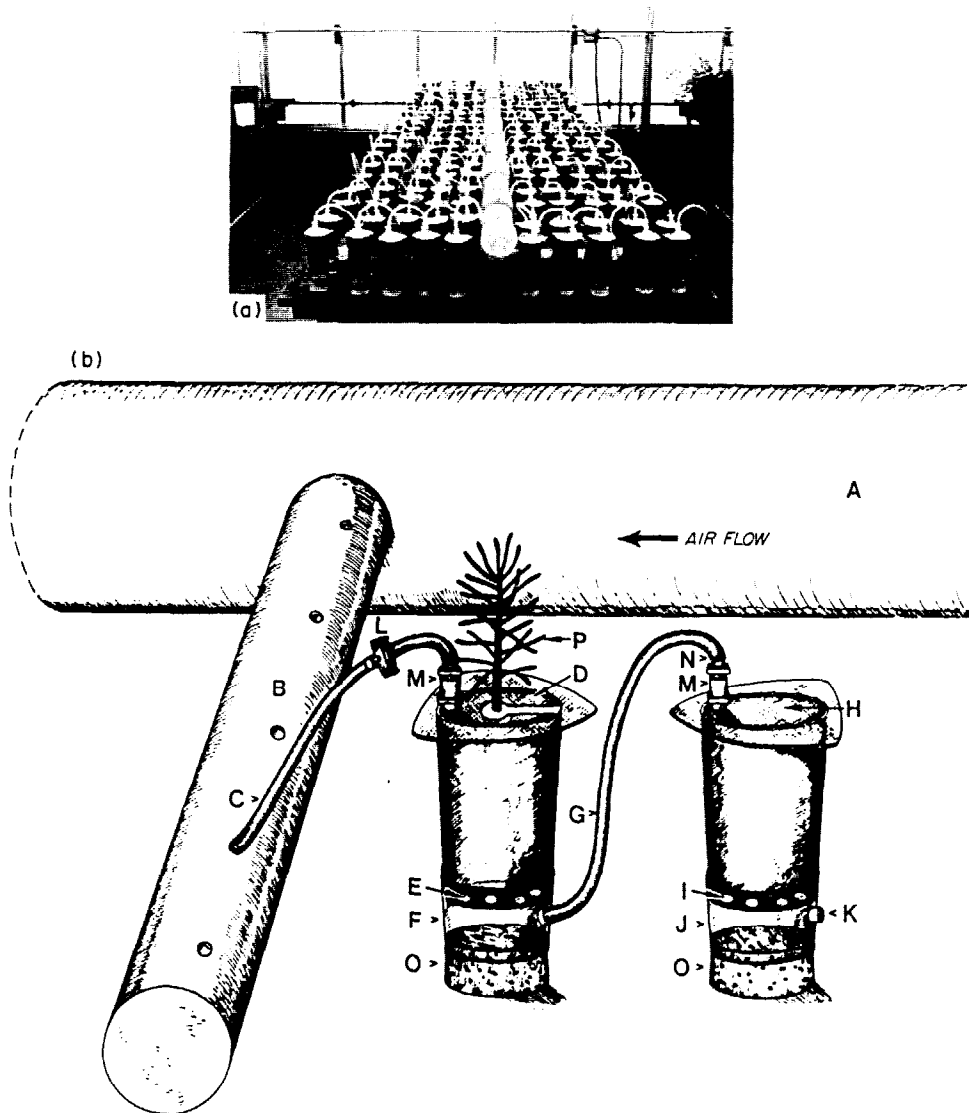


Fig. 1. Photo (a) and schematic diagram (b) of VES for purging the atmosphere from donor soils into receiver soils. (A) main manifold, (B) submanifold, (C) tygon tubing, (D) top of donor soil cup, (E) holes in the bottom of donor soil cup, (F) plastic sleeve, (G) tygon tubing, (H) top of receiver soil 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.

each site were randomly selected within a  $25 \times 25$  m sampling area. Prior to experimental use, pooled samples were sieved ( $< 2$  mm) and mixed with pasteurized ( $60^\circ\text{C}$  aerated steam for 30 min) river sand (3 soil:2 sand).

#### *Volatile exchange system*

A volatile exchange system (Fig. 1) 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) (Fig. 1, A) of the volatile exchange system (VES) and subsequently into ten submanifolds (5.1 cm i.d. PVC pipe) (Fig. 1, 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  $3\text{--}5 \text{ ml min}^{-1}$  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 of 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

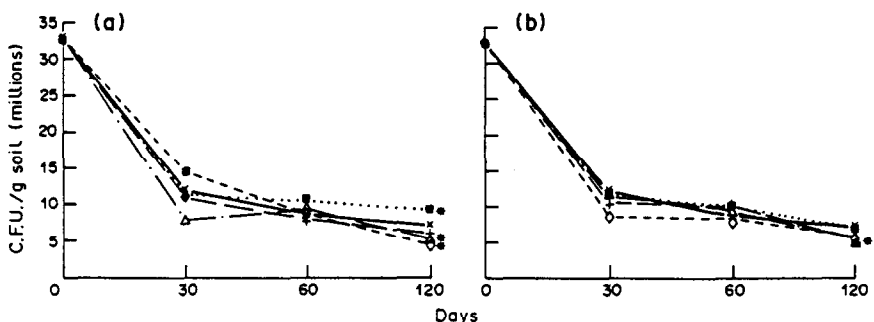


Fig. 2. Effect of volatiles from fresh (a) or pasteurized and fumigated (b) forest and nursery soils on the numbers of bacteria isolated from a receiver nursery soil. Values within an assay date followed by an asterisk are significantly different from the control,  $P < 0.05$  (Fisher's protected L.S.D. test). ■, F1; +, F2; ◇, N1; △, N2; and ×, control.

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 microbial profiles of the receiver soil were due to volatile effects alone. A 1 cm thick layer of chicken grit (gage No. 2; crushed quartz which passes through a 4 mm sieve) was placed on the bottom of cups and on the top of soil columns to assure that 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 inserted into the hole in the lid and 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

Forest soils F1 and F2, and nursery soils N1 and N2 were used as "donors" of soil volatiles, while a third nursery soil was used as a "receiver" soil in all cases. Sand was added to natural soils for improved porosity 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) (454 g of a 95% methyl bromide, 5% chloropicrin mixture and 12 l. soil spread 2 cm thick in a 2.5 m<sup>3</sup> 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 treated F1 and N1 donor soils were planted or not with a 30-day-old seedling of *Pseudotsuga menziesii* at day 30 of the experiment. Seedlings were grown from seed in flats in greenhouses at  $24 \pm 3^\circ\text{C}$  under ambient light and supplemented with high pressure sodium vapor lamps (average =  $300 \mu\text{E m}^{-2} \text{s}^{-1}$ ) and fertilized biweekly to saturation with 1/4 strength phosphorus Long Ashtons solution (Hewitt, 1966) prior to transplanting into the VES. Trees were not fertilized after being transplanted. Seedlings were harvested after terminating the experiment and scored to determine the percentage of short roots that were ectomycorrhizal.

There were eight donor-receiver cup pairs for each treatment which were placed in a completely randomized design. Controls were the third nursery receiver

soil mix used as a donor soil. All donor and receiver soil mixes were adjusted to  $-0.02 \text{ MPa}$  ( $1 \text{ b} = 0.1 \text{ MPa}$ ) (by misting soils with sterile distilled H<sub>2</sub>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, actinomycetes, *Fusarium*, extracellular chitinase producers, fluorescent pseudomonads and facultative anaerobes. Estimates of populations of microbial groups in receiver soils were made by serially diluting 75 mg dry wt equivalent of fresh receiver soil with 0.1% water agar and plating aliquots of appropriate dilutions on plates of tryptic soy agar +  $100 \mu\text{g g}^{-1}$  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 +  $1 \mu\text{g l}^{-1}$  resazurin (redox potential indicator dye) +  $275 \mu\text{g L-cysteine-HCl} \cdot \text{H}_2\text{O g}^{-1}$  (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 medium consists of: 980 ml H<sub>2</sub>O, 3.5 g colloidal chitin (Hsu and Lockwood, 1975), 16.0 g "gelrite" (Merck & Co., San Diego, Calif.), 7.2 g K<sub>2</sub>HPO<sub>4</sub> · 3H<sub>2</sub>O, 2.8 g KH<sub>2</sub>HPO<sub>4</sub> · 3H<sub>2</sub>O, 0.5 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 10 mg CaCl<sub>2</sub>, 5 mg FeSO<sub>4</sub> · 7H<sub>2</sub>O and 2.0 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> added from  $10.0 \text{ g} \times 100 \text{ ml}^{-1}$  dH<sub>2</sub>O stock solution autoclaved separately. A 6 ml sample of this preparation was poured, while hot, into plates containing solidified "gelrite" medium (8.0 g "gelrite" + 0.75 g MgSO<sub>4</sub> · 7H<sub>2</sub>O l<sup>-1</sup> dH<sub>2</sub>O). 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

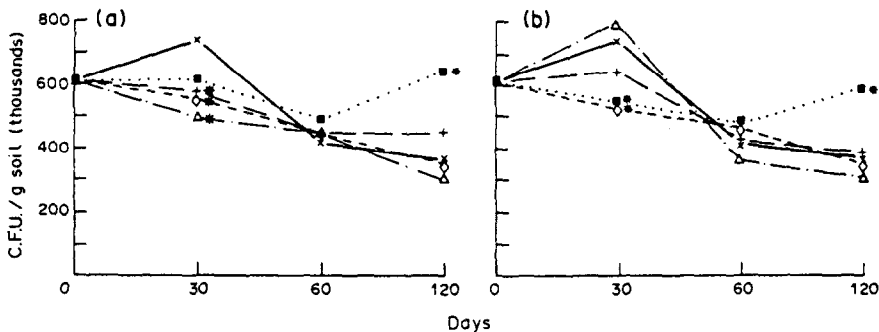


Fig. 3. Effect of volatiles from fresh (a) or pasteurized and fumigated (b) forest and nursery soils on the numbers of extracellular-chitinase-producing microorganisms isolated from a receiver nursery soil. Values within an assay date followed by an asterisk are significantly different from the control,  $P < 0.05$  (Fisher's protected L.S.D. test). ■, F1; +, F2; ◇, N1; △, N2; ×, control.

for facultative anaerobes 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 2a, b). At 120 days, fresh F1 forest 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. 2a). Also at 120 days, P-F N2 nursery soil volatiles had decreased bacterial populations in the receiver soil (Fig. 2b). 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 3a, b). For fresh soils, initial volatile-mediated declines in the populations of

chitinase-producing organisms at 30 days, were no longer apparent at 60 days, and populations were stimulated by fresh F1 forest soil volatiles after 120 days (Fig. 3a). Similar trends were seen for the P-F forest and nursery soils (Fig. 3b). 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 4a, b).

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 F1 forest or N1 nursery donor soils did not significantly influence receiver soil populations of actinomycetes at any assay date. Populations of extracellular chitinase-producing organisms were significantly decreased ( $P < 0.05$ ) by day 120 in soils receiving volatiles from P-F, seedling-planted F1 soil compared to fresh F1 soil not containing a seedling (Fig. 5a). Identical treatments of the N1 nursery soil resulted in no differences in volatile-mediated populations of chitinase-producing organisms (Fig. 5b). Facultative anaerobe populations in receiver soils were increased by volatiles from the P-F and seedling treatments of the F1 forest donor soil at the day 60 assay (Fig. 6a). This difference was no longer seen by

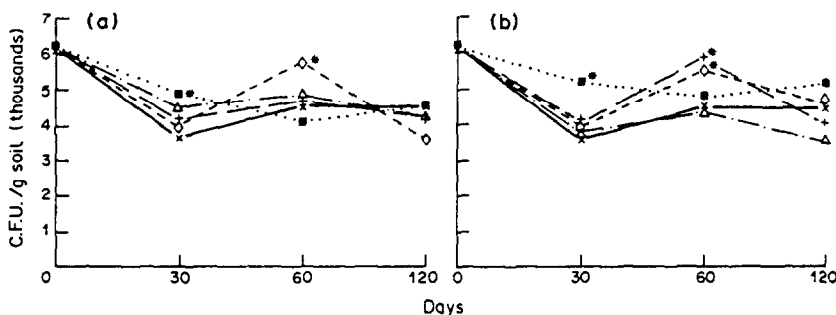


Fig. 4. Effect of volatiles from fresh (a) or pasteurized and fumigated (b) forest and nursery soils on the number of *Fusarium* propagules recovered from a receiver nursery soil. Values within an assay date followed by an asterisk are significantly different from the control,  $P < 0.05$  (Fisher's protected L.S.D. test). ■, F1; +, F2; ◇, N1; △, N2; ×, control.

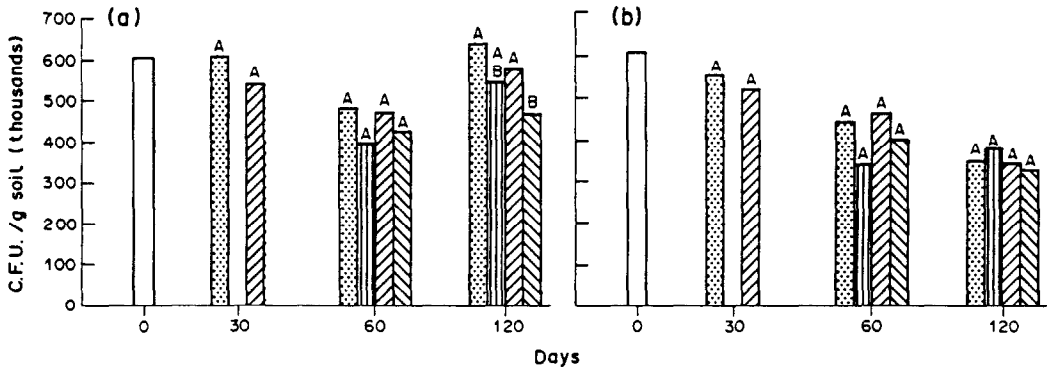


Fig. 5. Effect of volatiles from a fresh or pasteurized and fumigated forest (a) or nursery (b) soil, planted or not with a Douglas-fir seedling, on the numbers of extracellular-chitinase-producing microorganisms isolated from a receiver nursery soil. Values within an assay date not followed by the same letter are significantly different,  $P < 0.05$  (Fisher's protected L.S.D. test). □ Pre-experiment count, ▨ fresh F1, ▩ fresh F1 with seedling, ▧ P-F F1, ▦ P-F F1 with seedling.

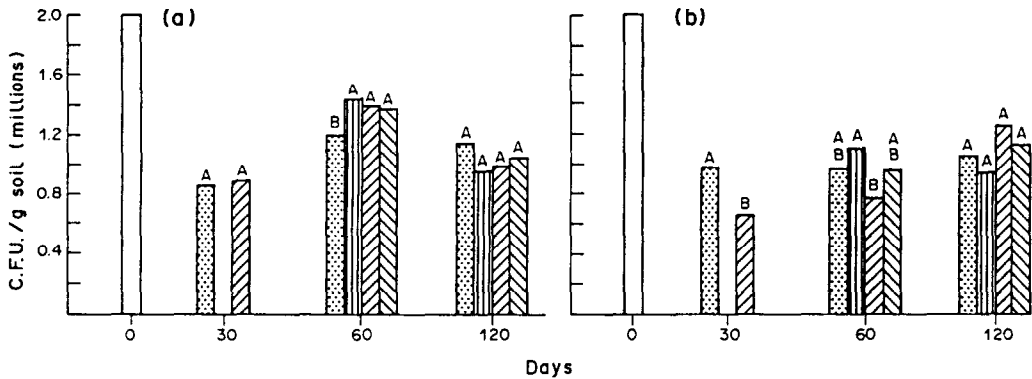


Fig. 6. Effect of volatiles from a fresh or pasteurized and fumigated forest (a) or nursery (b) soil, planted or not with a Douglas-fir seedling, on the numbers of facultative anaerobes isolated from a receiver nursery soil. Values within an assay date not followed by the same letter are significantly different,  $P < 0.05$  (Fisher's protected L.S.D. test). □ Pre-experiment count, ▨ fresh F1, ▩ fresh F1 with seedling, ▧ P-F F1, ▦ P-F F1 with seedling.

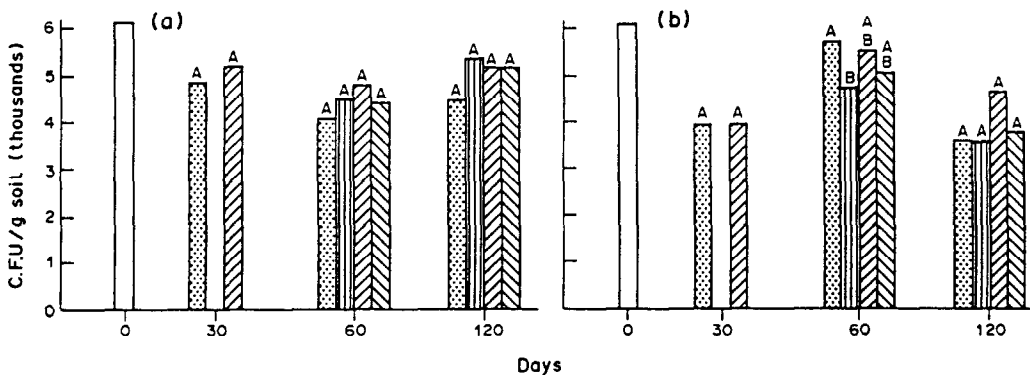


Fig. 7. Effect of volatiles from a fresh or pasteurized and fumigated forest (a) or nursery (b) soil, planted or not with a Douglas-fir seedling, on the number of *Fusarium* propagules recovered from a receiver nursery soil. Values within an assay date not followed by the same letter are significantly 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.

day 120. Similarly, an initial difference in the receiver soil population of facultative anaerobes exposed to volatiles from fresh vs P-F N1 nursery donor soil was only apparent during the first monitoring (Fig. 6b). Neither the P-F nor the seedling treatments of F1 donor soil resulted in volatile-mediated differences in the population of *Fusarium* in receiver soils (Fig. 7a). Conversely, volatiles from fresh N1 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. 7b), 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 F1 forest soil were mycorrhizal (average % short roots mycorrhizal =  $33 \pm 24$ ) 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 amounts 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 2-4). 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).  $\text{NH}_4^+$  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 ( $\text{NH}_3$ ) form (Schippers and Palm, 1973). 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 generally did not persist and were difficult to relate to *Fusarium* exclusion from native coniferous forest soils. Populations of *Fusarium* fluctuated considerably during the investigation 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 they 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, 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 volatile compounds from P-F donor soils differ from fresh soils in their influence on the receiver soil microbiota. 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 approx. 1/10 that of fresh soil were measured only 5 days after P-F treatment (D. A. Schisler, unpublished results). Our results may indicate only that the influence of volatiles from a qualitatively-changed donor soil microbiota on microbial populations 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*, remains undetermined.

Volatiles from soil planted with a Douglas-fir seedling inconsistently influenced receiver soil microbial populations. Though *in vitro* studies by Krupa and co-workers (Krupa and Fries, 1971; Krupa and Nyland, 1972; Krupa *et al.*, 1973) indicated that volatiles were produced by ectomycorrhizae that could be inhibitory to several root pathogenic fungi, and presumably could affect other microflora, *Fusarium* populations were never significantly altered. Perhaps our experimental conditions selected for ectomycorrhizal fungi which lacked the potential to influence *Fusarium* or were not representative of those active in native forest soils. Further, it seems probable that more time was needed for mycorrhizal establishment and for volatile-mediated decreases in soil *Fusarium* populations to occur, since it took several years for *Fusarium* populations in coniferous forest soils to decline in Smith's (1967) original study. It is also possible that concentrations of volatiles deleterious to *Fusarium* 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 *et al.*, 1986). Fluctuations in the effects of volatiles from seedling-planted donor soils on receiver soil

microbial populations could have resulted from temporal changes in the seedling volatile exudation pattern. 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 concentrations of soil volatiles *in vivo*. 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 *Fusarium* exclusion from coniferous forest soils is not readily determinable. *In vivo* studies using the VES and comparing the influence of volatiles from several Douglas-fir ectomycorrhizal fungi on populations of *Fusarium* and selected microbial groups are reported by Schisler and Linderman (1989).

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 added to or removed from experiments, allowing great flexibility in selecting 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.

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