Phytophthora cinnamomi as a Contributor to White Oak Decline in Mid-Atlantic United States Forests

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Abstract

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To evaluate *Phytophthora cinnamomi* as a cause of white oak (*Quercus alba*) decline in mid-Atlantic forests, sampling was conducted at 102 sites from 2011 to 2012. Soil and roots from healthy and declining white oak trees were collected. *Phytophthora* spp. were isolated using baiting and CFU of *P. cinnamomi* quantified using wet-sieving. Fine roots were scanned and measured. *Phytophthora* spp. were isolated from 43% of the sites. *P. cinnamomi* was common; six other species were isolated infrequently. Little difference in lesion size existed on white oak seedlings inoculated with 32 isolates of *P. cinnamomi*; only 13 isolates caused significant mortality. Soils from white oak versus

nine other hosts did not have significantly different CFU. *P. cinnamomi* was restricted to United States Department of Agriculture hardiness zones six and seven and never found in zone five. The presence of *Phytophthora* spp. in soil can be associated with white oak fine root health. When *Phytophthora* spp. were present, white oak trees in zones five and six had less fine roots. In mid-Atlantic oak forests, however, environmental conditions appear to play a key role in determining the impact of *P. cinnamomi* on the root system. *P. cinnamomi* alone does not appear to be a causal factor of white oak decline.

Decline of tree species in both the red and white oak groups is an ongoing occurrence in U.S. forests, and reports of oak decline have become more frequent (1,23,26). Oak decline is considered to be a disease complex, meaning eventual mortality occurs due to the interaction of abiotic and biotic factors over time, which often makes determining the cause of mortality difficult (26,39). Historically, oak species became dominant in eastern U.S. forests due to clear-cutting and burning, because oak species have the ability to quickly regenerate after disturbances (1,48,65). Once American chestnut populations were decimated by blight, oak also colonized former chestnut sites (48). Eventually, agricultural methods evolved and the widespread practices of clear-cutting and controlled burning were discontinued, resulting in forests becoming shadier and wetter as canopies filled in. The new climate conditions in many forests resulted in increased competition from species such as maple, cherry, and hemlock, which are better adapted to moist, low-light areas (48). In addition to changing forest composition, documented contributors to oak decline in the United States include inciting factors such as multiyear droughts, gypsy moth (*Lymantria dispar*) defoliation, and contributing factors such as boring insect damage and Armillaria root rot (17,21,37).

A greater incidence of decline was described on red oak compared with white oak, especially in the Appalachian Mountains and Ozark Highlands of Arkansas, Oklahoma, and Missouri (21,49,60). Causes of decline were most often described as prolonged drought, *Armillaria* spp. infection, and insect damage. However, several studies have noted that white oak (*Quercus alba*), in particular, has experienced significant decline in the eastern United States in recent years (1,10,23,46). Many of these trees are growing at sites with seemingly adequate resources; therefore, the loss of vigor is puzzling. Adverse long-term climate and site factors combined

with insect defoliation and boring are cited as the probable causes of white oak decline, while thorough investigation of soilborne pathogens is often lacking (21,36,38,59). The fungal pathogens usually associated with root mortality of white oak in U.S. forests are *Armillaria* spp.; little else is reported (15,37). In European oak declines, *Phytophthora* spp., especially *Phytophthora cinnamomi*, are proven contributors to the disease complex (8,9,13,14,16,34,52). The pathogen causes fine and lateral root mortality, as well as crown and trunk cankers of several oak species in Europe. *P. cinnamomi* also causes cankers on oak in Mexico, Florida, and California (2,25,61,70). Surveys by Balci and colleagues indicate that, although other species of *Phytophthora* are present, *P. cinnamomi* is the most widely distributed species in eastern U.S. oak forests (5,7). A recent study conducted in southern Ohio determined that declining white oak at sites infested with *P. cinnamomi* had significantly lower amounts of fine roots than white oak trees at noninfested sites, illustrating a tentative association between *P. cinnamomi* and white oak decline (10). Another study conducted in the same vicinity determined that population levels of *P. cinnamomi* were significantly higher in soil collected from declining white oak trees, providing further impetus to examine this possible association (46). This study was conducted to more thoroughly investigate the association of *P. cinnamomi* with white oak decline in the mid-Atlantic region. The role of *P. cinnamomi* in affecting root health and its distribution, host preferences, and differences in pathogenicity were also investigated in an effort to gather information for future management strategies.

Materials and Methods

Study site selection. Forest stands containing healthy and declining white oak were identified in collaboration with United States Department of Agriculture (USDA) Forest Service forest health and state department of agriculture personnel. Several sites were also selected from stands identified during previous surveys (5). In total, 102 sites in Delaware, Maryland, West Virginia, Ohio, Pennsylvania, New York, and Connecticut were sampled in 2011 (37 sites) and 2012 (65 sites) (Fig. 1). The majority of the sites were located in state-owned parks and forests (73 sites), with a further 24 sites located on private property and the final 5 sites on state university-owned land. Sites located in USDA hardiness zones five, six, and seven were selected in order to determine the

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possible northern range of *P. cinnamomi* (Fig. 1) (3). The average yearly range of soil temperatures for the past 10 years are 1.4 to 16.0, 1.2 to 25.3, and 4.9 to 27.6°C for zones five, six, and seven respectively (64). Eight sites that resulted in soil baits negative for *Phytophthora* in 2011 were sampled again in 2012 to determine if they were truly negative or if the inoculum level was too low for detection upon initial collection (5,20,67).

Sampling procedure by site. Sampling was generally restricted to spring and early summer each year to ensure that fine roots were harvested at roughly the same point in the white oak life cycle, as well because of the fact that increased isolation success of *Phytophthora* spp. occurs during spring (5). Sampling occurred from May to July 2011 and April to June 2012. The northernmost sites in New York and Connecticut were visited in September 2012, because high summer temperatures prevented continued sampling.

Oak trees sampled (408 in total) were an average of 91 years old and had an average dbh of 41 cm. Each site comprised two healthy and two declining white oak trees selected randomly within a 50-m radius. If only healthy or only declining white oak trees were present, four trees in the same condition were sampled. The surface layer of organic matter was scraped away with a pickaxe and four pits, roughly 30 by 30 by 30 cm in size, were dug in the cardinal directions approximately 1 to 1.5 m away from the trunk. Approximately 400 g of soil was collected from each soil pit and combined in a plastic resealable bag. All oak roots <1 cm in diameter were collected from the four soil pits for future scanning (10). Samples were refrigerated at 5°C or stored in coolers with ice until they were transported to the lab, then kept at 5°C until processing. Samples were processed within 2 months of collection date. Tree health was assessed based on the percentage of visible crown dieback. Crowns were rated based on the following criteria: class one, no decline symptoms, crown transparency less than 10 to

15%; class two, slight damage, some branch dieback and small gaps in lateral branch system, transparency 15 to 35%; class three, moderate twig and branch dieback, large gaps in lateral branch system, chlorosis and wilting of leaves, growth of epicormic shoots, transparency 35 to 55%; class four, severe branch dieback, many gaps in crown, chlorosis and loss of most leaves, many epicormic shoots, transparency 55 to 75%; and class five, dying tree, over 75% defoliated (5,9).

In 2012, soil was also collected from the most prevalent non-white oak possible alternate hosts of *Phytophthora* spp. within the same 50-m radius at each site, in a manner identical to the white oak soil sampling procedure. This was done in order to determine whether other plants were acting as alternate hosts or inoculum reservoirs of *P. cinnamomi*. Alternative hosts sampled included various shrub and tree species (Table 1).

Soil texture of each sample was determined in an attempt to evaluate any connection with the presence or absence of *Phytophthora* spp. in the soil at each site. In order to classify each soil, the California Soil Resource Lab's online SoilWeb mapping tool was used. Global positioning system coordinates were entered for each site and soil type was located on the SoilWeb maps (50).

Isolation of *Phytophthora* spp. from soil. Bags containing soil from the four soil pits were mixed thoroughly and 300 g of each soil sample was placed in a plastic container (19.5 by 14 by 9 cm) with lid, flooded with 500 ml of distilled water, and baited by floating *Q. robur* (English oak) leaflets on the water surface for 3 to 5 days at 17 to 18°C in the dark. Organic debris floating on the water surface was removed prior to placing the bait leaflets. In 2011, soil samples were first baited with rhododendron leaves (*Rhododendron maximum*) but, due to low isolation results, an additional 300 g of each negative soil sample was baited a second time with English oak leaflets. In 2012, all soil samples were

Fig. 1. Geographical distribution of *Phytophthora cinnamomi* and six other *Phytophthora* spp. Study site locations were mapped within United States Department of Agriculture plant hardiness zones.

baited using English oak leaflets. Discolored areas or lesions that developed on the bait leaflets were examined microscopically for the presence of sporangia characteristic of *Phytophthora* spp. and, if present, plated on clarified V8-based PARPNH (with pimaricin at 10 µg/liter, ampicillin at 200 µg/liter, rifampicin at 10 µg/ml, pentachloronitrobenzene at 25 µg/liter, nystatin at 50 µg/liter, and hymexazol at 50 µg/liter) media for isolation (5,9,10,32). Clarified V8 (cV8) was prepared by first adding 10 g of CaCO₃ to 1 liter of V8 juice, then spinning down the buffered vegetable juice in 50-ml centrifuge tubes at 4,000 rpm for 10 min to remove the solid vegetable matter.

Isolate characterization. Isolates of *Phytophthora* were identified based on both morphological features and molecular sequence data. Isolates were grown for 2 to 4 weeks on cV8 agar in order to observe hyphal morphology and chlamydospore and oospore characteristics. Subsets of heterothallic isolates were paired with A1 and A2 tester isolates of *P. cinnamomi* by placing mycelial plugs of known and unknown mating types on cV8 agar opposite one another (20). After 2 weeks, oospore formation was examined along the border where mycelia met. Sporangia for each isolate were produced by removing three to five agar plugs with mycelia from the leading edge of each colony growing on cV8 and flooding the plugs with nonsterile soil-extract solution (NS-SES). The agar plugs were checked for sporangia after incubating at room temperature overnight; if none were present, the plug containers were drained, new NS-SES was added, and the procedure repeated daily until sporangia were produced. Characteristics and sizes of 20 sporangia, chlamydospores, and oospores were measured and compared using a dichotomous key and species descriptions (20,41).

For molecular identification, isolates were grown in potato dextrose broth (Difco) for 5 to 10 days at 17°C on a laboratory bench top. Excess broth was removed using a pipet and mycelium was lyophilized for 12 h. For each isolate, approximately 0.1 mg of lyophilized mycelia was transferred to 0.2-ml polymerase chain reaction (PCR) strip tubes using a sterile tooth pick, and genomic DNA was extracted by adding 10 µl of Lyse and Go PCR Reagent (Thermo Scientific Pierce) to each tube and following the manufacturer's instructions for extraction. The internal transcribed spacer (ITS) region was amplified and sequenced (MCLAB) using primers ITS6 or ITS4 (68). Low-quality sequences were eliminated and the procedure was repeated with new material. Sequences were aligned and edited using Geneious Pro 5.5.6 Software (Biomatter Ltd.) and compared with known DNA sequences in the National Center for Biotechnology Information database using BLAST searches. GenBank accession numbers were obtained for representative isolates only.

Quantification of *P. cinnamomi* CFU. To quantify CFU of *P. cinnamomi* in soil, a modified wet-sieving method was used. Three 50-g subsamples of each soil sample positive for *P. cinnamomi* were homogenized in 300 ml of distilled water at low speed in a Waring laboratory blender for 5 to 30 s, depending on soil texture, because fine-textured soils required more time. The blended slurry was filtered and rinsed through nested 850-, 250-, 125- and 38-µm sieves in order to collect chlamydospores of *P. cinnamomi* (57). The material remaining on the 38-µm sieve was rinsed a final time and collected in 30 to 70 ml of distilled water in a 100-ml beaker, then plated on cV8-PARPNH selective medium (approximately 15 to 20 ml/plate). After incubation in complete darkness at 18°C for 3 days, petri dishes were rinsed free of soil and colonies of *P. cinnamomi* were counted (10,57). CFU were calculated based on colony count per 100 g of soil; data were corrected by calculating the dry weight of two 50-g subsamples for each soil sample analyzed.

Fine root processing. Collected roots were placed in a basin in the sink and rinsed with a hose to remove all soil. Any white oak roots were identified based on characteristic morphology and all others were discarded. Remaining white oak roots were scanned with the WinRHIZO Pro 5.0 program (Regent Instruments) to determine fine root (diameter of 0 to 1.5 mm) lengths and amounts (10).

Stem inoculations. *P. cinnamomi* isolates collected in 2011 were tested using a seedling stem inoculation assay to establish any differences in aggressiveness between the isolates. One-year-old white oak seedlings obtained from Maryland's John S. Ayton State Forest Tree Nursery were established in groups of 10 seedlings per 19-liter pot in the greenhouse prior to the start of the experiment. Efforts were made to select seedlings of similar size when transplanting. Overly large and small seedlings were excluded. Before the test began, seedlings were maintained using drip irrigation for 3 months. In all, 32 isolates of *P. cinnamomi* recovered from the rhizosphere soil of white oak trees in 2011 were used. A container of 10 seedlings was used for inoculation with each isolate (one replicate per isolate). Inoculum consisted of 6-mm plugs cut from the growing edge of 7-day-old colonies of *P. cinnamomi* grown on cV8 agar at 20°C. Control inoculum consisted of 6-mm plugs cut from sterile cV8 agar plates. Seedlings were wounded with a sterile razor blade about 3 cm above the soil line, inoculated with agar plugs, covered with a layer of sterile moist cotton, and sealed using Parafilm. After 2 months, the Parafilm and cotton were removed and bark tissue slightly shaved away to reveal any developing lesions (6). Seedlings that remained alive were measured and analyzed for lesion formation, but seedlings that died during the two-month incubation period were not included in lesion size analysis. Seedling mortality was monitored and analyzed separately.

Statistical analyses. Possible connections between the crown status of white oak, soil texture, and occurrence of *Phytophthora* spp. were analyzed using contingency tables and by conducting two-tailed Fisher's exact tests. Logistic regression analysis was conducted to evaluate any connection between the root status and crown dieback when *Phytophthora* spp. were present and absent. Stem lesion sizes on inoculated seedlings were analyzed using analysis of variance, and Tukey's multiple comparison test was performed to separate any differences between the isolates tested. The seedling mortality rates were analyzed using the Kruskal-Wallis nonparametric test. Significant differences were set at $P \leq 0.05$ and the software JMP 10.0.2. was used for all statistical evaluations (SAS Institute Inc., Cary, NC).

Table 1. *Phytophthora* spp. isolated from rhizosphere soil samples collected in mid-Atlantic oak forests from 2011 to 2012

Host	n	Positive samples	<i>Phytophthora</i> spp. ^a
<i>Acer pennsylvanicum</i>	1	0	...
<i>A. rubrum</i>	48	10	<i>Phytophthora cambivora</i> (1) <i>P. cinnamomi</i> (8) <i>P. quercetorum</i> (1)
<i>A. saccharum</i>	23	10	<i>P. cambivora</i> (3) <i>P. cinnamomi</i> (5) <i>P. plurivora</i> (2)
<i>Carya</i> sp.	2	0	...
<i>Castanea dentata</i>	2	0	...
<i>Fagus grandifolia</i>	11	4	<i>P. cinnamomi</i> (4)
<i>Hamamelis virginiana</i>	12	0	...
<i>Ilex opaca</i>	3	0	...
<i>Kalmia latifolia</i>	4	1	<i>P. cinnamomi</i> (1)
<i>Liriodendron tulipifera</i>	1	1	<i>P. cinnamomi</i> (1)
<i>Quercus alba</i>	408	91	<i>P. cambivora</i> (10) <i>P. cinnamomi</i> (72) <i>P. cryptogea</i> (1) <i>P. europaea</i> (4) <i>P. pini</i> (1) <i>P. plurivora</i> (1) <i>P. quercetorum</i> (2)
<i>Q. coccinea</i>	1	0	...
<i>Q. falcata</i>	2	0	...
<i>Q. prinus</i>	11	4	<i>P. cinnamomi</i> (4)
<i>Q. rubra</i>	37	5	<i>P. cinnamomi</i> (5)
<i>Q. velutina</i>	6	1	<i>P. cinnamomi</i> (1)
<i>Vaccinium</i> sp.	18	1	<i>P. cinnamomi</i> (1)

^a Numbers in parentheses represent isolates recovered from individual trees.

Results

Overall, an approximately equal number of healthy ($n = 200$) and declining ($n = 208$) white oak trees were sampled. Usually, declining trees were scattered within each stand; there were no sites with widespread areas of continuous decline.

Phytophthora spp.-associated bleeding trunk cankers were not detected at any of the sampling sites. When trees at each sampling site were inspected for other potential pathogens, no signs of *Armillaria* or *Biscogniauxia* spp. were observed. Although rhizomorphs of *Armillaria* were present at some sites, no fruiting structures or mycelial fans were evident on any sampled trees.

Isolation results and species assemblage. *Phytophthora* spp. were isolated from 43% of all sites sampled between 2011 and 2012 (44 positive out of 102 sites; Fig. 1). When eight sites that were *Phytophthora*-negative in 2011 were revisited in 2012, only one of these sites resulted in the isolation of a *Phytophthora* sp. *Phytophthora* spp. ultimately were isolated from soil from 10 of 17 plant species (Table 1). Trees in the genera *Acer*, *Fagus*, and *Quercus* commonly yielded *Phytophthora* spp. but *Phytophthora* spp. were almost never found beneath the understory shrub species *Hamamelis virginiana*, *Kalmia latifolia*, and *Vaccinium* sp.

In total, 622 soil samples were collected. This included soil from 408 white oak trees (32 of them resampled a second time) and 182 other hosts. In all, 101 samples yielded colonies of *Phytophthora* upon the first baiting, and a further 31 samples yielded positive results after they were air dried, reflooded, and baited a second time (Table 1). In 2011, two plant species were used for baiting. When rhododendron leaves resulted in no isolation of *Phytophthora* spp., the samples were rebaited using English oak leaf-

lets. This resulted in the isolation of *Phytophthora* spp. from an additional 12 soil samples.

Seven different species of *Phytophthora* were isolated during the study, all of which have been previously described (Table 1). *P. cinnamomi* (GenBank accession number KF271786) was the most frequently isolated species, accounting for 70% of *Phytophthora*-positive sites (31 sites in total). At five sites, *P. cinnamomi* was isolated together with one or more species of *Phytophthora*, though from soil from different hosts. When *P. cinnamomi*-infested sites were analyzed for frequency of isolation of the pathogen from plant species present, 47% of all plants at an infested site were predicted to harbor the pathogen (likelihood ratio $\chi^2 = 92.1$; $P < 0.0001$). The second most commonly encountered species was *P. cambivora* (GenBank accession number KF271790), which was isolated from seven sites. Other *Phytophthora* spp. were isolated sporadically and included *P. cryptogea* (GenBank accession number KF271791), *P. europaea* (GenBank accession number KF271788), *P. pini* (GenBank accession number KF271787), *P. plurivora* (GenBank accession number KF271789), and *P. quercetorum* (GenBank accession number KF271785) (Table 1). Only the A2 mating type was present when a subset of isolates of *P. cinnamomi* and all isolates of *P. cambivora* were tested. One isolate of *P. cambivora* was homothallic and oogonia characteristic of the species formed readily without an opposite mating type.

Incidence of *Phytophthora* in relation to USDA hardiness zones, soil type, and white oak crown status. *P. cinnamomi* was isolated only from sites in USDA hardiness zones six and seven, and not found further north in zone five (Fig. 1). Other *Phytophthora* spp. were isolated from all hardiness zones, including sites in zone five.

One or more species of *Phytophthora* were isolated from all soil types but the loamy sand collected from Delaware. *P. cinnamomi* was isolated more frequently from silt loam and loam-type soils, whereas the other *Phytophthora* spp. were distributed more evenly throughout the five *Phytophthora*-positive soil types (Fig. 2). *P. cinnamomi* was isolated approximately three times more often than other species of *Phytophthora* from silt loam soils, and nearly nine times more often than other species from loam-textured soils. When occurrences of *Phytophthora* spp. at infested sites were compared for soil textures across the study sites, only *P. cinnamomi* was more often associated with loam soils ($P = 0.028$). The occurrence of other *Phytophthora* spp. was not associated with silt loams ($P = 0.844$), silty clays ($P = 0.982$), or sandy loams ($P = 0.112$).

No associations were found between the crown symptoms and presence of *Phytophthora* spp. at infested sites during 2011 or 2012 (Table 2). During both years, *P. cinnamomi* was isolated approximately twice as often from soil from healthy white oak than from declining white oak. In contrast, other species of *Phytophthora* were isolated more often from declining trees than healthy trees, though this trend was not significant (Table 2).

CFU of *P. cinnamomi* in relation to crown dieback, host type, and hardiness zone. Numbers of *P. cinnamomi* CFU in soil were

Fig. 2. Frequency of isolation of *Phytophthora* from different soil types at infested sites. *Phytophthora* spp. excludes *Phytophthora cinnamomi* and represents all other species isolated, including *P. cambivora*, *P. cryptogea*, *P. europaea*, *P. pini*, *P. plurivora*, and *P. quercetorum*.

Table 2. Isolation frequency of *Phytophthora* spp. and *Phytophthora cinnamomi* associated with soil samples collected from white oak trees at infested sites

Year	Phytophthora status ^b	Crown status ^a		χ^2	$P > \chi^2$
		Healthy	Declining		
2011	Absent	16	18	...	...
	<i>P. cinnamomi</i>	15	11	0.667	0.4141
	<i>Phytophthora</i> spp.	2	6	1.287	0.2566
2012	Absent	53	27	...	...
	<i>P. cinnamomi</i>	48	23	0.031	0.8598
	<i>Phytophthora</i> spp.	12	10	1.023	0.3119
2011–12	Absent	69	45	...	...
	<i>P. cinnamomi</i>	63	34	0.438	0.5083
	<i>Phytophthora</i> spp.	14	16	1.868	0.1717

^a Declining trees were characterized as trees displaying >15% crown transparency, chlorosis of leaves, tip and shoot dieback, gaps in lateral branch system, and growth of epicormic shoots.

^b *Phytophthora* spp. include *P. cambivora*, *P. cryptogea*, *P. europaea*, *P. pini*, *P. plurivora*, and *P. quercetorum*.

quite variable over the study area, ranging from undetectable levels to a maximum of 118.5 CFU per 100 g of soil. In one instance, we isolated *P. cinnamomi* from soil collected from *Vaccinium* spp. with baiting; however, no viable CFU were detected with the wet-sieving method. The CFU from 2011 samples (average of 20 ± 31) were not significantly different from 2012 (average of 19 ± 36) ($P = 0.8037$). This was also not significantly different when the average CFU were compared between healthy and declining trees in both sampling years (2011: healthy = 19 ± 32 , declining = 22 ± 31 , $P = 0.8343$; 2012: healthy = 21 ± 27 , declining = 15 ± 25 , $P = 0.3955$). Similarly, no significant differences were detected in CFU of soils collected from diverse hosts sampled, though soil collected from the genera *Acer*, *Fagus*, and *Quercus* had the highest CFU levels (Table 3). When CFU were analyzed in relation to USDA hardiness zones, soils from the more southern zone seven had

Table 3. CFU and standard deviations (Stdev) of *Phytophthora cinnamomi* per 100 g of soil for various hosts sampled

Host	n	Mean $\pm$ Stdev
<i>Acer rubrum</i>	10	18 ± 37
<i>A. saccharum</i>	3	15 ± 5
<i>Fagus grandifolia</i>	3	34 ± 44
<i>Kalmia latifolia</i>	1	3
<i>Liriodendron tulipifera</i>	1	1
<i>Quercus alba</i>	73	18 ± 26
<i>Q. prinus</i>	4	41 ± 39
<i>Q. rubra</i>	3	28 ± 15
<i>Q. velutina</i>	1	34

Table 4. Mean colony counts (CFU) and standard deviations (Stdev) per 100 g of soil of *Phytophthora cinnamomi* in United States Department of Agriculture hardiness zones six and seven

Year, zone	n	Mean $\pm$ Stdev	F value	P > F
2011				
6	12	16.6 ± 29.6	0.29998	0.5891
7	14	23.3 ± 32.4	...	...
2012				
6	42	13.4 ± 23.3	4.1411	0.0455
7	32	25.6 ± 28.1	...	...
2011–12				
6	54	14.3 ± 24.6	4.0232	0.0476
7	46	24.9 ± 29.2	...	...

greater CFU than soil collected from the relatively cooler zone six; this was significant in the second year and also when the data for both years were combined (Table 4).

White oak fine root status in relation to sampling year, hardiness zone, crown status, and presence of *Phytophthora* spp. Total white oak fine roots (0 to 1.5 mm in diameter) collected in 2011 and 2012 differed significantly between sampling years. Therefore, fine root data were separately analyzed for each year. Far fewer fine roots were present in the second year of the study (average in 2011 = 3,457 cm and in 2012 = 2110 cm; $P < 0.0001$). Amounts of fine roots collected also differed between hardiness zones. In 2011, significantly fewer fine roots were detected in zone five compared with zones six and seven ($P < 0.0001$). This was also true in 2012 ($P < 0.0001$).

Mean fine root lengths varied between hardiness zones when the presence and absence of *Phytophthora* spp. was a factor (Table 5). Where *P. cinnamomi* was absent in zone five, trees affected by other *Phytophthora* spp. resulted in a significant decrease in fine root amounts during 2011 and 2012. In zone six, where *P. cinnamomi* was the most frequently isolated species, a decrease in fine roots in the presence of *Phytophthora* spp. was observed but was significant only in 2012. Although *P. cinnamomi* was also the most frequently isolated species in zone seven, white oak trees had significantly greater amounts of fine roots when *Phytophthora* spp. were present in both years of the study (Table 5). This was in contrast to zones five and six, where fewer roots were found when *Phytophthora* spp. were present.

In 2011, trees at sites where no *Phytophthora* spp. were detected had similar amounts of fine roots regardless of the crown status ($P = 0.8202$). In contrast, when *P. cinnamomi* or other *Phytophthora* spp. were present, a significant negative correlation was found between the crown status of trees and the amount of fine roots present ($P = 0.0022$ and $P = 0.0201$, respectively). In 2012 a different result was obtained. Regardless of the presence of *Phytophthora*, declining trees had fewer fine roots ($P = 0.0036$). Interestingly, more fine roots were present on declining trees when other *Phytophthora* spp. were present ($P = 0.0360$), although this was not significant for areas infested with *P. cinnamomi* ($P = 0.4712$).

Stem inoculation experiment. In all, 29 of 32 isolates caused lesions that were significantly different from the control inoculations (Fig. 3). Isolates that caused significant lesions differed slightly in their aggressiveness. However, stem lesion lengths did

Table 5. Mean total fine root lengths (cm) and standard deviations collected from four soil pits (30 $\times$ 30 $\times$ 30 cm) of white oak trees at sites infested with or free of *Phytophthora* in both sampling years and at different United States Department of Agriculture hardiness zones

Year, zone	Root diameter (mm)	Mean total fine root lengths (cm) ^a				F value	P > F ^b
		n	Absent	n	Present		
2011							
5	0–0.5	24	$1,123 \pm 543$	4	618 ± 422	3.106	0.0898
	0.5–1	24	456 ± 183	4	271 ± 191	3.4426	0.0749
	1–1.5	24	249 ± 91	4	144 ± 88	4.5713	0.0421
6	0–0.5	28	$3,010 \pm 2,244$	32	$2,658 \pm 1,482$	0.5265	0.471
	0.5–1	28	$1,069 \pm 738$	32	$1,040 \pm 541$	0.0311	0.8606
	1–1.5	28	541 ± 341	32	532 ± 269	0.0135	0.9079
7	0–0.5	24	$1,523 \pm 1,176$	24	$2,747 \pm 2,078$	6.3156	0.0155
	0.5–1	24	517 ± 391	24	$1,019 \pm 755$	8.3817	0.0058
	1–1.5	24	258 ± 191	24	498 ± 367	8.0792	0.0067
2012							
5	0–0.5	40	763 ± 77	8	393 ± 171	3.8826	0.0548
	0.5–1	40	354 ± 34	8	177 ± 77	4.3943	0.0416
	1–1.5	40	207 ± 20	8	100 ± 44	4.8909	0.032
6	0–0.5	40	$1,739 \pm 136$	56	$1,499 \pm 115$	1.8278	0.1796
	0.5–1	40	832 ± 59	56	663 ± 50	4.7402	0.032
	1–1.5	40	474 ± 32	56	363 ± 27	7.0935	0.0091
7	0–0.5	32	884 ± 782	28	$1,186 \pm 750$	2.3117	0.1338
	0.5–1	32	423 ± 367	28	617 ± 375	4.0941	0.0476
	1–1.5	32	237 ± 199	28	323 ± 186	2.9414	0.0917

^a *Phytophthora* spp. absent or present.

^b Significant differences are marked in bold ($P > 0.05$).

not always correlate with seedling mortality rates. Only 13 of the 32 isolates tested caused significant mortality to white oak seedlings. This discrepancy was best illustrated for the isolates MD-DK1-1 and WV-WVU1-3, which did not cause lesions that were significantly different from the controls but did cause significant seedling mortality (Fig. 3).

Discussion

Similar to previous studies in eastern U.S. and European oak forests, about half of the sites surveyed in our study were infested with *Phytophthora* spp. (5,9,32,34,66). The species assemblage of *Phytophthora* identified in our study was similar to previous oak forest surveys in the eastern United States, except for *P. cryptogea*, which has not been previously isolated (5,10,44). The species assemblage also largely overlaps with European oak forest surveys; the major difference is that *P. quercina* is the most commonly encountered species in Europe and *P. cinnamomi* is the most common species in eastern oak forests (8,9,29,31,32,34,66). In addition, *P. quercetorum* is only known to exist in U.S. oak forests (4).

Rebaiting soils after they were air dried improved isolation success. This procedure proved to be a useful method to stimulate germination of dormant structures of *Phytophthora* spp. Allowing soils to dry before baiting again may have also lowered populations of other competitive soil microorganisms, allowing any *Phytophthora* spp. present to be isolated more easily. Five species, including *P. cambivora*, *P. cinnamomi*, *P. cryptogea*, *P. europaea*, and *P. quercetorum*, were detected after this procedure. Such results were obtained in other studies and, thus, the procedure was suggested to increase isolation frequency of homothallic species that produce extensive oospores in culture media such as *P. quercina*, *P. quercetorum*, and *P. cactorum* (5,9,28,34). In our study, air-drying soil before rebaiting increased the isolation frequency of both hetero- and homothallic species. The heterothallic species *P. cinnamomi* was isolated in 18 instances only after soil was subject to air-drying after the first isolation attempt failed. In Western Australia, where *P. cinnamomi* is widespread in the eucalyptus forests, survival structures other than chlamydospores (oospores and hyphal aggregates) have been detected (35). Mimicking the periodically dry conditions of temperate areas when baiting may stimulate the growth of these various survival structures and

prove to be a useful method to detect *Phytophthora* spp. in eastern U.S. oak forests.

Many different plant species have been used to bait *Phytophthora* spp. from soil and, although this was not an objective of the study, we did observe that English oak leaflets were more sensitive as bait material; they resulted in twice the number of *Phytophthora* isolations compared with rhododendron leaves when both baits were used during the first year of the study. One reason for the increased isolation frequency could be the opportunity to examine English oak leaflets for sporangia of *Phytophthora* spp. with a compound microscope, whereas rhododendron leaves must be plated before the presence of *Phytophthora* spp. can be confirmed. Also, although whole English oak leaflets can be plated, it is only feasible to plate random sections of the water-soaked, necrotic tissue of rhododendron leaves, without knowing if the symptoms are caused by *Pythium* or *Phytophthora* spp. Particularly, if a higher *Pythium* population exists in a soil sample, any *Phytophthora* spp. present may be missed when baiting. Rhododendron leaf disks are commonly used to overcome this problem but wounding bait material favors *Pythium* spp. colonization, which can considerably reduce success in isolating *Phytophthora* spp. (41).

We noted that *P. cinnamomi* was isolated more frequently from soil types that hold more moisture and nutrients (e.g., silts and loams), though the California Soil Resource Lab's SoilWeb mapping tool is somewhat imprecise at specific locations. Nevertheless, our results correspond with similar studies. A study of *P. cinnamomi* causing disease on cork oak (*Q. suber*) in Portugal noted that, although *P. cinnamomi* was not significantly associated with any one soil type, it was more frequently isolated from soils with a higher silt or clay content (45). In Germany, a significant association was found between *Phytophthora* spp. and their presence in soils with a loamy, silty, or clayey texture but not for sandy or sandy loam soils (34). Similarly, in Swedish oak forests, *Phytophthora* spp. were isolated from all textures but sandy (29). In southern Ohio, soils free of *P. cinnamomi* also had a much higher percentage of sand than infested, clayey soils collected from a white oak stand (46). The more frequent isolation of *P. cinnamomi* from fine-textured soils may be due to a higher amount of calcium present in non-sandy soils, which have a higher cation exchange

Fig. 3. Average length of stem lesions (bars) and total number of 1-year-old *Quercus alba* seedlings killed (boxes) by *Phytophthora cinnamomi* after 2 months of incubation. Bars indicate standard deviations of lesion sizes. Gray bars and black squares indicate significant differences of mean lesion size or mortality rates, respectively, compared with controls. Black squares indicate total mortality for the group of 10 seedlings inoculated with each isolate. Column means not followed by the same letter are significantly different.

capacity (12,34). Calcium is a necessary element needed for both growth and reproduction of *Phytophthora* spp. (20), which may explain why soils with a finer texture result in a greater number of *Phytophthora* isolations. Although most oak forest studies in Europe and the United States could not detect *P. cinnamomi* in sandy soils, studies of the distribution of *P. cinnamomi* in sandy soils illustrate that the organism is not often found close to the soil surface (43,56). The pathogen was found in sandy soils in Australia at a depth of approximately 1 m and in North and South Carolina at 70 cm (44,55,56). Therefore, *P. cinnamomi* may be found far below the soil surface in soils with a higher percentage of sand. Sandy soils of Delaware and Maryland's Eastern Shore should be resampled for *P. cinnamomi* deeper in the soil profile; this procedure might reveal the presence of the pathogen in areas considered to be noninfested in the past.

At infested sites, when sampling was expanded to include other plant species in addition to white oak, *Phytophthora* spp. were detected from about half of the plant species examined. Although all hosts sampled in this study have known associations with various *Phytophthora* spp., *P. quercetorum* was isolated from soil collected from beneath red maple (*Acer rubrum*) for the first time, and new host associations for *P. cinnamomi* in soil included red maple, sugar maple (*A. saccharum*), American beech (*Fagus grandifolia*), and tulip poplar (*Liriodendron tulipifera*). *P. cambivora* and *P. plurivora* were also found in soil from sugar maple for the first time (22).

The *P. cinnamomi* CFU quantified for various hosts did not differ among the plants sampled but hosts that favor the population growth of *P. cinnamomi* are noted in other environments; yellow lupin (*Lupinus luteus*) in Spain and bull banksia (*Banksia grandis*) in Australia are two such examples (54,56). We could not relate CFU with crown dieback or root reduction in this study. In another study, when a white oak forest stand was sampled intensively, CFU levels were seven times greater on declining white oak trees than on oak trees without any crown dieback (10). The same conclusion was reached when CFU of declining white oak stands of the same forest site were quantified for 2 years and found to be significantly greater at declining trees than at healthy ones both years (46). The latter study also confirmed the field finding in an inoculum dose-response study using white oak seedlings and showed that greater inoculum levels resulted in significant root damage. Our study compared a wide variety of sites in an attempt to observe general trends but specific site conditions may have a significant impact on CFU levels, making a more general comparison difficult.

The aggressiveness of *P. cinnamomi* on white oak stems was illustrated in this study. Almost all isolates caused significant lesions on white oak seedlings. Furthermore, the isolates were nearly equally aggressive, a testament to the virulence of the *P. cinnamomi* population in eastern U.S. forests. Other studies have also shown the pathogenicity of *P. cinnamomi* isolates from the eastern United States on various oak trees (6,30,46) and, similar to our findings, little variation existed in aggressiveness of *P. cinnamomi* (18,61). Although little difference in pathogenicity was detected among the isolates, variation was found when seedling mortality rates were compared. It is possible that the isolates that caused more seedling mortality girdle stems quickly, rather than causing larger lesions to form. A longer incubation period could eliminate the observed differences between lesion size and mortality rates.

This study provides evidence that *P. cinnamomi*'s distribution in eastern U.S. forests may be linked to climate conditions. The distribution of *P. cinnamomi* was delineated by 40°N latitude, the approximate boundary between USDA hardiness zones five to the north and six to the south. This observation was previously noted but not evaluated (3,7,10). In our survey, 32 sites were located above 40°N latitude and *P. cinnamomi* was never found in these sites. Such limits were not present for the other *Phytophthora* spp. There was also a difference between zones six and seven in respect to CFU of *P. cinnamomi*: zone seven had twice the amount of CFU in soil. We hypothesize that these differences are related to en-

vironmental conditions. Soils north of 40°N latitude are not warm enough for a long enough time period each year for the population of *P. cinnamomi* to increase. Soils in hardiness zone five rarely rise above 20°C at any point during the year, instead remaining at approximately 15 to 17°C during the summer months (64). In contrast, soils in USDA hardiness zones six and seven both reach 24 to 27°C but soils in zone seven remain at this range for about 2 months longer each year than soils in zone six (64). In addition, soil temperatures during winter in zone seven are roughly 5°C warmer than in zone six. Therefore, it is likely that the soil temperatures in zone seven are the cause for the higher population levels observed for *P. cinnamomi*, and that lower temperatures in zone five prohibit the survival of the pathogen. If temperatures continue to rise due to climate change, *P. cinnamomi* might expand its range in mid-Atlantic oak forests and eventually move northward into zone five. Several past climate models using *P. cinnamomi* have determined that range expansion of the pathogen is likely if temperatures increase (11,13).

Unlike other oak decline studies that used crown symptoms as a way to measure the impact of *Phytophthora* spp., in this study, we evaluated the root system of white oak trees as a variable that might better demonstrate this interaction. When incidence of *Phytophthora* spp. was evaluated based on crown symptoms alone, no significant connection was found for either year; however, when data were analyzed including root status as a parameter, a different conclusion was reached. During our first sampling year, reduction of fine roots related to crown dieback at *Phytophthora* spp.-infested sites (*data not shown*). However, this connection was not present in the second year of the study. This result agrees with some of the previous findings (9,10,66) but also contradicts others where an association was noted based on the crown symptoms alone (8,16,34,45,53). The inconsistent association between *Phytophthora* spp. in soil, crown status, and root health of declining oak trees illustrates the dynamic changes that occur over time between the plant, pathogen, and environment, and sampling at different time periods provides only a snapshot of a longer progression of decline.

Evaluation of the root system in connection with environmental parameters as reflected by USDA hardiness zones gave a different perspective on the root dynamics of mature white oak trees in the mid-Atlantic region. A difference in fine root status emerged between sampling years regardless of whether or not the pathogen was present. Although fewer roots were available at *Phytophthora* spp.-infested trees during the first year, in the second year no such connection was found. It is imperative to state that environmental conditions were different between sampling years and an unusual early drought was experienced during the second year of sampling at many of the study sites. When the Palmer Drought Index for January to July of each sampling year was examined, drier conditions were present during the early part of the growing season in 2012 (19). It is possible that, in the first year of the study, more roots were killed because the environment was wetter and more suitable for longer infectious periods. During the second year, when drought occurred, the pathogen was probably not favored because the infectious cycle of *Phytophthora* spp. depends greatly on available water for dissemination of infectious zoospores and growth within host tissue. Several studies have noted that infection of hosts by *P. cinnamomi* under severe water stress results in growth inhibition of the pathogen (40,51,63).

The effect of environmental conditions on the interaction between host and pathogen became even clearer when data were separated by hardiness zone. In the northern zone five, white oak trees had fewer roots compared with the more southern zones six and seven. This was somewhat expected because cooler temperatures might have contributed to less root production by white oak trees (62). In zone five, presence of *Phytophthora* spp. significantly reduced the fine roots of trees at infested sites in both years. However, *Phytophthora* spp. were not consistently associated with root reduction in zone six. Only during the second year, when drought also occurred, was there a significant reduction. During the second

year, trees had overall fewer roots, which could be a reflection of early growing season drought. It is possible that the impact of *Phytophthora* spp. became more noticeable because of insufficient root amounts. This suggests that *Phytophthora* infection in combination with drought could become more important for root health. Site conditions were also suspected to be involved in *Q. alba* mortality caused by *P. cinnamomi* in Ohio (10). Similarly, in *P. cinnamomi*-infected *Q. suber* and *Q. ilex* forests in Europe, site conditions and unusual climatic events were thought to have contributed to a *P. cinnamomi*-induced oak decline (14,16,24,45,53). Such conclusions were also reached with pot experiments; damage to oak seedlings was shown to increase when they were subjected to drought or saturated conditions (33,40,42,46,53). Changing climate conditions in Europe that have resulted in warmer, wetter winters and droughty summers with periods of heavy rainfall are suspected to have changed the delicate balance between oak species and *P. cinnamomi* and may have led to current oak decline problems in certain locations (13,24). The climate in mid-Atlantic U.S. forests is not as warm as the Mediterranean region but, if drought events become more frequent, this phenomenon coupled with isolated periods of heavy rainfall throughout the year may result in previously unseen problems caused by *P. cinnamomi*.

The host–pathogen interaction appears to differ considerably in the warmest hardiness zone, seven, when compared with the two other zones. Although *P. cinnamomi* CFU were twice as high in this zone, infected trees also had greater amounts of fine roots during both years. This suggests that, despite the pathogen population being higher, fine root production as a host response was also better established in this zone. A similar plant response was also detected in artificial pot inoculation experiments. Tolerant citrus rootstocks were shown to regenerate more roots in the presence of *P. nicotianae* and *P. palmivora* (69). Similarly, beech trees infected with *Phytophthora* spp. had greater fine root lengths (47). More fine roots were also noted with various oak seedlings that remained alive after they had been exposed to *Phytophthora* spp. infection for 18 months under greenhouse conditions (Y. Balci, unpublished). Although the greater fine root amounts collected from zone seven may seem counterintuitive when a fine root pathogen such as *P. cinnamomi* is involved, this change illustrates that there is a definite plant response to the presence of the pathogen. In this region, it is possible that the presence of *P. cinnamomi* induces the formation of more fine roots, stressing the trees as they use stored energy for root production and potentially contributing to decline.

This study is the first to systematically collect fine roots from a large number of oak trees in mid-Atlantic forests to examine the possible effects of *P. cinnamomi* on the root system. We have shown that the presence of *Phytophthora* spp. can be associated with root health but environmental factors probably play a significant role in this interaction, which has not been previously documented under field settings. The dynamic interactions between host and pathogen in different climatic regions could explain why discrepancies exist among different studies and why we were unable to consistently associate crown symptoms with the presence of the pathogen in soil. Studies examining fine root health over multiple years could provide us with new insight if and when *P. cinnamomi* acts as a trigger of oak decline in areas where its biology is favored. Although we have shown that *P. cinnamomi* has a possible effect on white oak fine roots in this region, white oak decline is most likely not caused by this pathogen alone. Oak decline is often caused by the interplay of multiple factors conceptualized as predisposing, contributing, and inciting (39,58). Some of the most serious threats to white oak in the eastern United States include gypsy moth defoliation, insect borers, competition from successional plant species, drought, and root rot caused by *Armillaria* spp. (15,27,36,38). It is likely that white oak decline in mid-Atlantic forests is caused by the interaction of some combination of these factors, and that *P. cinnamomi* and, to a lesser extent, other sporadically found *Phytophthora* spp. are contributors to the decline complex as a whole.

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