Infection of Ectomycorrhizal and Nonmycorrhizal Roots of Shortleaf Pine by Nematodes and Phytophthora cinnamomi

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ABSTRACT

Ectomycorrhizae aseptically synthesized by *Pisolithus tinctorius* and *Thelephora terrestris* and nonmycorrhizal feeder roots on intact seedlings of *Pinus echinata* were placed in individual glass cylinders and inoculated with *Tylenchorhynchus claytoni* or *Helicotylenchus dihystera* and zoospores of *Phytophthora cinnamomi*. After 8 days of incubation, both *T. claytoni* and *H. dihystera* penetrated and migrated through the fungus mantle and Hartig net of ectomycorrhizae formed by each symbiont. Vascular tissues of ectomycorrhizae formed by both fungal symbionts were invaded by *T. claytoni*. *H. dihystera* disrupted the structural integrity of the fungus mantles of ectomycorrhizae, thereby creating infection courts for *P. cinnamomi*. Intracellular hyphae and vesicles of *P. cinnamomi* were found in cortex cells surrounded by the Hartig net of ectomycorrhizae parasitized by *H. dihystera*. *P. cinnamomi* did not infect ectomycorrhizae penetrated by *T. claytoni*. Results indicate that nematodes are able to modify resistance afforded pine hosts by fungus symbionts against *P. cinnamomi* attack.

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Additional key words: biological control, feeder root disease, disease complexes.

The fungus mantles of ectomycorrhizae of shortleaf pine (*Pinus echinata* Mill.) act as mechanical barriers which protect feeder root tissues against infection by *Phytophthora cinnamomi* Rands (7, 9, 10, 11). In addition, the Hartig net surrounding the cortical cell may protect these cells, either mechanically or chemically, from pathogenic fungi. Certain ectomycorrhizal fungi in pure culture, and in ectomycorrhizal association, produce antibiotics which inhibit growth of many fungal root pathogens, including *P. cinnamomi*. (8).

Plant parasitic nematodes are associated with the feeder roots of pines in natural stands (13), pine plantations (16), and seedling nurseries (2). Spiral nematodes, especially *Helicotylenchus dihystera* (Cobb) Sher are commonly found in soil around the roots of shortleaf pines showing symptoms of littleleaf disease (13). Hopper (2, 3) observed certain stunt nematodes particularly *Tylenchorhynchus claytoni* Steiner, around and in the roots of injured pine seedlings. Ruehle (14) reported that *H. dihystera* and *T. claytoni* are parasitic on the roots of shortleaf pine seedlings.

Spiral and stunt nematodes are usually classed as ectoparasites, but they may also feed on the roots of a few hosts as endoparasites (5). Although these nematodes feed and reproduce on pine seedlings, their mode of feeding on feeder roots of pine, and especially on ectomycorrhizae, has not been reported. Feeder roots of shortleaf pine are ectomycorrhizal under natural field conditions. Since the studies concerning root protection by ectomycorrhizae against root pathogens were

conducted in nematode-free environments, the effect of nematodes on ectomycorrhizae and the ability of ectomycorrhizae to protect the root cells against fungal pathogens following nematode parasitism has not been investigated. An endoparasitic nematode, *Hoplolaimus galeatus* Cobb, readily parasitized ectomycorrhizae of both loblolly and shortleaf pines in greenhouse tests (15). The susceptibility of these parasitized ectomycorrhizae to fungal pathogens was not investigated.

The purpose of the present study was to determine the manner of feeding by spiral (*H. dihystera*) and stunt (*T. claytoni*) nematodes on ectomycorrhizae of shortleaf pine, and to determine whether the feeding of these nematodes modifies the resistance of ectomycorrhizae to attack by zoospores of *P. cinnamomi*.

Pisolithus tinctorius (Pers.) Coker and Couch (isolate 49) and *Thelephora terrestris* (Ehrh.) Fr. (isolate 2) were selected as test fungal symbionts because they have a wide pine host range and are important fungal symbionts of southern pines, In pure culture, neither fungus produces antibiotics inhibitory to the growth of *P. cinnamomi*. Antibiotic production by fungal symbionts would confound the results of studies dealing with fungus-nematode complexes. *P. cinnamomi* was used as the fungal pathogen because it readily infects feeder roots of shortleaf pine and has been used in previous studies of resistance of ectomycorrhizae to pathogenic infections (1, 7, 10, 11).

MATERIALS AND METHODS.—The research techniques have been described in detail in previous papers (7, 10, 11). Shortleaf pine seeds were germinated aseptically and planted in 2-liter glass jars containing vermiculite and peat moss moistened with Melin-Norkrans' nutrient solution (9). The jars were placed in a water bath maintained at 23 C in a greenhouse. The fungal symbionts were grown for 30 days at 25 C in petri plates containing modified Melin-Norkrans' agar medium (6). Twenty-one days after planting, randomly selected aseptic seedlings were inoculated with an 8-mm diam mycelial disk of either *T. terrestris* or *P. tinctorius*. Noninoculated seedlings were used for nonmycorrhizal controls. The seedling jars were replaced in the water bath, and substrated temp during ectomycorrhizal synthesis averaged 23 C. Seedlings received a minimum of 54 klx of light during a 13-17-hr photoperiod.

After 3 mo, seedlings were carefully removed from the jars and their roots placed in modified (150 mm × 25 mm) plastic petri plates (10). Intact root segments of three types (ectomycorrhizae, nonmycorrhizal short roots, and nonmycorrhizal lateral root tips) were placed in individual glass inoculation cylinders and sealed with a nontoxic silicone compound (7). Feeder roots in the inoculation cylinders were covered with acid-washed, dry, white sand. The remaining root system was covered with sand and moistened with 45 ml of the inorganic salts of Melin-Norkrans' nutrient solution.

Both spiral and stunt nematodes were reared on roots of potted cotton plants in the greenhouse. Nematode inoculum was obtained from pot cultures by the elutriator-pan method (12). One-ml suspensions of 200 *H. dihystera*, 100 *T. claytoni*, or control inocula were added to individual root inoculation cylinders. The control inoculum was prepared by passing the remaining nematode suspension through a 160 mesh/cm screen to remove all plant-parasitic nematodes.

Phytophthora cinnamomi was grown in petri dishes containing V-8:oatmeal agar medium at 25 C in complete darkness. After 20 days, mycelial mats were stripped from the agar surface, quartered, and floated on a nonsterile soil leachate for 7 days at 25 C in darkness (7). The mats of mycelium were then washed in cool tap water, placed in sterile distilled water, and chilled for 45 min at 5 C to trigger zoospore release from sporangia. After 1.5 h at room temp, maximum zoospore release was obtained. A zoospore suspension was diluted and standardized using a Coulter particle counter to contain 100,000 zoospores per ml. One-ml suspensions of zoospores were added to each root inoculation cylinder designated to receive *P. cinnamomi*. One ml of 0.1% nonsterile soil leachate was added to each of the remaining cylinders to standardize populations of other soil microorganisms. After inoculation, the petri plates containing seedling root systems were wrapped in aluminum foil. Seedling stems and foliage protruded from the wrapped plates. All plants were incubated in a growth chamber adjusted for 12 h of light (25 klx) at 24 C and 12 h of darkness at 16 C.

Three treatments: (i) *P. cinnamomi* alone, (ii) a nematode species alone (either *H. dihystera* or *T. claytoni*), or (iii) *P. cinnamomi* with a nematode species, were placed randomly in inoculation cylinders containing ectomycorrhizae formed by *P. tinctorius* or *T. terrestris* on each of seven seedlings per ectomycorrhizal condition. These treatment combinations were each placed in a minimum of four cylinders. Nonmycorrhizal short roots and lateral root tips receiving the identical treatments were placed in four cylinders per root type on each of seven nonmycorrhizal seedlings. Between 19 and 43 ectomycorrhizae formed by either symbiont and 16 and 25 nonmycorrhizal short roots, as well as six nonmycorrhizal lateral root tips per treatment, were contained on lateral roots placed in the inoculation cylinders for each treatment.

After 8 days of incubation, the root segments were removed from the inoculation cylinders and fixed in weak Formalin-acetic acid (4). Root segments 1 cm long were dehydrated, embedded in hard paraffin, serially sectioned at 12 μ, and stained in safranine-fast green for histological examination (10). Segments from two lateral root tips with nematodes protruding were fixed in acetone-alcohol, cleared in saturated chloral hydrate, stained with 5% acid fuchsin-lactophenol, and cleared in lactophenol (15).

RESULTS.—Both *Tylenchorhynchus claytoni* and *Helicotylenchus dihystera* fed as migratory endoparasites on ectomycorrhizae. They penetrated and migrated through the fungus mantles and Hartig nets of ectomycorrhizae formed by *Thelephora terrestris* and *Pisolithus tinctorius* and the cortex and vascular tissues of nonmycorrhizal short roots and lateral roots.

T. claytoni migrated through the cortex and disrupted the vascular elements of approximately 14% of the ectomycorrhizae formed by *T. terrestris*, 20% of the ectomycorrhizae formed by *P. tinctorius*, 22% of the nonmycorrhizal short roots, and 10% of the lateral root tips (Table 1). They were observed to feed primarily on the meristematic regions of each root type, but were also

TABLE 1. Infection of feeder roots of shortleaf pine by *Tylenchorhynchus claytoni* and *Phytophthora cinnamomi*[a]

Treatments	Number of feeder roots		
		infected by	
	inoculated	*P. cinnamoni*	nematodes
Ectomycorrhizae:			
Thelephora terrestris:			
P. cinnamomi	19	0	0
T. claytoni	35	0	6
P. cinnamomi + *T. claytoni*	29	0	3
Pisolithus tinctorius:			
P. cinnamomi	38	0	0
T. claytoni	34	0	11
P. cinnamomi + *T. claytoni*	43	0	3
Nonmycorrhizal short roots:			
P. cinnamomi	25	0	0
T. claytoni	20	0	1
P. cinnamomi + *T. claytoni*	16	1	7
Nonmycorrhizal lateral short roots:			
P. cinnamomi	6	0	0
T. claytoni	6	0	0
P. cinnamomi + *T. claytoni*	6	1	1

[a]Noninoculated feeder root segments were free of infection by either *T. claytoni* or *P. cinnamomi.*

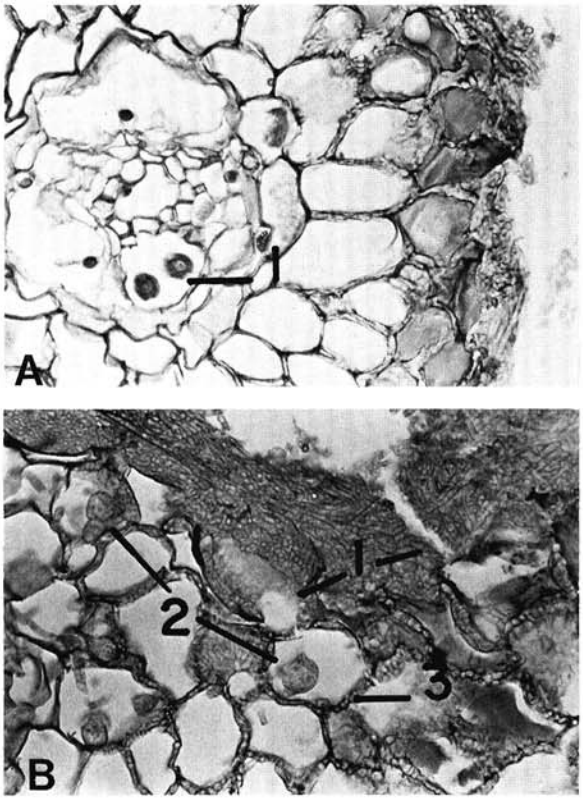

Fig. 1-(A, B). A) Cross section of an ectomycorrhiza formed by *Pisolithus tinctorius* with (1) *Tylenchorhynchus claytoni* in the vascular tissue. **B)** Tangential section of an ectomycorrhiza formed by *P. tinctorius* with vesicles of *Phytophthora cinnamomi* in cortical cells surrounded by the Hartig net. Note breaks in the fungus mantle caused by *Helicotylenchus dihystera* (1). vesicles of *P. cinnamomi* (2), and the Hartig net (3).

found in the cortex and vascular elements (Fig. 1). Nematodes were not observed penetrating the intact endodermis, and apparently entered the vascular tissues through undifferentiated tissue immediately basipetal to the subapical meristem. *T. claytoni* apparently migrated in and out of the cortex of lateral root tips. Six nematodes were observed on and within 1 mm of the root cap of a whole mount of one lateral root. In another, about 30 nematodes were in, or attached to, the regions of subapical meristems, elongation, and maturation. *Phytophthora cinnamomi* did not infect any of the four root types either with or without *T. claytoni.* Saprophagous nematodes inadvertently added to root cylinders were found associated with *T. claytoni* in disrupted vascular and cortical tissues, but were not found in association with *H. dihystera.*

H. dihystera parasitized 36% of the ectomycorrhizae formed by *T. terrestris*, 25% of the ectomycorrhizae formed by *P. tinctorius*, 25% of the nonmycorrhizal short roots, and 20% of the lateral roots (Table 2). This nematode caused lesions in both ectomycorrhizal and nonmycorrhizal feeder roots. Some lesions were shallow, one to two cortical cells deep, and others extended to the endodermis. This nematode entered the roots at natural breaks in the epidermis at the base of short root initials, or directly penetrated the epidermis of the lateral roots supporting the feeder roots. Ectomycorrhizae were frequently attacked in the forks of the branched mycorrhizal rootlets.

P. cinnamomi, in the absence of *H. dihystera*, infected the cortex of 25% of the nonmycorrhizal short roots, 17% of the lateral roots, and none of the ectomycorrhizae. When *H. dihystera* was present, however, *P. cinnamomi* was found in 27% of the ectomycorrhizae formed by *T. terrestris*, 36% of the ectomycorrhizae formed by *P. tinctorius*, 28% of the nonmycorrhizal short roots, and 50% of the lateral roots. *H. dihystera* caused breaks and

TABLE 2. Infection of feeder roots of shortleaf pine by *Helicotylenchus dihystera* and *Phytophthora cinnamomi*[a]

Treatments	Number of feeder roots		
		infected by	
	inoculated	*P. cinnamomi*	nematodes
Ectomycorrhizae:			
Thelephora terrestris:			
P. cinnamomi	19	0	0
H. dihystera	26	0	1
P. cinnamomi + *H. dihystera*	29	4	15
Pisolithus tinctorius:			
P. cinnamomi	38	0	0
H. dihystera	36	0	7
P. cinnamomi + *H. dihystera*	43	4	11
Nonmycorrhizal short roots:			
P. cinnamomi	25	6	0
H. dihystera	18	0	1
P. cinnamomi + *H. dihystera*	16	2	7
Nonmycorrhizal lateral root tips:			
P. cinnamomi	6	0	0
H. dihystera	5	0	0
P. cinnamomi + *H. dihystera*	6	1	2

[a]Noninoculated feeder root segments were free from infection by either *H. dihystera* or *P. cinnamomi*.

galleries in the fungus mantle and cortex of ectomycorrhizae. *P. cinnamomi* established infections in these nematode feeding sites. Intracellular hyphae and vesicles of *P. cinnamomi* were found in the cortical cells surrounded by the Hartig net of ectomycorrhizae, especially ectomycorrhizae formed by *P.* tinctorius (Fig. 1). *P. cinnamomi* was also found in the vascular tissues of nonmycorrhizal roots parasitized by *H. dihystera*. *P. cinnamomi* was not detected in ectomycorrhizae with intact fungus mantles.

DISCUSSION.—*H. dihystera* fed primarily on nonmeristematic regions of feeder roots. Their feeding, therefore, may not directly interfere with root extension and absorption of nutritients. By disrupting the structural integrity of ectomycorrhizae and mature feeder roots, however, *H. dihystera* may increase the severity of feeder root diseases caused by fungal pathogens such as *P. cinnamomi*. Therefore, spiral nematodes may be more important in predisposing feeder roots to fungal disease, rather than by acting as primary pathogens.

The feeding habits of *T. claytoni* indicate that it is a potential root pathogen, as reported by Hopper (2, 3). *T. claytoni* attacked the vascular elements and meristematically active regions of ectomycorrhizal and nonmycorrhizal feeder roots. This type of parasitism may prevent root extension by destruction of the root meristem, limiting nutrient absorption, and possibly, translocation of nutrients. Saprophagous nematodes found in the vascular elements and cortex are suspected as following the stunt nematodes into the roots, but their role in root damage is not understood.

Our results support the previous conclusion (7,9,10,11) that mature ectomycorrhizae are resistant to infection by *P. cinnamomi*. However, when *H. dihystera* attacked ectomycorrhizae, *P. cinnamomi* was able to infect ectomycorrhizae.

Breaks in the fungus mantle caused by nematode penetration provided points of entry for *P. cinnamomi*. The pathogen was able to bypass the mechanical barrier created by the fungus mantle of ectomycorrhizae by following the nematode migration routes through the mantle. Spiral nematodes may play a major role in root disease complexes involving *P. cinnamomi* by creating portals through which the pathogen can become established.

A high incidence of root infection by zoospores of *P. cinnamomi* did not occur in this experiment. It is assumed that static electrical charges which were readily observable in the acid-washed, dry sand activated a significant quantity of zoospores, thereby reducing the degree of root infection. However, even with a low amount of root infections, the role of *H. dihystera* in predisposing ectomycorrhizae to infection by *P. cinnamomi* was apparent.

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