Populations of Phytophthora cinnamomi and Pythium spp. Under Shortleaf and Loblolly Pines in Littleleaf Disease Sites

William J. Otrosina and Donald H. Marx

Graduate Research Assistant, School of Forest Resources, University of Georgia, Athens 30602; and Principal Plant Pathologist, U.S. Department of Agriculture (USDA), Forest Service, Southeastern Forest Experiment Station, Forestry Sciences Laboratory, Athens, Georgia 30602.

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ABSTRACT

Soil populations of *Phytophthora cinnamomi* and *Pythium* spp. in stands of *Pinus echinata* and *P. taeda* in the Georgia Piedmont district were determined using two selective agar media and an apple trap technique. Littleleaf disease symptoms were moderate-to-severe on *P. echinata* and absent on *P. taeda* trees. Soils in most test sites were of the Cecil clay series; one site of each pine species was on Madison clay. *P. cinnamomi* was isolated from soil under both pine species, but recovery was more consistent under *P. echinata*. *Pythium* spp. of the *P. irregulare-P. debaryanum* complex were isolated more consistently from soil under *P. taeda* than from under *P. echinata*. Seasonal differences in soil populations of the different fungi were not detected. Seasonal differences were detected in the capacity of soil extracts to induce sporangium production in *P. cinnamomi* from under both pine species, but significant differences were not detected between pine species. Extracts from surface soil (0-8 cm) had greater sporangium-inducing capacity than extracts from soil from various depths (10-45 cm) in soil profiles.

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Littleleaf disease of shortleaf (*Pinus echinata* Mill.) and loblolly (*Pinus taeda* L.) pines is reportedly caused by *Phytophthora cinnamomi* Rands on severely eroded, clay sites. Zoospores of *P. cinnamomi* infect and kill the feeder roots and impair the ability of the trees to absorb water and nutrients. Although the fungus is relatively common in forest soils in the southeastern United States, the disease is associated mainly with soil series having poor internal drainage and low fertility. Trees younger than 20 years of age rarely exhibit symptoms of littleleaf disease, but symptom expression increases with tree age. Symptoms of the disease resemble those of nitrogen deficiency, and usually affected trees die within 6 years after the onset of crown symptoms (4).

Campbell (2) first detected *Phytophthora cinnamomi* in soils under shortleaf pine with littleleaf disease by using an apple trap technique that is qualitative and rather specific for *Phytophthora* spp. Later, Campbell and coworkers (3, 4) used this procedure on soil under healthy and diseased shortleaf pine and found no significant seasonal variation in soil populations of *P. cinnamomi*. However, since individual propagules of *P. cinnamomi* were not enumerated, their results do not necessarily preclude the existence of quantitative seasonal differences.

Since the earlier work on littleleaf disease, several quantitative techniques which involve direct counting of chlamydospores (21), plant or plant-part baits (1, 6, 28), and selective agar media (25) have been developed for enumeration of soil populations of *P. cinnamomi* and *Pythium* spp. Campbell and Hendrix (5) used a selective agar medium and the apple trap technique and found *Pythium irregulare* Buis. to be very common under pine of the southeastern United States. Later, Hendrix and Campbell (12) used the above techniques, as well as another selective agar medium (9), and found the *P. irregulare-P. debaryanum* Hesse group to be generally distributed throughout soils of the United States. These reports strongly implicate *Pythium* spp. in littleleaf disease of shortleaf and loblolly pines. Mehrlich (22) first noted the relationship between the presence of nonsterile soil extracts and sporangium production of *P. cinnamomi*. Since that report, various workers (17, 20, 27) found that the sporangium-inducing substance is a byproduct of certain soil bacteria, and that it varied with soil type and between summer and autumn (7).

Interactions among several factors have not been investigated, namely inoculum density of *P. cinnamomi* and *Pythium* spp. in the soil in relation to amount of littleleaf disease symptoms on the two affected pine species and the influence of soil extracts on sporangium formation of *P. cinnamomi*. The purposes of the study described here are to determine the seasonal populations of *Phytophthora cinnamomi* and *Pythium* spp. in soils under shortleaf and loblolly pines in littleleaf disease sites, and to determine the possible seasonal differences in the capacity of soil extracts from under these pine species to induce sporangium formation in *P. cinnamomi*.

MATERIALS AND METHODS.—*Description of sites.*—Three loblolly and three shortleaf pine stands were selected; two stands of each pine species were in Madison County, Georgia, and one of each pine species was in Elbert County, Georgia. All shortleaf pine stands were natural stands with an understory composed of primarily grasses, brambles, or honeysuckle. Two loblolly pine stands were plantations, and one was a natural stand.

Two of the nine loblolly pine plots had grasses and briers as understory vegetation; these two plots were on the same site. In general, the loblolly pine stands had deeper, more friable soils than those with shortleaf pine. Roots were present in the deepest pit level (45 cm) on all stands, but the majority of feeder roots were above the compact clay zone (18-20 cm) in the soil profile. Shortleaf pines averaged 25-32 years of age, 19 cm in diameter, and 17 mm of radial growth during the past 10 years. Littleleaf index varied from 3.0 to 4.5 on a scale of 0 to 5, with 5 being the most severe. Soil series were mainly Cecil with a mixture of the closely related Madison series in one loblolly and one shortleaf pine stand. Loblolly pines averaged 23 years of age, 22 cm in diameter, and 36 mm of radial growth during the past 10 years. Littleleaf disease symptoms were absent on all loblolly pines.

Three 18 m × 18 m plots were installed on each site. The plots were subdivided diagonally into four equal subplots. One subplot was randomly chosen in each plot and a soil pit about 0.6 m × 1.5 m × 0.6 m deep was dug. Age, diameter at breast height (D.B.H.), and the preceding 10 years' radial growth of the dominant trees in each plot were measured and the understory vegetation and soil conditions were recorded.

Sampling and experimental procedures.—Soil samples were taken during midsummer, fall, winter, and spring of 1971-1972. Surface litter was removed and five surface subsamples were taken randomly with a trowel to a depth of 8 cm. These subsamples were combined into one sample for each of the pit-free subplots in each plot. Samples from the soil pits were taken at depths of 10, 30, and 45 cm. Three horizontal subsamples were taken and combined to make one sample at each depth. Prior to sampling, the pits were refaced for 0.3 m to expose fresh soil. Soil samples were stored in an ice chest in the field and then transferred to a cold storage room. They were processed within 24 hours of collection. One-hundred-eight soil samples were obtained in each of the four seasons.

Samples were mixed and passed through an 8-mm (mesh openings) screen to eliminate large stones and organic debris. Thirty grams (wet weight) of each sample were mixed with 100 ml of 0.3% agar and 1-ml aliquots were placed on each of 10 plates of the selective medium developed by Flowers and Hendrix (9) and Kerr's medium (15) as modified by Hendrix and Kuhlman (13). Details of the procedures for inoculating plates and obtaining dry weights of soil samples have been described (18).

After incubation for 36 hours, colonies of *P. cinnamomi* and *Pythium* spp. were counted. Representative colonies were plated on hempseed agar for confirmation of fungal identity. Colony counts were converted to propagules per gram of air-dried soil.

Three surface samples and three pit samples from a randomly selected plot in each pine stand were assayed by Campbell's apple trap technique (2). Soil was placed in two holes bored in each of three apples for each soil sample. When a firm, brown rot appeared on an apple, usually 5-10 days after inoculation, bits of apple from the edge of the rot were plated on cornmeal agar to confirm the presence or absence of *P. cinnamomi.*

Determination of sporangium-inducing capacity of soils.—Three surface and three pit samples from a randomly selected plot in each pine stand were tested to determine the capacity of the soil to induce sporangium production in *P. cinnamomi.* Duplicate 25-g subsamples were prepared from each soil sample. One subsample was added to a flask containing 200 ml of sterile distilled water and incubated in darkness at 25 C. The flasks were agitated weekly. The other subsample was air-dried and weighed to enable conversion of the data to an air-dried status. After 30 days, 20 ml of the cleared upper portion of the soil extract in each flask was pipetted into sterile petri plates. Three plates were used for each soil extract. *P. cinnamomi* (isolate 49) was grown for 20 days on V-8 juice-oatmeal agar (20) at 25 C in darkness. Mycelia were stripped from the agar surface in 1 cm^2 mats, and three mats were placed in each plate. Similarly, three mats were placed in each of three plates containing 20 ml of sterile distilled water and three mats in each of three plates containing 20 ml of laboratory soil extract to serve as controls. After incubation for one week at 25 C in darkness, the mycelial mats were transferred to plates which contained 20 ml of distilled water filtered through a 0.2-μm Millipore filter. The plates were chilled at 5 C for 30 minutes, and allowed to warm at room temperature to stimulate discharge of zoospores from sporangia.

Maximal zoospore release was obtained after 1.5 hours, after which two drops of 1% $HgCl_2$ were added to each plate to kill the zoospores. Mycelial mats were placed in tared aluminum weighing dishes, oven-dried for 24 hours and weighed. A sample of 100 zoospores from selected samples were measured and their size distributions and volumes were determined to set threshold limits on a Model B Coulter particle counter (24). Before counting, 1 ml of zoospore suspension from each sample was diluted with 9 ml of filtered 0.85% NaCl (w/v) solution. The manometer was set to draw 1/2 ml of this suspension through a 100-μm aperture in the counter. Each sample was counted three times. Counts were converted to numbers of zoospores per mg of mycelium per gram of air-dried soil. Analyses of variance were made on all data.

Soil samples from one plot in each stand, consisting of a surface sample and three pit samples, were analyzed from the summer and winter samples for pH, K, P, Ca, Mg, Zn, Fe, Mn, and Al using the double-acid extraction procedure (Soil Testing Laboratory, Cooperative Extension Service, University of Georgia, Athens).

RESULTS.—*Mineral nutrient status and pH of soils.*—Soils under shortleaf pine had lower amounts of the major elements (except magnesium) than soils under loblolly pine. This element was greater in the lowest horizons of the soil profile in the shortleaf (160 μg/g) than in the loblolly pine stands (85 μg/g). Approximately 110 μg/g magnesium was present in surface samples. Phosphorus was uniform throughout soil depths in the shortleaf pine sites (4 μg/g) for both seasons, but in the loblolly pine sites it tended to increase with depth in summer (from 6 to 17 μg/g) and decrease with depth in winter (from 7 to 4 μ/g). The pH increased with depth in shortleaf (from 5.0 to 5.7) and loblolly (from 5.1 to 5.5) pine sites. The most striking seasonal differences were with aluminum and zinc. Zinc tended to have higher winter values (9 μg/g) than summer values (1 μg/g), and aluminum lower winter values (120 μg/g) than summer values (300 μg/g) in soil under shortleaf and loblolly

TABLE 1. Comparison of two selective media and the apple trap isolation technique percent recovery and propagules per gram of air-dried soil of *Phytophthora cinnamomi* and *Pythium* spp. sampled from under shortleaf and loblolly pines[a]

Isolation techniques and *Pinus* spp.	Soil depth (cm)	Percent recovery		Propagules per gram of soil	
		P. cinnamomi	*Pythium* spp.	*P. cinnamomi*	*Pythium* spp.
Flowers-Hendrix' selective medium					
Pinus taeda	0-8	1.8	27	<1	2.0
(Loblolly pine)	10	8.3	49	<1	5.2
	30	2.8	33	<1	2.4
	45	0	17	0	<1
Pinus echinata	0-8	18.5	2.5	<1	<1
(Shortleaf Pine)	10	16.6	0	<1	0
	30	11.1	0	<1	0
	45	2.8	2.8	<1	<1
Modified Kerr's selective medium					
Pinus taeda	0-8	2.7	27	<1	4.0
(Loblolly Pine)	10	5.5	28	<1	5.1
	30	2.7	19	<1	2.3
	45	0	14	0	<1
Pinus echinata	0-8	18.5	7.4	<1	<1
(Shortleaf Pine)	10	13.8	0	<1	0
	30	11.1	2.8	<1	<1
	45	0	2.8	0	<1
Campbells' apple trap technique					
Pinus taeda	0-8	2.3	2.7		
(Loblolly Pine)	10	2.7	0		
	30	5.6	3.8		
	45	2.7	0		
Pinus echinata	0-8	45.0	0.5		
(Shortleaf Pine)	10	24.0	0		
	30	17.0	0		
	45	14.0	0		

[a]Seasonal samples were combined because no significant differences were found between seasons in numbers of propagules of *Pythium* spp. and *Phytophthora cinnamomi* per gram of air-dried soil.

pines. Certain elements decreased with increasing depth in the soil profile—calcium (175 to 70 μg/g), iron (60 to 25 μg/g), manganese (30 to 7 μg/g), and potassium (45 to 30 μg/g).

Population densities of Phytophthora cinnamomi and Pythium spp.—Recovery of *P. cinnamomi* and *Pythium* spp. varied widely among plots and subplots in both shortleaf and loblolly pine stands; therefore, data from the plots were combined and expressed as an average for each site. Analysis for seasonal differences in populations of these fungi under each tree species was done by using a one-way F test, which tested for differences within each pine species. Based on this analysis, populations of *P. cinnamomi* and *Pythium* species were not significantly influenced by seasons, therefore, data (Table 1) are presented as an average of all seasons.

Phytophthora cinnamomi was present in a greater number of samples and in greater quantity per gram of soil from all soil depths in shortleaf than in loblolly pine stands, regardless of isolation technique. A reversal of this finding was obtained with *Pythium* spp. These fungi occurred in a greater number of samples and had higher densities in loblolly than shortleaf pine stands, regardless of isolation technique.

Significant differences in numbers of positive samples or densities of *P. cinnamomi* and *Pythium* spp. at different soil depths were not found, although there was a general trend of decreasing populations with increasing depth. On most sites, tree roots were observed at all soil depths, with the majority of feeder roots located in the upper 20 cm of soil.

Pythium spp. of the *P. irregulare-P. debaryanum* complex were those most often isolated from both loblolly and shortleaf pine stands, regardless of isolation technique. *Pythium ultimum* Trow was isolated from two loblolly pine samples during the summer on Flowers' and Hendrix' medium. Unidentified *Pythium* spp. were isolated once from a loblolly sample during the spring on modified Kerr's medium, and once from a loblolly pine and once from a shortleaf pine sample during the fall with the apple trap technique.

Few Pythium isolates were consistently recovered by the apple trap technique. *Phytophthora cinnamomi* isolations by the apple trap technique were poorly

correlated with those by either of the selective agar media in the same sample. Both selective agar media were equally sensitive for isolating *P. cinnamomi* and *Pythium* spp. Propagules of *P. cinnamomi* originated from chlamydospores and vesiculated hyphae, and those of *Pythium* spp. from oospores and chlamydospores. These observations were based on microscopic examination of fungal colonies.

Sporangium-inducing capacity of soils.—There were no significant differences between the ability of soil extracts under shortleaf and loblolly pines to induce sporangium production in *P. cinnamomi* (Table 2). Extracts of surface samples of shortleaf pine collected in the fall, winter, and spring induced significantly more sporangia and zoospores than those collected during the summer. Surface samples from loblolly pine collected in the winter were significantly more active than the summer samples. Seasonal differences were not detected in the sporangium-inducing capacity of samples from various depths under shortleaf pine. Winter samples from loblolly pine were significantly more active than fall samples collected at depths of 10 and 45 cm. Although not consistently significant, surface samples from under shortleaf and loblolly pines were generally more active in inducing the production of sporangia and zoospores in *P. cinnamomi* than samples collected at various depths.

TABLE 2. Production of zoospores by *Phytophthora cinnamomi* in extracts of soil from under shortleaf and loblolly pines collected during four seasons from fall 1971 through summer 1972[a]

Season	Soil depth (cm)	Zoospores/mg mycelium/gm air-dried soil: Loblolly pine	Shortleaf pine
Summer	0-8	426 A	327 ACDX
	10	370	110 BX
	30	350	216
	45	300	80 BX
Fall	0-8	973 X	840 AX
	10	163 CX	587
	30	100 X	128 X
	45	90 BX	200 X
Winter	0-8	1,426 AX	1,453 C
	10	757 CX	576
	30	360 X	653
	45	1,000 B	686
Spring	0-8	766	810 DX
	10	1,063	1,180
	30	663	373
	45	800	1,343 X

[a]Means sharing the same letter A, B, C, or D within a pine species are significantly different between seasons by F test ($P = 0.05$). Means at 10, 30, and 45 cm depth followed by X within a pine species and season are significantly different from the 0-8 cm sample, $P = 0.05$. Quantity of zoospores is expressed as number produced per milligram of oven-dried mycelium per gram of air-dried soil. The data are averages of those from the three shortleaf pine sites and the three loblolly pine sites.

DISCUSSION.—*Phytophthora cinnamomi* was found in the soil under both species of pine, but recovery was more consistent and at higher populations under the shortleaf pine than the loblolly pine. This result is to be expected, since shortleaf pine is more susceptible to *P. cinnamomi* than loblolly pine (26).

The sporadic isolation of *P. cinnamomi* and the variability between plots and sites may occur because *P. cinnamomi* is a root-inhabiting fungus. Its distribution in the soil is confined generally to the region of susceptible roots (10), resulting in uneven distribution. Hendrix and Kuhlman (13) also experienced sporadic recovery of *P. cinnamomi* in littleleaf pine sites using the dilution plate technique. They pointed out that the pattern of recovery of the fungus may be affected by the relatively small amount of soil actually assayed for the fungus. In comparing the apple trap technique to that of the dilution plate, it becomes apparent that size of sample is important. In this experiment, the apple trap technique tended to yield more consistent numbers of *P. cinnamomi* than the dilution plate technique. This may have been due to variations in the amount of soil assayed. Approximately 60 g of soil per sample is assayed with the apple trap technique versus 3 g of soil per sample used in the dilution plate technique. It appears that in assaying populations of this fungus by the dilution plate technique, investigation of optimum sample sizes and procedures should be done as well as decreasing soil dilutions.

The absence of significant seasonal differences in populations of *P. cinnamomi* and *Pythium* spp. also may have been due to unusual meteorological conditions. Meterological data during the sampling period (1971-1972) indicated a milder winter and cooler summer than normal. These conditions may have influenced new root growth and the seasonal populations of these fungi.

In our study, no relationship was found between disease severity and amount of feeder root pathogen. These results agree with those of a study by Campbell and Copeland (4) in which the apple trap technique was used. They suggested that the lack of correlation between *P. cinnamomi* and disease symptoms may be due to the reduction in susceptible roots associated with declining trees.

Pythium spp. of the *P. irregulare-P. debaryanum* complex were the most frequently isolated species in soil under both species of pine. Populations of this fungus were considerably higher under loblolly pine than shortleaf pine. Although the loblolly pines showed no symptoms of decline, the *Pythium irregulare-P. debaryanum* complex has been associated with decline of loblolly pine (16). The pattern of recovery of *Pythium* from the dilution plates was similar to that of *P. cinnamomi*, except for one loblolly pine stand. On this site, which supported a vigorously growing plantation, comparatively high populations (9-46 propagules/g) of the *P. irregulare-P. debaryanum* complex were consistently recovered from the soil. These results indicated that these fungi may be soil-inhabiting (10) on this site. These findings are analogous to those of Mircetich (23), who investigated the influence of populations of *Pythium* spp. in peach orchard soils on the development of peach tree decline disease. He found no correlation between soil populations of *Pythium* spp. and incidence of disease. He also found that *Pythium vexans* d By, *P. irregulare*, and *P. ultimum* had considerable saprophytic ability to colonize dead roots of peach. However, Hendrix et al. (14) found a strong correlation

between soil populations of *Pythium* spp. in peach orchards and incidence of peach tree decline in Georgia. Although in our study the feeder roots of the trees on the loblolly pine site with high populations of the *P. irregulare-P. debaryanum* complex were not examined for disease, colonies of this fungus were observed developing from small root fragments on the dilution plates. It may be that *P. irregulare*, *P. debaryanum*, and other *Pythium* spp. are causing feeder root damage, but this young plantation apparently can regenerate an adequate feeder root system.

Pythium spp. of the *P. irregulare-P. debaryanum* complex are pathogenic to loblolly and shortleaf pine seedlings (11). Little is known about their effects on the health and vigor of mature forest trees, although the shortleaf pine site on which recovery of *Pythium* was most consistent had higher mortality of timber than did the other shortleaf sites (R. Cleaver, District Forester, The Catawba Timber Company, Elberton, Georgia, *personal communication*). Campbell and Hendrix (5) suggested that the presence of these and other *Pythium* spp. may be associated with understory vegetation as well as tree species. Copeland and McAlpine (8) noted a relationship between type of ground cover and amount of littleleaf disease in the Piedmont district shortleaf pine stands. Sites with honeysuckle, brambles, and vines tended to have fewer trees with littleleaf symptoms than those having grasses, brambles, and vines. Total nitrogen was found to be greater in sites with honeysuckle. These workers suggested that ground-cover type may serve as an indicator of soil nitrogen level.

Marx and Bryan (19) noted that soil extracts prepared from soil under loblolly pine were less active in inducing sporulation of *P. cinnamomi* than soil extracts from under shortleaf pine. They also noted a qualitative difference in the soil bacterial flora between these pine species. Chee and Newhook (7) found that soil extracts from various locations in New Zealand differed in their sporangium-inducing capacities. The lack of detection of differences in sporangium-inducing capacity between shortleaf and loblolly pine soils in this study may be due to widely divergent site and ground vegetation characteristics within each pine species. The effect of varying soil types, age of trees, and presence or absence of ground cover types may result in differences in microbial flora, which could tend to offset differences in sporangium-inducing capacity between the two pine species. This implies that the differences in sporangium-inducing capacity of the soils from the two pine species may not be due to the presence of the pine per se, but may be due in part to other factors.

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