ABSTRACT

Title of Document: DISTRIBUTION AND INVOLVEMENT OF
                      PHYTOPHTHORA CINNAMOMI IN WHITE
                      OAK (*QUERCUS ALBA*) DECLINE IN MID-
                      ATLANTIC UNITED STATES FORESTS.

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                      2013

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The association of *Phytophthora cinnamomi* with declining white oaks was studied at 102 sites in mid-Atlantic oak forests. Seven *Phytophthora* species were isolated from 44 sites. *P. cinnamomi* was restricted to USDA plant hardiness zones six and seven, and *P. cinnamomi* propagules in soil were significantly higher in zone seven than in zone six. When white oak fine root lengths of infected and *Phytophthora*-free trees were compared, infected trees had significantly lower fine root amounts. However, infected trees in zone seven had more fine roots. Little difference in pathogenicity between 32 *P. cinnamomi* isolates was found during white oak seedling stem inoculations. Fine root lengths of inoculated white and red oak seedlings decreased most during the spring. Propagule density in soilless potting media decreased with increasing temperature, except at 8°C and 16°C. These studies demonstrated that the impact, survival and spread of *P. cinnamomi* are strongly linked to environmental conditions and host species.

DISTRIBUTION AND INVOLVEMENT OF *PHYTOPHTHORA CINNAMOMI*
IN WHITE OAK (*QUERCUS ALBA*) DECLINE IN MID-ATLANTIC UNITED
STATES FORESTS.

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2013

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Dedication

This thesis is dedicated to Paul Dilworth. Thanks for sticking with me and always

believing in me, even when I wasn't so sure.

Acknowledgements

I would like to thank my adviser, Dr. Yilmaz Balci, for his guidance, endless creativity, and the support I have received while completing this thesis project. Thank you also to my other committee members Drs. Karen Rane, Arvydas Grybauskas, and Kathryne Everts for their many ideas and constructive comments in both the planning stages of the research project and the ultimate documentation of same. I would not have been able to complete my extensive field, greenhouse, and laboratory work without the support from Dr. John C. Bienapfl, Dr. Ailton Reis, Melissa Breiner, Steven Crisafulli, Patrick Di Bello, Blaine Ford, Danilo Reis Gonçalves, Rachel Kierzewski, and Lam Thuy Vi Tran Ho. I must also acknowledge the USDA-Forest Service and various state forestry personnel for funding and extensive support in locating sites and field sampling. Thanks also to Dr. Jennifer Himmelstein for letting me practice countless presentations for her, and for some great times in the cycling studio and the grad office!

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Chapter 1: Introduction

History

The arrival of the chestnut blight fungus combined with drastically altered land-use patterns after the arrival of Europeans in North America resulted in a profound shift in the dominant tree species in eastern US forests, from American chestnut (*Castanea dentata*) to various oak species (genus: *Quercus*) (1, 83). Where once millions of acres of mature chestnut trees thrived, now mixed oak forests dominate and sustain the landscape (82). In the eastern US alone, upland oak species cover 63.7 million hectares, or 43% of all timberland (83). On the land east of the Mississippi river, oaks make up about 23% of all species by volume (128). Oak populations also exploded after early settlers cleared land for farming by controlled burning and felling timber for construction and charcoal production to support the growing iron industry (1, 83).Once land-use methods changed in eastern forests, hardy, acorn-producing oak species, quick to colonize land after disturbances, thrived. Now, however, forest management trends have shifted to fire prevention, thus resulting in slowly changing growing conditions in eastern US forests. With so few blazes to promote the growth of fire-tolerant species like oaks, forests have become shadier and wetter, ultimately allowing succession by fast-growing species more adapted to the new conditions, such as maple, birch, and hemlock (1, 94)

Oak Ecology

Oak trees are long-lived (trees over 500 years old are not rare), drought tolerant, and adaptable, growing anywhere from low, moist bottomlands to the sides of dry, steep slopes with poor and rocky soil (7, 53). Approximately 400 different oak species inhabit the planet, each belonging to the white, red, or intermediate oak group (53, 83). The red and white oak groups

contain both evergreen and deciduous species, while the intermediate oak group contains only

evergreen species (53). Species in the white oak group have leaves with rounded margins and

acorns that mature the same year they are produced. Species in the red oak group have leaves

with pointed margins and acorns that mature the second year after they are produced (7).

Generally, species in the red oak group grow at higher elevations and under less than optimal

growing conditions, often in poor, droughty soil. The intermediate group is comprised of six

species which are found only in North America and also require two years for acorns to fully

mature (53). The most common oak species found in mid-Atlantic US forests are black

(*Quercus velutina*), bur (*Q. macrocarpa*), chestnut (*Q. prinus*), northern red (*Q. rubra*), post (*Q. stellata*), scarlet (*Q. coccinea*), southern red (*Q. falcata*), and white (*Q. alba*) (23, 53). Northern

red oaks in particular exist in large numbers in northeastern forests and are one of the most

commercially valuable oak species (53).

One reason that oaks are so prevalent in eastern US forests is that each species is adapted

to slightly different growing conditions, making virtually every part of forests suitable for

growth. Chestnut oak is a species that grows well at higher elevations, along ridge tops and

slopes. Chestnut oak thrives in well-drained soils but is quite drought tolerant, and so is often

found growing on drier sites with rocky, acidic soil (26). Northern red oak can be found at lower

elevations than chestnut oak, including rocky outcrops, middle slopes, ravines, coves, valley

floors, and the edges of floodplains. Northern red oak usually grows in moist soils, but will

establish in drier, more acidic sites as well. The species is adapted to growing in clayey, loamy,

sandy, and gravely soils (126). White oak is most often found at low elevations in somewhat

more mesic sites, including valley floors, bottomlands, along streams, in coves, and on sandy

plains. This species will not grow well in shallow, dry soil or in poorly drained bottomland areas.

White oaks thrive in silt/clay loams and fine sand and are also common on rocky soils (126).

Black and scarlet oaks are well-suited to drier upland forests with sandy and/or gravelly soils;

these species also colonize disturbed sites well (25, 27). The forested regions of the northeastern

United States have many different geologic and hydrologic features, guaranteeing the growth of

a wide variety of oak species adapted to different ecosystems.

Oak Decline

While oaks are generally considered to be hardy trees, they are not immune to damage

caused by environmental stresses, insects, or disease. Since the early twentieth century, a decline

syndrome has been observed affecting forest oak stands (46, 132). Oak decline is caused by a

complex of abiotic and biotic factors that interact to cause decline and eventual death of trees

(46, 114, 118, 123, 133). General consensus on the progression of oak decline is that

predisposing site conditions such as nutrient-poor soil, inadequate moisture availability, species

that are not genetically adapted to the growing conditions of the site, and advanced stand age are

all initial stressors. These factors combine with one or more short-term inciting event(s) such as

severe drought or insect defoliation, which then leave oak trees less able to withstand attack from

weaker contributing factors like secondary insect and fungal pathogens that eventually result in

mortality (73, 83, 113). Symptoms of oak decline include reduced growth, twig and branch

dieback, canopy thinning, and undersized or chlorotic foliage that may scorch or senesce early

(114). Declining oaks can survive for a number of years before succumbing, but may die in as

little as two to three years if severe stressors like debilitating drought and/or repeated insect

defoliation are present (118).

Oak decline is a difficult problem to diagnose and manage because the disease has many

possible combinations of contributing factors. Attention must be paid to both longer trends over

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time and the presence of more recent stressors in order to properly diagnose the decline syndrome. Knowing how stressors interact is essential in the case of decline complexes. Major stressful events will cause oaks to use stored nutrients for regeneration, which causes physical and chemical changes that reduce host defenses and vigor, and can also signal insects and pathogens to attack (114, 132). Several studies over the years have examined the combined effects of multiple causal factors that ultimately result in oak decline. For example, several years of severe drought can combine with a gypsy moth outbreak and *Armillaria* root rot to cause widespread mortality of oaks in US forest settings (133). In a case of oak decline in northwestern Germany, the interaction of several years of drought, frost events, and significant insect defoliation were examined and determined to be the cause of the problem (123). In southern Sweden, suspected causes of oak decline included drought, advanced tree age, and possible insect damage (115). A review of oak decline studies from around the world concluded that in the vast majority of cases, instances of oak decline were caused by a combination of stressors, including a primary triggering event followed by invasion by secondary insects and or/fungal pathogens (46)

Oak decline around the world

Oak decline is an international issue. Triggering factors include drought, frost, insect damage, secondary fungal infection, air pollution, excess moisture, poor site conditions, excess nitrogen, mistletoe infestation, and improper silvicultural manipulations (46). Perhaps most notably, infections caused by *Phytophthora spp.* have been important contributing factors to oak decline in many countries around the world, while information on the same association in US forests is scarce (10, 13-15, 20, 21, 43, 58, 60, 63, 89, 91, 115, 121).

Oak decline in the United States

Oak decline is not a new phenomenon in eastern US forests. Reports of oak decline date from the mid-1850s and continue to present, with reports of worsening conditions occurring from about 1980-2000 (1, 42, 46, 95). Oak decline in the eastern US has been reported in the Appalachian and Ozark Mountains, the New England states, and the mid-Atlantic states (1, 34, 118). Past observations of oak decline in the US suggest that species in the red oak group (northern red, scarlet, and black) are more susceptible to oak decline, as they have been the more severely affected group thus far. However, recent data illustrates the fact that white oak mortality is now also on the rise and merits further investigation (1, 40, 42, 65, 118, 119). Decline has been noted most recently in white oak stands in the mid-Atlantic and northeastern states, including Maryland, Pennsylvania, Ohio, Delaware, and West Virginia (1, 15, 42, 91).

Causes of oak decline in the United States

Drought, excess moisture, and frost damage

One of the most important events that weakens trees and triggers oak decline is drought, especially severe drought that occurs repeatedly over a period of several years (34, 70, 83, 116, 119). Indeed, many reports of oak decline come several years after drought affects a stand (64, 119). Species in the red oak group most affected by drought include red, scarlet, black, and pin oaks; white and chestnut oaks are most usually affected in the white oak group (133). Oak trees most susceptible to drought can be found growing in locations with unpredictable moisture availability- on ridge tops, slope sides, in wet areas, and on thin, rocky, or dry soils (64, 133). In fact, reports of oak decline more severely affecting species in the red oak group may be due to the fact that red oak group species are more adapted and likely to grow on less suitable sites. In the Ozark Highlands in the southern United States, reports of oak decline preferentially affecting

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red oak species were explained by the fact that red oaks had colonized less-than-optimal growing

sites (64, 65, 90). Sites with poorly drained, compacted, or waterlogged soils may also

predispose oaks to oak decline, though this stressor seems to be less important than dry

conditions (133). Of course, poorly drained soils reduce root survival and can also provide

conditions conducive to growth of pathogens like *Phytophthora*; both situations that can also

contribute to oak decline. Finally, spring frosts can cause significant tissue damage and may

factor into oak decline (118). Frosts can occasionally occur late enough in the spring to cause

harm newly formed xylem vessels and the young leaves they supply nutrients to, further

reducing tree vigor (123). The aforementioned abiotic factors are considered predisposing and

inciting factors, as they weaken oaks and increase the trees' susceptibility to secondary agents

that can aggressively attack stressed trees, leading to eventual mortality.

Insect damage

Another significant contributor to oak decline is insect damage, particularly from

defoliators and borers (118). After several seasons of significant insect damage, oaks are much

more prone to secondary fungi that attack stressed trees. Oaks are vulnerable to several kinds of

insects; the most important are the gypsy moth (*Lymantria dispar*), a serious invasive defoliator,

and the two-lined chestnut borer (*Agrilus bilineatus*), which creates galleries that girdle branches

and trunks (71, 118). Oaks are also fed on by leafrollers and leaftiers (114).

A major insect defoliator of oaks in eastern US forests is the gypsy moth. The insect has

been spreading steadily westward since its accidental introduction to the United States in Boston,

Massachusetts in the late 1860s, resulting in episodes of serious damage to forest trees during the

summer months (5). Major forest outbreaks occur least every ten years and usually take place in

forest stands that are composed of over 20% of the preferred host (83). Gypsy moths

preferentially attack oak and aspen trees, but will feed on many other species as well (71). The current western boundaries of the infestation are Wisconsin, Illinois, West Virginia, and North Carolina (83). The mixed-oak forests of the eastern US that are most susceptible to defoliation include the southern Appalachians, the Ozarks, and the northern lake states (71).

The effects of severe, repeated defoliation by the gypsy moth can have profound consequences on the health of a tree. Gypsy moth larvae are voracious feeders, occasionally consuming so many leaves in one season that trees are forced to produce a second flush, depleting necessary nutrients stored in the roots (114). Several successive years of heavy defoliation are enough to kill an oak tree; the likelihood of mortality rises when other biotic and abiotic stressors are present (53). Gypsy moth is a proven cause of oak mortality in US forests. Studies have shown that oak mortality is much higher in defoliated stands than undamaged stands, and that more damage is done to trees with healthy crown ratings during a defoliation event (39, 45).

Defoliation also leads to a reduction in acorn production, which affects not only the wildlife that depend on acorns for consumption, but also the potential of oaks to produce a new generation of seedlings for species regeneration within a forest. In cases of less severe defoliation, acorn production may be reduced by as much as 50-100%. When gypsy moth populations are high and cause heavy defoliation, acorn production can be virtually halted, with no yield at all in those years (83). Reduced acorn production can also contribute to the alteration of forest species composition. When oaks become less competitive due to reduced acorn production and/or vigor, faster-growing species like maples that are less susceptible to gypsy moth feeding take the opportunity to colonize available space, further compromising the ability of oaks to remain competitive within forest stands (53, 71, 83). Fortunately, the implementation

of two biological control methods (a fungus and a virus that infect and kill gypsy moth caterpillars) has resulted in much less damage from gypsy moth in recent years, and should continue to be viable control strategies (47, 100).

The two-lined chestnut borer is a pest of *Quercus, Castanea,* and *Fagus* species. Infestations of two-lined chestnut borer often follow episodes of defoliation, but the insects will also attack healthy trees (54). Weakened trees support larger populations of borers; in some cases, two to three years of heavy borer infestation are enough to cause mortality. The insects prefer to feed on chestnut, white, black, red, scarlet, and burr oaks, but damage many other species as well. Two-lined chestnut borers lay their eggs in oak bark crevices, enabling larvae to chew through the bark and create an ever-widening network of tunnels that eventually girdle the trunk, disrupting the flow of nutrients within the tree (118). Feeding by the two-lined chestnut borer usually begins in the crown and moves down the trunk; thus affected trees die from top to bottom. Oaks infested with two-lined chestnut borers exhibit branch dieback and foliage that may be stunted and discolored (54). Once oaks have been affected by a primary stressor such as drought, two-lined chestnut borer injury is often enough to cause mortality.

Oaks are also affected to a lesser extent by the oak leafroller (*Archips semiferana*), the leaftier (*Croesia albicomana*), and the oak leaftier (*Croesia semipurpurana*) (54). The oak leafroller webs leaves together and then feeds, causing remaining vegetation to look tattered and frayed. High population levels can cause serious defoliation, but the range of the insect is limited to deciduous oaks. Leaftiers can enter unopened buds and feed on new leaves, as well as fold and web leaves together to feed. Large leaftier populations can destroy most of the new buds on a tree, but this insect is a less serious pest than the gypsy moth and the two lined-chestnut borer (54).

Secondary fungi

Once oaks have been subjected to the stresses of drought, excess soil moisture, frost, and insect damage, they are often unable to defend against colonization by opportunistic fungi. The fungal genus most commonly associated with oak decline is *Armillaria*, which attacks tree roots and collars. *Armillaria* has a world-wide distribution and includes species that act as saprophytes and species that act as parasites (106). *Armillaria* spp. attack both conifers and deciduous trees, and while more commonly associated with stressed trees, certain species of the fungus will infect healthy trees as well (106). *Armillaria* infection is usually associated with the presence of other stress factors, most notably drought, waterlogging, and insect defoliation, and is capable of causing serious mortality in forest stands (106, 114). In the US in particular, several studies have shown the relationship between drought, *Armillaria* infection, and oak decline (64, 66). One possible reason the fungus is so proficient at attacking stressed trees is its ability to sense chemical changes within the tree, including the conversion of root starches to sugars for aboveground growth after defoliation, or the production of ethanol by roots in anaerobic soils (106, 118, 132). Due to the widespread forest distribution and capability of *Armillaria* to attack trees regardless of health status, the fungus can be a major contributor to oak decline.

Hypoxylon canker, caused by two species of the fungus *Biscogniauxia* spp. (*B. atropunctata* and *B. mediterranea*), is one of the most common fungal cankers associated with oak decline (132). The fungus is an opportunistic pathogen that invades stressed and dying trees and forms cankers characterized by a hard, black fungal stroma with embedded fruiting bodies. In some cases, hypoxylon cankers may become severe enough to girdle the affected tree (114). In several oak decline studies in the Mediterranean region, hypoxylon canker (*B. mediterranea*) resulted in increased mortality of water-stressed trees (130). Hypoxylon also infects stressed or

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wounded trees in the United States and often leads to mortality; the fungus affects primarily oak, but also aspen, beech, maple, and other tree species (3, 4). Given its cosmopolitan distribution and pathogenicity to stressed oak trees, hypoxylon canker could be a possible contributor to oak decline in northeastern US forests.

Several other fungi have been identified as contributors to oak decline. Gottschalk and Wargo's 1996 review article identifies *Diplodia* spp*., Ceratocystis* spp*., Ophiostoma* spp*., Ganoderma* spp*., Phomopsis* spp*.,* and others as being associated with oak decline around the world. *Botryosphaeria* spp. have also been implicated in oak decline as an agent causing twig and branch dieback, but the exact relationship of many of these secondary fungi with oak decline is not known (132). Once stressed trees have been infected with a secondary fungal root rot and/or trunk canker, mortality often follows.

Tree age

In addition to other stressors, tree age has also been suggested as a contributing factor to oak decline. In the past, oak tree age was regulated by environmental disturbances, especially forest fires and deer browsing, most stands were uneven-aged. Now, oak forests are much more heavily managed and the past system of death and replacement has been disrupted, allowing large numbers of oaks to mature together and consequently compete for the same resources (46). It has been suggested that stands of very old oaks may lose vigor at approximately the same time, allowing secondary factors to attack and kill the weakened trees, leading to large areas of oak decline (46, 53). In the Ozark mountains in the southern United States, tree age was suggested as a cause of decline of black and scarlet oaks, as large groups had colonized less than optimal sites, matured at the same time, and then began to decline together, perhaps due to competition for resources (64). In southern Sweden, tree age and drought were found to be contributing factors to

oak decline, as even-aged stands were observed to be declining together (115). While tree age may contribute to oak decline, tree age is considered less significant than the more damaging causes of drought, defoliation, and disease.

<u>*Phytophthora* as a cause of oak decline</u>

Many reports of oak decline in Europe include information on *Phytophthora* species as contributing factors; those most commonly found causing disease on oaks include *Phytophthora quercina, P. cambivora, P. cactorum, P. gonapodyides, P. cinnamomi,* and species within the *P. citricola* complex (13, 14, 20, 43, 58, 60, 63). Surveys of oak forest soils for *Phytophthora* spp. have been completed in Austria, Turkey, Germany, Italy, France, Spain, Portugal, Hungary, Sweden, and Slovenia (13, 14, 21, 43, 55, 60, 89, 115). *Phytophthora* spp. involved in oak decline in Europe cause several common symptoms, including fine root mortality, lesions on larger lateral roots, and occasional cankers on collars and trunks (55, 60). *Phytophthora* is suspected to be one of the main causes of Iberian oak decline, and combines with other detrimental factors such as drought or flooded soils to initiate decline symptoms (21). *P. cinnamomi* in particular has been isolated during many studies examining the possible relationship between *Phytophthora spp.* and oak decline. Significant reduction in fine root amounts, and collar and root lesions have been found on oaks growing in soils infested with *P. cinnamomi*. In some cases, the pathogen was isolated directly from root lesions, in others, from soil (20). Overall, *Phytophthora* species have been studied rather extensively as contributors to oak decline in Europe.

In the eastern United States, however, similar studies are scarce; the earliest was published less than ten years ago. In the eastern US, areas of significant oak decline have been observed and forest soils have been sampled to determine which *Phytophthora* species, if any,

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may be associated with the declining trees (8-10, 15, 91). *P. cinnamomi* has consistently been the most commonly isolated species in mid-Atlantic forests, and has been specifically associated with a white oak decline event in southern Ohio (15, 91). *P. cinnamomi* is also being studied as a cause of oak decline in California (44). Elsewhere in the US, however, researchers have not examined the possible involvement of *Phytophthora* spp. when conducting oak decline studies, and so further work on the subject is needed. The strong proven association of *Phytophthora* spp. with oak decline in European countries suggests that determining whether or not the genus is contributing to oak decline via root disturbance in mid-Atlantic forests might lead to a more accurate explanation of oak decline causes in the US.

Phytophthora cinnamomi

P. cinnamomi is one of the most ecologically and economically important species of *Phytophthora*, an aggressive soil-inhabiting pathogen found in most temperate and tropical regions world-wide, with over 5,000 known hosts (24, 38, 142). *P. cinnamomi* was first isolated and identified in 1922 causing stripe cankers on cinnamon trees in Sumatra by R. D. Rands (142). Since its characterization, the pathogen has been found world-wide causing disease on a wide variety of hosts. Common hosts include fir, chestnut, pine, oak, Eucalyptus, avocado, cinnamon, pineapple, *Prunus* spp., and ericaceous plants (38, 114). The pathogen causes rot of both fine and thicker roots, as well as collar rot, bleeding trunk cankers, stripe cankers, stem and root lesions, and wilting on woody hosts (38, 107, 114).

Perhaps the most dramatic example of the possible effects of *P. cinnamomi* has been the occurrence of dieback in the jarrah (*Eucalyptus marginata*) forest in southwestern Australia. *P. cinnamomi* is not native to the country, and as a result most native species in the forest are quite susceptible to infection, including trees, shrubs, and herbs (49, 107). It is assumed that the

pathogen was introduced to Australia in the early 1900s and spread by the timber industry. By the 1920s and 1930s, areas of dieback were observed in the forest, but it took another 30 years to attribute the problem to *P. cinnamomi.* In the meantime, infested soil was spread through the forest as additional roads were built and logging continued. Now, the pathogen has infested large sections of the forest and is attacking and rapidly killing many important native plants (49). According to one account, at least 75% of plants in the jarrah forest of Western Australia are vulnerable to *P. cinnamomi* infection (135). *P. cinnamomi* is also causing significant mortality in other parts of the world, including a mixed-oak forest in the state of Colima, Mexico. This particular area of infection expanded from 50 hectares in 1992 to over 300 hectares in 1997, with no signs of slowing (121). In the southeastern United States, *P. cinnamomi* causes root rot of shortleaf, loblolly, Virginia, pitch, slash, and longleaf pines, resulting in littleleaf disease in conjunction with waterlogged soil and soil nutrient deficiencies (49, 88). Littleleaf disease is quite destructive; annual losses are estimated to be over $15 million, with 35% of the commercial range of shortleaf pine affected (88). *P. cinnamomi* has also been found damaging oaks in the northeastern and western US (15, 44). In Europe, *P. cinnamomi* has been implicated in decline of cork and holm oak in recent years (43, 89, 104).

P. cinnamomi has been found in nurseries, Christmas tree plantations, landscapes, and forest soils and is capable of surviving for many years in the absence of a host by the formation of asexual resting structures called chlamydospores (38). Crandall first reported the positive identification of *P. cinnamomi* root rot in the United States on nursery stock (chestnut and various conifers), but reports of native chestnut and chinquapin mortality in southern US forests in the early 1800s are thought to have been caused by *P. cinnamomi* as well (31, 124). Recent sampling of eastern US oak forest soils has yielded a variety of *Phytophthora* spp., the most

commonly isolated by far being *P. cinnamomi* (found at approximately 70% of *Phytophthora-*infested sites sampled),and less frequently, *P.cambivora, P. citricola*, and *P. europaea* (10). Higher population densities of *P. cinnamomi* were detected in soil sampled from declining white oak sites. Oaks in northeastern US forests growing in soil infested with *P. cinnamomi* were also found to have 2.5 times less roots than their healthier counterparts, similar to the findings of fine root loss of trees growing in *Phytophthora*-infested soil in European studies (15). These preliminary studies in eastern US forests illustrate the fact that an organism that is extremely pathogenic to the roots of woody hosts is present, and could have thousands of acres of susceptible hosts in eastern US forests.

Effects of temperature on *P. cinnamomi*

P. cinnamomi is considered a moderate temperature species when it comes to growth and survival in soil and host tissues (142). Like other *Phytophthora* spp., low temperatures in winter cause a decrease or cessation in pathogen activity, warmer temperatures and moist conditions in spring are optimal for pathogen growth and infection, and high temperatures in summer cause inactivation of the organism as the upper limit of tolerance is reached (37). Average temperature ranges for the pathogen have been given based on studies of mycelium and chlamydospores cultured on Petri plates and in soil. Zentmyer reviewed numerous studies conducted with different isolates of *P. cinnamomi* and concluded that 5ºC is the lowest temperature at which any growth occurs; temperatures of at least 10ºC are required for any significant growth. Optimum growth of the pathogen occurs at 20-32.5ºC, and maximum growth from 30-36ºC (141, 142). While Zentmyer was able to summarize results from many growth tests to list optimum temperature ranges, the studies he reviewed indicate the presence of significant variation in growth rates between the isolates tested at different temperatures.

Optimal growth temperatures for *P. cinnamomi* explain why the pathogen causes different disease symptoms in different parts of the world. *P. cinnamomi* is considered to be an aggressive pathogen, and as such, is capable of causing aboveground symptoms in conducive climates. The most common aboveground symptom that *P. cinnamomi* causes on oaks in warmer environments is bleeding trunk cankers. Reports from warm climates exist around the world of the pathogen causing bleeding trunk cankers, rot and mortality of fine roots, and necrotic lesions on larger roots of oak. *P. cinnamomi* has been associated with bleeding cankers on declining oaks in France, Spain, South Africa, and Mexico (21, 74, 97, 121). In several oak decline complexes in Europe, *P. cinnamomi* has been baited from soil and also has been associated with fine root loss and the presence of lesions on larger roots (21, 74). Warmer states in the US also report the finding of bleeding trunk cankers caused by *P. cinnamomi* on healthy and declining oak trees. In Florida, bleeding cankers have been observed on the trunks of laurel oaks (16). In California, a state with a more Mediterranean-type climate, declining coast live oak trees in forest settings were found to have bleeding cankers and fine root mortality caused by *P. cinnamomi* (44).

The climate in the northeastern US, however, is quite different from sub-tropical and Mediterranean areas. *P. cinnamomi* has been associated with root mortality of declining oaks in eastern US forests, but aboveground symptoms caused by the pathogen have not been observed. In several studies on oak decline in the northeastern US, Balci and colleagues were unable to find any trunk cankers or lesions on larger roots caused by *P. cinnamomi,* nor were they able to isolate the pathogen in culture from fine roots of declining red or white oaks (10, 15). Research suggests that *P. cinnamomi* causes aboveground symptoms in warmer climates, while remaining primarily in the root zone in climates with colder winters like the northeastern US. As winters

are much colder in the mid-Atlantic region than in the Mediterranean climates where *P. cinnamomi* is often a problem, a theoretical northern boundary to the spread of *P. cinnamomi* in the US probably exists, and is estimated to be at approximately 40° N latitude. Currently, the most northern forest sites proven to be infested with *P. cinnamomi* roughly correspond to the boundary between USDA hardiness zones five and six illustrated on the USDA's plant hardiness zone map (10). The lower soil temperatures characteristic of zone five may have a negative effect on the ability of *P. cinnamomi* propagules to germinate and infect susceptible hosts during the year, as soil temperatures may not be conducive for long enough time periods.

Function and low-temperature germination of *P. cinnamomi* chlamydospores

Phytophthora cinnamomi is one of the species of *Phytophthora* that readily form an abundance of chlamydospores, both in host tissue and in culture (38). As sporangia and zoospores are both short-lived, and sexual reproduction of oospores occurs only when the opposite mating type is present (the *P. cinnamomi* population is dominated by the A2 mating type in the US), chlamydospores are considered to be the pathogen's primary means of long-term survival in northeastern US forest soils. Chlamydospores of *P. cinnamomi* are able to survive saprophytically in soil or in dead plant tissue for long periods of time, ensuring the multi-year survival of the pathogen, even without the presence of a host (142). Though chlamydospores of *P. cinnamomi* have thinner walls than chlamydospores formed by other *Phytophthora* species, the walls do thicken over time, and older chlamydospores of *P. cinnamomi* have been found to have vacuoles containing a high percentage of lipid material (80, 142). Chlamydospores germinate when a combination of the correct moisture level and availability of necessary exogenous nutrients allow (142).

Much of the research on chlamydospores of *P. cinnamomi* focuses on nutrient and moisture levels required for germination, as the pathogen is quite important in warm locations where tropical crops are grown and winter survival is less of an issue. Fewer studies have been conducted to examine the effects of low temperature exposure on survival of chlamydospores in soil and plant tissue. *P. cinnamomi* will not survive prolonged exposure to temperatures below 0ºC, though chlamydospores that have been exposed to 0ºC briefly (ie., less than two weeks) have remained viable (17, 141, 142). In a multi-year nursery study, chlamydospores in soil infected with *P. cinnamomi* from a nursery were inactivated after winter temperatures dropped below 0ºC. Temperatures did not drop below 0ºC during another year of the study, and chlamydospores were still viable, though at a reduced rate (17).

The response of *P. cinnamomi* to low soil temperatures is an important consideration when it comes to the pathogen's involvement in oak decline in northeast US forests. Evidence suggests that *P. cinnamomi* would not be found in soils that drop below 0ºC during the winter months, because such low temperatures would essentially inactivate any chlamydospores, therefore removing the inoculum from the soil (17). Previous studies indicate that *P. cinnamomi* causes significant root infection at 15ºC on several different hosts, but little to no infection occurs below that temperature; optimum temperatures for the development of root rot are from 19-27ºC (110, 142). In Shew and Benson's 1983 study on *P. cinnamomi* root rot of Fraser fir, they found that 16-25ºC provided the best conditions for infection, and that while minor infection occurred at 12 and 14ºC, no mortality was observed. If low soil temperatures for the majority of the year in northern soils inhibit chlamydospore germination, no disease should be observed even if the pathogen is present. Production of chlamydospores occurs anywhere from 12-30 ºC, with optimum temperatures for production at 21-24ºC (142). The minimum temperature range

required for germination of chlamydospores is 9-12ºC, with optimum temperatures between 18-30ºC (142). Three climate zones as defined by the USDA are present in the study area; five, six, and seven (USDA Plant Hardiness Zone Map). Soil Climate Analysis Network data indicates that in zone five, where soils infested with *P. cinnamomi* have not yet been found, soil temperatures are at or above 15ºC for an average of 27 days per year. In zone six, where numerous sites infested with *P. cinnamomi* exist, soil temperatures are at or above 15ºC for an average of 149 days per year. *P. cinnamomi* is also found in zone seven, where soil temperatures are at or above 15ºC for an average of 207 days per year. Currently, it seems that *P. cinnamomi* has a defined northern range in the eastern US based on low soil temperatures that limit pathogen spread.

Possible effects of current climate change on *P. cinnamomi*

The study of climate change, and especially its possible effects on range expansion of plant pathogens, is a contemporary topic. Current climate models predict a rise of 0.9° to 3.5°C in global temperature by the year 2100, as well as a rise in precipitation levels (28). Models developed to predict changes in plant pathogen distribution and virulence state that range expansion, infection of new hosts, winter survival, changes in inoculum production and density, and severity of resulting disease are all candidates for change due to a general increase in global temperatures (20, 28, 75, 120).

In the case of *P. cinnamomi*, warming of the climate could mean the difference between the pathogen being inactivated by low temperatures or overwintering to cause further damage. In Europe, *P. cinnamomi* causes symptoms both above and below ground. The pathogen contributes to the oak decline complex by causing root rot and as trunk cankers (20). Climate models have been developed to predict the influence of warmer temperatures on both kinds of disease. *P.*

cinnamomi trunk cankers are found on *Quercus robur* and *Q. rubra* in southern France and are perennial (18). Cold winter frosts kill the inoculum for the year, resulting in little growth of the pathogen, as evidenced by measuring the canker margins in the phloem. When winters are too warm, the pathogen is able to survive within the tissue and the canker increases in size during the year (18, 75). Climate models predict that as temperatures rise and are more favorable to pathogen survival through the winter, the range of *P. cinnamomi* as a canker-causing pathogen in southern France will likely increase to both the north and the east (18, 74, 75).

P. cinnamomi is a significant contributor to decline of oak species in Europe as a causal agent of extensive fine root rot (20). Temperatures and precipitation levels are expected to rise in the future, and an increase in sudden heavy rain events is also predicted. The resulting warmer, wetter soils may favor *Phytophthora* infection, especially of woody plant roots (120). Temperate forest soils, which rarely freeze during winter at present, will most likely be even warmer in the future and allow for better pathogen survival in the root zone (18). As the global climate changes, more extreme weather patterns are expected. Cycles of alternately saturated and dry soils stress oak forests, making the trees more susceptible to secondary infection. The combination of warmer winters, wetter soils, and general higher soil temperatures may interact in the future to produce conditions quite favorable for high inoculum production and associated increased disease severity of *Phytophthora* root rot in European oak forests (20, 120). Recent data shows that *Phytophthora cinnamomi* may be a contributing factor to oak decline in eastern US forests, so climate model conclusions dealing with range expansion and increased disease may become a concern in the future.

Research Objectives

The objectives of the research were:

1. To determine the distribution of *P. cinnamomi*, a possible northern boundary in the mid-Atlantic region to the spread of the pathogen at approximately 40ºN latitude, and the effects of temperature on *P. cinnamomi* propagules in soil.

2. To determine the effects of *P. cinnamomi* on white oak root systems.

To achieve these objectives, 102 sites in seven mid-Atlantic states were sampled from 2011-2012. Soil and roots from healthy and declining white oaks were collected, soils were baited for *Phytophthora* spp. with English oak leaflets, and roots were washed and scanned to quantify lengths. *P. cinnamomi* propagules were quantified for all positive soils. A white oak seedling stem inoculation experiment was used to examine the aggressiveness of all *P. cinnamomi* isolates collected in 2011, in order to choose the most virulent isolate for use in future soil inoculation experiments. White and red oak seedlings were soil-inoculated with *P. cinnamomi* and incubated in the greenhouse at a constant temperature for ten months; a subset of seedlings was harvested every 30 days for 300 days, and root systems were washed and scanned to quantify changes in root amounts. Finally, white oak seedlings were also soil-inoculated with *P. cinnamomi* and incubated at different temperatures and durations indicative of actual climate conditions in USDA hardiness zones five, six, and seven, the zones present in the mid-Atlantic study region. *P. cinnamomi* propagules in each pot were quantified at the beginning and the end of the experiment.

Chapter 2: *Phytophthora cinnamomi* as a contributor to white oak decline in mid-Atlantic United States forests

Taken in part from: McConnell, M. E. and Y. Balci. *Phytophthora cinnamomi* as a contributor to white oak decline in mid-Atlantic United States forests. *Plant Disease*. In press.

Introduction

Decline of tree species in both the red and white oak groups is an ongoing occurrence in United States forests, and reports of oak decline have become more frequent (1, 42, 46). 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 (46, 73). Historically, oak species became dominant in eastern US forests due clear-cutting and burning, because these trees have the ability to quickly regenerate after disturbances (1, 94, 129). Once American chestnut populations were decimated by chestnut blight, oaks also colonized former chestnut sites (94). 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 like maple, cherry, and hemlock, which are better adapted to moist, low-light growing conditions (94). In addition to changing forest composition, documented contributors to oak decline in the US include inciting factors such as multi-year droughts, gypsy moth (*Lymantria dispar*) defoliation, and contributing factors such as boring insect damage and *Armillaria* root rot (33, 40, 66).

A greater incidence of decline has historically been described on red oaks compared to white oaks, especially in the Appalachian Mountains and Ozark Highlands of Arkansas, Oklahoma, and Missouri (40, 95, 119). Causes of red oak decline were most often described as prolonged drought, *Armillaria* infection, and insect damage. However, several studies have noted

that white oak (*Quercus alba*) in particular has experienced significant decline in the eastern US in recent years (1, 15, 42, 91). Many of these trees are growing at sites with seemingly adequate resources, so 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 (40, 64, 70, 117). The fungal pathogens usually associated with root mortality of white oaks in US forests are *Armillaria* species; little else is reported (22, 66). In oak decline in Europe, *Phytophthora* species, especially *P. cinnamomi*, are proven contributors to the decline complex (13, 14, 20, 21, 30, 60, 103). 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 oaks in Mexico, Florida, and California (2, 44, 121, 140). Surveys by Balci and colleagues indicate that while other species of *Phytophthora* are present, *P. cinnamomi* is the most widely distributed species in eastern US oak forests (10, 12). A recent study conducted in southern Ohio determined that declining white oaks at sites infested with *P. cinnamomi* had significantly lower amounts of fine roots than white oaks at non-infested sites, illustrating a tentative association between *P. cinnamomi* and white oak decline (15). Another study conducted in the same vicinity determined that population levels of *P. cinnamomi* were significantly higher in soil collected from declining white oaks, providing further impetus to examine this possible association (91). 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, its distribution, host preferences, and differences in pathogenicity were also examined in an effort to gather information for future management strategies.

Study site selection

Forest stands containing healthy and declining white oaks were identified in collaboration with USDA Forest Service forest health and state department of agriculture personnel. Several sites were also selected from stands identified during previous surveys (10). A total of 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 five sites on state university-owned land. Sites located in USDA hardiness zones five, six, and seven were selected in order to determine the possible northern range of *P. cinnamomi* (Fig. 1) (6). The average yearly range of soil temperatures at a depth of 8-20 cm for the past ten years were 1.4-16.0°C, 1.2-25.3°C, and 4.9-27.6°C for zones five, six, and seven respectively (127). 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 (10, 38, 135).

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 as the fact that increased isolation success of *Phytophthora* species occurs during spring (10). 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, as high summer temperatures that year prevented continued sampling.

Oak trees sampled (408 in total) were an average of 91 years old and had an average diameter at breast height (dbh) of 41 cm. Each site was comprised of two healthy and two declining white oaks selected randomly within a 50 m radius. If only healthy or only declining white oaks 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 30x30x30 cm, were dug in the cardinal directions ca. 1-1.5 m away from the trunk. Approximately 400 g of soil was collected from each soil pit, for a total of approximately 2 kg of soil combined in a plastic re-sealable bag. All oak roots <1 cm in diameter were collected from the four soil pits for future scanning (15). Fine roots were collected from each soil pit by discarding any larger lateral roots (>1 cm). Samples were refrigerated at 5ºC or stored in coolers with ice until they were transported to the lab, then kept at 5ºC until processing. Samples were processed within two 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%, class five, dying tree, over 75% defoliated (10, 14).

In 2012, soil was also collected from the most prevalent non-white oak possible alternate hosts of *Phytophthora* within the same 50 m radius at each site identical to the white oak soil sampling procedure. This was done in order to determine if 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* species 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 (98).

Isolation of *Phytophthora* spp. from soil

Bags containing soil from the four soil pits were mixed thoroughly and 300 g of each soil sample were placed in a plastic container (19.5x14x9 cm) with lid, flooded with 500 ml of distilled water, and baited by floating English oak (*Quercus robur*) leaflets on the water surface for 3 to 5 days at 17 to 18ºC 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 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*, and if present, plated on clarified V8-based PARPNH (with 10 µg/liter pimaricin, 200 µg/liter ampicillin, 10 µg/ml rifampicin, 25 µg/liter pentachloronitrobenzene [PCNB], 50 µg/liter nystatin, and 50 µg/liter hymexazol) media for isolation (10, 14, 15, 58). Clarified V8 (cV8) was prepared by first adding 10 g $CaCO_3$ to 1 liter of V8 juice, then spinning down the buffered vegetable juice in 50 ml centrifuge tubes at 4,000 rpm for 10 minutes 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 two to four weeks on cV8 agar in order to observe hyphal, chlamydospore, and oospore morphology. A subset of heterothallic isolates were paired with A1 and A2 tester isolates of *P. cinnamomi* by placing mycelial plugs of known and unknown mating type on cV8 agar opposite one another (38). After two 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 non-sterile soil extract solution (NS-SES). The agar plugs were checked for sporangia after incubation 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 (38, 77).

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 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, Rockford, IL) to each tube and following the manufacturer's instructions for extraction. The internal transcribed spacer (ITS) region was amplified and sequenced (MCLAB, San Francisco, CA) using primers ITS6/ITS4 (138). 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., Auckland, NZ) and compared to known DNA sequences in the NCBI database using BLAST searches. GenBank Accession numbers were obtained for representative isolates only.

Quantification of *P. cinnamomi* colony-forming units

To quantify colony forming units (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 distilled water at low speed in a Waring laboratory blender for five to 30 seconds depending on soil texture, as 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 propagules of *P. cinnamomi* (111). The material remaining on the 38 μm sieve was rinsed a final time and collected in 30 to 70 ml distilled water in a 100 ml beaker, then plated on cV8-PARPNH selective medium (approximately 15 to 20 ml per plate). After incubation in complete darkness at 18ºC for three days, Petri dishes were rinsed free of soil and colonies of *P. cinnamomi* counted (15, 111). Propagule densities were calculated based on colony count per 100 g of soil; data was 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 placed in a pre-measured plastic tray and scanned with the WinRHIZO Pro 5.0 program (Regent Instruments, Canada) to determine total fine root (diameter 0 to1.5 mm) lengths (15). Samples consisted of many fine roots, requiring multiple

trays of roots to be scanned per sample. The total fine root length for that particular sample was then calculated by adding individual scans.

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 ten 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 three months. Thirty-two isolates of *P. cinnamomi* recovered from the rhizosphere soil of white oak trees in 2011 were used. A container of ten seedlings was used for inoculation with each isolate (one replicate per isolate). Inoculum consisted of six mm plugs cut from the growing edge of seven day old colonies of *P. cinnamomi* grown on cV8 agar at 20°C. Control inoculum consisted of six mm plugs cut from sterile cV8 agar plates. Seedlings were wounded with a sterile razor blade about three cm above the soil line, inoculated with agar plugs, covered with a layer of sterile moist cotton, and sealed using Parafilm. After two months, the Parafilm and cotton were removed and bark tissue slightly shaved away to reveal the edges of any developing lesions (11). 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 oaks, soil texture and occurrence of *Phytophthora* 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* was present and absent. Stem lesion sizes on

inoculated seedlings were analyzed using ANOVA, 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 non-parametric test. Significant differences were set at *P*

≤ 0.05 and the software JMP® 10.0.2 was used for all statistical evaluations (SAS Institute Inc.,

Cary, NC).

Results

Overall, an approximately equal number of healthy (200) and declining (208) white oaks

were sampled. Usually, declining trees were scattered within each stand; there were no sites with

widespread areas of continuous decline.

Phytophthora-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, infection

caused by *Armillaria* spp. and *Biscogniauxia* spp. were not observed. While rhizomorphs of

Armillaria were present at some sites, no fruiting structures or mycelial fans were evident on any

sampled trees.

Isolation results and species identified

Phytophthora was isolated from 43% of all sites sampled between 2011 and 2012 (44

positive out of 102 total sites) (Fig. 1). When eight sites that were *Phytophthora*-negative in

2011 were resampled in 2012, only one of these sites resulted in the isolation of a *Phytophthora*

species. *Phytophthora* was ultimately isolated from soil from 10 out of 17 plant species (Table

1). Trees in the genera *Acer*, *Fagus*, and *Quercus* commonly yielded *Phytophthora*, but

Phytophthora was almost never found beneath the understory shrub species *Hamamelis virginiana*, *Kalmia latifolia*, and *Vaccinium sp*.
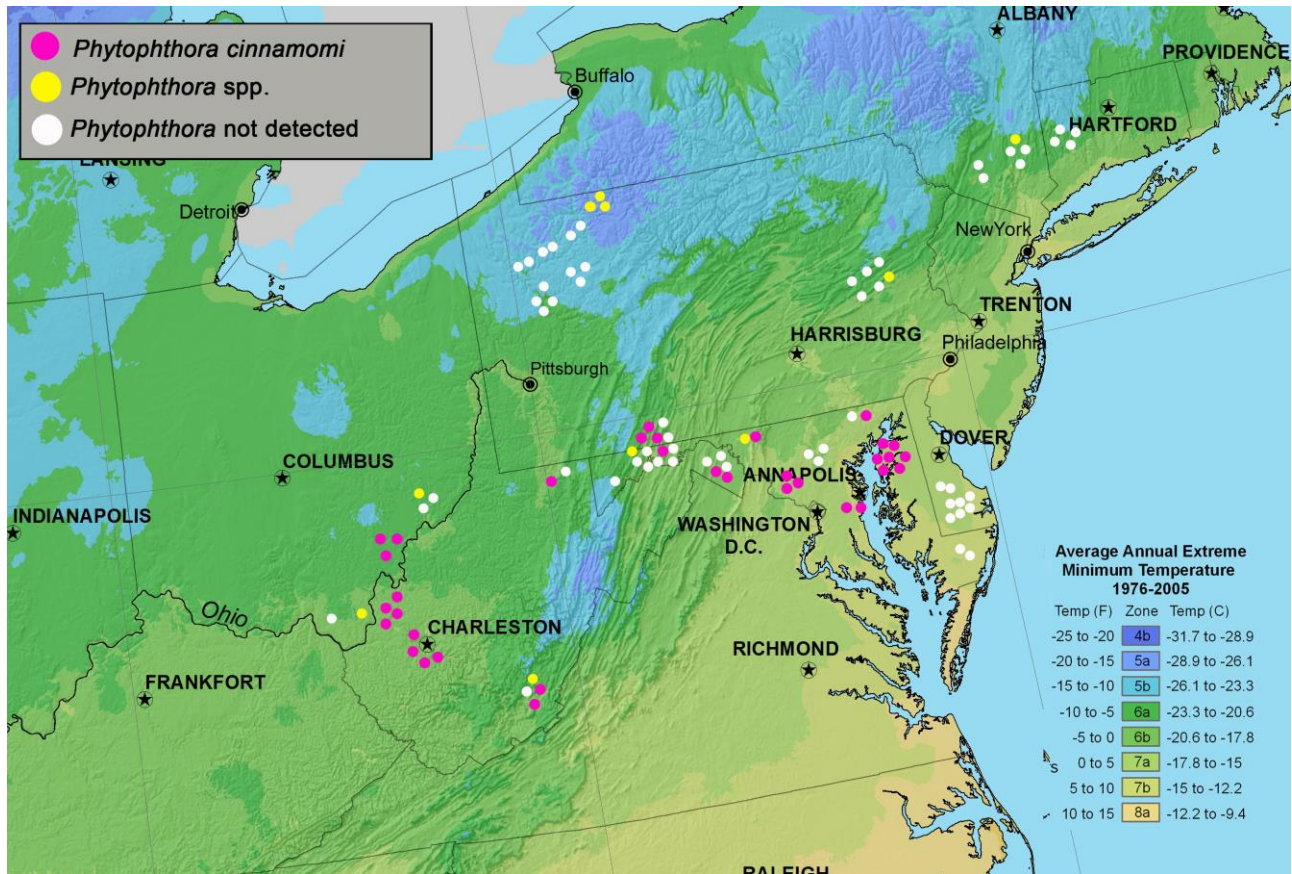
Figure 1. Geographical distribution of *Phytophthora cinnamomi* and six other *Phytophthora* species. Study site locations were mapped within USDA plant hardiness zones.

In total, 622 soil samples were collected. This included soil from 408 white oaks, 32 of them resampled a second time, as well as 182 other hosts. One hundred and one samples yielded colonies of *Phytophthora* upon the first baiting, and a further 31 samples yielded positive results after they were air-dried, re-flooded, 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*, the samples were re-baited using English oak leaflets. This resulted in the isolation of *Phytophthora* 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: 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: KF271790), which was isolated from seven sites. Other *Phytophthora* species were isolated sporadically and included *P. cryptogea* (GenBank Accession: KF271791), *P. europaea* (GenBank Accession: KF271788), *P. pini* (GenBank Accession: KF271787), *P. plurivora* (GenBank Accession: KF271789), and *P. quercetorum* (GenBank Accession: 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.

Table 1. *Phytophthora* species isolated from rhizosphere soil samples collected in Mid-Atlantic oak forests from 2011-2012.

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

[a] Numbers in parentheses represent isolates recovered from individual trees.

Incidence of *Phytophthora* spp. 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* species 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, while the other *Phytophthora* species 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 occurrence of *Phytophthora* species at infested sites analyzed in relation to soil textures across the study sites, only *P. cinnamomi* was more often associated with loam soils ($P < 0.028$). The occurrence of other *Phytophthora* species was not associated with silt loams ($P < 0.844$), silty clays ($P < 0.982$), or sandy loams ($P < 0.112$).
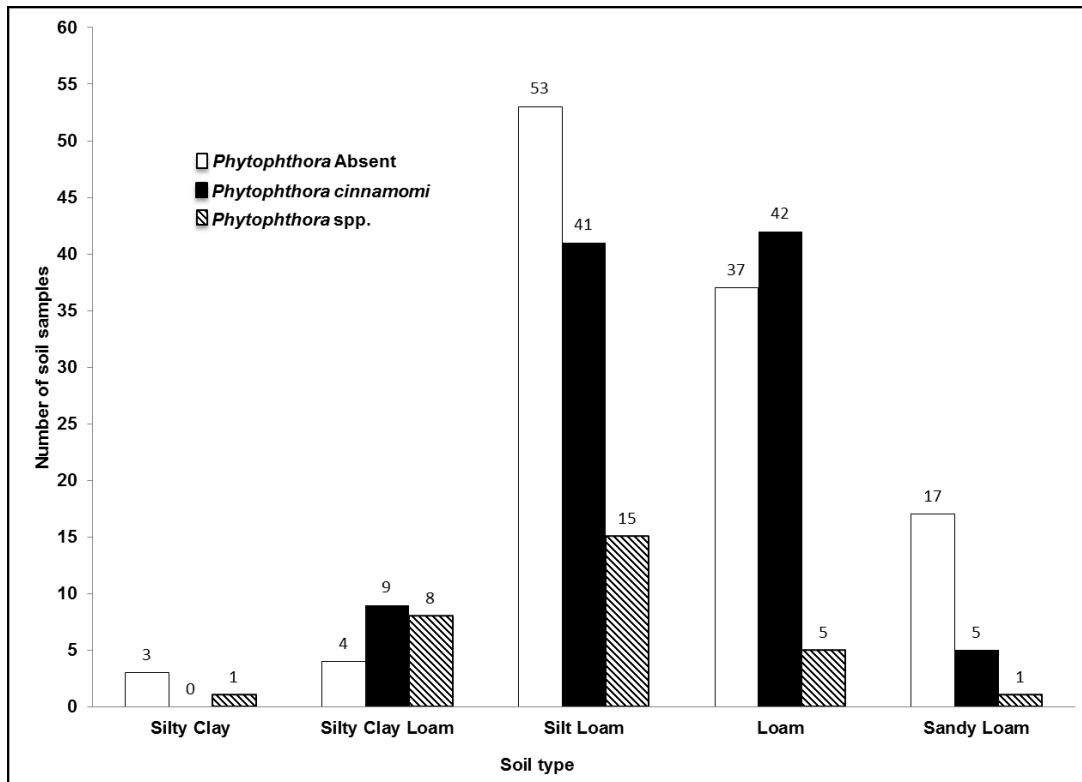
Figure 2. Frequency of isolation of *Phytophthora* from different soil types at infested sites. "*Phytophthora* spp." excludes *Phytophthora cinnamomi* and represents all other species isolated, including *Phytophthora cambivora*, *Phytophthora cryptogea*, *Phytophthora europaea*, *Phytophthora pini, Phytophthora plurivora,* and *Phytophthora quercetorum.*

No associations were found between crown condition and presence of *Phytophthora* 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 oaks than from declining white oaks. In contrast, other species of *Phytophthora* were isolated more often from declining trees than healthy trees, though this trend was not significant (Table 2).

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

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

[a] *Phytophthora* spp. include *P. cambivora*, *P. cryptogea*, *P. europaea*, *P. pini, P. plurivora*, and *P. quercetorum*
[b] 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

Colony-forming units of *P. cinnamomi* in relation to crown dieback, host type, and hardiness zone

P. cinnamomi propagule levels (colony-forming units) in soil were 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* with baiting, however, no viable CFU were detected with the wet-sieving method. The CFU from 2011 samples (Avg: 20 ± 31) were not significantly different from 2012 (Avg: 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, respectively (Table 3). When CFU were analyzed in relation to USDA hardiness zones, soils from the more

southern zone seven had 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 was combined (Table 4).

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

Host	*n*	Mean ± Stdev
Acer rubrum	10	18 ± 37
Acer saccharum	3	15 ± 5
Fagus grandifolia	3	34 ± 44
Kalmia latifolia	1	3
Liriodendron tulipifera	1	1
Quercus alba	73	18 ± 26
Quercus prinus	4	41 ± 39
Quercus rubra	3	28 ± 15
Quercus velutina	1	34

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

Year	USDA Hardiness Zone	*n*	Mean ± 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		

White oak fine root status in relation to sampling year, hardiness zone, crown status, and presence of *Phytophthora* spp.

Total white oak fine roots (0-1.5 mm in diameter) collected in 2011 and 2012 differed significantly between sampling years. Therefore, fine root data was separately analyzed for each year. Far fewer fine roots were present in the second year of the study (Avg: 2011= 3457 cm*, Avg: 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 to zones six and seven (*P*= <0.0001). This was also true in 2012 (*P* < <0.0001).

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

In 2011, trees at sites where no *Phytophthora* was detected had similar amounts of fine roots regardless of the crown status (*P* < 0.8202). In contrast, when *P. cinnamomi* or other *Phytophthora* species were present, a significant negative relationship 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

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were present on declining trees when other *Phytophthora* species were present ($P < 0.0360$),

although this was not significant for sites infested with *P. cinnamomi* ($P < 0.4712$).

Stem inoculation experiment

Twenty-nine out 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 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).

Table 5. Mean total fine root lengths (cm) and standard deviations collected from four soil pits (30 cm x 30 cm x 30 cm) of white oak

trees at sites infested with or free of *Phytophthora* in both sampling years and at different USDA hardiness zones.

Year	Hardiness Zone	Root Diameter (mm)	n	Mean Total Fine Root Lengths (cm)				
				Phytophthora Absent	n	*Phytophthora* Present	F value	*P > F*
2011	5	0-0.5	24	1123 ± 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**[a]
	6	0-0.5	28	3010 ± 2244	32	2658 ± 1482	0.5265	0.471
		0.5-1	28	1069 ± 738	32	1040 ± 541	0.0311	0.8606
		1-1.5	28	541 ± 341	32	532 ± 269	0.0135	0.9079
	7	0-0.5	24	1523 ± 1176	24	2747 ± 2078	6.3156	0.0155
		0.5-1	24	517 ± 391	24	1019 ± 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	1739 ± 136	56	1499 ± 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	1186 ± 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] Significant differences are marked in bold ($P > 0.05$).
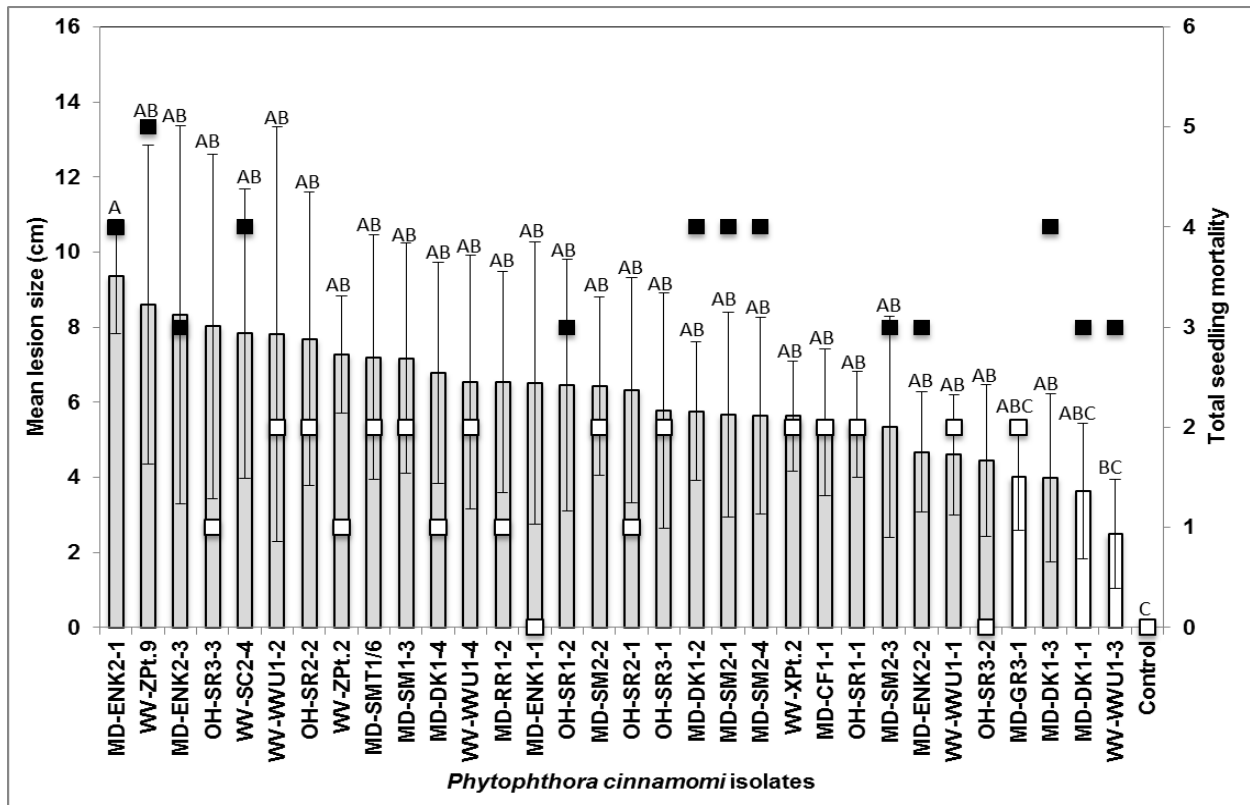
Figure 3. Average length of stem lesions (bars) and total number of one-year-old *Quercus alba* seedlings killed (boxes) by *Phytophthora cinnamomi* after two months of incubation. Error bars indicate standard deviations of lesion sizes. Gray bars and black squares indicate significant differences of mean lesion size or mortality rates compared to controls, respectively. Black squares indicate total mortality for the group of 10 seedlings inoculated with each isolate. Column means not designated by the same letter are significantly different.

Discussion

Similar to previous studies in eastern US and European oak forests, about half of the sites surveyed in our study were infested with *Phytophthora* (10, 14, 58, 59, 131). The species of *Phytophthora* identified in our study were similar to previous oak forest surveys in the eastern US except for *P. cryptogea*, which has not been previously isolated (10, 15, 85). The species isolated also largely overlap 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 (13, 14, 55, 57, 58, 60, 131). In addition, *P. quercetorum* is only known to exist in US oak forests (8).

Re-baiting 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* 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 like *P. quercina*, *P. quercetorum*, and *P. cactorum* (10, 14, 52, 60). In our study, air-drying soil before re-baiting increased the isolation frequency of both hetero- and homothallic species. The heterothallic species *P. cinnamomi* was isolated in eighteen instances only after soil was subject to air-drying after the first isolation attempt failed. In Western Australia, where *P. cinnamomi* is widespread in eucalyptus forests, other survival structures besides chlamydospores (oospores and hyphal aggregates) have been detected (62). 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 US oak forests.

Many different plant species have been used to bait *Phytophthora* from soil, and while 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 than 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* with a compound microscope, while rhododendron leaves must be plated before the presence of *Phytophthora* can be confirmed. Also, while 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* species. Particularly, if a higher *Pythium* population exists in a soil sample, any *Phytophthora* present may be missed when baiting. Rhododendron leaf disks are commonly used to overcome this problem, but wounding bait material favors *Pythium* colonization, which can also considerably reduce success in isolating *Phytophthora* spp. (77).

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. Never the less, our results correspond with similar studies. A study of *P. cinnamomi* causing disease on cork oak (*Quercus suber*) in Portugal noted that while *P. cinnamomi* was not significantly associated with any one soil type, it was more frequently isolated from soils with a higher silt/clay content (89). In Germany, a significant association was found between *Phytophthora* species and their presence in soils with a loamy, silty, or clayey texture, but not for sandy or sandy loam soils (60). Similarly, in Swedish oak forests, *Phytophthora* spp. were isolated from all textures but sandy (55). 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 (91). 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 capacity (19, 60). Calcium is a necessary element needed for both growth and reproduction of *Phytophthora* species (38), which may explain why soils with a finer texture

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result in a greater number of *Phytophthora* isolations. While most oak forest studies in Europe and the US 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 (84, 109). 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 (85, 108, 109). 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 non-infested in the past.

At infested sites, when sampling was expanded to include other plant species in addition to white oaks, *Phytophthora* was detected from about half of the plant species sampled. While all hosts sampled in this study have known associations with various *Phytophthora* species, *P. quercetorum* was isolated from soil collected from beneath red maple (*Acer rubrum*) for the first time; new host associations for *P. cinnamomi* in soil included red maple, sugar maple (*Acer 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 (41).

P. cinnamomi propagule levels 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 (105, 109). 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 at declining white oaks than at oaks without any crown dieback (15). The same conclusion was reached when CFU of declining white oak

stands of the same forest site were quantified for two years and found to be significantly greater at declining trees than at healthy ones both years (91). 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 had 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 lesion development on white oak seedlings. Furthermore, the isolates were nearly equally aggressive, a testament to the virulence of the *P. cinnamomi* population in eastern US forests. Other studies have also shown the pathogenicity of *P. cinnamomi* isolates from the eastern US on various oaks (11, 56, 91) and, similar to our findings, little variation existed in aggressiveness of *P. cinnamomi* (35, 121). While 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 the distribution of *P. cinnamomi* in eastern US 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 (6, 12, 15). In our survey, 32 sites were located above 40°N latitude and *P. cinnamomi* was never found at these sites. Such limits were not present for the other *Phytophthora* species. 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 environmental conditions. Soils north of 40°N latitude may not be 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 15-17°C during the summer months (127). In contrast, soils in USDA hardiness zones six and seven both reach 24-27°C, but soils in zone seven remain at this range for about two months longer each year than soils in zone six (127). In addition, soil temperatures during winter in zone seven are roughly five degrees 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 inhibit the survival of the pathogen. If temperatures continue to rise due to climate change, the range of *P. cinnamomi* might expand 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 (18, 20).

Unlike other oak decline studies that used crown symptoms as a way to measure the impact of *Phytophthora*, in this study we evaluated the root system of white oaks as a variable that might better demonstrate this interaction. When incidence of *Phytophthora* was evaluated based on crown symptoms alone, no significant connection was found for either year, however, when data was analyzed including root status as a parameter, a different conclusion was reached. During our first sampling year, reduction of fine roots was related to crown dieback at *Phytophthora*-infested sites (data not shown). However, this connection was not present in the second year of the study. This result agrees with some previous research (14, 15, 131), but also contradicts other studies where an association was noted based on the crown symptoms alone (13, 30, 60, 89, 104). The inconsistent association between the presence of *Phytophthora* species

in soil, the crown status and root health of declining oaks illustrates the dynamic changes that occur over time between the plant, pathogen, and environment, and the fact that 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 of the root dynamics of mature white oaks in the mid-Atlantic region. A difference in fine root status emerged between sampling years regardless of whether or not the pathogen was present. While fewer roots were available at *Phytophthora*-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 occurred 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 (36). 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 since the infectious cycle of *Phytophthora* 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 (78, 101, 125).

The effect of environmental conditions on the interaction between host and pathogen became even clearer when data was separated by hardiness zone. In the northern zone five, white oaks had less roots compared to the more southern zones six and seven. This was somewhat expected since cooler temperatures might have contributed to less root production by white oaks (122). In zone five, presence of *Phytophthora* spp. significantly reduced the fine roots of trees at

infested sites in both years. However, *Phytophthora* was 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* 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 (15). 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 (21, 30, 43, 89, 104). Such conclusions were also reached during pot experiments; damage to oak seedlings was shown to increase when they were subjected to drought or saturated conditions (59, 78, 79, 91, 104). 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 (20, 43). The climate in mid-Atlantic US forests is not as warm as Mediterranean Europe, but if drought events become more frequent, and 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, compared to the two other zones. While *P. cinnamomi* population levels were twice as high in this zone, infected trees had also 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 *Phytophthora nicotianae* and *Phytophthora palmivora* (139). Similarly, beech trees infected with *Phytophthora* spp. had greater fine root lengths (92). More fine roots were also noted with various oak seedlings that remained alive after they had been exposed to *Phytophthora* infection for eighteen months under greenhouse conditions (Y. Balci, *unpublished*). While the greater fine root amounts collected from zone seven may seem counterintuitive when a fine root pathogen like *P. cinnamomi* is involved, this observation 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 production of more fine roots, thereby 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 oaks in mid-Atlantic forests to examine the possible effects of *P. cinnamomi* on the root system. We have shown that the presence of *Phytophthora* 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 when and if *P. cinnamomi* acts as a trigger of oak decline in areas where its biology is favored. While 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 results from the interplay of multiple factors classified as predisposing, contributing and

inciting (73, 113). Some of the most serious threats to white oak in the eastern US include gypsy moth defoliation, insect borers, competition from successional plant species, drought, and root rot caused by *Armillaria* spp. (22, 51, 64, 70). 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 isolated *Phytophthora* spp. are contributors to the decline complex as a whole.

Chapter 3: Fine root dynamics of oak seedlings in response to *Phytophthora cinnamomi* infection under different temperatures and durations

Introduction

Oak decline in the eastern US is most commonly reported in the southeastern states, west to Missouri, and north into the mid-Atlantic region (40, 42, 64, 66, 96). In most of these reports, species within the red oak group were reported as being commonly affected. However, more recently, decline of white oaks in eastern US forests has been described (1, 15, 81, 91). Many of the declining white oaks are growing under seemingly adequate conditions, making the observed mortality all the more puzzling. Causes of oak decline in the eastern US are thought to include multiple predisposing, inciting, and contributing factors (73, 113). The most common predisposing factor is advanced tree age, and the most common inciting factor is drought; contributing factors include insect defoliation and borer damage, as well as *Armillaria* root infection. Few other root pathogens have been investigated as contributors to the decline complex. Recent surveys exploring the possible involvement of *Phytophthora* yielded a diverse array of species; the most commonly isolated was *P. cinnamomi* (10, 12, 81). The pathogen is widely distributed in mid-Atlantic oak forests but has not been found above 40°N latitude, which is the approximate boundary between USDA plant hardiness zones five and six. *Phytophthora* species, and in some cases *P. cinnamomi* specifically, have proven to be direct contributors to oak declines in Europe (13, 14, 58, 61, 89, 104). In Mediterranean Europe, *P. cinnamomi* causes fine root rot and can also result in the formation of lesions on larger lateral roots and trunks of oaks (74, 101).

In the eastern US, the involvement of *P. cinnamomi* was specifically investigated in connection to white oak decline (Balci et al. 2010a; McConnell and Balci 2013; Nagle et al.

2010). While these studies provided evidence that *P. cinnamomi* is causing root mortality, the impact of the pathogen on fine roots was also greatly influenced by environmental parameters as reflected by plant hardiness zones (McConnell and Balci 2013). It is possible that lower soil temperatures are critical in determining the amount of oak root mortality caused by *P. cinnamomi* in each hardiness zone, as well as why the northward spread of *P. cinnamomi* is restricted in the region (Balci et al. 2007; McConnell and Balci 2013). In order to examine the biology of *P. cinnamomi*, we specifically examined the effects of exposure to various temperatures on the survival of the pathogen. Oak seedlings were inoculated with soilless potting media infested with *P. cinnamomi* and fine root lengths of white (*Q. alba*) and red (*Q. rubra*) oaks were monitored every month for ten months. In addition, *P. cinnamomi* CFU were quantified before and after exposure to five different temperatures and 12 incubation periods representative of actual soil temperatures present in the mid-Atlantic region.

Materials and Methods

Plant material

In early April 2012, one-year old bare-root red and white oak seedlings from Maryland's John S. Ayton State Tree Nursery were transplanted into 15 x 41 cm (experiment one) or 10 x 36 cm (experiment two) Treepots™ (Steuwe & Sons, Corvallis, OR) filled with soilless potting mix (Metro-Mix, 30-40% composted pine bark, composted peanut hulls, Canadian Sphagnum peat moss, dolomite lime). All seedlings were grown outdoors with regular irrigation for six months prior to the commencement of the experiment. Seedlings were then moved into a greenhouse with environmental controls set at 14-17°C during the day and 8-11°C during the night one month before inoculation. Seedlings grown in individual pots were randomly selected and placed in groups of three (fine root monitoring experiment) or in groups of seven (population level

experiment) in 19 liter containers to hold the tall pots upright and lined with a set of two white plastic bags for water retention.

Inoculum production and inoculation

Inoculum was prepared by adding ten 6-mm agar plugs with mycelia cut from the leading edge of a one-week-old *P. cinnamomi* isolate grown on clarified V8 agar (cV8) (10 g CaCO$_3$ per 1 liter of V8 juice spun down at 4,000 rpm for 10 min., 100 ml clarified V8 juice in 900 ml dH$_2$O with 10 g agar) to a flask containing sterile rice grains. Rice grains were prepared by autoclaving 50 g of rice and 36 ml of dH$_2$O for 20 minutes at 121°C twice in Erlenmeyer flasks, cooling between each cycle. After the agar plugs with mycelia were added, flasks were incubated for three weeks at 20°C and mixed by shaking once each week to ensure uniform colonization of the grains (Balci et al. 2008). Before use, rice grains were examined visually for the presence of mycelium and ten randomly chosen rice grains were plated on cV8 to check for colonization by *P. cinnamomi*. Control inoculum consisted of sterile rice grains inoculated with plugs of V8 agar. The isolate MD-ENK2-1 was used for inoculation, as it was determined to be the most aggressive on stems of white oak seedlings out of 32 isolates collected from mid-Atlantic forests (McConnell and Balci 2013). Approximately 2 g of rice grain inoculum was added to the potting mix around each seedling by distributing the grains in a depression made by a metal rod to a depth of 10 cm. Each hole was filled in with surrounding media after the grains were added. One month after the initial inoculation, all seedlings were inoculated a second time following the same procedure in order to assure the presence of *P. cinnamomi* in each pot. Immediately after each inoculation, pots were flooded for two weeks, and then watered periodically to keep potting media evenly moist for the remainder of the experiments.

Determination of white and red oak seedling root changes

This experiment was conducted in a greenhouse set to heat at 14°C and cool at 17°C during the day, and to heat at 8°C and cool at 11°C during the night. The monthly average temperature in the greenhouse section was 14.2°C during the first incubation period (December), and ranged from 22.2°C to 24.7°C during the 60 to 180 day incubation periods (January to May). During the 210 to 300 day incubation periods (June to September) in summer, the monthly average ranged from 26.9°C to 28.5°C. The experiment began on November 9, 2012 and ended on September 3, 2013. After each pot was inoculated with colonized rice grains, both red and white oaks were kept in the same greenhouse, though inoculated and control treatments were placed on separate benches. Ten incubation periods were used; the first incubation treatment was 30 days and each successive incubation was increased by 30 days, resulting in a final incubation period of 300 days. Each month, fifteen seedlings from each treatment (white oak + inoculum, white oak + sterile rice grains, red oak + inoculum, red oak + sterile rice grains) were harvested to examine changes in fine root lengths. Seedlings were randomly chosen from the bulk of potted seedlings and assigned randomly to each treatment when relocated indoors at the greenhouse. In total, 300 white and 297 red oak seedlings were used. At the time of harvest, seedlings were carefully removed from their pots, excess potting media was shaken off, and the root systems washed with tap water to remove all remaining potting media. All roots were collected in a basin and fine roots were separated from larger roots, placed in a pre-measured plastic tray, and scanned with the WinRHIZO Pro 5.0 software (Regent Instruments, Quebec) to measure fine root lengths. Samples often consisted of a large volume of roots, requiring the addition of multiple fine root scans to obtain total fine root lengths for one sample. If lesions were visible on the taproot or larger lateral roots, small necrotic sections were cultured on V8-based PARPNH

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selective media (with 10 μg/liter pimaricin, 200 μg/liter ampicillin, 10 μg/ml rifampicin, 25 μg/liter pentachloronitrobenzene [PCNB], 50 μg/liter nystatin, and 50 μg/liter hymexazol) in an attempt to re-isolate *P. cinnamomi* (11).

Effects of temperature/incubation duration on *P. cinnamomi* propagule quantity

This experiment was conducted using climate chambers (Environmental Growth Chambers, Chagrin Falls, OH). White oak seedlings were incubated for different periods at various temperatures in soilless media and infested with *P. cinnamomi*. For this experiment, 180 white oak seedlings were used; each treatment consisted of 15 seedlings. Treatment durations and temperatures were determined by analyzing soil temperature data collected from weather stations in the Natural Resource Conservation Service's Soil Climate Analysis Network (127). Soil temperature data was obtained from specific weather stations located in USDA hardiness zones five, six, and seven, the zones present in the mid-Atlantic region where *P. cinnamomi* was previously isolated (zones six and seven) or absent (zone five) (Table 6) (Balci et al. 2007; McConnell and Balci 2013).

Table 6. Average annual soil temperature and duration at each USDA hardiness zone from 2000 to 2010.

Hardiness zone[a]	Temperature range (°C)	Temperature[b]	Days[c]
Five	1-5	2	126
	5-10	7	73
	10-15	13	100
	>15	16	27
Six	1-5	3	85
	5-10	8	58
	10-15	13	58
	>15	21	149
Seven	1-5	4	6

5-10	8	66
10-15	12	75
>15	22	207

[a]: Climate data was obtained from weather stations representing the hardiness zones, including Lye Brook Falls (zone 5), Mahantango Creek (zone 6), and Tidewater AREC (zone 7).
[b]: Average annual soil temperature at a given range.
[c]: Average number of days the soil was at a given temperature for a one-year period.

Published *P. cinnamomi* growth temperatures and the average soil temperatures in different hardiness zones were used when designing this experiment. According to Zentmyer (1980), 5°C is the lowest temperature for mycelial growth, 9-12°C is the lowest range for chlamydospore germination, and 15°C is the lowest temperature where significant root infection is possible on several hosts. Based on these temperatures, four temperature ranges were identified, 1-5°C, 5-10°C, 10-15°C, and >15°C. For each of the temperature ranges, actual average yearly temperatures for the last decade were determined for each of the plant hardiness zones using Soil Climate Analysis Network data (Table 6) (Tolsdorf 2013). The average number of days per year when soil temperatures were within the given temperature ranges were identified in order to determine the incubation period for each average temperature (Table 6). Ultimately, four different timed temperature treatments were created for each of the three hardiness zones present in the mid-Atlantic region (Table 7). Separate treatments began at various points during the winter dormancy period, including November 2012, and February and March 2013.

Population levels (colony-forming units, or CFU) of *P. cinnamomi* were quantified from the root zone of the potted seedlings. The population in each pot containing a white oak seedling were quantified before and after each temperature/incubation treatment. For initial population determination, after inoculation and the initial two-week flooding period, approximately 50 g of potting mix was collected by hand in a re-sealable plastic bag from the root zone in each pot to a

depth of 5-15 cm and 6 cm from the stem. For the final population determination, all remaining media in each pot was shaken from the roots and collected in a re-sealable plastic bag. Potting mix samples from each seedling were stored at 4°C and processed within six weeks of collection. CFU were quantified using a modified wet-sieving method. After thoroughly mixing each potting mix sample inside the plastic bag, a 10 g subsample of potting media was taken and homogenized in 60 ml dH_2O at low speed in a Waring laboratory blender for 15-30 seconds. The blended slurry was filtered through nested 850, 250, 125- and 38 µm sieves in order to collect *P. cinnamomi* propagules. The material remaining on the 38 µm sieve was rinsed into a 100 ml beaker in approx. 20 ml distilled water. The soil suspension was then plated on two Petri dishes containing V8-PARPNH selective medium. After incubation in complete darkness at 17ºC for two days, Petri dishes were rinsed free of potting media and colonies of *P. cinnamomi* counted for two subsequent days (Balci et al. 2010a; McConnell and Balci 2013; Shew and Benson 1982). The total population in each pot was quantified using three replicates, utilizing 30 g of potting media in total. The remaining 20 g was used to determine the dry weight of the media. Two 10 g subsamples were dried in an oven at 60ºC to identify the total dry weight, and then CFU quantity per 100 g of dry soil was calculated.

Table 7. Temperature/duration combinations used to incubate inoculated white oak seedlings during experiment the propagule density experiment.

Hardiness Zone	Incubation period (days) at each temperature				
	3°C	8°C	13°C	16°C	21°C
5	126	73	100	27	n/a
6	85	58	58	n/a	149
7	6	66	75	n/a	207

Effects of temperature on *P. cinnamomi* in vitro

Cultures of *P. cinnamomi* isolated from zone six (five isolates) and zone seven (six isolates), including MD-ENK2-1, the isolate used for the inoculation experiments, were tested for their growth rates at different temperatures *in vitro* (Table 8). Using a 6 mm cork borer, agar plugs containing mycelium from 8-day old cultures grown on CV8 were transferred aseptically to Petri plates (8 cm diameter) containing CV8 (one plug per plate) and allowed to grow at room temperature for two days. The colony edges were then marked at four perpendicular points and the plates were placed in incubators at 4°C, 8°C, 13°C, 16°C, 21°C, and 25°C to approximate the growth chamber temperatures used in experiment two. A minimum of two replicates of each isolate was tested at each temperature. Isolates were incubated at a specific temperature and marked before the colony reached the edge of the plate. The daily growth rate at each of the four directions was measured and the average was divided by the number of incubation days to obtain daily growth rate (mm/day).

Table 8. Daily growth rate ± stdv of *Phytophthora cinnamomi* mycelium on clarified V8 juice agar at various temperatures. Letters indicate significant differences among the isolates according to ANOVA.

| Zone	Isolate	Colony growth rate (mm/day)											
		4°C		8°C		13°C		16°C		21°C		25°C	
6	WV-CS2-4	0 ± 0	A	1.4 ± 0.3	BC	9 ± 0.5	C	11.5 ± 0.2	C-E	16.9 ± 0.4	C	23.0 ± 0.1	C
6	MD-GR12-1	0 ± 0	A	1.5 ± 0.5	A-C	7.9 ± 0.1	CD	11.3 ± 0.1	C-F	17.1 ± 0.5	C	22.8 ± 0.4	C
6	WV-KW1-1	0 ± 0	A	1.6 ± 0.1	A-C	8.3 ± 0.8	CD	9.6 ± 0.4	F	17.3 ± 0.8	C	26.8 ± 0.7	B
6	WV-SC2-4	0 ± 0	A	1.6 ± 0.2	A-C	11.1 ± 0.8	B	14.7 ± 0	B	27.8 ± 0.4	B	27.5 ± 1.1	B
6	OH-SR3-1	0 ± 0	A	1.4 ± 0.1	A-C	13.9 ± 0.2	A	17.8 ± 0	A	27.8 ± 0.4	B	32.0 ± 0.1	A
7	MD-DK1-4	0 ± 0	A	1.5 ± 0.4	A-C	7.3 ± 0.7	CD	9.8 ± 0.9	EF	15.5 ± 0.5	C	23.1 ± 0.2	C
7	MD-DK2-2	0 ± 0	A	1.5 ± 0.3	A-C	8.2 ± 0.2	CD	10.3 ± 0.2	D-F	16.1 ± 0.6	C	23.6 ± 0.2	C
7	MD-ENK2-1	0 ± 0	A	1.3 ± 0.4	BC	6.6 ± 0.1	D	11.7 ± 0.5	CD	17.5 ± 0	C	19.1 ± 0.9	D
7	MD-ENK5-4	0 ± 0	A	2.5 ± 0.3	A	11.7 ± 0	B	12.5 ± 0.7	C	28.0 ± 0.7	B	33.6 ± 0.5	A
7	MD-RR1-2	0 ± 0	A	0.7 ± 0.1	C	7.8 ± 0.7	CD	10.8 ± 0.5	C-F	17.3 ± 0.7	C	22.4 ± 0.9	C
7	MD-SM2-3	0 ± 0	A	2.1 ± 0.1	AB	8.2 ± 0.5	CD	19.3 ± 0	A	33.8 ± 0.4	A	32.1 ± 0.2	A

Total fine root lengths were checked for normal distribution and equal variances, and if not, values were root or log transformed. Any significant differences between root lengths of inoculated white and red oak seedlings were compared to control seedlings using analysis of variance (ANOVA). Similarly, total root lengths of white and red oak seedlings after the ten different incubation periods were pooled and compared to the controls using ANOVA. A standard least square test was used to analyze the effects of incubation time and *P. cinnamomi* inoculation on total fine root lengths of red and white oak seedlings. The CFU levels at the beginning and end of the experiment after each temperature/duration treatment were separately analyzed. ANOVA was used to separate any differences in isolate growth rates at a particular temperature during *in vitro* growth experiments. Figures illustrating the trends in CFU quantities after exposure to different treatments were created using JMP Graph Builder. 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).

Results

White and red oak fine root status over 10 months

Mean fine root lengths of control and inoculated seedlings varied greatly during the ten different incubation periods. Over the course of the experiment, white and red oak fine root lengths appeared to correspond with normal seasonal growth patterns (Fig. 4 and 5). During the dormant period from December to February (white oak) or March (red oak), fine root lengths were lowest. As the temperature began to

rise, fine root lengths began to increase with a peak during April (white oak) or June (red oak). The impact of *P. cinnamomi* also became apparent after this period of growth in spring (Fig. 4 and 5). For example, inoculated white oak seedlings displayed greater amounts of fine root loss at the beginning of the growing season, resulting in significant differences in March (120 incubation days; $P < 0.0340$) and May (180 incubation days; $P < 0.0364$); a reduction in fine root lengths of 49% and 31%, respectively (Fig. 4). Plant responses (though not statistically significant) were also observed later in the experiment (after 210 incubation days) when infected seedlings produced equal or greater lengths of fine roots than controls (e.g. 270 and 300 incubation days) (Fig. 4 and 5). Over the course of the entire experiment, when white oak fine roots from the ten incubation periods were combined, inoculated white oak seedlings had 2% less fine roots compared to the controls, although this was not statistically significant (*F* ratio: 0.5131; $P < 0.4744$).
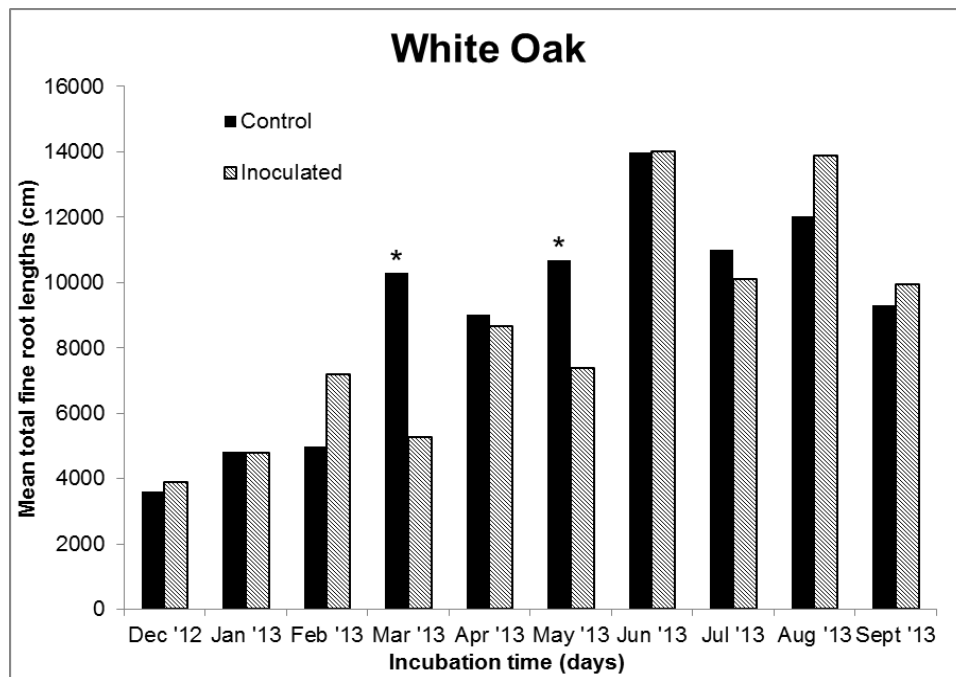
Fig. 4. Mean total fine root (0-1.5 mm diameter) lengths of inoculated and non-inoculated white oak seedlings harvested every 30 days for 300 days. Asterisks indicate significant differences according to ANOVA ($P < 0.05$)
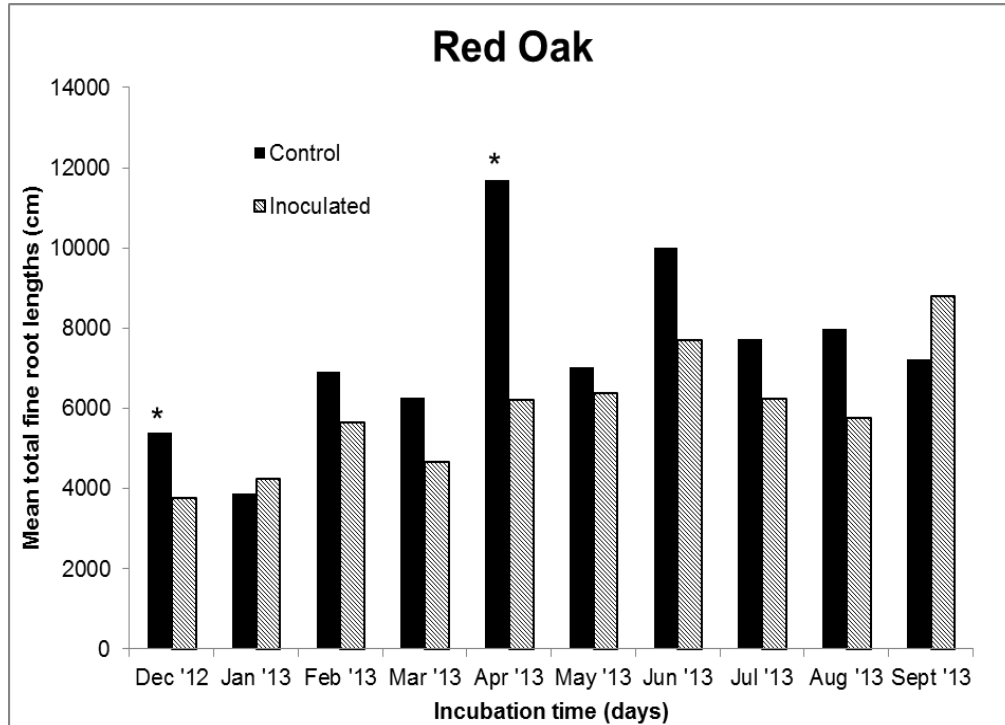
Fig. 5. Mean total fine root (0-1.5 mm diameter) lengths of inoculated and non-inoculated red oak seedlings harvested every 30 days for 300 days. Asterisks indicate significant differences according to ANOVA ($P < 0.05$)

While inoculated white oaks appeared to produce relatively similar amounts of fine roots compared to the controls during most of the incubation periods in response to *P. cinnamomi* infection, this was not always observed with red oaks. Fine root amounts from inoculated red oak seedlings were always lower (though this trend was not statistically significant) than the control seedlings, with the exceptions of the January (60 days of incubation) and September (300 days of incubation) harvest periods (Fig. 5). Significant differences, however, were only detected during

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December (30 incubation days; $P < 0.0352$) and April (150 incubation days; $P < 0.0006$) (Fig. 5). During these time periods, infected seedlings had differences in fine root amounts of 30% and 47%, respectively. Over the course of the entire experiment, when root lengths from the ten incubation periods were analyzed together, inoculated red oak seedlings had 17% less fine roots compared to control seedlings (F ratio: 12.3187; $P < 0.0005$).

In an effects test, the incubation time was a significant factor in influencing total fine root lengths of both oak species (Table 9). When the effect of *P. cinnamomi* was analyzed as a factor affecting available fine root lengths, the pathogen only significantly affected red oak seedlings. Likewise, the combined effect of *P. cinnamomi* and incubation time was a significant factor that affected total fine root lengths of red oaks but not white oaks (Table 9).

When the lateral and taproots of white and red oak seedlings were visually examined for necrotic lesions, more necrotic areas were detected on red oak seedlings. During the experiment, 15 inoculated red oaks developed lesions covering 25% or more of the taproot, and when cultured on PARPHN, *P. cinnamomi* was recovered from two of the inoculated seedlings. In contrast, only two inoculated white oak seedlings (out of four cultured) developed similar large lesions, and one resulted in a successful isolation of *P. cinnamomi*. While we observed some control seedlings (three red and two white oak seedlings) with necrotic areas, *P. cinnamomi* was never isolated from any control seedlings.

Table 9. The effects of *Phytophthora cinnamomi* inoculation and incubation time on total fine root amounts of red and white oak seedlings.

Plant species	Effect	DF	F Ratio	$P > F$
Quercus rubra	Incubation time	9	7.0397	**<0.0001**
	Inoculated vs. Control	1	13.8229	**0.0002**
	Incubation time * Inoculated vs. Control	9	2.1299	**0.0272**
Quercus alba	Incubation time	9	15.4126	**<0.0001**
	Inoculated vs. Control	1	0.7476	0.3880
	Incubation time * Inoculated vs. Control	9	1.7265	0.0828

Effects of temperature on *P. cinnamomi* propagule quantities

Temperature significantly influenced *P. cinnamomi* propagule quantities. Quantities at the beginning of the experiment ranged from 0 to 2278 propagules per 100 g of dry soil and were quite variable from pot to pot. However, average starting propagule levels for the five different temperature treatments (3°C, 8°C, 13°C, 16°C, and 21°C) were 310, 298, 445, 436, and 496, and were not significantly different ($F = 1.4521$; $P < 0.2189$). By the end of the experiment, propagule levels ranged from 0 to 1018 propagules per 100 g of dry soil, and significant differences occurred after incubation at different temperature/exposure treatments ($F = 30.3963$; $P < 0.0001$). When the effect of exposure time at a particular temperature was analyzed, all durations at a particular temperature resulted in either a significant (13°C and 21°C; $P < 0.0001$ and $P < 0.0001$, respectively), or not significant (8°C and 16°C; $P < 0.5455$ and $P < 0.1021$, respectively) decrease in CFU quantity. The only exception was the 85-day incubation period at 3°C; propagule levels decreased, but this change was not significant ($P < 0.1016$). When the starting and ending propagule levels were compared for each temperature treatment, a significant reduction in propagule

quantity occurred at all temperatures ($P < 0.0001$) except 8°C and 16°C ($P < 0.7789$ and $P < 0.1865$, respectively) (Fig. 6).

Isolate growth test

All eleven isolates tested on CV8 medium showed an increase in growth at higher incubation temperatures (Table 8). At the two lowest temperatures, 4°C and 8°C, little or no growth was recorded. Growth rates began to increase at 13°C, and one isolate from zone six and one isolate from zone seven (SC2-4 and SM2-3) reached their optimum growth temperatures at 21°C, while growth of the remaining isolates continued to increase at 25°C. MD-ENK2-1, the isolate used in the soil inoculations for both experiments, proved to be one of the more slower-growing isolates tested, but still had an optimum temperature at or beyond 25°C. Significant differences in growth rate existed between some isolates at every temperature but 4°C, where no growth occurred. Growth rates of each isolate also varied when they were compared by their hardiness zone of origin.
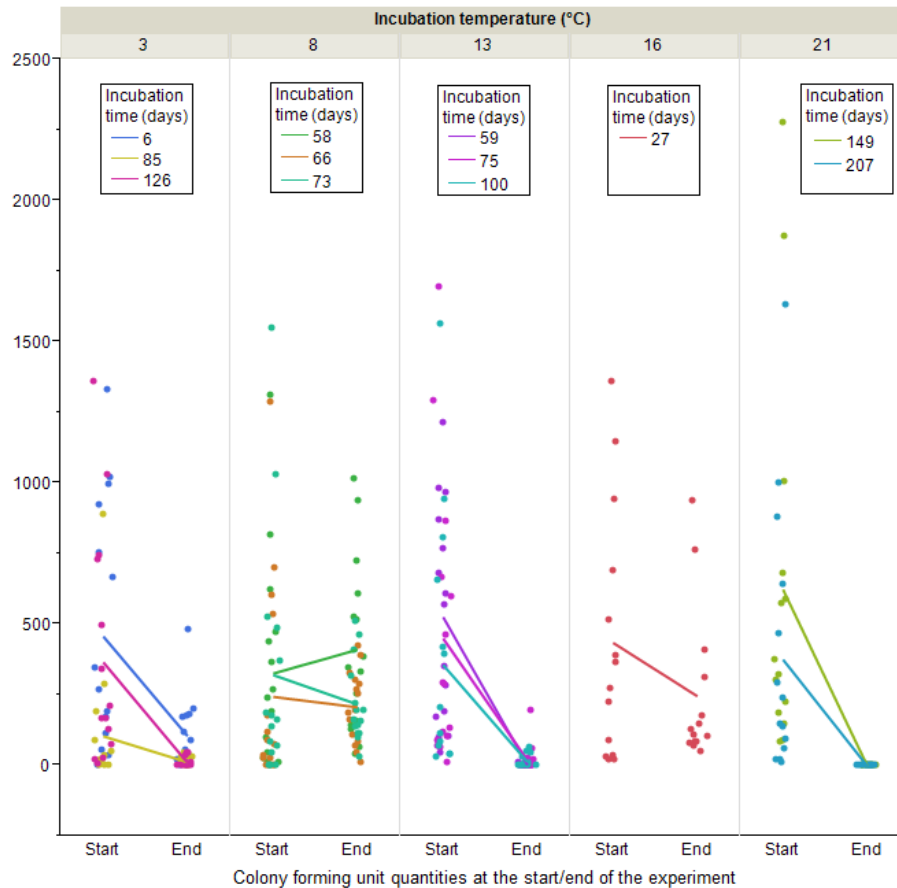
Fig. 6. *Phytophthora cinnamomi* colony forming unit quantities at the beginning and end of each temperature/incubation treatment.

<u>*Discussion*</u>

Our inoculation experiment designed to examine fine root lengths of oak seedlings over a ten-month incubation period demonstrated that while *P. cinnamomi* alone causes damage to fine roots, host phenology plays a significant role in determining root health. Numerous other inoculation experiments have shown that *P. cinnamomi* causes significant damage to the root and stem tissue of inoculated oak seedlings (11, 76, 78, 79, 101, 102). The primary aim of these studies was to determine the aggressiveness of the pathogen; thus, plant responses over time were

not considered. In our study, however, we observed that seedling root responses change over time, and that the impact of the pathogen was most noticeable when the greatest amounts of fine roots were produced. In addition, plant responses took much longer to appear and were evident only after incubation for an extended time period.

Oak species responded differently to *P. cinnamomi* fine root infection. While inoculated white oaks experienced a limited amount of fine root loss over the course of the experiment, inoculated red oaks experienced significant fine root reduction. Indeed, inoculation with *P. cinnamomi* was not a significant parameter overall when it came to white oak fine root changes. In a previous study utilizing species from both the red (*Quercus* section *Lobatae*) and white (*Quercus* section *Quercus*) oak groups that were soil-inoculated with *P. cinnamomi*, seedlings in the white oak group were less susceptible to pathogen damage than those in the red oak group (Balci et al. 2008). In eastern US forests, decline of species in the red oak group has been observed and reported more frequently than decline of white oak species, especially in the southeastern part of the country (40, 112, 119). Our comparative root inoculation experiments suggest that this scenario might be occurring at *P. cinnamomi*-infested declining field sites. In addition, red oaks more often colonize droughty, nutrient poor sites (64). As a result, we hypothesize that at infested sites, red oaks may be under greater stress due to increased fine root loss and are thus more likely to be declining than white oaks.

During our inoculation experiments, we did observe a limited amount of taproot infection. In these inoculation experiments, we only flooded our seedlings once before resuming regular watering, which might have been less conducive to

pathogen development. This may explain why we observed limited taproot infections as compared to numerous other inoculation experiments (Balci et al. 2008; Jung et al. 1996; Jönsson et al. 2003; Nagle et al. 2010; Robin et al. 2001). In these studies, severe root disease was observed after *Phytophthora* soil inoculation when regular flooding and/or drought was incorporated into the watering regime. However, our finding agrees with field findings from central Appalachian oak forests. When root systems of red and white oaks at numerous infested sites were excavated and isolation was attempted, a limited amount of necrotic lesions were detected, and few yielded positive isolation of *P. cinnamomi* despite extensive plating (Balci et al. 2010a, b, McConnell & Balci 2013).

We observed clear changes in fine root amounts due to the presence of *P. cinnamomi*, but root changes due to typical plant phenology were also evident. After inoculation in winter 2012, the first spike in growth of red and white oak fine roots occurred during March and April, coinciding with spring bud-break and the growth of new leaves. Spring is known to be the first major period of root elongation during the white oak growing season (122). Interestingly, this period was also when a significant reduction in fine root amounts of inoculated seedlings occurred for both species. Several studies examining tree root survival concluded that newly produced fine roots are most susceptible to infection, degradation, and mortality, especially in warmer soils, possibly due to the vulnerability of newly formed root tissue (50, 134). Our data suggest that these newly-formed early-season fine roots are most susceptible to *P. cinnamomi* infection. This is perhaps another reason why greater isolation success is achieved in the field when samples are collected during spring rather than mid-

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summer or fall (Balci et al. 2007). In addition to fine roots possibly being more susceptible during this time, this period represents the time of year when soils are cool, moist, and apparently more conducive to supporting *Phytophthora* populations, as shown in our CFU quantification experiment.

Most incubation periods for soil inoculation experiments involving *Phytophthora* are much shorter than our ten-month trial. Shorter incubation periods fail to illustrate the changes in inoculated root systems over time. In numerous experiments that soil-inoculated oaks with *Phytophthora* spp., significant damage to root systems was nearly always observed (Balci et al. 2008; Jung et al. 1996; Jönsson et al. 2003; Marcais et al. 1996; Robin et al. 2001; Robin and Desprez-Loustau 1998). Indeed, this was also the case during our experiment, when inoculated red and white oaks had less fine roots than controls approximately 140 days into the study. However, by the end of our experiment, root systems of both species were showing signs of recovery, as root amounts from inoculated seedlings roughly equaled or surpassed control root systems. Shorter incubation times will certainly answer the question of whether or not the pathogen can infect the host and damage roots, but only longer incubation periods can illustrate how the root system responds to infection over time as well as more accurately illustrate the susceptibility of a particular species.

To our knowledge, this is the first time a study has been done on the effects of temperature on *P. cinnamomi* propagules (or CFU) in soil in the presence of an oak host. The significant reductions in propagule quantities that we observed in our experiment were probably due to a combination of low incubation temperature and in

some cases, the incubation duration. The significant decrease in propagule quantity in pots incubated at 3°C was most likely due to temperature, because a steep decline in propagule quantity occurred even during the shortest incubation time of six days. The effects of incubation at 3°C on *P. cinnamomi* have not been tested before, but in forest soils infested with *P. cinnamomi* in Australia and the US state of Hawaii, it has been observed that the pathogen is not active in soil at temperatures below 10°C, or when soils become very dry (67, 135-137). Also, in our growth tests on artificial culture media, *P. cinnamomi* showed no growth below 5°C (Zentmyer 1980). We assume that incubation at such a low temperature had an almost immediate negative effect on the pathogen, both on the mycelial growth and available propagules in soil. Significant reductions in CFU also occurred in pots incubated at 13°C and 21°C. These reductions in propagule quantity may have occurred due to prolonged incubation time. When Kuhlman (69) inoculated forest and agricultural soil with *P. cinnamomi* and incubated the soil in bottles for 18 months, pathogen survival in all bottles was "moderately poor," even in the presence of a susceptible Douglas fir seedlings. Also, chlamydospores of *P. cinnamomi* rarely formed under favorable conditions in inoculated pot tests, instead, more mycelium grew on the surface of available roots (Weste and Ruppin 1977). Propagule quantities in pots incubated at 8°C were the most puzzling, as they remained approximately equal after two months of incubation from the beginning to the end of the experiment. It is not clear why propagule quantities remained constant at this temperature. One possible explanation is that 8°C represents the optimum soil temperature, where new fine roots are still produced but fewer antagonistic organisms are active, allowing *P. cinnamomi* to

reproduce successfully. Knowledge on the effects of soil microbial community on *P. cinnamomi* at different temperatures will greatly aid us in understanding the mechanism of survival of this pathogen in eastern oak forests.

In potting media, propagule densities significantly decreased with increasing temperatures, while mycelial growth steadily increased in Petri dish experiments under the same conditions. This finding significantly contradicts our attempts to define the optimum growth temperature of *P. cinnamomi*, and other *Phytophthora* spp., which are primarily based on mycelial growth on artificial growth media. Thus, our data suggests that when identifying forest areas at risk, propagules of *Phytophthora* should be examined in the context of the natural setting in order to reveal the true biological significance of temperature.

Low temperatures and prolonged incubation periods at certain temperatures had a negative effect on the survival of *P. cinnamomi* in potting media despite the presence of a susceptible host. The decrease in viable propagules at the lowest soil temperature provides additional support for the hypothesis that the northward spread of *P. cinnamomi* in the mid-Atlantic region is limited by cooler soil temperatures that inhibit pathogen growth. Although *P. cinnamomi* is causing fine root damage, its impact on oaks appears to be tempered by the varying level of susceptible oak species present in eastern oak forests. Also, our experiments were conducted over a one-year period, but the likelihood of longer-term fine root survival in the presence of the pathogen is unknown; studies in other systems indicate that root lifespans are shortened (68). We have shown that *P. cinnamomi* can cause significant damage to oak fine roots, and therefore is likely involved in oak decline in mid-Atlantic US

forests. Its impact possibly becomes more detrimental when trees are already stressed due to unusual weather pattern or site conditions. Continuous fine root loss caused by *P. cinnamomi* can become more critical if in combination with other decline-causing factors. Long-term studies targeting the significance of *P. cinnamomi*-mediated fine root loss in the presence of other stressors common to oak decline complexes like drought could better explain the decline phenomenon at infested sites.

Chapter 4: Discussion and future directions

General discussion

Over the course of this research project, I sought to analyze the impact of *P. cinnamomi* on white oak roots collected from naturally infested forest soil, as well as on oak seedling roots artificially inoculated in the greenhouse. I also mapped the distribution of *P. cinnamomi* in mid-Atlantic forests and analyzed connections between the presence of the pathogen in soil and white oak root and crown health. Finally, I investigated the effects of temperature on *P. cinnamomi* population levels in soilless potting media and growth on artificial culture media to test the hypothesis that the spread of *P. cinnamomi* northward is limited by soil temperature.

Fine root lengths from infested and *P. cinnamomi*-free trees varied; this variation was linked both to geography and site-specific growing conditions. Environmental effects on both root growth and root loss were evident. Overall, regardless of whether *Phytophthora* spp. was present or absent, less fine roots were collected from the northernmost zone, five, compared to zones six and seven, possibly due to the fact that there is less fine root turnover in cooler soils (50). During the seedling inoculation experiment, seasonal effects on fine root growth were readily observed, with a spike in growth of control plants during spring when temperatures began to warm. This was also the time period that fine root lengths of inoculated root systems were significantly reduced, as the newly formed fine roots were likely quite susceptible to *P. cinnamomi*.

In northern field sites (e.g. plant hardiness zones five and six), a distinct reduction in fine roots existed between the infested and *P. cinnamomi*-free trees.

Generally, soils in zones five and six were cooler and wetter than those in zone seven, perhaps providing an adequate soil environment for *Phytophthora* to cause greater amounts of fine root damage. At field sites in southern locations (e.g. zone seven), infected trees, surprisingly, had more fine roots than non-infested trees. One possible explanation could be that white oaks sampled in zone seven may have produced more fine roots in response to drought, as has been noted in field studies of several oak species (29, 72, 122). In addition higher soil temperatures could favor greater root production (50, 99). In the field, significant differences between root amounts from infested and non-infested sites were more evident in the second, drier year of the study. Another explanation for why more roots are produced in this zone could be that the trees are responding to the presence of *P. cinnamomi*, as has happened in a *Phytophthora nicotianae*-citrus system (68). In zone seven, propagule levels were double the levels present in zone six. The higher population of *P. cinnamomi* in zone seven might have caused more fine root damage and thus resulted in the response of elevated fine root production. A combination of these factors might also explain the field observations. Overall, our field and greenhouse observations suggest that oak fine root dynamics are influenced by the interaction of species phenology, site-level environmental conditions, and the presence of *Phytophthora*, rather than one particular determining factor.

Field results did not show any significant associations between fine root lengths and *P. cinnamomi* CFU quantities, or between crown condition, fine root length, and CFU level. This is in contrast to previous studies, which noted that higher *P. cinnamomi* population levels in forest soil and artificial inoculation experiments

corresponded with more severe damage to white oak roots (15, 91). The lack of an observed connection between fine roots and *P. cinnamomi* CFU may have been due to the fact that sampled sites in this study included a wide variety of environmental conditions, rather than a single state forest. No significant associations between the presence of *P. cinnamomi*, CFU quantity, and crown health were noted in inoculated seedlings used in this study either. These observations corresponded well with previous findings (15). It appears that in the case of white oak decline in the mid-Atlantic region, crown condition is not a precise predictor of fine root status, and that instead fine root amounts may give a more complete picture of tree health.

CFU levels after incubation at various temperatures were very different from population levels in field soils. CFU levels were lowest at the lowest incubation temperature, but also decreased at the three highest temperatures tested, in contrast to what was observed in the field. Since the highest CFU quantities in field soil were always from the warmest hardiness zone (seven), the expectation was that during controlled settings, pots incubated at 21°C would also have the highest CFU quantities. In addition, *P. cinnamomi* grew readily on artificial media at 13°C and 16°C during the growth test, suggesting higher temperatures would be more favorable to the pathogen. Instead, the long incubation times at 13°C and 21°C had a negative effect on *P. cinnamomi* survival in the soil environment, and resulted in a significant reduction in the number of viable propagules present. The only temperature at which CFU quantities did not significantly differ after incubation was 8°C; population levels remained nearly equal after incubation. This discrepancy in growth on culture plates and soilless media with white oak present suggests that growth tests are not accurate

in predicting the behavior of *P. cinnamomi* in the soil environment. The experimental results indicate that belowground, the optimum temperature for the pathogen maybe around 8°C, much cooler than temperatures indicated by mycelial growth in culture. Increased root growth of white oak in spring has also been noted at this temperature (122); perhaps *P. cinnamomi* is adapted for optimum growth during the same period when vulnerable root tissue is available to infect. The constant incubation temperatures and long incubation durations used for the experiment did not reflect actual field conditions, which are much more dynamic. Indeed, population levels of *P. cinnamomi* quantified from field soil have been highly variable (81, 91). In order to develop a more accurate picture of the biology of *P. cinnamomi* in mid-Atlantic forests, continuous monitoring of CFU levels at selected forest sites could be helpful, as the pathogen would be subject to changes in the soil environment and quantifying viable propagules would be more accurate.

Another possible explanation for the sharp decline of *P. cinnamomi* populations in pots observed during the growth chamber experiment is the fact that *P. cinnamomi* is known to be a notoriously poor competitor in the presence of a diverse microbial community (135, 142). The regular watering regime and use of a soilless potting mix rich in organic matter during the experiment may have supported a community of microbial species that ultimately had a detrimental effect on the *P. cinnamomi* population within each container, leading to the observed disparity in growth rates on culture media and propagule quantity. *Phytophthora* species in general seem to thrive in certain soils and not others. Soil texture, host type, nutrient and moisture availability, and the microbial community are known to influence

Phytophthora spp. survival and establishment at a particular site. For example, over the course of the field research, *P. cinnamomi* was most often isolated from fine-textured soils such as loams and silt loams (81). This finding is in agreement with other *Phytophthora*-related surveys (14, 61, 89). Moist, fine-textured, nutrient-rich soils can support *Phytophthora* populations, but they also encourage a wide variety of microbial growth that can be detrimental to the pathogen, causing hyphal lysis and aborted sporangia (38, 93). Soils with high microbial populations that negatively affect *Phytophthora* spp. are considered to be suppressive, while more nutrient-poor soils prone to waterlogging are conducive to disease (108, 135). It is important that soil type be taken into account at each area infested with *P. cinnamomi* in mid-Atlantic forests, as this site characteristic in concert with others like soil moisture and temperature may determine the severity of damage the pathogen is able to cause to white oak fine roots.

Neither this study nor any previous study could locate forest sites infested with *P. cinnamomi* above 40°N latitude, approximately the boundary between the plant hardiness zone 6 and 5 (10, 81). This observation does not seem to have anything to do with differences in soil types in the study region. The most common soil orders in the mid-Atlantic are alfisols, ultisols, and inceptisols (19). These soils are found both above and below 40°N latitude, and within hardiness zones five, six, and seven. It is therefore unlikely that soil order affects the distribution of *P. cinnamomi*. In addition, *P. cinnamomi* was most commonly isolated from silt loams, loams, and sandy loam-type soils, all of which are fairly evenly distributed across both hardiness zones five and six. In fact, temperature-based experiments provided

evidence that the proposed northern boundary is more likely related to soil temperature than soil type. During Petri dish experiments, growth was nonexistent at 4°C, than steadily increased as temperature increased. Similarly, a significant decrease in viable *P. cinnamomi* CFU in soilless potting media also occurred after incubation at 3°C, regardless of incubation duration, and population levels merely remained constant. Soils in zone five reach temperatures above 15ºC for an average of just 27 days per year (127), which may result in the inability of *P. cinnamomi* to survive or increase its population level, and thus explain the pathogen's absence in north of USDA hardiness zone six.

While the spread of *P. cinnamomi* northward is probably currently limited by temperature, range expansion in eastern US forests may occur if global climate change results in rising temperatures across the region (20). Several European climate change models involving *P. cinnamomi* have been published and indicate that range expansion of the pathogen in forests is likely, as well the development of a warmer and wetter soil environment for rapid pathogen reproduction, faster rates of infection and spread within hosts, and greater expression of aboveground disease (18, 20). The strong environmental connection found in the field and during pot inoculations supports the view that *P. cinnamomi* could become a more important pathogen in the future if mid-Atlantic forest soils warm, resulting in more favorable conditions for the pathogen but not the host.

Until recently, chlamydospores have been considered to be the primary survival structure and most common propagule of *P. cinnamomi* present in soil (80, 142). However, recent work in Australian forests has revealed the presence of stroma-

like hyphal aggregations and abundant selfed oospores within host tissue despite the lack of the required opposite mating type. In contrast, few chlamydospores were found in sampled soil. Instead, when the material from which colonies of *P. cinnamomi* originated during soil plating assays was examined, it was determined that germination was occurring from hyphal aggregations or oospores within pieces of organic matter (32, 62). In this study, the designation "colony-forming units" was often used in quantifying *P. cinnamomi* populations, as the pathogen structure from which each colony originated was not determined. Previously, the majority of colonies originating from mid-Atlantic forest soil samples arose from chlamydospores deposited onto the surface of culture plates (81). However, when culture plates from the growth chamber experiments were examined microscopically, the majority of *P. cinnamomi* colonies appeared to originate from small pieces of organic matter on the surface of the media. These pieces of organic matter were not examined further to determine the type of propagule that had given rise to each colony. It is possible that the colonies did not originate from chlamydospores, as has been commonly assumed, but instead grew from oospores or hyphal aggregations. Further study of the biology of *P. cinnamomi* in eastern US forests is necessary to determine the most common survival structures and what the primary survival of *P. cinnamomi* is in eastern oak forest soils.

Limitations in experimental design

Both the stem and soil inoculation experiments require replication to reveal any errors due to differences in the greenhouse chamber environment that might have not been accounted for. For example, during the stem inoculation experiment to test

the aggressiveness of *P. cinnamomi* isolates, each isolate was only used to inoculate ten seedlings, all of which were in the same pot. Each pot corresponding to one isolate was randomly placed on the greenhouse bench, but because only one pot was used per isolate, small differences in temperature along the bench might have affected lesion growth. What we measured as a significant difference between isolates might have in fact been the result of differences in growing conditions in the greenhouse room used for the experiment. Similarly, the seedling root inoculation experiment involving white and red oak seedlings might have been influenced by the temperature variation inside the greenhouse section from December when the experiment began to September next year when the experiment ended (14°C to 28.5°C). While there were only minimal temperature fluctuations in the environmental chambers (0.1°C to 2.4°C) used to test CFU levels at various temperatures, replication for this experiment is also needed to give more confidence in the results obtained.

Another possible limitation that might have affected the experimental results is due to the inoculation method used. The addition of rice grain inoculum at only one point in each pot might have resulted in less-than-uniform colonization of the potting media by *P. cinnamomi* in inoculated pots. Instead of adding inoculum in this manner, it would perhaps be better to uniformly mix inoculum with the bulk of potting media being used for that particular experiment before transplanting the seedlings used. That way, plant roots would come in contact with the pathogen at nearly every point in the pot, and impact of the pathogen might be better assessed.

The use of soilless potting media instead of field soil for the controlled experiments examining oak roots and *P. cinnamomi* population levels also may have

had a profound effect on the experimental results. The potting media used for both experiments was SunGro's Metro-Mix PX1, which contains gypsum and dolomite lime, in addition to composted pine bark, peanut hulls, and sphagnum moss. Gypsum is calcium sulfate ($CaSO_4 \cdot 2H_2O$), and dolomite lime is calcium magnesium carbonate ($CaMg(CO_3)_2$). Studies on the use of both these compounds to control disease caused by *P. cinnamomi* show that they are effective in reducing infection and inhibiting the production of sporangia (48, 86, 87). High levels of calcium have also been noted in soils suppressive to *P. cinnamomi* in the field (37). The use of this particular potting medium may have inhibited population growth of the pathogen during the experiments, despite our efforts to create a suitable environment for reproduction immediately following inoculation. Some negative effects on oak fine root lengths were noted, but *P. cinnamomi* was recovered infrequently from oak roots, and few disease symptoms were observed above or below-ground on the seedlings used for these experiments. It is possible that the growing medium inhibited *P. cinnamomi,* and that we might have seen more dramatic or different results had we used another type of growth media.

Future directions

Further research is needed to continue the work described in this thesis and to more fully develop our understanding of the role of *P. cinnamomi* in white oak decline in the mid-Atlantic region. First, additional field sampling to collect soil and roots is necessary. In this study, environmental conditions in the study region differed between both sampling years. Sampling the same sites for several years and keeping records of soil moisture and temperature at each site could provide a clearer picture of

root response to changing soil conditions and pathogen presence. Greenhouse experiments testing the effects of combinations of soil moisture and the presence of *P. cinnamomi* on white oak root systems would also be helpful in explaining field observations.

It is imperative that the biology of *P. cinnamomi* in mid-Atlantic oak forests be studied. *Phytophthora* researchers are operating under the assumption that chlamydospores are the primary survival structure of the pathogen in soil, but the recent work in Australia contradicts this theory. We need to know what type of *P. cinnamomi* survival structures are most common in the mid-Atlantic region, where they are located within the soil (freely in the soil matrix, within organic debris, or within infected host tissue), what their temperature parameters are, and how long they survive. Knowing the main structure (e.g. chlamydospores, oospores, hyphal aggregates) that enables *P. cinnamomi* to drive its inoculum density to significant levels in the soil environment could aid in developing future management strategies. Knowing what environmental factors influence its spread and survival will help us greatly improve our predictions on the effects of *P. cinnamomi* on oak decline as climate change continues.

Global climate change is a current and evolving issue, and will almost certainly have an effect on host-pathogen interactions. Climate models predict worsening disease as temperatures rise and sudden heavy rainfall events become more common, resulting in a more hospitable environment for pathogens. Range expansion of forest pathogens is likely, as well as the possibility of more reproductive cycles per year and a longer period of time to infect and cause disease. Over the

course of this research, fine root reduction in the presence of *P. cinnamomi* in hardiness zones six and seven was observed, as was fine root reduction caused by other *Phytophthora* species in zone five, where *P. cinnamomi* has not been detected. According to Soil Climate Analysis Network data, soil temperatures in zone five do not rise above 15°C for long periods each year, while soil temperatures in zones six and seven often remain above 20°C during the summer months. It is quite likely that as soils warm in response to climate change, the current boundary of *P. cinnamomi* at 40°N latitude in oak forests will likely expand northward. Examination of soil temperature data collected in the future could indicate whether soil temperatures in zone five are rising, and then subsequent sampling for *P. cinnamomi* could be conducted.

The ultimate goal of this thesis project was to define the role of *P. cinnamomi* in white oak decline in mid-Atlantic US forests. Prior to this and other related studies, *Armillaria* was the only fine root pathogen that had been examined as a contributor to oak decline in the eastern US. Having observed white oak fine root loss in the presence of *P. cinnamomi* in the field and under controlled experimental conditions, *P. cinnamomi* can be classified as another root pathogen involved in the decline complex. *P. cinnamomi*, however, does not appear be a sole cause of white oak mortality, but could make trees more susceptible to other decline factors, the most important likely being drought. Root damage done by the pathogen probably depends heavily on how favorable conditions are for infection and reproduction within a given year; some years white oaks will experience more significant damage than others. *P. cinnamomi*, then, is capable of stressing white oaks over successive years and can be

classified as a contributor to white oak decline. In other instances, *P. cinnamomi* may not act as a long-term stressor but as a secondary invader after decline has begun, by causing root damage over time after trees are already stressed by other adverse conditions. Overall, root damage caused by *P. cinnamomi* most likely depends heavily on local environmental conditions, occurs over a longer period of time, and could act as a long-term predisposing or a contributing stressor to trees already experiencing white oak decline in the mid-Atlantic region.

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