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TRACKING A TREE-KILLER: IMPROVING DETECTION AND CHARACTERIZING SPECIES DISTRIBUTION OF *PHYTOPHTHORA CINNAMOMI* IN APPALACHIAN FORESTS

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TRACKING A TREE-KILLER: IMPROVING DETECTION AND CHARACTERIZING SPECIES DISTRIBUTION OF *PHYTOPHTHORA CINNAMOMI* IN APPALACHIAN FORESTS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
Kenton Lee Sena
Lexington, Kentucky

Director: Dr. Chris Barton,
Professor of Forest Hydrology and Watershed Management
Lexington, Kentucky
2018

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ABSTRACT OF DISSERTATION

TRACKING A TREE-KILLER: IMPROVING DETECTION AND CHARACTERIZING SPECIES DISTRIBUTION OF *PHYTOPHTHORA CINNAMOMI* IN APPALACHIAN FORESTS

Phytophthora cinnamomi is a soil-borne oomycete pathogen causing root rot in susceptible host species. *P. cinnamomi* is thought to have originated in Southeast Asia, but has since been introduced to many regions around the world, where it causes dramatic declines in many forest tree species. In the eastern US, the primary susceptible tree species of concern are American chestnut (*Castanea dentata*), white oak (*Quercus alba*), and shortleaf pine (*Pinus echinata*). American chestnut, functionally eliminated in the early 1900s by the rapidly acting chestnut blight (*Cryphonectria parasitica*), has been the subject of decades-long breeding efforts aimed at improving chestnut resistance to chestnut blight. To improve chestnut restoration success, and restoration of other susceptible species, the distribution patterns of *P. cinnamomi* on a landscape scale must be better understood. This project was initiated to develop an improved method for detecting *P. cinnamomi* to permit high-throughput screening of forest soils, and to implement the improved detection approach in characterizing the distribution patterns of *P. cinnamomi* in developing soils on reclaimed surface mines in eastern Kentucky, as well as mature forest soils within an undisturbed watershed in a reference-quality eastern Kentucky forest. We developed an improved detection method using a molecular DNA-amplification approach (PCR), which demonstrated similar sensitivity to traditional culture-based methods, but required less time and space than traditional methods. We used this detection approach to screen soils from a chronosequence of reclaimed surface mines (reclaimed at different points in time) to evaluate whether reclaimed surface mined sites become favorable for *P. cinnamomi* colonization over time. Our analysis detected *P. cinnamomi* at the two older sites (reclaimed in 1997 and 2003), but we did not detect *P. cinnamomi* at the two newer sites sampled (reclaimed in 2005 and 2007). These results suggest that surface mined sites become favorable for *P. cinnamomi* colonization over time, and should not be considered permanently "Phytophthora-free." We also collected ~200 samples from a watershed in UK's Robinson Forest, from plots representing a gradient of topographic position, slope, and aspect. This survey indicated that *P. cinnamomi* distribution in forests is complex and can be difficult to predict; however, *P. cinnamomi* was detected in both drier upslope sites and in moister drainage sites.

KEYWORDS: Invasive species, oomycete, American chestnut, shortleaf pine, soilborne forest pathogen

KENTON LEE SENA

April 13, 2018

TRACKING A TREE-KILLER: IMPROVING DETECTION AND CHARACTERIZING SPECIES DISTRIBUTION OF *PHYTOPHTHORA CINNAMOMI* IN APPALACHIAN FORESTS

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April 13, 2018

DEDICATION

This dissertation is dedicated to my wife Susanna, my parents Mark and Anita, and my dear friends and family in the Christian Fellowship churches of Lexington. Without your love and support, this would not have been possible.

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CHAPTER 1: *Phytophthora cinnamomi* as a driver of forest change: implications for conservation and management†.

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1.1. Abstract

Phytophthora cinnamomi is a soil-borne plant pathogen of global significance, threatening many forest tree species around the world. In contrast to other well-known tree pathogens, *P. cinnamomi* is a generalist pathogen that, in many cases, causes less immediately obvious symptoms, making *P. cinnamomi* more difficult to diagnose. This creates special challenges for those trying to assess and manage diseases caused by *P. cinnamomi*. *P. cinnamomi* affects a wide range of tree species across the world including chestnuts, particularly American and European chestnuts, *Eucalyptus* and *Banksia* species in Australia, and oaks in Mediterranean Europe. We believe that forest professionals should incorporate an understanding of the diseases caused by *P. cinnamomi* in conservation, management, and restoration of threatened ecosystems dominated by *P. cinnamomi*-affected tree species. Here we review the impact of *P. cinnamomi* on forest ecosystems internationally and suggest three major strategies for improving forest resilience to *P. cinnamomi*: 1) Improving site quality to reduce risk of *P. cinnamomi*-related disease, 2) Genetically improving threatened species to improve resistance to *P. cinnamomi*, and 3) Restricting further spread (especially by nursery trade) of *P. cinnamomi*.

1.2. Introduction

Phytophthora cinnamomi (Pc) is a pathogenic invasive species that causes tree disease in forest ecosystems around the world (Burgess *et al.*, 2017). Unlike specialized pathogens, such as chestnut blight (*Cryphonectria parasitica*) or Dutch elm disease (*Ophiostoma novo-ulmi*), Pc is a generalist pathogen with a broad host range. Despite the widespread impact of Pc across the globe, it is relatively unrecognized by land managers and forest professionals. This may be due to the diverse range of diseases that Pc can cause on different plant species, many of which are

not immediately deadly but contribute to poor establishment or decline and mortality over time.

While Pc is widespread in North America, Europe, and Australia, its precise origin remains unclear. Based on analyses of population diversity, scientists hypothesize that Pc originated in Taiwan (Ko *et al.*, 1978) or Papua New Guinea (Shepherd, 1975; Arentz and Simpson, 1986). Pc has invaded forests around the world causing a wide variety of diseases on many host plants. In some places Pc seems to be a relatively recent arrival, while evidence suggests it has been established for many decades in other locations. The magnitude of Pc impact in these systems ranges from high mortality to few noticeable symptoms.

Recent research has implicated Pc in an increasing range of tree species declines; however, its impact has largely been overshadowed by other more visible forest health threats that cause widespread and immediate mortality (e.g., Anagnostakis 2001). Given changing climate and land-use patterns, we predict that Pc will become increasingly relevant for forest management. To increase awareness and understanding of Pc, here we provide an introduction to the pathogen, focused on its impact on forest ecosystems. In addition, we outline a three-part management plan for minimizing Pc-associated disease and decline in the future, based on improving site quality, tree breeding for resistance to Pc, and preventing Pc spread.

1.3. Diseases caused by Pc in global forests

The earliest signs of Pc invasion in North America were reports of chestnut decline in the southeast US in the late 19th century (Corsa, 1896). Pc was officially implicated in root rot of American chestnut (also known as ink disease) and littleleaf disease in shortleaf and loblolly pines in the mid-1900s (Crandall *et al.*, 1945; Campbell, 1949; Campbell and Copeland, 1954), and later fir mortality especially in North Carolina (McKeever and Chastagner, 2016). In the western US, Pc was first reported as causing root rot in avocado plantations in the 1940s (Wager, 1942). Pc was also associated with ohia declines in Hawaii (Kliejunas and Ko, 1976a, b) in the 1970s. More recently, several studies have implicated Pc in oak decline in the eastern US (Balci *et al.*, 2010; McConnell and Balci, 2014, 2015). Pc was also recently associated with walnut decline in Chile (Guajardo *et al.*, 2017).

In Europe, Pc was documented causing ink disease in chestnut in the late 19th century (Day, 1938; Crandall *et al.*, 1945; Brasier, 1996). Ink disease, a root disease causing wilt and eventual tree death in chestnut, has since been found to be caused by several *Phytophthora* species including *Phytophthora cambivora* and Pc (Choupina *et al.*, 2014). More recently,

interest in Pc has resurged with rising *Phytophthora*-associated declines in oaks and chestnuts in France (Robin *et al.*, 1998), Austria (Balci and Halmschlager, 2003), Italy (Vettraino *et al.*, 2001; Vettraino *et al.*, 2002; Scanu *et al.*, 2013), Spain (Rodríguez-Molina *et al.*, 2005), and Portugal (Brasier *et al.*, 1993; Moreira *et al.*, 1999).

In Australia, Pc is most noted for the large-scale epidemics it has caused in *Eucalyptus* and *Banksia* species, especially in the *Eucalyptus marginata* (Jarrah) forests of Western Australia (Podger *et al.*, 1965; Davison and Tay, 1987; Shearer *et al.*, 1987) and the forests of east and southeast Australia (Weste and Taylor, 1971; McLennan *et al.*, 1973; Weste and Marks, 1974). Records of Pc-caused jarrah dieback in Australia date back to the early-mid 1900s (Newhook and Podger, 1972). Pc has recently been associated with root rot in Chinese chestnut and blueberry in China (Lan *et al.*, 2016a, b). Pc is also associated with macadamia root rot in Kenya (Mbaka, 2013), and avocado root rot in South Africa (Engelbrecht and Van den Berg, 2013; Reeksting *et al.*, 2014).

1.4. Pc as a driver of forest ecosystem change

1.4.1. Australia

Pc has driven extensive declines of susceptible species in multiple forest ecosytems in Australia. In Western Australia, 2284 plant species are classified as susceptible to Pc, and 800 species are classified as highly susceptible (Shearer *et al.*, 2004). Of particular concern in this region are Jarrah forests and *Banksia* woodlands, characterized by a number of Pc-susceptible species (Shearer and Dillon, 1996a, b). The jarrah forest type in Western Australia is dominated by jarrah (*E. marginata)*, which comprises 68% of trees taller than 1.8m in the ecosystem (Podger, 1972). Jarrah is highly susceptible to infection by Pc, with 52% cumulative mortality in disease patches and nearly 100% of trees showing symptoms. Disease symptoms in jarrah include chlorosis and crown thinning, followed by epicormic sprouting. While mortality rates of infected jarrah are relatively low (2-5% per year in affected stands), growth rates are significantly reduced by infection (5-6 fold) (Podger, 1972). *Banksia spp*., which dominate the understory in this ecosystem, exhibit even higher mortality (92%). Disease spread in the landscape is rapid when conditions are favorable, and is most rapid (40m/yr) downslope along drainage areas (Podger, 1972). Anthropogenic activity, especially road building and soil movement, is thought to accelerate Pc spread in the landscape (Dawson and Weste, 1985). By altering species composition of these woodlands, Pc also drives changes in habitat and food

resource availability for wildlife, especially sensitive endemic specialist species such as the honey possum (*Tarsipes rostratus*), dependent on nectar and pollen of *Banksia spp*. (Dundas *et al.*, 2016).

A major driver of land-use change in Western Australia is bauxite mining. Hardy et al. (1996) reported that 450 ha was surface mined for bauxite and reclaimed annually (Nichols *et al.*, 1985; Hardy *et al.*, 1996). Restoration of these mined sites presents both challenges and opportunities for the broader effort to address jarrah forest decline; early restoration of these mined sites did not attempt to reforest with jarrah, due to high mortality rates related to Pc infection. More recently, efforts have been focused on restoring the native forest community composition (Hardy *et al.*, 1996), necessitating the development of techniques to address Pc-caused mortality. Hardy et al. (1996) observed high survival (85-92%) in jarrah on reclaimed mine sites, with observed mortality and disease symptoms strongly associated with poor soil drainage leading to ponding (Hardy *et al.*, 1996), conditions generally considered favorable to Pc. Overall, the type of altered soil structure associated with mining (specifically, breaking up a subsurface crust), is thought to enhance soil drainage, reduce ponding, and reduce Pc disease incidence. In their 2007 paper, Koch and Samsa reported high survival rates of jarrah (>80%) planted in bauxite mine restoration efforts, even where Pc is present (Koch and Samsa, 2007), suggesting that maintenance of adequate soil drainage may inhibit Pc growth, thereby limiting Pc infection and disease severity and permitting acceptable levels of survival of susceptible species for restoration efforts.

Screening of jarrah suggests that jarrah susceptibility to Pc is variable, and that genetic resistance to Pc is present to varying degrees in natural populations (Stukely & Crane, 1994). Jarrah seedlings can be screened for resistance at an early growth stage as resistance in seedlings is correlated with in-field survival and growth (Stukely et al., 2007). Continued research elucidating genetic mechanisms for resistance to Pc in jarrah and other susceptible species in this ecosystem will inform development of varieties with improved resistance, useful for ecosystem restoration.

Pc is also credited with driving ecosystem change in southeast Australia, shifting plant communities from shrubby sclerophyll forests to open grassy woodlands (Weste and Marks, 1974; Weste, 1981), with reduced tree and shrub species richness and increased annual plant species richness (McDougall *et al.*, 2002b). Pc infestation in the Brisbane Ranges National Park increased from 1% to 31% in just a decade (1970-1981), an indication of the potential for Pc to

rapidly expand its range. This expansion was associated with human activity, especially road building, and movement along natural drainage ways (Dawson and Weste, 1985). It is likely that Pc propagules were transported in contaminated soil and gravel as part of the road building process, and subsequently flowed along drainage systems, causing additional infestations downstream. Susceptible species that persist at dieback sites, as well as infected but asymptomatic herbaceous species, serve as an inoculum reservoir for Pc, maintaining high propagule levels throughout disease cycles (McDougall *et al.*, 2002b; Crone *et al.*, 2013a; Crone *et al.*, 2013b). The persistence of high inoculum levels at infected sites, even after elimination of most susceptible species, suggests that vegetative community composition in invaded areas will be permanently altered toward dominance by Pc-tolerant or resistant species (Weste, 1981; Weste, 2003).

1.4.2. Europe

Pc has been credited to varying degrees with decline in both *Quercus suber* (cork oak) and *Q. ilex* (holm oak) in Mediterranean Europe (Robin *et al.*, 1998; Scanu *et al.*, 2013). In a laboratory setting, Pc inoculation led to 100% mortality of holm oak seedlings across a range of incubation temperatures (17-26 °C) (Martín-García *et al.*, 2014), and significant mortality in cork oak acorns (Rodríguez-Molina *et al.*, 2002). Maurel et al. (2001) observed 67% root loss and 10% mortality in holm oak seedlings inoculated with Pc (Maurel *et al.*, 2001a). Similarly, Robin et al. reported 85-95% root losses in holm oak and cork oak after inoculation with Pc (Robin *et al.*, 2001), and Serrano et al. (2015) observed increasing disease severity in cork oak with increasing Pc concentration (Serrano *et al.*, 2015).

While Pc can cause holm oak and cork oak mortality in controlled conditions, correlations of Pc infection with declines of these species in the environment are complex and tied to environmental conditions, especially soil moisture. Holm oak trees in decline exhibited reduced fine root biomass, stomatal conductance, and leaf water potential when compared to non-declining trees (Corcobado *et al.*, 2013). Holm oak and cork oak seedlings infected with Pc were more susceptible to drought, likely due to combined effects of reduced root mass and degraded vascular tissue, especially when drought followed temporary flooding (Moreira *et al.*, 1999; Corcobado *et al.*, 2014a). Consistent with these observations, mortality of holm oak is seasonally variable, with high mortality in summer and low mortality in autumn and winter (Rodríguez-Molina *et al.*, 2005).

Stands in decline tended to be associated with finer-textured soils, and decline symptoms appeared to be exacerbated by waterlogging and drought (Corcobado *et al.*, 2013). In addition, mycorrhizal colonization of roots is inversely associated with decline, with stands in decline exhibiting lower mycorrhizal colonization rates than non-declining stands (Corcobado *et al.*, 2014a; Corcobado *et al.*, 2014b; Corcobado *et al.*, 2015). While the association is clear, it is unknown whether low mycorrhizal colonization leads to decline, or whether stands in decline are unable to support high mycorrhizal infection rates. These and other studies suggest that Pc infection causes a resource strain on holm oak and cork oak by reducing fine root biomass and requiring resource allocation to root regrowth. In turn, this stress reduces host resilience to inclement environmental conditions, especially drought (da Clara and de Almeida Ribeiro, 2013; Moricca *et al.*, 2016) and may play a role in multi-species decline complexes including those involving mycorrhizal fungi.

Pc also causes root rot in European chestnut (*Castanea sativa*), which represents an important industry in Western Europe. Chestnut is highly susceptible to Pc, exhibiting rapid mortality in inoculation experiments (Maurel *et al.*, 2001b). In the early 1900s, Pc was identified as a causal agent in chestnut ink disease of chestnut, (Day, 1938) and ink disease was recognized as the most important disease of chestnut (Vannini and Vettraino, 2001). However, mortality of chestnut caused by Pc was overshadowed through the middle of the 20th century by the devastating chestnut blight (*Cryphonectria parasitica*). More recently, interest in ink disease occurrence patterns on chestnut has resurged as ink disease has increased in European chestnut orchards over the past few decades while resistance breeding programs and hypovirulence management approaches have decreased the significance of chesnut blight (Vannini and Vettraino, 2001; Vettraino *et al.*, 2001).

Landscape scale analyses of chestnut groves in Portugal indicated that the chestnut population had increased by 18.5% from 1995-2002 due to establishment of new plantations, but declined after 2002 as new plantations were unable to keep up with population reduction by Pc (Martins *et al.*, 2007). Surveys of ink disease progression in chestnut groves in Italy demonstrated a correlation of disease severity with nearness to natural drainage areas, supporting the widely recognized association of Pc with poorly drained, moist soils (Vannini *et al.*, 2010). Ongoing European chestnut restoration is concerned with identifying areas unsuitable for Pc incidence and prioritizing those for chestnut planting (Dal Maso and Montecchio, 2015). Also, characterization of genetic resistance to Pc is a priority for developing improved chestnut

varieties. Investigations of chestnut transcriptomes have elucidated genetic differences between resistant and susceptible chestnut species upon challenge by Pc (Santos *et al.*, 2015a; Santos *et al.*, 2015b; Serrazina *et al.*, 2015). Recently, eight candidate resistance genes have been identified, providing direction for continued investigation into specific mechanisms for resistance (Santos *et al.*, 2017). Further research is necessary to fully characterize the resistance pathways in chestnut so that chestnut varieties with improved resistance can be developed for restoration.

1.4.3. North America

Similar to its role in European chestnut declines, Pc causes devastating ink disease (also called *Phytophthora* root rot) in American chestnut (*Castanea dentata*). Decline of American chestnut in the southeast US, with symptoms matching ink disease, was observed in the late 1800s (Corsa, 1896). American chestnut was dying out in the southern part of its range, and the disease front was slowly moving north. In the mid 1900s Pc was identified as the causal agent of the disease epidemic (Crandall *et al.*, 1945); however, by that time ink disease had been overshadowed by chestnut blight, which swept through the entire range of American chestnut, killing trees back to the ground (Anagnostakis, 2001). Ink disease once again resurfaced as a particular concern in the southeast US when early chestnut restoration efforts experienced high mortality rates because of *Phytophthora* root rot (Brosi, 2001). Similar to observations from other regions, ink disease in the eastern US was associated with low topographic position and moist, poorly drained soils (Rhoades *et al.*, 2003). Because American chestnut was historically found on ridges and well-drained soils prior to mortality from chestnut blight, it was thought that restoration efforts with blight-resistant trees could target these areas of the landscape that are potentially less suitable for Pc (Rhoades *et al.*, 2003).

The historical range of American chestnut included the eastern coalfields, which have been extensively deforested and surface-mined for coal. As such, surface-mined land in Appalachia presents an important opportunity for reforestation generally, and restoration of American chestnut in particular. Mine soils from surface mines reclaimed using recently developed forestry reclamation procedures (Zipper *et al.*, 2011) tested negative for Pc incidence (Adank *et al.*, 2008; Hiremath *et al.*, 2013), suggesting that reclaimed surface mines could present a “Pc-free” environment, at least temporarily, ideal for American chestnut restoration with blight-resistant trees (French *et al.*, 2007). Additional research is required to evaluate whether Pc may eventually colonize reclaimed surface mined lands, or whether reclaimed

surface mine soils are unsuitable for Pc indefinitely. If Pc does successfully recolonize these sites, additional studies will be required to assess whether ink disease can be predicted by Pc presence alone, or by a complex of Pc presence and environmental conditions, as consistent with disease in jarrah on reclaimed mined land in Western Australia.

Current understanding of Pc distribution in the eastern US suggests that the pathogen is widespread in eastern forests, ranging at least as far north as Ohio (Hwang *et al.*, 2009; Meadows and Jeffers, 2011; Balci *et al.*, 2013). However, better understanding of Pc distribution in the eastern US will be critical for effective restoration of American chestnut in the region. For example, drier ridge-top sites must be evaluated for both Pc incidence and disease risk. In addition, improving chestnut resistance to Pc will enhance chestnut restoration potential. Efforts are underway to develop chestnut varieties that exhibit resistance to Pc (Jeffers *et al.*, 2009; Nelson *et al.*, 2012; Olukolu *et al.*, 2012; Zhebentyayeva *et al.*, 2013).

Pc, along with *P. citricola* and *P. cambivora*, has also been associated with observations of white oak (*Q. alba*) decline in Ohio, although this association is more tenuous than Pc-associated decline in Mediterranean oak species (Balci *et al.*, 2010). Pc inoculation caused root rot in greenhouse trials (Nagle *et al.*, 2010; McConnell and Balci, 2015), but associations of Pc with patterns of decline in the field are less clear. Pc infection is correlated with reduced fine root length in stands (Balci *et al.*, 2010; McConnell and Balci, 2014), but not significantly associated with crown decline or mortality. While Pc incidence is not consistently associated with high soil moisture in these studies, flooding was found to increase root disease severity (Nagle *et al.*, 2010), and infected white oaks appeared to be more sensitive to drought conditions (McConnell and Balci, 2014). Thus, while Pc can cause root rot in white oaks it is not strongly associated with tree mortality, and not as clearly associated with white oak decline as it is in holm and cork oak decline.

Recently, Pc has been implicated in shifts in species composition of forests in northern California (Swiecki and Bernhardt, 2017). Some of the affected species, especially Ione manzanita (*Arcostaphylos myrtifolia*) and pallid manzanita (*Arcostaphylos pallida*), are highly endemic species, and declines in their isolated populations may be detrimental to species survival (Swiecki and Bernhardt, 2017).

1.5. Ecology and Epidemiology

1.5.1. Mechanisms of infection

Pc appears to infect hosts primarily through asexual heterokont zoospores (Byrt and Grant, 1979; Hardham, 2001, 2005; Ridge *et al.*, 2014). Zoospores are motile and capable of swimming through water on plant surfaces, in bodies of water (streams, lakes, irrigation ponds), and in inundated soil. Because zoospore production is favored by increasing soil moisture, zoospore abundance is higher in poorly drained soils, leading to increased disease pressure on host plants (Sterne *et al.*, 1977a; Sterne *et al.*, 1977b). As in other *Phytophthora* species, Pc zoospores are capable of movement toward chemical cues, chemotaxis, which assists them in locating viable sites of infection (Hardham, 2005; O'Gara *et al.*, 2015).

Pc zoospore taxis, encystment, and germination target emerging roots, where the cuticle is disrupted and/or not yet fully formed (O'Gara *et al.*, 2015; Redondo *et al.*, 2015), although zoospores are known to be able to penetrate unwounded periderm (O'Gara *et al.*, 1996; O'Gara *et al.*, 1997). Penetration of host tissue can be enabled by secretion of cell-wall degrading enzymes, such as polygalacturonases (Götesson *et al.*, 2002). Pc hyphae rapidly penetrate epidermal and cortical cells, both intracellularly and intercellularly (Cahill *et al.*, 1989; Redondo *et al.*, 2015; Ruiz Gómez *et al.*, 2015), developing haustoria and stromata consistent with hemibiotrophic behavior (Redondo *et al.*, 2015; Ruiz Gómez *et al.*, 2015). Once hyphae penetrate phloem cells, they rapidly elongate and shift to necrotrophic behavior, causing cell wall degradation, phloem blockage (Cahill *et al.*, 1989; Davison *et al.*, 1994; Redondo *et al.*, 2015; Ruiz Gómez *et al.*, 2015) and discoloration associated with oxidated polyphenols (Tippett *et al.*, 1983). In general, Pc infection is associated with loss of fine roots (Corcobado *et al.*, 2013; Corcobado *et al.*, 2014a; McConnell and Balci, 2015; Ruiz Gómez *et al.*, 2015). Physiologically, Pc infection has been associated with altered plant water status, reducing predawn leaf water potential and stomatal conductance (Dawson and Weste, 1982; Maurel *et al.*, 2001a; Maurel *et al.*, 2001b; Robin *et al.*, 2001), although this relationship is not consistently observed. For example, Turco *et al.* (2004) found no significant relationship between Pc infection and plant water physiological condition. Aboveground symptoms of Pc root rot include chlorosis, wilting, stunting, thinning and whole plant death (Podger, 1972; Cahill *et al.*, 1989).

1.5.2. Detection of Pc from environmental samples

Understanding where Pc is present and causing disease, especially in forest ecosystems, is essential for developing conservation and management strategies. Pc is typically detected by isolating from soil or water samples, or plant material. Susceptible plant material is used to bait Pc from the sample, and the bait is subsequently transferred to selective media for isolation of

the pathogen. This method relies on active production of zoospores by Pc and is ineffective for quantifying Pc abundance in a sample or detecting Pc that is inactive or dead. Pc has been successfully isolated from baits including blue lupine roots (Podger, 1972; Pratt and Heather, 1972; Kliejunas and Ko, 1976a, b; Blowes *et al.*, 1982; Moreira *et al.*, 1999), rhododendron leaves (Shew and Benson, 1982), oak leaves (Balci et al. 2010), and *Camellia japonica* leaves (Meadows and Jeffers, 2011). Infected baits are transferred to amended agar media selective for *Phytophthora spp*., such as PARP, PARPH, and PCH. PARP is prepared by amending an agar (V8, cornmeal, or potato dextrose) with ampicillin, rifampicin, pentachloronitrobenzene (PCNB), and pimaricin. PARPH includes the amendment of hymexazol to the PARP formulation (Jeffers and Martin, 1986). Similarly, PCH media is prepared by amending a dextrose agar with PCNB, pimaricin, chloramphenicol, and hymexazol (Shew and Benson, 1982).

While baiting and culturing is the standard method used for detecting Pc from environmental samples, it can be insensitive. Experiments on Pc detection from stem-inoculated jarrah in Australia indicated that up to 11% of samples containing viable Pc yielded false negatives in first-round culturing on selective media (Hüberli *et al.*, 2000). Another study in Australia observed dramatic variability across individual baiting techniques and soil types (McDougall *et al.*, 2002a). More recently, several studies have demonstrated reliable detection of Pc using polymerase chain reaction (PCR) on DNA extracted from infected plants and infested soil samples. Williams et al. (2009) found that a nested PCR assay targeting the internal transcribed spacer (ITS) region dramatically improved sensitivity of detection from soil from 0-10% for conventional baiting with rose petal discs and culturing on NARPH media to 90-100% (Williams *et al.*, 2009). A number of PCR assays have been developed for detection of Pc, and several of these demonstrate high sensitivity and specificity (Kunadiya *et al.*, 2017).

1.5.3. Pc distribution on the landscape

Pc distribution in soil is controlled by environmental conditions, primarily moisture and temperature. In jarrah forest soils collected for lab analyses, Pc survival in root fragments was related to soil moisture, with lower Pc survival in drier treatments (Old *et al.*, 1984). In Oregon, Pc hyphal growth was optimal in 43-58% moisture (Kuhlman, 1964). While Pc survival increases from dry soil to moist soil, Pc survival decreases in flooded or submerged soils (Stolzy *et al.*, 1967; Hwang and Ko, 1978). These results are consistent with Pc colony growth in the lab; Pc hyphal growth increases with oxygen concentration to a plateau, with anaerobic conditions unfavorable for Pc growth (Davison and Tay, 1986). It is important to note that optimal

conditions for Pc hyphal growth are different from those supporting zoospore production although both are relevant to Pc distribution and local abundance.

The influence of soil moisture on Pc distribution is also observable at the landscape scale. Surveys of Pc distribution in Australia, Italy, and Portugal found that incidence of disease caused by Pc was associated with natural drainage areas and poorly drained soils (Weste and Marks, 1974; Moreira *et al.*, 1999; Moreira and Martins, 2005; Vannini *et al.*, 2010; Duque-Lazo *et al.*, 2016). In addition to spatial variability, Pc inoculum levels have been shown to vary with temporal patterns in soil moisture, with higher isolation frequency in spring than summer (Shearer *et al.*, 2010). Pc is capable of producing structures (e.g., chlamydospores, stromata, oospores) that permit survival in dry conditions, enabling Pc to persist across a range of soil moisture conditions (Dawson and Weste, 1985; Robin and Desprez-Loustau, 1998). Because of this, Pc presence on the landscape does not necessarily indicate that Pc disease will be severe in susceptible hosts. In addition to investigating the patterns of distribution of Pc propagules on the landscape, it is necessary to clarify how these patterns relate to actual disease incidence in susceptible species.

Temperature is another important control on Pc distribution, especially at the landscape scale. Both lab and field trials demonstrate that Pc does not survive freezing temperatures well (Benson, 1982). In culture, hyphal growth is optimal at 20-32.5 °C, with minimal growth at low temperatures (5-16 °C) and high temperatures (30-36 °C) (Zentmyer *et al.*, 1976). In general, disease severity in susceptible species (e.g., *Eucalyptus marginata* and *Banksia grandis*) corresponds to these patterns, with disease severity highest at intermediate temperatures (10-30 °C) (Kuhlman, 1964; Halsall and Williams, 1984; Shearer *et al.*, 1987). Landscape features such as canopy cover and aspect can dramatically influence soil temperature and, in some cases, alter soil favorability for Pc (Moreira and Martins, 2005; Shearer *et al.*, 2012). Most landscape-scale spatial analyses conclude that winter temperature, and thus latitude, is a principle factor controlling Pc global distribution. However, Pc continues to be isolated further north than previously thought (e.g., Chavarriaga *et al.*, 2007; Balci *et al.*, 2010) and models of potential future Pc distribution under climate change projections suggest that rising winter temperatures will increase the area suitable for Pc survival (Brasier, 1996; Bergot *et al.*, 2004; Thompson *et al.*, 2014).

Pc is capable of surviving in the environment for long periods of time, even in unfavorable conditions. In Oregon, Pc was recovered from forest soils 19 months after

infestation (Kuhlman, 1964). In Australian soils with low organic matter content and low soil matric potential, Pc survives for shorter periods of time. For example, one study found that few Pc chlamydospores survive for even 2-4 months in unfavorable soil while Pc in more favorable soils remains viable up to 8-10 months (Weste and Vithanage, 1979). In Australia, Pc was isolated from the feces of feral pigs fed plant material artificially infested with Pc (Li *et al.*, 2014). Recently, studies have reported that Pc is capable of surviving harsh environmental conditions in symptomless herbaceous hosts (Crone *et al.*, 2013a; Crone *et al.*, 2013b). While previous research has focused on symptomatic woody hosts, these studies suggest that Pc employs a range of host-interaction strategies. The potential for Pc to infect and dormantly survive in asymptomatic hosts is a critical point of future investigation, especially in other regions where Pc is of concern, such as the eastern US.

At small spatial scales, Pc incidence is typically clustered but sometimes randomly distributed. Pryce et al. (2002) found Pc in 20m radius plots across their study region, but detected Pc in only 56% of samples within plots (Pryce *et al.*, 2002). Similarly, Meadows and Jeffers (2011) found Pc in 7 of 9 plots in their study region, but as few as 14% of samples within plots were positive (Meadows and Jeffers, 2011). Since these detection methods (baiting) measure active zoospore production in a laboratory context as a proxy for Pc presence, it is unclear whether the observed results are due to a limited number of Pc propagules or variability in zoosporgenesis. To reduce the risk of false negatives in Pc detection in the environment, multiple subsamples should be collected at each sampling point (Pryce *et al.*, 2002).

1.5.4. Interactions of Pc with other soil microbes

Soil microbial community composition has been demonstrated to influence Pc growth and the ability of Pc to infect host plants (Broadbent and Baker, 1974; Marks and Smith, 1981; Old *et al.*, 1984). Suppression of Pc has been correlated with microbial activity (e.g., microbial enzymatic activity) and populations of specific microbial groups (e.g., endospore-forming bacteria, actinomycetes) (Halsall, 1982a, b; Malajczuk, 1988; You *et al.*, 1996). In other oomycete pathosystems, particularly *Pythium*, bacterial communities have been found to create disease suppressive soils by altering the chemotaxis of zoospores and interrupting the germination of encysted zoospores (Jack, 2010; Chen et al., 2012; Carr & Nelson, 2013). While the specific mechanisms of suppressive activity are uncertain, some studies suggest that compounds secreted by some microbes may directly degrade Pc (El-Tarabily *et al.*, 1996).

Mycorrhizal populations are also associated with reduced risk of infection by Pc (Ross and Marx, 1972; Malajczuk, 1979). Ectomycorrhizal fungi may reduce the ability of Pc germ tubes to penetrate host tissue, potentially by physically blocking infection sites on host roots (Malajczuk, 1988). However, in field settings, the relationship between mycorrhizal colonization and infection by Pc is not well understood. In *Quercus ilex* populations in Spain, Pc-associated oak decline was also associated with reduced ectomycorrhizal colonization rates; however, it is unclear whether the oak decline caused mycorrhizal decline, or whether mycorrhizal decline increased susceptibility to Pc infection (Corcobado *et al.*, 2014a; Corcobado *et al.*, 2014b; Corcobado *et al.*, 2015).

Finally, the potential for multiple pathogens to act synergistically to drive decline in susceptible species has been suggested in some studies. For example, Pc and *Diplodia corticola* were found to act synergistically to drive decline in holm oak (Linaldeddu *et al.*, 2014). Recent studies utilizing metagenomic tools to investigate communities of *Phytophthora spp*. associated with declining holm oak found that the most commonly detected *Phytophthora* taxon was previously unidentified and has not been cultured (Català *et al.*, 2016). The possibility that decline of oak and other susceptible species can be driven by Pc together with other pathogens, including previously unstudied *Phytophthora* species, has yet to be explored thoroughly. In a recent study on the simultaneous infection of holm oak by multiple *Phytophthora* species, different *Phytophthora spp*. did not cause more severe symptoms when infecting together. Instead, infection by a less virulent *Phytophthora* species (e.g., *P. gonapodyides*) reduced disease severity caused by subsequent *Pc* infection (Corcobado *et al.*, 2017).

1.5.5. Options for controlling and eliminating Pc

Options for controlling Pc in forest ecosystems are limited. Several studies have explored the potential for potassium phosphonate (phosphite) application to control infection by Pc. While phosphite successfully reduces infection rates in treated plants, effectiveness declines over time and applications must be repeated (Hardy *et al.*, 2001; Tynan *et al.*, 2001; Wilkinson *et al.*, 2001; Daniel *et al.*, 2005). The fungicide fosetyl-aluminum reduces Pc growth in culture and disease severity in plants (González *et al.*, 2017). Treatment with copper salts improves host resistance to Pc infection, but effectiveness declines over time and repeated applications are required (Keast *et al.*, 1985). Extract of *Phlomis purpurea*, an understory plant in Mediterranean oak forests, also reduces growth of Pc hyphae on agar, as well as infection severity in cork oak roots, over short timescales (Neves *et al.*, 2014). Fire has also been explored

as a potential control option (Dawson and Weste, 1985; Moore *et al.*, 2015), but Pc has been isolated from soils even after fire (McLaughlin *et al.*, 2009), indicating that fire is not likely to provide an effective control option. Perhaps the most effective control option demonstrated to date is an aggressive spot eradication of Pc, utilizing host removal, fumigation, and fungicide application (Dunstan *et al.*, 2010). Dunstan et al. (2010) found this intensive approach useful for 1) eradicating Pc from localized infestations, 2) restricting further spread of infestations too extensive to completely eradicate, and 3) protecting at-risk populations of susceptible species.

1.5.6. Mechanisms whereby Pc spreads on the landscape

Pc spreads naturally by movement of zoospores through soil water, especially via surface flow. In favorable conditions, such as downslope along natural drainage ways, Pc spread can be as rapid as 40m/yr (Podger, 1972). Pc spread is accelerated by movement of soil, such as in roadbuilding and other construction activities (Dawson and Weste, 1985). Pc is spread over great distances by the movement of infected nursery species (Jung *et al.*, 2016; Beaulieu *et al.*, 2017). Unfortunately, sometimes even species selected for restoration efforts are infected, spreading the pathogen to sites of conservation/restoration priority (Swiecki and Bernhardt, 2017).

1.5.7. Priorities for further research

Detection: Pc is conventionally detected from soils using a time-consuming and potentially insensitive baiting and culturing approach. Soil samples are flooded with water to stimulate zoospore production and baited with plant material from susceptible species (e.g., rhododendron, lupine, rose, oak, etc.). Zoospores infect the bait, causing necrotic lesions to form. These can be excised and transferred to selective media, followed by subsequent transfers to additional media for morphological identification and/or DNA confirmation. This approach is standard, but is unsuitable for the rapid, large-scale screening of environmental samples required for conservation and management. A conventional approach can take weeks to return a screening result and this delay can present challenges for time-constrained conservation and management scenarios. Additionally, studies have identified that conventional detection can be insensitive, returning negative results even when the pathogen is known to be present (e.g., Huberli et al. 2000; McDougall et al. 2002a). This insensitivity could arise from the fact that a conventional approach includes at least two selective steps—baiting and growth on selective media. These steps can lead to poor detection sensitivity when dealing with soils and environmental conditions inhibiting zoospore production or increasing fungicide sensitivity.

Development of detection methods with improved sensitivity and reduced time requirements could dramatically improve understanding of Pc distribution and potential invasion patterns on a landscape scale with meaningful implications for conservation and management. Development of PCR and related molecular detection assays has improved detection sensitivity in screening plant samples, but these methods have not been broadly applied to screening of soils at landscape scales (e.g., Kunadiya et al. 2017). Further development of sensitive molecular assays for use in rapid, high-throughput screening of soil samples is essential.

Distribution: In addition to improved detection methodology, more detailed understanding of Pc distribution and potential invasion patterns on landscape scales is critical for effective conservation and management of ecosystems threatened by Pc. Early understanding of Pc distribution was tied to disease incidence patterns. For example, disease caused by Pc was associated with low-lying, poorly drained soils in studies in Europe and Australia (Weste and Marks, 1974; Moreira *et al.*, 1999; Moreira and Martins, 2005; Vannini *et al.*, 2010; Duque-Lazo *et al.*, 2016). However, Pc has also been detected in higher, drier soils where disease was not significant or not recorded (Shea and Dell, 1981). Thus, in addition to understanding the distribution of Pc on the landscape, it is essential to understand the conditions in which Pc can cause disease in susceptible species. While these conditions have been well-described in some forest ecosystems, particularly in Australia and Europe, the distribution patterns of Pc are not well known in forests in the eastern US. Finally, the recent discovery of Pc occuring in symptomless herbaceous species (Crone et al. 2013a, b) is a turning point for the understanding of Pc in forests. Pc distribution is not controlled (and cannot be predicted) only by the presence of susceptible symptomatic woody hosts—even forbs showing no symptoms of infection may serve as refugia for Pc. A wide range of plants in forest systems of concern, especially eastern US forests, should be screened to determine if they are capable of serving as symptomless hosts for Pc.

Control: Perhaps the most well-studied option for control of Pc in the environment is phosphite, which has been successfully applied at multiple spatial scales for control of Pc. Hardy et al. (2001) report that phosphite application was common in natural ecosystems, ranging from trunk injections to foliar sprays. These applications can reduce mortality in susceptible plants (Shearer and Fairman, 2007), and can reduce continued spread of Pc-related disease on the landscape (Shearer et al. 2004). However, the precise mechanisms whereby phosphite confers resistance have been unclear. Phosphite appears to cause some direct inhibition of Pc growth,

but does not cause Pc mortality (Smillie et al. 1989; Jackson et al. 2000). Additional research continues to clarify the physiological links between phosphite application and plant defense mechanisms. For example, in *Xanthorrhoea australis*, phosphite treatment enhanced the plant defence response to Pc infection, preventing Pc hyphae from reaching vascular tissue (Daniel et al. 2005). Phosphite appears to enhance plant defence through stimulation of the auxin pathway (Eshragi et al. 2014). While phosphite significantly improves plant resistance to Pc infection, the effects taper off over time, and reapplications are required to sustain long-term resistance (Tynan et al. 2001; Wilkinson et al. 2001). Continued research elucidating the defense pathways stimulated by phosphite is essential for improving long-term control of Pc disease in forest ecosystems, both improving management strategies utilizing phosphite and genetically enhancing natural defense pathways.

1.6. Implications for conservation and management

Pc has been introduced to highly susceptible forest ecosystems around the world, especially the eucalpytus and banksia woodlands of Australia, the oak woodlands of Mediterranean Europe, and chestnut species in both the Eastern US and Europe. In these systems, the pathogen has driven declines in susceptible species, leading to long-term changes in species composition and ecosystem structure. With recent reports highlighting that Pc is capable of persisting in asymptomatic herbaceous hosts, as well as residual susceptible species, and with no effective strategies for eliminating the pathogen from the landscape, it is clear that Pc is not going away. Thus, plans for moving forward with forest management, conservation, and restoration in affected ecosystems must incorporate mechanisms for improving forest resilience to Pc.

Research in affected forest ecosystems suggests some direction for conservation and management. First, observations of the association between disease severity and soil conditions suggest that poorly drained soils with consistently high soil moisture may be unfavorable for susceptible species. For example, chestnuts, oaks, and eucalyptus growing in consistently moist soils, poorly drained and/or in low-lying areas, exhibited greater disease severity than those growing in drier soils (Davison and Tay, 1987; Rhoades *et al.*, 2003; Moreira and Martins, 2005). Conservation and management strategies should take advantage of these associations, prioritizing conservation of forest communities in drier soils, which may be predisposed to greater resilience to Pc, and thus more successful conservation. In addition, where land-use change and degradation require intentional reforestation (e.g., surface mined land), site

preparation techniques must ensure adequate soil drainage (Koch and Samsa, 2007; Adank *et al.*, 2008).

Second, the genetic potential for resistance to infection by Pc has been largely unexplored for most susceptible forest species. Preliminary investigations into *Eucalyptus marginata* suggest that individuals demonstrating resistance to Pc can be used for selective breeding, leading to the development of improved varieties with high Pc resistance or tolerance (Stukely and Crane, 1994; Stukely *et al.*, 2007). Significant research programs are also underway to develop improved *Castanea* varieties with resistance to Pc. Decades of selective breeding targeting resistance to chestnut blight have produced improved blight-resistant varieties, and screening of these varieties for Pc resistance has identified potential Pc resistance genes (Zhebentyayeva *et al.*, 2013). Recent research in Europe has identified candidate resistance genes in European chestnut, providing direction for further research elucidating Pc resistance mechanisms (Santos *et al.*, 2017). Development of genetically improved varieties, through traditional breeding methods as well as more advanced genomic technologies, presents a major opportunity for conservation and restoration of susceptible species.

Third, the current and potential future distribution of Pc and Pc-related disease on the landscape must be understood. Current distribution of Pc in its introduced ranges must be elucidated, especially in areas where disease progression on the landscape is not obvious. For example, the current distribution of Pc in the southeastern US at multiple spatial scales is unclear, making the development of management recommendations challenging. Areas without Pc currently present should be prioritized for conservation efforts; these areas may represent long-term refugia for communities of Pc-susceptible species, if Pc spread into these areas can be prevented. At small spatial scales (e.g., within-watersheds), variation in site suitability for Pc and Pc-related disease must be elucidated to provide direction in prioritizing planting of susceptible species. Finally, mechanisms whereby Pc spreads, especially across great distances, must be understood and controlled. Movement of infested soils and Pc-infected plants via nursery trade must be controlled.

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CHAPTER 2: Detection of *Phytophthora cinnamomi* in forest soils by PCR on DNA extracted from leaf disc baits

2.1. Abstract

Phytopthora cinnamomi Rands causes root rot in a number of important forest tree species around the world, including American chestnut (*Castanea dentata*) and shortleaf pine (*Pinus echinata*). Conventional methods for detecting *P. cinnamomi* in forest soils may require too much time and space to permit wide-scale and long-term screening of the large sample numbers required for landscape scale distribution analysis. This project compared conventional detection methods (baiting with full rhododendron leaves or leaf discs and subsequent culturing on selective media) with a molecular detection method using DNA extracted from leaf baits. These methods were comparable, and the DNA-based method was correlated with culture-based methods. In a field-validation screening using the leaf bait PCR method, *P. cinnamomi* was found across a range of topographic conditions, including dry ridge-top sites and moist lowland sites. Soil texture analysis supports the traditional association of *P. cinnamomi* with finer-textured soils. Further large-scale surveys are necessary to elucidate landscape-scale distribution patterns in eastern US forests.

2.2. A dangerous and clandestine pathogen

Phytophthora cinnamomi Rands is an oomycete pathogen of global significance that originated in Southeast Asia, but has been introduced around the world (Arentz & Simpson 1986; Ko et al. 1978). *P. cinnamomi* is a generalist pathogen with a broad host range, but is perhaps most well known as the causal agent of declines in jarrah (*Eucalpytus marginata*) and other forest types in west Australia (Podger 1972; Shearer & Dillon 1995; Shearer & Dillon 1996), cork oak (*Quercus suber*) and holm oak (*Quercus ilex*) woodlands in Mediterranean Europe (Moreira et al. 1999; Robin et al. 1998; Scanu et al. 2013), and chestnut species (*Castanea dentata* and *Castanea sativa*) in North America and Europe (Day 1938; Milburn & Gravatt 1932), as well as avocado (*Persea americana*) and macadamia (*Macadamia spp.*) (Akinsanmi et al. 2016; Pagliaccia et al. 2013; Wager 1942).

The earliest records of *P. cinnamomi*-related disease in the US were reports of American chestnut decline in the southeast in the early-mid 1800s (Corsa 1896). Subsequently, the devastating chestnut blight fungus (*Cryphonectria parasitica*) was introduced to the eastern US

and rapidly killed back chestnuts throughout their range, functionally eliminating this dominant canopy species (Anagnostakis 2001). While a significant research effort was dedicated to investigating disease patterns of chestnut blight, *P. cinnamomi* continued to advance on the landscape with relatively little monitoring. After decades of breeding aimed at introgressing chestnut blight resistance from Chinese chestnut (*Castanea mollissima*) into American chestnut (Diskin et al. 2006), early plantings of varieties with improved blight resistance experienced high mortality caused by *P. cinnamomi* (Jacobs 2007; Rhoades et al. 2003). This spurred a resurgence of interest in *P. cinnamomi* and its associated disease in American chestnut. Current research efforts are focused at improving American chestnut genetic resistance to *P. cinnamomi* (Olukolu et al. 2012; Zhebentyayeva et al. 2013); however, improved understanding of distribution patterns of *P. cinnamomi* on the landscape is also critical to an informed restoration effort (Jacobs 2007; Sena et al., 2018).

While several studies have demonstrated that *P. cinnamomi* is widespread in the eastern US, relatively little is known about how *P. cinnamomi* distribution is constrained by environmental factors on the landscape scale in Appalachia. Campbell and Hendrix (1967) found that *P. cinnamomi* was widely distributed in forest soils in the southern Appalachians (isolating it from 5 of 43 samples in mountain hardwood/conifer stands and from 14 of 31 samples in coastal pine stands) but did not identify spatial patterns of distribution. Similarly, Sharpe (2017) isolated *P. cinnamomi* from 34% (120 of 353) of soil samples collected from forest plots in North Carolina, Tennessee, Virginia, and South Carolina, and Pinchot et al. (2017) detected *P. cinnamomi* in 100% of soil samples collected from an American chestnut study site in southeastern Kentucky. Generally, disease caused by *P. cinnamomi* is associated with moist, poorly drained soils (Dawson & Weste 1985; Keith et al. 2012; Vannini et al. 2010); however, *P. cinnamomi* has been successfully isolated from drier ridge-top soils in some cases (Shea & Dell 1981). *P. cinnamomi* is capable of surviving periods of sustained drought by producing survival structures such as chlamydospores (Kuhlman 1964; Old et al. 1984; Weste & Vithanage 1979). Chlamydospores are globose structures that can form within root tissue, but also on the surface of roots and other organic matter, and can be thick- or thin-walled. Thick-walled chlamydospores inside root tissue are thought to be the most resistant to drought and other inclement conditions (McCarren et al. 2005). In addition, *P. cinnamomi* was recently discovered in asymptomatic herbaceous understory plants in Australia, suggesting that its relationships with host plants are more complicated than previously understood (Crone et al. 2013a, b). At

broad landscape scales, *P. cinnamomi* is limited by freezing temperatures (Burgess et al. 2017); in the eastern US, *P. cinnamomi* has been detected in forest soils as far north as Ohio and Pennsylvania, United States Department of Agriculture Hardiness Zone 6 (McConnell & Balci 2014).

At relatively small spatial scales, *P. cinnamomi* is randomly distributed, with variable detection even within a 1 m^2 area (Meadows & Jeffers 2011; Pryce et al. 2002). Thus, rigorous subsampling is required to reduce risk of false negative detection results. However, in addition to improved sampling design, some studies suggest a need for improved detection sensitivity. Conventionally, *P. cinnamomi* is detected from soils by flooding soil with sterile water, baiting with susceptible plant material (e.g., rhododendron leaf), culturing infected bait material on selective media (agar amended with antibiotics and fungicides), and identifying *P. cinnamomi* by morphological characteristics and/or DNA sequence (Jeffers & Martin 1986). However, some studies have demonstrated that baiting and culturing can be insensitive, returning a high frequency of false negative results (Hüberli et al. 2000; McDougall et al. 2002). Finally, conventional methods require large volumes of soil (e.g., ~300 ml), and may be inconvenient for large-scale screening efforts or for screening of sites that are not readily accessible by road. With the development of molecular microbiology, improved methods have been established for screening potentially infected plant material. Conventional baiting and culturing detection methods are time-consuming and may be insensitive, while DNA-based detection methods may be both more rapid and more sensitive. Several polymerase chain reaction (PCR) methods have been developed for use in detecting *P. cinnamomi* from infected plant material (Kunadiya et al. 2017). Somewhat less attention has been paid to developing PCR-based methods for screening soils; however, a recent European study successfully implemented a nested touchdown PCR assay for rapid detection of *P. cinnamomi* from DNA extracted directly from chestnut plantation soils, with detection frequencies comparable to standard baiting and culturing (Langrell et al. 2011). DNA-based methods may also permit high-throughput screening of large numbers of samples by reducing the soil sample volume (as well as time and space) required for screening.

This project was initiated to compare detection assays for use in high-throughput screening of small-volume soil samples. Specifically, conventional baiting and culturing methods (one with full leaf baits and one with leaf disc baits, both followed by isolation on PARP media) were compared to a modified baiting method (baiting with leaf discs, followed by DNA extraction and screening with *P. cinnamomi*-specific PCR), and a soil-DNA method (soil DNA

extraction followed by screening with *P. cinnamomi*-specific PCR). To our knowledge, this is the first study screening DNA extracted from incubated leaf baits for detection of *P. cinnamomi* from soils. This methods comparison analysis was conducted using soils collected from a series of plots representing a gradient of soil and topographic variables in an eastern Kentucky watershed, providing insight into *P. cinnamomi* landscape distribution patterns.

2.3. Soil sampling and *P. cinnamomi* detection assays

Soil samples were collected from continuous forest inventory (CFI) plots in the 1500 hectare Clemons Fork watershed, University of Kentucky Robinson Forest, Breathitt County, Kentucky, in October-November 2016. CFI plots were selected for this analysis to make use of an extensive existing dataset (plots are surveyed periodically for a suite of topographic and vegetative parameters); the specific 47 plots surveyed were chosen to maximize accessibility (e.g., proximity to roads/trails) and minimize sampling time required. Samples were collected using a sampling spade (sterilized with 70% ethanol between plots), to a depth of ~10 cm where possible (soils were frequently very rocky and/or shallow). Six subsamples were collected within a ~1 m^2 area at plot center and combined in the field, and stored in plastic sampling bags at 4 °C until processed (1-2 weeks).

In the lab, soils were aliquoted in appropriate volumes for comparison of detection methods (Figure 2.1). For the full leaf baiting method, a 40 ml aliquot of soil was flooded with sterile water in a soil sample bag, and baited with a rhododendron leaf that had been surface sterilized by wiping with 70% ethanol. Samples were incubated in a growth chamber in the dark at 27 °C for one week. Lesions were excised from rhododendron leaves and transferred to PARP media (clarified v8 agar was amended with pimaricin, ampicillin, rifamycin, and PCNB), as described by Jeffers & Martin (1986). If lesions were not present (or if fewer than 3 lesions were present), segments of seemingly healthy tissue were excised and transferred to PARP media. A total of 3 segments were transferred to PARP media for each full leaf bait. After five days, hyphal tips were transferred to water agar and incubated for one week at 27 °C. A final transfer was made to clarified v8 agar for morphological identification.

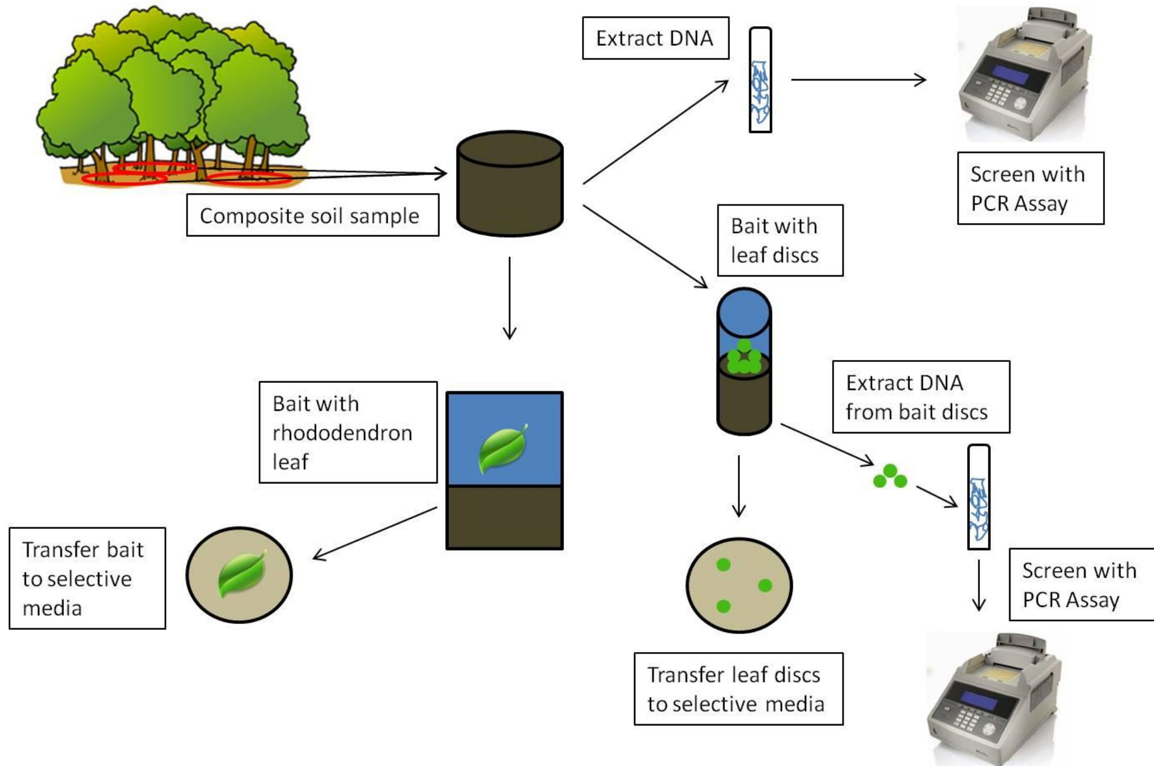


Figure 2.1: Overview of assays for detection of *P. cinnamomi* from forest soils selected for comparison in this study: DNA extraction from soil and amplification by *P. cinnamomi*-specific PCR, baiting with full rhododendron leaves and subsequent culturing on selective media, baiting with rhododendron leaf discs and subsequent culturing on selective media, and baiting with rhododendron leaf discs and subsequent DNA extraction and amplification by *P. cinnamomi*-specific PCR.

For the leaf disc baiting method, a 40 mL aliquot of soil was flooded with sterile water in a sterile 50 mL tube, and baited with leaf discs. Leaf discs (~6 mm diameter) were prepared by hole-punching rhododendron leaves that were surface sterilized by wiping with 70% ethanol. Six leaf discs were incubated in each flooded sample in a growth chamber in the dark at 27 °C for one week; three leaf discs were cultured as described above, and three leaf discs were frozen in 1.5 ml tubes for subsequent DNA extraction. Samples for which *P. cinnamomi* was isolated from at least one disc/piece were considered positive. Identification of isolates from leaf disc baits and full leaf baits was confirmed by *P. cinnamomi*-specific PCR as described below.

DNA was extracted from leaf disc baits (one DNA extraction per soil sample) using the Qiagen DNeasy UltraClean Microbial DNA Extraction Kit. Leaf discs (three per sample) were transferred to a bead tube, with 300 μl Microbead Solution and 50 μl solution MD1, and vortexed at maximum speed for 20 minutes using a platform vortexer attachment. After vortexing, 12 μl of 1 mg/ml Proteinase K was added to each sample, and samples were incubated overnight at room temperature. Supernatant was transferred to a clean 1.5 ml tube, and subsequent extraction steps were carried out according to manufacturer instructions. Amplifiable DNA was confirmed for each DNA extraction using ITS1-ITS4 primers, which amplify DNA from fungi and oomycetes (White et al. 1990). Reactions were prepared in 12.5 μl volumes, with 1 μl genomic DNA and 11.5 μl master mix containing 1.25 μl 10× PCR buffer, 1.25 μl of 2 mM dNTPs, 0.75 μl of 50 mM $MgCl_2$, 0.5 μl of 10 μM of each primer (ITS1 and ITS4), 0.0625 μl of Immolase Taq polymerase (Bioline, at 5U/μl), and 7.1875 μl water. Thermocycling conditions were: 94 °C for 10 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, with a final extension step at 72 °C for 5 min.

DNA was screened for *P. cinnamomi* using a PCR assay (primers Ycin3F: GTCCTATTCGCCTGTTGGAA and Ycin4R: GGTTTTCTCTACATAACCATCCTATAA) targeting a 300 bp segment of the Ypt gene developed by Schena et al. (2008) and recommended for *P. cinnamomi*-specificity by Kunadiya et al. (2017). Schena et al. (2008) tested these primers for specificity using genomic DNA from 73 isolates representing 35 species of the genus *Phytophthora* and 9 species of *Pythium*. Kunadiya et al. (2017) tested these primers against 11 species from Phytophthora clade 7, and one species from each of the other clades. Both studies reported these primers as specific to *P. cinnamomi*.

P. cinnamomi-specific PCR reactions (using primers Ycin3F and Ycin4R) were 25 μl containing 1.5 μl DNA, and 23.5 μl master mix (2.5 μl of 10× PCR Buffer, 2.5 μl of 2 mM dNTPs,

0.75 μl of 50 mM $MgCl_2$, 1 μl of 10 μM each primer, and 1 U Immolase Taq polymerase, and 15.55 μl water). Thermocycling conditions were: 95 °C for 10 min, followed by 42 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 15 s, and a final extension step of 72 °C for 10 min. PCR amplicons were visualized using agarose gel electrophoresis (1.5% m/v). Samples were screened in duplicate with positive controls, *P. cinnamomi* isolate RF5 (isolated from Robinson Forest, GenBank Accession #MF966152) 1.5×10^{-2} ng/PCR (the limit of detection), and no-template negative controls (Figure 2.2).

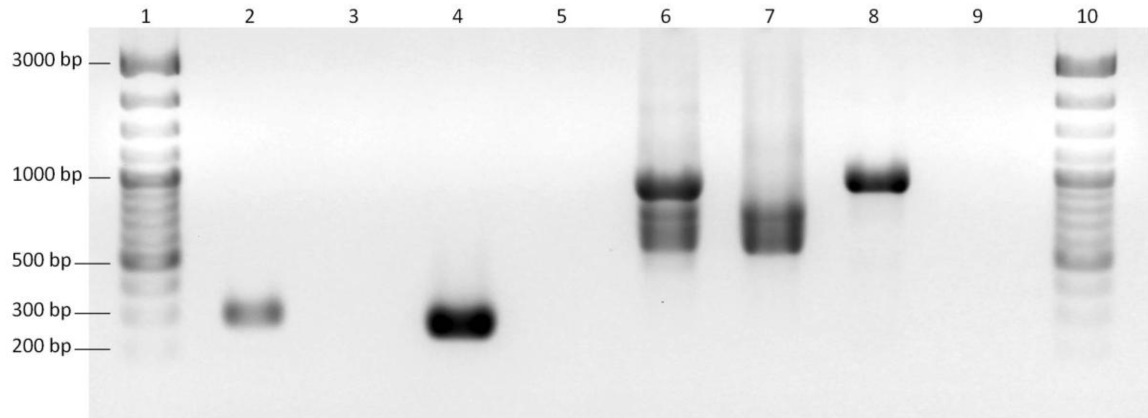


Figure 2.2: Gel (1.5% *m/v* agarose) image showing *Phytophthora cinnamomi*-specific PCR (Lanes 2-5, 300 bp fragment, using primers Ycin3F and Ycin4R) and confirmation of amplifiable DNA (Lanes 6-9, using primers ITS1 and ITS4): Marker (Lanes 1 and 10), *P. cinnamomi* positive control, isolate RF5 [1.5×10^{-2} ng/PCR] (2 and 8), No DNA template (3 and 9), Plot 360, *P. cinnamomi*-positive, (4 and 6), Plot 126, *P. cinnamomi*-negative, (5 and 7).

For the soil DNA detection method, DNA was extracted from 0.25 g soil (Qiagen PowerSoil DNA Extraction Kit, according to manufacturer instructions) and screened using the assay described above (one DNA extraction per soil sample). Detection methods were compared for effectiveness by assessing correlation of screening results with consensus positives and negatives (results given by the majority of methods).

2.4. Soil physical and chemical characteristics

In addition to screening for *P. cinnamomi*, soils were analyzed for the following physical and chemical parameters: pH, P, K, Ca, Mg, Zn, soil organic matter (SOM), total N, texture, and field capacity. Soil pH was measured in a 1:1 soil:water paste (Soil and Plant Analysis Council 2000). Concentrations of P, K, Ca, Mg, and Zn were measured by Mehlich III extraction and analysis by ICP (Soil and Plant Analysis Council, 2000; chapters 3, 6, and 7). SOM and total N were quantified by combustion using a LECO instrument (Nelson and Sommers 1982). Particle size distribution was evaluated by the micropipette method (Miller & Miller 1987), and field capacity was evaluated by the pressure plate method (Topp et al. 1993). Differences in soil physical and chemical data between samples with *P. cinnamomi* detected and samples with *P. cinnamomi* not detected (by any of the three assays) were assessed using a t-test, assuming unequal variances (SAS 9.3, PROC TTEST).

2.5. Comparison of *P. cinnamomi* detection methods

P. cinnamomi detection frequency varied across detection methods—direct soil DNA extraction and amplification was ineffective (none of the 47 samples tested were identified as positive), and results from the soil DNA extraction and amplification method were not included in further analysis. Thus, only results from the full leaf bait and culture, leaf disc bait and culture, and leaf disc bait and PCR methods are presented below. Of the 47 samples screened, 35 samples were negative by at least two of the three assays and considered consensus negatives, and 12 samples were screened as positive by at least two assays and considered consensus positives (Table 2.1). Samples screened as negative by one method but positive by one or two of the other methods were considered false negative results. Screening results in disagreement with the consensus result were termed “nonconsensus” positives or negatives. The full leaf bait and culture method returned 6 false negatives, 5 nonconsensus positives, and 2 nonconsensus negatives, and exhibited the lowest strength of correlation with consensus results (Figure 2.3, R^2 = 0.4172). The leaf disc bait and culture method returned 11 false negatives, 1 nonconsensus

positive, and 3 nonconsensus negatives, and exhibited better correlation to consensus results (R^2 = 0.5908). Finally, the leaf disc bait and PCR method returned 6 false negatives, 3 nonconsensus positives, and no nonconsensus negatives, and exhibited the highest correlation with consensus results (R^2 = 0.7314).

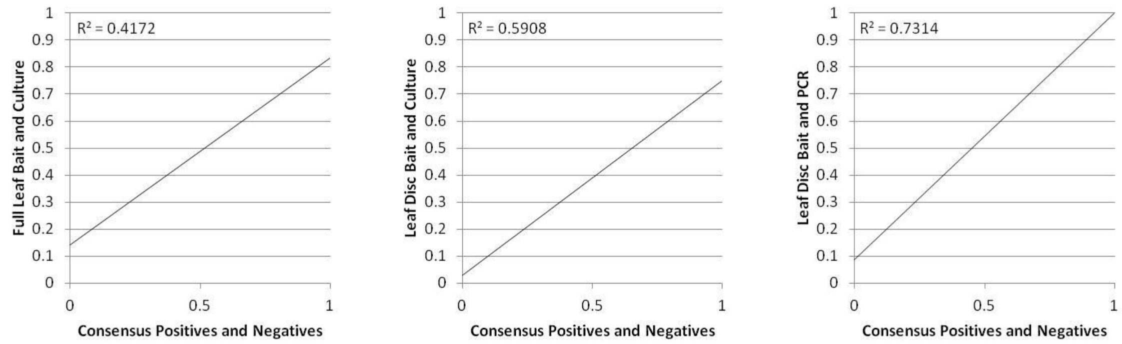


Figure 2.3: Correlations of screening results of *P. cinnamomi* detection assays with consensus screening results (0 = not detected, 1 = detected): full leaf bait and culture, leaf disc bait and culture, and leaf disc bait and PCR.

Table 2.1: Assay screening results for forest soils collected from 47 plots in Robinson Forest, Eastern Kentucky, USA (+ = *P. cinnamomi* detected, - = *P. cinnamomi* not detected).

Plot #	Full Leat Bait and Culture	Leaf Disc Bait and Culture	Leaf Disc Bait and PCR	Consensus* Positive or Negative
102	+	-	-	-
103	-	-	-	-
104	-	+	+	+
113	-	-	-	-
114	-	-	+	-
116	-	-	+	-
117	+	+	+	+
118	+	+	+	+
119	+	+	+	+
120	+	-	+	+
126	+	-	-	-
127	-	-	-	-
128	-	-	-	-
129	-	-	-	-
130	+	-	+	+
131	-	-	-	-
132	-	-	-	-
143	-	-	-	-
144	-	-	-	-
145	+	+	+	+
146	-	-	-	-
160	-	-	-	-
161	-	-	-	-
163	-	-	-	-
178	-	-	-	-
179	-	-	-	-
181	-	-	-	-
184	-	-	-	-
185	-	-	-	-
194	-	-	-	-
195	+	+	+	+
198	+	-	+	+
199	+	+	+	+
226	-	-	-	-
240	-	+	-	-
241	+	-	-	-

Table 2.1 (continued)

Plot #	Full Leat Bait and Culture	Leaf Disc Bait and Culture	Leaf Disc Bait and PCR	Consensus* Positive or Negative
345	-	-	-	-
346	-	-	-	-
351	-	-	-	-
355	-	-	-	-
356	-	+	+	+
357	-	-	-	-
360	-	-	+	-
361	+	-	-	-
367	-	-	-	-
368	+	-	-	-
369	+	+	+	+
Plots Positive	15	10	15	12
Plots Negative	32	37	32	35
False Negatives†	6	11	6	
Nonconsensus‡ Positives	5	1	3	
Nonconsensus‡ Negatives	2	3	0	

*Consensus: result from the majority of the assay methods.

†False negative: plot screened as negative by one method but positive by one or two of the other methods.

‡Nonconsensus: result indicates a screening result given by one method in disagreement with the other two methods.

Screening of DNA extracted from soil was unsuccessful at detecting *P. cinnamomi*. This is likely due to the limited capacity of the soil DNA extraction kit, which only extracted DNA from very small soil samples (~0.25 g). Because *P. cinnamomi* incidence is highly variable across small spatial scales, the likelihood of *P. cinnamomi* propagules being included in any given 0.25 g aliquot is low. In addition, if *P. cinnamomi* were present in the aliquot, the concentration of target DNA in the soil DNA extract may be below the detection limit of the PCR method. Development of efficient DNA extraction technology for purification of high-quality DNA from larger volumes of soil would make this method more effective. Langrell et al. (2011) reported high-quality DNA extraction from 10 g soil samples using a CTAB/chloroform method; however, we chose to use the commercial kit to increase throughput and maximize reproducibility. PCR inhibition is a major concern when working with environmental samples (Hedman and Radstrom, 2013); to minimize this risk, we selected a DNA extraction kit (Qiagen PowerSoil) recommended for reducing PCR inhibitors in soil DNA extracts, and we confirmed presence of amplifiable DNA in each soil DNA extract using ITS1-ITS4 primers as described above. In spite of these steps, PCR inhibition may still have been an impediment in the soil DNA method; absence of PCR inhibition should be confirmed in subsequent attempts at developing a soil DNA-based method. In addition, continued development of sensitive molecular detection methods would permit detection of lower concentrations of target DNA, also improving method sensitivity. Use of high-sensitivity nested assays with DNA extracted from large volumes of soil may provide the level of sensitivity necessary for reliable detection of low propagule concentration in soils.

P. cinnamomi detection frequency by the remaining three methods was similar, ranging from 10-15 positives and 32-37 negatives, with 12 consensus positives and 35 consensus negatives. The leaf disc bait and PCR method and the full leaf bait and culture methods both returned 6 false negatives, while the leaf disc bait and culture method returned 11 false negatives. The leaf disc bait and PCR method returned the fewest nonconsensus results, with three nonconsensus positive and zero nonconsensus negatives. The full leaf bait and culture method returned the greatest number of nonconsensus results, with 5 nonconsensus positives and 2 nonconsensus negatives.

P. cinnamomi detection by amplification of DNA extracted from leaf baits returned a greater number of consensus positive results and fewer false negatives and nonconsensus negatives than detection by culturing leaf disc baits. DNA-based microbial detection methods have been demonstrated to be more sensitive than traditional culturing methods for detection

of *P. cinnamomi* from infected plant material (Huberli et al. 2000; Williams et al. 2009). Culturing is necessarily selective and will not successfully detect all propagules present. In contrast, DNA-amplification based methods are capable of detecting propagules that may not be successfully cultured, including dead or stressed propagules, and are not affected by competing microbes.

While sensitivity was similar between the leaf disc bait and PCR method and the full leaf bait and culture method (15 positives, 32 negatives, and 6 false negatives each), the leaf disc bait and PCR method was more convenient, and required less operator time, elapsed time, and lab space. Traditional baiting and culturing is relatively time-constrained—cultures must be transferred to new media within a particular window of time, and likely transferred multiple times to produce isolates for identification. Each transfer step requires preparation of sterile media and sterile space, and can require hours of technician time. In contrast, the bait-PCR method was flexible and convenient—after the baiting step, bait discs were stored at -20°C until it was convenient to extract DNA and proceed with PCR. In addition, traditional baiting and culturing can require relatively large volumes of soil, with large space requirements for storage and incubation. In contrast, the bait-PCR method using 50 ml tubes required very little storage and incubation space during the baiting phase (50 ml tubes could be stacked neatly in racks), and even less space after baiting (bait discs were transferred to 1.5 ml tubes). The similarity between the leaf disc bait and PCR method and the full leaf bait and culture method suggests that the space and time requirements of screening for *P. cinnamomi* can be reduced without sacrificing sensitivity. If incubation space is not limiting, and if propagule density is low, the volume of soil used for baiting can be increased to improve sensitivity. Subsequently, extracting DNA from baits and proceeding with PCR reduces space and time required for screening, and improves flexibility.

2.6. P. cinnamomi distribution within the Clemons Fork Watershed

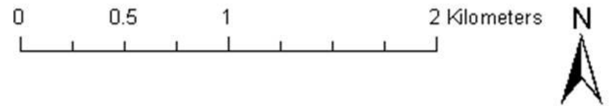
In this study, *P. cinnamomi* was detected (by one or more screening methods) in a total of 21 plots out of 47 sampled (44%). Plots in which *P. cinnamomi* was detected ranged from xeric ridge-top sites to sites located in natural drainage areas, including plots near perennial streams (Figure 2.4). While *P. cinnamomi*-associated disease is typically thought to be spatially restricted to moist, poorly drained soils (Dawson & Weste 1985; Keith et al. 2012; Vannini et al. 2010), the pathogen itself has also been recovered from dry sites with little to no observable disease symptoms (Shea and Dell 1981). Our results support the observation that *P. cinnamomi* occurs across a variety of environmental conditions; however, further research will be necessary

to evaluate the impact *P. cinnamomi* has on susceptible hosts across this range of environmental conditions.

Detection Results

Detected

Not Detected



N

Digital Elevation Model

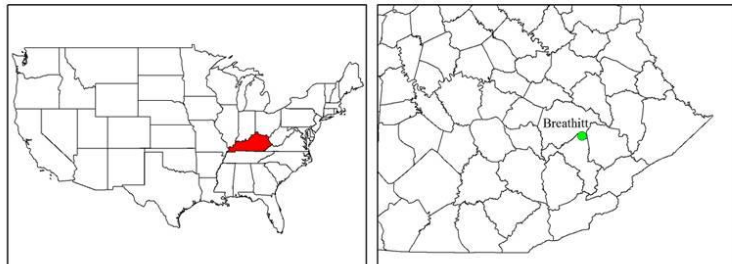


Figure 2.4: *P. cinnamomi* distribution map, depicting screening results for 47 samples collected within the Clemons Fork Watershed, Robinson Forest, Kentucky, USA.

The soil physical and chemical variables measured may shed some light on environmental constraints to *P. cinnamomi* distribution (Table 2.2). Soils in which *P. cinnamomi* was detected were characterized by lower pH, suggesting a tolerance for acidic soils. *P. cinnamomi* also tended to be detected in soils with lower cation concentrations (Ca, Mg, and Zn), which may relate to osmotic sensitivity of zoospores (Byrt et al. 1982). Finally, soil texture appeared to differ between these sample groups, with soils in which *P. cinnamomi* was detected characterized by lower percent sand and higher percent silt and clay. These results are consistent with traditional associations of *P. cinnamomi* with fine-textured soils (Dawson & Weste 1985; Keith et al. 2012; Vannini et al. 2010). Broader surveys are necessary to characterize landscape-scale distribution patterns of *P. cinnamomi* in central Appalachian forest soils.

Table 2.2: Soil physical and chemical characteristics (means ± SE) of soil samples in which *P. cinnamomi* was detected or not detected (N.D.) by any of three screening assays. p-value of t-tests (unequal variance assumed) are shown, with $p < 0.05$ considered significant.

	Detected	N.D.	*p-value*
Soil pH	3.80 ± 0.12	4.31 ± 0.14	**0.01**
P (mg/kg)	10.9 ± 1.1	13.7 ± 1.2	0.09
K (mg/kg)	90.3 ± 6.7	110.6 ± 10.5	0.11
Ca (mg/kg)	320.3 ± 60.7	709.5 ± 124.0	**0.008**
Mg (mg/kg)	79.5 ± 8.0	138.6 ± 20.9	**0.01**
Zn (mg/kg)	2.4 ± 0.24	3.2 ± 0.45	**0.001**
SOM (%)	7.72 ± 0.92	7.26 ± 0.58	0.67
Total N (%)	0.215 ± 0.02	0.233 ± 0.02	0.53
% Sand	44.6 ± 3.6	59.7 ± 2.3	**0.001**
% Silt	41.8 ± 3.0	29.6 ± 1.8	**0.001**
% Clay	13.6 ± 0.82	10.8 ± 0.53	**0.007**
% Fines	55.4 ± 3.6	40.3 ± 2.3	**0.001**
Plant Available Water (%)	21.1 ± 1.6	16.8 ± 0.94	**0.03**
Field Capacity (%)	36.7 ± 2.2	32.0 ± 1.3	0.08
Wilting Point Water (%)	15.6 ± 0.93	15.2 ± 0.90	0.73

2.7. Recommendations for Practitioners

This study suggests that detection assays for *P. cinnamomi* can be further developed to reduce time and space required for screening, without sacrificing sensitivity. Specifically, our study supports the use of PCR on DNA extracted from leaf disc baits for high-throughput detection of *P. cinnamomi* from relatively small soil samples. This approach can be customized to meet specific investigator needs—for example, sample size used for baiting can be increased to improve sensitivity, if lab space is not limited. In addition, this study demonstrates that *P. cinnamomi* is not restricted to moist lowland soils, but is also capable of survival in dry ridge-top soils. This suggests that dry ridge-top sites are not necessarily Phytophthora-free and may not be ideal sites for restoration of susceptible species.

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CHAPTER 3: *Phytophthora cinnamomi* colonized reclaimed surface mined sites in Eastern Kentucky: implications for restoration of susceptible species†

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3.1 Abstract

Appalachian forests are threatened by a number of factors, especially introduced pests and pathogens. Among these is *Phytophthora cinnamomi*, a soil-borne oomycete pathogen known to cause root rot in American chestnut, shortleaf pine, and other native tree species. This study was initiated to characterize the incidence of *P. cinnamomi* on surface mined lands in eastern Kentucky, USA, representing a range of time since reclamation (10, 12, 15, and 20 years since reclamation). Incidence of *P. cinnamomi* was correlated to soil properties including overall soil development, as indicated by a variety of measured soil physical and chemical parameters, especially the accumulation of soil organic carbon. *P. cinnamomi* was detected in only two of the four site ages studied, aged 15 and 20 years since reclamation. These sites were generally characterized by higher organic matter accumulation than the younger sites in which *P. cinnamomi* was not detected. These results demonstrate that *P. cinnamomi* is capable of colonizing reclaimed mine sites in Appalachia; additional research is necessary to determine the impact of *P. cinnamomi* on susceptible tree species at these sites.

3.2. Introduction

Appalachian forests are threatened by many stressors, including climate change (McLaughlin et al. 2007; Butler et al. 2015) land use change (Gragson and Bolstad, 2006; Cormier et al. 2013; Wickham et al. 2013), and invasive pests and pathogens (Anagnostakis, 2001; Flower et al. 2013). Forest restoration and management efforts must be informed by a clear understanding of these and other impacts to ensure forest health and resilience in the future (Nelson et al. 2012). The American chestnut (*Castanea dentata* (Marsh.) Borkh.) story is a well-known example of the dramatic effects of invasive pathogens. American chestnut was once a

dominant canopy species throughout the Appalachian region, which includes the states of West Virginia and parts of Alabama, Georgia, Kentucky, Maryland, Mississippi, New York, North Carolina, Ohio, Pennsylvania, South Carolina, Tennessee, and Virginia. Composing 50% or more of the forest canopy over parts of its range, American chestnut was functionally eliminated from eastern forests in only a few decades in the early 1900's by the introduced fungal pathogen causing chestnut blight, *Cryphonectria parasitica* (Murr.) Barr (Anagnostakis, 2001; Paillet, 2002; Rigling and Prospero, 2018). Thanks to breeding targeted at introducing resistance genes from blight-resistant Chinese chestnut (*Castanea mollissima* Blume) into American chestnut, American chestnut varieties with reasonable levels of blight resistance are becoming available for use in restoration plantings (Diskin et al. 2006).

Unfortunately, even before the introduction of chestnut blight, *Phytophthora cinnamomi* Rands, a pathogen causing ink disease in American chestnut, had been introduced in the southeastern US and had been slowly causing American chestnut decline in the mid-late 1800s (Corsa, 1896; Milburn and Gravatt, 1932). Thought to have originated from Papua New Guinea or Taiwan (but since distributed globally) (Arentz, 2017), *P. cinnamomi* is a soil-borne oomycete pathogen with >2000 susceptible species (Shearer et al. 2004), with disease symptoms including black exudate staining infected roots, chlorotic leaves, and thinning crowns (Anagnostakis, 2001). The pathogen continued its slow march northward, overshadowed by the much more dramatic activity of chestnut blight (Anagnostakis, 2001). Unfortunately, genes conferring resistance to chestnut blight do not necessarily also confer resistance to *P. cinnamomi*; thus, some of the early blight-resistant American chestnut varieties were killed by *P. cinnamomi* when planted (Rhoades et al. 2003; Jeffers et al. 2009). These early observations have led researchers to focus on identifying genes conferring resistance to *P. cinnamomi* and developing strategies for improving blight-resistant varieties to also be *P. cinnamomi* resistant (Olukolu et al. 2012; Zhebentyayeva et al. 2013). In addition to the genetic improvement of susceptible hosts, such as American chestnut, a recent review recommended the construction of a more robust dataset on *P. cinnamomi* distribution in eastern US forests (Sena et al. 2018a).

P. cinnamomi is broadly distributed globally. At very small spatial scales (i.e., ~1 m^2), the pathogen appears to be somewhat randomly distributed; although it may be present within a given square meter soil patch, it is likely to be detected in only a fraction of samples collected within that patch (Pryce et al. 2002; Wilson et al. 2003; Meadows et al. 2011). Pathogen propagules are also capable of moving vertically within the soil profile, retreating to depth to

survive inclement conditions (McLaughlin et al. 2009; Meadows et al. 2011). Across broader spatial scales, *P. cinnamomi* is thought to be associated with moist soils at low topographic positions, such as drainages. Conversely, the pathogen is generally thought to be absent from higher and drier soils (Dawson and Weste, 1985; Pryce et al. 2002; Wilson et al. 2003; Rodriguez-Molina et al. 2005). However, *P. cinnamomi* has been isolated from dry ridge-top soils in Australia (Shea and Dell, 1981) and eastern Kentucky (Sena et al. 2018b), suggesting that environmental conditions limiting *P. cinnamomi* and its distribution are complex. *P. cinnamomi* has been present in the southeastern US for over 150 years (Corsa et al. 1896), and is known to be widely distributed in the Appalachian region (Pinchot et al. 2017; Sharpe, 2017), but topographic and soil factors controlling its distribution at smaller spatial scales (e.g., within a watershed) have not been elucidated in any detail. These patterns must be well-understood before informed decisions about the restoration of susceptible tree species can be made.

In addition to introductions of non-native pests and pathogens, Appalachian forests have been degraded by a legacy of surface mining for coal. An estimated 600,000 ha of formerly forested land in Appalachia have been converted to novel grassland systems, characterized by high soil compaction and vegetative competition, and generally unfavorable for colonization by native trees (Zipper et al. 2011a,b; Zipper et al. 2013). These grassland patches perpetuate negative impacts of surface mining into the future, increasing forest fragmentation, increasing species invasion opportunity, and inhibiting site productivity and carbon storage (Wickham et al. 2006; Amichev et al. 2008; Zipper et al. 2011b; Wickham et al. 2013).

This situation prompted researchers, regulators, and industry practitioners to develop a set of recommendations for reforestation at surface mined sites, termed the Forestry Reclamation Approach (FRA, Adams, 2017). These recommendations encourage minimal spoil compaction and reduced vegetative competition, improving the growth and survival of native trees over traditional reclamation practices (Wilson-Kokes et al. 2013a,b; Sena et al. 2015). These mine spoils, while initially devoid of organic matter, can support rapid tree growth and organic matter accumulation, and are considered soils after reclamation (Amichev et al. 2008). Yellow poplar (Liriodendron tulipifera L.) and white oak (Quercus alba L.) planted in FRA plots exhibited growth rates similar to the growth of these species in naturally regenerating clear cuts (Cotton et al. 2012). A chronosequence study in West Virginia found that soil organic carbon (SOC) accumulation in mine soils could be predicted by a logarithmic model, with 75% of 50-year SOC stock (13.3 Mg ha^{-1}) accumulating in the first ten years (Chaudhuri et al. 2012). Other

studies have found even higher SOC accumulation—stocks of 19.2 Mg ha^{-1} 13 years after reclamation and 38 Mg ha^{-1} 25 years after reclamation at sites in Ohio (Amichev et al. 2008; Shrestha and Lal, 2010), and 16.8 Mg ha^{-1} 16 years after reclamation at sites in Kentucky (Amichev et al. 2008). Together with SOC accumulation, above-ground biomass and litter accumulation contribute to overall ecosystem C sequestration, projected at 140.8–162.3 Mg ha^{-1} after 60 years in forest reclamation sites from Kentucky, Ohio, Indiana, and Illinois (Amichev et al. 2008).

Because these mine soils are typically not reclaimed with native topsoil, *P. cinnamomi* and other soil-borne pathogens are generally thought to be initially absent from these sites (French et al. 2007; McCarthy et al. 2008; Fields-Johnson et al. 2012; Hiremath et al. 2013). FRA sites may also be unfavorable for *P. cinnamomi* for some time after reclamation due to high infiltration rates, low moisture retention, and relatively high temperatures associated with low shade, low organic matter, and little to no clay content (Taylor et al. 2009a,b; Sena et al. 2014). *P. cinnamomi* was not detected in mine reforestation plots in southeastern Kentucky one and three years after reclamation (Adank et al. 2008), and was also not detected in a number of sites 3–20 years after reclamation in Ohio (Hiremath et al. 2013). However, a recent chronosequence study in eastern Kentucky suggests that rates of microbial activity (assayed by dehydrogenase activity), microbial biomass C and N, litter decomposition rates, and CO_2 efflux in mined sites eight years after reclamation were similar to native forests 12 years after clear-cutting (Littlefield et al. 2013). It is possible that *P. cinnamomi* can colonize these sites over time, along with other soil microorganisms, as soil development progresses. This study was initiated to evaluate whether *P. cinnamomi* colonizes FRA sites representing a chronosequence of time since reclamation in eastern Kentucky, and relate this incidence to soil development parameters, especially SOC accumulation.

3.3. Methods and Materials

Eight reclaimed surface mined sites of varying ages (two sites each reclaimed in 1997, 2003, 2005, and 2007, representing ages 20, 15, 12, and 10 years since reclamation) in eastern Kentucky were selected for screening for the presence of *P. cinnamomi* (Figure 3.1).

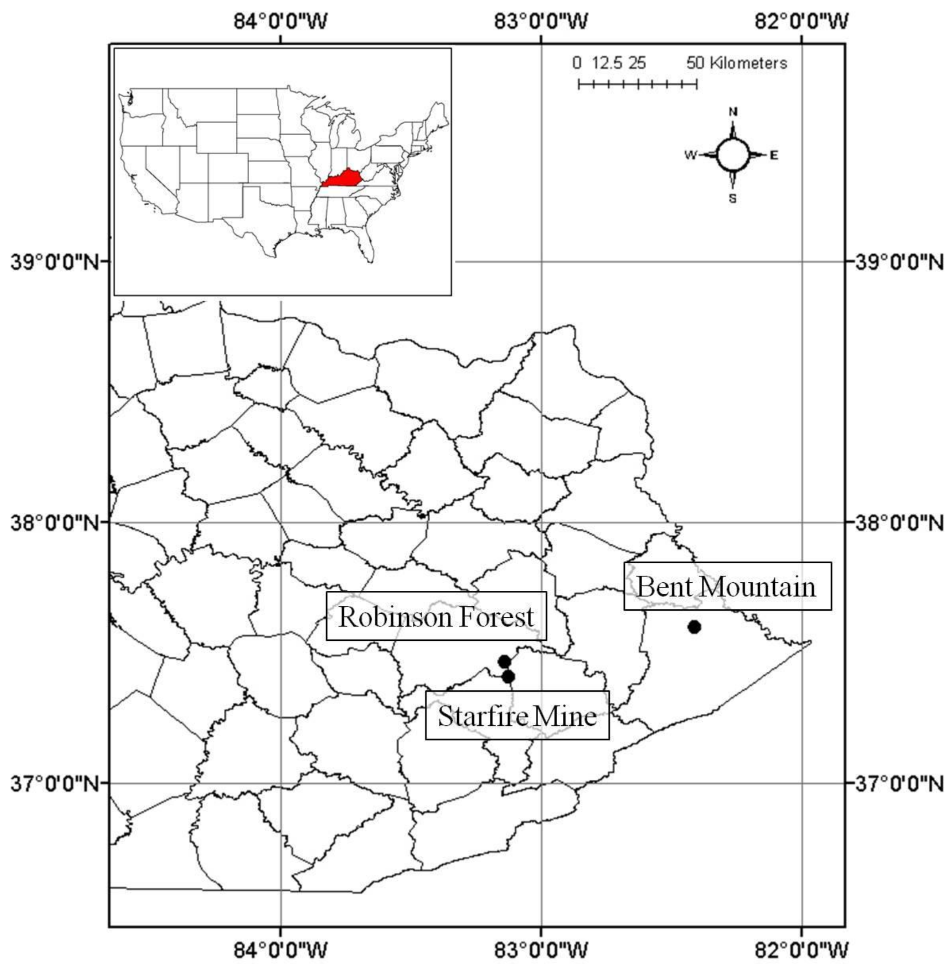

Figure 3.1. Location of reclaimed mine sites and unmined forest control, Breathitt (Robinson Forest), Perry (Starfire Mine), and Pike (Bent Mountain) Counties, Eastern Kentucky, USA.

Sites reclaimed in 1997 and 2003 were located at Starfire Mine (Breathitt County, Kentucky, 37.40939° N, −83.1229° W), and sites reclaimed in 2005 and 2007 were located at Bent Mountain Mine (Pike County, Kentucky, 37.60023 N, −82.40848 W). Climate in this region is temperate humid continental, with an average temperature of 13.3 °C (USDA 2004). Average annual precipitation in Robinson Forest (adjacent to the Starfire Mine site) is 117.5 cm (Cherry, 2006), and average annual precipitation in Pike County is 114 cm (Taylor et al. 2009a). Typical temperatures range from 18–30 °C in summer and −5–6 °C in winter at Robinson Forest, and from 18–32 °C in summer and −4–7 °C in winter in Pike County (Taylor et al. 2009a). Sites were constructed using low-compaction spoil placement techniques (Adams, 2017), with overburden sourced from the Breathitt formation, which is dominated by sandstones and shale (Daniels et al. 2016). For site details, see Cotton et al. (2012) for the 1997 site, Littlefield et al. (2013) for the 2003 site, Agouridis et al. (2012) for the 2005 site, and Miller et al. (2012) for the 2007 site. Similar tree species were planted at each of these sites during reclamation, and white oak was present at all sites at the time of sampling. Because these sites were reclaimed using similar techniques with spoil from the same geologic formation, and are subject to similar weather conditions, we employed a site-for-time substitution (chronosequence approach) to evaluate trends over time (Littlefield et al. 2013).

Twenty soil samples were collected underneath white oak at each site in October–November 2017, for a total of 160 samples, and all 160 samples were screened independently. Samples for *P. cinnamomi* screening were collected in 50 mL tubes from the top 5 cm of soil. Samples were screened using a rapid screening approach developed in our laboratory (Sena et al. 2018b). Briefly, ~40 mL soil samples were flooded with sterile water in 50 mL tubes, and baited with six ~6 mm diameter rhododendron leaf discs for five to seven days. Baits were then removed and frozen in 1.5 mL tubes for subsequent DNA extraction. DNA was extracted from baits using the DNeasy UltraClean Microbial DNA Extraction Kit [Qiagen]. Amplifiable DNA was confirmed for each DNA extraction using universal ITS1–ITS4 primers (White et al. 1990). DNA was screened for *P. cinnamomi* using a conventional PCR assay with primers Ycin3F and Ycin4R targeting a 300 bp fragment of the Ypt gene recommended for *P. cinnamomi* specificity (Schena et al. 2008; Kunadiya et al. 2017). Samples were screened in duplicate with positive controls, *P. cinnamomi* isolate RF5 (isolated from Robinson Forest, GenBank Accession #MF966152) limit of detection 15×10^{-2} ng per PCR, and no template negative controls. Primer annealing temperatures were 55 °C and 58 °C for the ITS and Ypt PCRs, respectively, with PCR amplicons

visualized using agarose gel electrophoresis, 1.5% (m/v). *P. cinnamomi* incidence was assessed as % of total samples (20 samples screened per site) screened as positive.

In addition to screening for *P. cinnamomi*, a number of soil physical and chemical parameters were assessed at these reclaimed mine sites, as well as a mature white oak stand in Robinson Forest (Breathitt and Perry Counties, Kentucky) selected as an unmined forested control. Three soil samples per site age (sampled to 10 cm using a soil probe) were collected for chemical and physical analyses, and data were averaged by site age (10, 12, 15, and 20 years since reclamation), to permit direct comparison to data reported by previous studies (Maharaj et al. 2007b). Particle size was assessed by quantifying the mineral grain size distribution, and sand, silt, and clay fractions as defined by the Wentworth Scale (Wentworth, 1922). These samples were dried at ~75 °C for 24–48 h, gently disaggregated, wet-sieved through 2 and 0.5 mm sieves, and treated with dilute H_2O_2 to destroy organic binding agents (Hillier, 2001; Yeager et al. 2005). Samples were then analyzed using a Malvern Mastersizer S-2000, a laser-optical particle size characterization instrument capable of accurately resolving particles over a size range of 0.02 to 2000 μm.

Concentrations of Al, Mn, Fe, Mg, K, Ca, and Na were assessed by inductively coupled plasma mass spectrometry (ICPMS) after samples were completely dissolved using concentrated acids (HF, HCl, and HNO_3) over heat. Bulk density was assessed by the excavation method (Grossman and Reinsch, 2002), and SOC was measured using an LECO CHN 2000 analyzer after an acid pretreatment (HCl). Although conventional SOC assessment (using a LECO analyzer) follows an acid pretreatment to remove inorganic carbon (e.g., carbonate minerals), this step has been known to incompletely eliminate carbonates from carbonate-rich mine soils, and can overestimate SOC (Maharaj et al. 2007a,b). Thus, soil organic matter (SOM) was evaluated by the thermogravimetric method—this method more accurately differentiates “new organic carbon” contributed by biomass from “old organic carbon” contributed by coal fragments or inorganic carbon contributed by carbonate minerals (Maharaj et al. 2007a,b). $\delta^{13}C$ (‰) was measured after HCl pretreatment (and was thus reflective of organic C only) on a Thermo-Finnigan Delta XP Isotope Ratio Mass Spectrometer.

Soil physical and chemical data (means by site age) were analyzed by regression, together with data from previous studies at these sites where available (Maharaj et al. 2007b; Adank et al. 2008; Agouridis et al. 2012; Cotton et al. 2012; Miller et al. 2012; Littlefield et al. 2013; Sena et al. 2014), with years since reclamation as the main effect (PROC GLM, SAS 9.3).

Both linear and quadratic relationships were tested for each variable; data were interpreted using a quadratic regression if the quadratic factor was significant ($p < 0.05$), but were interpreted using a linear regression if the quadratic factor was insignificant ($p > 0.05$). Sand and silt data were available from these sites at times representing 0, 1, 2, 3, and 8 years after reclamation (Maharaj et al. 2007b; Adank et al. 2008; Agouridis et al. 2012; Cotton et al. 2012; Miller et al. 2012; Littlefield et al. 2013; Sena et al. 2014). SOM, SOC, and $\delta^{13}C$ data were available for these sites at times representing 0, 2, 3, and 8 years since reclamation (Maharaj et al. 2007b; Littlefield et al. 2013). *P. cinnamomi* incidence was interpreted in light of trends over time in soil physical or chemical parameters to provide insight into *P. cinnamomi* colonization of these sites.

3.4. Results

Soil particle size distribution was significantly correlated ($p < 0.05$) with time since reclamation for % sand and % silt (Figure 3.2). Sand decreased from 70% in new mine soils to <40% in mine soils 12–20 years after reclamation. In contrast, silt increased from 20% in new mine soils to 55% in mine soils 12–20 years after reclamation. This shift in particle size distribution toward a dominance of silt may be related to increasing trends in concentrations of some of the metal analytes evaluated in this study (e.g., Al, Fe, Mg, and Ca, Table 3.1). Decreased soil particle size corresponds to dramatically increased soil surface area and reactivity, which very likely increases the sorption of these and other cations (Sposito, 1989; Essington, 2004).

Table 3.1. Soil physical and chemical characteristics (means ± SE).

Years Since Reclamation					Robinson Forest
	10	12	15	20	
Al (ppm)	36,800 ± 640	36,800 ± 1200	46,600 ± 7200	48,100 ± 9800	50,800 ± 5000
Mn (ppm)	184 ± 8.5	316 ± 25	321 ± 38	259 ± 39	997 ± 150
Fe (ppm)	9770 ± 140	17,800 ± 960	17,400 ± 2500	19,800 ± 3400	25,600 ± 2000
Mg (ppm)	1420 ± 37	2280 ± 63	2700 ± 550	3300 ± 680	1980 ± 230
K (ppm)	13,500 ± 400	12,400 ± 800	15,400 ± 2500	14,600 ± 2700	12,900 ± 1400
Ca (ppm)	489 ± 36	1040 ± 130	1540 ± 440	1220 ± 62	455 ± 110
Na (ppm)	3880 ± 31	2740 ± 120	2650 ± 220	1800 ± 590	833 ± 57
% Sand	60.6 ± 3.3	37.5 ± 1.9	48.4 ± 5.9	38.1 ± 2.1	27.3 ± 3.7
% Silt	35.9 ± 3.0	56.4 ± 2.2	47.1 ± 5.8	55.1 ± 2.0	62.6 ± 3.6
% Clay	3.45 ± 0.38	6.07 ± 0.48	4.53 ± 0.33	6.80 ± 1.6	10.1 ± 0.28

Total SOC, determined using an LECO analyzer, also demonstrated a significant correlation with time since reclamation, increasing from very low levels in new mine soils (0.1–0.2% in two to three year old soils) to >3.0% in 15–20 year old soils, nearing the SOC levels in unmined forest soils (Figure 3.3). Similarly, "new organic carbon" represented by SOM measured by the thermogravimetric method increased from 0.03–0.10% in zero to two year old mine soils to 1.5–2.2% in 15–20 year old mine soils, nearing SOM levels in unmined forest soils (Figure 3.4). SOC concentrations measured by the LECO analyzer tended to be higher than SOM measured by thermogravimetry (Figure 3.5).

While organic carbon concentrations increased with time since reclamation, $\delta^{13}C$ values decreased with time since reclamation, approximating $\delta^{13}C$ values in unmined forest soils (Figure 3.6). This relationship between $\delta^{13}C$ and SOM is negative and quadratic, with lower $\delta^{13}C$ values and higher SOM concentrations in the older mine soils, nearing levels in unmined forest soils (Figure 3.7). Total SOM stocks (Mg C ha^{-1}) exhibited different trends across sites, suggesting some differences in site quality, likely related to differences in regional geology and site construction (Figure 3.8) (Maharaj et al. 2007b). However, at both sites, SOM stocks approached 20 Mg ha^{-1} by eight to 12 years after reclamation.

P. cinnamomi was detected at all four Starfire sites (15 and 20 years after reclamation), but was not detected in any of the Bent Mountain sites (10 and 12 years after reclamation, Figure 3.9). Of the samples screened at the Starfire sites, 27.5% of samples (range: 5–50%) at the 15-year old sites were positive, and 12.5% (range: 10–15%) of samples at the 20-year old sites were positive. These sites tended to have lower % sand and higher % silt, as well as increased SOC and SOM stocks, compared to the younger Bent Mountain sites.

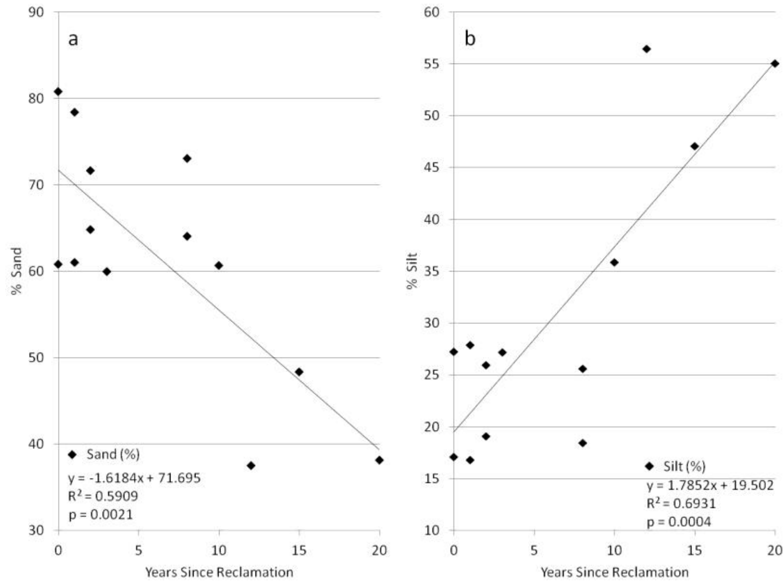

Figure 3.2. Changes in (a) % sand and (b) % silt in mine soils over time since reclamation.

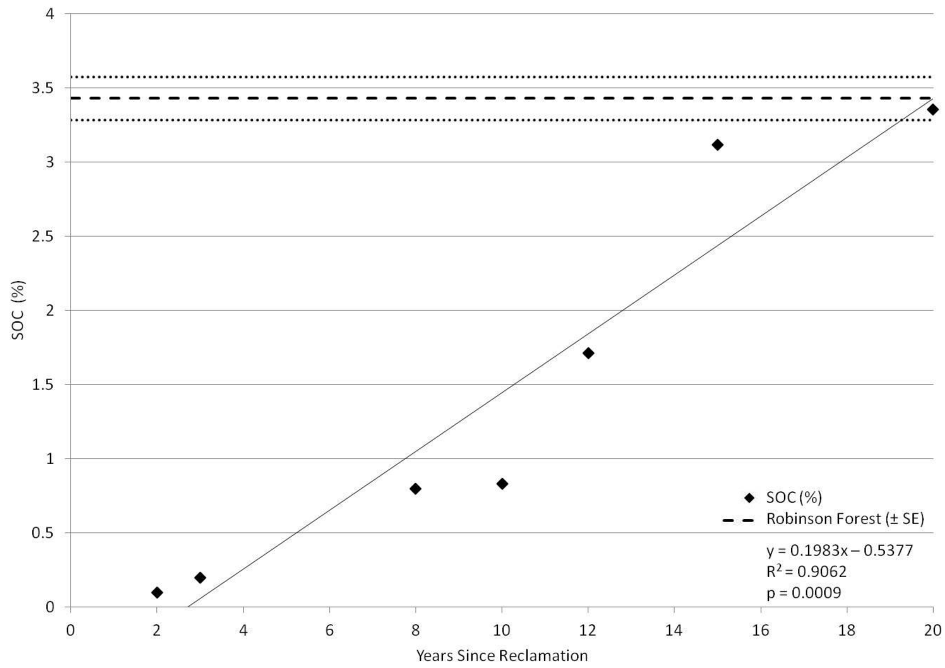

Figure 3.3. Changes in soil organic carbon (SOC; LECO analysis) in mine soils over time since reclamation, with mean % SOC (± SE) for Robinson Forest plotted for reference.

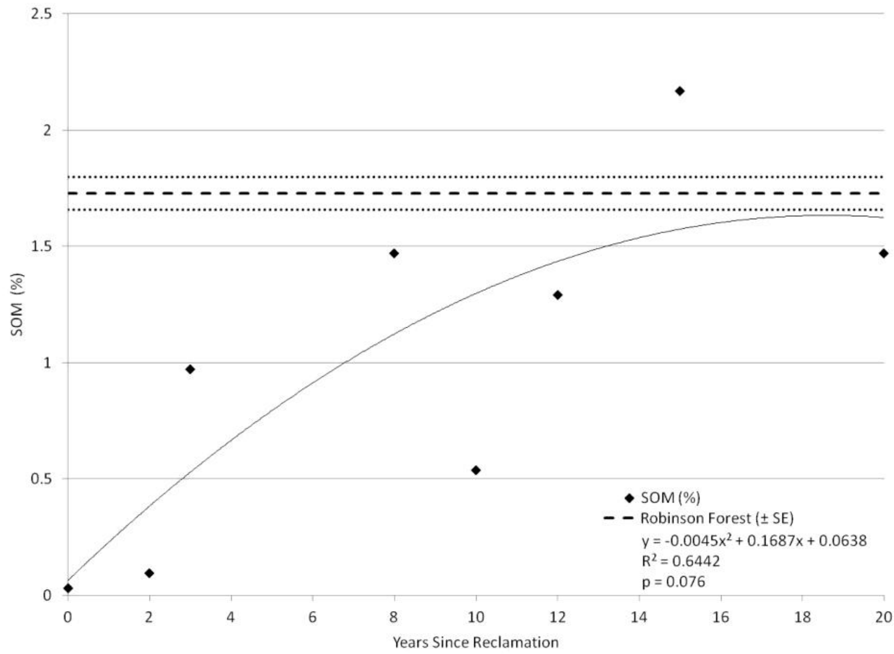

Figure 3.4. Changes in soil organic matter (SOM; thermogravimetric analysis) in mine soils over time since reclamation, with mean % SOM (± SE) for Robinson Forest plotted for reference.

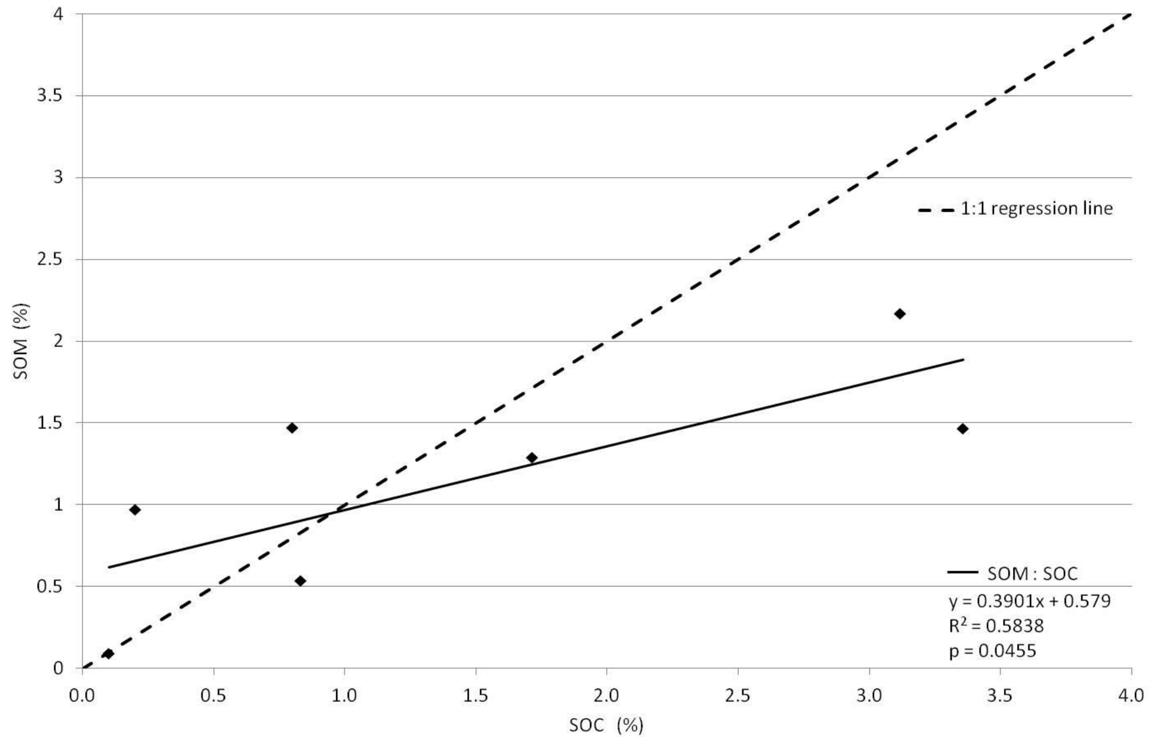

Figure 3.5. Correlation of SOC (LECO analysis) and SOM (thermogravimetric analysis), compared to a 1:1 reference line.

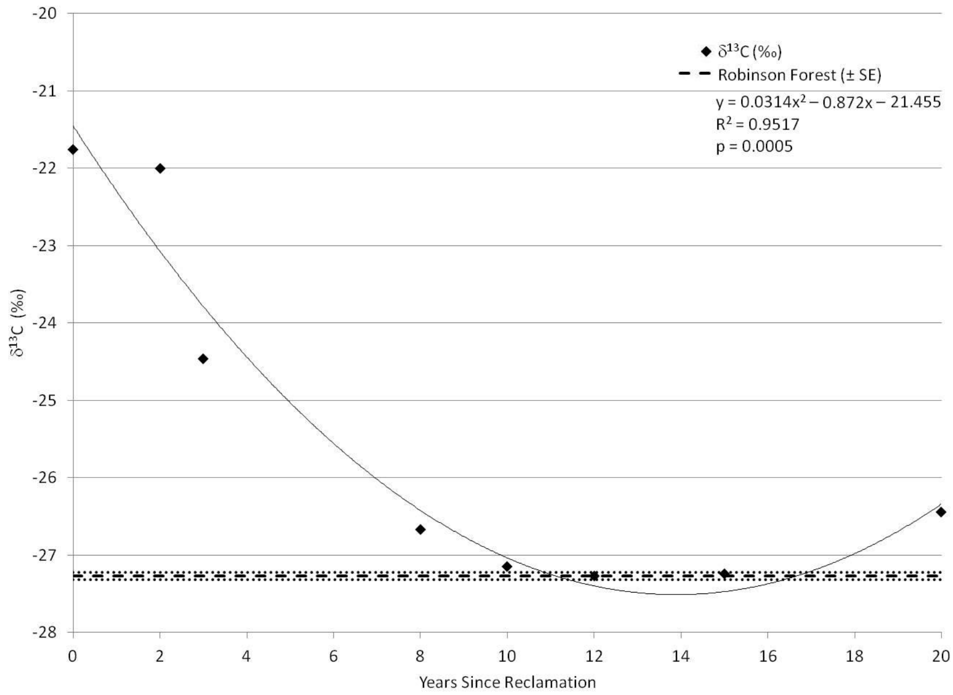

Figure 3.6. Changes in $\delta^{13}C$ (‰) in mine soils over time since reclamation, with mean $\delta^{13}C$ (± SE) for soil from a mature white oak stand in Robinson Forest plotted for reference.

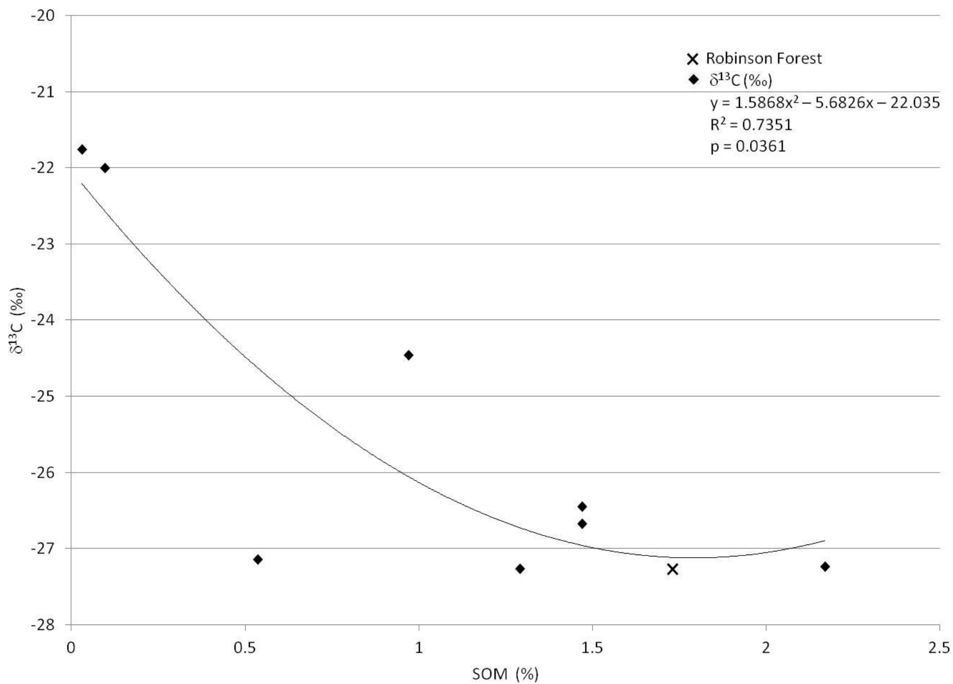

Figure 3.7. Correlation between % SOM (thermogravimetric analysis) and $\delta^{13}C$ (‰) in mine soils representing a range of time since reclamation, with Robinson Forest plotted for reference.

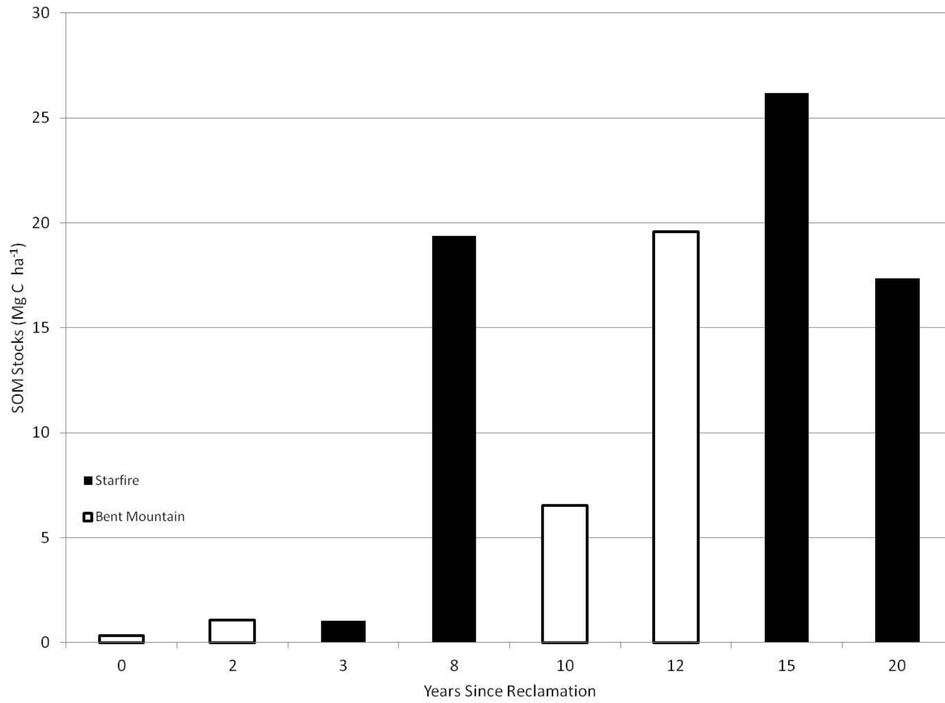

Figure 3.8. SOM stocks in mine soils over time since reclamation. Rates of accumulation of SOM in Bent Mountain sites appeared to lag slightly behind those of Starfire Mine sites.

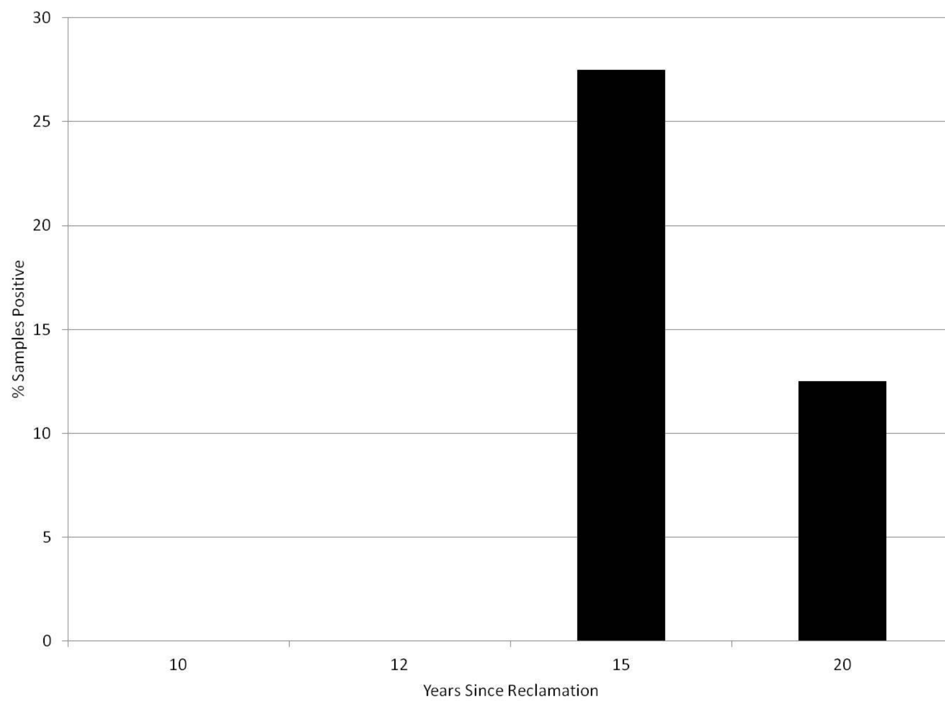

Figure 3.9. Incidence of *Phytophthora cinnamomi* in mine soils over time since reclamation.

3.5. Discussion

Soil particle size shifts are consistent with those observed by previous studies at these sites, generally reporting increased fines and decreased coarse fractions with time (Miller et al. 2012; Sena et al. 2015). Over time, this shift in particle size is certain to increase soil reactivity, reflected in the increasing concentrations of metals seen in this study (Sposito, 1989; Essington, 2004). Increased SOC and SOM concentrations with time since reclamation are also consistent with other studies investigating mine soil development (Maharaj et al. 2007b; Amichev et al. 2008; Chaudhuri et al. 2012). Values of SOC (LECO analyzer) tended to be higher than values of SOM (thermogravimetric method) in this study. Acid (HCl) pretreatment followed by measurement of SOC on a LECO analyzer can inadequately remove inorganic carbon by incompletely dissolving carbonate minerals in carbonate-rich mine soils. LECO analysis also does not distinguish "old organic carbon" in coal fragments from "new organic carbon" in biomass, which can also lead to the overestimation of SOC (Maharaj et al. 2007a,b). The thermogravimetric method used in this study to assess SOM more accurately differentiates "new organic carbon" in biomass from inorganic carbon (e.g., carbonate minerals) and "old organic carbon" (e.g., coal fragments); thus, values of SOM (thermogravimetric method) are expected to be lower than those of SOC (LECO analyzer) (Maharaj et al. 2007a,b). SOM stocks at the sites included in this study (20 Mg ha^{-1} by eight to 12 years after reclamation) were similar to those reported by other studies on reclaimed sites in Appalachia, ranging from 13.3 Mg ha^{-1} over 10 years in West Virginia (Chaudhuri et al. 2012), to 26 Mg ha^{-1} after 10 years in Ohio (Amichev et al. 2008; Shrestha and Lal, 2010).

P. cinnamomi was only detected at sites 15 and 20 years after reclamation, demonstrating that *P. cinnamomi* is capable of colonizing FRA reclaimed sites over time. The older sites where *P. cinnamomi* was detected were located on Starfire Mine (Perry County), while the younger sites where *P. cinnamomi* was not detected were located at Bent Mountain (Pike County). While some differences in site quality are suggested by observed differences in SOM accumulation, *P. cinnamomi* has been documented in areas adjacent to both mine sites, isolated from soils in Robinson Forest (adjacent to Starfire Mine, Breathitt County) (Rhoades et al. 2003; Adank et al. 2008) and from a dead American chestnut seedling at a nearby reforestation plot on Bent Mountain (Pike County; French, 2017)—suggesting that sources of *P. cinnamomi* are available for colonization at all sites. A parallel study in Robinson Forest detected *P. cinnamomi* in 45% of screened samples (Sena et al. 2018b), suggesting that the incidence of *P. cinnamomi* in unmined forest may be higher than the frequencies reported for reclaimed mined

land in this study. Future surveys will clarify whether *P. cinnamomi* becomes more prolific in these sites over time.

Infected seedlings may be an important source of contamination on these sites—a California study documented the introduction of *P. cinnamomi* to previously uninfested sites via seedlings used in restoration plantings (Swiecki and Bernhardt, 2017). As mentioned above, *P. cinnamomi* was detected on a dead American chestnut in a restoration planting on Bent Mountain; it is unknown whether this seedling was infected before planting (i.e., with propagules from the nursery at which it was grown) or after planting (i.e., with propagules already present at the site)(French, 2017). Nurseries supplying seedlings for the restoration of these surface mined areas should be screened for the presence of *P. cinnamomi* to reduce the risk of contaminating restoration sites.

To our knowledge, this is the first study reporting *P. cinnamomi* incidence at FRA sites. In a previous study, *P. cinnamomi* was not detected at a site in eastern Kentucky (the 10 year old site in this study) during the first season after spoil placement (Adank et al. 2008). *P. cinnamomi* was also not detected at a series of reclaimed sites in Ohio ranging from three to 20 years since reclamation (Hiremath et al. 2013). These researchers did not report surveys of adjacent forest soils for *P. cinnamomi*; thus, while *P. cinnamomi* had been previously reported in more southerly regions of Ohio (Balci et al. 2010), it is unknown whether *P. cinnamomi* is present in unmined forests in their study area.

More generally, a recent study in eastern Kentucky found that some microbial community metrics, such as microbial biomass C and N, and microbial activity (assessed by dehydrogenase activity), in mine soils eight years after reclamation, were similar to regenerating clear cut soils in unmined forests (Littlefield et al. 2013). The current study supports observations that microbial community development occurs over time since reclamation, alongside plant community and soil development, including invasion by individual plant pathogens such as *P. cinnamomi*.

In previous studies on FRA-reclaimed sites, such as one of the Bent Mountain sites referenced here, forest development occurred rapidly on favorable mine soils, with planted trees achieving partial canopy closure after only nine growing seasons (Sena et al. 2015). Alongside tree growth and canopy closure, these researchers also reported the development of a litter layer and colonization by shade-tolerant understory species (Sena et al. 2015). Over time, shading provided by canopy closure and moisture storage provided by accumulating litter are

expected to moderate soil moisture conditions, increasing site favorability for colonization by soil microbes. *P. cinnamomi* is thought to prefer moist soils—development of conditions increasing soil moisture may improve site quality for *P. cinnamomi* (Hwang and Ko, 1978). However, the presence of *P. cinnamomi* alone is not sufficient to cause disease in some susceptible hosts. For example, although *P. cinnamomi* was widespread at reclaimed bauxite mine sites in Western Australia, root rot in susceptible jarrah (*Eucalyptus marginata*) was related to high moisture conditions in poorly drained sites with ponding rainwater (Hardy et al. 1996). In these systems, researchers recommended intentional site preparation (e.g., deep tillage to improve drainage) to reduce ponding and reduce *P. cinnamomi* infection risk (Koch and Samsa, 2007). FRA sites are constructed with low-compaction spoil placement techniques, and are characterized by high infiltration and low runoff rates (Taylor et al. 2009a,b; Sena et al. 2014; Adams, 2017). Although *P. cinnamomi* was detected on 15- and 20-year old FRA sites, no above-ground symptoms of Phytophthora root rot were observed in the chestnuts and white oak growing at these sites; additional studies on roots of these species will be necessary to definitively document infection status at these sites. Although *P. cinnamomi* is present, it is unclear whether or not conditions at these sites are conducive for the development of Phytophthora root rot in susceptible species. Also, follow-up studies will be required to assess whether *P. cinnamomi* will eventually colonize the 10- and 12-year old sites screened in this study in which *P. cinnamomi* was not detected.

3.6. Conclusions

These data suggest that site quality at FRA-reclaimed mine sites is sufficient by 15 years after reclamation for colonization by *P. cinnamomi*. To our knowledge, this is the first study documenting *P. cinnamomi* colonization of FRA-reclaimed mine sites, and demonstrates that these sites do not remain “Phytophthora-free” over time. Additional research will be necessary to clarify the impact of *P. cinnamomi* on susceptible hosts at these sites. While *P. cinnamomi* was detected at these sites, it is unclear whether or not environmental conditions are conducive to the development of *P. cinnamomi*-related disease in susceptible hosts (such as white oak). Finally, potential routes of invasion of *P. cinnamomi* onto reclaimed mine sites should be assessed—especially distinguishing whether *P. cinnamomi* is more likely to colonize sites via infected seedlings used in plantings, or by the transport of propagules from adjacent infested forest sites.

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Chapter 4: Landscape variables influence *Phytophthora cinnamomi* distribution within a forested Kentucky watershed

4.1. Introduction

Phytophthora cinnamomi is a soil-borne oomycete pathogen causing disease in a wide variety of forest tree species around the world (Sena et al., 2018). Thought to have originated in Southeast Asia (Ko et al., 1978, Arentz & Simpson, 1986), *P. cinnamomi* has been introduced throughout the world, and has been associated with dramatic declines in Eucalyptus trees in Australia (Podger, 1972, Shearer & Dillon, 1996, McDougall et al., 2002), and oaks and chestnuts in Europe (Vannini & Vettraino, 2001, Vettraino et al., 2002). In the eastern U.S., *P. cinnamomi* is associated with root rot in American chestnut (*Castanea dentata*)(Anagnostakis, 2001) and little leaf disease in shortleaf pine (*Pinus echinata*)(Campbell & Copeland, 1954).

American chestnut, once a dominant forest canopy species throughout the eastern U.S., has suffered greatly at the hands of introduced pathogens (Paillet, 2002, Rigling & Prospero, 2018). In the early 1900s, the fungal pathogen *Cryphonectria parasitica*, causal agent of chestnut blight, swept through the eastern US forests, killing chestnut back to the ground and functionally eliminating it from forest ecosystems (Anagnostakis, 2001). Previously, *P. cinnamomi* had been introduced to the southeast U.S. in the mid-late 1800s (Corsa, 1896), but was more subtle in its impacts and was not the subject of intensive study in eastern U.S. forests until relatively recently (Hwang et al., 2009, Meadows et al., 2011, Meadows & Jeffers, 2011). Thanks to extensive breeding efforts introducing disease resistance from Chinese chestnut, blight-resistant American chestnut varieties are now available (Diskin et al., 2006), and are the subject of additional selection targeting *P. cinnamomi* resistance (Jeffers et al., 2009, Zhebentyayeva et al., 2013; Santos et al., 2015). In addition to developing host genetic resistance to *P. cinnamomi*, restoration efforts should be informed by improved understanding of how *P. cinnamomi* is distributed on the landscape in eastern U.S. forests (Sena et al., 2018).

At small spatial scales, *P. cinnamomi* distribution is thought to be related to soil moisture, presence of susceptible host species, and composition of soil microbial communities. *P. cinnamomi* survival is higher in moist soils than dry or flooded soils (Kuhlman, 1964, Hwang & Ko, 1978, Weste & Vithanage, 1979), although *P. cinnamomi* can survive prolonged periods of drought by producing survival structures such as chlamydospores, or by colonizing root fragments (Old et al., 1984, Jung et al., 2013). *P. cinnamomi* is also known as a poor saprophyte; thus, distribution is thought to be related to presence of susceptible host species (Crone et al., 2014). However, a recent study in Australia found that *P. cinnamomi* infects herbaceous understory plants without causing disease (Crone et al., 2013a, Crone et al., 2013b), suggesting that the relationship of *P. cinnamomi* and hosts is more complex than previously thought. Finally, a significant body of research has related *P. cinnamomi* survival to soil microbial communities (Halsall, 1982, Malajczuk et al., 1983), suggesting that presence of microbes including endospore-forming bacteria (Aryantha et al., 2000) and actinomycetes (Broadbent & Baker, 1974, You et al., 1996), or even microbial activity in general (Nesbitt et al., 1979), can suppress *P. cinnamomi* growth or survival. Additionally, some studies suggest that infection of host roots by ectomycorrhizal fungi can reduce vulnerability to infection by *P. cinnamomi* (Corcobado et al., 2014).

At the landscape scale, disease caused by *P. cinnamomi* has traditionally been associated with moist, low-lying areas, such as drainages (Dawson & Weste, 1985, Wilson et al., 2003, Vannini et al., 2010, Keith et al., 2012), although it has also been isolated from drier ridge-top soils in some cases (Shea & Dell, 1981). *P. cinnamomi* has poor tolerance for freezing temperatures (Bergot et al., 2004); in the eastern US, *P. cinnamomi* has been detected in forests ranging north to Pennsylvania and Ohio (Balci et al., 2013, McConnell & Balci, 2014). In the southern Appalachians, *P. cinnamomi* was isolated from 12% of samples in mountain

pine/hardwood stands and 45% of samples from coastal pine stands (Campbell & Hendrix, 1967), and from 34% of forest soil samples in another study in south-central Appalachia (Sharpe, 2017). A recent study in an American chestnut planting site in eastern Kentucky found *P. cinnamomi* in 100% of soil samples collected (Pinchot et al., 2017). However, these studies did not provide insight into patterns of *P. cinnamomi* distribution at the watershed scale. This study was initiated to characterize the distribution patterns of *P. cinnamomi* within a watershed in eastern Kentucky, with specific interest in identifying relevant soil and landscape factors.

4.2. Methods and Materials

This study evaluated soils from the Little Millseat watershed in Robinson Forest, an approximately 6,000 ha research forest which is located in parts of Breathitt, Knott, and Perry counties, Kentucky, in the Appalachian Coalfields. This section of the Cumberland Plateau is characterized by steep slopes (25-60%), with elevation differences ranging from 150 to 300 m (Smalley, 1986) and well-drained residuum or colluvial soils derived from sandstone, shale, and siltstone parent material (Kalisz et al., 1987). The underlying geology in the region consists of interbedded sandstone, siltstone, shale, and coal of the Breathitt formation of the Lower to Middle Pennsylvania age (McDowell et al., 1981, Wunsch, 1993). Vegetation in Robinson Forest is characterized as mixed-mesophytic forest (Braun, 1950) and dominated by more than 50 woody species including oak (*Quercus* sp.), hickory (*Carya* sp.), yellow-poplar (*Liriodendron tulipifera*), and American beech (*Fagus grandifolia*) (Carpenter & Rumsey, 1976).

The Little Millseat watershed encompasses an area of 79 ha and is situated entirely in Breathitt County. Soil samples were collected from plots evaluated for patterns in soil respiration rates by a previous study (Abnee et al., 2004). Four samples were collected in 50 ml tubes from the corners of a square meter plot centered on the coordinates from Abnee et al. (2004). All samples (4 per each of 48 plots) were screened according to the method described by

Sena et al. (Chapter 2). Briefly, ~40 ml samples in 50 ml tubes were flooded with sterile water and baited with rhododendron leaf discs for 5 days. Leaf discs were stored at -4 °C until it was convenient to proceed with DNA extraction. DNA was extracted from leaf discs using QIAGEN DNeasy UltraClean Microbial DNA extraction kit, with an added proteinase K digestion step (Chapter 2). Presence of amplifiable DNA was confirmed in every sample using universal ITS1-ITS4 primers (White et al., 1990). Samples were screened for *P. cinnamomi* using published primers Ycin3F and Ycin4R, which target a 300 bp segment of the Ypt gene (Schena et al., 2008, Kunadiya et al., 2017). Samples were screened in duplicate with positive controls, *P. cinnamomi* isolate RF5 (isolated from Robinson Forest, GenBank Accession #MF966152) 1.5×10^{-2} ng/PCR, and no template negative controls. PCR amplicons were visualized by agarose gel electrophoresis (1.5% m/v).

The soils dataset reported for these sites by Abnee et al. (2004) was screened to identify variables with potential for predicting site suitability for *P. cinanmomi*. In addition to these soil physical and chemical data, ten potential landscape predictor variables were selected. These included elevation and several elevation derivatives: slope, curvature (total, profile, contour, and tangential), solar radiation, Beers aspect (Beers et al., 1966), specific catchment area, and topographic wetness index (TWI). Elevation derivatives were generated using a mosaicked 10-meter digital elevation model (DEM; NED tiles acquired from USDA Geospatial Data Gateway). The majority of elevation derivatives (slope, curvature, solar radiation, and Beers aspect) were generated in ArcMap (ESRI, 2017). Tangential curvature was computed by multiplying contour curvature and slope (Abnee et al., 2004). Beers aspect was computed using the Topography Toolbox for ArcGIS. The remaining elevation derivatives (specific catchment area and topographic wetness index) were generated in R (Team, 2017) using the “raster” (Hijmans, 2016) and “RSAGA” (Brenning, 2008) packages. The “RSAGA” package provides access to a more

realistic flow-routing algorithm (multiple flow direction; (Freeman, 1991, Quinn et al., 1991)) than is available in ArcMap.

Logistic regression was used to identify variables that were successful predictors of *P. cinnamomi* distribution in the Little Millseat watershed (PROC LOGISTIC, SAS 9.3). Contrary to expectations, soil respiration rates and soil organic matter from Abnee et al. (2004) were not related to *P. cinnamomi* incidence; the only parameters from Abnee et al. (2004) related to *P. cinnamomi* incidence were elevation and Ca, but even these did not accurately predict *P. cinnamomi* incidence (AIC = 51.7). Because elevation was the only landscape variable that was able to explain *P. cinnamomi* distribution across the entire watershed, further steps were taken to (1) generate potential vegetative predictor variables via species distribution modeling and (2) explore potential predictor variables when the data were grouped by aspect (southwest-facing and northeast-facing). Species distribution models were developed to predict the abundance of nine species (*Quercus montana, Oxydendrum arboreum, Quercus velutina, Acer saccharum, Carya tomentosa, Fraxinus americana, Quercus rubra, Nyssa sylvatica, and Fagus grandifolia*) using abundance (count) data for understory/midstory size classes at 72 hundredth-acre plots in the Little Millseat watershed (Rasp, unpublished data). These models were then applied to generate a continuous raster surface of predicted abundance values for each species. Predicted abundance values were then extracted from the raster surface for each plot and evaluated as predictor variables in logistic regression models.

4.3. Results

P. cinnamomi was detected in 12 of the 48 plots screened, three on the northeast-facing slope and nine on the southwest-facing slope. *P. cinnamomi* distribution on the northeast-facing slope was accurately predicted by TWI, with all positive plots characterized by low TWI (Figure 4.1, AIC = 5.78). *P. cinnamomi* distribution on the southwest-facing slope was not accurately

predicted by landscape variables, but logistic regression using species distribution models of nine understory tree species suggested that *P. cinnamomi* incidence was related to predicted abundances of *N. sylvatica* and *F. grandifolia* (Figure 4.2, AIC = 30.3).

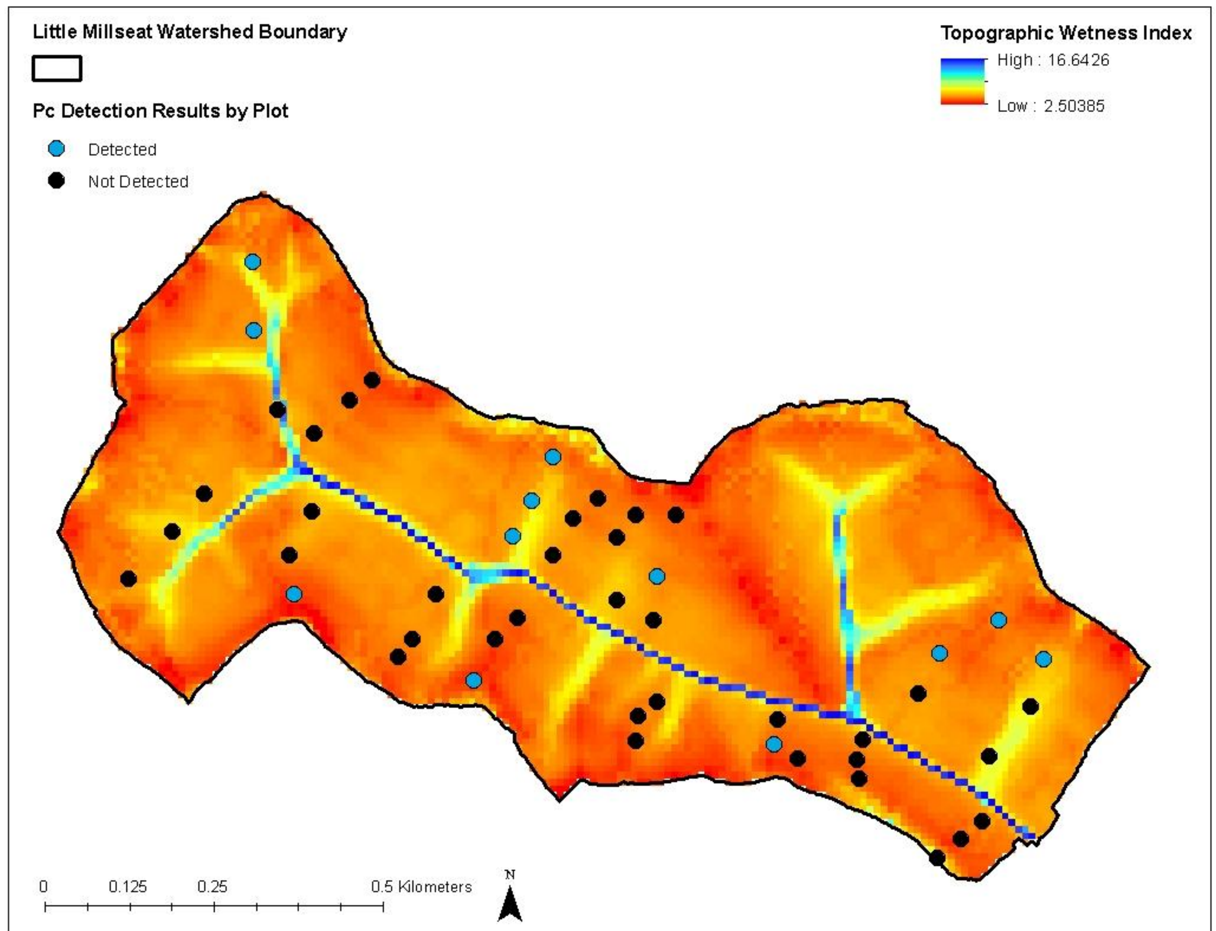

Figure 4.1: *P. cinnamomi* on the northeast-facing slope is associated with low Topographic Wetness Index (TWI) (AIC = 5.78), but TWI does not accurately predict *P. cinnamomi* incidence on the southwest-facing slope.

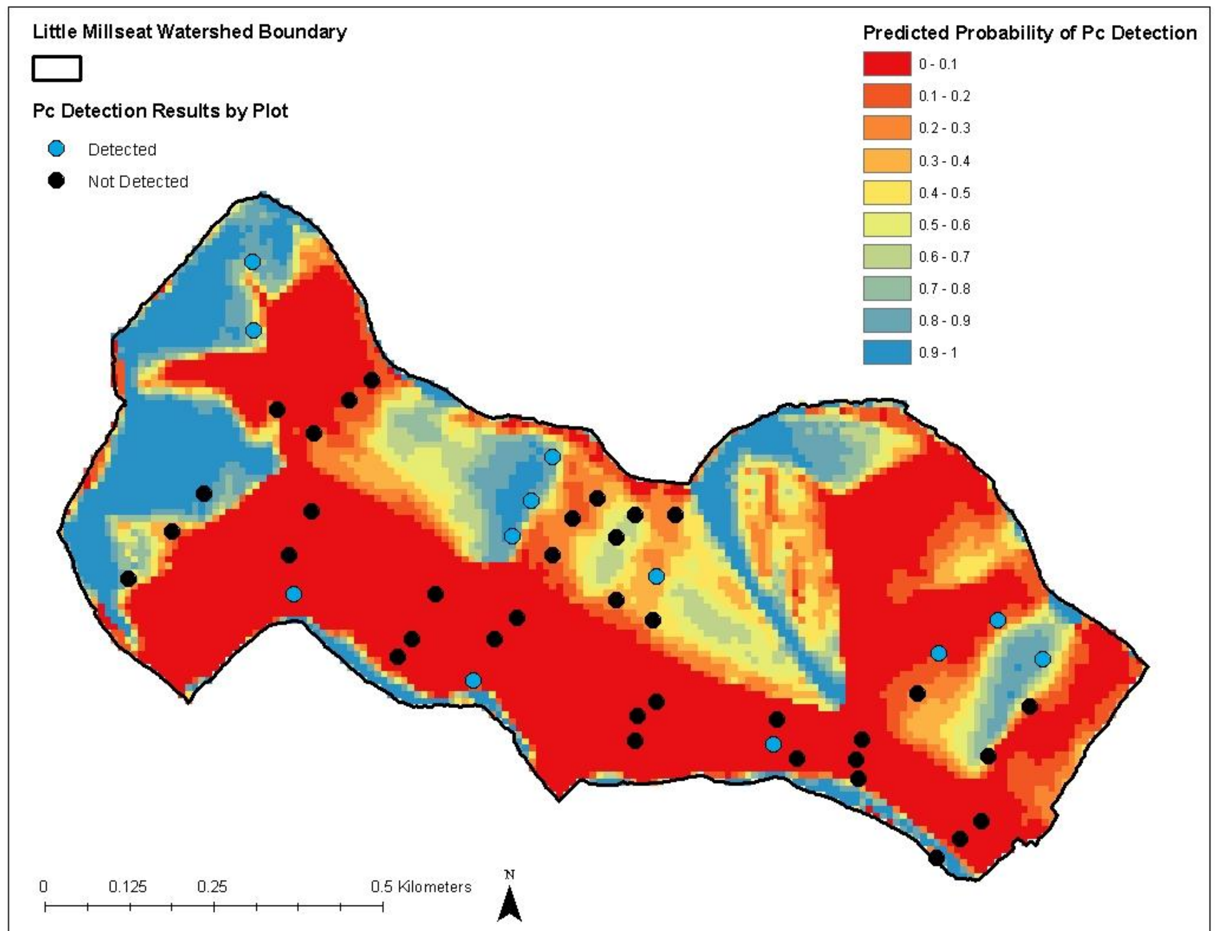

Figure 4.2: Probability of *P. cinnamomi* detection (southwest-facing slope) modeled with predicted abundances of *N. sylvatica* and *F. grandifolia* as parameters (AIC = 30.3). This model does not accurately predict *P. cinnamomi* incidence on the northeast-facing slope.

4.4. Discussion

In this study, *P. cinnamomi* was detected in 25% of samples, somewhat similar to other studies in the southern and south-central Appalachian regions detecting *P. cinnamomi* at frequencies ranging from 12-45% of samples (Campbell & Hendrix, 1967, Sharpe, 2017). A preliminary study in Robinson Forest by the same authors detected *P. cinnamomi* in 45% of samples from the Clemons Fork watershed (of which Little Millseat is a subwatershed) (Chapter 2). However, this detection frequency is less than that reported by Pinchot et al. (2017), who found *P. cinnamomi* in all samples screened in an experimental American chestnut planting in the Daniel Boone National Forest, Kentucky.

P. cinnamomi distribution has been traditionally associated with moist, low-lying soils in drainages (Vannini et al., 2010). However, the pathogen has also been isolated from drier soils at higher relative topographic position (Shea & Dell, 1981), and is known to be able to persist in soils typically considered unfavorably dry (Jung et al., 2013). In this study, *P. cinnamomi* on the northeast-facing slope was detected only in plots located at upslope positions, and was actually associated with low TWI.

Visual assessment of *P. cinnamomi* on the southwest-facing slope suggested that *P. cinnamomi* incidence was associated with drains; however, TWI and other landscape variables did not accurately predict *P. cinnamomi* distribution on this slope. Species distribution models were constructed for nine candidate understory tree species, and logistic regression related *P. cinnamomi* incidence on the southwest-facing slope with higher abundance of *N. sylvatica* and lower abundance of *F. grandifolia*. While *P. cinnamomi* distribution on the southwest-facing slope exhibited slight associations with predicted abundances of *N. sylvatica* and *F. grandifolia*, these species are not thought of as susceptible to infection by *P. cinnamomi*. This association may be indicative of site conditions favoring distribution of these plant species rather than specific associations with these particular species.

Because *P. cinnamomi* produces zoospores, like other oomycetes, that are capable of movement by water, the groups of plots testing positive for *P. cinnamomi* near the head of the watershed may violate assumptions of independence (i.e., populations of *P. cinnamomi* in upslope positions in these drains may serve as "source" populations for downslope positions in the drain, perhaps increasing probability of *P. cinnamomi* detection in what otherwise may be less favorable conditions.) Additional surveys could target these drains, characterizing *P. cinnamomi* incidence at shorter intervals, to elucidate whether a source-sink metapopulation model could be used to describe these sample groups.

Abnee et al. (2004) reported strong aspect effects on potential respiration rates (PRR) in Little Millseat, observing higher respiration rates on the northeast-facing slope than the southwest-facing slope, which they related to fertility (higher extractable nutrients, total N, and pH on the northeast-facing slope). Additionally, they reported stronger predictive models relating PRR to soil and landscape parameters on the northeast-facing slope than on the southwest-facing slope. On the northeast-facing slope, the best model included extractable Mg and Zn, specific catchment area, and slope aspect, with an R^2_{adj} = 0.620. In contrast, the best model predicting PRR on the southwest-facing slope included extractable P and Zn and tangential curvature, with a lower R^2_{adj} = 0.413 (Abnee et al., 2004).

In the present study, we observed that distribution of *P. cinnamomi* could be predicted more accurately on the northeast-facing slope than on the southwest-facing slope. *P. cinnamomi* was found in higher slope positions on the northeast-facing slope, which are associated with higher organic matter content (Abnee et al. 2014). Conversely, downslope positions on the northeast-facing slope were associated with higher microbial activity (Abnee et al., 2004); soils favorable for microbial activity generally and activity of some species in particular (e.g., actinomycetes, endospore-forming bacteria), can be unfavorable for *P.*

cinnamomi (Broadbent & Baker, 1974, Nesbitt et al., 1979, Halsall, 1982, Malajczuk et al., 1983, You et al., 1996, Aryantha et al., 2000). On the drier southwest-facing slope, it is likely that moisture and organic matter were more limiting, generating a more heterogeneous soil landscape leading to a more patchy and unpredictable distribution of soil conditions favorable for *P. cinnamomi*. Observed patterns of *P. cinnamomi* incidence on this slope could have been constrained by microsite moisture and organic matter characteristics rather than larger-scale (i.e., 10 m) landscape patterns extractable from DEM. It is possible that *P. cinnamomi* incidence in this watershed is constrained between moisture (e.g., slight association with drains on the dry slope) and competition from other microbes (e.g., restriction to upslope positions on the wet slope).

4.5. Conclusions

The association of *P. cinnamomi* with drier sites (low TWI) on the "wet" northeast-facing slope is unexpected, differing with the traditional association of *P. cinnamomi* and diseases it causes with low-lying soils in drainages. Similarly, distribution of *P. cinnamomi* on the southwest-facing slope is not closely associated with moisture regimes, with five detections located in drains (high-moisture) and another three detections on a knob (low-moisture). *P. cinnamomi* was also not associated with distributions of susceptible species of concern, such as white oak (*Quercus alba*). In other studies, *P. cinnamomi* was found at high incidence rates in American chestnut plantings (Brosi, 2001, Rhoades et al., 2003, Pinchot et al., 2017), suggesting that the presence of highly susceptible species could lead to increased abundance of *P. cinnamomi* in some forest systems.

Pc is not associated with disease across all environmental conditions. In Europe, researchers found that disease caused by *P. cinnamomi* is typically associated with moist soils in drains (Vannini et al., 2010). Similarly, in Australia, *P. cinnamomi* was detected across a

reclaimed mine site, but jarrah dieback (*Eucalyptus marginata*) was associated with poorly drained landscape areas where rainwater ponded (Hardy et al., 1996). Further studies in Robinson Forest will be necessary to clarify the environmental conditions in which *P. cinnamomi* will cause disease in susceptible species (especially American chestnut and white oak).

This study suggests that *P. cinnamomi* distribution within the Little Millseat watershed is complex, and not easily predicted by landscape variables alone. Additional surveys will be necessary to clarify microsite conditions favoring *P. cinnamomi* incidence in this watershed, and in the Appalachian region more broadly. Specifically, these surveys should characterize overstory vegetative communities in addition to understory communities, as well as soil chemistry (especially soil organic carbon) and particle size. Microbial community characterization would also be informative, including general assessments of microbial activity and specific assessments targeting individual microbial groups with known positive or negative association with *P. cinnamomi*.

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Chapter 5: Summary and Conclusions

Phytophthora cinnamomi is a soil-borne oomycete pathogen causing root rot in susceptible host species. *P. cinnamomi* is thought to have originated in Southeast Asia, but has since been introduced to many regions around the world, where it causes dramatic declines in many forest tree species. In the eastern US, the primary susceptible tree species of concern are American chestnut (*Castanea dentata*), white oak (*Quercus alba*), and shortleaf pine (*Pinus echinata*). American chestnut, functionally eliminated in the early 1900s by the rapidly acting chestnut blight (*Cryphonectria parasitica*), has been the subject of decades-long breeding efforts aimed at improving chestnut resistance to chestnut blight. Now, breeding efforts have expanded to include development of resistance to Phytophthora root rot (PRR) caused by *P. cinnamomi*.

In addition to breeding efforts targeting improved host resistance, chestnut restoration success (and restoration of other susceptible species) requires clearer understanding of the distribution patterns of *P. cinnamomi* on a landscape scale. One objective of this project was to develop an improved method for detecting *P. cinnamomi* to permit rapid screening of large numbers of forest soil samples. Our method utilized *P. cinnamomi*-specific PCR to screen DNA extracted from leaf baits incubated in flooded soil samples. This PCR-based assay reduced time and space required by conventional culture-based methods, but produced screening results similar to those returned by the conventional approach. Thus, our improved assay alleviated time and space constraints without sacrificing sensitivity.

We then screened soils from a chronosequence of reclaimed surface mines (reclaimed at different points in time) to evaluate whether these sites become suitable for *P. cinnamomi* colonization over time. In addition to screening these sites for *P. cinnamomi*, we evaluated general soil development patterns in this chronosequence, especially noting that organic matter levels in these soils approximate near-surface organic matter content in native forest soils after 15-20 years. Our analysis detected *P. cinnamomi* at the two older sites (reclaimed in 1997 and 2003), but not at the two newer sites (reclaimed in 2005 and 2007). These results suggest that surface mined sites become favorable for *P. cinnamomi* colonization over time, and should not be considered permanently "Phytophthora-free." We suggest that the harsh soil environmental conditions on newly reclaimed mine sites are alleviated over time, especially as planted trees achieve canopy closure and as a litter layer develops, shading the soil and regulating soil moisture.

Finally, we collected ~200 samples from a watershed in UK's Robinson Forest, from plots representing a gradient of topographic position, slope, and aspect. This survey indicated that *P. cinnamomi* distribution in forests is complex and can be difficult to predict; however, *P. cinnamomi* was detected in both drier upslope sites and in moister drainage sites. These results suggest that *P. cinnamomi* is distributed across a range of topographic and environmental conditions in eastern Kentucky forests; however, it is unclear whether *P. cinnamomi* is capable of causing disease in susceptible species across this range of environmental conditions. Specifically, it is essential to clarify whether *P. cinnamomi* will cause disease in American chestnut (or other species of interest) in both drier upslope locations and moister downslope locations. Additionally, further research will be necessary to develop more accurate models for predicting *P. cinnamomi* incidence in forests.

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VITA

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1. **Sena, Kenton*,** Kevin Yeager, Tyler Dreaden, and Chris Barton. 2018. "*Phytophthora cinnamomi* colonized reclaimed surface mined sites in Eastern Kentucky: implications for restoration of susceptible species." *Forests* 9:203. doi: 10.3390/f9040203
2. **Sena, Kenton***, Ben Goff, David Davis, and S. Ray Smith. 2018. "Switchgrass growth and forage quality trends provide insight for management." In Press: *Crop, Forage, and Turfgrass Management*.
3. **Sena, Kenton***, Ellen Crocker, Paul Vincelli, and Chris Barton. 2018. "*Phytophthora cinnamomi* as a driver of forest change: Implications for conservation and management." *Forest Ecology and Management*. 409:799-807.
4. Sidhu, Jatinder*, **Kenton Sena**, Leonie Hodgers, Andrew Palmer, and Simon Toze. 2018. "Comparative enteric viruses and coliphage removal during wastewater treatment processes in a sub-tropical environment." *Science of the Total Environment.* 616:669-677.
5. Bell, Geoff, **Kenton Sena***, Chris Barton, and Michael French. 2017. "Establishing pine monocultures and mixed pine-hardwood stands on reclaimed surface mined land in eastern Kentucky: implications for forest resilience in a changing climate." *Forests* 8:375.
6. **Sena, Kenton***, Christopher D. Barton, Sarah Hall, Patrick Angel, Carmen Agouridis, and Richard Warner. 2015. Influence of Spoil Type on Afforestation Success and Natural Vegetative Recolonization on a Surface Coal Mine in Eastern Kentucky. *Restoration Ecology* 23:131-138. (Impact Factor: **1.838**)
7. **Sena, Kenton***, Christopher D. Barton, Patrick Angel, Carmen Agouridis, Richard Warner. 2014. Influence of spoil type on chemistry and hydrology of interflow on a surface coal mine in eastern Kentucky. *Water, Air, and Soil Pollution* 225:1-14. (2014 Impact Factor: **1.554**)