Mechanisms Inhibiting Damping-off Pathogens of Slash Pine Seedlings with a Formulated Soil Amendment

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ABSTRACT

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Suppressiveness of soil amended with SF-21 mixture to *Rhizoctonia solani* and *Pythium aphanidermatum* was tested with a modified pine stem-segment colonization method and germ tube growth, respectively. Soil amended with 1% (w/w) SF-21 did not immediately inhibit hyphal growth and stem-segment colonization by *R. solani*, but 7 days after amendment it significantly inhibited the fungus. The population of other fungi increased significantly 4 days or more after application of SF-21. The density of this population was correlated with suppression of stem-segment colonization by *R. solani* in amended soil. Stem-segment colonization by *R. solani* increased 30–40% after amended soil was autoclaved, but the inhibitory effect returned 7 days after infestation with *Trichoderma harzianum* or *Penicillium oxalicum* or both. Infestations with *T. harzianum* also restored suppressiveness to autoclaved nonamended soil, whereas infestations of autoclaved nonamended soil with *P. oxalicum* did not. Increasing the population of *T. harzianum* or *P. oxalicum* or both in amended soil at day 0 to about the same level as that in amended soil at day 7 immediately rendered it suppressive to stem colonization by *R. solani*. In addition, amended soil at day 0 directly inhibited and lysed germ tubes of *P. aphanidermatum*, and at day 7 after amendment was even more effective. Autoclaved amended soil with or without added *T. harzianum* or *P. oxalicum* or both suppressed germ tube elongation by *P. aphanidermatum*. However, inhibition of germ tube elongation was partially nullified in amended soil mixed with benomyl at 50 μg/g soil after 7 days. Soil amended with SF-21 and incubated for 7 days produced inhibitory substances that diffused into a synthetic medium and inhibited hyphal growth of *R. solani* and *P. aphanidermatum* by 22 and 28%, respectively, compared with diffusates from nonamended soil. When the pH of amended soil was adjusted from 4.3 to 5.8, the suppressive effect on colonization by *R. solani* was completely nullified, but the soil remained partially inhibitory to germ tube elongation by *P. aphanidermatum*. Population density of *Trichoderma* spp. and *Penicillium* spp. in amended soils was negatively correlated ($r = -0.91$), with pH values from 4 to 6. The capacity of $Al_2(SO_4)_3$ to inhibit growth of *R. solani* and *P. aphanidermatum* in water agar was much greater at pH 4 than at pH 6. *P. aphanidermatum* was more sensitive to $Al_2(SO_4)_3$ at pH 4 than was *R. solani*. Sporangial formation by *P. aphanidermatum* was inhibited by water extracts of milled pine bark-water mixtures of 2:1–1:1, v/v, whereas extracts from 1:2–1:50, v/v, enhanced zoospore release. A 50 μg/ml alcohol extract from Virginia or shortleaf pine bark more strongly inhibited sporulation by *P. aphanidermatum* than did that from loblolly pine bark. We conclude that the inhibitory effects of SF-21 on specific pathogens differ. Effects on *R. solani* are indirect through proliferation of the microbial species, especially *Trichoderma harzianum* and *Penicillium oxalicum*. Effects on *P. aphanidermatum* are direct by the inorganic and organic components of SF-21 and indirect by reduced soil pH and stimulation of the microbial population.

Additional keywords: biocontrol, pine nursery.

Organic amendments are recommended for limiting pathogen populations by producing conditions unfavorable to pathogens in cultivated soil (15). Incorporation of farmyard manure, peat moss, pine sawdust, pine bark, and pine litter into nursery soil suppresses damping-off of pine seedlings (9,23,32). For hundreds of years it has been routine practice for farmers in mainland China to apply organic amendments to the soil. These components are considered fertilizers, but it was suggested recently that these organic amendments might be the reason for the general absence of important root diseases on agricultural crops in China (6). Soil amendments are considered by many plant pathologists to have promise for control of soilborne diseases without seriously polluting the soil with pesticides (26).

Organic amendments may affect soilborne fungi indirectly through their influence on the soil microflora (10). Papavizas and Davey (28) showed that populations of soil microorganisms, particularly actinomycetes, antagonistic to *Rhizoctonia solani* Kühn significantly increased during the suppression of Rhizoctonia disease of beans by organic amendment. Suppression of Pythium damping-off by amendment of container media with hardwood bark was attributed to increases in populations of mesophilic microorganisms, increased microbial activity, reduced concentrations of available nutrients, and a high degree of microstasis (3). SF-21, a soil amendment consisting of milled pine bark and several inorganic materials, was developed for controlling damping-off of slash pine seedlings (*Pinus elliottii* Engelm. var. *elliottii*) (14). SF-21 stimulates the fungus flora, especially *Trichoderma* spp. and *Penicillium* spp. (14), but the exact mechanisms associated with suppression of the pathogens have not been identified. Our objective was to investigate the characteristics and the mechanisms of suppression to *R. solani* and *Pythium aphanidermatum* (Edson) Fitz. in soil amended with SF-21.

MATERIALS AND METHODS

Soil and SF-21 mixture. Sandy soil (14) was sifted through a 2-mm-mesh screen and stored at 6% (w/w) moisture content in polyethylene bags. The SF-21 mixture (pH 2.8) (150 g of $Al_2(SO_4)_3$, 25 g of KCl, 30 g of $CaCl_2$, 10 g of triple superphosphate, 35 g of $(NH_4)_2SO_4$, 750 g of milled pine bark, and 750 ml of 10% glycerine) (14) was prepared by soaking milled pine bark in 10% glycerine solution for 1 hr and then mixing evenly with

other chemicals. The mixture was air-dried for 1 wk and stored in polyethylene bags. Amended soil contained 1% (w/w) SF-21 mixture.

Fungal cultures. The sources of isolates of *R. solani* (BB-08) AG-4, a binucleate *Rhizoctonia* sp. (BR) CAG-3, *P. aphanidermatum* (VW-06 and VWB-01), *Trichoderma harzianum* Rifai (TH-06), and *Pencillium oxalicum* Currie et Thom (PA-01) have been described (14). All isolates were maintained on potato-dextrose agar (PDA) or V-8 juice agar (V-8).

Bioassay. The pine stem-segment colonization method (13) was modified to estimate suppressiveness of amended soil to *R. solani* and BR. A 15-g soil sample was dispensed into a 5.5-cm-diameter petri dish to make a soil plate. Soil moisture was brought to 12% (w/w) with sterile deionized water. A 1-cm-diameter disk from a 5-day-old culture of *R. solani* or BR on 2% water agar (WA) was placed on the center of the soil plate. Ten pine stem segments were inserted vertically into the soil, approximately 1 cm from the agar disk, for baiting *R. solani* and BR. In each experiment four replicates of each soil plate were used to observe stem segment colonization by *R. solani* and BR on amended and nonamended soils. After incubation for 48 hr at 25 C, stem segments were recovered and washed for 3 min with 1% NaOCl, blotted with paper towels, and placed on WA containing 300 μg/ml streptomycin sulfate. After incubation at 25 C for 24 hr, stem segments with signs of *R. solani* and BR were counted under a microscope at 150×.

Germ tube length was used to determine suppressiveness of amended soil to *P. aphanidermatum*. Zoospores of *P. aphanidermatum* were produced by placing 10 1-cm-diameter disks from 5-day-old cultures on V-8 agar in a 15-cm-diameter petri dish containing 50 ml of sterilized distilled water at 25 C (14). After 18 hr, the water was carefully decanted and centrifuged at 1,500 *g* for 5 min to concentrate the zoospores. A 5-ml suspension of encysted zoospores was obtained from the bottom of the polyethylene centrifuge tube (10 cm high, 2.0 cm i.d.) and mixed thoroughly with 5 ml of 40% V-8 juice. The V-8 juice was prepared by filtering the diluted juice through Whatman No. 1 filter paper and adjusting the filtrate to pH 6.5 with 1 N NaOH. The encysted zoospore-V-8 juice suspension (0.7 ml) was spread evenly on the surface of a soil with 12.5% (w/w) water content in a 5.5-cm-diameter petri dish and incubated at 25 C. After 4 hr, 0.1 ml of water with germinating zoospores was withdrawn from the soil surface and stained with cotton blue on a glass slide. The total length of germ tubes from 50 zoospores was determined under a microscope at 300×. Four replicates were used in each experiment of the germ tube method. After a 6-hr incubation, average percent lysis of germ tubes was determined by estimating the amount of lysis in each germ tube (0, 20, 40, 60, 80, and 100%).

Population of fungal flora in soils. Soil suspensions were prepared by mixing 10 g of soil with 90 ml of sterile 0.1% water agar (Difco Laboratories, Detroit, MI). Serial dilutions (10^{-3}, 10^{-4}, 10^{-5}) were plated in quadruplicate on selective media. Peptone-dextrose-rose bengal agar (17) was used for determining population densities of fungal flora, and peptone-dextrose-rose bengal-PCNB agar (22) for *Trichoderma* and *Penicillium* spp.

Soil amendment regimes and suppression of the pathogens, incubation vs. heat. Amended or nonamended soil (100-g lots) adjusted to 12% (w/w) water content in 250-ml beakers were covered with aluminum foil and incubated for 0 or 7 days at 25 C. Then, half of these soils were sterilized in the autoclave (121 C) for 20 min. After cooling, soil plates from the various soil treatments were used to test growth and colonization of *R. solani* and the length and relative lysis of germ tubes of *P. aphanidermatum*. For measurement of the growth of *R. solani* on the soil surface, 1-cm-diameter disks from a 5-day-old culture on 2% WA were placed on the surface of soil plates (19). The three longest hyphae (mm) of *R. solani* on the soil surface of each soil plate were measured with a dissecting microscope after 20 hr. A randomized complete block design was used with four replicates of each soil plate per treatment.

Incubation time. A 200-g portion of amended or nonamended soil was placed in a 15-cm-diameter petri dish, brought to 12% water content, and incubated for 0, 2, 4, 6, or 8 days at 25 C. Amended and nonamended soils incubated for various times were used to determine stem-segment colonization by *R. solani* and germ tube length of *P. aphanidermatum* and to assay other fungal flora population densities. Petri dishes with soil were put in a completely randomized design with four replicates per incubation period in the growth chamber.

Microbial effect. Spore suspensions of *T. harzianum* and *P. oxalicum* were prepared by scraping conidia from 2-wk-old cultures on malt extract agar (20 g of malt extract [Difco], 20 g of glucose, 1 g of peptone [Difco], 20 g of agar, and 1,000 ml of water) in 200 ml of sterile distilled water. After vigorous stirring and filtration through two layers of cheesecloth, the filtrate was centrifuged (10,000 *g* for 10 min) and the pellet resuspended in sterile distilled water. Spore concentrations were determined with the aid of a hemacytometer. In a 250-ml beaker, 100-g lots of autoclaved amended soil, autoclaved nonamended soil, and nonamended soil were infested with a 10-ml spore suspension (2×10^7 spores per milliliter) of *T. harzianum* or *P. oxalicum* or both and incubated for 7 days at 25 C. Ten milliliters of sterilized deionized water was added to amended or nonamended soil, with or without autoclaving, as controls. The suppressiveness of these soils to *R. solani* and *P. aphanidermatum* was determined by the methods described previously.

***Trichoderma* and *Penicillium* as key factors.** Amended soil was infested with *T. harzianum* or *P. oxalicum* or both to increase the population densities of these fungi to 2×10^6 colony-forming units (cfu)/g dry soil (about the same population level as that in amended soil after 7-day incubation). Amended and nonamended soils without infestation were used as relative controls. Colonization by *R. solani* and BR in soils was tested immediately after infestation by modified stem-segment colonization method. A randomized complete block design was used with four replicates of each treatment for a pathogen.

Selective microbial inhibitors. Amended or nonamended soil samples (100 g) were mixed thoroughly with either 10 ml of distilled water containing 50 mg of streptomycin sulfate, 10 mg of benomyl (50WP, Dupont Chemical Corp., Wilmington, DE), or 25 mg of rose bengal, and put into 250-ml beakers. The beakers were covered with aluminum foil and incubated for 7 days at 25 C. Four subsamples from each treatment were used to make four soil plates in a completely randomized design for assessing germ tube length of *P. aphanidermatum*.

Diffusion of inhibitory substances from amended soils. After incubation at 25 C for 0 or 7 days, blocks ($2.5 \times 2.5 \times 0.8$ cm) of amended or nonamended soil with 12.5% (w/w) water content were put on Millipore filter paper (Millipore Corp., Bedford, MA) (0.2-μm pores) on a solidified synthetic medium (SM) (25) at pH 5 in 5.5-cm-diameter petri dishes. Filter paper without a soil block was used as a control. After incubation for 24 hr at 25 C, the soil block and filter paper were removed and a fungal disk (0.4-cm diameter) of *R. solani* or *P. aphanidermatum* from 5-day-old culture on SM was placed in the center of each dish. After 24 hr of growth at 25 C, average colony diameter was obtained from six dishes per fungus per treatment in a completely randomized design.

pH effect on pathogens. The pH values of amended and nonamended soils were 4.3 and 5.8, respectively. To determine if changes in soil pH can cause amended and nonamended soils to be either suppressive or conducive to the pathogens, the soil pH of amended and nonamended soils was adjusted. The amounts of 1 N NaOH or HCl needed to adjust the pH of each soil type to 4.3 and 5.8 was determined by mixing these soils with 1:1 (w/v) with deionized water and titrating to the desired pH. Soil plates from both amended or nonamended soil with pH of 4.3 and 5.8 after a 10-day incubation at 25 C were used for testing colonization of *R. solani* and germ tube length of *P. aphanidermatum*. A randomized complete block design was used with four replicates of each treatment for a pathogen.

pH effect on fungal competitors. Amended soil was adjusted with 1 N NaOH or HCl to pH 4.0, 4.5, 5.0, 5.5, and 6.0. After

a 10-day incubation at 25 C, the total population density of *Trichoderma* and *Penicillium* spp. was determined on peptone-dextrose-rose bengal-PCNB agar (22). A randomized complete block design was used with eight replicates of each pH value. Population densities were transformed to $\log_{10}$ before analysis.

$Al_2(SO_4)_3$ effect on the growth of the pathogens. Petri dishes of 2% WA with 0, 10, 100, 500, 1,000, or 1,500 μg/ml of $Al_2(SO_4)_3$ were prepared and adjusted to pH 4 or 6 with 1 N NaOH or HCl before solidification. Each concentration of $Al_2(SO_4)_3$ at pH 4 and 6 was prepared in quadruplicate for both *R. solani* and *P. aphanidermatum*. An agar disk (0.6 cm diameter) from the margin of a 5-day-old WA culture of a pathogen was placed in the center of each 9-cm-diameter dish. After 24 hr of growth at 25 C, average colony diameter was obtained from four dishes per fungus per treatment.

Effect of pine bark extracts on zoospore production. Milled pine bark (60% *P. taeda* and 40% *P. echinata* Mill. from Fernacre Farms, Washington, GA) that had been composted for more than 9 mo had a pH of 4.4 and a bulk density of 0.23 g/cm^3. Water-soluble extracts of bark were prepared by soaking 2,000 cm^3 of milled pine bark in 1 L of sterile deionized water for 1 hr at 25 C and filtering through two layers of sterile cheesecloth. Particles in the filtrate were removed by centrifugation at 4,000 *g* for 5 min (30), followed by filtration through sterile Whatman No. 1 filter paper and a sterile Millipore filter (0.45-μm pore size). This extract was designated a 100% concentration. Sterile deionized water was used to dilute the concentrate into 75, 50, 25, 10, 5, and 1% solutions. Sterile deionized water was the control.

Alcohol extracts of pine bark (supplied by Dr. F. A. Pokorny, University of Georgia) from Virginia pine (*P. virginiana* Mill.), shortleaf pine, and loblolly pine, respectively, were dissolved in 95% alcohol and then diluted with sterile deionized water into 100, 50, and 10 μg/ml solutions. Alcohol (95%) diluted in deionized water and deionized water alone were used as controls. Zoospore production by isolates VWB-01 and VW-06 of *P. aphanidermatum* was determined (14) in solutions of both water- and alcohol-soluble extracts.

Data analysis. All experiments were performed twice, with similar results. Data from the second trials are presented. Analyses of variance or regression analyses were run on the SAS/STAT System for Personal Computers (SAS Institute Inc., Cary, NC). Means were separated by the Student's *t* test or Duncan's multiple range test.

TABLE 1. Effect of various soil amendment regimes on hyphal growth and stem-segment colonization by *Rhizoctonia solani* (AG-4) and zoospore germ tube length and lysis of *Pythium aphanidermatum* at 25 C

Soil treatment[w]	*R. solani*[x]		*P. aphanidermatum*[y]	
	Hyphal growth (mm)	Stem-segment colonization (%)	Germ tube length (μm)	Germ tube lysis (%)
Amended with SF-21 incubated 7 days	2.6 c[z]	5 c	93 c	24 a
Amended with SF-21 incubated 7 days, then autoclaved	4.8 b	83 a	64 d	6 c
Amended with SF-21 incubated 0 days	5.9 ab	43 b	116 b	15 b
Amended with SF-21 incubated 0 days, then autoclaved	4.9 b	73 a	53 d	8 c
Nonamended incubated 7 days	7.3 a	38 b	190 a	16 b
Nonamended autoclaved	6.7 a	85 a	202 a	6 c

[w] Soil amended with 1% (w/w) SF-21, which consists of 150 g of $Al_2(SO_4)_3$, 25 g of KCl, 30 g of $CaCl_2$, 10 g of triple superphosphate, 35 g of $(NH_4)_2SO_4$, 750 g of milled pine bark, and 750 ml of 10% glycerine.

[x] Hyphal growth values were obtained from four replicates; in each replicate, after a 20-hr incubation, the three longest hyphae on the soil surface were measured and averaged. Ten stem segments were inserted into soil in each 5.5-cm-diameter petri dish, approximately 1 cm from an agar disk with *R. solani*, and incubated for 48 hr.

[y] Germ tube length and lysis of zoospores were measured 4 and 6 hr, respectively, after encysted zoospores were placed on soil surface.

[z] Means ($n = 4$) followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

RESULTS

Soil amendment and suppression of the pathogens, incubation vs. heat. Soil amended with 1% (w/w) SF-21 did not immediately inhibit hyphal growth and stem-segment colonization by *R. solani* (Table 1). Amended soil significantly inhibited *R. solani* 7 days after SF-21 application compared with nonamended soil. Autoclaving completely nullified suppression of *R. solani* by amended soil. In addition, amended soil (0 days) reduced germ tube length and caused germ tube lyses in *P. aphanidermatum* compared with nonamended soils, and was even more effective 7 days after treatment (Table 1). Autoclaving enhanced the suppressiveness of amended soil towards germ tube growth of *P. aphanidermatum*.

Incubation time. Inhibition of stem-segment colonization by *R. solani* was detected 2 days after amendment as compared with colonization in nonamended soil (Table 2). Moreover, it was even greater 6 and 8 days after treatment. The population density of microbial fungi was increased significantly in amended soil compared with nonamended soil after 2 days of incubation. Fungal counts in amended soils were dramatically higher after 4 days than after 0 or 2 days of incubation. After 4 days of incubation, stem-segment colonization by *R. solani* and growth of germ tubes of *P. aphanidermatum* were suppressed more than in soil assayed immediately after amendment.

Microbial effect. Incubation for 7 days of autoclaved amended soil infested with *T. harzianum* or *P. oxalicum* or both at 2×10^6

TABLE 2. Length of incubation of soil amended or nonamended with SF-21 mixture in relation to stem-segment colonization by *Rhizoctonia solani*, length of zoospore germ tube of *Pythium aphanidermatum*, and other fungal population densities[x]

Days after soil amendment	Stem segments colonized by *R. solani* (%)		Germ tube of *P. aphanidermatum* (μm)		Population of fungal flora[y] cfu/g of dry soil ($\times 10^4$)	
	Amended	Nonamended	Amended	Nonamended	Amended	Nonamended
0	35	43	98*[z]	205	5	3.8
2	23*	40	92*	199	10*	4.1
4	20*	43	83*	210	123*	3.7
6	10*	35	86*	213	116*	4.2
8	13*	38	79*	209	176*	4.0

[x] The relationship of incubation period (X) with stem segments colonized by *R. solani* (Y) was quadratic in amended soil ($Y = 34.7 - 6.1X + 0.4X^2$, $P < 0.05$); with population densities of other fungi, (Y) was linear in amended soil ($Y = -4 + 22.4X$, $P < 0.01$). Regressions of stem segments colonized by *R. solani* and population densities of other fungi in nonamended soil, or of germ tube length of *P. aphanidermatum* in both amended and nonamended soils were not significant ($P = 0.05$).

[y] Population densities of fungal flora were determined on peptone-dextrose-rose bengal agar.

[z] Means ($n = 4$) with an asterisk are significantly different between columns of amended and nonamended soils for stem-segment colonization, germ tube length, or fungal flora population for each date at $P = 0.05$ based on Student's *t* test.

cfu/g dry soil resulted in stem-segment colonization levels of 8–13% compared with 5% in the amended nonautoclaved soil (Table 3). Infestation with *T. harzianum* reduced stem-segment colonization in autoclaved nonamended soil more than in the autoclaved nonamended control, whereas *P. oxalicum* did not. Suppression of germ tube length of *P. aphanidermatum* was significantly reduced in amended soil, autoclaved amended soil infested with *T. harzianum* or *P. oxalicum* or both, and autoclaved amended soil compared with nonamended soils (Table 3). *T. harzianum* and *P. oxalicum* did not suppress *P. aphanidermatum* in nonamended soil or autoclaved nonamended soil.

TABLE 3. Ability of *Trichoderma harzianum* and *Penicillium oxalicum* to restore suppressiveness to *Rhizoctonia solani* and *Pythium aphanidermatum* in autoclaved amended soil, autoclaved nonamended soil, and nonamended soil

Treatment[x]	Stem segments colonized by *R. solani* (%)[y]	Germ tube length of *P. aphanidermatum* (μm)[y]
Soil amended with 1% SF-21 (control)	5 d[z]	96 e
Soil amended with 1% SF-21, autoclaved, then infested with		
None	65 a	69 f
T. harzianum (TH-06)	8 d	113 d
P. oxalicum (PA-01)	10 d	112 d
TH-06 & PA-01	13 d	99 de
Autoclaved nonamended soil infested with		
None	50 b	194 abc
T. harzianum (TH-06)	28 c	199 ab
P. oxalicum (PA-01)	50 b	192 abc
TH-06 & PA-01	30 c	205 a
Nonamended soil infested with		
None	35 c	182 c
T. harzianum (TH-06)	30 c	187 bc
P. oxalicum (PA-01)	30 c	192 abc
TH-06 & PA-01	28 c	186 bc

[x]Soils were or were not infested with *T. harzianum* and/or *P. oxalicum* at 2×10^6 cfu/g dry soil and then incubated for 7 days at 25 C.

[y]Ten stem segments were inserted into soil in each 5.5-cm-diameter petri dish approximately 1 cm from an agar disk with *R. solani* and incubated for 48 hr; germ tube length was measured 4 hr after encysted zoospores were placed on soil.

[z]Means ($n = 4$) within columns followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test.

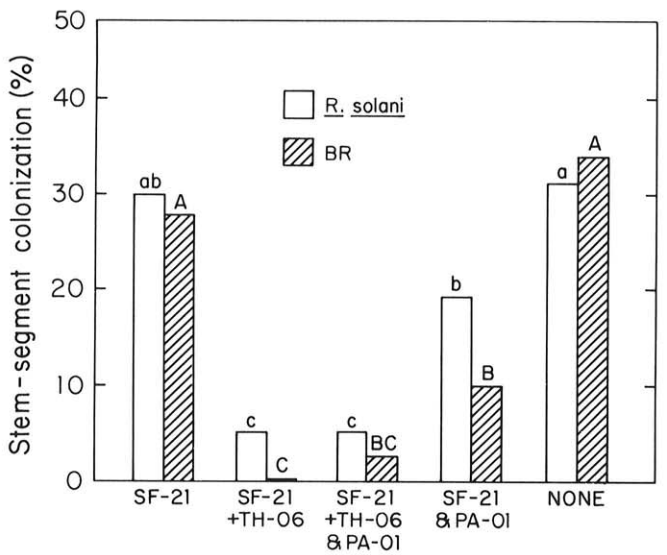

Fig. 1. Inhibition of colonization of pine stem-segments at day 0 by *Rhizoctonia solani* and a binucleate *Rhizoctonia* sp. (BR) after amendment of nonfumigated soil with SF-21 mixture infested by adding spores of *Trichoderma harzianum* (TH-06) or *Penicillium oxalicum* (PA-01) or both. Stems were incubated 48 hr in amended soil. Means ($n = 4$) (bars) for the same fungus pathogen with the same letter do not differ significantly; $P = 0.05$ according to Duncan's multiple range test.

Trichoderma and Penicillium as key factors. Addition of *T. harzianum* or *P. oxalicum* or both to the amended soil inhibited stem-segment colonization by *R. solani* and BR at day 0 (Fig. 1) equivalent to that observed for *R. solani* in amended soil with a 7-day incubation (Tables 1 and 3). *P. oxalicum* was less effective in reducing colonization at day 0 in amended soil.

Selective microbial inhibitors. Only benomyl affected germ tube growth of *P. aphanidermatum*. The length of germ tubes of *P. aphanidermatum* averaged 111 μm after 7 days of incubation of SF-21 amended soil with benomyl at 50 μg/g of soil compared with 85 μm for the control. Rose bengal at 250 μg/g and streptomycin sulfate at 500 μg/g were not effective in reducing suppressiveness of amended soils: the inhibitors did not reduce germ tube growth of *P. aphanidermatum* in nonamended soil at the concentrations tested (data not shown).

Diffusion of inhibitory substances from amended soils. The diffusates from amended soils into SM plates inhibited growth of *R. solani* and *P. aphanidermatum* more than did diffusates from nonamended soil (Table 4). Diffusates from amended soil at day 0 did not differ from diffusates from nonamended soil; both reduced growth of *P. aphanidermatum*, but not of *R. solani*,

TABLE 4. Effect of inhibitory substances diffused from soils amended with SF-21 mixture through Millipore filter paper on growth of *Rhizoctonia solani* and *Pythium aphanidermatum* on solidified synthetic medium (SM)[x]

Treatment[y]	Colony diameter (mm)[z]	
	R. solani	*P. aphanidermatum*
Soil amended with 1% SF-21 mixture incubated 7 days	26.3 ± 1.2 b	23.8 ± 2.0 c
Soil amended with 1% SF-21 mixture incubated 0 days	33.5 ± 1.0 a	31.7 ± 2.5 b
Soil without amendment (control)	33.7 ± 1.5 a	33.0 ± 2.6 b
Filter paper only (control)	33.5 ± 1.6 a	42.0 ± 2.4 a

[x]Solidified synthetic medium (25).

[y]Soil blocks (2.5 × 2.5 × 0.8 cm) were placed on Millipore filter paper (0.2-μm pore size) placed on SM in petri dishes (5.5-cm diameter) at 25 C for 24 hr. Then, soil blocks and the filter papers were removed and a fungal disk (0.4-cm diameter) of *R. solani* or *P. aphanidermatum* from 5-day-old cultures on SM was placed in the center of each plate. Colony diameter was determined 24 hr later.

[z]Means, ± standard deviations ($n = 6$), within columns followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test.

TABLE 5. Effect of pH of soils nonamended or amended with 1% SF-21 mixture on stem-segment colonization by *Rhizoctonia solani* and length of zoospore germ tubes of *Pythium aphanidermatum*

Soil treatment[w]	pH	Stem segments colonized by *R. solani*[x] (%)	Length of zoospore germ tube of *P. aphanidermatum*[y] (μm)
Amended	4.3	13 c[z]	80 d
Amended	5.8	55 a	115 c
Nonamended	4.3	45 ab	141 b
Nonamended	5.8	38 b	175 a

[w]After pH adjustment, amended and nonamended soils were remoistened to 12% (w/w) water content and incubated for 10 days at 25 C.

[x]Ten stem segments were inserted into soil in each 5.5-cm-diameter petri dish approximately 1 cm from an agar disk with 5-day-old *R. solani* and incubated for 48 hr.

[y]Fifty germ tubes were measured and averaged for each replicate 4 hr after encysted zoospores were placed on soil plates.

[z]Means ($n = 4$) within columns followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test.

more than the filter paper control.

Effect of soil pH and amendment on pathogens and microbial populations. Amended soil at pH 4.3 was more inhibitory to *R. solani* and *P. aphanidermatum* than amended soil at pH 5.8 (Table 5). In nonamended soils, colonization by *R. solani* was not significantly different at pH 4.3 and 5.8, but germ tube length of *P. aphanidermatum* was significantly reduced at pH 4.3 compared with pH 5.8. Amended soil at pH 4.3 was most inhibitory to both fungi.

pH effect on competitors. Soil pH affected the total amount of *Trichoderma* and *Penicillium* spp. in amended soil. There was a negative correlation ($r = -0.91$) in amended soil between pH 4 and 6 with the total population density of *Trichoderma* and *Penicillium* spp. (Fig. 2). When the pH of amended soil was adjusted to 5.5 or 6, *Geotrichum* spp. were more frequent than *Trichoderma* and *Penicillium* spp. (data not shown).

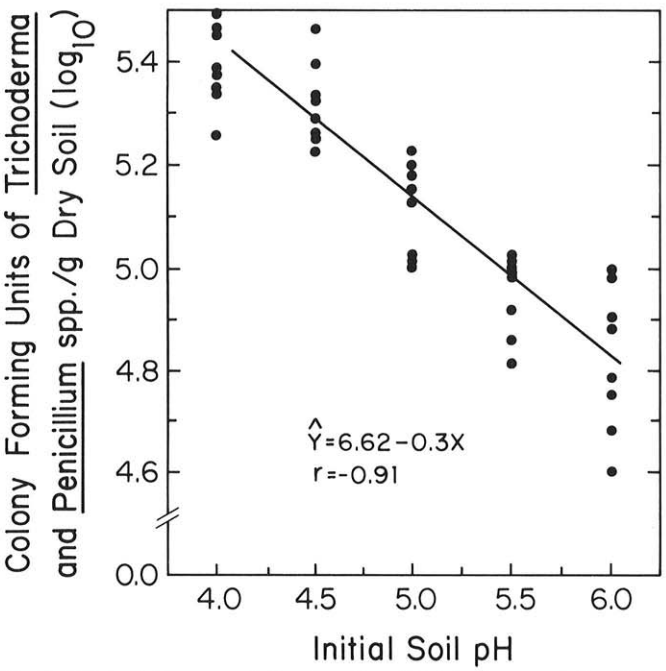

Fig. 2. Effect of pH on population densities of *Trichoderma* and *Pencillium* spp. in soils amended with SF-21 mixture after 10 days incubation at 25 C.

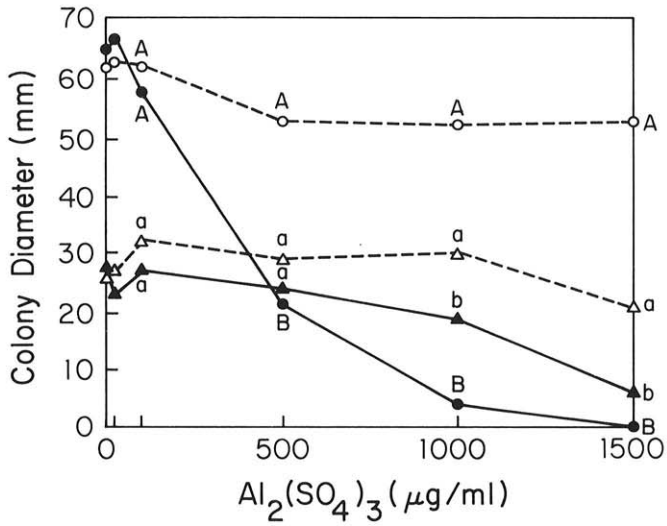

Fig. 3. Effect of different $Al_2(SO_4)_3$ concentrations on growth of *Rhizoctonia solani* (▲) and *Pythium aphanidermatum* (·) on 2% water agar at pH 4 (solid line) or pH 6 (broken line) after 24 hr at 25 C. Means ($n = 4$) within concentrations and fungus with the same letter do not differ significantly at $P = 0.05$ according to Student's t test.

Effect of $Al_2(SO_4)_3$ on pathogen growth. When 2% water agar was adjusted to pH 4, $Al_2(SO_4)_3$ at 500 and 1,000 μg/ml significantly suppressed hyphal growth of *P. aphanidermatum* and of *R. solani*, respectively, compared with growth at pH 6.0 (Fig. 3). *P. aphanidermatum* was more sensitive to $Al_2(SO_4)_3$ at pH 4 than was *R. solani*.

Effect of pine bark extracts on zoospore production. *Water extracts.* Zoospore formation by *P. aphanidermatum* was inhibited in pine bark extract at 50–100% (100% concentration

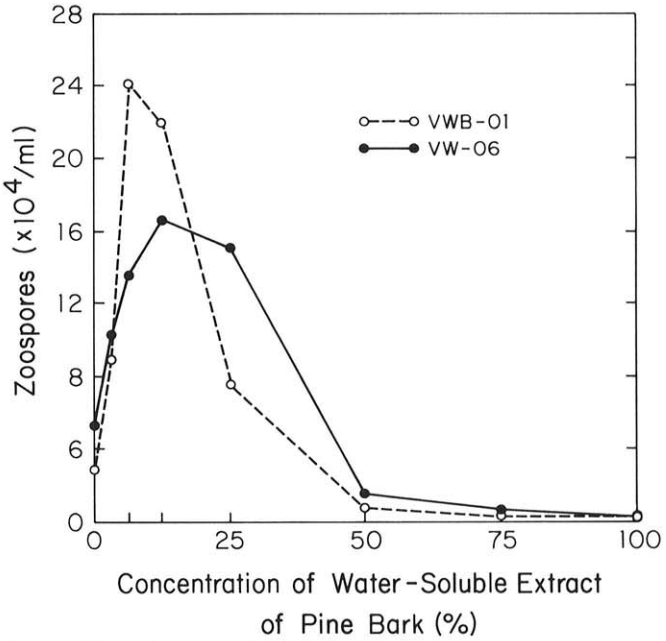

Fig. 4. Effect of pine bark water-extract concentrations on zoospore production by *Pythium aphanidermatum* (isolates VWB-01, VW-06). Values represent average of four replicates 22 hr after first flooding at 25 C. Extract was prepared by soaking 2,000 cm^3 of milled pine bark with 1 L of sterile deionized water for 1 hr (100% concentration).

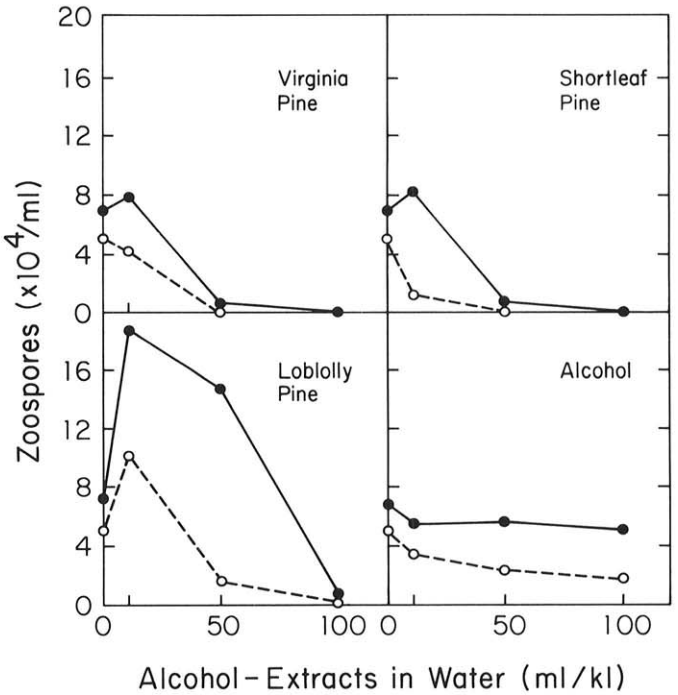

Fig. 5. Zoospore production by *Pythium aphanidermatum* (isolates VW-06 [∘----∘] VWB-01 [•—•]) in water with several concentrations of alcohol extracts from bark of three pine species. Alcohol (95%) diluted in deionized water and deionized water alone were used as controls. Values represent average of four replicates 22 hr after first flooding.

= 2:1, v/v = pine bark/water) (Fig. 4). In general, zoospore production was significantly reduced in extracts ≥50%, whereas extracts from 1–25% enhanced zoospore release.

Alcohol extractives. Alcohol soluble extracts from three pine species varied in suppressiveness to zoospore production by *P. aphanidermatum*. The 10 and 50 μg/ml alcohol extracts of loblolly pine in water stimulated zoospore production by *P. aphanidermatum*, isolate VW-06, whereas alcohol extracts of shortleaf and Virginia pine stimulated this isolate only at 10 ppm (Fig. 5).

DISCUSSION

Amendment of soil with SF-21 inhibited pine stem-segment colonization by *R. solani* and germ tube growth of *P. aphanidermatum* and, also, induced germ tube lysis of *P. aphanidermatum*. Four indications that the inhibitory effect of the SF-21 mixture-amended soil was associated with microbial activity are: 1) the inhibitory effect towards *R. solani* was nullified by sterilization of the amended soil; 2) inhibition was restored to autoclaved amended soil after infestation with *T. harzianum* and *P. oxalicum*; 3) the amended soil required an incubation period to inhibit *R. solani*, whereas incubation enhanced inhibition of *P. aphanidermatum*; and 4) the partially reduced suppression of amended soil to *P. aphanidermatum* by benomyl.

T. harzianum and *P. oxalicum* are the two predominant fungi in amended soil (14), and their hyperparasitic nature is well known (6,27). Boosalis (1) reported that *R. solani* added to unsterilized field soil could be parasitized by *Trichoderma* sp. and *P. vermiculatum*. In our study, *T. harzianum* and *P. oxalicum* restored suppressiveness to autoclaved amended soil (Table 3) and immediately established suppression of the amended soil to *R. solani* (Fig. 1). Apparently, these two fungi play a major role in the suppressiveness of amended soil to *R. solani*.

Autoclaved amended soil slowed germ tube growth of *P. aphanidermatum*, and we used microbial inhibitors to determine what group of microorganisms was involved. The addition of benomyl to the amended soil increased the length of germ tubes. Benomyl inhibits fungi but not *Pythium* spp., bacteria, or actinomycetes, whereas rose bengal and streptomycin inhibit bacteria and actinomycetes but not fungi (18). Benomyl strongly inhibits *Trichoderma* in culture, even at concentrations as low as 0.5 mg/L (27). In our study, green mold covered amended soils mixed with rose bengal and streptomycin after a 7-day incubation, suggesting that green molds, especially *Trichoderma* spp. and *Penicillium* spp., are involved in inhibition of germ tube growth of *P. aphanidermatum*. Although *T. harzianum* and *P. oxalicum* were associated with suppressiveness of the amended soil to *R. solani* and *P. aphanidermatum*, the microbiological factor alone was not sufficient to produce the inhibitory effect, because addition of *T. harzianum* and *P. oxalicum* in nonamended soil did not cause it to become suppressive (Table 3). Apparently, *T. harzianum* and *P. oxalicum* used some factors in SF-21 to maintain their growth and reproductive activity to exert suppressiveness, which supports the food base theory of effective biocontrol (26,27).

SF-21 is acidic and reduces soil pH. Soil pH primarily is determined by the base-exchange components of the soil. The solubility and availability to plants and microorganisms of many chemicals in soil is influenced strongly by pH (2). Low soil pH limits the development of damping-off of pine seedlings caused by *R. solani* and *Pythium* spp. and also contributes to suppression of these two pathogens (16). *Trichoderma* spp. are known to be strongly favored by acidic conditions (29). Chet and Baker (4) indicated that the antagonism by *Trichoderma* was more readily enhanced in acidic than in alkaline soils. Therefore, SF-21 helps to control damping-off of pine seedlings by lowering soil pH while aiding growth of pine seedlings, which favor low pH under proper fertilizer regimes in forest nursery soils (16). *Trichoderma* spp. are also abundant in forest nursery soils (8).

The optimum pH for growth of *Trichoderma* spp. ranges from 3.7 to 4.7 (7). Adjusting soil pH from alkaline to acid resulted in an increase in the population density of *Trichoderma* during radish monoculture to a level that suppressed *R. solani* (22). In our study, acidification to pH 4 of soil amended with SF-21 aided multiplication of *Trichoderma* spp. and *Penicillium* spp. compared with the soil at pH 6 (Fig. 2). Weindling (33) compared pH growth curves of *T. lignorum* and *R. solani* and found alkaline reactions were favorable to *Rhizoctonia* but unfavorable to *Trichoderma*. When pH was increased in the medium, *T. lignorum* gradually lost its ability to parasitize *Rhizoctonia*. Production of the antibiotic gliotoxin by *T. viride* (= *Gliocladium*?) was much higher from wheat straw in acidic soil than in alkaline soil or compost (35). Increased antibiotic production in soil may have a role in causing injury to *R. solani* (34). Chet and Baker (4,5) showed that *Trichoderma* spp. produce cellulase, β-(1-3)-glucanase, and chitinase, which degrade the glucans in the walls of *Pythium* spp. and the chitin and glucans in the walls of *R. solani*. In our study, we found that inhibitory substances could diffuse from soils amended with SF-21 to inhibit hyphal growth of *R. solani* and *P. aphanidermatum* (Table 4) and to lyse germ tubes of *P. aphanidermatum* (Table 1). These phenomena indicate that the inhibitory substances in amended soil may be related to exudates or metabolites of *Trichoderma* spp. and *Pencillium* spp., production of which are stimulated by SF-21.

Muchovej et al (24) indicated that manipulation of exchangeable soil aluminum was useful in the control of Phytophthora blight of pepper because Al^{+3} ion may retard the development of *P. capsici* in soil. Free Al^{+3} ion in soil, which is responsible for inhibition of *Neurospora tetrasperma* (20), is an abiotic factor that contributes to the suppression of *R. solani* in Onomea soil (21). In addition, increase in aluminum solubility and toxicity is followed by a decrease in pH (2). In our study, $Al_2(SO_4)_3$ was more toxic to *P. aphanidermatum* and *R. solani* at pH 4 than at pH 6 (Fig. 3).

After adjustment to pH 5.8, amended soil completely lost its inhibitory effect to *R. solani*, but it could still inhibit germ tube growth of *P. aphanidermatum* (Table 5). Also, milled pine bark, a major component of SF-21, inhibited *P. aphanidermatum*. Extracts of southern pine bark contain tannins, resins, waxes, saponins, starches, simple carbohydrates, and alkaloids (31). Hoitink and Kuter (11) indicated that the most toxic compounds purified from bark composts are ethyl esters of C_{18} hydroxylated organic acids. Therefore, in our study, the reduction of zoospore release of *P. aphanidermatum* in water-soluble extracts and alcohol extracts may be due to the presence of chemical compounds with fungicidal activity, such as reported for inhibition of *Phytophthora* spp. in composted bark (12,30).

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