Growth of Ectomycorrhizal and Nonmycorrhizal Shortleaf Pine Seedlings in Soil with Phytophthora cinnamomi

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Number 6 in a series of studies on the influence of ectomycorrhizal fungi on the resistance of pine roots to pathogenic infections.

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ABSTRACT

In a fiberglass-covered, air-filtered growth room, shortleaf pine seedlings (*Pinus echinata*) with ectomycorrhizae formed by *Pisolithus tinctorius* or *Cenococcum graniforme* grew as well in nonsterile soil with *Phytophthora cinnamomi* (20 propagules/g) as did nonmycorrhizal pine seedlings in nonsterile soil without the pathogen. Nonmycorrhizal seedlings grew well in the absence of the pathogen, but exhibited obvious symptoms (chlorosis, reduced top and root growth) of feeder root disease in soil with *P. cinnamomi*. Mature ectomycorrhizae were not infected by the pathogen, whereas significant numbers of nonmycorrhizal roots were infected. Ectomycorrhizal development was inversely related to the number of susceptible, nonmycorrhizal feeder roots available for attack by *P. cinnamomi*.

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Additional key words: biological control of feeder root disease, stabilizing fungal pathogen inoculum.

It has been demonstrated (5, 8, 9) that ectomycorrhizae formed by a variety of fungal symbionts on shortleaf (*Pinus echinata*) and loblolly (*P. taeda*) pine seedlings were resistant to infections by zoospores and vegetative mycelium of *Phytophthora cinnamomi*. Nonmycorrhizal lateral and short roots were heavily infected by both forms of the pathogen. On the basis of these results, the ecological significance of ectomycorrhizae was extended from a primarily physiological function benefiting plant growth to include their role as biological deterrents against pathogens which infect succulent feeder roots. These reports were concerned only with the resistance or susceptibility of individual roots to pathogenic infection, and not to the complete syndrome of feeder root disease. Before the biological control concept can be applied to the solution of any problem on feeder root disease, it must first be determined whether or not the presence of ectomycorrhizae on root systems of susceptible plants decreases the amount of susceptible root tissue and, consequently, causes a significant decrease in symptoms (i.e., reduction in foliar weight, stunted root system, chlorosis, etc.) common to feeder root disease.

Wingfield (14) worked with aseptic seedlings of loblolly pine and observed that ectomycorrhizae formed by *Pisolithus tinctorius* improved survival of seedlings growing with the root pathogen *Rhizoctonia solani*. Richard et al. (11) studied the interaction between the ectomycorrhizal fungus *Suillus granulatus* and the root pathogen *Mycelium radicis atrovirens* on aseptic seedlings of *Picea mariana*. Seedlings inoculated only with *Suillus granulatus* grew well, whereas those inoculated with the pathogen alone were chlorotic and severely stunted. Observations on the seedlings in the latter group revealed that the pathogen initially infected the root collar, and eventually was detected in the lateral roots, short roots, and root hairs. The pathogen was also detected in the cortex of the feeder roots, often penetrating to the endodermis. However, when *S. granulatus* was inoculated simultaneously with the pathogen, the seedlings did not become chlorotic or stunted. These seedlings grew as well as those with the mycorrhizal fungus alone.

The latter two reports are based on research carried out under germ-free conditions, and, accordingly, the application of the results to natural systems may be limited. The only available research carried out under nonsterile test conditions is that of Ross & Marx (12). In a greenhouse pot study, they found that seedlings of the Ocala race of sand pine (*Pinus clausa*) were protected against *P. cinnamomi* by ectomycorrhizae formed by *P. tinctorius*. Nonmycorrhizal pine seedlings infected by *P. cinnamomi* exhibited massive necrosis of the feeder roots, and only 40% survived after 2 months. Nonmycorrhizal roots on pine seedlings with ectomycorrhizae were also infected by the pathogen. Seventy percent of the mycorrhizal seedlings survived after 2 months. Cortical tissues in the mycorrhizal roots were free of *P. cinnamomi*, verifying their resistance to the pathogen. However, considering the high susceptibility of nonmycorrhizal roots of sand pine to *P. cinnamomi*, the low proportion (25%) of the feeder roots in an ectomycorrhizal condition was too small to define clearly the significance of ectomycorrhizae to control of feeder root disease.

The purpose of the present research, therefore, was to study the susceptibility or resistance of shortleaf pine (*Pinus echinata* Mill.) seedlings without and with ectomycorrhizae formed by *Pisolithus tinctorius* (Pers.) Coker & Couch and *Cenococcum*

graniforme (Sow.) Ferd. & Winge to feeder root disease caused by *Phytophthora cinnamomi* Rands under nonsterile soil conditions.

MATERIALS AND METHODS.—*Growth room for mycorrhizal synthesis.*—This study was conducted in a growth room (Fig. 1) which is a larger modification of a prototype previously reported (6). The purpose of the room is to grow plants without contamination by airborne basidiospores of ectomycorrhizal fungi. It was constructed of 170-g (6-oz) clear fiberglass attached to extruded 7.5-cm (3-in) steel beams on approximately 3.3-m (4-ft) centers. The fiberglass was attached on both the inside and the outside of the steel beams with a resilient rubber mastic and rivets. Doors, exhaust fans, electrical outlets, and a thermistor-based telethermometer were installed as in the prototype, as were the arrangement of the pre-entry and the main growth rooms.

The room was continuously air-conditioned, and the air was cleaned with three electronic air cleaners installed in the return air ductwork. The growth room and its contents (concrete mixer, benches, water hoses, pots, saucers, flats, labels, etc.) were sterilized with formaldehyde gas after the entrance door and outside exhaust fan were sealed with polyethylene plastic. Paraformaldehyde powder (720 g) was depolymerized to formaldehyde gas in an electric frying pan heated to 215 C. The quantity of paraformaldehyde was equal to ca. 0.01 g/m^3 (0.3 g/ft^3) of air, as recommended by Taylor et al. (13). Prior to sterilization, 24 paper strips (8 mm X 2.5 cm) impregnated with endospores of *Bacillus subtilis* (*globigii*) in paper envelopes (Spordex, American Sterilizer Co., Erie, Pa.) were placed at various locations in the growth room to check sterility. After 16-hr contact time, the gas was exhausted through the fan system. The bacterial spore strips were removed and cultured in nutrient broth for 72 hr at 30 C. All exposed spore strips were negative for growth of *B. subtilis*, indicating that the gas sterilization was successful.

All personnel followed the procedures for entry into the growth room as described earlier (6). All soil mixtures used in the room were steam-treated 3 times on alternate days for 6 hr each at 80 C in 10-liter porcelain buckets covered with aluminum foil. The buckets of soil mixture were brought directly from the steamer and put in the growth room without cooling to eliminate possible microbial contamination.

Light intensity in the main growth room was approximately 80% of that measured inside the greenhouse, or ca. 7,000 ft-c (76 Klux).

Stabilization, detection, and standardization of Phytophthora cinnamomi in artificially infested soil.—Several pilot studies were undertaken to devise methods of standardizing inoculum of *P. cinnamomi* in soil. Inoculum of the pathogen was grown for 30 to 40 days at 25 C (6) in 2-liter jars containing 1.5 liters of grade 4 vermiculite and finely divided peat moss moistened with 500 ml of modified Melin-Norkrans' (4) liquid medium. Microscopic

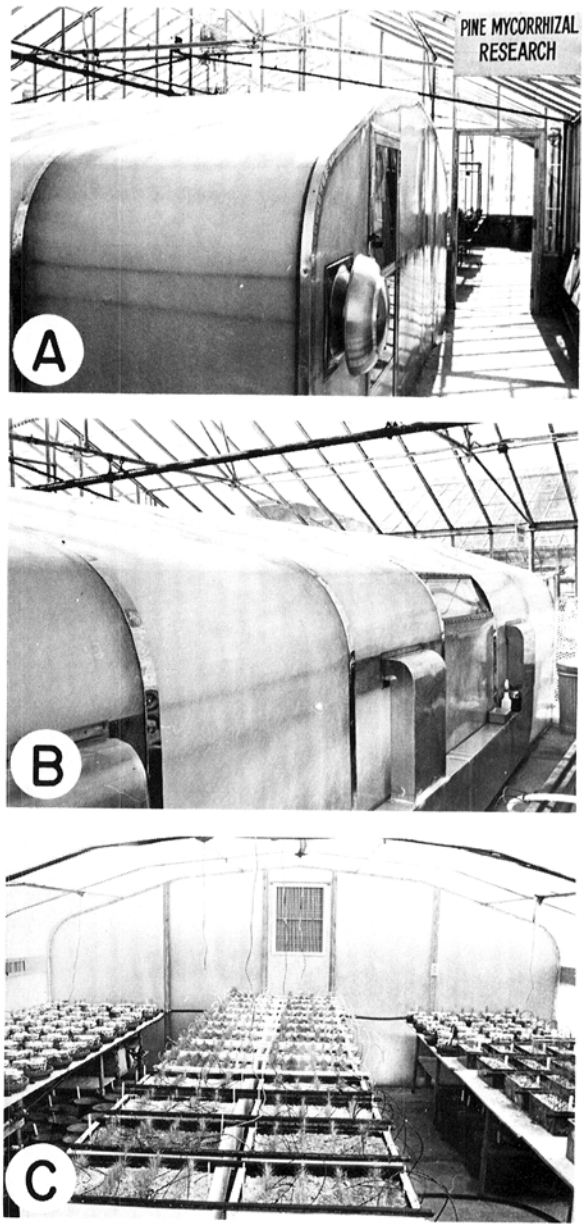

Fig. 1. Front (A), side (B), and inside (C) view of the growth room. Note door and exhaust fan for transference of air pressure in (A), supply air vents and viewport in (B), and bench arrangement and location of telethermometer temperature probes hanging from ceiling in (C).

examination of the inoculum revealed an abundance of chlamydospores and vesiculated hyphae of *P. cinnamomi* interlaced in particles of vermiculite and peat moss. In certain tests, we leached the inoculum to remove unassimilated nutrients by adding and decanting 1-liter volumes of cool tap water 4 or 5 times from each jar.

The soil mixture used in these studies was a 2:1:1 volume ratio of sandy loam, grade 4 vermiculite, and peat moss. The soil and vermiculite were

steam-treated as previously described, and the peat moss was autoclaved separately, then added to the other components. Steam-treating peat moss with the other components released toxins inhibitory to microorganisms. The resulting mixture (pH 5.3) contained ca. 15 μg/g phosphorus, 40 μg/g potassium, 90 μg/g calcium, and 80 μg/g magnesium (Soil Testing Laboratory, Cooperative Extension Service, University of Georgia, Athens). The soil mixture and the inoculum were placed in a concrete mixer with a capacity of 0.16 m^3. The mixer was loaded with sufficient soil mixture to fill ten 20-cm clay pots plus 1.5 liters of inoculum of *P. cinnamomi*. It was necessary to run the mixer for 15 min in order for proper blending of inoculum with the soil mixture. Jars of identical substrate and nutrients, but without *P. cinnamomi*, served as control inocula. Test inoculum-soil mixtures were placed in 20-cm clay pots on greenhouse benches and watered twice weekly. Soil temperatures in the greenhouse ranged between 18 and 30 C.

Modified Kerr's medium (2) and the medium recently developed by Flowers & Hendrix (1) for the selective isolation of *Phytophthora* and *Pythium* spp. from soil were used in these studies. Soil samples were removed from three locations in each pot and mixed thoroughly. Twenty-five g of test soil were placed in 100 ml of 0.3% water agar in a 200-ml beaker. The soil suspension was mixed vigorously on a magnetic stirrer for 20 sec, and ca. 1 ml was removed and placed on an agar plate with a small dipper. The slurry was spread evenly over the surface of the agar. Ten replicate plates/soil sample were used. With the dipper procedure, 10 subsamples/soil sample could be plated, whereas only one subsample could be obtained from each sample using a 10-ml pipette. Ten 1-ml subsamples/sample were also placed in a tared petri dish lid, air-dried for 48 hr, and weighed. These soil weights were used to convert the colony counts obtained from the agar plates to propagules of *P. cinnamomi*/g of air-dried soil.

Nonsterile leachate of forest soil (450 cm^3 of forest clay soil mixed thoroughly in 10 liters of distilled water and incubated at room temperature for 1 month) was added to artificially infested soil in certain tests to create a nonsterile soil system comparable to natural soil, and also to aid in stabilization of *P. cinnamomi*. Autoclaved soil leachates were used as a comparison. Fifty-ml volumes of leachate were added to the clay pots.

Growth of pine seedlings in soil infested with Phytophthora cinnamomi.—Shortleaf pine seed were surface-sterilized in H_2O_2, planted in flats (12.5 X 30 X 7.5 cm deep) of steamed soil mixture, as previously described, and grown in the fiberglass growth room. Each flat was fertilized with 100 ml of Melin-Norkrans' (MN) salt solution (6). After 7 weeks, seedlings which had attained approximately the same size were removed from the flats and planted in plastic pots (5 X 5 X 10 cm deep) containing inoculum of *P. tinctorius, C. graniforme*, or control medium at a 1:3 volume ratio with the steamed soil mixture. Inoculum of the symbionts or control medium was prepared and mixed with steamed soil as in the previously described technique with *P. cinnamomi*, except that the inocula of the symbionts were grown for 6 months and not leached to remove unassimilated nutrients. Twenty-five ml of MN salts were added to each plastic pot 3 weeks after planting, and the pots were watered twice weekly.

After 8 weeks, eight pine seedlings from the nonmycorrhizal and eight from each of the ectomycorrhizal treatments were randomly selected and growth measurements recorded. The remaining seedlings were used for transplanting into soil with or without *P. cinnamomi*.

Inoculum of *P. cinnamomi* was leached and stabilized with nonsterile soil leachate for 40 days in individual 20-cm clay pots in the growth room as previously described. Modified Kerr's medium (2) was used to detect *P. cinnamomi* in the test soil. In order to obtain similar inoculum densities of *P. cinnamomi* in all pots, the contents of some pots with different densities of the pathogen were mixed with those of other pots. For example, the contents of a pot having 15 propagules/g of the pathogen were mixed with those of another pot having 25 propagules/g to obtain a mixture of ca. 20 propagules/g. Inoculum density of *P. cinnamomi* was stabilized at 20 ± 3 propagules/g of air-dried soil/pot. This figure is an average of three separate assays for the pathogen from each pot; the pots were sampled between 30 and 40 days after adding the inoculum to the soil mixture. Most propagules of *P. cinnamomi* observed on the agar medium were chlamydospores and vesiculated hyphae. Control soil without *P. cinnamomi* was prepared as previously described.

Nonmycorrhizal and ectomycorrhizal pine seedlings in the small plastic pots were transplanted singly into soil either infested or noninfested with *P. cinnamomi*. Prior to transplanting, we washed the soil from the roots of the test seedlings. The experimental design of the study consisted of shortleaf pine seedlings planted in soil with or without *P. cinnamomi*, and developing without ectomycorrhizae (nonmycorrhizal) or with ectomycorrhizae formed by *Pisolithus tinctorius* or *Cencoccum graniforme*. Each of the six treatments was replicated 8 times. The pots were randomized monthly on a bench in the growth room and watered twice weekly. Soil temperatures in the growth room ranged between 24 and 28 C. Mean day-length was 14 hr (range 13 to 16 hr).

After 4 months, the pine seedlings were removed from the pots and the soil was assayed for *P. cinnamomi*. Seedlings were measured for height, foliar-stem dry weight (after drying for 48 hr at 90 C), root dry weight, number of lateral roots, and percent of feeder roots that were ectomycorrhizal. The percentage of ectomycorrhizal feeder roots was determined as previously described (7). Analyses of variance were made on all data, and significant differences were evaluated with Duncan's multiple range test at the 99% confidence limit. Six lateral root segments, each with several nonmycorrhizal short roots or ectomycorrhizae and three lateral root tips, were removed from each seedling and prepared

TABLE 1. Recovery of *Phytophthora cinnamomi* from artificially infested soil

	Propagules recovered/g air-dried soil							
	Medium 1[a]				Medium 2[b]			
	Nonsterile soil leachate[c]		Autoclaved soil leachate[d]		Nonsterile soil leachate		Autoclaved soil leachate	
Days of incubation	Leached inoculum[e]	Nonleached inoculum[f]	Leached inoculum	Nonleached inoculum	Leached inoculum	Nonleached inoculum	Leached inoculum	Nonleached inoculum
0	18	27	16	22	36	37	33	46
3	22	34	27	35	47	48	54	77
9	34	42	62	71	72	<150	79	<150
15	47	37	52	84	78	<150	84	<150
20	51	24	59	77	88	<150	97	<150
30	38	10	39	31	58	92	63	104
40	35	7	33	22	51	47	54	74

[a] Modified Kerr's medium.
[b] Flowers & Hendrix's medium (1).
[c] Leachate of forest soil (450 cm^3 in 10 liters water) containing a variety of microorganisms.
[d] Same as [c] except autoclaved.
[e] Inoculum of *P. cinnamomi* leached with water to remove unassimilated nutrients.
[f] Nonleached inoculum of *P. cinnamomi.*

for histological examination (8). Representative soil samples were analyzed and averaged 26 μg/g phosphorus, 63 μg/g potassium, 178 μg/g calcium, and 122 μg/g magnesium. Soil reaction averaged pH 5.3.

RESULTS.–Table 1 shows the effect of nonsterile and autoclaved soil leachate on leached and nonleached inoculum of *P. cinnamomi* at different periods of time. Apparently, the microorganisms in the nonsterile soil leachate stabilized the inoculum of the pathogen more rapidly than did the autoclaved soil leachate. Leaching the inoculum also reduced the amount of *P. cinnamomi*, especially in the nonsterile system. Either the leaching removed propagules of *P. cinnamomi* directly from the jars of inoculum or the absence of the unassimilated nutrients removed by leaching prevented the pathogen from growing vegetatively in the soil.

Stabilization of pathogen inoculum is important if test results are to be correctly interpreted. If a study were initiated immediately after mixing inoculum with the soil, and if *P. cinnamomi* increased rapidly in density 3 or 4 times, as is the case in Table 1, then the increase in inoculum levels could be inaccurately construed as due to a test variable.

It is apparent from Table 1 that the medium developed by Flowers & Hendrix (1) was more effective in recovering *P. cinnamomi* from soil than was the modified Kerr's medium (2). Less variation in colony counts within treatments, however, was experienced with the latter medium.

At the time of transplanting the pine seedlings into test soil, *Pisolithus tinctorius* formed ectomycorrhizae on 55% of the feeder roots and *Cenococcum graniforme* on 48%. Seedlings grown in control soil did not have ectomycorrhizae, but ca. 15% of the feeder roots were dichotomously branched. Significant growth differences between seedlings with or without ectomycorrhizae were not detected at this time (Table 2).

Ectomycorrhizae formed by *P. tinctorius* were typically yellow-gold and complex coralloid (8). Ectomycorrhizae formed by *C. graniforme* were jet-black, and either pinnate or bifurcate. The white and brown types of ectomycorrhizae reported by Park (10) to be formed by *C. graniforme* on seedlings of *Tilia americana* were not observed on these pine seedlings.

Nonmycorrhizal seedlings of shortleaf pine grown with *P. cinnamomi* were chlorotic, and had significantly less foliar-stem and root dry weights and fewer lateral roots than did corresponding seedlings grown in soil without the pathogen (Table 2). Differences in height growth were not significant. During the 4-month exposure to *P. cinnamomi*, the weights of nonmycorrhizal seedlings increased in comparison with their weights at transplant time. However, the number of lateral roots/seedling did not change significantly between these two periods, indicating damage by *P. cinnamomi*. The inoculum density of *P. cinnamomi* did not change significantly in soil with nonmycorrhizal seedlings.

Seedlings ectomycorrhizal with either *P. tinctorius* or *C. graniforme* were not chlorotic, and grew as well in soil with *P. cinnamomi* as they did in soil without the pathogen (Table 2). Ectomycorrhizal development by both fungal symbionts during the 4-month test period increased by over 50%. Inoculum density of *P. cinnamomi* decreased ca. 35 to 40% in both ectomycorrhizal treatments. Mycorrhizal seedlings were larger than the nonmycorrhizal seedlings with or without *P. cinnamomi* in all weight comparisons. Ectomycorrhizal seedlings in the presence or absence of *P. cinnamomi* and nonmycorrhizal seedlings in the absence of the pathogen developed comparable numbers of lateral roots during the 4-month test.

TABLE 2. Growth of shortleaf pine seedlings with and without ectomycorrhizae in the presence and absence of *Phytophthora cinnamomi*

Test condition	Height, cm	Foliar-stem dry wt, mg	Root dry wt, mg	No. lateral roots	% Ectomycorrhizae	Propagules/g of *P. cinnamomi*
			Seedlings ectomycorrhizal with Pisolithus tinctorius			
Transplant time	6.2 a,1[a]	75 a,1	48 a,1	11 a,1	55 a,1	19 a,1
After 4 months with *P. cinnamomi*	7.8 b,1	203 b,1	134 b,1	21 b,1	90 b,1	11 b,1
After 4 months without *P. cinnamomi*	7.4 b,1	185 b,1	131 b,1	23 b,1	86 b,1	
			Seedlings ectomycorrhizal with Cenococcum graniforme			
Transplant time	6.0 a,1	80 a,1	56 a,1	13 a,1	48 a,1	20 a,1
After 4 months with *P. cinnamomi*	6.5 a,2	126 b,2	141 b,1	17 b,1	76 b,2	13 b,1
After 4 months without *P. cinnamomi*	6.4 a,2	115 b,2	137 b,1	19 b,1	70 b,2	
			Nonmycorrhizal seedlings			
Transplant time	5.4 a,1	62 a,1	51 a,1	10 a,1		21 a,1
After 4 months with *P. cinnamomi*	5.0 a,3	74 b,3	86 b,2	9 a,2		19 a,2
After 4 months without *P. cinnamomi*	5.5 a,3	99 c,3	114 c,2	23 b,1		

[a] For seedlings in the same ectomycorrhizal condition but different test conditions, means with a common letter (a, b, or c) are not significantly different ($P \leq .01$). Means with a common number (1, 2, or 3) are not significantly different for seedlings in different ectomycorrhizal conditions but the same test conditions.

Intracellular hyphae of *P. cinnamomi* were present in meristematic and cortical tissues of ca. 65% of the nonmycorrhizal lateral and short roots examined from either nonmycorrhizal or ectomycorrhizal seedlings. Infection by *P. cinnamomi* was observed in only 6 of 486 ectomycorrhizae examined. These infections, as were those previously reported (5, 8, 9), occurred at the meristem of ectomycorrhizae formed by *C. graniforme*, and did not progress in cortex tissues surrounded by the Hartig-net. Thickness of the fungus mantle of ectomycorrhizae formed by *P. tinctorius* averaged 58 μ, and the Hartig-net developed to the endodermis. Thickness of the fungus mantle of ectomycorrhizae formed by *C. graniforme* averaged 34 μ, and the Hartig-net penetrated at least two cortical cell layers deep.

DISCUSSION.—These results showed that ectomycorrhizal seedlings of shortleaf pine did not exhibit symptoms (i.e., chlorosis, reduced foliar-stem and root weights, and a few lateral roots) of feeder root disease caused by *P. cinnamomi* as did nonmycorrhizal seedlings. Even though short roots and tips of nonmycorrhizal lateral roots on ectomycorrhizal seedlings were infected by the pathogen, the amounts of available root tissue susceptible to infection were too low (between 10 and 24%) in comparison with those of the resistant ectomycorrhizae (between 76 and 90%) to account for sufficient infection by *P. cinnamomi* to cause disease symptoms. In other words, because 76 to 90% of the feeder roots were in the resistant ectomycorrhizal condition, the only available tissue susceptible to attack was the few remaining nonmycorrhizal roots, and the consequence of these infections was not measurable.

The significant reduction in inoculum density of *P. cinnamomi* in both ectomycorrhizal treatments, but not in the nonmycorrhizal treatment, requires explanation. Propagules of the pathogen were apparently stimulated to germinate by root exudates from nonmycorrhizal roots, and either zoospores or vegetative mycelia were able to establish infection. Subsequent to infection of nonmycorrhizal roots, more propagules of the pathogen were synthesized, thereby preventing depletion of inoculum density. However, propagules in the sphere of influence of ectomycorrhizae may have germinated but failed to synthesize new propagules because significant root infection did not occur. Propagules may have been killed directly on the root surface by volatile antibiotics, terpenes, and sequiterpenes produced by ectomycorrhizae (3), or perhaps by antagonistic rhizosphere microorganisms. Any of these possibilities would account for the depletion of inoculum density of *P. cinnamomi*. Because neither *P. tinctorius* nor *C. graniforme* consistently produces nonvolatile antibiotics inhibitory to *P. cinnamomi* (4, 9), this mechanism for the resistance of ectomycorrhizae cannot be used to explain these results.

This research supports earlier conclusions that ectomycorrhizae, via their resistance to pathogen infections, serve not only as physiological benefits to plant health but also as biological deterrents to organisms causing feeder root disease. Any research aimed at investigating root disease, and in particular feeder root disease, should take into consideration the value of ectomycorrhizae in controlling root infection and subsequent expression of the symptoms of feeder root disease.

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