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Recovery of Phytophthora Ramorum and Other Phytophthora Spp. in a Forest Adjacent to a Mississippi Ornamental Plant Nursery

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Recovery of *Phytophthora ramorum* and other *Phytophthora* spp. in a forest adjacent to a Mississippi ornamental plant nursery

By

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Recovery of *Phytophthora ramorum* and other *Phytophthora* spp. in a forest adjacent to

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The movement of the exotic and destructive plant pathogen *Phytophthora ramorum* into unquarantined areas via the plant nursery trade provides a potential outlet for transmission into eastern United States forests. A two-year survey of *Phytophthora* species in a forest adjacent to an ornamental plant nursery in Mississippi isolated *P. ramorum* 20 times from water and once from vegetation, with an additional detection of 14 *Phytophthora* species and one provisional species. Isolates were recovered from soil, water, and vegetation using baiting and filtering techniques, and verified by their DNA through Polymerase Chain Reaction (PCR) followed by genomic sequencing. This study confirms the ability of *P. ramorum* to sustain itself in Mississippi, although disease progression appears to be inhibited by the relatively small window of favorable environmental conditions.

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CHAPTER I

INTRODUCTION

Biology

Phytophthora ramorum Werres, De Cock, and Man in't Veld is an Oomycete organism classified in the kingdom Chromista within the family Pythiaceae (Werres et al. 2001; Jeffers 2005). Oomycetes share unique morphological characteristics such as coenocytic mycelium, cell walls made of glucan and cellulose, a diploid thallus, zoospores with heterokont flagella, and sexual recombination by oogamy and the formation of oospores (Blackwell 1949; Waterhouse 1970; Jeffers 2005). *Phytophthora* spp. are primary pathogens of numerous woody plants, with limited saprobic abilities (Erwin and Ribeiro 1996; Jeffers 2005). Of the 10 phylogenetic clades recognized in the genus *Phytophthora, P. ramorum* is in Clade 8 with its closest relative *Phytophthora lateralis* Tucker & Milbrath, distinguishing itself from the majority of other members in the group by its heterothallism and aerial etiology (Kroon et al. 2012).

As a heterothallic organism, *P. ramorum* requires two compatible partners for the production of sexual spores (Werres et al. 2001). Sexual gametangia have been produced *in vitro*, though with difficulty and largely unsuccessfully (Brasier and Kirk 2004; Boutet et al. 2009). With the absence of a suitable mating type in nature, *P. ramorum* relies on the asexual reproduction of sporangia and chlamydospores as infection and survival structures (Davidson et al. 2003; Fitchner et al. 2007). *P. ramorum* is unique as it is one of the few *Phytophthoras* that have deciduous sporangia along with the prolific formation of large chlamydospores (Werres et al. 2001). Cardinal temperatures for proliferation are between 2-28ºC, with optimum growth between 16-26ºC. Optimum temperatures for chlamydospore production are between 14-26ºC, and sporangia production between 16-22ºC (Browning and Tooley 2008).

P. ramorum produces mostly ellipsoid, semi-papillate, caducous sporangia that release approximately 30 motile, bi-flagellate zoospores (Werres et al. 2001; Widmer 2009). Sporangia can directly propagate from hyphae or chlamydospores, releasing zoospores and acting as the major agent of pathogen dispersal (Hardham 2007). At high temperatures zoospores may not discharge and direct germination of the sporangia may occur, though increased pathogenicity has been observed when zoospores are individually released (Widmer 2009). When zoospores are successfully discharged, wind and rain are believed to disperse the spores in a forest canopy where a film of water can allow them to swim to new infection sites (Tooley et al. 2009). Zoospores, which are chemotactically attracted to their hosts, encyst onto the tissue of the plant and insert a germ tube into the epidermis cells to instigate infection (Hardham 2007). Zoospores can persist in the environment for days if conditions are favorable, with increased infection rates observed on plant tissue 48 hours after inoculation (Judelson and Blanco 2005; Moralejo and Decals 2011).

Chlamydospores are defined as perennating walled spores filled with energy reserves, delimited from the parental mycelium by a septum (Blackwell 1949). *P. ramorum* develops larger, thicker-walled chlamydospores compared to other *Phytophthora* spp., with the thickest walled chlamydospores documented from

Rhododendron Linnaeus leaf tissue, where a single 28-day-old lesion may produce up to 10,000 spores (Smith et al. 2007). Viable chlamydospores have been recovered from both sand and leaf tissue after exposure to 0°C and 30°C for 7 days (Tooley et al. 2009). The robustness of the spores can be attributed to the survival of the pathogen during unfavorable conditions in plant material, soil, and water (Smith et al. 2007). *P. ramorum* chlamydospores have been able to persist with easy recovery for over one year in nursery potting mix, with extremely low recovery (1.4-2.3%) at 654 days (Shishkoff 2007). However, initiation of the yearly disease cycle from soil inoculum may not be significant in nature, as disease progression has only been attributed from infected leaves and twigs (Fichtner 2007; Eyre et al. 2013).

Introduction into the United States

P. ramorum was first observed in 1993 when a highly infectious, yet previously unknown *Phytophthora* sp. was associated with a foliar blight on nursery-grown rhododendron plants in Germany and the Netherlands (Werres et al. 2001). In 1995, the San Francisco bay area experienced abrupt mortality of tanoak trees (*Lithocarpus densiflorus* (Hook. & Arn.) Rehd.), and by 1999 tens of thousands of trees, including *Quercus* spp., had succumbed to the unknown pathogen (Garbelotto et al. 2001). In 2000, University of California researchers isolated an unknown *Phytophthora* species from an infected coast live oak (*Quercus agrifolia* Nee.) (McPherson et al. 2000). In 2002 *Phytophthora ramorum* was officially identified as the causal agent, and given the name Sudden Oak Death (SOD) by the press because of the perceived rapid mortality of diseased trees, though subsequent research has shown that the symptom progression takes many years before instigating death (Rizzo et al. 2002).

P. ramorum was first confirmed from a nursery in 2001, when the owner of a Santa Cruz county, California nursery observed tree dieback in the forest adjacent to their property (Tjosvold et al. 2008; Frankel 2008). The first quarantine for *P. ramorum* was issued from Oregon in 2001, and shortly after Canada banned the import of nursery stock of all oaks, tanoak, *Rhododendron*, and *Vaccinium* spp. from any area infected by *P. ramorum* in the United States (Frankel 2008). In July 2001 Curry County, Oregon confirmed a 40 acre wild-land infestation of tanoaks located in the Rogue River-Siskiyou National Forest, hundreds of miles away from the nearest infestation (Goheen et al. 2012). In 2002 APHIS issued a federal regulation for domestic interstate shipments of any *P. ramorum* host material from counties in California that were known to harbor the pathogen. Success was limited though, and by 2003 infested nursery stock was detected at 20 nurseries in Oregon, Washington, and British Columbia, Canada (Garbelotto and Rizzo 2005; Frankel 2008). Nurseries only located within quarantined areas were audited, leaving many nurseries unregulated. This allowed two large southern California nurseries, and one in Oregon, to ship millions of potentially infected plants to over 1200 nurseries in 39 states (Frankel 2008).

Concerns of SOD spreading to the east coast of the United States via the nursery trade became a reality in 2004 when APHIS conducted a national nursery survey and the pathogen was recovered from 171 nurseries in 20 states (Frankel 2008). Audits reveal that nurseries in Florida, Maryland, North Carolina, Louisiana, Texas, Tennessee, Georgia, Arkansas, Pennsylvania, Alabama, Virginia, New Mexico, and Colorado had positive plants from trace-forward nursery stock all originating from Monrovia nursery, located in southern California (California Oak Mortality Task Force 2014). With a cost of over 15 million dollars, APHIS eradicated over 1.6 million plants in attempts of mitigating the possible spread of *P. ramorum* through the domestic and international nursery trade (Alexander 2005).

Lineages

The lack of resistance within hosts, combined with the absence of native mating types, suggests that *P. ramorum* is exotic and introduced into North America from imported nursery stock on multiple occasions (Garbelotto et al. 2001a ; Werres and Kaminski 2005; Hansen 2009; Grunwald et al. 2009). This introduction is evident of the historically long-distance dispersal of *Phytophthora* spp. through the movement of infected plant material, as most *Phytophthora* spp. evolved in isolated geographical areas (Goss et al. 2009a). *P. ramorum* has four genetically distinct clonal lineages, EU1, EU2, NA1, and NA2, which are genetically and phenotypically distinct from each other, indicating that they evolved in isolation from each other for possibly hundreds of thousands of years, and then introduced into the United States independently (Goss et al. 2009b; Mascheretti et al. 2009). The common ancestral population is still unclear, yet research conducted in Taiwan proposes that *P. ramorum* may have originated in East Asia, where its closest relative, *P. lateralis* has been isolated from *Chamaecyparis* trees with an apparent degree of immunity (Brasier et al. 2010).

P. ramorum is self-sterile, requiring two mating types for sexual reproduction (Werres et al. 2001). European lineages carry exclusively the A1 mating type, while the North American lineages exclusively carry the A2 mating type (Garbellotto and Hayden 2012). Lineage EU1 has been isolated from nurseries in Canada, Washington, Oregon, and California, with a unidirectional migration from Europe to North America (Hansen et al. 2003; Gross et al. 2009a). The EU2 lineage was discovered in 2007, and is currently restricted to Ireland and Scotland with the epithet "Sudden Larch Death", where an estimated half a million Japanese larch (*Larix kaempferi* (Lamb.) Carr.) trees have been infected (Brasier and Webber 2010; Poucke et al. 2012). Failed quarantine efforts has allowed the NA1 lineage to be detected in nurseries around the country, and because of its transmission into the forest in the San Francisco Bay area, it is attributed to the expansive forest mortality in California and Oregon (Dart et al. 2007; Goss et al. 2009a). Lineage NA2 has only been documented in California, British Columbia, and Washington nurseries (Ivors et al. 2006; Grunwald et al. 2009).

With the evident distribution of both mating types in nurseries, there is a concern about the possibility of sexual reproduction, though there has been no evidence of this even when both mating types are present (Boutet et al. 2009; Goss et al. 2009a). This leads to speculation that each mating type has been isolated from each other for so long that the sexual reproductive system may not be functional (Brasier and Kirk 2004; Werres and Kaminksi 2005).

Symptoms

P. ramorum causes two types of diseases; lethal bole cankers, and leaf spots and branch dieback, depending on what host is infected (Garbelotto and Hayden 2012). Points of entry into the plant are from lenticels, stomata, and wounds (Florance 2002). As of August 2013, *P. ramorum* has an extensive host list with 46 proven host species, and over 90 associated species, many of which are common nursery plants (USDA APHIS 2013a). Regardless of the host, symptoms have only been observed on parts of the plant above the ground, rather than roots, contrasting the infection pattern from the majority of other *Phytophthora* spp. associated with oaks (Garbelotto et al. 2003; Rizzo et al. 2005).

Dark, discolored cankers usually develop on the bole of trees before foliar dieback is apparent, damaging the vascular tissue and girdling the hydraulic conductivity, resulting in decline and eventually death (Rizzo et al. 2002; Rizzo et al. 2005; Grunwald et al. 2008). If death is not immediate, secondary infection organisms such as decay fungi and bark beetles have invaded live oak and tanoak trees (McPherson et al. 2010). Lethal cankers develop on especially vulnerable species such as tanoak , coast live oak, and California black oak (*Q. kelloggii* Newb.) (Davidson et al. 2003; Hayden et al. 2011). Cankers on oaks usually develop on the lower trunk, and trees less than 10cm in diameter at breast height are almost never infected, yet tanoaks may be infected at any age or size (Grunwald et al. 2008). This may correlate to plant vigor and slightly higher resistance in *Quercus* spp., as experiments have shown lower inoculum levels are needed to infect tanoak than true oak species (Hansen et al. 2005).

Leaf blight and branch dieback, appropriately called "ramorum blight" affects a large number of hosts or associated plant species, where necrotic lesions form on small twigs, leaf petioles, and leaves (Davidson et al. 2003). Leaf spots form where water has accumulated on the leaf, and can progress into other parts of the plant via the petiole (Davidson et al. 2003). Ramorum blight weakens the plant, and in rare occasions may kill it, but more importantly serves as an inoculum reservoir for the pathogen to spread in favorable environments. Ramorum blight has been documented on forest tree species such as redwood (*Sequoia sempervirens* (D. Don) Endl.), California bay laurel (*Umbellularia californica* (Hook. & Arn.) Nutt.), Douglas fir (*Pseudotsuga menziesii*

(Mirb.) Franco), true firs (*Abies* spp.), tanoak, and yew (*Taxus* spp.), as well as prevalent and highly susceptible nursery plants such as rhododendron, camellia, viburnum, and pieris (Parke et al. 2003; Tubajika et al. 2006).

While *P. ramorum* is considered a generalist pathogen, isolates from "dead-end" hosts such as oak are less pathogenic compared to isolates from transmissive hosts such as California bay laurel, suggesting intense competition among isolates for leaf infection on epidemiologically significant hosts (Huberli and Garbelotto 2011). Combined with the knowledge that larger leaf lesions exhibit higher sporulation rates, and high concentrations of inoculum are necessary for transmission between hosts, this may have a positive correlation with the relatively slow disease progression in tanoak stands with little or no bay laurel, despite the fact there is minimal variation in resistance in tanoak (Hayden et al. 2011). Additionally, host pathogenicity and disease lineage are linked, with the NA1 lineage being more aggressive on camellia than the NA2 lineage, possibly explaining differences in dispersal rates among different hosts and lineages across the globe (Eyre et al. 2014).

Economic impact

The current and potential economic impact of *P. ramorum* on the nursery and forestry industry in the United States is substantial. Historically, *Phytophthora* spp. are some of the most eminent and economically significant diseases of ornamental nursery stock worldwide (Jones and Lambe 1982; Erwin and Ribeiro 1996). Nurseries must go to exacerbating lengths for monitoring, and if audited with *P. ramorum* infected stock, financially burdening quarantine and eradication measures must be enacted (USDA APHIS 2013b). Even after mitigation steps have been implemented, inoculum may be present in soil and water around the nursery, requiring additional monitoring from regulatory agencies (USDA APHIS 2010).

In the United States, invasive plant pathogens are estimated to inflict $21 billion per year in crop losses, with an additional half a billion spent on fungicides in management efforts, and $7 billion lost in forest product industries (Pimental et al. 2004). Although often considered as undesirable lumber in the past, there is growing demand for tanoak for flooring, with wood esthetics similar to eastern oak species. The loss of mature tanoak to the forest product industry could ravage a potentially growing market, where tanoak constitutes 6% of the tree volume in California (Shelly and Quarles 2012; Bowcutt 2014). California's forests are valued at over $500 million for the forest product industry (US Government Accountability Office 2006). Employing the current eradication program, *P. ramorum* may cost the forest industry approximately $31,178,402 in Oregon alone (Hall and Albers 2009). Estimates from USDA Forest Service predict the threat to commercial timber could exceed $30 billion if *P. ramorum* established itself in eastern deciduous forests (US Government Accountability Office 2006).

Between the years 2000-2005, USDA expenditures related to SOD eradication was over $50 million (US Government Accountability Office 2006). The financial impacts to California's nursery industry from quarantine efforts are estimated to be $4.3 million solely for the month of March 2014 (Frankel 2008). The eradication of over one million infected camellias at one southern California nursery was worth $9 million alone (Alexander 2005). Quarantine efforts in Washington State are estimated to cost a mean loss of $13,220 per nursery over a two-year period (Dart and Chastagner 2007). Canada banned the importation of plant crops from Oregon in 2001, with a state sales loss of $15-20 million (Frankel 2008). The impact on urban areas are also costly, as the estimated expenditure for tree removal and decreased property values attributed to SOD in California could reach $142.5 million over a ten year period (Kovacs et al. 2010).

Ecological impact

Historically, invasive forest pathogens have been shown to disrupt forest productivity, increase erosion and leaching of nutrients, stimulate decomposition, reduce wildlife habitat, and shift species composition (Orwig 2002; Lovett et al. 2006; Meentemeyer et al. 2008b). Parker et al. 1999 argues that the total impact of an invasive pathogen is determined by the range, abundance, and the per-capita or per-biomass effect of the pathogen.   To minimize the impact of an invasive pathogen, researchers need to monitor the changes in geographic distributions, predict the environmental conditions that promote the spread of the disease, and address the long-term ecological and evolutionary responses to an ecosystem invasion (Crowl et al. 2008).

The short-term and long-term ecological impacts of *P. ramorum* on native forests are difficult to calculate due the relatively short time period the pathogen has been established in the forest, along with variances in disturbance, land history, and forest type (Rizzo et al. 2005; Meentemeyer et al. 2008b ; Grunwald et al. 2008b). For example, heterogeneous landscapes with high species diversity may reduce disease severity compared to homogenous forests that have been impacted by fire or anthropogenic influence in the past (Haas et al. 2011) Additionally, shifting climate patterns regulate the yearly variation of annual rainfall, with increased precipitation directly correlated to increased disease transmission in multiple forest types (Davidson et al. 2011).

The absence of mature tanoak trees, which produce the largest acorn crops in Pacific coast forests, will undoubtingly have consequential long-term effects on wildlife habitat and food resources, though short-term production may be boosted from girdled trees inflicted with SOD (Hadj-Chikh et al. 2005; Bowcutt 2014). Additionally, the decline of tanoak in forests has been shown to curtail the abundance of ectomycorrhizal fungi associated with this species, where 127 ectomycorrhizal taxa were observed at a single site of tanoak, many of which are important food sources for wildlife (Bergemann et al. 2013). The reduction of symbiotic partners may increase forests vulnerability to other oomycete pathogens, as ectomycorrhizal fungi have been shown to reduce the impacts of *Phytophthora cinnamomi* Rands (Marx 1969).

Coastal forests of California that have suffered extensive tan-oak mortality from *P. ramorum* have experienced an increased rate of community change, which may accelerate the prevalence and dominance of bay laurel and redwood in certain forest types (Maloney et al. 2005; Cobb et al. 2013). *P. ramorum* does not significantly injure bay laurel, and the increase of bay laurel in forests will most likely favor disease progression, as bay laurel serves as the major inoculum reservoir in affected forests, with a direct correlation between tanoak mortality and the presence of bay laurel (Cobb et al. 2010; Davidson et al. 2011; Beh et al. 2012; DiLeo et al. 2014). Additionally, bay laurel has much higher rates of nitrogen in its leaves compared to tanoak, and a shift in species composition may alter the litter fall chemistry, increasing soil NO_3 and nitrogen availability, which would most likely escalate overall litter decomposition rates (Cobb et al. 2013).

The impact of *P. ramorum* on wild land fire behavior is difficult to assess due to the long time scales, with ecological influence strongly correlated to the stage of disease progression (Valachovic et al. 2009; Metz et al. 2011). Moisture content of tanoak leaves is significantly reduced during disease development, with deceased trees only having 5.8% moisture content during fire season (Kuljian and Varner 2010). These leaves then persist on the tree for several years after death, acting as ladders and increasing the probability of a crown fire within the initial years of infection (Lee 2009). Subsequently, additional increased coarse woody debris from tanoak mortality may escalate the intensity of fires, especially where pre-disease host biomass is high, though this progression may take years before impacts are noticeable (Valachovic 2011; Cobb et al. 2012). Furthermore, the altered forest structure and increased availability of surface and aerial fuels from SOD impacted forests may intensify mortality on resilient species such as coast redwood, which are highly resistant to fire and disease (Metz et al. 2013).

Nursery transmission

The presence of *P. ramorum* in the nursery industry creates an elaborate and difficult situation to manage. The large amount of susceptible host species complicates regulations, especially because symptoms may be latent and not easily detected (Cave et al. 2008). Common nursery species such as rhododendron, camellia, and viburnum have high infection rates, with certain cultivars more susceptible than others (Shishkoff 2006; Linderman et al. 2006; Grunwald et al. 2008a). In addition, common weeds of container nurseries such as northern willow herb (*Epilobium ciliatum* Raf.) and fireweed (*Chamerion augustifolium* L.) are susceptible to infection, boosting host density in nurseries and escalating the possibility of undetected inoculum (Shishkoff 2012). While the threat of *P. ramorum* to ornamental nursery stock is minimal, the presence of the pathogen in the nursery trade increases the risk of transmission to native forests in non-quarantined areas (Jeffers et al. 2009; Yakabe et al. 2009).

The optimal conditions of nurseries and greenhouses can increase the inoculum level of *Phytophthora* spp. within a few days or weeks, acting as a catalyst for propagule dispersal into the surrounding environment via water and soil (Erwin and Ribeiro 1996; Ristaino 2000; Jeffers et al. 2009). *P. ramorum* can stay viable in container potting soil for over one year, serving as a primary source of inoculum for root and foliar infections during rain events, as well as a vector for transportation and long-term survival (Shishkoff 2007; Parke and Lewis 2007; Tjosvold et al. 2009). Additionally, the use of irrigation systems in nurseries facilitates the dispersal and contact of *Phytophthora* propagules to other host plants (Werres et al. 2007; Tjosvold et al. 2008; Hong et al. 2008). The metabolic properties of most *Phytophthora* spp. make isolation and detection more difficult than "true fungi", and ubiquitous secondary invader pathogens may inaccurately be attributed to the primary invasion of *Phytophthora* (Erwin and Ribeiro 1996). A classic example of this is *P. cinnamomi*, which can grow saprophytically with no above ground symptoms for up to 6 years in container soil until conditions are favorable (Hwang and Ko 1978; Hardham 2005). This lack of transparency allows *Phytophthora* spp. to be transported into new areas without being detected.

Failed enforcement of regulations in the past have resulted in the importation of exotic Oomycetes such as *Phytophthora lateralis*, which has destroyed a multi-million dollar ornamental cedar industry on the west coast, along with the degradation of the ecological and economically significant Port Orford cedar (*Chamaecyparis lawsoniana*

(A. Murray) Parl.) in its native range (Hansen et al. 2000; Oh and Hansen 2007). Other Oomycetes such as *Phytophthora kernoviae* Braiser, which is currently restricted to England, if introduced into the United States could be devastating to native oak forests as host and dispersal properties are similar to *P. ramorum* (Braiser et al. 2005; Fichtner et al. 2012). Furthermore, the advent of globalization has increased the rate and distance plant material is being transported, increasing the possibly of introducing exotic Oomycetes from their native origin. There are estimates that there may be as many as 100-500 *Phytophthora* spp. unknown to science (Braiser 2007).

Depending on the climate and host prevalence, escaped inoculum can survive in the environment around a nursery, establishing itself in soil, water, or vegetation and dispersing into new habitats (Osterbauer et al. 2005; Jeffers et al. 2009; Elliott et al. 2012). Oomycetes thrive in aquatic environments, and isolation from runoff, irrigation ponds, and waterways is an effective method of determining the presence of inoculum (Hwang et al. 2007; Jeffers et al. 2009). Furthermore, isolation from waterways can help identify the source of inoculum, especially in natural ecosystems with multiple tributaries, as the source can be attributed upstream from the collection (Hwang et al. 2009; Sutton et al. 2009). Once a species of *Phytophthora* has been introduced into a stream or river, it is nearly impossible to eradicate without considerable environmental damage (Chastagner et al. 2009).

The aquatic ecology of *P. ramorum* is still relatively unknown, but *Phytophthora* propagules can be recovered from waterways even when conditions are not conducive for sporulation on land (Aram and Rizzo 2012). Though *P. ramorum* has been recovered from fresh leaf litter submerged in streams, it appears incapable of colonizing degraded leaf litter, suggesting its role as a saprophyte in riparian areas are limited (Aram and Rizzo 2012). However, periodic inundation of hydrophilic vegetation does create an avenue for transmission from water to terrestrial plants, although this is only known to have occurred twice outside quarantined areas in both Washington and Mississippi (Davidson et al. 2011; Elliott et al. 2012; Aram and Rizzo 2012).

Phytophthora **spp. residing in southeastern United States environments**

The initiation of the SOD national detection survey, along with recent advances in molecular technology, has provided new insight on the *Phytophthora* populations within nurseries and natural ecosystems in the southeastern United States. Since 2000, there have been over 51 newly described taxa of *Phytophthora*, many unintentionally discovered during efforts to recover *P. ramorum* (Brasier 2007). Clarity towards the prevalence, habitat niches, and distribution of these species in nurseries and ecosystems is improving. Species of *Phytophthora* in ITS Clade 6, including *Phytophthora gonapodyides* (Petersen) Buisman, *Phytophthora megasperma* Drechsler, and *Phytophthora chlamydospora* sp. *nov.* Hansen, have seen a recent surge in species declaration, and appear to have a widespread distribution in riparian ecosystems along with common recovery from irrigation systems (Braiser 2007; Hansen et al. 2015).

A recent survey of the ornamental horticulture industry carried out in six states across the southeastern United States detected *Phytophthora nicotianae* Breda de Haan, *Phytophthora hydropathica* Hong & Gallegly, and *P. gonapodyides* as the most prevalent species affiliated with plants, irrigation water, and streams (Olson et al. 2013). In Virginia, a statewide survey recovered *Phytophthora cactorum* (Leb. & Cohn) Schröeter, *Phytophthora citricola* Sawada, *Phytophthora citrophthora* (R.E. Smith & E.H. Smith)

Leonian, *Phytophthora drechsleri* Tucker, *P. megasperma, P. nicotianae, Phytophthora palmivora* Butler, *Phytophthora syringae* Klebahn, and *Phytophthora tropicalis* Aragaki & Uchida from irrigation systems (Bush et al. 2006). In 2006, water and soil from six nurseries in Florida and three nurseries in South Carolina recovered *P. cinnamomi, P. citricola, P. palmivora*, and *P. gonapodyides*, with *P. ramorum* detected at low levels from a retention basin at the Florida nursery (Wamishe et al. 2007). A 2003 survey of 14 woody ornamental nurseries in North Carolina recovered *P. citricola* and *Phytophthora cambivora* (Petri) Buisman most frequently from *Rhododendron* and *Pieris* spp. (Warfield et al. 2008). A similar survey was conducted in Tennessee during 2004-2005, with *P. citricola* and *P. citrophthora* accounting for 66% of the isolates recovered (Donahoo and Lamour 2008). Additionally, a 2001-2002 survey of floriculture greenhouses in North Carolina isolated *Phytophthora cryptogea* Pethybridge & Lafferty, *P. nicotianae*, and *P. palmivora* from a variety of ornamental plants (Hwang and Benson 2005).

The recovery of *Phytophthora* spp. in natural ecosystems via stream surveys helps researchers quantify the population communities that reside in eastern forests (Oak et al. 2007; Rizzo and Fichtner 2007; Hwang et al. 2009). Common *Phytophthora* spp. that attack trees and shrubs include *P. cinnamomi, P. cactorum, P. cambivora, P. citricola, P. cryptogea,* and *P. palmivora* (Jeffers 2005). Many of these organisms reside in the United States as previously introduced species that have "naturalized" and can routinely be recovered from areas with a favorable climate and accessible host species (Erwin and Ribeiro 1996).

In 2008 a survey of eight watersheds in eastern Tennessee resulted in the isolation of *P. citricola, P. citrophthora, Phytophthora irrigata,* and *P. hydropathica* (Hulvey et al. 2010). A 2010-2011 survey of sixteen streams in middle and eastern Tennessee recovered *P. cryptogea, P. hydropathica, P. irrigata* Hong & Gallegly*, P. gonapodyides, Phytophthora lacustris* Brasier*,* and *Phytophthora polonica* Belbahri, with *P. cryptogea* isolated the most (Shrestha et al. 2013). In Georgia, *P. cinnamomi, P. gonapodyides, P. citricola*, and *P. nicotianae* were most commonly recovered from forest streams during a 2006 survey (Adams 2008). In the Appalachian mountains of North Carolina, *P. cinnamomi, P. citricola, P. citrophthora, Phytophthora heveae* Thomps., and *Phytophthora pseudosyringae* Jung & Delatour were recovered from streams during a 2007 survey (Hwang et al. 2009). In 2003-2004, a soil survey from oak dominated forests in nine eastern and north-central states resulted in the new description of *Phytophthora quercetorum* sp. *nov* Balci & Balci, as well as the recovery of *P. cinnamomi, P. citricola, Phytophthora europaea* Hansen & Jung*,* and *P. cambivora*, with *P. cinnamomi* recovered in 69.4% of the sites surveyed (Balci et al. 2007; Balci et al. 2008).

Threat to eastern forests

The perseverance of the pathogen combined with the complexity of the nursery industry raises concern about the susceptibility of eastern forests to sudden oak death. The effect of *P. ramorum* could be devastating to the east, with oak-hickory/oak-pine type forests covering 160.3 million acres, or 43 percent of eastern timberland (Moser et al. 2005). The risk assessment for *P. ramorum* to spread into the environment is highly favorable in much of the southern Appalachians and Gulf states (Koch and Smith 2007).

Other areas, such as the Ozark Plateau of Missouri and Arkansas, contain both the highest host susceptibility and density in the country (Gottschalk et al. 2002). Furthermore, the pathogens tolerance to freezing temperatures (-5^{o}C for 24 h with no impact, -25^{o}C for 24h with minimal impact) demonstrates the potential of establishment in northern latitudes (Browning et al. 2008).

The disposition of native eastern plant species to *P. ramorum* has been tested *in vitro* to determine the susceptibility of hosts if the pathogen establishes itself in eastern forests (Linderman et al. 2007; Tooley and Kyde 2007; Tooley and Browning 2009). Plant species in the Ericaceae and the Fagaceae families are especially vulnerable to infection (Davidson et al. 2003; Tooley et al. 2004). Plants with high levels of infection exhibited symptoms on >80% of their leaves, including black cherry (*Prunus serotina* Ehrh.), mountain laurel (*Kalmia latifolia* L.), black walnut (*Juglan nigra* L.), southern dogwood (*Cornus florida* L.), sassafras (*Sassafras albidium* (Nutt.) Nees.), black locust (*Robinia pseudoacacia* L.), and multiple sumac species (*Rhus* spp.) (Linderman et al. 2007; Tooley and Browning 2009). Some species may act as inoculum reservoirs in forests, much like California bay laurel, including serviceberry (*Amelanchier Canadensis* L.), Japanese honeysuckle (*Lonicera japonica* Thunb.), and southern dogwood, all of which have high rates of sporangia production, with black locust generating the most sporangia at 2,001 sporangia per cm^2 lesion area (Tooley and Browning 2009).

Dominant eastern canopy trees in the *Quercus* genus are also susceptible, especially species in the live and red oak (Erythrobalanus) subgenus. Susceptible white oak (Leucobalanus) species include chestnut oak (*Quercus prinus* L.) and white oak (*Quercus alba* L.), while red oak species include northern red oak (*Quercus rubra* L.), black oak (*Quercus veluntina* Lam.), scarlet (*Quercus coccinea* Muenchh.), and pin oak (*Quercus palustris* Muenchh.) (Tooley and Kyde 2007; Spaulding 2011). One prediction estimates a 26-40% decline of oak in forests over the next 50 years in eastern forests infected with *P. ramorum*, with red maple (*Acer rubrum* L.) and tulip tree (*Liriodendron tulipifera* L.) succeeding in gaps formerly inhabited by oak species (Spaulding 2011). Chestnut oak had higher infection rates than tanoak, and combined with its ubiquitous presence in the southern Appalachians, along with its common association with understory hosts such as rhododendron and mountain laurel, it is a species of particular concern (Tooley and Kyde 2007).

***Phytophthora ramorum* in Mississippi**

The climate and flora of the Gulf coast is also conducive for the establishment of *P. ramorum*. The climate suitability for *P. ramorum* in southern Mississippi is among the highest in the east coast (Venette and Cohen 2006; Kelly et al. 2007). Inoculation tests have shown high susceptibility to native Gulf coast trees such as yaupon *(Ilex vomitoria* Aiton.) and southern magnolia (*Magnolia grandiflora* L.). Other native plants such as spicebush (*Lindera benzoin* L.), sweet bay (*Magnolia virginiana* L.), and Virginia creeper (*Parthenocissus quinquefolia* L.), have been proven to harbor the pathogen (Preuett et al. 2013).

Site History

In 2007, as a result of the USDA Animal and Plant Health Inspection Service (APHIS) national *P. ramorum* regulatory program, *P. ramorum* tested positive on nursery stock at an ornamental plant nursery located in Rankin County, Mississippi. As a request from APHIS, sampling was conducted by Clemson University, Mississippi State University, and Forest Service personal in 2007-2008 to assess the amount of residual inoculum present in and around the nursery. Over the two-year period, *P. ramorum* was confirmed by PCR diagnostics from water samples 16 times from the drainage ditch, and four times from Hog Creek. Additionally, a riparian willow located along the nursery drainage was confirmed positive for *P. ramorum,* though ensuing sampling did not recover the pathogen (Dr. Steven Jeffers, unpublished data). More recent baiting of the drainage by the Forest Service has confirmed *P. ramorum* positives by PCR diagnostics on 3/22/2013 and 4/15/2014 (Dr. John Nowak, Forest Service, personal communication).

Mississippi's forests are now at risk for a sudden oak death introduction, and consequently, comprehensive research needs to be devoted towards determining the source and amount of inoculum that allows positive stream baits to persist. With consecutive detections year after year, *P. ramorum* has evidently been transported to Mississippi, yet nobody knows to what degree, and if it has the potential to evolve into an epidemic. This study monitored the progression of the pathogen using both terrestrial and aquatic sampling to determine the amount of inoculum that is being released into the environment, and the impact it has on local vegetation. This attention will provide clarity towards the ecological context around plant nurseries in the southeast, while preventing *P. ramorum* from establishing itself in the eastern United States.

CHAPTER II

MATERIALS AND METHODS

Site Description

The ornamental plant nursery is located in the suburbs northeast of Jackson, Mississippi. Two sides of the nursery's property boundary is native forest with a multitude of host species. A water drainage originates from the shade houses where plant stock is located, and meanders through the property before passing a large irrigation reservoir where additional discharge is released. About 100 meters from the nursery property boundary the drainage flows into Hog Creek (figures 2.1 & 2.2). Hog Creek meanders through a mesic oak-pine forest community for roughly 1.13 miles before it converges with the Pearl River. The mid-seral forest adjacent to the drainage is abundant with vegetation, much of it exotic, which is intermittently flooded during periods of rainfall (table 2.2). Two riparian *Rhododendron* and four *Vaccinium* bushes are located within the study area. The dynamic hydrology of the drainage can be attributed to the significant water-level fluctuations between seasons, which periodically displace the sandy loam soils of the area. Average annual precipitation in Jackson, MS from 1896 to present is 54.77 inches, and the annual temperature average is 67.3°F (National Oceanic and Atmospheric Administration 2012).

Figure 2.1 Satellite imagery of the nursery property

Figure 2.2 Satellite imagery of the sampling area

Table 2.1 Plant genera located within the sampling area

Riparian plant species		**Flood plain plant species**	
Acer sp.	*Platanus* sp.	*Acer* sp.	*Smilax* sp.
Albizia sp.	*Quercus* sp.	*Albizia* sp.	*Symplocos* sp.
Amaranthus sp.	*Rhododendron* sp.	*Carpinus* sp.	*Toxicodendron* sp.
Ampelopsis sp.	*Rubus* sp.	*Carya* sp.	*Trillium* sp.
Arundinaria sp.	*Salix* sp.	*Cornus* sp.	*Ulmus* sp.
Betula sp.	*Sambucus* sp.	*Crataegus* sp.	*Vaccinium* sp.
Carpinus sp.	*Senecio* sp.	*Fagus* sp.	*Viola* sp.
Chasmanthium sp.	*Smilax* sp.	*Ilex* sp.	*Xanthium* sp.
Commelina sp.	*Toxicodendron* sp.	*Ligustrum* sp.	
Ilex sp.	*Tradescantia* sp.	*Lonicera* sp.	
Ligustrum sp.	*Ulmus* sp.	*Parthenocissus* sp.	
Liquidambar sp.	*Vaccinium* sp.	*Podophyllum* sp.	
Lonicera sp.	*Viola* sp.	*Quercus* sp.	
Persea sp.	*Vitis* sp.	*Rubus* sp.	

Protocols

Soil baiting complied with the APHIS soil and container mix protocol, revised in November 2010 (USDA APHIS 2010). Stream baiting followed the APHIS *in vitro* water sampling "bottle of bait" protocol, designed by Steve Oak of the USDA Forest Service and revised in March 2014 (USDA APHIS 2014). Vegetation sampling adhered to the APHIS inspection and sampling protocol for nurseries, revised in August 2014, though sampling did not take place on nursery property (USDA APHIS 2014b). Water filtering was adopted from the APHIS water sampling protocol, designed by Hwang, Oak, and Jeffers 2007. Selective media formulas (PARPH-V8 and PAR-V8) were defined by Dr. Steven Jeffers at Clemson University (Jeffers and Martin 1986; Ferguson and Jeffers 1999).

Stream baiting

Every sampling trip water temperature was taken four times at different water depths at each sampling site and averaged together. Two, 1-liter plastic bottles were filled with 900 ml of stream water in 100 ml aliquots from the nursery drainage ditch. One bottle was collected at mid-point of the ditch, the other bottle at the convergence of the drainage with Hog Creek. An additional two bottles were used to collect stream water from Hog Creek at the convergence with the nursery drainage and 50 meters downstream from that point. Ten leaf disks and one intact asymptomatic rhododendron leaf were added to each of the bottles. The *Rhododendron maximum* L. leaves were picked from plant stock located on Mississippi State University campus, and were not sprayed with any fungicides or pesticides. Bottles were capped and put on their side so vegetation was floating on the water surface, and then incubated in the dark for 72 hours at 20^{o}C.

After three days the intact leaf was removed, wrapped in a damp paper towel moistened with distilled water, and then put into a gallon-size Ziploc® bag with ample air for incubation up to an additional 7 days at 20^{o}C. Four disks were removed and blotted with a paper towel before culturing on PARPH-V8 media and incubated at 20^{o}C. Growth was then hyphal-tipped onto PAR-V8 media for isolation. Once isolates from the PAR-V8 media reached the edge of the Petri plate, they were swabbed with a sterilized toothpick and placed in a micro-tube to be boiled for 20 minutes in 500 µl of 10mM Tris-CL buffer (pH 7.5), followed by three minutes of vortexing to extract Deoxyribonucleic acid (DNA) for PCR (Kong et al. 2004). The remaining six leaf disks from each bottle were combined into a single micro tube for DNA extraction and PCR (refer to methods below).

The intact leaf was removed from the incubator after symptoms were visible, usually anywhere from 5-7 days, and lesions were punched into ¼- inch disks. Four disks were cultured onto PARPH-V8, hyphal-tipped onto PAR-V8 for isolation, and mycelia was swabbed and boiled for DNA extraction and PCR. Remaining disks from the intact leaf were placed into a micro-tube for DNA extraction and PCR. Leftover tissue from the intact leaf was labeled and frozen for future diagnostics.

Water Filtering

Water filtering was incorporated into the study in October of 2014. Two liters of water were collected in 100 ml aliquots from the nursery drainage ditch. Aliquots were taken from eddies and pools located along the stream. One liter of water was collected from the nursery boundary to mid-way of the drainage; the other liter was collected from mid-way to the convergence of Hog Creek. Water samples were placed in a cooler and immediately filtered in the lab, about 4 hours from collection. Water was processed using a 47-mm, 250 ml Nalgene® filter funnel with clamp, and a hand-operated vacuum pump. About 100 ml of clean water was used per filter, while 50-75 ml was used for turbid water. Clean water was filtered through a 3-µm Nuclepore© polycarbonate membrane while dirtier water was filtered through a 5-µm Durapore© polyvinylidene fluoride membrane. The walls of the funnel were rinsed with distilled water after each aliquot to dislodge spores adhering to the sides. Large organic debris was removed from the filters with forceps before inverting the filter onto PARPH-V8 media petri plates. Plates were then incubated for three days at 20^{o}C. After three days, filters were removed and gently rinsed with tap water to remove fine detritus. Colony morphology was then observed under a dissection microscope for the following 72 hours and distinguished colonies were hyphal-tipped onto PAR-V8 media for isolation. To reduce redundant isolations, morphologically identical colonies on PARPH-V8 were grouped together and one representative was taken. Rate of growth, morphology, and the occurrence of reproductive structures were noted. Once isolates from the PAR-V8 media reached the edge of the petri plate, mycelia was swabbed with a sterilized toothpick and placed in a micro-tube to be boiled for 20 minutes in 500 µl of 10mM Tris-CL buffer (pH 7.5), followed by three minutes of vortexing to extract DNA for PCR (refer to methods below). Sporangia produced on the surface of the agar from *P. ramorum* isolates were directly pipetted into a micro-tube for DNA extraction.

Soil baiting

Soil samples were collected bi-weekly using a 2x10 cm Oakfield© soil sampler. Soil temperature was taken at four different locations in each composite sample area and averaged together. Six composite samples comprising 10 core samples each were taken from the drainage ditch for a total of 60 core samples constituting about 6 liters of soil per trip. Composite samples were collected from the same designated area each trip (Figure 2.3). Each core sample was configured in a staggered manner; one sample was taken close to the waterline, then moved 50cm downstream and 25cm away from the waterline, where the next sample was taken, and then 50cm downstream and back to the waterline, where the next sample was taken. This procedure continued until the designated sampling area ended and where the next composite sample began. Additionally, rhizosphere soil was sampled around deceased and symptomatic vegetation located in the alluvial plain. The six, 1-liter composite soil samples were then thoroughly mixed and placed in a cooler and taken back to the lab to be baited.

The soil sample was dispersed into three, 500 ml plastic containers with a soil depth of approximately 1-2 cm and flooded with distilled water until the water line was 2.5cm above the soil line. The solution was then stirred and allowed to settle before 15 rhododendron leaf discs were added stomata-side down to each container. If the leaf disks sank more disks were added until 15 floating disks were visible. The containers were then covered and put into a dark incubator at 20°C for 72 hours. After three days, four bait pieces from each container were removed and blotted with a paper towel. The four pieces were then cultured on PARPH-V8 media and incubated at 20°C until growth was observed. Growth was then hyphal-tipped onto PAR-V8 media for isolation. Six additional bait pieces from each container were placed in a micro-tube and reserved for DNA extraction. Once isolates from the PAR-V8 media reached the edge of the Petri plate, they were swabbed with a sterilized toothpick and placed in a micro-tube to be boiled for 20 minutes in 500 µl of 10mM Tris-CL buffer (pH 7.5), followed by three minutes of vortexing to extract DNA for PCR (refer to methods below). After the soil had been baited a small sample was preserved for possible future baiting, and any remaining soil was then sterilized in an autoclave.

Figure 2.3 Soil scheme for the sampling area

Vegetation sampling

Vegetation sampling was performed bi-weekly during the appropriate seasons of the year. Symptomatic leaves, twig and branch cankers, and roots were collected from various plant species (see table 2.2). Additionally, discolored lesions from the bole of a riparian oak tree on Hog Creek were repeatedly sampled. Root samples were taken from riparian plants that were exposed to seasonal flooding. Root samples were surfaced sterilized with 70% ethanol, observed for any symptoms, and cultured onto PARPH-V8 media. Growth was then hyphal-tipped onto PAR-V8 media for isolation. Once isolates from the PAR-V8 media reached the edge of the petri plate, mycelia was swabbed with a sterilized toothpick and placed in a micro-tube to be boiled for 20 minutes in 500 µl of 10mM Tris-CL buffer (pH 7.5), followed by three minutes of vortexing to extract DNA for PCR. Foliar sampling was conducted on symptomatic plants anywhere in the vicinity of the drainage ditch or Hog Creek. Highly susceptible host species located in the flood plain were repeatedly sampled throughout the study. Vegetation samples were surfaced sterilized with 70% ethanol before being punched into leaf discs. Disks were then cultured onto PARPH-V8, hyphal-tipped onto PAR-V8 for isolation, and the mycelia was swabbed and incubated at 65°C for two hours for DNA extraction and PCR (refer to methods below). Additional foliar tissue was frozen for future diagnostics.

Table 2.2 GPS locations of the plants sampled

Plant species	**North**	**West**
Box elder (*Acer negundo)*	32.33852	90.09709
Cane grass (*Arundinaria* sp.)	32.33855	90.09691
Ditch azalea (*Rhododendron* sp.)	32.33861	90.09714
Black willow (*Salix nigra*)	32.33846	90.09723
Hog creek azalea (*Rhododendron* sp.)	32.33884	90.09766
Hickory (*Carya* sp.)	32.33852	90.09686
Willow oak (*Quercus phellos*)	32.33846	90.09711
Swamp chestnut oak (*Quercus michauxii*)	32.33861	90.09702
Sweetgum (*Liquidambar styraciflua*)	32.33859	90.09701
Blueberry *(Vaccinium* sp.)	32.33856	90.09692
Elm (*Ulmus* sp.)	32.33862	90.09703
Water oak saplings (*Quercus nigra*)	32.33855	90.09691

DNA extraction from leaf discs and mycelia

Phytophthora isolates used for the enzyme digest were received from Dr. Steven Jeffers, Department of Agricultural, Forest, and Environmental Sciences, Clemson University, and grown in a sterile liquid nutrient broth, placed on an oscillator for 14 days, and the mycelium was deposited into extraction tubes containing sterile glass beads for maceration. Isolates derived from environmental baiting methods were grown on PAR-V8 media until the colony reached the edge of the petri plate, and were then swabbed with a sterile toothpick and placed in an extraction tube. Additionally, from each collection, six leaf discs from each Bottle of Bait (BOB) water bait bottle and six leaf disks from each soil baiting container were cut in half and placed into extraction tubes containing sterile glass beads.

The leaf disks and mycelia were macerated in 800µl of CTAB lysis buffer (2% cis-trimethyl ammonium boric acid, 100mM Tris, 20mM Na_2EDTA, 1.4 M NaCl, and 1% polyvinylpyrolidine,pH 8.0) for three cycles on a Biospec© Mini Beadbeater mill (Bartlesville, OK) for three minutes at maximum speed each cycle. After maceration, 20µl Rnase A was added to each sample, and samples were incubated in a water bath at 65º C for two hours. DNA was extracted following the Nucleospin© Plant II Kit (Machery Nagel, Duren, Germany) protocol. The DNA samples were eluted with 30µl of preheated PE buffer.

DNA Clean-up and Concentration

Extracted DNA from the toothpick swabs and from the leaf discs were further processed in order to clean-up and concentrate the DNA. Two different genomic DNA clean-up kits were used. Initially the Nucleospin© gDNA Clean-Up Kit (Machery Nagel, Duren, Germany) was used following the manufacturer's protocol. When this kit could not be purchased, the Ambion Wizard© Genomic DNA Clean-up Kit (Ambion, Austin, TX) was used following that manufacturer's protocol.

Polymerase Chain Reaction (PCR) Amplification

After DNA extraction and clean-up, each sample was amplified by PCR using an Eppendorf ©Thermocycler (Hamburg, Germany). The primers used were *Phytophthora* specific A2-forward (5'-ACTTTCCACGTGAACCGTTTCAA) and I2-reverse (5'-GATATCAGGTCCAATTGAGATGC) designed by Drenth et al. 2006. A hotstart protocol, which included genomic DNA plus molecular-grade water, was made up to 10µl in a 0.2µl PCR tube and placed in a thermocycler for a five minute 94°C hot start. Once the five minutes were met, 40µl of Master Mix (consisting of 5 mM Promega Go Taq© Buffer, 1.5 mM MgCl, 0.2 mM each primer, 0.2 mM dNTP mix, 1µg bovine serum albumin, and 1.25 units of Promega Go Taq© polymerase; Promega Corporation, Madison, WI) was added to each PCR tube and the amplification continued for 35 cycles of 30 seconds at 94°C, 45 seconds at 61°C, and 2 minutes at 72°C with a final 10 minute 72°C extension. The amplification product was visualized by 1.5% agarose gel electrophoresis run in Sodium Boric Acid (SBA) buffer pH 8.0, with added GelRed™ (Lonza, Allendale, NJ) using a Biorad GelDoc™ XR+ (Hercules, CA).

Restriction digests of amplicon

The PCR amplicon from above was digested with three different restriction enzymes, *Msp1*, *Rsa1*, and *Taq1* (Promega, Madison, WI). Digestion was conducted in 20µl volumes, consisting of 10 µl of PCR product, 2 µl 10x appropriate restriction buffer, 6.8 µl water, 0.2 µg bovine serum albumin, and 1µl restriction enzyme. Samples were incubated in a water bath for 2-3 hours at 37°C for *Msp1* and *Rsa1*, and 65°C for *Taq1*. After digestion, samples were frozen at -20°C until they were run on a 2% agarose gel with a 100 bp ladder to obtain the digest fingerprints (Drenth et al. 2006). Study samples were compared to digests of known species. These species included *Phytophthora mississippiae*, obtained from Dr. Warren Copes of the USDA Agricultural Research Service, and *P. citricola, P. cryptogea, P. palmivora, P. citrophthora, P. nicotianae, P. cinnamomi,* and *P. gonapodyides,* obtained from Dr. Steve Jeffers, Clemson University. DNA from these species were extracted according to the leaf disc protocol described above, amplified by PCR as described above, and digested as described. In addition, DNA from *P. ramorum* was also amplified and digested. Fingerprint patterns of the unknown study samples were compared to the known species fingerprint patterns and grouped.

Sequencing of PCR product for species identification

Select PCR products were prepared for sequencing following the protocol of Eurofin-Operon sequencing center (Louisville, KY). The PCR product was mixed with either the forward or reverse primer at the appropriate concentrations stated by the company and the plates were sent to the Eurofin-Operon sequencing center. The sequence data received were cleaned (primer sequences removed), and forward and reverse sections were aligned using FinchTV v1.4.0 (Geospiza, Seattle, WA). BioEdit v7.2.5 (Hall 1999) was used to generate a consensus sequence, and compared via BLASTnt to the NCBI database

CHAPTER III

PHYTOPHTHORA SPECIES RECOVERED

Phytophthora ramorum

Phytophthora ramorum was first identified by morphological characteristics and later confirmed by sequencing (figure 3.2). *Phytophthora ramorum* was isolated in May of 2014 from necrotic lesions of a *Vaccinium* leaf located in the drainage ditch. It was additionally isolated 18 times from filtered water, and twice from a BOB intact leaf. All isolations were from the drainage ditch deriving from nursery property (figures 3.1 & 3.3).

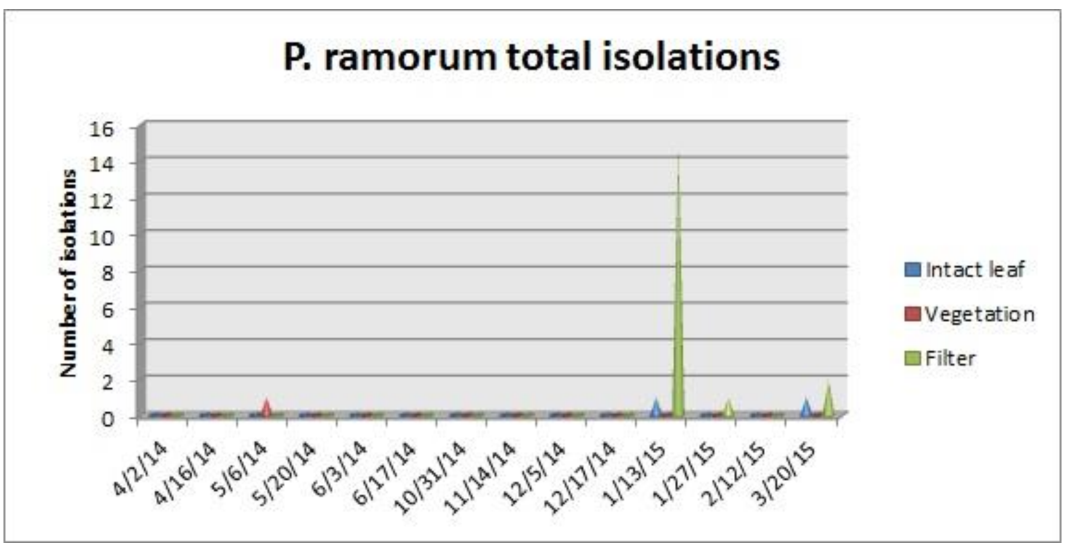

Figure 3.1 Total isolations of *Phytophthora ramorum*

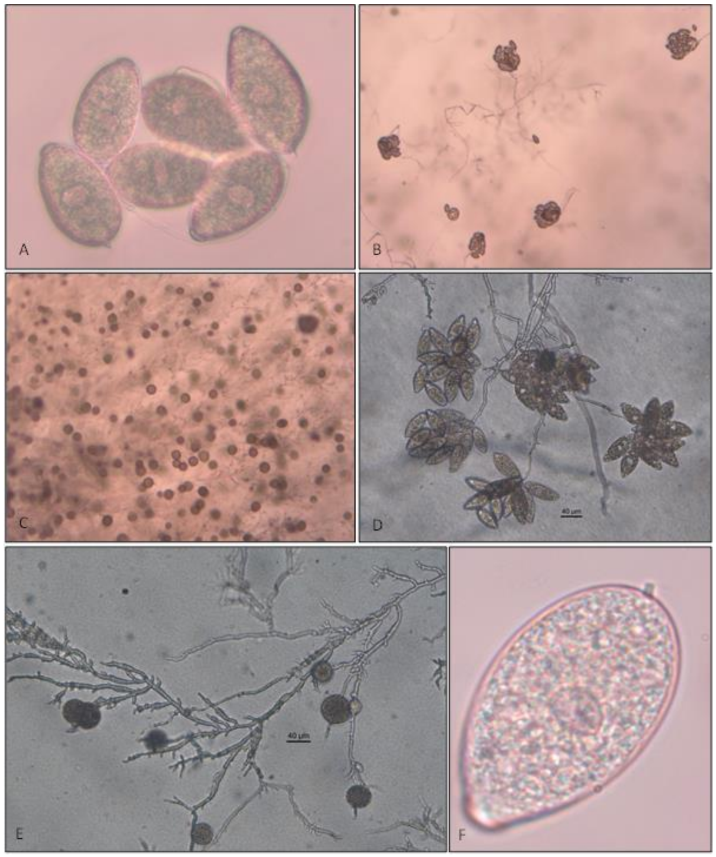

Figure 3.2 Morphology of *Phytophthora ramorum*

(A) packet of semi-papillate sporangia (B) packets of sporangia on the surface of agar (C) abundance of chlamydospores (D) sympodial packets of sporangia (E) coralloid hyphae and large chlamydospores (F) caducous sporangia showing pedicel length

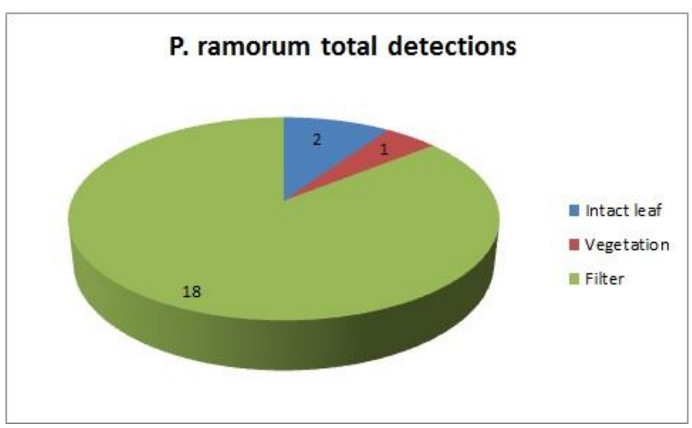

Figure 3.3 Retrieval methods for *Phytophthora ramorum*

Phytophthora gonapodyides

Phytophthora gonapodyides Petersen was first described in 1910 as a minor pathogen of a wide variety of woody plants (Erwin and Riberio 1996). Considered the most prevalent *Phytophthora* is the world, it is commonly isolated from aquatic habitats in the United States (Hansen et al. 2007; Hwang et al. 2007). It has previously been recovered from nursery irrigation systems around the southeast United States, including Mississippi (Ghimire et al. 2011; Olson et al. 2013; Copes et al. 2015).

Phytophthora gonapodyides is heterothallic and placed in Clade 6, with its closest relative *P. megasperma* (Martin et al. 2014). Sporangia were observed as non-papillate, ellipsoid, ovoid, to obpyriform, with internal and external proliferation (figure 3.6). Sporangia lacked caducity. Hyphal swellings were not observed, though bulbous, irregularly branched hyphae was distinct in isolates. Chlamydospores were not observed.

P. gonapodyides was isolated and verified by sequencing from necrotic lesions of a *Vaccinium* leaf, soil from area 5 in the drainage ditch, a BOB intact leaf from the drainage ditch, and twice from a BOB leaf disk from both the drainage ditch and Hog Creek. Enzyme digest results detected *P. gonapodyides* an additional 167 times, with 24 of those detections deriving from Hog Creek (figures 3.4 & 3.5).

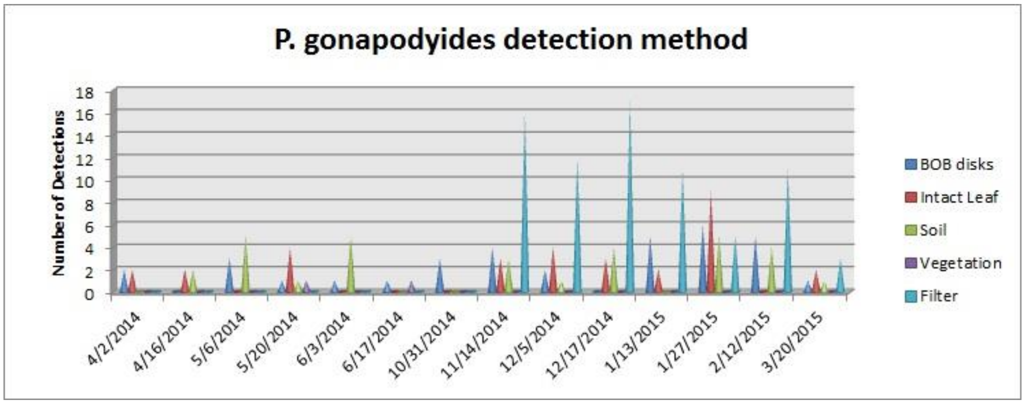

Figure 3.4 Total detections for *Phytophthora gonapodyides*

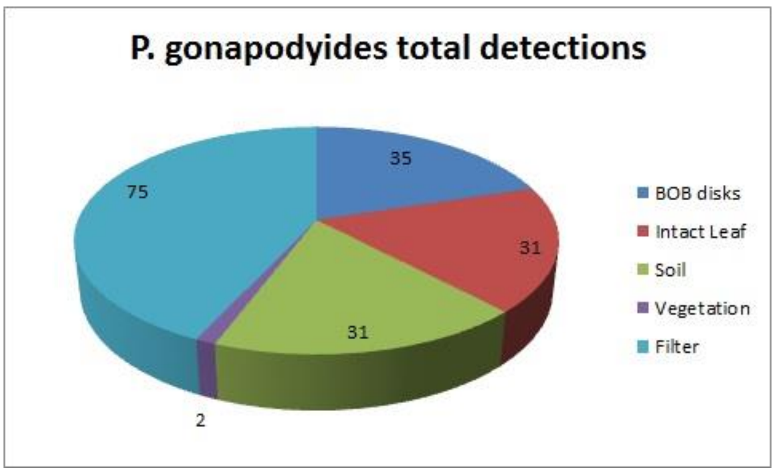

Figure 3.5 Retrieval methods for *Phytophthora gonapodyides*

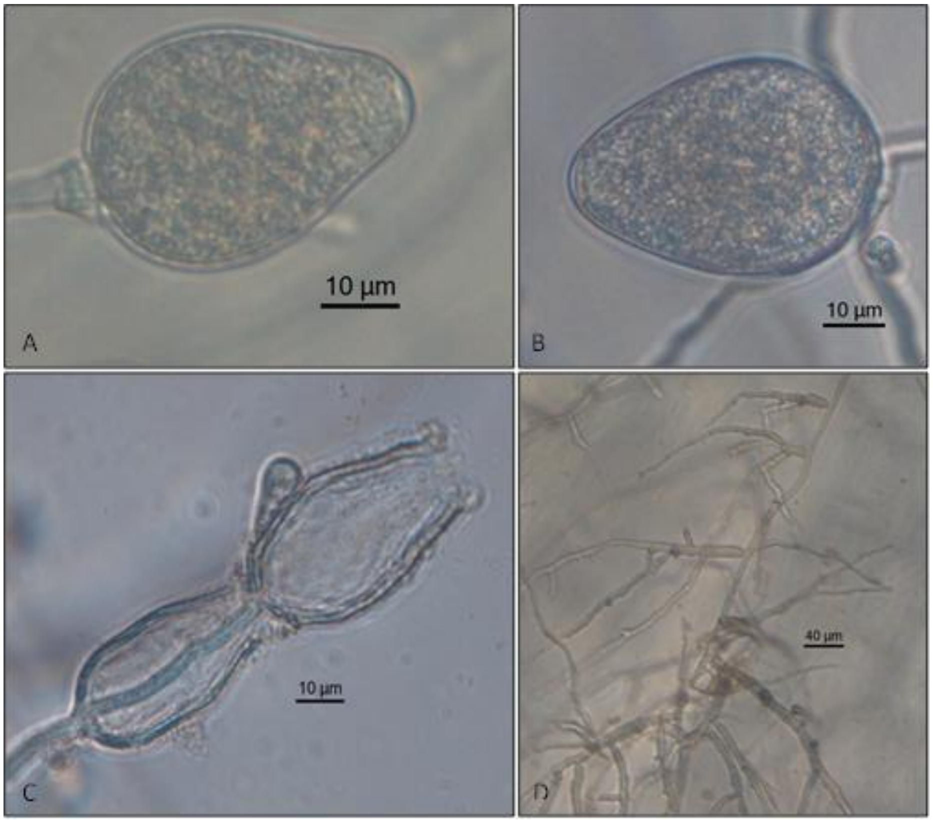

Figure 3.6 Morphology of *Phytophthora gonapodyides*

(A&B) obpyriform and ovoid, non-papillate sporangia (C) internal and external proliferation (D) bulbous, irregularly branched hyphae

***Phytophthora chlamydospora* sp. *nov*.**

Phytophthora chlamydospora sp. *nov*. Hansen, Reeser, Sutton, Braiser formally known as *Phytophthora* taxon Pgchlamydo, is designated as a Clade 6 species. It is ubiquitously found worldwide in streams and forest soil, and considered the second most abundant *Phytophthora* species in the world, serving as a weak pathogen of woody plants (Hansen et al. 2007).

P. chlamydospora sp. *nov.* is heterothallic. Sporangia were simple, mostly ovoid but occasionally obpyriform, non-papillate with a slight apical thickening, non-caducous, with internal proliferation (figure 3.8). Globose to subglobose hyphal swellings were observed, along with terminal and intercalary chlamydospores. Coiled mycelia was noted in V8 agar.

P. chlamydospora sp. *nov.* was isolated and confirmed by sequencing 65 times (figures 3.7 & 3.9). It was recovered six times from filtering water from the drainage ditch, and once from filtered water from Hog Creek. It was isolated 14 times from BOB leaf disks, with seven of those isolations deriving from Hog creek. Additionally it was recovered from soil 14 times. Isolations from vegetation include a horn beam (*Carpinus caroliniana* Walt.) twig canker, a black willow (*Salix nigra* Marsh.) leaf, a *Quercus phellos* L. leaf, a *Vaccinium* leaf, both the ditch and Hog Creek *Rhododendrons*, and a stem canker from a *Quercus nigra* L. sapling located in the drainage ditch.

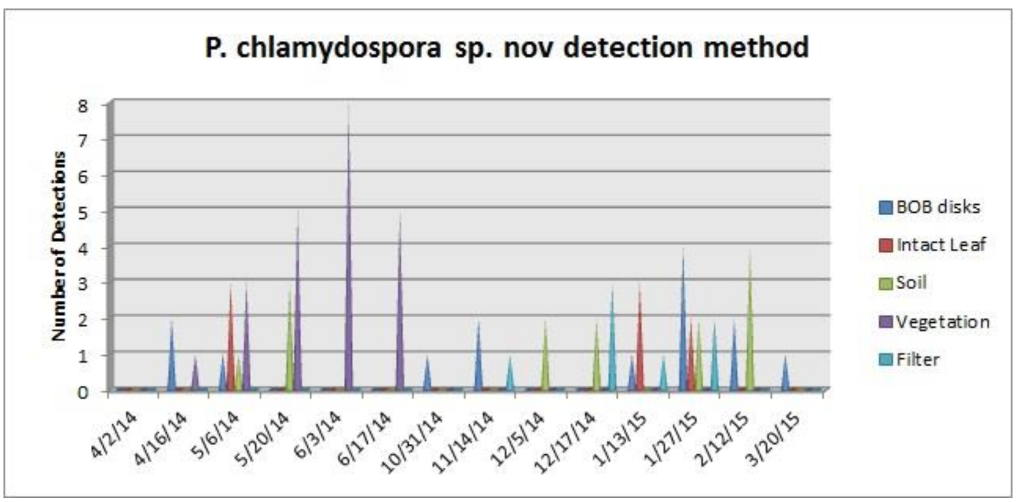

Figure 3.7 Total isolations for *Phytophthora chlamydospora* sp. *nov*.

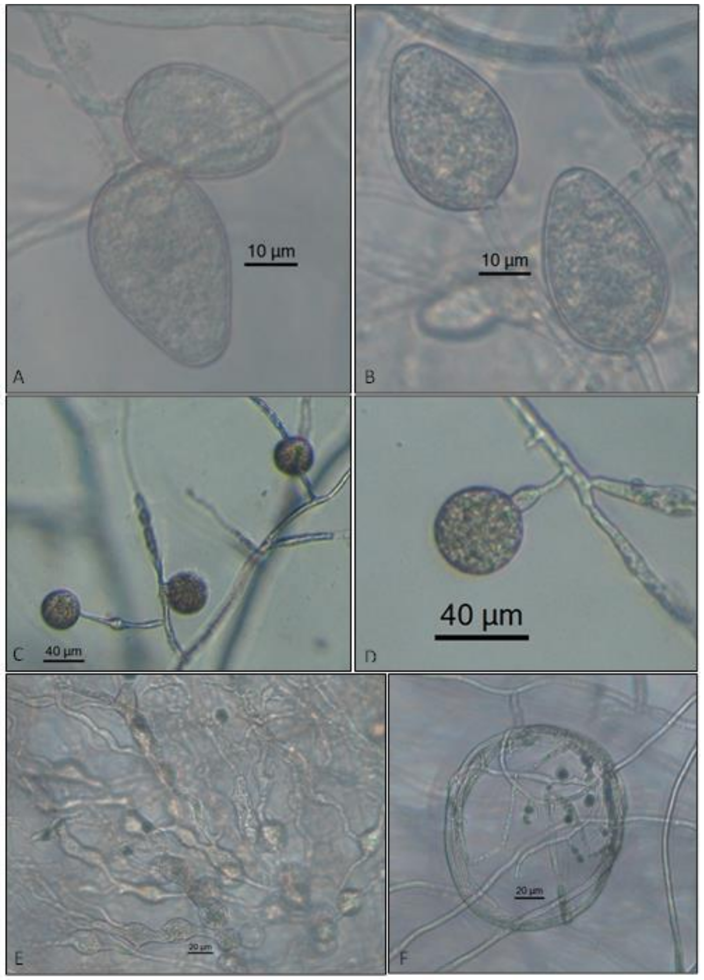

Figure 3.8 Morphology of *Phytophthora chlamydospora* sp. *nov*

(A&B) ovoid and ellipsoid non-papillate sporangia (C&D) terminal and intercalary chlamydospores (E) hyphal swellings (F) coiled hyphae

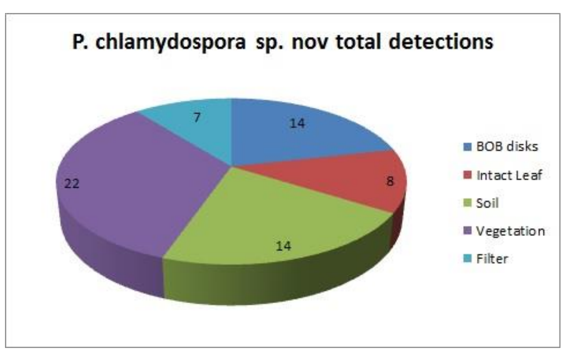

Figure 3.9 Retrieval methods for *Phytophthora chlamydospora* sp. *nov*.

***Phytophthora mississippiae* sp. *nov*..**

Phytophthora mississippiae sp. *nov.* Yang, Copes, Hong is a recently described species that was isolated from irrigation water at an ornamental plant nursery in Mississippi in 2012. *P. mississippiae* sp. *nov.* is designated in Clade 6 with *Phytophthora thermophile* Jung, Stukely, Burgess as a close relative (Yang et al. 2013).

Few sporangia formed after submerging 7 day-old V8 agar plugs in a non-sterile soil extract solution (NSSES, 15g soil/1 L distilled water) under fluorescent lighting at room temperature. Rhododendron disks were also inoculated with the isolate and floated on both NSSES and distilled water with few reproduction structures observed. Isolate 57J3 was received by Dr. Warren Copes of the USDA Agricultural Research Service. Sporangia that did form were obpyriform and obovoid, non-papillate with a slight apical thickening, and not caducous (figure 3.11). Mycelium in V8 agar was appressed.

P. mississippiae sp. *nov*. was detected 62 times from the enzyme digest and 11 times from sequencing. Fourteen detections were from BOB leaf disks, 16 from a BOB intact leaf, 16 from soil, 16 from filtering water, and once from necrotic lesions of a *Vaccinium* leaf (figures 3.10 & 3.12).

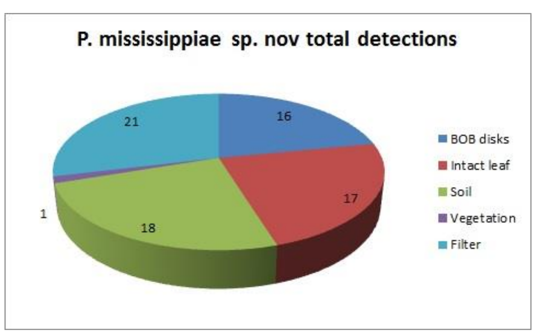

Figure 3.10 Retrieval methods for *Phytophthora mississippiae* sp. *nov.*

Figure 3.11 Sporangia of *Phytophthora mississippiae* sp. *nov*.

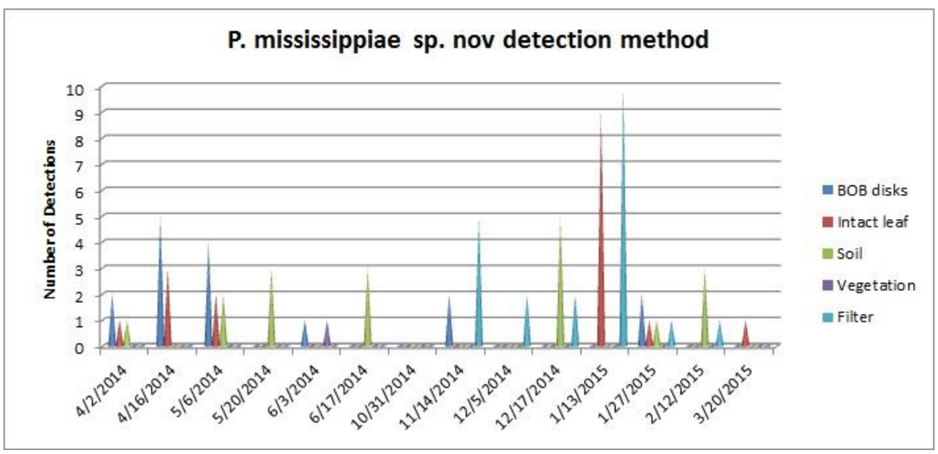

Figure 3.12 Total detections for *Phytophthora mississippiae* sp. *nov*.

Phytophthora cambivora

Phytophthora cambivora Petri was first described in 1917, and since then has been documented to affect over 30 different species of woody plants, most commonly inciting root rot (Erwin and Riberio 1996). Its soil-born nature has allowed it to be documented in low frequency from oak forests in the eastern and central United States

(Balci et al. 2007). It has also been recovered from ornamental nursery plants (Bienapfl and Balci 2014).

P. cambivora is heterothallic, and is placed in Clade 7 (Martin et al. 2014). Morphology was not observed. *P. cambivora* was isolated from necrotic leaf spots from a *Vaccinium* as well as a stem canker from cane grass (*Arundinaria* sp.), both located in the drainage ditch.

Phytophthora cinnamomi

Phytophthora cinnamomi Rands is an economically devastating soil-born root rot pathogen of ornamental trees, agricultural crops, fruit trees, and forest plantations, with a plant host list of over 1,000 species (Erwin and Ribeiro 1996). Thought to have originated in Papua New Guinea, due to its rapid spread in the plant trade industry over the past century it is now has the most expansive host range of any *Phytophthora* species (Robin et al. 2012).

In Mississippi, *P. cinnamomi* is a devastating root rot of highbush blueberry (*Vaccinium corymbosum* L.), an economically vital crop for the southern part of the state (Smith 2012). *P. cinnamomi* has also been associated with root and crown rot of peach trees in Mississippi (Haygood et al. 1986). Economically important pine trees in Mississippi are also affected by *P. cinnamomi*, nicknamed Littleleaf disease from the shortening and yellowing of the needles once the tree is infected (Mistretta 1984). Additionally, *P. cinnamomi* has also been isolated from eastern oak species in both Florida and the central eastern United States (Barnard 2006; Eggers et al. 2012).

P. cinnamomi is a heterothallic species in Clade 7 (Martin et al. 2014). Distinctive coralloid mycelium and abundant hyphal swellings were common in isolates (figure 3.15). Abundant globose, thin-walled chlamydospores were present, ranging from 35-45µm in size. Non-papillate sporangia were ovoid, obpyriform, or ellipsoid with a slight apical thickening. Sporangia were not deciduous and were only observed to be borne singularly. Aerial mycelium was noted in V8 agar Petri dishes.

P. cinnamomi was isolated from the drainage ditch six times from a necrotic black willow (*Salix nigra*) leaf, three times from swamp chestnut oak (*Quercus michauxii* Nutt.) petiole canker and leaves, three times from necrotic leaf lesions from a willow oak (*Quercus phellos*), and once from a water oak (*Quercus nigra)* sapling root. Additionally it was recovered seven times from filtering water and once from an intact leaf collected from the drainage ditch (figures 3.13 & 3.14).

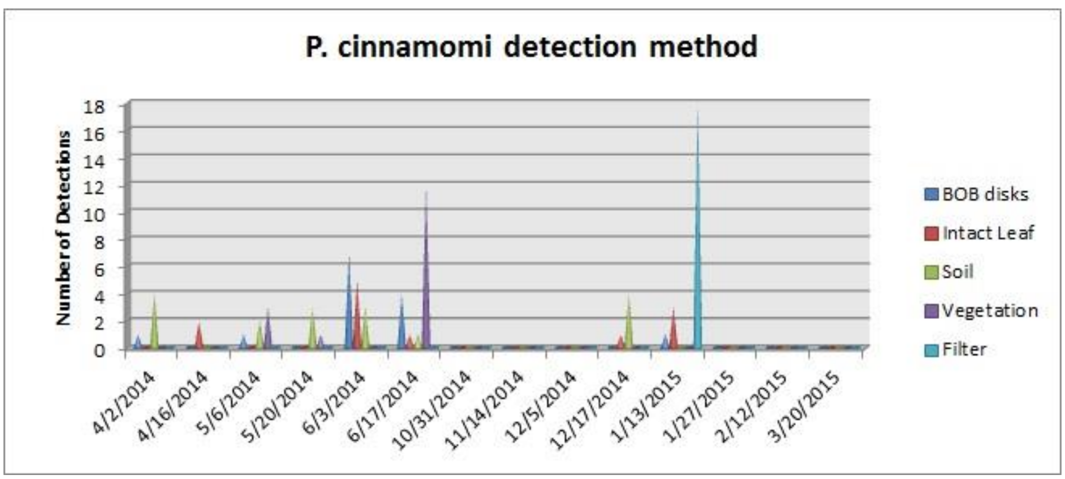

Figure 3.13 Total detections for *Phytophthora cinnamomi*

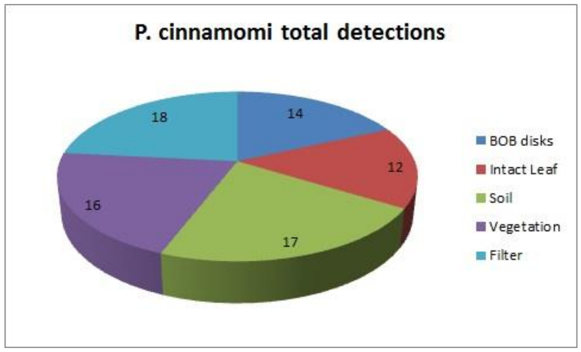

Figure 3.14 Retrieval methods for *Phytophthora cinnamomi*

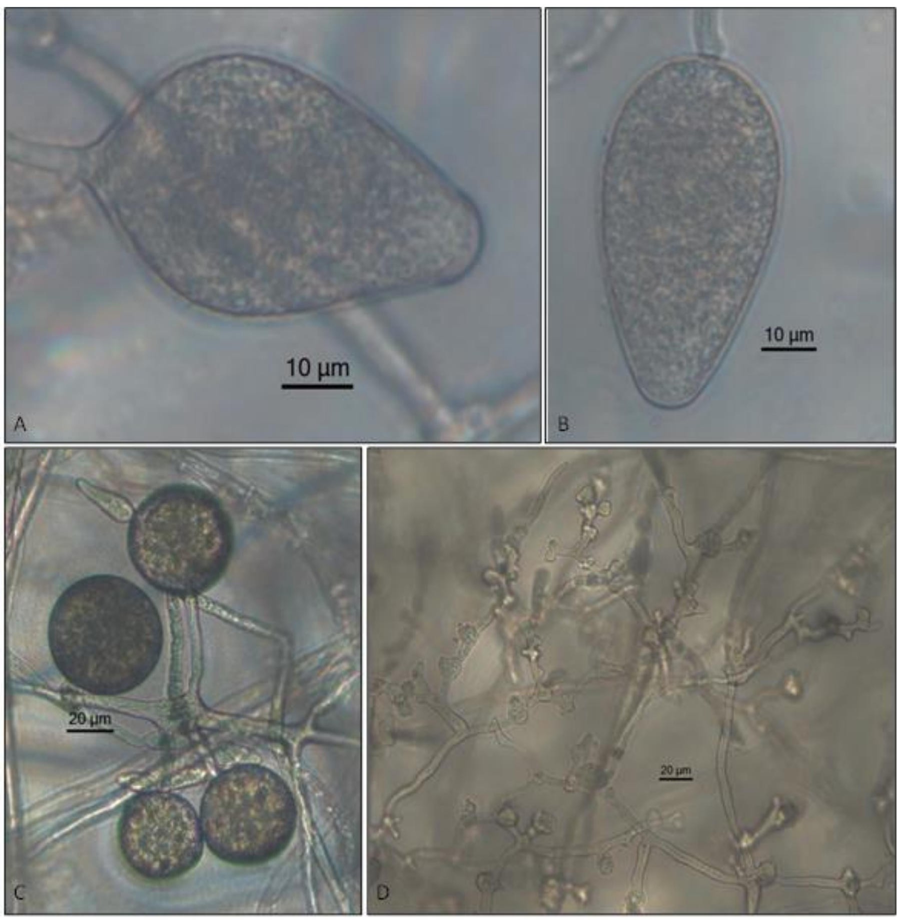

Figure 3.15 Morphology of *Phytophthora cinnamomi*

(A&B) ovoid, singular, non-papillate sporangia with apical thickening (C) globose, large chlamydospores (D) hyphal swellings

Phytophthora cryptogea

Phytophthora cryptogea Pethybridge & Lafferty has been isolated from over 100 species of woody plants in 23 different families (Erwin and Ribeiro 1996). The fact that is recovered from both forest streams and irrigation systems suggests it is capable of surviving without a host (Erwin and Ribeiro 1996; Shrestha et al. 2013). This survival mechanism has allowed *P. cryptogea* to become a significant pathogen in domestic floral and ornamental greenhouses (Macdonald et al. 1994; Ferguson and Jeffers 1999; Hwang and Benson 2005; Bush et al. 2006; Olson et al. 2011).

Phytophthora cryptogea is in Clade 8, and morphologically similar to *Phytophthora drechsleri* Tucker, though *P. drechsleri* has a higher maximum growing temperature (Erwin and Riberio 1996; Martin et al. 2014). *P. cryptogea* is heterothallic, and produces abundant obpyriform to ovoid, non-papillate, non-caducous sporangia, much of them germinating from internal proliferation (figure 3.16). Chlamydospores and hyphal swellings were absent. Aerial mycelium was noted in V8 agar.

P. cryptogea was isolated three times during the study. It was cultured from necrotic leaf spots on a sweetgum (*Liquidambar styraciflua* L.) and a hickory (*Carya* sp.) located in the flood plain of the drainage ditch, and from a BOB leaf disk collected from the drainage ditch.

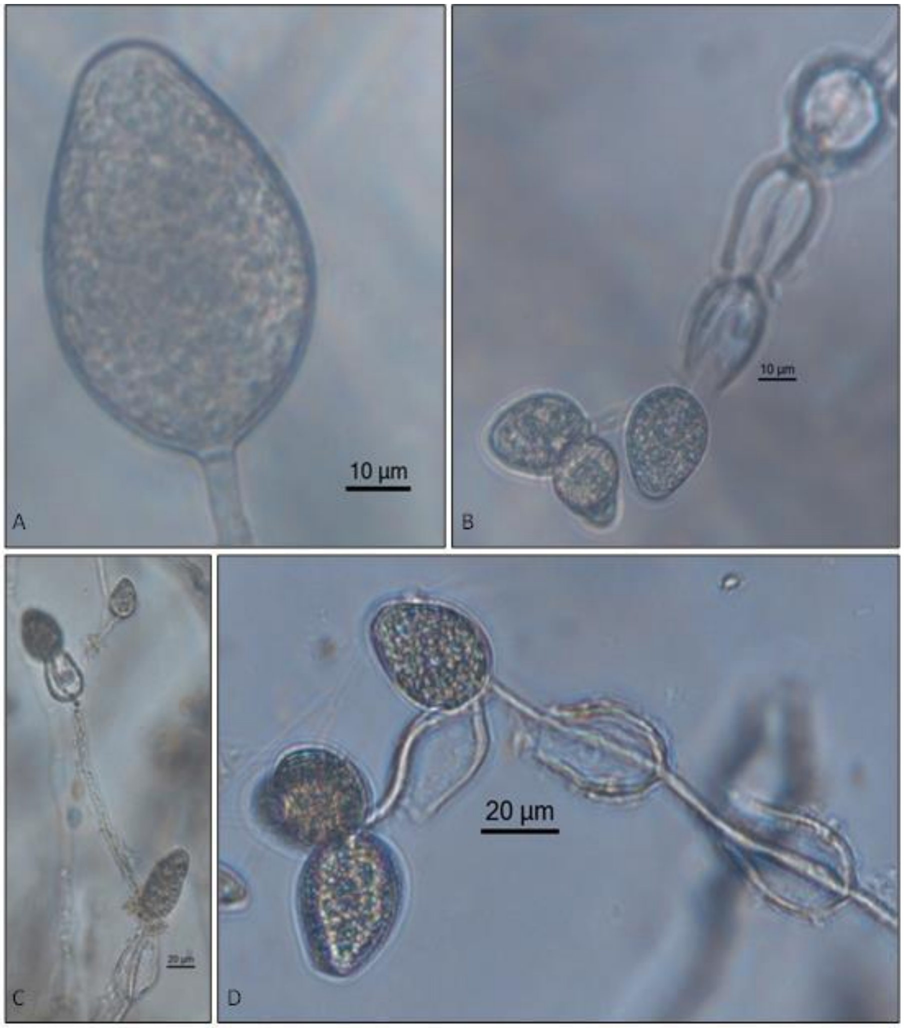

Figure 3.16 Morphology of *Phytophthora cryptogea*

(A) ovoid, singular, non-papillate sporangia (B-D) internal proliferation with variable-shaped sporangia

Phytophthora citricola

Phytophthora citricola Sawada has been reported to infect over 50 species of woody plants since first being described in 1927(Erwin and Ribeiro 1996). Widely distributed in the United States, it is commonly isolated in eastern ornamental plant nurseries as well as from oak forest soils from the eastern United States (Benson and

Jones 1980; Ferguson and Jeffers 1999; Dart et al. 2007; Balci et al. 2007; Warfield et al. 2008; Donahoo and Lamour 2008).

P. citricola is a homothallic species designated in Clade 2 (Martin et al. 2014). Sporangia were semi-papillate, non-caducous, and extremely variable, ranging from ovoid, reniform, obpyriform, with some bifurcated and exhibiting bi-papillation (figure 3.17). Spherical oospores with paragynous antheridia were observed. *P. citricola* was isolated from a swamp chestnut oak leaf (*Quercus michauxii*) and once from a necrotic leaf lesion of a *Rhododendron* located in the drainage ditch.

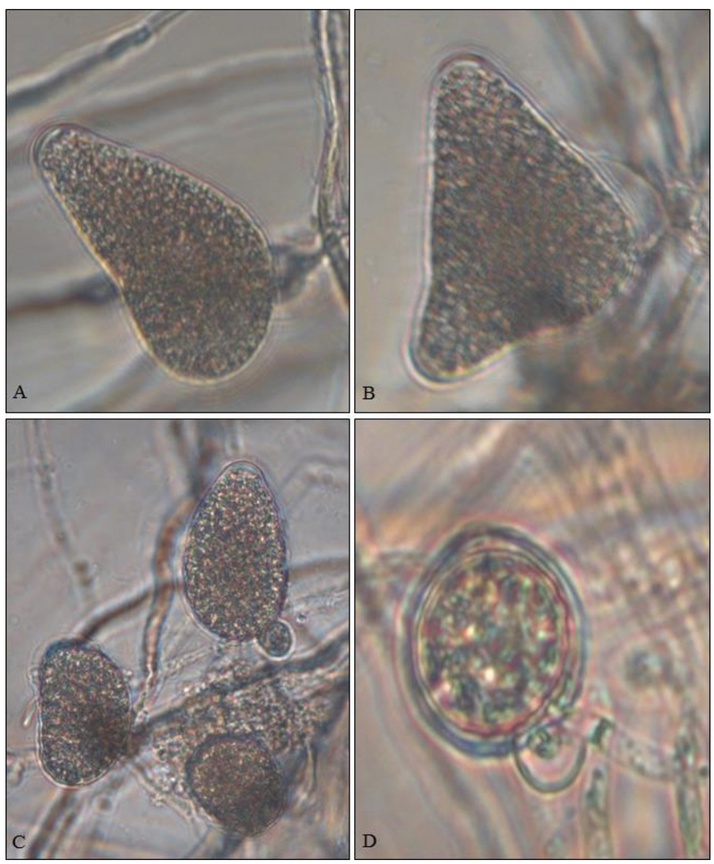

Figure 3.17 Morphology of *Phytophthora citricola*

(A-C) reniform, bifurcated, and ovoid semi-papillate sporangia (D) plerotic oospore with paragynous antheridia

Phytophthora rosacearum

Phytophthora rosacearum Hansen, Wilcox, Reeser is a recently described species in the *P. megasperma* complex, which are morphologically similar but differentiated by phylogenetics. Morphologically it can be distinguished from *P. megasperma* by its high optimum growth temperature (36°C) and small oospores (29-34 µm diameter) (Hansen et al. 2009).

P. rosacearum is homothallic species in Clade 6 (Martin et al. 2014). It is named after its recovery from rosaceous fruit trees in California. It is unknown if it resides naturally in Mississippi or was transplanted from nursery stock. Morphology was not observed. *P. rosacearum* was detected from a box elder (*Acer negundo* L.) petiole canker, twice from necrotic leaf spots on an elm (*Ulmus* sp.), once from leaf necrosis from the ditch *Rhododendron*, and once from a *Quercus nigra* sapling root located in the drainage ditch.

Phytophthora europaea

Phytophthora europaea Hansen & Jung has previously been isolated from rhizosphere soil samples from *Quercus* spp. in both France and Germany, acting as a weak pathogen on English oak (*Quercus robur* L.) seedlings (Jung et al. 2002). In 2003 and 2004 it was recovered from soil from oak forest ecosystems in five central and eastern states of the United States (Balci et al. 2007). No other information is available regarding the organism's distribution.

P. europaea is homothallic and placed in Clade 7 (Martin et al. 2014). Morphology was not observed. *P. europaea* was recovered once from a BOB leaf disk from Hog Creek. This isolate had a 92% query cover match with a 170 max score out of

170 total score with previous isolates BBA 12/02-3, IBL/2011/21, IBL/2011/20, BR1072, and VI_1-2P in the NCBI BLAST database.

Phytophthora citrophthora

Phytophthora citrophthora Smith & Smith has been documented to infect over 75 woody host species, with a presence in fruit orchards, nurseries, and tree plantations (Erwin and Ribeiro 1996). It has been recovered from nursery irrigation water in ornamental plant nurseries as well as forest streams in the eastern United States (Ferguson and Jeffers 1999; Bush et al. 2006; Hwang et al. 2007; Donahoo and Lamour 2008; Hulvey et al. 2010).

P. citrophthora is a heterothallic species designated in Clade 2 (Martin et al. 2014). Few chlamydospores were observed, which were both terminal and intercalary and about 38x35μm in size (figure 3.18). The abundant sporangia were papillate, lacking caducity, with variable shapes ranging from spherical, ovoid, to ellipsoid, and born singularly and sympodially.

P. citrophthora was isolated from drainage ditch water by a BOB leaf disk and intact leaf, as well as from necrotic leaf lesions of a *Rhododendron* bush (figure 3.19). It was also isolated from Hog Creek twice from a BOB intact leaf. Enzyme digest results show the additional detection of *P. citrophthora* 10 times throughout the study, with four of those detections deriving from Hog Creek.

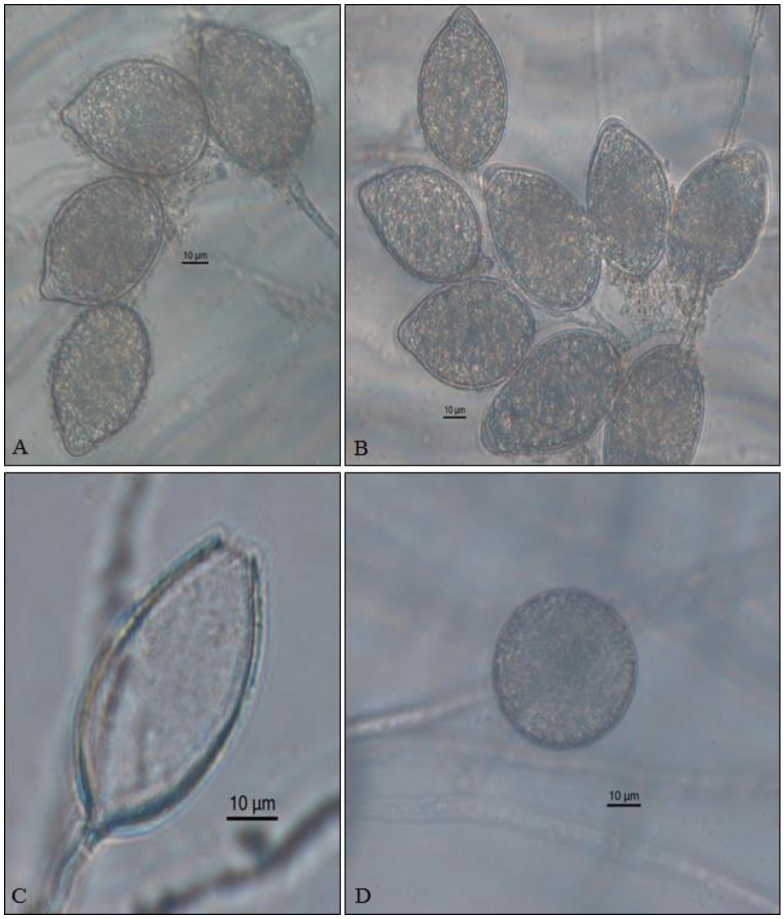

Figure 3.18 Morphology of *Phytophthora citrophthora*

(A&B) sympodial, ovoid, papillate sporangia (C) opening width of an empty sporangia (D) chlamydospore

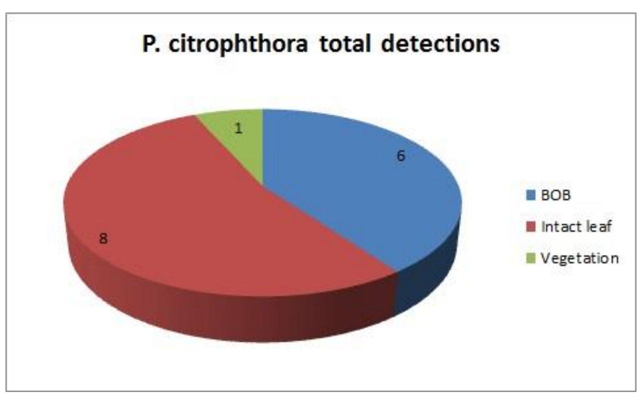

Figure 3.19 Retrieval methods of *Phytophthora citrophthora*

Phytophthora hydropathica

Phytophthora hydropathica Hong & Gallegly was recently described from isolates recovered from nursery irrigation water in both Virginia and Maryland (Hong et al. 2010). Previously it had been recovered as a provisional species from nursery plant foliage and stream water in eastern Tennessee (Hulvey et al. 2010). *P. hydropathica's* dissemination in irrigation water, along with its ability to tolerate high summer temperatures, leads to a potentially serious pathogen of greenhouse plants. Leaf necrosis on rhododendron, and collar rot of mountain laurel (*Kalmia latifolia* L.), along with pathogenicity towards numerous other economical crops has been demonstrated (Hong et al. 2010).

P. hydropathica is a heterothallic species in Clade 9 (Martin et al. 2014). Morphology was not observed. *P. hydropathica* was detected from a necrotic *Rhododendron* leaf located in the flood plain as well as a BOB intact leaf from drainage ditch water.

Phytophthora riparia

Phytophthora riparia Hansen, Reeser, Sutton is a recently described species isolated from forest streams in Oregon, California, and Alaska. It is designated in Clade 6 with its closest relative P. taxon salixsoil, though is morphologically similar to *P. gonapodyides* (Hansen et al. 2012). It is unknown if *P. riparia* naturally resides in Mississippi or was transplanted here from imported nursery stock.

P. riparia is heterothallic, exhibiting the general characteristics of most Clade 6 species. Sporangia formed singularly, were mostly ovoid but occasionally obpyriform, non-papillate, non-caducous, with a slight apical thickening (figure 3.20). Sporangia displayed internal extended proliferation occasionally. Chlamydospores were not produced in culture. Additionally, coiled mycelium and hyphal swellings were observed in V8 agar. *P. riparia* was isolated once from filtered water from the drainage ditch and once from soil from area four in the drainage ditch.

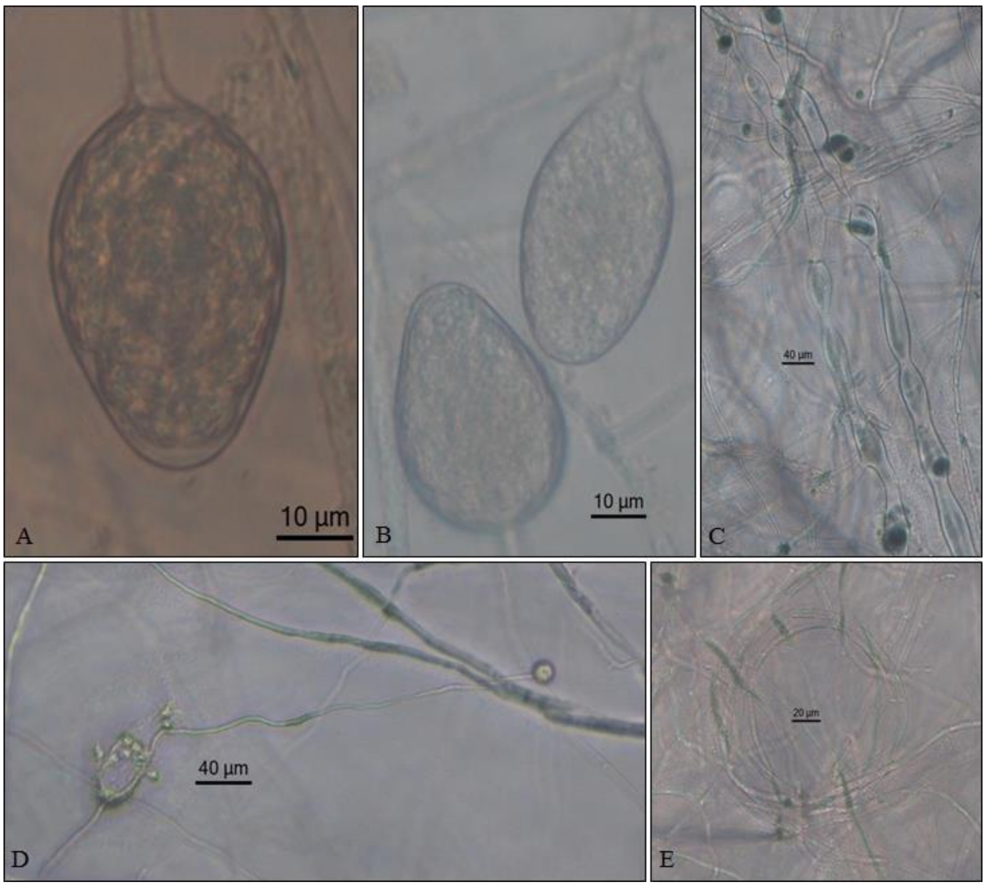

Figure 3.20 Morphology of *Phytophthora riparia*

(A&B) ovoid, singular, non-papillate sporangia (C) hyphal swellings (D) internal proliferation (E) coiled hyphae

Phytophthora megasperma

Phytophthora megasperma Drechsler is a unique taxon composed of multiple biotypes each based on protein profiles, pathogenicity, and morphology (Hansen and

Marx 1991; Erwin and Ribeiro 1996). Braiser et al. 2003 divided the *P. megasperma/gonapodyides* clade into 11 phenotypic taxa, many being undescribed. Improvements in molecular techniques have brought taxonomic clarity within the complex, separating unrelated species while confirming the legitimacy of *P. megasperma sensu strictu* (Hansen et al. 2009: Hansen et al. 2015).

P. megasperma is homothallic species in Clade 6 (Martin et al. 2014). Ovoid, obpyriform, non-papillate, non-caducous sporangia with internal proliferation were observed in abundance (figure 3.21). Oospores were prevalent and variable in size, with an average of about 30 µm, which did not compare with the size of the oospores (>40µm) originally described by Drechsler. Oospores were about 3 months old when observed. Sequence data also has a 100% query cover with *Phytophthora borealis* Hansen, Sutton, Reeser isolate AKWA73.2-0708, which has been described partially heterothallic, producing smaller oospores when paired with *P. cryptogea* A2 tester (Hansen et al. 2012). Various authors state that oospore size within the *P. megasperma* complex are highly variable and dependent on the host, and are not reliable criteria for separating varieties (Erwin and Ribeiro 1996). Therefore it is not known whether this is an isolate of *P. borealis* or a variety of *P. megasperma*. Isolate T10 A.29 was only recovered once from stream water in the drainage ditch.

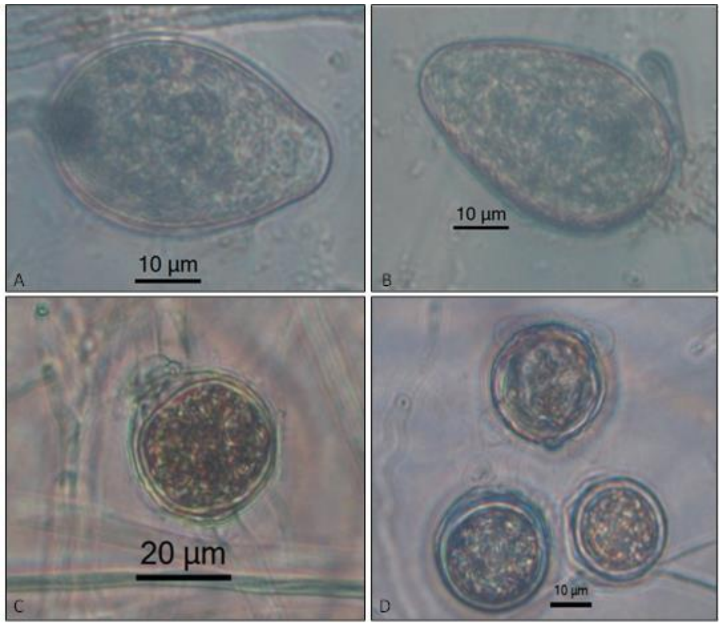

Figure 3.21 Morphology of *Phytophthora megasperma*

(A&B) ovoid, non-papillate sporangia (C&D) 3 month-old oospores

Phytophthora syringae

Phytophthora syringae Klebahn was first described in 1905. Since then it has been documented to infect 31 plants in 19 genera, especially in the Rosaceae family (Erwin and Riberio 1996). *P. syringae* has been isolated from nursery irrigation systems from around the country (Bush et al. 2006; Hulvey et al. 2010; Ghimire et al. 2011; Bienapfl and Balci 2014).

P. syringae is a homothallic species placed in Clade 8 (Martin et al. 2014). Even though the species is sexually fertile, oospores were not observed, resembling the results of Doster and Bostock 1988, which suggest the use of vegetable oils to stimulate the production of oospores in culture. Sporangia were semi-papillate, ranging from ovoid, obpyriform, globose, to ellipsoid, and non-caducous. Chlamydospores were not produced (figure 3.22). Catenulate hyphal swellings were observed in V8 agar.

P. syringae was isolated five times in February 2015 by filtering water from the drainage ditch, with a 95% query cover and maximum score of 1375 to isolates 85-52, 87-8, Ps-79, and BBA 12/99-5 in the NCBI BLAST database.

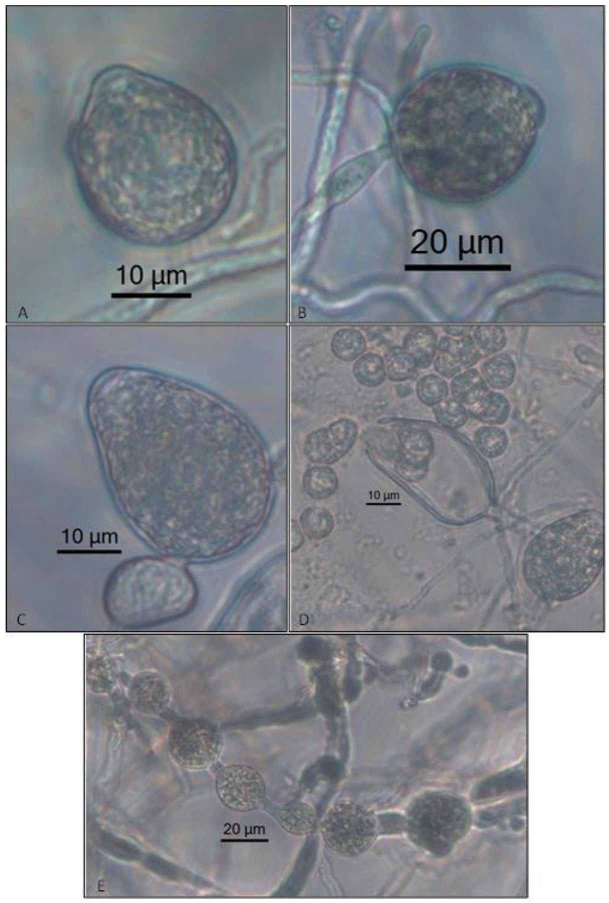

Figure 3.22 Morphology of *Phytophthora syringae*

(A-C) globose and ovoid semi-papillate sporangia (D) sporangia releasing zoospores (E) catenulate hyphal swellings

***Phytophthora* taxon oaksoil**

Phytophthora taxon oaksoil is an undescribed species designated in Clade 6 by Braiser et al. 2003. It has been recovered from soil in France and streams from Oregon

(Braiser et al. 2003; Reeser et al. 2011). P. taxon oaksoil is sterile with an optimum growth temperature of 25°C (Braiser et al. 2003).

Sporangia were ovoid, non-papillate, non-caducous, and varied in size with an average of about 26x40µm (figure 3.23). Internal proliferation from sporangia was observed. No chlamydospores formed in V8 agar. Mycelium on V8 agar was appressed.

P. taxon oaksoil was isolated from a BOB leaf disk collected from Hog Creek. Additionally it was recovered from discolored lesions on a trunk of an oak tree located adjacent to Hog Creek and periodically subjected to flooding. Of the numerous attempts at culturing samples from the oak tree this was the only *Phytophthora* sp. recovered.

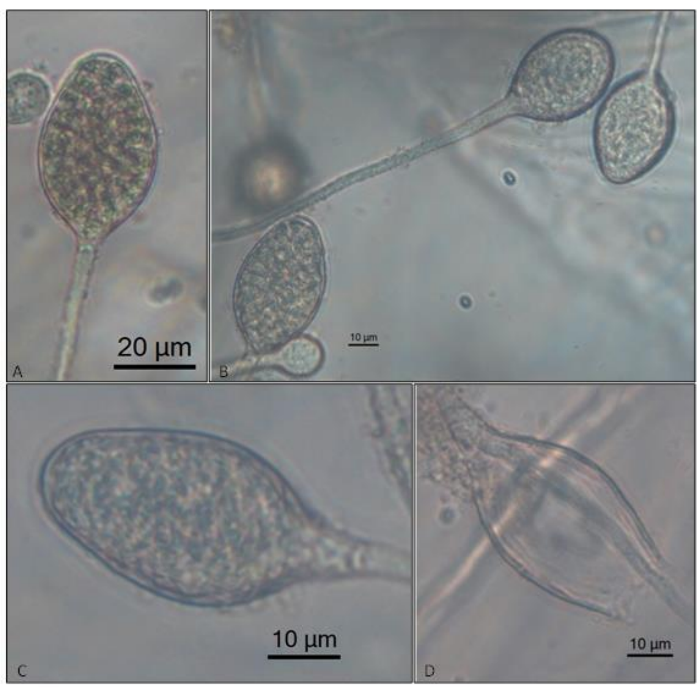

Figure 3.23 Morphology of *Phytophthora* taxon oaksoil

(A-C) singular, ovoid to ellipsoid non-papillate sporangia (D) width of empty sporangia with internal proliferation

CHAPTER IV

DISCUSSION

The detection of 16 species of *Phytophthora* during this study validates the ability of an ornamental plant nursery to sustain and transmit Oomycete pathogens into new environments. While the species richness in this study may be attributed to the heterogeneity of plant hosts and regions the nursery stock was imported from, the abundance of each species released into the environment may be restricted by the local climate. Therefore the overall evenness of species recovered during this study was inconsistent (figure 4.1); suggesting that the diversity of *Phytophthora* spp. discharged into the environment in adverse conditions may be low, but can balloon during favorable periods. The predisposed nature of these organisms to reproduce with little impetus reinforces the significance of appropriate management practices in plant nurseries.

In addition to climate, particular recovery methods may be exclusive towards certain species depending on their etiology. It is not surprising that *P. gonapodyides*, an aquatic species with saprobic capabilities, was the most common species isolated from filtering stream water (figure 4.2). Species that propagate prolific sporangia, especially deciduous sporangia such as *P. ramorum*, will release greater motile zoospores into the environment and contribute to higher aquatic recoveries, even though the emanating infection may be marginal. Both *P. citrophthora* and *P. syringae* were isolated numerous times, but from only one sampling trip, suggesting their presence in the environment is irregular and may be attributed to current nursery stock. *P. cinnamomi* was consistently recovered from soil, water, and vegetation over the two-year period, indicating a perpetual presence in the local environment, possibly because of the organism's capability to survive as a saprophyte in moist soil for indefinite periods of time.

Furthermore, different laboratory methods produced different results. Identity confirmation was more conclusive when DNA was extracted from isolated cultures opposed to inoculated, intact leaf disks. DNA results from the majority of intact leaf disks were overwhelmed by Clade 6 species. Isolating species before DNA extraction not only allowed for morphological observation, but also distinguished species against the ubiquitous "cloud" of Clade 6 species that acted as "background noise". In this study, isolation from filtered water resulted in the greatest precision towards species identification.

P. ramorum was isolated 20 times from water throughout this study, with the optimum months to sample from late-November to January and from mid-February to March. Sixteen isolations were derived from 44°F water, one isolation from 50.4°F water, and three isolations were from 68°F water. All isolations were within the cardinal temperatures for proliferation, but with the exception of three, were well outside the optimum temperature range for sporangia production. Acclimated species with broad cardinal temperatures may release inoculum more frequently compared to species with narrow cardinal temperatures. Some species were recovered when temperatures were far from being ideal, provoking speculation on how and where these organisms complete their life cycle (figure 4.3). *P. riparia* was isolated from 45°F water even though its optimal temperature *in vitro* is close to 86°F. This may be a result of a superficial microclimate produced from the nursery, in addition to certain species thermal flexibility and possible capitalization towards favorable environmental niches.

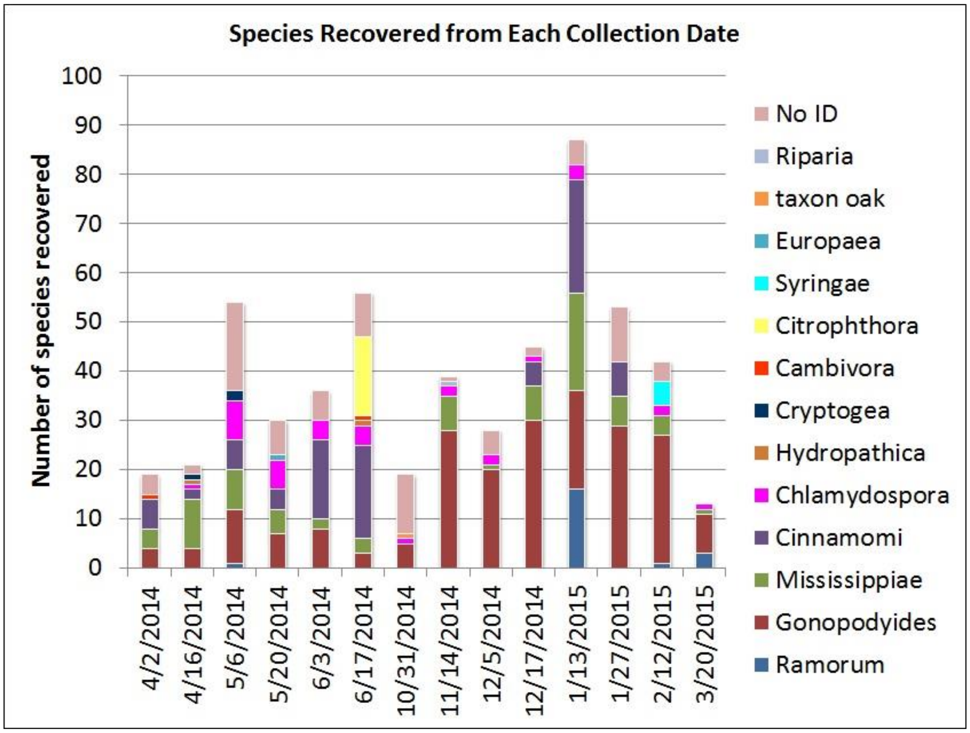

Figure 4.1 Variety of species from each collection date

The sampling trip with the most isolations (1/13/15) was immediately after heavy rains, with the highest water level recorded over the two-year time period (figure 4.3). Therefore, the highest inoculum loads released into the environment may not only be dependent on seasonal temperatures, but also the amount of local precipitation. The increase of inoculum in waterways during heavy rain events suggests 1) the possible inundation of infected vegetation or the aquatic deposition of residual inoculum from runoff, or 2) the increased production of sporangia from infected vegetation which naturally disseminates into streams.

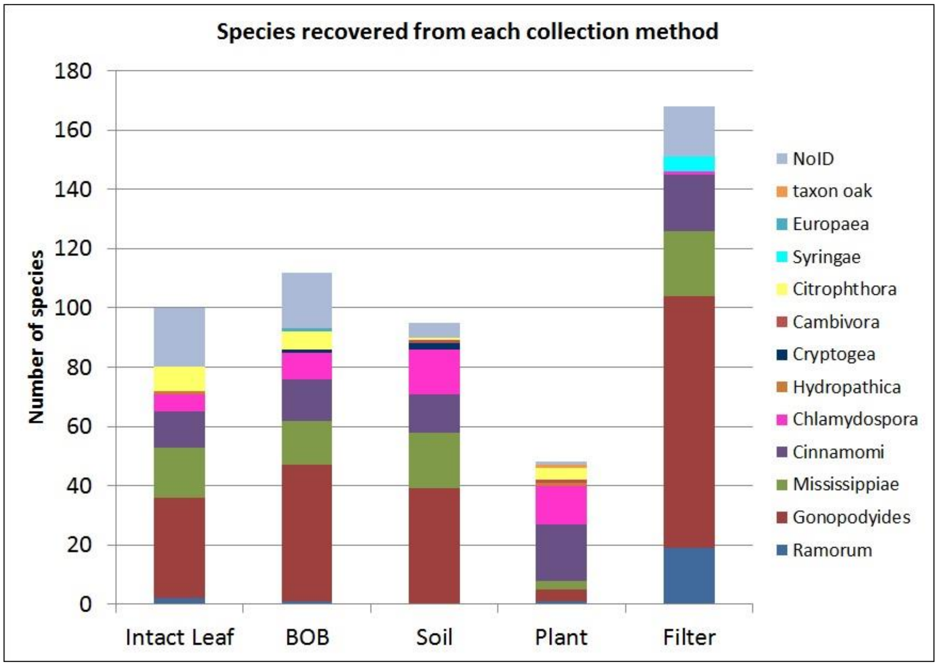

Figure 4.2 Variety of species from each collection method

The infection of riparian, associated host plants by *P. ramorum*, resulting in a biotrophic "ramorum blight" scenario, could sustain a reservoir of inoculum that is deposited into streams during rain events without inciting obvious plant mortality. The sampling area contains seven regulated plant host species and four associated plant host species. *P. ramorum* was isolated once from a surfaced-sterilized *Vaccinium* leaf that was submerged during periods of flooding, demonstrating that the pathogen can and has infected riparian vegetation. This is also supported by the fact that Dr. Steven Jeffers detected the pathogen from a riparian willow in 2010.

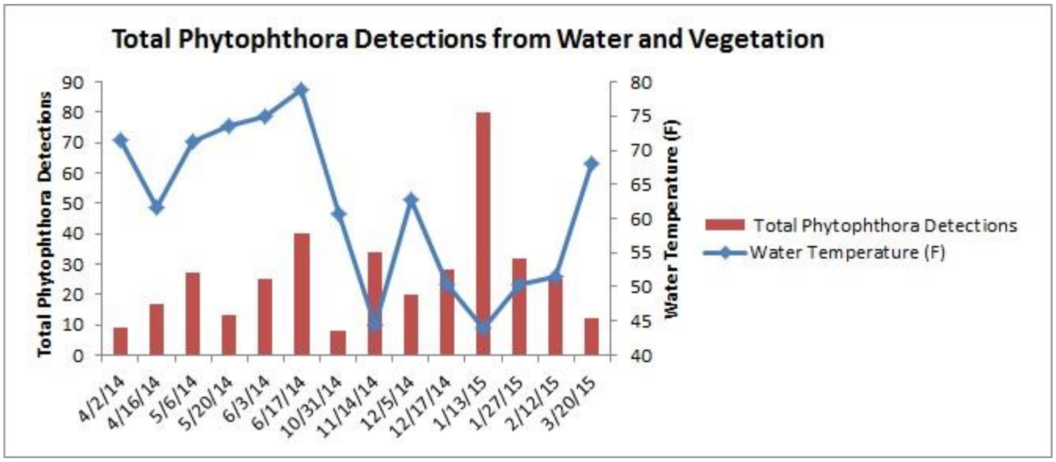

Figure 4.3 Total *Phytophthora* spp. collected from water and vegetation

Disease progression may be hindered by unfavorable seasonal fluctuations in climate, which would prevent the necessary accumulation of inoculum needed for an epidemic, but would allow substantial sporulation during favorable climatic windows. The isolation of *P. ramorum* 16 times during January from two liters of stream water proves that inoculum levels are extremely high at certain months of the year. Additionally, the vegetative dormancy of native plants during the winter months may substantially reduce the transmission rate of *P. ramorum* into the environment, as the disease cycle in forests is mostly attributed to foliar infections. More research is needed to see if exotic vegetation plays a role in the persistence and reproduction of *P. ramorum*, as exotic foliage persists long into winter months, and may contribute to the high levels of inoculum during the months of December and January.

CHAPTER V

CONCLUSION

This study demonstrates that even after mitigation attempts, residual inoculum of *P. ramorum* can persist in the environment for years. Although *P. ramorum* has prevailed for 8 years in central Mississippi, there is no evidence of substantial vegetative dieback associated with this disease. This may be attributed to 1) the relatively small climatic window in Mississippi that is conducive for the pathogen to sporulate, 2) the precipitation frequency, and 3) the correlation of plant dormancy with the months of the year that are favorable for disease progression.

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