DEVELOPING A LAMP ASSAY FOR THE RAPID AND ACCURATE IN-FIELD DETECTION OF THE LAUREL WILT PATHOGEN

by

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(Under the Direction of Caterina Villari)

ABSTRACT

Laurel wilt is an invasive, fatal vascular wilt disease that is devastating lauraceous species in the southeastern United States. It is caused by *Raffaelea lauricola*, the mycangial symbiont of *Xyleborus glabratus*, and trees infected with the pathogen experience wilted foliage, sapwood discoloration, and rapid mortality. Since its introduction in 2002 near Savannah, Georgia, laurel wilt disease has quickly spread as far as Florida, Kentucky, North Carolina, and Texas. Current operations to prevent the spread of laurel wilt disease are delayed by a week or more by laboratory procedures to confirm the diagnosis. To mitigate damage and improve disease management, we developed a loop-mediated isothermal amplification (LAMP) assay for the rapid in-field detection of the pathogen. Our species-specific assay is capable of amplifying as little as 0.5 pg of fungal DNA, as few as 50 spores, and can detect the pathogen directly from wood tissue in as little as 12 days post inoculation, even when testing crude DNA extracts. It can also detect the pathogen directly from the beetle host. To verify the robustness of the assay, we tested naturally infected wood samples from across the

Southeast and successfully confirmed the presence or absence of the disease, even in those cases where visual symptoms were ambiguous. Finally, we validated the assay for use on a portable device directly at point-of-care, showing how the use of LAMP allows rapid disease confirmation within an hour of locating a potentially diseased plant. This study provides a successful example that should foster the use of LAMP based technology to rapidly diagnose laurel wilt disease directly in-field, ultimately enabling better disease management.

INDEX WORDS: LAMP, Laurel wilt disease, Redbay, Sassafras, Avocado, *Raffaelea lauricola*, *Xyleborus glabratus*, Vascular wilt disease, Forest Health and Protection

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DEDICATION

For my mother, Marlene Smith Hamilton, who was always the most ardent supporter of anything I choose to undertake. I am only where I am today because of your love and encouragement. You were my rock and anchor. This work is wholly dedicated to your memory and I dearly hope that it would have made you proud. Thank you for always sacrificing your own needs to ensure the happiness and wellbeing of my siblings and me.

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CHAPTER 1

THESIS INTRODUCTION AND LITERATURE REVIEW

1.1 Laurel Wilt Disease Background

Laurel wilt disease has emerged as a deadly, invasive vascular wilt disease afflicting lauraceous species in the southeastern United States, including ecologically and economically important species like redbay (*Persea borbonia* (L.) Spreng), sassafras (*Sassafras albidum* (Nutt.) Nees), and avocado (*Persea americana*) (Fraedrich, et al. 2008). The cause of laurel wilt disease is *Raffaelea lauricola* (T.C. Harr. Fraedrich & Aghayeva), an ambrosia fungus and nutritional symbiont of *Xyleborus glabratus* Eichoff, the redbay ambrosia beetle, both of which are native to southern Asia (Fraedrich, et al. 2008, Harrington, et al. 2011). Since their introduction, laurel wilt disease has spread rapidly through the southeastern United States resulting in the death of millions of trees (Hughes, et al. 2017b). In addition to the movement of the vector by flight, anthropocentric movement of infested firewood and packaging material likely contributes to the spread of laurel wilt disease over long distances (Hughes, et al. 2015). All North American members of the Lauraceae family are susceptible hosts (Hughes, et al. 2015), and because many of these species can be found throughout the Southeast (Gramling 2010), laurel wilt disease has the potential to continue to spread further north and west, generating a shift in species composition, changing forest structure and detrimentally impacting forest communities (Nielsen and Rieske 2015). The susceptibility of Central

American Lauraceae, on the other hand, is less well understood and requires further research (Ploetz, et al. 2017b). Laurel wilt is also a danger to the commercial avocado industry in Florida, which is valued at $30 million annually (Pisani 2015), and has the potential to endanger other commercial avocado regions such as those in Mexico and California. As of 2017, about 3% of commercial avocado production in Florida had already been affected by laurel wilt (Ploetz, et al. 2017b), and this percentage has likely increased since that time.

1.2 Vascular Wilt Diseases and Host Response

Laurel wilt disease is a destructive vascular wilt disease. Vascular wilt diseases are among of the most devastating plant diseases, capable of systemically infecting any plant with vascular tissue (Dimond 1970) and can be caused by fungi, bacteria, or viruses (Pearce 1996, Pouzoulet, et al. 2014). Dutch elm disease, for instance, which is the most notorious vascular wilt disease of trees, quickly attained epidemic proportions killing millions of elms in Europe and North America, and plainly illustrating how thoroughly vascular wilt diseases can affect naïve species (Brasier and Webber 2019). Vascular wilt pathogens colonize the xylem tissues impacting their conducting capability (Bishop and Cooper 1984). This results in the rapid wilting of twigs and leaves, along with sapwood discoloration and weakening and loss of function of limbs and stems, often followed by the death of the whole plant or sections of it (Bishop and Cooper 1984). The quick progression of wilting is a result of the loss of hydraulic conductivity and turgor pressure of the vascular tissue, which prevents the transport of water and nutrients from roots to photosynthetic tissues of a plant (Pearce 1996, Inch and Ploetz 2011). The loss of water

transporting capabilities is in part directly caused by the presence of a pathogen within the xylem tissue of susceptible host plants, as mycelia and spores of fungal pathogens can obstruct xylem channels (Agrios 2005). The production and oxidation of plant defensive compounds, specifically phenolics, and the breakdown of plant cells by fungal pathogens can also contribute to the loss of xylem function and is responsible for the characteristic sapwood discoloration often associated with vascular wilts (Davis, et al. 1953). Phytotoxins (e.g. fusaric acid and other secondary metabolites) produced by vascular fungal pathogens can also directly affect plant processes like cell growth, membrane permeability, and apoptosis, leading to wilt symptoms. These low molecular weight substances can also travel through the vascular system to reach photosynthetic tissues and directly cause the mortality of leaves (Agrios 2005, Bani, et al. 2014, Pouzoulet, et al. 2014).

In an attempt to halt an infection, host plant species produce gums, gels and balloon-like structures called tyloses, which are outgrowths from parenchyma cells along xylem vessels that are produced to reduce pathogen spread in the vascular system (Pearce 1996). In resistant host species, tylosis production is sufficiently rapid to effectively reduce pathogen spread, while in susceptible species, their production is not effective for limiting pathogen spread but is nonetheless effective in restricting water movement in the vascular system (Bishop and Cooper 1984). If enough xylem channels are blocked or compromised, the tissue above the blockage desiccates and dies, resulting in wilt symptoms that can affect individual limbs or entire crowns (De Micco, et al. 2016). In avocado, it has been shown that plants inoculated with *R. lauricola* lose hydraulic conductivity exponentially as the proportion of diseased non-functional xylem tissue

increases, and tree mortality sharply rises as the amount of functional xylem decreases below 10% (Inch and Ploetz 2011, Ploetz, et al. 2017b).

1.3 The Fungus: *Raffaelea Lauricola*

Raffaelea lauricola, the causal agent of laurel wilt disease, belongs to the genus *Raffaelea* (family Ophiostomataceae), which is comprised of ambrosia fungi (Fraedrich, et al. 2008, Harrington, et al. 2008). Ambrosia fungi are nutritional symbionts of wood boring beetles, with which they have co-evolved to form a tight mutualistic relationship: the fungi grow and produce abundant conidia inside the tunnels and galleries excavated by the beetles, and constitute the primary food source for the beetles' larvae (Batra 1963b, Batra 1963a, Batra 1967). It has been shown that the removal of conidia by feeding beetles can actually stimulate further growth and sporulation of the fungus (Batra 1963a). In turn, ambrosia fungi are dependent upon their symbiotic insect species for dissemination and penetration of their plant host tissues (Batra 1963b). Ambrosia beetle symbionts are rarely aggressive pathogens (Harrington and Fraedrich 2010), but recently, in part due to the increased introduction of non-native species into naïve areas and climate change, some species (e.g., *R. lauricola*, *R. quercivora*,) have shown to be highly aggressive on non-native susceptible hosts (Ploetz, et al. 2013). *Raffaelea lauricola* and *R. quercivora* are the first ambrosia fungi recognized as causal agents of lethal vascular wilt diseases in non-native host plants (Kubono and Ito 2002, Harrington and Fraedrich 2010).

Raffaelea lauricola was first described in 2008 after being isolated in 2004 from the sapwood of dead and dying redbay trees in Georgia and South Carolina (Fraedrich, et

al. 2008, Harrington, et al. 2008), and pathogenicity tests determined that the species was highly pathogenic to redbay (Fraedrich, et al. 2008). It was also determined that *R. lauricola* is a nutritional symbiont of *X. glabratus*, the invasive redbay ambrosia beetle (Fraedrich 2008, Harrington, et al. 2008), as well as its most commonly associated fungus (Harrington and Fraedrich 2010, Campbell, et al. 2016). However, *R. lauricola* has now been documented on several other ambrosia beetle species as well, including *X. bispinatus*, which is commonly associated with avocado (Ploetz, et al. 2017c). At this time, *R. lauricola* is only known to reproduce asexually (Dreaden, et al. 2019), however, the species possesses both the MAT-1 and MAT-2 genes, suggesting that sexual reproduction is theoretically possible and both alleles are found in abundance in the native range of the fungus (Wuest, et al. 2017). The species' growth habit is dimorphic depending on if it is found in a host plant or its vector, which is common among many ambrosia fungi (Beaver 1989, Fraedrich, et al. 2008). In the host plant, or when cultured on growth media, the fungus is in a hyphal/mycelial growth phase and produces small translucent conidophores borne on sporodochia (Fraedrich, et al. 2008). The conidia are produced holoblastically and are very small, translucent, and variable in shape, ranging from spherical to oval to elongated (Harrington, et al. 2008). However, when in the beetle vector, it lives as a budding yeast inside specific structures called mycangia (Fraedrich, et al. 2008), which can potentially carry thousands of viable spores (Harrington and Fraedrich 2010). *Raffaelea lauricola* grows optimally between temperatures of 16 and 26 °C, with no growth readily apparent when temperatures exceed 30 °C. It also prefers slightly acidic conditions of pH ≤ 6.8 (Zhou, et al. 2018).

The fungus systemically moves and reproduces in the vascular system of the plant, including leaf tissue, and causes the characteristic symptoms of laurel wilt (Fraedrich, et al. 2008). The rate of spread through redbay and avocado saplings in experimental conditions is rapid and the pathogen can be detected systemically after just five days (Fraedrich, et al. 2015a).

Being that *R. lauricola* is an ambrosia fungus, its high pathogenicity on American Lauraceae, even though they are naïve hosts, was not expected, but recent studies have begun to elucidate the mechanisms that enable this fungus to be such an effective pathogen. The genome of *R. lauricola* encodes plant polysaccharide degradation enzymes that function as effectors, as well as other proteins that are correlated to virulence in other fungal pathogens (Zhang, et al. 2018). Caballero, et al. (2019) identified over 650 predicted proteins in two *R. lauricola* isolates including small-secreted proteins, which are thought to interact with host plant cells, proteases, ABC transporters to be protected from host defenses, and peroxidases and laccases for lignin degradation among others. Many of these gene families are orthologous to other virulent *Ophiostoma* plant pathogens (Caballero, et al. 2019). However, no known phytotoxins have been associated with *R. lauricola* at this time.

1.4 The Beetle: *Xyleborus Glabratus*

Xyleborus glabratus (tribe: Xyleborini) (Figure 1.1) is a minute ambrosia beetle that is native to southeastern Asia (Japan, Taiwan, India, China, Bangladesh, and Myanmar), where it is associated with Asian members of the Lauraceae family (Wood and Bright 1992). Ambrosia beetles have historically been considered secondary pests

and caused only limited damage to trees (Batra 1963b), but recently there have been several incidences of large scale damage caused by ambrosia beetles (as summarized in Ploetz, et al. 2013). Ambrosia beetles colonize host plants by boring deep into the xylem, where nutrient conditions are poor, to establish galleries and lay their eggs (Batra 1967). However, ambrosia beetles do not directly feed on host plant tissue, but instead feed on symbiotic fungi—a feeding habit known as mycetophagy (Beaver 1989). Ambrosia fungi thus play and essential role in the lifecycle of the beetles by concentrating nutrients, especially nitrogen and phosphorus, and minerals and readily providing them to grazing beetles (Klepzig and Six 2004, Filipiak and Weiner 2014). Sterols are also scarce in xylem tissue, and symbiotic beetles coopt fungal ergosterol during their biosynthesis of hormones, growth, and development (Clark and Bloch 1959). Ambrosia beetles are attracted to and associated with a primary dominant fungal symbiont (Hulcr, et al. 2011), but other fungal species can also form associations and interactions (Kostovcik, et al. 2015). Each ambrosia beetle species does not feed exclusively on a single ambrosia fungus, especially as adults (Batra 1963a, Beaver 1989), and ambrosia fungi, in turn, can be carried by multiple species of ambrosia beetle or even other types of insects (Simmons, et al. 2016). However, the primary symbionts facilitate the quicker growth and higher overall fitness and fecundity of ambrosia beetles (Biedermann, et al. 2013).

Xyleborus glabratus is slender, 2.1-2.4 mm long, and dark brown to black with numerous indentations on its declivity (Rabaglia, et al. 2006). Xyleborini siblings inbreed and produce both larger diploid adult females, which have mycangia and are flight capable, and dwarfed, flightless males, which develop from unfertilized eggs via parthenogenesis (Kirkendall 1993). This results in a highly skewed sex ratio in the

species (Maner, et al. 2013). The peak activity of *X. glabratus* is usually in early September and brood development can take between 50 and 60 days; however, there are no distinct generations (Hanula, et al. 2008, Brar, et al. 2013) and galleries can be active for over a year with beetles emerging during all months of the year (Maner, et al. 2013).

Xyleborus glabratus was first detected during a survey in 2002, in funnel traps at Port Wentworth outside of Savannah, Georgia, (Haack 2006, Rabaglia, et al. 2006). Multiple species of *Raffaelea* have been documented on *X. glabratus* (Harrington and Fraedrich 2010, Campbell, et al. 2016), but whether other species can replace *R. lauricola* as the primary symbiont requires further study. Yeast species are also found commonly in association with *X. glabratus,* but they are probably gut microflora and not nutritional symbionts (Harrington and Fraedrich 2010). *Xyleborus glabratus* females are thought to start boring into lower portions of stems of healthy hosts, but then abandon the tunnels before egg laying (Fraedrich, et al. 2008, Harrington, et al. 2008). During this colonization attempt, however, beetles inoculate the host trees with *R. lauricola,* which then quickly spreads through the sapwood leading to development of laurel wilt disease symptoms, and ultimately providing a more suitable environment within the xylem for future colonization by *X. glabratus* beetles and their developing brood (Fraedrich, et al. 2008). This is a stark contrast to many ambrosia beetles, which usually attack and colonize only damaged or unhealthy plants (Batra 1963a, Wood 1982), while in the southeastern U.S., *X. glabratus* can readily attack healthy trees as well (Fraedrich, et al. 2008, Hughes, et al. 2017a).

Early research with *X. glabratus* and Lauraceae host plants demonstrated that the beetle is similarly attracted to infested and uninfested trees, but is not attracted to nonhost

species. Beetle population levels are dependent on their proximity to colonized trees, which suggests that their populations might decrease significantly after host trees have been decimated (Hanula, et al. 2008). However, it has also been show that *X. glabratus* can still persist at low population levels on small diameter trees, by colonizing the lowest part of the stem (Maner, et al. 2014). While pheromone production occurs in many species of ambrosia beetles, *X. glabratus* appears to not produces any aggregation pheromones (Hanula, et al. 2008).

Experiments with swampbay (*Persea palustris* (Rafinesque) Sargent) found that *X. glabratus* is initially strongly repelled by leaf volatiles of trees recently infected by *R. lauricola*, but is then strongly attracted to them several days after the infection (Martini, et al. 2017). During the course of infection, levels of foliar volatile compounds associated with plant defense, including methyl salicylate, increase, and Martini, et al. (2017) hypothesized this might be the reason why some females abandon the initial galleries on healthy trees, as observed by Fraedrich, et al. (2008). Declining levels of volatiles coincides with the decline of host xylem function, and with a decrease in the release of defensive metabolites (Martini, et al. 2017). Contrary to expectations, *X. glabratus* is not attracted to ethanol, a common tree stress semiochemical (Hanula and Sullivan 2008), but is highly attracted to plant volatiles of healthy redbay and swampbay, including α-copaene (Martini, et al. 2015). Interestingly, α-copaene is normally detectable only in woody tissue in *Persea* species, while being absent from leaf volatiles of healthy trees (Martini, et al. 2015). However, α-copaene also becomes present in foliar volatile emissions after infection with *R. lauricola*, and this factor, in combination with declining

methyl salicylate levels, could explain why *X. glabratus* is attracted to these plants after the initial temporary repellency (Martini, et al. 2017).

1.5 The Hosts: Lauraceae

The Lauraceae family, also known as the laurel family, is a large and diverse family including approximately 2,850 species in 45 genera of woody plants that are found throughout the world (Chanderbali, et al. 2001, Christenhusz and Byng 2016). In tropical and subtropical regions, where they are mostly found, lauraceous species are dominant components of poor soil lowland forests, and one of the most species rich families at intermediate elevations (up to 3,000 m) (Gentry 1988). The species diversity of Lauraceae is highest in Asia and South America (Chanderbali, et al. 2001), but the family is well represented in the Western Hemisphere, and the United States has nine native tree or shrub species in addition to several introduced or naturalized species, including a vine (Coder 2012). Madagascar and Australia have a rich species diversity of Lauraceae as well (van der Werff 1991).

The Lauraceae family, which takes its name from the famous Grecian laurel, *Laurus nobilis*, is a highly heterogeneous group, but most members are aromatic shrubs or moderate height trees with persistent evergreen leaves, although some species, like sassafras, are deciduous (Watson and Dallwitz 1992-onwards). The flowers of this family are generally small and inconspicuous, trimerous, and can be either monoecious or dioecious (van der Werff and Richter 1996). The fruits are green colored at first but then become darkly pigmented and are one-seeded berry-like or drupaceous, with a tough outer receptacle, like that of avocado (van der Werff and Richter 1996).

The family is ecologically important, as the fruit and leaves of its species are a source of forage and are consumed by wildlife including songbirds and deer (Brendemuehl 1990, Griggs 1990, Hughes, et al. 2015). Several species of wildlife depend wholly on lauraceous species for their lifecycle including the Palamedes Swallowtail (*Papilio Palamedes* Drury) and Spicebush Swallowtail (*Papilio troilus* L.), which utilize these plants as their sole food source throughout development (Gramling 2010).

Many members of the laurel family, like cinnamon, sassafras, and camphor, produce recognizable aromatic compounds that are used is cosmetics, medicine, and culinary arts (Coder 2012). However, the most economically and agriculturally important species is by far avocado, which is a significant crop in Florida, California and Mexico, and is cultivated worldwide. In 2014 alone, 5 million metric tonnes of avocado were produced worldwide at a value of $9.17 billion (Chanderbali, et al. 2001, Pisani 2015, Ploetz, et al. 2017a). Although limited in use, the wood from various lauraceous species has an unique dark pigmentation and has been used for utensil making, cabinetry, and veneer (Schroeder 1976, Coder 2012). Lastly, Native Americans value redbay and sassafras for their medicinal use, and sassafras is considered sacred by many tribes (Entsminger 2010, Coder 2012).

1.6 Development of Disease in Different Hosts

The first externally visual symptom of laurel wilt disease is wilting foliage and dieback of branches or stems, which can be rapid and begin as soon as five days after infection (Fraedrich, et al. 2008). In redbay (and closely related swampbay), infected

branches exhibit reddish to brown discolored leaves within 1-2 weeks after infection, while later in the disease cycle, systemic wilting of the entire crown and rapid death of trees occur (Figure 1.2). A characteristic feature in redbay is that wilted leaves persist on branches even after death of the host tree (Fraedrich, et al. 2008). Beneath the phelloderm, the vividly discolored sapwood of the stems and branches is readily apparent and begins along the outside edges initially, but soon darkens and spreads more profusely inward throughout the stem (Fraedrich, et al. 2008). Typically, small diameter (~0.75mm) beetle entrance holes are found in segments of branches and stems which then exhibit sapwood discoloration (Fraedrich, et al. 2008) (Figure 1.3). Redbay is the species most impacted by laurel wilt disease and typically all individuals within a stand over a certain stem diameter succumb quickly upon being infected (Fraedrich, et al. 2008).

The progression of laurel wilt disease in other lauraceous species is similar to that of redbay, with some notable variations. In sassafras, for example, which is a deciduous species, wilted foliage turns yellow, orange, or red, mimicking early autumn senescence, and eventually falls to the ground (Cameron, et al. 2010). The patterns of wood discoloration are also different in sassafras, with oftentimes a lack of discoloration in the outermost growth ring of sapwood, which could imply some attempts of the host to fight back the disease (Cameron, et al. 2010) (Figure 1.4). Additionally, laurel wilt disease incidence in sassafras seems to be more random and haphazard than in redbay (Cameron, et al. 2010).

Similar to sassafras, avocado wilted leaves defoliate after 2-3 months, and the plants exhibits dark sapwood discoloration (Inch and Ploetz 2011). While avocado is attractive to *X. glalbratus* and is readily attacked and colonized, the fecundity of the

beetle is lower when compared to other hosts (Brar, et al. 2013). Both avocado and sassafras form root-grafts and the pathogen can be spread via these connections (Ploetz, et al. 2012, Ploetz, et al. 2017a). All cultivars of avocado are susceptible to laurel wilt; however, the disease progresses more slowly depending on the avocado cultivar (cultivars with a Guatemalan or Mexican ancestry are less susceptible than those of West Indian ancestry) and overall avocado may be slightly less susceptible to laurel wilt disease than redbay and sassafras (Mayfield, et al. 2008b, Ploetz, et al. 2012), even though high mortality still occurs (Inch, et al. 2012). Unfortunately, Simmonds, the most commonly grown cultivar in Florida, is among the most susceptible, but the Hass cultivar, which is more common in California, is more tolerant of the disease (Ploetz, et al. 2012).

Compared to other lauraceous species, camphor tree (*Cinnamomum camphora* (L.) J. Presl), which is native to Asia, is resistant to laurel wilt disease (Fraedrich, et al. 2015a). While the pathogen can be isolated from the xylem of inoculated trees, visual wilting of leaves is rare and sapwood discoloration is patchy and limited to the wood adjacent to inoculation points (Fraedrich, et al. 2015a, Campbell, et al. 2017). Camphor tree often shows branch dieback only when challenged with multiple inoculations of *R. lauricola*, and mortality and systemic spread is rare to non-existent when the tree is inoculated at a single point (Fraedrich, et al. 2015a). In a recent study, it has been observed that camphor tree exhibits a high degree of sapwood discoloration even at very low levels of pathogen colonization, but the zones of discoloration are concentrated around the inoculation points on the plant (Campbell, et al. 2017). This vivid but limited discoloration could be due to the production of higher levels of defensive compounds by

camphor tree (Campbell, et al. 2017). In turn, *R. lauricola* propagates at a much lower rate through camphor tree compared to American Lauraceae. This could also be due to the differences in xylem lumina size, but the defensive mechanisms are not fully understood and warrants further study (Fraedrich, et al. 2015a, Campbell, et al. 2017).

In most species, larger diameter trees are usually the first to wilt and die, probably because they are more attractive to *X. glabratus* (Fraedrich, et al. 2008, Ploetz, et al. 2012, Mayfield and Brownie 2013). *Xyleborus glabratus* uses stem diameter silhouettes as a host finding cue, which could explain why larger diameter trees are the hardest hit by laurel wilt (Mayfield and Brownie 2013). Even though smaller diameter trees or sprouts initially survive, they eventually become susceptible as they grow (Fraedrich, et al. 2008), and are typically killed before they reach reproductive age, making the species at risk of becoming functionally extinct in areas hit by the disease (Evans, et al. 2013).

1.7 The Spread of Laurel Wilt Disease

The species most severely affected by laurel wilt are redbay and sassafras, and both have extensive ranges in the eastern United States. Redbay can be found from the Virginia coast to southern Florida and as far west as eastern Texas (Koch and Smith 2008), while sassafras has a historical range from Maine to Florida and from Michigan to Texas (Randolph 2017). If laurel wilt maintains its destructive trajectory, the native ranges of these species could be catastrophically altered, especially in the face of increased global temperatures as a result of climate change (Formby, et al. 2017).

It is thought that *R. lauricola* and its vector arrived in the United States within wood packaging material from Asia (Fraedrich, et al. 2008, Harrington, et al. 2008), and

the characterization of fungi associated with beetles collected in the United States, Japan, and Taiwan supports this hypothesis (Harrington, et al. 2011). A subsequent phylogenetic analysis suggests that *R. lauricola* was probably introduced to the United States in a single introduction event, as the United States population exhibits genetic uniformity, in addition to possessing only the MAT-2 mating type (Wuest, et al. 2017). Although *X. glabratus* was known to be associated with the Lauraceae family in Asia (Wood and Bright 1992), *R. lauricola* was previously undiscovered until it was detected in wilted redbay trees in the United States, and presumably the pathogen does not cause noticeable disease in its native range (Fraedrich, et al. 2008). However, *R. lauricola* has been recently detected and confirmed as causing laurel wilt disease on introduced avocado in Myanmar, representing the first incidence of laurel wilt disease outside of the southeastern United States, and ironically in the native range of the pathogen and vector (Ploetz, et al. 2016). Recent inoculation experiments conducted on Asian Lauraceae show that *R. lauricola* can infect co-evolved host species without causing mortality or the development of major symptoms, suggesting that, as expected, co-evolved host species are much more resistant than their North American counterparts (Shih, et al. 2018).

Since its introduction near Savannah, Georgia in 2002 (Fraedrich, et al. 2008), laurel wilt has spread rapidly across the southeastern United States, in part due to the flight of its vector over short distances, and in part due to human transport of infested woody material over longer distances (Figure 1.5). As of 2008, the disease was estimated to be spreading at a rate of 54.8 km/yr. (Koch and Smith 2008); however, this projected rate of spread was an underestimation and most likely did not take into account anthropogenic spread due to movement of infected wood (Randolph 2017). Although

initially confirmed only on redbay in Georgia and South Carolina, by the summer of 2005 the disease was found in neighboring Florida (Haack 2006, Fraedrich, et al. 2008). By 2009, laurel wilt disease was reported on redbay in Mississippi (Riggins, et al. 2011), on sassafras in Alabama (Bates, et al. 2013), and on redbay in southeastern North Carolina in 2011 (North Carolina Forest Service 2012). Then, infected sassafras was discovered in Louisiana in 2014 (Fraedrich, et al. 2015b) and, in 2015, wilting redbay trees in eastern Texas were confirmed to be infected (Menard, et al. 2016) along with the confirmation of the pathogen affecting sassafras in Arkansas (Olatinwo, et al. 2016). Laurel wilt has also been documented on sassafras for the first time in North Carolina, representing the most northerly spread of the disease along the Atlantic coast of the United States as of 2018 (Mayfield, et al. 2019). Currently, there are intensive monitoring programs in place to monitor the spread of laurel wilt disease further north and east into Tennessee and surrounding states, but as a matter of fact, the disease has already been detected in Kentucky and Tennessee on sassafras as recently as 2019 (Loyd, et al. 2020) (Figure 1.5).

As for the other members of the Lauraceae family susceptible to the disease, the pathogen has been detected on avocado and camphor tree in Florida (Mayfield, et al. 2008c, Smith, et al. 2009), while Fraedrich et al. (2016) reported that laurel wilt had been discovered on spicebush (*Lindera benzoin* (L.) Blume) as well as pondberry (*Lindera melissifolia* (Walter) Blume) and pondspice (*Litsea aestivalis* (L.) Fernald) (2011); both of the latter species are currently listed as threatened or endangered (Gramling 2010). There are several other susceptible lauraceous species across the contiguous United States and Europe (Hughes, et al. 2015), but many of them, such as California laurel (*Umbellularia californica (*Hook. & Arn.) Nutt.), are well outside of the current impact

area of *R. lauricola*. These species have been shown to be susceptible in controlled inoculation experiments (Fraedrich 2008), highlighting the need for vigilance and improved management of laurel wilt disease.

1.8 Management of Laurel Wilt Disease

Since laurel wilt disease is already widespread and established in the United States, eradication efforts are not viable anymore, and current management aims instead at slowing the spread of disease to new areas (Hughes, et al. 2015). This is obtained by focusing on the monitoring of the insect vector and on the rapid detection and rapid removal of infested host plants (Hughes, et al. 2015). Unfortunately, this approach has not been successful thus far. In particular, early detection of the disease is the most crucial, and currently the most inefficient step for the successful management of laurel wilt disease, as trees need to be promptly removed before the new generation of insect beetle vectors can emerge (Hughes, et al. 2015). Typically, detection has focused on trapping efforts for *X. glabratus* and on finding symptomatic trees, which requires constant surveillance efforts to delineate the leading edge of the disease (Hughes, et al. 2015). Symptomatic leaves of avocado can be detected with the use of visible-near infrared (Sankaran, et al. 2012), and low altitude aerial spectral imaging can differentiate symptomatic and asymptomatic avocado plants (Abdulridha, et al. 2018). Recent research has shown that dogs can be trained to detect the volatiles emitted by *R. lauricola* even before disease symptoms are visible in avocado (Mendel, et al. 2018), but further validation of the method and a cost/benefit evaluation of the approach are still pending before dogs could be actually deployed in laurel wilt disease surveillance. While rapid

detection of symptomatic trees is feasible in avocado orchards and urban areas, which have high human frequentation, it is much more difficult in natural environments (Ploetz, et al. 2017a) and the aforementioned detection methods have not been tested on other species.

Upon discovering trees potentially infected with laurel wilt disease, a diagnosis confirmation process must take place to confirm the presence of *R. lauricola*. The required steps include packaging and shipping samples to a diagnostic laboratory, cultural plating for isolation, DNA extraction, and PCR based molecular identification (Hughes, et al. 2015). Morphological attributes of *R. lauricola* can be referenced from Harrington, et al. (2008) to rule out suspected fungi that are visually distinct. However, a molecular based approach is required to actually confirm the identity of the pathogen. The currently adopted method uses a primer set designed on a microsatellite region unique to *R. lauricola* known as CHK (Dreaden, et al. 2014). The CHK primers are species-specific, thus a positive PCR result verified by gel electrophoresis does not require additional sequencing of the product. The large subunit ribosomal (28S) primers LROR, LR5, and L3 primers, on the other hand, which have been used in other instances, require the sequencing of the PCR product to confirm *R. lauricola* identity (Wuest, et al. 2017). Finally, Mayfield et al. (2018) also used the beta-tubulin region (Glass and Donaldson 1995) for the confirmation of *R. lauricola* from infected sassafras, but this region too required sequencing of the PCR product.

Whichever primer set is used, the whole procedure is still time consuming due to the requirement of obtaining a pure mycelial culture before performing PCR analysis. The success of isolating the fungus from symptomatic tissues depend on several factors,

including the proper handling and shipment of samples to prevent the desiccation and mortality of fungi during transit. Once samples are received at the diagnostic laboratory, it can take at least a week to process samples and obtain sufficient pure mycelial growth to perform DNA extractions. An alternative molecular approach that can confirm a disease diagnosis more quickly, and possibly without the need to isolate the pathogen in pure culture, would greatly reduce diagnostic times and would advance the implementation of management strategies designed to slow the spread of laurel wilt disease (Hughes, et al. 2015, Rabaglia, et al. 2019).

As mentioned previously, α-copaene lures are readily attractive to *X. glabratus* beetles and these work well in conjunction with Lindgren funnel traps to monitor beetle populations, however, α-copaene is expensive and difficult to produce at the scale needed for effective monitoring operations (Hughes, et al. 2015). Cubeb oil, on the other hand, a natural essential oil derived from the berries of *Piper cubeba* L., contains large fractions of α-copaene and is a more cost effective lure (Hanula, et al. 2013, Kendra, et al. 2016). Manuka oil is attractive to *X. glabratus* as well, but is effective for as little as two weeks in the field (Hanula, et al. 2013). .

High value trees, such as those on private properties or urban landscapes, can be protected with the application of the systemic fungicide propiconazole; however, the fungicide has to be reapplied on a yearly basis (Mayfield, et al. 2008a), and loses efficacy with subsequent applications (Ploetz, et al. 2017a). Due to cost constraints, fungicide applications are not a feasible management option in a forest setting.

There are several biocontrol options that have been recently reported as potentially valuable alternatives to fungicides for management of laurel wilt disease on

high value trees. *Beauveria bassiana,* for instance, is an entomopathogenic fungus that can be externally applied to trees, and is capable of increasing the mortality of *Xyleborus bispinatus*, a secondary vector of *R. lauricola* in avocado (Zhou, et al. 2018). *Xyleborus bispinatus* is a better colonizer of avocado than *X. glabratus,* thus using *B. bassiana* as a biocontrol agent in avocado plantations could slow the spread of laurel wilt disease in this commodity (Zhou, et al. 2018). *Acaromyces ingoldii,* on the other hand, is an antagonistic fungus to *R. lauricola*, and via the production of secondary metabolites, it is capable of significantly reducing the growth of the pathogen when present on the host plant before the inoculation of *R. lauricola* (Olatinwo and Fraedrich 2019). Additionally, 50 strains of bacteria have been found to be strong antagonistic toward *R. lauricola* (Dunlap, et al. 2017), pointing out the need of further studies for the discovery of other potential biological control options.

1.9 Loop-Mediated Isothermal Amplification (LAMP)

1.9.1 *The Principle of LAMP*

Loop-mediated isothermal amplification (LAMP) is a recently developed molecular technique that can provide a field-portable alternative to traditional laboratory PCR-based approaches. Notomi, et al. (2000) first described LAMP as an alternative approach to rectify some of the shortcomings of other nucleic acid amplification procedures, such as expensive equipment or low specificity. Similarly to PCR, in LAMP reactions, a template DNA is targeted by primers and amplified by a polymerase, and the products can subsequently be visualized for diagnostic purposes. However, LAMP has several characteristic differences from PCR. Specifically, LAMP features at least four

primers that recognize six segments of target DNA sequences (Notomi, et al. 2000), whereas PCR typically uses just two (i.e., forward and reverse primer). The primers for LAMP are composed of two outer primers, which are similar to standard PCR primers, in addition to two hybrid inner primers (Notomi, et al. 2000). These hybrid inner primers, known as forward inner primer (FIP) and backward inner primer (BIP), are designed in a way to feature self-complimentary sequences in the amplification product, and initiate the production of the LAMP starting structure in conjunction with the outer primers, F3 and B3 (Notomi, et al. 2000) (Figure 1.6A). Both FIP and BIP have specific sequences, F2 and F1c and B2 and B1c, respectively, which can be optionally separated by a thymine bridge. F2 and B2 anneal to the corresponding F2c and B2c regions on the target DNA and allow binding of the polymerase and synthesis of a new strand (Figure 1.6B). F3 and B3 will then anneal externally to F3c and B3c, respectively (Figure 1.6C) and because the LAMP polymerase has high strand displacement activity, the new forming strands will release the previous F2/B2-initiated ones (Figure 1.6D). F1c and B1c will thus anneal to either F1 or B1 of the now released strands. Once the process has taken place in both directions for a given strand, the resulting structure is a stem-loop structure, also referred to as a dumbbell, that serves as the actual starting structure for the rest of the LAMP reaction (Notomi, et al. 2000) (Figure 1.6E). These structures will allow annealing of either the FIP or BIP primers on the outside of one of their dumbbell loops, which carry F2c or B2c sequences, respectively, and thus the continuation of exponential amplification of the structure isothermally. The final products of LAMP amplification consist of a mix of zigzag-like and cauliflower-like structures with multiple inverted repeats of the target region (Notomi, et al. 2000).

1.9.2 *LAMP Advantages Compared to PCR*

A key difference between LAMP and PCR is that the former, as its name implies, can be performed under isothermal conditions, as the reaction proceeds at a single temperature (Notomi, et al. 2000). In fact, due to the high strand displacement activity of the LAMP DNA polymerase and the nature of the dumbbell structures, temperature cycling is not required to either melt DNA, or allow annealing of the primers and initiation of polymerase activity (Notomi, et al. 2000). The standard polymerase used in LAMP is *Bst*, which has been chosen because of its heat stability and strand displacement activity, in addition to being less sensitive to inhibitors (Notomi, et al. 2000). This polymerase, which has an optimal temperature of 60-65 °C, is derived from *Geobacillus stearothermophilus* (Stenesh and Roe 1972), a thermophilic bacterium that grows at 55-65 °C (Aliotta, et al. 1996). *Bst* polymerase is also capable of amplifying very minute amounts of starting target DNA (Mead, et al. 1991), making LAMP assays successful even when DNA concentrations are very low. Indeed, LAMP reactions can be initiated with as little as six copies of target DNA and results in billions of copies in under an hour (Notomi, et al. 2000).

Another advantage of LAMP over PCR is its potential for higher specificity in amplifying target sequences, due to the fact that LAMP uses four primers that must successfully recognize and anneal to six distinct regions of the target DNA for the reaction to initiate (Notomi, et al. 2000). Moreover, LAMP reactions do not seem to be inhibited my non-target DNA either in terms of amplification efficiency or background

noise (Notomi, et al. 2000). The robustness of LAMP in terms of high sensitivity, specificity and low sensitivity to inhibitors make this method ideal in instances where low quality template or low titer volumes arise, as in the case of crude DNA extracts, which despite minimal processing and purification can still be used as a testing template (Kaneko, et al. 2007, Francois, et al. 2011).

While the originally described LAMP reaction was already rapid compared to PCR, additional optimization further reduced the time required to perform a reaction. For instance, initially reactions started with a heat denaturing step (Notomi, et al. 2000); however, it was later demonstrated that LAMP reactions proceed equally as well with nondenatured template (Nagamine, et al. 2001). Furthermore, Nagamine, et al. (2002) developed additional primers, termed loop primers (loop F and loop B), that reduce the reaction time to less than half of an original reaction. While a LAMP reaction without loop primers can produce detectable amounts of DNA in under an hour (Notomi, et al. 2000), the addition of loop primers can facilitate detection in less than 30 minutes (Nagamine, et al. 2002). Loop primer sequences are complementary to the region of a single strand loop between F1 and F2 or B1 and B2, respectively, where they anneal in the 5' direction (Nagamine, et al. 2002). *Bst* polymerase binds to the loop primer and begin synthesizing a complementary strand which is then displaced by extension from other strands as the reaction proceeds, participating in the formation of products that do not form in the original LAMP reaction (Nagamine, et al. 2002). To put it simply, loop primers take advantage of single stranded loop structures that would be otherwise be unutilized in the original LAMP reaction, and provide additional binding sites for the polymerase, which vastly increases the rate of DNA amplification.

1.9.3 *Visualization of LAMP products*

When LAMP was first described, the method to visualize a positive result was the digestion of LAMP products with restriction enzymes followed by electrophoresis and probe hybridization via Southern Blot (Notomi, et al. 2000). However, due to the high stability of LAMP products and their potential to form aerosols, all types of results visualization that require handling of the product poses a serious risk for cross contamination and are strongly not recommended (Kubota, et al. 2011, Niessen 2015, Le and Vu 2017). Additionally, substantial time and material investment is required to perform electrophoresis with LAMP products (Kubota, et al. 2011). An alternative method to visualize the results is the measure of the turbidity of a reaction, which is due to the accumulation of the by-product magnesium pyrophosphate, and can be correlated with the amount of DNA amplification that has taken place (Mori, et al. 2001). Changes in turbidity can be measured in real-time (Mori, et al. 2004). LAMP products can also be visualized by the addition of fluorescent or colorimetric dyes. Ethidium bromide or SYBR-type dyes, for instance, intercalate with amplified DNA and can be measured in real-time by monitoring the increase in fluorescence (Nagamine, et al. 2002). Hydroxy naphthol blue, on the other hand, is a colorimetric indicator of alkaline earth metals, and can chelate magnesium ions to produce a color change from violet to sky blue in the presence of magnesium pyrophosphate, indicating DNA amplification (Goto, et al. 2009). Hydroxy naphthol blue does not allow for real-time monitoring but has the advantage that

it can be easily detected by naked eye, thus allowing for result visualization without the need of additional equipment (Goto, et al. 2009).

The confirmation methods discussed above, however, are all non-specific as they either detect ds-DNA or are predicated on the presence of by-products of the amplification reaction, no matter what the amplified sequence might actually be. Thus, any method that can detect target specific products would, by default, greatly increase the diagnostic capability of a LAMP assay. One such method is the use of fluorescent assimilating probes that are designed to anneal only to specific portions of LAMP products, and thus will emit fluorescence only when the correct DNA has been targeted and amplified (Kubota, et al. 2011). Assimilating probes, or labeled oligonucleotides probes, are composed of a target DNA complementary portion at the 3' end and a fluorophore-carrying tail at the 5' end, which binds to a complementary quencher strand (Kubota, et al. 2011). As polymerase facilitates extension from an inner primer, the assimilating probe, which has annealed to either FIP or BIP associated loops, disassociate from the quencher, allowing the fluorophore to emit light upon being excited (Kubota, et al. 2011). As the number of loops in a reaction increases, the number of annealed assimilating probes increases in turn, and the growing intensity of light emitted by the fluorophore can be monitored in real-time. The emission intensity can also be correlated to the number of initial copies of target DNA, allowing quantitative LAMP assays (Kubota, et al. 2011).

1.9.4 *LAMP and the Detection of Plant Pathogens*

LAMP is routinely used for both clinical and point-of-care diagnostic tests, providing rapid results and informing decision making in many different disciplines (Niessen 2015, Notomi, et al. 2015, Le and Vu 2017). In fact, the features of LAMP make it a model assay for rapid, portable point-of-care diagnostic testing due to its reaction speed, robustness when using rudimentary prepared samples, and the ease at which it can be performed by inexperienced practitioners. In the field of plant pathology and diagnostics, LAMP assays have been successfully utilized to identify pathogenic bacteria (e.g. Larrea-Sarmiento, et al. 2018), viruses (e.g. Congdon, et al. 2019), oomycetes (e.g. Dai, et al. 2019), fungi (e.g. Villari, et al. 2017), phytoplasmas (e.g. Kogovsek, et al. 2015), and nematodes (e.g. Meng, et al. 2018). The early detection of pathogens is crucial to limit their spread and prevent epidemics or outbreaks, thus protecting valuable commodities and resources (Congdon, et al. 2019, Luchi, et al. 2020). This is especially relevant in light of the growing risk of invasive species, due to increased globalization and trade (National Invasive Species Council 2016).

Food security and the detection of fungal phytopathogens, in particular, is one area where LAMP has proved particularly useful. For example, *Fusarium graminearum*, a major mycotoxin producer in wheat, and *Fusarium mangniferae*, the causal agent of mango malformation disease, can be detected using a LAMP assay (Niessen and Vogel 2010, Pu, et al. 2014). Similarly, the generalist plant pathogens *Botrytis cinerea*, which causes grey mould, and *Sclerotinia sclerotiorum*, can be rapidly identified within plant material with LAMP (Tomlinson, et al. 2010, Duan, et al. 2013). More recently, LAMP has been utilized in the early detection of *Uromyces betae*, the cause of sugar beet rust

disease, from both infected plant tissue and airborne spore sampling (Kaczmarek, et al. 2019).

Recently, LAMP has seen use in turf-grass and orchard trees as well. In turf-grass, LAMP has been employed to detect airborne *Magnaporthe oryzae* spores in perennial ryegrass (*Lolium perenne*) fields before the appearance of visual symptoms of gray leaf spot (Villari, et al. 2017). *Guignardia citricarpa* (Kiely), the pathogenic fungus that causes citrus black spot disease, is a regulated pathogen in both the United States and Europe, and can be successfully detected in less than 40 minutes when assayed with LAMP (Tomlinson, et al. 2013). Finally, three separate LAMP assays have rapidly and accurately detected root pathogens (*Calonectria ilicicola*, *Dactylonectria macrodidyma*, and the *Dactylonectria* genus) within root tissues of avocado in Australia in under 30 minutes (Parkinson, et al. 2019).

Forest pathogens cause extensive damage to forest trees and can incur high economic and ecological costs (Santini, et al. 2013). Although to a lesser extent, LAMP has been valuable in rapidly detecting forest pathogens as well. In addition to the detection of the pine wood nematodes, *Bursaphelenchus xylophilus* (Leal, et al. 2015, Meng, et al. 2018), LAMP assays have been utilized in forestry settings to confirm the presence of Scots pine blue stain fungi *Ophiostoma clavatum* and *O. brunneo-ciliatum* directly from the surface of their beetle vector, *Ips acuminatus* (Villari, et al. 2013). Further, LAMP technology has reduced the confirmation time of *Hymenoscyphus fraxineus*, the causal agent of ash dieback affecting *Fraxinus excelsior* in Europe, from 3-4 weeks to less than an hour (Harrison, et al. 2017). In Italy, the introduced fungus *Heterobasidion irregulare* can be detected from colonized wood in less than 40 minutes

with an appropriate assay (Sillo, et al. 2018). Lastly, *Xylella fastidiosa*, *Ceratocystis platani*, and *Phytophthora ramorum*, three important pathogens causing severe damage to both orchard and forest trees worldwide, can now be rapidly detected with LAMP (Aglietti, et al. 2019).

1.10 Aims of this Thesis

Laurel wilt is devastating lauraceous species throughout the southern United States. From an ecological perspective, Lauraceae are members of diverse ecosystems, and the spread of this invasive pathogen could severely diminish or even extirpate some species from the Americas (Fraedrich, et al. 2011). The effects of this ecosystem change can be profound, but are largely difficult to predict. As of 2017, over 320 million redbay trees, estimated at 1/3 of the total population, have been killed by laurel wilt since its introduction in 2002 (Hughes, et al. 2017b). Considering the extensive range of redbay and sassafras, forest communities will experience large scale changes in the structure and function of these ecosystems that no longer have mature individuals of these species. From an economic perspective, avocado is the most important commercial lauraceous species and is cultivated throughout the world. If laurel wilt continues to affect avocado orchards in Florida, and expands into other major avocado production areas in California and Mexico, the impact of the disease could be economically disastrous, and the repercussions could be felt not just in the United States but across the world.

Unfortunately, a burdensome diagnosis confirmation process slows the implementation of management strategies that could reduce the spread of laurel wilt. Most restrictive is the prerequisite of a pure mycelial culture of *R. lauricola* for molecular

identification, which can extend laboratory based diagnostic testing to a week or more. Eliminating the necessity of pure fungal cultures by promptly detecting *R. lauricola* from plant tissue directly in-field will quickly inform management decisions in less time than is currently required to even just ship suspected samples to an external laboratory. LAMP is a molecular technique that allows for quick in-field analysis of target DNA sequences and remedies the time constraining issues associated with the laboratory analyses currently needed to confirm a diagnosis. LAMP is rapid, highly specific, and has a detection limit comparable or better than traditional PCR. The greatest advantage of LAMP is that it can be performed in-field with small, portable devices (Ebert, et al. 2010, Jenkins, et al. 2011). The use of LAMP for the early detection of laurel wilt would greatly reduce the time currently needed to confirm a laurel wilt disease diagnosis and allow for a timely implementation of the containment procedures, which are a crucial part of the success of integrated pest management strategies. Therefore, the goal of this thesis is to develop and validate a LAMP assay for the rapid and accurate molecular identification of the laurel wilt pathogen directly in the field.

The second chapter of this thesis outlines and describes the process of design and optimization of primers that are species-specific to *R. lauricola,* the laurel wilt disease pathogen. Our objectives were specifically to: 1) design primers that target unique sequences of *R. lauricola* and validate them against *Raffaelea* congeners, other fungi, and redbay host DNA, 2) determine the earliest time after infection that the assay can detect *R. lauricola* from artificially inoculated redbay saplings, and 3) test the assays suitability to successfully amplify *R. lauricola* DNA directly from host plant tissue using both high quality DNA and crude DNA extracts, and from *X. glabratus* beetle tissue using crude

DNA. Achievement of these objectives will reduce the diagnostic time required to confirm a laurel wilt disease diagnosis from as much as two weeks to as little as just one or two hours after receiving infected samples.

The third chapter of this thesis focuses on the use of the LAMP assay in conditions that are comparable to those that would be encountered if the assay was to be implemented for actual laurel wilt detection by surveillance personnel. Specific objectives were to: 1) to test the performance of the LAMP assay in a laboratory setting using both high quality and crude DNA extracts of naturally infected samples collected from multiple states by surveillance personnel affiliated with both federal and state forestry agencies, and 2) to conduct in-field confirmation of the presence of *R. lauricola* in symptomatic trees in various states using a portable LAMP device. Achievement of these objectives will provide a confirmation that the developed LAMP assay is actually capable of being successfully implemented directly at point-of-care by surveillance personnel, with the potential to further reduce the diagnostic time for laurel wilt disease to less than an hour from the identification of a symptomatic tree.

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Figure Legend

Figure 1.1. Redbay Ambrosia beetle (*Xyleborus glabratus*). Credit: Joseph Benzel, Screening Aids, USDA APHIS PPQ, Bugwood.ord (left) and Michael C. Thomas, Florida Department of Agriculture and Consumer Services (right).

Figure 1.2. A young redbay tree (left) in Sesquicentennial Park, South Carolina showing characteristic leaf wilt associated with laurel wilt disease.

Figure 1.3. A redbay infested by laurel wilt disease in Mount Olive, North Carolina. Beetle entrance holes surrounded by sapwood discoloration can be seen once the bark is removed.

Figure 1.4. A cross section of a laurel wilt diseased sassafras branch showing sapwood discoloration. The lack of discoloration in the outermost ring of the sapwood is often observed in this species.

Figure 1.5. Distribution of counties with laurel wilt disease by year of initial detection. As of December, 2019, the disease has crossed the Appalachian Mountains and has been reported as infecting sassafras in Kentucky and Tennessee. Credit: USDA Forest Service.

Figure 1.6. Loop mediated isothermal amplification (LAMP). Credit: Eiken Chemical Co., Ltd.

Figure 1.1

Figure 1.2

Figure 1.3

Figure 1.4

Figure 1.5

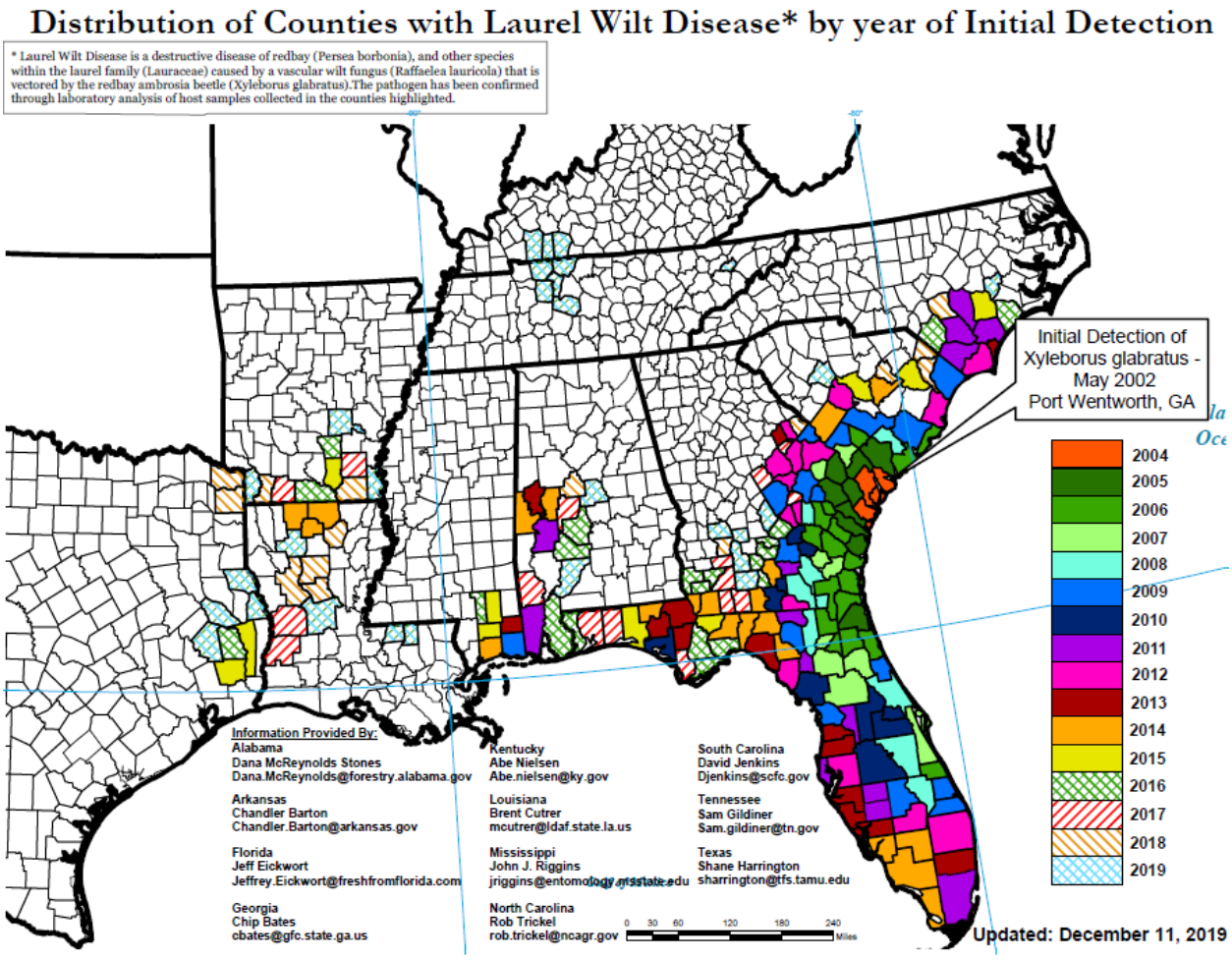
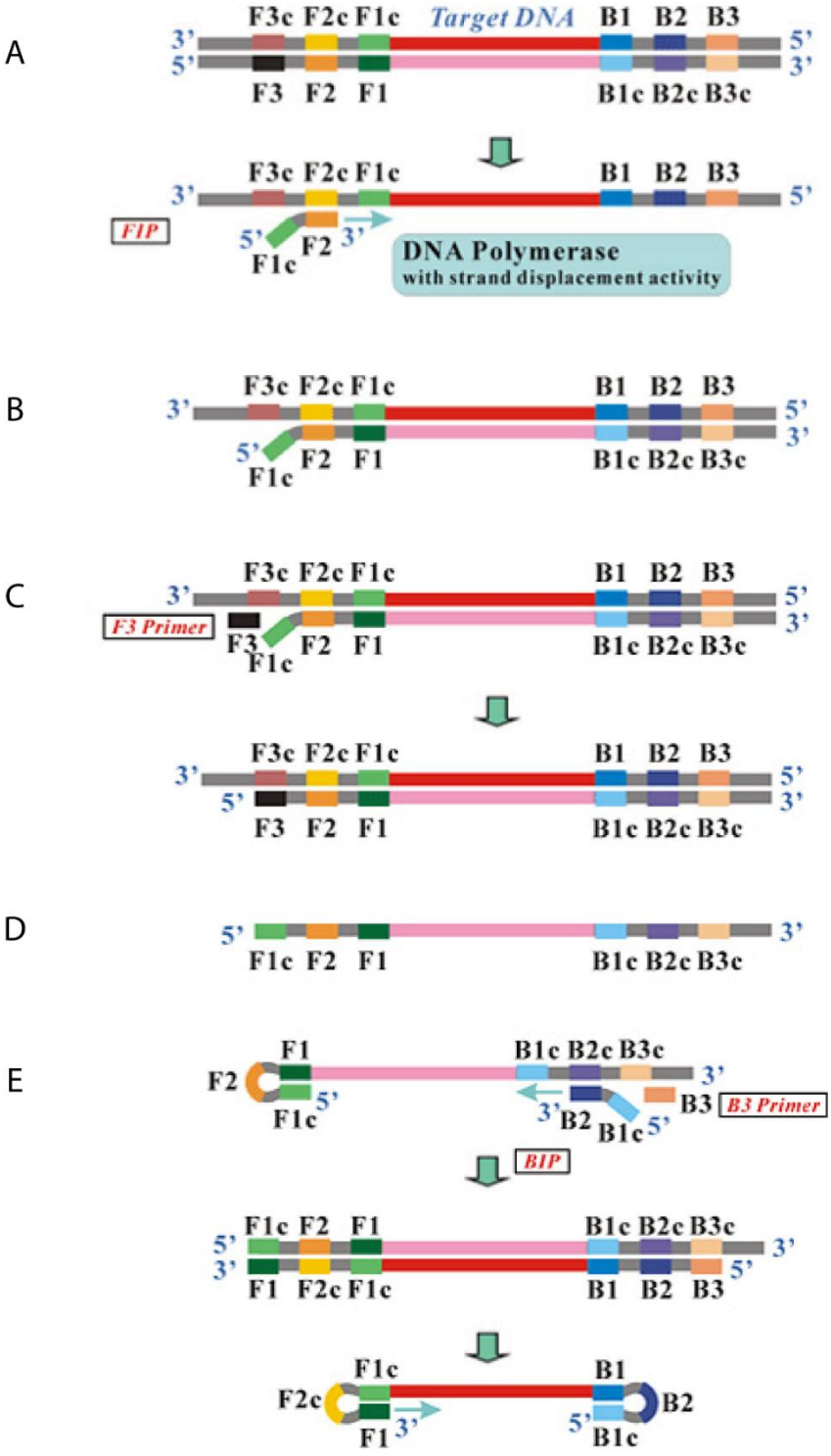

Distribution of Counties with Laurel Wilt Disease* by year of Initial Detection
* Laurel Wilt Disease is a destructive disease of redbay (Persea borbonia), and other species within the laurel family (Lauraceae) caused by a vascular wilt fungus (Raffaelea lauricola) that is vectored by the redbay ambrosia beetle (Xyleborus glabratus).The pathogen has been confirmed through laboratory analysis of host samples collected in the counties highlighted.
Initial Detection of Xyleborus glabratus - May 2002 Port Wentworth, GA
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
Information Provided By:
Alabama
Dana McReynolds Stones
Dana.McReynolds@forestry.alabama.gov
Arkansas
Chandler Barton
Chandler.Barton@arkansas.gov
Florida
Jeff Eickwort
Jeffrey.Eickwort@freshfromflorida.com
Georgia
Chip Bates
cbates@gfc.state.ga.us
Kentucky
Abe Nielsen
Abe.nielsen@ky.gov
Louisiana
Brent Cutrer
mcutrer@ldaf.state.la.us
Mississippi
John J. Riggins
jriggins@entomology.msstate.edu
North Carolina
Rob Trickel
rob.trickel@ncagr.gov
South Carolina
David Jenkins
Djenkins@scfc.gov
Tennessee
Sam Gildiner
Sam.gildiner@tn.gov
Texas
Shane Harrington
sharrington@tfs.tamu.edu
0 30 60 120 180 240
Miles
Updated: December 11, 2019

Figure 1.6


A
F3c F2c F1c
Target DNA
B1 B2 B3
3'
5'
5'
3'
F3 F2 F1
B1c B2c B3c
F3c F2c F1c
B1 B2 B3
3'
5'
FIP
5'
F2
3'
F1c
DNA Polymerase
with strand displacement activity
B
F3c F2c F1c
B1 B2 B3
3'
5'
3'
5'
F2
F1
F1c
B1c B2c B3c
C
F3c F2c F1c
B1 B2 B3
3'
5'
3'
F3 Primer
F3
F2
F1
F1c
B1c B2c B3c
F3c F2c F1c
B1 B2 B3
3'
5'
5'
3'
F3 F2 F1
B1c B2c B3c
D
5'
3'
F1c F2 F1
B1c B2c B3c
E
F1
F2
B1c B2c B3c
3'
5'
F1c
3'
B2
B3
B3 Primer
B1c
5'
BIP
F1c F2 F1
B1c B2c B3c
5'
3'
3'
F1 F2c F1c
B1 B2 B3
5'
F1c
B1
F2c
B2
F1
3'
5'
B1c

CHAPTER 2

RAPID DETECTION OF *RAFFAELEA LAURICOLA* DIRECTLY FROM HOST PLANT AND BEETLE VECTOR TISSUES USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION.[1]

[1]Hamilton, J.L., J.N. Workman, C.J. Nairn, S.W. Fraedrich, and C. Villari. Submitted for publication to *Plant Disease*.

Abstract

Since its introduction in 2002, laurel wilt disease has devastated indigenous lauraceous species in the southeastern United States. The causal agent is a fungal pathogen, *Raffaelea lauricola*, which after being introduced into the xylem of trees by its vector beetle, *Xyleborus glabratus*, results in a fatal vascular wilt. Rapid detection and accurate diagnosis of infections is paramount to the successful implementation of disease management strategies. Current management operations to prevent the spread of laurel wilt disease are largely delayed by time-consuming laboratory procedures to confirm the diagnosis. In order to greatly speed up the operations, we developed a loop-mediated isothermal amplification (LAMP) species-specific assay that targets the β-tubulin gene region of *R. lauricola*, and allows for the rapid detection of the pathogen directly from host plant and beetle tissues. The assay is capable of amplifying as low as 0.5 pg fungal DNA and as few as 50 conidia. The assay is also capable of detecting *R. lauricola* directly from wood tissue of artificially inoculated redbay saplings as early as 10 and 12 days post inoculation, when testing high quality and crude DNA extracts, respectively. Finally, crude DNA extracts of individual adult female *X. glabratus* beetles were assayed and the pathogen was detected from all specimens. This assay greatly reduces the time required to confirm a laurel wilt diagnosis, and because LAMP technology is well suited to provide point-of-care testing, it has the potential to expedite and facilitate implementation of management operations in response to disease outbreaks.

INDEX WORDS: Laurel wilt disease, LAMP, *Xyleborus glabratus*, *Persea borbonia*, *Sassafras albidum*, early detection rapid response, crude DNA

2.1 Introduction

Non-native plant diseases have the ability to drastically alter the composition of forests worldwide and early detection is key to implement a rapid management response and contain their spread (Aglietti, et al. 2019, Luchi, et al. 2020). Laurel wilt disease is a deadly, invasive vascular disease afflicting all lauraceous species in the southeastern United States, including ecologically important forest species like redbay (*Persea borbonia* (L.) Spreng) and sassafras (*Sassafras albidum* (Nutt.) Nees), the threatened pondberry (*Lindera melissifolia* (Walter) Blume) and the endangered pondspice (*Litsea aestivalis* (L.) Fernald), and the economically important crop avocado (*Persea americana*) (Fraedrich, et al. 2008, Fraedrich, et al. 2011, Ploetz, et al. 2017a). The causal agent of laurel wilt disease is *Raffaelea lauricola* (T.C. Harr. Fraedrich & Aghayeva), a mycangial fungus and nutritional symbiont of *Xyleborus glabratus* Eichoff, the redbay ambrosia beetle (Harrington, et al. 2008). Both the fungus and the beetle vector are indigenous to southeastern Asia where they are primarily secondary pests and are not known to cause a vascular wilt in healthy native hosts (Fraedrich, et al. 2008, Harrington, et al. 2011, Shih, et al. 2018). In their non-native range, however, they are readily capable of quickly infecting, and ultimately killing, healthy plants.

Since its first detection in 2002 near Savannah, Georgia, laurel wilt disease has spread rapidly through the southeastern coastal plain and beyond, causing the deaths of millions of trees (Hughes, et al. 2017), with the potential to spread even further wherever susceptible lauraceous hosts can be found (Gramling 2010). The rapid spread of laurel wilt disease to redbay in Texas (Menard, et al. 2016), to sassafras in Arkansas (Olatinwo, et al. 2016) and North Carolina (Mayfield, et al. 2019), and very recently to sassafras in

Kentucky and Tennessee (Loyd, et al. 2020) highlight the need for improved monitoring, rapid diagnostics, and aggressive management of this destructive, invasive disease. Additionally, laurel wilt disease is a direct threat to the commercial avocado industry in Florida, which is valued at $30 million annually (Pisani 2015), and is a potential threat to avocado production in other areas such as Mexico and California. Avocado plantations in Myanmar have recently developed symptoms of laurel wilt disease, indicating the first instance of the disease outside of the southeastern U.S. (Ploetz, et al. 2016).

The introduction of *R. lauricola* into the xylem elicits the abundant production of tyloses, which are ineffective for preventing the systemic movement of the fungus (Inch, et al. 2012, Hughes, et al. 2015). However, the combined effects of the breakdown of host cells, the presence of fungal spores, and the production of tyloses ultimately block the transport of water and nutrients from roots resulting in the loss of hydraulic conductivity and turgor pressure in vascular tissue (Pearce 1996, Inch and Ploetz 2011). Symptoms of laurel wilt disease include leaf wilt, vivid brown to black sapwood discoloration, and eventually host death in as fast as a few months (Fraedrich, et al. 2008). These symptoms, however, are fairly common to wilt diseases and abiotic stressors (Dimond 1970), and thus a more definitive verification in addition to a visual diagnosis is required.

Currently, laurel wilt disease is managed through detection of symptomatic host plants, laboratory confirmation of the diagnosis, and removal of infected material, as well as through monitoring of the beetle vector and wood products movement restrictions (Dreaden, et al. 2014b, Hughes, et al. 2015). Early detection of the disease is a crucial step for its successful management (National Invasive Species Council 2016), but current

strategies relying on morphological and molecular confirmation of the diagnosis are time consuming. In particular, the confirmation of laurel wilt disease relies on external laboratories and time-intensive procedures, specifically: isolation of a pure culture of the fungus, which can take up to a week, followed by DNA extraction and species-specific PCR amplification (Dreaden, et al. 2014b). Moreover, no PCR based molecular method is available to reliably confirm the presence of *R. lauricola* directly in the host tissues, emphasizing the need for alternative approaches that can reliably and rapidly detect *R. lauricola*, possibly directly in the field, from plant tissues and insect vectors.

LAMP (loop-mediated isothermal amplification) is a molecular technique that facilitates rapid detection of target DNA sequences (Notomi, et al. 2000) in an hour or less compared with days or weeks associated with conventional laboratory confirmation (Le and Vu 2017). LAMP utilizes multiple primers that bind to distinct sections of target DNA, making the assay highly specific and sensitive (Notomi, et al. 2000). Further, in contrast to conventional PCR, its polymerase enzyme is less sensitive to reaction inhibitors, allowing for the use of crude DNA template (Poon, et al. 2006, Kaneko, et al. 2007, Kogovsek, et al. 2015). LAMP reactions are isothermal, thereby removing the requirement for a bulky, laboratory-bound, power-intensive thermocycler (Notomi, et al. 2000), and allowing for the use of small, field portable instrumentation (Niessen 2015, but see Hole and Nfon 2019 and Thomas, et al. 2019 for recent examples of portable PCR thermocyclers). Finally, LAMP is also rapid, being capable of amplifying target DNA to detectable levels in as little time as 20 min (Villari, et al. 2017), and visualization of results can be performed in real-time using fluorescent assimilating probes (Jenkins, et al. 2011, Kubota, et al. 2011). LAMP is commonly used for point-of-care testing and rapid

diagnosis confirmation because of its portability, and quick and accurate diagnostic capabilities (Niessen 2015). Previous research has shown that LAMP is capable of detecting fungal pathogens under a wide range of conditions in both clinical and agricultural settings (e.g. Endo, et al. 2004, Niessen and Vogel 2010, Villari, et al. 2017, King, et al. 2018). However, the technique has only recently been used in forestry settings to detect pathogens directly from host plant tissue (e.g. Sillo, et al. 2018, Aglietti, et al. 2019, Dai, et al. 2019) or from insect vectors (e.g. Villari, et al. 2013, Meng, et al. 2018).

The aim of this study was to develop a LAMP assay for the rapid and accurate molecular detection of the laurel wilt disease pathogen directly from host tissues. Specifically, our objectives were to (*i*) develop and validate a *R. lauricola* species-specific LAMP assay, (*ii*) determine the earliest time after host infection that the assay can detect *R. lauricola* in host sapwood, and (*iii*) test the assay's suitability for amplification of the target species directly within host plant tissue using both high quality and crude DNA, and from beetle tissues using crude DNA.

2.2 Materials and Methods

2.2.1 *Outline of the experiment*

To fulfill objective (*i*), the LAMP assay was first developed and validated using high quality DNA from pure fungal isolates. To fulfill objectives (*ii*) and (*iii*), a time course experiment with artificially inoculated redbay saplings was performed, and plant tissues collected from the experiment were tested with the LAMP assay using both high

quality and crude DNA extracts. In addition, to fulfill objective (*iii*), crude extracts from female *X. glabratus* specimens were tested.

2.2.2 *Fungal Isolates and DNA Extraction*

Fungal isolates used for the development and validation of the LAMP assay are listed in Table 2.1. Different types of extraction procedures were used for the different components of the study. High quality DNA from both fungal mycelia and plant tissues was extracted using the Qiagen DNeasy plant mini kit (QIAGEN, Germantown, MD, USA) as per the manufacture's protocol. Mycelia of the pure fungal isolates was collected from fresh cultures grown on potato dextrose agar (Becton, Dickinson and Company, Sparks, Maryland, USA) plates covered with a sterile cellophane disk. All samples were ground in liquid nitrogen prior to DNA extraction. Presence of DNA in each sample was assessed by gel electrophoresis, and approximate DNA concentrations were quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

To extract crude DNA from plant tissues, a flame-sterilized scalpel was used to shave off 15-20 mg of wood tissue after debarking, which was then placed into a 1.5 mL microcentrifuge tube along with 300 µL prepared 5% Chelex 100 solution (BioRad, California, USA). To extract crude DNA from *X. glabratus*, whole beetles were manually ground with a sterile micropestle in a 1.5 mL microcentrifuge tube with 50 µL of 5% Chelex 100 solution. Crude extraction samples were then boiled for 5 min, vortexed for 15 s, boiled for an additional 5 min, and vortexed again for 15 s. Before testing, which occurred on the same day as extraction, samples were centrifuged at 3,884 rcf in a mini

centrifuge (VWR, Radnor, PA, USA) for 30s and only the supernatant was used in the LAMP reaction.

2.2.3 *Development of a R. lauricola species-specific LAMP assay*

Two different LAMP primer sets were designed targeting separately the *R. lauricola* β-tubulin (BT) gene region (GenBank accession number KJ909302) (Dreaden, et al. 2014a) and the CHK microsatellite region (GenBank accession number KF381410), the latter of which is currently used for PCR based identification of *R. lauricola* (Dreaden, et al. 2014b). BLAST analysis of the BT gene sequences from *R. lauricola* was performed using the National Center for Biotechnology Information (NCBI) database (Zhang, et al. 2000), and the most similar sequences, in addition to those of the host species, were aligned in order to select an appropriately divergent region of the *R. lauricola* BT gene for primer design. Sequence alignment was performed using ClustalW, in MEGA version 7 software (Kumar, et al. 2016). The CHK microsatellite is taxon-specific to *R. lauricola* (Dreaden, et al. 2014b). Primers were then designed using PrimerExplorer (v. 4.0: Eiken Chemical Co., Tokyo), as per Notomi, et al. (2000) and Nagamine, et al. (2002), and a FAM fluorescent assimilating probe and associated quencher were designed against the backward loop of the amplicon, as per Kubota, et al. (2011). However, preliminary testing showed that the CHK primers cross-reacted with redbay host DNA, and were excluded from further analysis. Primers, probes, and quencher were synthesized by IDT (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). Sequences of the BT primer set are reported in Table 2.2.

Lamp reactions were performed as per Villari, et al. (2017) with modifications as follows. Each 25 μL reaction contained 15 μL 1x no-dye Isothermal Master Mix (Optigene, Horsham, UK), 2.8 μM of internal primers BT-FIP and BT-BIP, 0.28 μM of external primers BT-F3 and BT-B3, 0.8 μM F-Loop primer, 0.092 μM assimilating probe fluorescent (FAM) strand, 0.184 μM quencher strand, 1.3 μL molecular grade water (Thermo Fisher Scientific), and 5 μL template. Reactions were carried out in 0.2 mL optically clear PCR eight-tube strips (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific) and monitored in real-time. Reactions were programmed with the following conditions: 65° C for 60 1-minute cycles with fluorescence reading every minute followed by a denaturing step at 85° C for 5 min to halt the reaction and deactivate the polymerase. Each sample was tested in triplicate and a no template control was included in each run, while high quality DNA from a known *R. lauricola* isolate (i.e., FS-0001, FS-0004, or FS-0006—FS-0017) was used as a positive control. A result was considered positive if two out of three replicates resulted in amplification.

After verifying that the BT primer set was not cross-reacting with host plant DNA, specificity of the LAMP assay was tested with high quality DNA extracted from the isolates described in Table 1. Assay sensitivity was determined by testing a 1 ng to 0.01 pg serial dilution of *R. lauricola* isolate FS-0009 DNA. Additionally, to estimate a more biologically relevant sensitivity threshold, dilutions of *R. lauricola* spore suspensions were tested. Spores of isolate FS-0001 were collected by covering the surface of colonized malt extract agar (VWR) amended with 200 ppm cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) and 100 ppm streptomycin (Sigma-Aldrich)

(CSMA) (Harrington and Fraedrich 2010) plates with 10 mL of molecular grade water, and gently scraping the agar surface and mycelium with a sterilely gloved finger or a sterile glass spreader. The suspension was then collected and filtered through sterile cheese cloth. Conidia concentration was estimated under magnification using a hemacytometer (Thermo Fisher Scientific) and adjusted to 1,000, 250, 100 and 10 conidia/µL. Spore suspension dilutions were then boiled for 5 min and vortexed for 15 s prior to testing with the LAMP assay as previously described.

2.2.4 *Time Course Experiment*

To determine the earliest time after host infection that the LAMP assay is capable of detecting *R. lauricola* within plant tissues, thirty-six 2-3 year old redbay saplings were placed in a walk-in growth chamber (Conviron Model GR48, Controlled Environments Ltd., Winnipeg, Manitoba, Canada) in August 2018. Saplings were grown at 25° C on a 14/10 h day/night cycle at ambient humidity for two weeks before being subjected to the following treatments: 18 saplings (average height 117.78 cm ± 4.04 cm standard error; diameter 12.28 mm ± 0.41 mm standard error) were randomly selected to receive a *R. lauricola* spore suspension, while the remaining 18 were used as negative controls (height 116.89 cm, ± 2.97 cm standard error; diameter 12.79 mm ± 0.35 mm standard error). Two holes at a 45º inclination were drilled with a 2.5 mm flame-sterilized bit into the opposite sides of each sapling's stem, 5-7 cm above the soil line. Each drilled hole was either inoculated with 10 µL (20 µL per plant) spore suspension or sterile water, before being wrapped in Parafilm® (Bemis Company, Inc., Neenah, WI, USA). The spore suspension was prepared following the procedure previously described, and diluted

to 5,000 conidia/µL so that treatment saplings received in total 100,000 conidia. Beginning two days after inoculation and continuing at two-day intervals for up to 12 days after inoculation, three inoculated and three control saplings were randomly selected and destructively sampled by obtaining a 10 cm stem segment at 50 cm above the inoculation point using flame-sterilized pruning shears. Saplings were also visually inspected for the presence of wilting or vascular streaking at each sampling time. Collected stem segments were stored at -20° C until extraction and testing. We initially planned to include sampling and analysis of leaf samples as well, but preliminary testing showed inconsistency in the results, hence leaves were dropped from further testing. An additional 10 cm stem segment was also taken at 60 cm above the inoculation point to confirm colonization by *R. lauricola* via plating as described below. High quality DNA was extracted from each stem segment with a Qiagen DNeasy plant mini kit and crude DNA extracts were obtained with the 5% Chelex 100 extraction method as previously described. LAMP assays were performed as previously described.

Processing of the samples to determine *R. lauricola* colonization of stems consisted of debarking samples, rinsing with 95% EtOH, flame sterilization, and plating onto CSMA plates. Plates were then wrapped in Parafilm® and incubated at 25º C on a 14/10 h day/night cycle in a Precision Model FU019ARW2 Dual Program Illuminated Incubator (Thermo Fisher Scientific) until *R. lauricola* growth was observed or a minimum of two-weeks had passed.

2.2.5 *Capability of the LAMP assay to detect R. lauricola DNA in X. glabratus beetles*

To test the capability of the LAMP assay to detect *R. lauricola* directly from the insect vector, 11 female *X. glabratus* beetles were collected from infected sassafras bolts at the Conway Cemetery Historic State park in Lafayette County, Arkansas and assayed individually. Each beetle was stored separately in a 1.5 mL centrifuge tube and kept at -20º C until extraction. Crude DNA extracts from beetles were obtained with the 5% Chelex 100 extraction method and LAMP was performed as previously described, with the exception that only 1 µL was used in the reaction to avoid excessive background noise.

2.3 Results

2.3.1 *Specificity of β-tubulin LAMP assay to R. lauricola*

The BT LAMP assay tested positive for all *R. lauricola* isolates evaluated from both beetles and plants, regardless of their geographic origin (Table 1). No amplification was observed when the assay was tested against 20 other *Raffealea* species, more distantly related fungal and oomycete species (Table 1), nor redbay host DNA.

2.3.2 *Sensitivity of β-tubulin LAMP assay*

Using serial dilutions of *R. lauricola* DNA (Figure 1A), all technical replicates amplified within 16 min at levels as low as 0.5 pg DNA. Only one out of three technical replicates amplified with 0.05 pg DNA. When tested with *R. lauricola* spore suspensions (Figure 1B), all technical replicates with 500 to 5,000 conidia amplified within 12 min, and those with 50 conidia amplified within 20 min.

2.3.3 *Time Course Experiment*

The LAMP assay detected *R. lauricola* in redbay xylem as early as 10 days post inoculation (dpi), with all technical replicates for two out of three samples testing positive using high quality DNA (Table 2.3). However, no amplification was observed in 10 dpi samples using crude DNA (Table 2.3). By 12 dpi, the LAMP assay successfully amplified all three inoculated replicate saplings with both high quality (Figure 2A) and crude DNA extracts (Figure 2B). In the test with crude DNA extracts, amplification was delayed by approximately 10 min for the majority of the reactions, but all successful reactions occurred within 40 min (Figure 2B). For one of the samples (i.e., R17), only two out of three technical replicates successfully amplified. No amplification was observed for any sample earlier than 10 dpi. All control samples tested negative for both high quality or crude DNA extracts.

Full crown wilt was not observed in any of the saplings inoculated with *R. lauricola* for the duration of the experiment. External symptoms of laurel wilt disease were first evident at 12 dpi, but were observed in only one out of the three inoculated saplings, whose leaves had just begun to wilt and discolor (Table 2.3). At this time, all inoculated saplings exhibited internal symptoms at the 50 cm sampling height that appeared primarily as a faint sapwood discoloration (Table 2.3, Figure 2.3). All control samples remained asymptomatic and showed no evidence of either wilting or vascular discoloration.

In inoculated redbay saplings, *R. lauricola* was recovered from samples collected 60cm above the inoculation point as early as 4 dpi (Table 2.3). *R. lauricola* was not recovered from any control saplings.

2.3.4 *Detecting R. lauricola DNA in X. glabratus beetles*

Amplification was observed for all technical replicates from all eleven female *X. glabratus* adult beetles tested with the LAMP assay. Amplification occurred in less than 10 min when using crude DNA extracts from beetles and was only delayed approximately two minutes compared to a high quality fungal DNA positive control.

2.4 Discussion

This study describes the development of the first LAMP assay for the detection of *R. lauricola*, the causal agent of laurel wilt disease, directly form host plant tissues and using crude DNA extracts. The BT LAMP assay is species-specific and capable of detecting *R. lauricola* DNA regardless of its origin, showing no cross-reaction with other closely related *Raffaelea* species or redbay host DNA. The assay is sensitive and capable of detecting target DNA at levels as low as 0.5 pg in less than 16 min, and fungal conidia at levels as low as 50 conidia within 20 min. Moreover, the BT LAMP assay can successfully detect *R. lauricola* associated with *X. glabratus* females with minimal DNA extraction processing and reaction times of less than 10 min. The ability of the BT assay to detect as little as 50 conidia is well within the range typically found within a single *X. glabratus* female, which may harbor from between 1,000 and 30,000 conidia (Harrington and Fraedrich 2010). This potentially makes the assay useful for laurel wilt disease

pathogen detection from other ambrosia beetle species which are known to harbor *R. lauricola*, but at much lower quantities, such as *Xyleborus bispinatus* (Ploetz, et al. 2017b). Additionally, thanks to the capability of detecting *R. lauricola* directly from the beetle vector, the assay provides an alternative method to confirm the presence of the pathogen in an area (i.e., by testing trapped insects). This could be particularly useful in those instances when *X. glabratus* beetles are trapped during routine monitoring, but no symptomatic trees have been found.

The ability to detect *R. lauricola* within host plant tissues is crucial for the rapid detection of laurel wilt disease. Similarly, developing a crude DNA extraction protocol to enable direct testing of host tissue samples with LAMP assays is a major step for "in-field" diagnosis. Our results show that the assay is capable of detecting the pathogen 50 cm above the inoculation point in artificially inoculated saplings as soon as 10 dpi if using high quality DNA, and as early as 12 dpi if using crude DNA, which coincides with the development of the first external and internal symptoms in plants. It is notable that our assay was capable of detecting *R. lauricola* in two experimental saplings that had yet to exhibit external symptoms of laurel wilt disease. This is particularly relevant given that in disease monitoring operations, field personnel will most likely sample wilting or otherwise externally symptomatic trees, hence minimizing the risk of false negatives. Additional experimentation will be required to determine if the assay can detect *R. lauricola* before the appearance of visual symptoms in larger, more mature trees, or if other tissue types are appropriate for testing in more advanced cases of the disease.

When using crude DNA extracts, amplification commenced slightly later compared to high quality DNA extracts. However, the delayed response time associated

with the rapid, crude DNA extraction protocol is inconsequential when compared to the time required to culture *R. lauricola* and perform a high quality DNA extraction from pure-culture mycelium for a traditional PCR based confirmation.

Previous research in this disease system has shown that *R. lauricola* can be recovered from infected wood tissue at various stem heights several days after laboratory inoculations (Fraedrich, et al. 2015). In our time course experiment, we were able to re-isolate *R. lauricola* at 60cm above the inoculation point as soon as 4 dpi, which is six days before we could detect the pathogen with the LAMP assay. It should be noted, however, that the number of reproductively viable units (i.e. conidia) needed to successfully culture *R. lauricola* from infected wood (in theory as low as one viable spore) is significantly less than the number of spores that were detectable with the LAMP assay (~50).

The current standard procedure to process a suspected laurel wilt disease sample and provide a confirmation of the diagnosis relies on an external laboratory performing fungal isolations from symptomatic tissues followed by DNA extraction and traditional PCR (Hughes, et al. 2015). This laboratory based process can provide a response in approximately one week (Dreaden, et al. 2014b). However, because the *R. lauricola* specific PCR primers used in the traditional assay do not produce consistent amplification directly from diseased wood tissues (Dreaden, et al. 2014b), the time required for diagnosis confirmation can often be extended beyond a week when there are difficulties isolating the pathogen. The LAMP assay developed in this study addresses both the time constraint and the issue of testing directly from the host plant or insect vector, while maintaining high specificity and sensitivity. This demonstrates that the time required to

confirm laurel wilt disease can be significantly reduced, speeding up the implementation of management strategies that aim to slow the spread of laurel wilt disease.

Future work will focus on the validation of the LAMP assay for field implementation by utilization of portable LAMP devices. The use of portable devices is crucial in the field of forestry where remote distances, large areas, and finite laboratory capabilities limit the utilization of traditional techniques. The implementation of the LAMP assay developed in this study would enable forest and orchard managers to confirm the presence of laurel wilt disease directly in-field in as little as an hour compared with a week or more required for existing procedures. This assay facilitates the recently proposed framework for effective early warning and rapid response to successfully mitigate the impact of invasive pathogens of forest ecosystems (Aglietti, et al. 2019, Luchi, et al. 2020).

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Table 2.1. Fungal isolates used in this study to validate the specificity of the LAMP primers.

Species	Isolation Host	Original Location	Collector	Isolate Name	Year of Isolation	LAMP Result
Raffaelea lauricola	*Lindera benzoin*	SC	S. Fraedrich	FS-0017	2007	+
	Persea borbonia	SC	S. Fraedrich	FS-0001	2004	+
		TX	S. Fraedrich	FS-0002	2014	+
		SC	S. Fraedrich	FS-0008	2004	+
		SC	S. Fraedrich	FS-0009	2007	+
		SC	S. Fraedrich	FS-0010	2007	+
		MS	S. Fraedrich	FS-0011	2015	+
		TX	S. Fraedrich	FS-0015	2014	+
		TX	S. Fraedrich	FS-0016	2014	+
		GA	S. Fraedrich	FS-0018	2007	+
		GA	S. Fraedrich	FS-0019	2007	+
		GA	S. Fraedrich	FS-0020	2007	+
		GA	S. Fraedrich	FS-0021	2007	+
		GA	T. Harrington	C2246	2005	+
		GA	T. Harrington	C2245	2005	+
		GA	T. Harrington	C4073	-	+
		GA	T. Harrington	C2208	-	+
	Sassafras albidum	AL	S. Fraedrich	FS-0003	2015	+
		GA	S. Fraedrich	FS-0012	2016	+
		GA	S. Fraedrich	FS-0013	2016	+
		GA	S. Fraedrich	FS-0014	2014	+
		NC	S. Fraedrich	CV2018 003	2018	+
		NC	S. Fraedrich	CV2018 004	2018	+
		AL	T. Harrington	C2953	2012	+
		GA	T. Harrington	C2212	-	+
	Xyleborus glabratus	SC	S. Fraedrich	FS-0004	2007	+
		SC	S. Fraedrich	FS-0005	2007	+
		SC	S. Fraedrich	FS-0006	2007	+
		SC	S. Fraedrich	FS-0007	2007	+
R. aguacate	-	FL	J. Smith/T. Dreaden	PL1004	-	–
R. albimanens	*Platypus externedentatus*	S. Africa	T. Harrington	C2223	1969	–
R. amasae	*Amasa concitatus*	Taiwan	T. Harrington	C2750	2000	–
R. ambrosiae	*P. cylindrus*	England	T. Harrington	C2225	1963	–
R. arxii	*X. volvulus*	FL	D. Carillo/L. Cruz	Ph 24-3 MEA	2015	–
R. brunnea	-	FL	J. Hulcr	CV2018 026	2018	–
R. canadensis	*P. wilsonii*	Canada	T. Harrington	C2233	1966	–
R. ellipticospora	*X. glabratus*	SC	T. Harrington	C2395	-	–
R. fusca		FL	D. Carillo/L. Cruz	AH3-M2	2016	–

R. gnathotrichi	*Gnathotrichus retusus*	CO	T. Harrington	C2219	1965	–
R. montetyi	*P. cylindrus*	France	T. Harrington	C2221	1993	–
R. quercivora	*P. quercivorus*	Japan	T. Harrington	C2526	-	–
R. santoroi	*P. mutatus*	Argentina	T. Harrington	C2748	1966	–
R. sp. PL1001	*X. volvulus*	FL	D. Carillo/L. Cruz	Ph 24-2 MEA	2015	–
R. subalba	*X. bispinatus*	FL	D. Carillo/L. Cruz	AFH M1	2016	–
	X. volvulus	FL	D. Carillo/L. Cruz	A2-1 P	2016	–
R. subfusca	*X. bispinatus*	FL	D. Carillo/L. Cruz	A M2 G	2016	–
	X. volvulus	FL	D. Carillo/L. Cruz	A3-1M	2016	–
R. sulcati	*G. sulcatus*	Canada	T. Harrington	C2234	1970	–
R. sulphurea	*Xyleborinus saxeseni*	KS	T. Harrington	C593	1967	–
R. tritirachium	*Quercus sp.*	PA	T. Harrington	C2222	-	–
R. xyleborina	*X. glabratus*	FL	D. Carillo/L. Cruz	R. PL6099	2016	–
Leptographium profanum	*Hylobius pales*	GA	M. Buland	CV2017 305	2017	–
Ophiostoma ips	*Hylobius porculus*	GA	M. Buland	CV2017 308	2017	–
Nigrospora oryzae	*Pinus taeda*	GA	A. Niyas	CV2018 018	2017	–
Phytophthora cinnamomi	*Castanea dentata*	GA	A. Niyas	CV2017 095	2018	–

Table 2.2. LAMP primer set and probes used for the detection of *Raffaelea lauricola*. Primers are designed against the β-tubulin (BT) gene region.

Primer or probe	Sequence (5'-3')
LAMP Primer set	
BT-F3	GTGT ATGT GTCC CTGC TGAA
BT-B3	GCTC GAGA TCGA CGAG GA
BT-FIP	CTCA TGCG CTCA AGCT GGAG CTGC CTTC TCTA ACGT GATG C
BT-BIP	CCAG CCAC TGGG CAAA GTGA AACG TACT TGTT GCCA GAGG
BT-F Loop	CCGA CGTG CCAT TGTA CCT
Assimilating Probe[a]	
BT-FAM, fluorescent strand	FAM - ACGC TGAG GACC CGGA TGCG AATG CGGA TGCG GATG CCGA TACC AAAA ACGA GCAA GCAT CT
BT-Q, Quench strand	TCGG CATC CGCA TCCG CATT CGCA TCCG GGTC CTCA GCGT - BHQ

[a]Assimilating probe was designed as per Kubota, et al. (2011). Underlined fragment of BT-FAM acts as backward loop primer. FAM, 6-carboxyfluorescein. BHQ, Black Hole Quencher-1 (Biosearch Technologies, Novato, CA).

Table 2.3. Results of the time course experiment.

Sample ID[a]	Days Post Inoculation	Presence of Leaf Wilt	Presence of Wood Discoloration	Positive Fungal Isolation	LAMP with High Quality DNA[b]	LAMP with Crude DNA[b]
R1	2	-	-	-	0/3	0/3
R2		-	-	-	0/3	0/3
R3		-	-	-	0/3	0/3
C1		-	-	-	0/3	0/3
C2		-	-	-	0/3	0/3
C3		-	-	-	0/3	0/3
R4	4	-	-	+	0/3	0/3
R5		-	-	+	0/3	0/3
R6		-	-	-	0/3	0/3
C4		-	-	-	0/3	0/3
C5		-	-	-	0/3	0/3
C6		-	-	-	0/3	0/3
R7	6	-	-	+	0/3	0/3
R8		-	-	-	0/3	0/3
R9		-	-	-	0/3	0/3
C7		-	-	-	0/3	0/3
C8		-	-	-	0/3	0/3
C9		-	-	-	0/3	0/3
R10	8	-	-	+	0/3	0/3
R11		-	-	+	0/3	0/3
R12		-	-	-	0/3	0/3
C10		-	-	-	0/3	0/3
C11		-	-	-	0/3	0/3
C12		-	-	-	0/3	0/3
R13	10	-	-	+	1/3	0/3
R14		-	-	+	**2/3**	0/3
R15		-	-	+	**2/3**	0/3
C13		-	-	-	0/3	0/3
C14		-	-	-	0/3	0/3
C15		-	-	-	0/3	0/3
R16	12	-	+	+	**3/3**	**3/3**
R17		-	+	+	**3/3**	**2/3**
R18		+	+	+	**3/3**	**3/3**
C16		-	-	-	0/3	0/3
C17		-	-	-	0/3	0/3
C18		-	-	-	0/3	0/3

[a]Sample names starting with R indicate inoculated samples; sample names starting with C indicate control samples. [b]Results are reported as number of positive reactions out of three technical replicates. Positive observations are highlighted in bold.

Figure Legend

Figure 2.1. Sensitivity test of the BT LAMP assay using high quality DNA of *Raffaelea lauricola* mycelium (A) and crude DNA of *R. lauricola* conidia suspensions (B). Each reaction was run in three replicates. sp., spores; NTC, no template control.

Figure 2.2. Results of the BT LAMP assay on redbay saplings inoculated with *Raffaelea lauricola* and sampled 12 days post inoculation. Template of the reaction was either high quality kit extracted DNA (A) or 5% Chelex crude extracted DNA (B). Each reaction was run in three replicates. NTC, no template control; PC, positive control.

Figure 2.3. Redbay stem sections 12 days post inoculation with either *Raffaelea lauricola* (top row) or sterile water (bottom row), sampled 50 cm above the inoculation point. Inoculated samples exhibit a faint discoloration of the wood, while control samples show no discoloration. Scale bar, 2 mm.

Figure 2.1

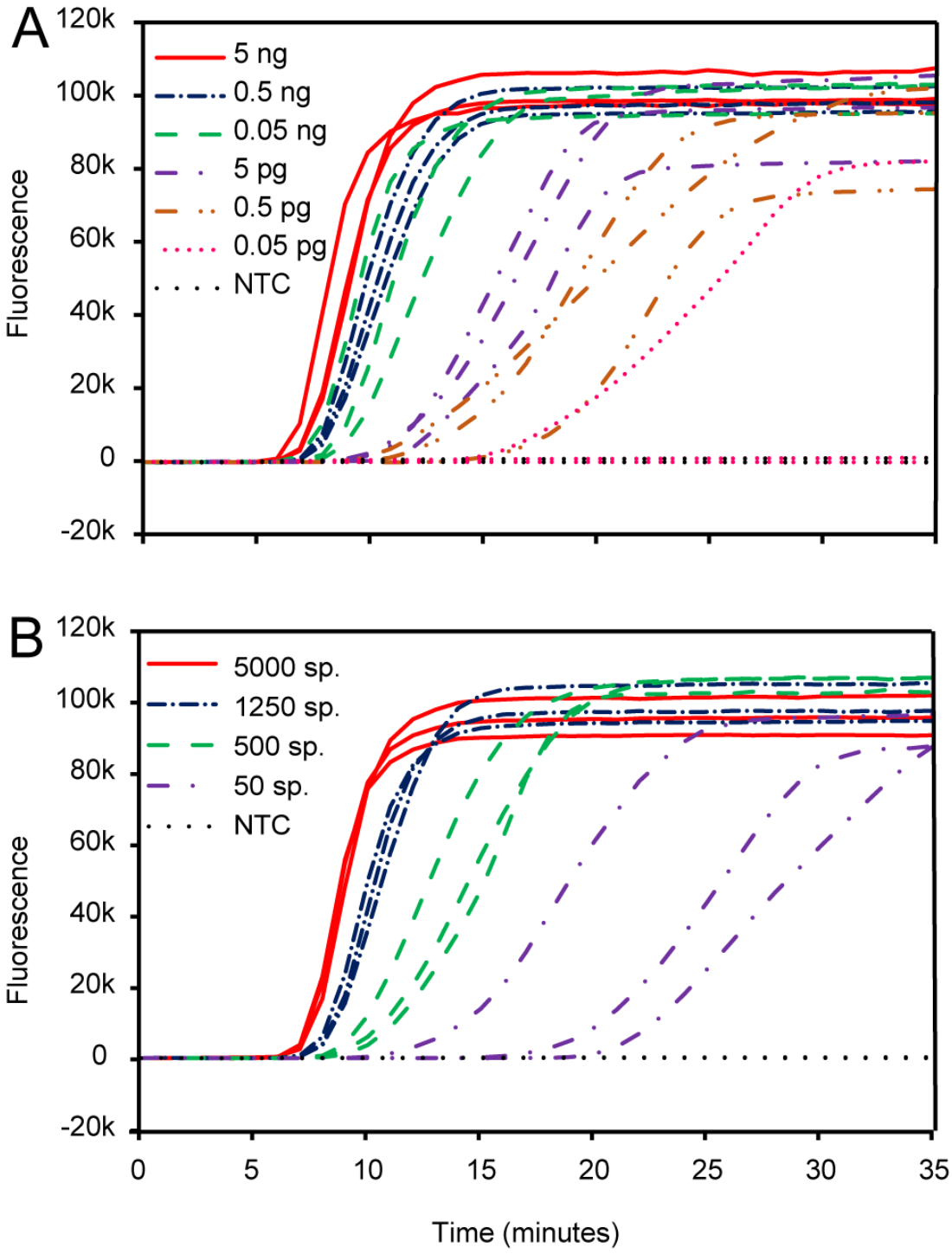
A
120k
100k
80k
60k
40k
20k
0
-20k
Fluorescence
5 ng
0.5 ng
0.05 ng
5 pg
0.5 pg
0.05 pg
NTC
B
120k
100k
80k
60k
40k
20k
0
-20k
Fluorescence
5000 sp.
1250 sp.
500 sp.
50 sp.
NTC
0
5
10
15
20
25
30
35
Time (minutes)

Figure 2.2

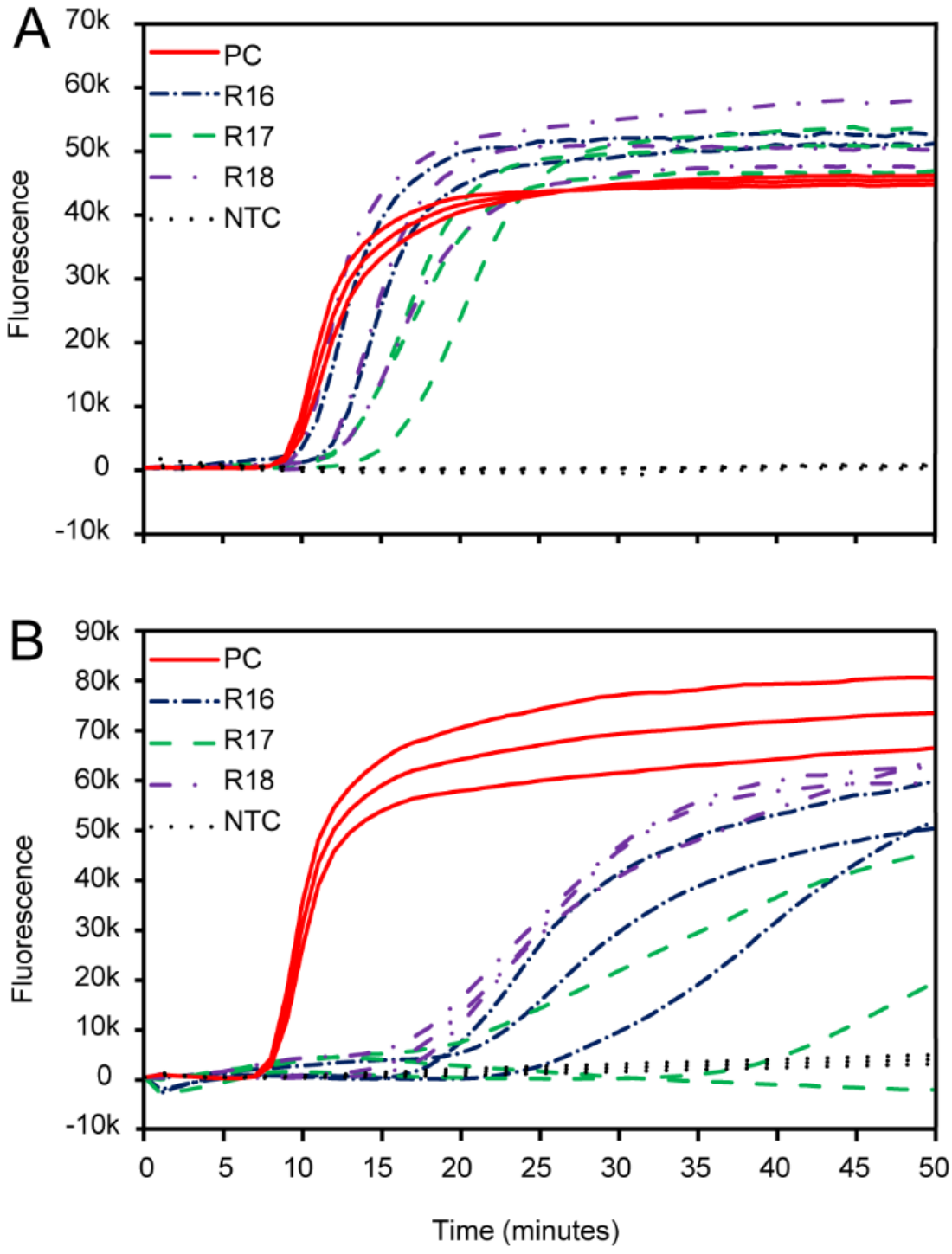
A
70k
60k
50k
40k
30k
20k
10k
0
-10k
Fluorescence
PC
R16
R17
R18
NTC
B
90k
80k
70k
60k
50k
40k
30k
20k
10k
0
-10k
Fluorescence
PC
R16
R17
R18
NTC
0
5
10
15
20
25
30
35
40
45
50
Time (minutes)

Figure 2.3

CHAPTER 3

IMPLEMENTATION OF A FIELD-PORTABLE DIAGNOSTIC APPROACH TO DETECT LAUREL WILT DISEASE: HOW TO CONFIRM A DIAGNOSIS IN MINUTES INSTEAD OF DAYS[2]

[2]Hamilton, J.L., S.W. Fraedrich, C.J. Nairn, A.E. Mayfield, and C. Villari. To be submitted for publication to *Arboriculture & Urban Forestry*.

Abstract

Laurel wilt disease is a rapid, systemic, vascular wilt that has caused extensive mortality of lauraceous species in the southeastern United States. The responsible invasive fungus, *Raffaelea lauricola*, is a symbiont of the invasive beetle vector *Xyleborus glabratus*, which was first detected in the USA in 2002. Early diagnosis of laurel wilt is imperative for rapid and efficient disease management, however a setback for current operations is the necessity for lengthy laboratory procedures to confirm the presence of *R. lauricola*. Here, we validated the robustness and field-portability of a recently developed species-specific loop-mediated isothermal amplification (LAMP) assay, with the overall goal to eliminate the need for laboratory confirmation of laurel wilt diagnoses. We first tested the assay on a benchtop equipment with both redbay and sassafras samples collected from six southeastern states. The assay successfully detected *R. lauricola* directly from symptomatic wood tissue using both high quality and crude DNA extracts. The assay was also able to distinguish between *R. lauricola* and other agents that cause similar symptoms. We then tested the assay directly in the field, using a Genie® III portable LAMP device. We again assayed symptomatic wood samples from both redbay and sassafras across the Southeast and consistently detected *R. lauricola* directly from symptomatic tissue using crude DNA extracts. Results of this study confirmed that the tested field deployable LAMP assay can rapidly and accurately detect *R. lauricola* in symptomatic trees in as little as an hour. LAMP technology is well suited for in-field implementation and this study serves as an incentive for the further use of this technology in the field of forest pathology.

INDEX WORDS: Loop-mediated isothermal amplification (LAMP), early Detection and Rapid Response, crude DNA, *Raffaelea lauricola*, in-field

3.1 Introduction

The forest biome covers over 30% of the Earth's surface, yet is shrinking every year (FAO 2018). Forests provide a multitude of ecosystem services including carbon sequestration, water retention, erosion and soil stabilization, and protection from weather events among many other essential functions (Sakals, et al. 2006, Streck and Scholz 2006). Urban forests and community trees also provide benefits ranging from improved air quality, noise reduction, aesthetics and recreational opportunities, and are important components of urban landscapes (Anyanwu and Kanu 2006, Coder 2011). In turn, any agent causing damage or mortality to trees reduces and interrupts these valuable ecosystem services (Bonello, et al. 2020).

Pathogens are one of the greatest threats to forests and individual trees, and fungi and fungi-like organisms in particular cause the greatest amount of damage (Santini, et al. 2013), especially when invasive such as in the cases of Chestnut blight and Dutch elm disease, both of which resulted in the deaths of millions of trees (Freinkel 2007, Martín, et al. 2018). Besides their devastating effects on ecosystems, the cost of managing invasive species can reach into the millions (National Invasive Species Council 2016). Thus, efforts to protect trees from invasive fungal diseases is paramount to ensure the preservation of the benefits they provide. In specific, early detection, followed by rapid response, are among the crucial steps of frontline defense to contain invasive species and

mitigate their damage (Lamarche, et al. 2015, National Invasive Species Council 2016, Eschen, et al. 2018).

Laurel wilt disease is an invasive, fatal vascular wilt disease that has resulted in extensive mortality among North American members of the Lauraceae (Fraedrich, et al. 2008). The species most commonly affected are redbay (*Persea borbonia* (L.) Spreng), sassafras (*Sassafras albidum* (Nutt.) Nees), and avocado (*Persea americana),* which are ecologically and economically important forest, urban, and agriculture trees (Fraedrich, et al. 2008, Ploetz, et al. 2012). The disease is caused by an ambrosia fungus, *Raffaelea lauricola*, which is carried by a beetle vector, *Xyleborus glabratus* (redbay ambrosia beetle) (Fraedrich 2008, Harrington, et al. 2008). Although *R. lauricola* is just a secondary pest of dying and unhealthy trees in their native Asian range (Shih, et al. 2018), in North America the fungus is extremely pathogenic to all naïve lauraceous species (Hughes, et al. 2015), including those currently outside of impacted areas, such as California laurel (*Umbellularia californica* (Hook. & Arn.) Nutt.) (e.g. Fraedrich 2008). Upon being introduced to Georgia and South Carolina in 2002 (Fraedrich, et al. 2008), laurel wilt disease has quickly spread west to Texas (Menard, et al. 2016), along the Atlantic Coast up to North Carolina (North Carolina Forest Service 2012), and has recently crossed the Appalachian Mountains into Tennessee and Kentucky (Loyd, et al. 2020).

Since the disease is already widely established in the United States, eradication efforts are no longer viable and current management aims instead at early detection and removal of the infected trees, in the effort of slowing the spread of laurel wilt disease to new areas (Hughes, et al. 2015). Early detection, in particular, is the most crucial and

currently the most inefficient step for the successful management of the disease, as trees need to be promptly removed before the new generation of beetle vectors can emerge (Hughes, et al. 2015). The guidelines in place to confirm the disease involves a lengthy process including the shipment of a suspected sample to an equipped laboratory and the isolation of a pure fungal culture, followed by DNA extraction and species-specific PCR (Hughes, et al. 2015). This is a time intensive process, often taking a week or more, and it significantly delays disease management implementation. Furthermore, there are currently no PCR protocols that can reliably detect *R. lauricola* directly from host tissues, thus imposing the requirement for fungal isolation before any test can be performed (Dreaden, et al. 2014), and emphasizing the need for a diagnostic test that can quickly and accurately detect *R. lauricola* directly from host tissues, ideally in the field.

Loop-mediated isothermal amplification (LAMP) is a newer molecular technique (Notomi, et al. 2000) that has the capability of providing point-of-care diagnostic testing (Niessen 2015). LAMP is less sensitive to inhibitors than PCR (Francois, et al. 2011), allowing for the use of crude DNA extracts, which can be rapidly obtained in field conditions (Kogovsek, et al. 2015, Colombari, et al. 2016). LAMP reactions are also isothermal, thereby removing the requirement for a thermocycler (Notomi, et al. 2000). While LAMP reactions can be performed on a simple hot plate, portable devices that feature a heating element along with fluorescence based detection are also available for real-time monitoring (Ebert, et al. 2010, Jenkins, et al. 2011). The use of portable LAMP devices fulfills the criteria for Early Detection and Rapid Response as outlined by the U.S. Department of the Interior for safeguarding natural resources (The U.S. Department of the Interior 2016), and would be a useful tool for the rapid detection of invasive

pathogens such as *R. lauricola*. LAMP assays have been shown to successfully diagnose plant diseases ranging from bacterial (Keremane, et al. 2015, Ocenar, et al. 2019), to viral (e.g. Congdon, et al. 2019), and fungal pathogens (e.g. Tomlinson, et al. 2010, Villari, et al. 2013, Aglietti, et al. 2019).

We recently designed a species-specific LAMP assay for the molecular detection of *R. lauricola* directly from host tissues in less than 20 min. of reaction time, and as early as 12 days post inoculation when using crude DNA extracts (Hamilton, et al. submitted). However, the assay has been only tested with artificially inoculated, and otherwise clean redbay samples, and only on a benchtop instrument in a laboratory setting. Hence, further testing is needed to make certain the approach is still suitable for field use, when samples might be colonized by multiple organisms, and when the equipment available in a laboratory setting is not at hand. Building upon our previous work, the aim of this study was to validate the performance of the recently designed *R. lauricola* species-specific LAMP assay for use in conditions comparable to the ones actually encountered by laurel wilt surveillance personnel. With respect to this, we divided the study in two successive phases with the specific objectives of (*i*) testing the suitability of the LAMP assay when using both high quality and crude DNA extracts of naturally infected sassafras and redbay wood samples in a laboratory setting, and (*ii*) determining the reliability of the LAMP assay when conducted directly in the field with portable equipment.

3.2 Materials and Methods

3.2.1 *Objective I: Testing of naturally infected samples*

3.2.1.1 *Sample collection*

Redbay and sassafras samples were collected in summer and autumn of 2018 at six locations in six different states of the southeastern United States, including Arkansas, Georgia, Louisiana, North Carolina, South Carolina and Texas. At each location, two sites were selected: one where laurel wilt disease is known to occur and one where the disease is not known to occur. From each diseased site, five symptomatic and five non-symptomatic trees were sampled (Table 3.1), while at the non-diseased sites only five non-symptomatic trees were sampled (Table 3.2). The only exceptions to this sampling design were that (i) in South Carolina, a non-diseased site could be not be found, and (ii) at the Louisiana location, only three trees instead of five were sampled per symptom type per site. Samples were comprised of branch or stem sprouts sections up to 10 cm long, and ranged between 4 mm and 15 mm in diameter. Sampling was achieved in collaboration with the local state and federal forestry agencies, who identified and collected the samples during their routine surveillance operations, and then shipped them overnight on ice to the University of Georgia. Once received, samples were stored at -20° C until further processing.

3.2.1.2 *DNA extraction in the laboratory*

A portion of the debarked branch was used to obtain both high quality and crude DNA extracts, which were prepared one at a time in a laminar flow hood to avoid cross contamination. DNA of samples from symptomatic trees were extracted both with high quality and crude extraction protocols, while samples from non-symptomatic trees, either from diseased or non-diseased sites, were extracted only with the crude extraction

protocol. For high quality DNA, samples were ground in liquid nitrogen and extracted with a Qiagen DNeasy plant mini kit (QIAGEN, Germantown, MD, USA) as per the manufacture's protocol. Crude DNA extracts were obtained following the protocol described in Hamilton, et al. (submitted). Briefly, 15-20 mg of wood tissue was shaved from samples with a scalpel after debarking and added to a 1.5 mL microcentrifuge tube containing 300 mL 5% Chelex 100 (BioRad, California, USA). Crude extraction samples were then boiled for 5 min., vortexed for 15 s, boiled again for 5 min., and vortexed again for 15 s, before being spun down in a mini-centrifuge (VWR, Radnor, PA, USA) at 3,884 x g for 30 s. The supernatant was used as template in the LAMP reactions.

3.2.1.3 *LAMP assay on a benchtop equipment*

LAMP reactions were performed as per Hamilton, et al. (submitted) with modifications. Each 25 μL reaction contained 15 μL 1x no-dye Isothermal Master Mix (Optigene, Horsham, UK), 2.8 μM of internal primers BT-FIP and BT-BIP, 0.28 μM of external primers BT-F3 and BT-B3, 0.8 μM F-Loop primer, 0.092 μM assimilating probe fluorescent (FAM) strand, 0.184 μM quencher strand, and either 5 μL of high quality or 1 μL of crude DNA template, and was brought up to volume in molecular grade water (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were carried out in 0.2 mL optically clear PCR eight-tube strips (Thermo Fisher Scientific), and run on a benchtop StepOnePlus Real-Time PCR system (Thermo Fisher Scientific) programmed with the following conditions: preheat to 65° C for 1 min., 65° C for 30 min. with fluorescence reading every minute, followed by a denaturing step at 85° C for 5 min. to halt the reaction and deactivate the polymerase. Each sample was run in triplicate, and each

reaction included high quality DNA of *R. lauricola* isolate FS-0009 (Hamilton, et al. submitted) as a positive control, in addition to a no-template negative control. A result was considered positive if two out of three technical replicates amplified.

3.2.1.4 *Fungal isolation*

To verify the presence of the pathogen in each tested sample, a portion of tissue of each sample was plated onto amended malt extract agar. Processing consisted of debarking the sample and surface sterilizing it by dipping it in 10% bleach for 10 s, then 70% EtOH for 10 s, and finally in sterile water for 10 s. After the sample was allowed to air dry, it was placed onto malt extract agar (VWR) amended with 200 ppm cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) and 100 ppm streptomycin (Sigma-Aldrich) (CSMA) (Harrington and Fraedrich 2010). Plates were wrapped with parafilm (Bemis, Neenah, WI, USA) and incubated at 25 °C in an Isotemp® incubator (Model 655D, Thermo Fisher Scientific) until *R. lauricola* growth was observed or a minimum of two-weeks had passed.

3.2.2 *Objective II: In field validation of the LAMP assay*

3.2.2.1 *Sample collection*

Redbay and sassafras samples were assayed in-field during the autumn and winter of 2019 and 2020 in five different southeastern states, including Georgia, Kentucky, North Carolina, South Carolina, and Tennessee (Table 3.3). At each location, testing was performed at a single site on a single day, with the exception of Georgia, where two sites were sampled for redbay and sassafras, but still on the same day. Site locating and

sampling was likewise achieved in collaboration with local state and federal forestry agencies. At least six individual, potentially diseased trees were selected in each state based on visual symptoms, including evident leaf wilt and sapwood discoloration upon debarking. Collected samples consisted of sapwood tissue from the main stem of larger trees or entire sections of stems from sprouts and saplings.

3.2.2.2 *DNA extraction in the field*

For in-field crude DNA extractions, we used a protocol similar to that previously used in the laboratory, but with some adaptations for use in the field. In particular, we wanted the protocol to be workable with minimal equipment and to avoid an open flame. Stem wood samples were acquired from visually symptomatic host trees only, and tools were disinfected with 10% bleach and 70% ethanol in between each sample. For smaller diameter trees, a hand saw was used to fell trees and expose discolored sapwood, and whole stem sections were then collected. For larger diameter trees, that could not be readily felled, a hatchet was used to remove bark and expose discolored sapwood, and samples were collected with a pocket knife. From each tree, part of the collected wood sample was processed for crude DNA extraction and LAMP analysis directly on-site, and the other portion of the sample was stored in a clean resealable plastic bag and transported in a cooler with ice packs to the laboratory for further analyses. One to three thinly shaved discolored pieces of sapwood (comparable in terms of weight to the 15-20 mg used for the crude extraction protocol in the laboratory) were placed into a previously prepared extraction tube containing 300 mL 5% Chelex 100. When the wood discoloration was particularly dark, less wood was used in the reaction to avoid excessive

background noise and inhibition of the LAMP assays. Extraction tubes were then placed in a floating tube rack and boiled in a low wattage 110 v 0.5 L electric kettle (DCIGNA190327, DCIGNA, Wuhan, China). The kettle was either plugged into an electricity supply when available (e.g. at the state forestry building nearby the sampling location in Tennessee) or into a 1500 W power inverter (Model # HD1500, Krieger, Fort Lauderdale, FL, USA) connected to the battery of a running vehicle. The lid was removed from the kettle to prevent accumulation of vapor, which would trigger its turning off. Samples were boiled for 5 min., manually shaken for 10 s, boiled for an additional 5 min., shaken again, and then stored in a cooler with ice packs until testing. Prior to testing, samples were centrifuged at 2,000 x g for 10 s on a Corning LSE #6770 mini-centrifuge (Corning, NY, USA) connected to the same power source previously used for the kettle, and only the supernatant was used as template in the LAMP reactions.

3.2.2.3 *LAMP assay on a portable device*

Preliminary testing was performed using a BioRanger™ Platform (Diagenetix, Honolulu, HI, USA), but because the device functioning was not consistent (data not shown), we decided to proceed with a Genie® III (Optigene) portable LAMP device. LAMP reactions in the field were performed using the same crude DNA reaction mix as described above, which had been prepared beforehand (24-36 hr. prior to testing) and already aliquoted into strips. Ready-to-use reaction strips, with each including a positive control and a negative control as described above, were double wrapped in aluminum foil to avoid photodegradation of the probes, and stored at 4° C until use. During travel, reagents were constantly kept on ice. After adding 1μL of template, LAMP reactions

were performed on the Genie® III (Optigene) device programmed with the following conditions: 65° C for 30 min. with the default fluorescence reading (15 s) followed by an inactivation step at 85° C for 5 min. to halt the reaction and deactivate the polymerase.

3.2.2.4 *Verification of field results*

Each field collected sample was transported back to the laboratory to verify the actual presence of *R. lauricola*. A portion of tissue of each sample was plated onto amended malt extract agar as previously described, and mycelial growth of *R. lauricola* (or its absence) was recorded. Discrepancies among field LAMP assay results and plating were further assessed as following: (i) if a sample tested positive according to the in-field LAMP assay, but *R. lauricola* was not recovered when plated, a high quality DNA extraction followed by a LAMP assay on the benchtop equipment was performed following the protocol previously described; (ii) if a sample tested negative according to the in-field LAMP assay, but *R. lauricola* was recovered when plated, a crude DNA extraction with less starting material, to reduce potential inhibition, was performed in the laboratory, followed by a LAMP assay on the portable device, as previously described.

3.2.2.5 *Statistical Analysis*

To determine the accuracy of the in-field LAMP assay compared to the actual presence of *R. lauricola* in the samples, the true positive rate (i.e. sensitivity) (Yerushalmy 1947) was calculated using Microsoft Excel ® 2013 (Ver 15.0.5215.1000).

3.3 Results

3.3.1 *Objective I: Testing of naturally infected samples*

3.3.1.1 *LAMP testing in the laboratory*

The results of the laboratory validation of naturally infected samples collected in diseased locations are reported in Table 3.1, while the samples collected in the non-diseased locations are reported in Table 3.2. Of the symptomatic samples we received, four out of five samples from both North Carolina and South Carolina and three out of five samples from both Arkansas and Georgia tested positive for laurel wilt disease, while none of the symptomatic samples collected in Louisiana or Texas tested positive for the disease. All results were consistent when comparing high quality or crude DNA extracts. None of the non-symptomatic samples we received, for which only crude DNA was tested, gave a positive result with the LAMP assay, regardless if they were collected from a diseased or non-disease location.

3.3.1.2 *Fungal isolation*

Results of the fungal isolations were all consistent with the results of the LAMP assay (Tables 3.1 and 3.2): growth of *R. lauricola* was only observed for those samples that tested positive with the LAMP assay, while no growth was observed when the results of the LAMP assay were negative.

3.3.2 *Objective II: In field validation of the LAMP assay*

3.3.2.1 *LAMP assay on a portable device*

The results of the in-field testing of symptomatic samples are reported in Table 3.3 and an example of individual runs results from each state is shown in Figure 3.1. The LAMP assay was capable of successfully detecting *R. lauricola* directly at the sites where trees were identified, using a field appropriate crude extraction protocol and a Genie® III LAMP device. No amplification in the negative control wells was observed and all positive controls amplified successfully. In Georgia, three out of four redbay and three out of four sassafras tested positive for laurel wilt disease, while in South Carolina, three out of five redbay and three out of three sassafras tested positive. In North Carolina, four out of six redbay tested positive, while in both Tennessee and Kentucky, six out of six sassafras tested positive.

3.3.2.2 *Verification of field results*

Results of the fungal isolations were mostly consistent with the results of the LAMP assay (Table 3.3), but discrepancy in some of the results was observed. Specifically, *R. lauricola* could not be isolated from two of the sassafras samples that tested positive with LAMP at the Georgia site, and for one of the sassafras samples that tested positive at the South Carolina site. High quality DNA of these samples was extracted in the laboratory and tested again with a LAMP assay on a benchtop equipment, and a positive result was observed in all cases (Figure 3.2), suggesting that the results previously observed in the field were a true positive. On the other hand, the two redbay samples that tested negative in the North Carolina site, and one of the redbay samples that tested negative in the South Carolina site resulted in growth of *R. lauricola* once plated, indicating that the results previously observed in the field were a false negative. To further assess if the false

negative was potentially due to inhibition of the reaction, a new crude extraction with less starting plant material was performed in the laboratory, followed by a LAMP reaction on the portable device. Results of this second reaction were all positives for the presence of *R. lauricola* (Table 3.3), confirming that the false negative results observed in the field were probably due to inhibition of the reaction. Overall true positive rate of the LAMP assays performed in field conditions was 90.32%.

3.4 Discussion

This study reports the implementation of a LAMP based assay for the rapid and accurate molecular detection of the laurel wilt disease pathogen, *R. lauricola*, directly in-field without the reliance on an external laboratory. Building upon previous work that developed the species-specific LAMP primers (Hamilton, et al. submitted), we were able to show that the assay is capable of correctly detecting the pathogen even in naturally infected samples, when multiple organisms might be colonizing the tissues. Moreover, we were able to validate the reliability of the LAMP assay when conducted directly in the field on portable equipment. True positive rate of the in-field testing was 90.32%, which is satisfactory, and comes with the great advantage that the diagnostic response (which included sampling of identified trees, crude DNA extraction and LAMP reaction) was completed within an hour of arriving on-site. This is a huge improvement when compared to previous confirmation strategies that could take up to a week or more (Dreaden, et al. 2014, Hughes, et al. 2015), and exemplify how the implementation of LAMP could provide a robust detection tool that could ultimately improve integrated pest management of laurel wilt disease. Indeed, by providing an accurate diagnosis at the point of infection,

management practices, including for instance the decision to conduct sanitation removals of infected trees, could be made without delay.

Due to the generic features of laurel wilt disease symptoms (Fraedrich, et al. 2008), the disease is often misdiagnosed in the field. In our experiment, for instance, we received several putatively infected redbay and sassafras samples collected by different personnel involved in the disease surveillance operations. All of these putatively infected samples displayed some degree of wilting and sapwood discoloration which resembled infection by *R. lauricola*, yet many tested negative, pointing out how diagnosis based on visual symptoms still needs to be confirmed, which so far had to rely on external laboratories. Particularly misleading are the symptoms observed in lauraceous species that have been colonized by a different beetle, *Xylosandrus compactus*, which also results in sapwood discoloration (Chong, et al. 2009).

One of the challenges of performing point-of-care diagnostic testing is the limited control over sample quality. In our experiment, for instance, we had three false negatives. When samples were retested in the laboratory, the reaction worked properly and samples tested positive, as they should have reacted in the field. The only difference in these cases between the protocol used in the field and the protocol used in the laboratory, was that in the laboratory we used less starting material for the extraction, which strongly suggests that the reactions in the field failed due to the presence of inhibitors in the template. The possibility that the false negatives in the field were due to degradation of the reagents is not plausible because the positive controls always amplified correctly. The presence of inhibitors in naturally infected, darkly pigmented samples was a common issue that we encountered as we were troubleshooting the crude DNA extraction protocol. This early

finding prompted us to decrease the amount of wood used during extraction and to reduce the volume of template per reaction from 5 µL to 1 µL, compared to the protocol described in Hamilton, et al. (submitted). Despite the presence of inhibitors, however, extracting from discolored wood tissues is still preferred, as it maximizes the probability of pathogen detection.

Three of the sassafras samples that tested positive for the presence of *R. lauricola*, both in the field and with further assessment in the laboratory, failed to exhibit growth when plated on growing media. This might be because the fungus was no longer viable, while its DNA was still present. The in-field testing phase of this study was conducted in the autumn and winter seasons, when sassafras has already shed its leaves, which made it difficult to determine the age of the infection on sampled tress. The three samples in question may have been long dead, and secondary colonizers might have already superseded *R. lauricola*. Yet, the LAMP assay was still able to detect the DNA of the pathogen.

Future work should focus on further optimization of in-field LAMP based diagnostic procedures. This includes, for instance, the development of commercially available lyophilized ready-to-use reagents and a more efficient crude DNA extraction protocol, preferably without the need of a dedicated power source (see for instance Aglietti, et al. 2019). LAMP technology is already appreciated because it lacks the need to extensively train the personnel performing the assay (Tomlinson, et al. 2013, Thiessen, et al. 2018). These improvements could reduce even further the technical expertise needed and increase the ease and portability of the assays. In conclusion, this study provides a successful example that should foster the use of LAMP based technology to

rapidly diagnose laurel wilt disease or other forest pathogens directly in-field, ultimately enabling better disease management. The capability of LAMP to provide rapid and accurate in-field confirmation of forest diseases, in fact, successfully addresses the requirements of a framework for an effective early detection and rapid response system to mitigate the impact of these damaging agents (Luchi, et al. 2020).

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Table 3.1: Results of the laboratory LAMP assay testing and corresponding validation of samples collected from diseased sites. Results are reported as number of positive assays or fungal isolations out of the total samples tested.

State	Collecting Agency	Site name	Host species	Symptomatic samples			Non-symptomatic samples	
				High Quality DNA LAMP	Crude DNA LAMP	Fungal Isolation	Crude DNA LAMP	Fungal Isolation
Arkansas	Arkansas Department of Agriculture, Forestry Division	Conway Cemetery Historic State Park	Sassafras	3/5	3/5	3/5	0/5	0/5
Georgia	Georgia Forestry Commission	Toomsboro	Redbay	3/5	3/5	3/5	0/5	0/5
Louisiana	United States Forest Service	Kisatchie National Forest	Sassafras	0/3	0/3	0/3	0/3	0/3
North Carolina	North Carolina Forest Service	Cabin Lake Road	Redbay	4/5	4/5	4/5	0/5	0/5
South Carolina	South Carolina Forestry Commission	Sesquicent-ennial State Park	Redbay	4/5	4/5	4/5	0/5	0/5
Texas	United States Forest Service	John H. Kirby State Park	Redbay	0/5	0/5	0/5	0/5	0/5

Table 3.2: Results of the laboratory LAMP assay testing and corresponding validation of samples collected from non-diseased sites. Non-diseased sites could not be located in South Carolina. Results are reported as number of positive assays or fungal isolations out of the total samples tested.

State	Collecting Agency	Site name	Host species	Crude DNA LAMP	Fungal Isolation
Arkansas	Arkansas Department of Agriculture, Forestry Division	Beirne Bottom Road	Sassafras	0/5	0/5
Georgia	Georgia Forestry Commission	University of Georgia	Redbay	0/5	0/5
Louisiana	United States Forest Service	Fishville	Sassafras	0/3	0/3
North Carolina	North Carolina Forest Service	Ditchbank Road	Redbay	0/5	0/5
Texas	United States Forest Service	The Woodlands	Redbay	0/5	0/5

Table 3.3: Results of the in-field LAMP assay testing and corresponding laboratory validation.

State	Date	Site name	Host species	Sample Number	In-field LAMP	Fungal Isolation[a]	Further Assessment[b]
Georgia	18 Nov 2019	Di-Lane Wildlife Management Area	Redbay	1	-	-	
				2	+	+	
				3	+	+	
				4	+	+	
		Mitchell Road	Sassafras	1	-	-	
				2	+	-[a]	+
				3	+	+	
				4	+	-[a]	+
Kentucky	11 Mar 2020	Fort Campbell	Sassafras	1	+	+	
				2	+	+	
				3	+	+	
				4	+	+	
				5	+	+	
				6	+	+	
North Carolina	04 Mar 2020	Mount Olive	Redbay	1	+	+	
				2	+	+	
				3	+	+	
				4	+	+	
				5	-	+[a]	+
				6	-	+[a]	+
South Carolina	26 Nov 2019	Sesquicentennial State Park	Redbay	1	-	+[a]	+
				2	+	+	
				3	-	-	
				4	+	+	
				5	+	+	
			Sassafras	1	+	-[a]	+
				2	+	+	
				3	+	+	
Tennessee	23 Jan 2020	Cherokee Park	Sassafras	1	+	+	
				2	+	+	
				3	+	+	
				4	+	+	
				5	+	+	
				6	+	+	

[a]Discording results between in-field LAMP assays and fungal isolations, which prompted a further assessment of the sample. [b]Samples that were positive in-field but negative when plated were further assessed by high quality DNA extraction followed by a LAMP assay on a benchtop equipment; samples that were negative in-field but positive when plated were further assessed by a crude DNA extraction followed by a LAMP assay on a portable device.

Figure Legend

Figure 3.1. Examples of results of the in-field tests performed on a Genie® III portable LAMP device using symptomatic samples from (A) Georgia (GA), (B) Kentucky (KY), (C) North Carolina (NC), (D) South Carolina (SC), and (E) Tennessee (TN). Template of each reaction was 1 μL of the 5% Chelex 100 crude extracted DNA. Each reaction was run in three technical replicates. NTC, no template control; PC, positive control.

Figure 3.2. Example of a laboratory assessment of discording results between in-field LAMP assay using a portable device and fungal plating. Samples that tested positive in the field, but that did not result in growth of *Raffaelea lauricola* when plated in growing media, were further assessed by high quality DNA extraction followed by a LAMP assay on a benchtop equipment. Each reaction was run in three technical replicates. GA, Georgia; SC, South Carolina; NTC, no template control; PC, positive control.

Figure 3.1

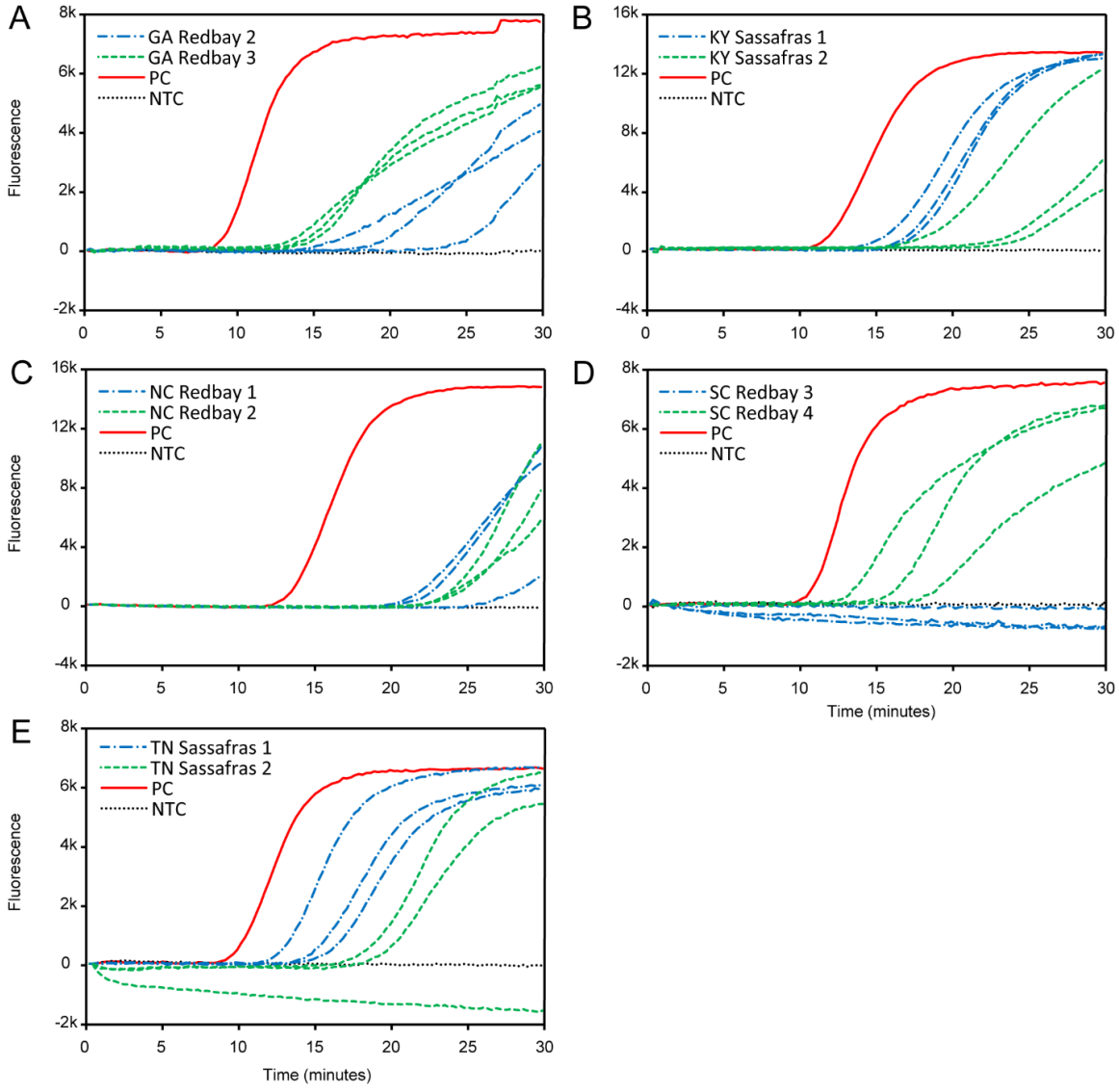
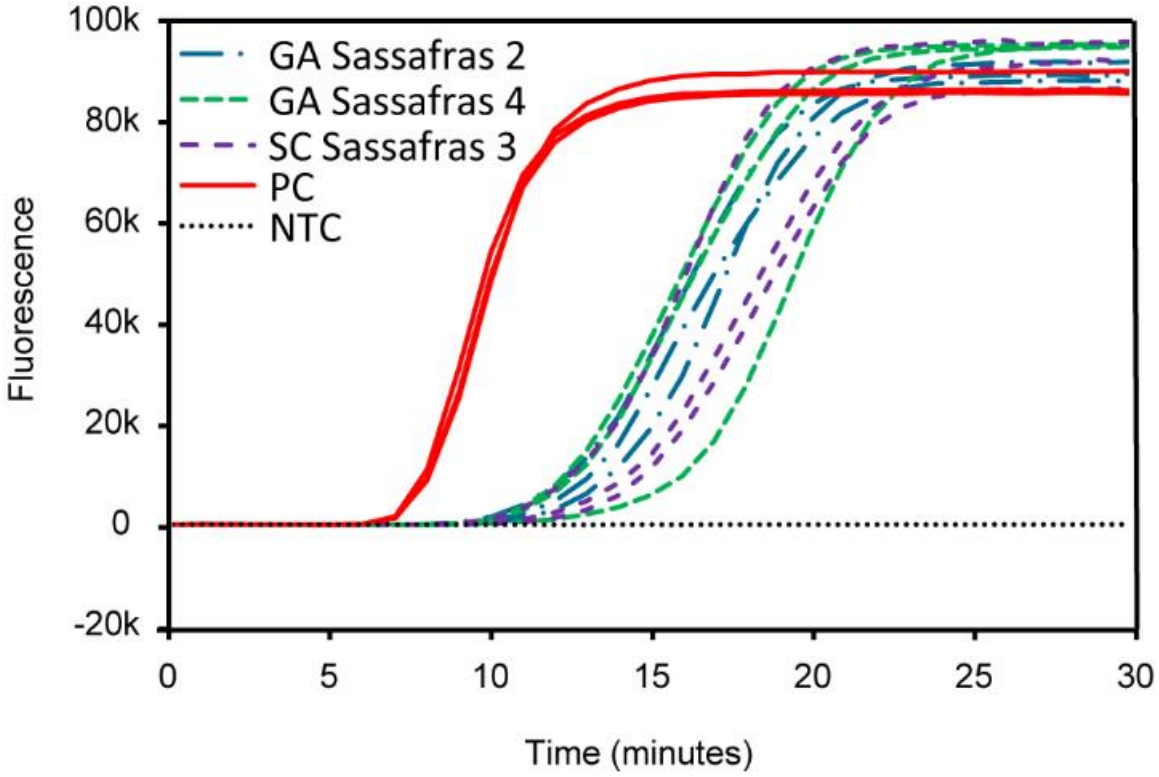

A
GA Redbay 2
GA Redbay 3
PC
NTC
Fluorescence
B
KY Sassafras 1
KY Sassafras 2
PC
NTC
C
NC Redbay 1
NC Redbay 2
PC
NTC
Fluorescence
D
SC Redbay 3
SC Redbay 4
PC
NTC
Time (minutes)
E
TN Sassafras 1
TN Sassafras 2
PC
NTC
Fluorescence
Time (minutes)

Figure 3.2


100k
80k
60k
40k
20k
0
-20k
Fluorescence
0
5
10
15
20
25
30
Time (minutes)
GA Sassafras 2
GA Sassafras 4
SC Sassafras 3
PC
NTC

CHAPTER 4

THESIS CONCLUSIONS

4.1 Conclusions

Since the introduction of *Xyleborus glabratus* Eichoff and *Raffaelea lauricola* (T.C. Harr. Fraedrich & Aghayeva) near Savannah, Georgia in 2002, millions of lauraceous trees have died in the southeastern United States (Fraedrich, et al. 2008, Hughes, et al. 2015). Laurel wilt disease has spread quickly, jeopardizing the health of forests further away from the coast as it continues its destructive trajectory (Olatinwo, et al. 2016, Mayfield, et al. 2019, Loyd, et al. 2020). Virulent invasive species disturb the balance of natural ecosystems in ways that are difficult to predict, thus their containment is a priority (Santini, et al. 2013, Bonello, et al. 2020). Early detection systems, in particular, would be paramount to containing their spread and preserving forest health (Aglietti, et al. 2019, Luchi, et al. 2020). Unfortunately, in the case of laurel wilt, a lengthy process is currently required to accurately confirm the diagnosis, and any management actions are thus delayed by days (Dreaden, et al. 2014, Hughes, et al. 2015). This thesis sought to develop a loop-mediated isothermal amplification (LAMP) assay (Notomi, et al. 2000, Nagamine, et al. 2002, Kubota, et al. 2011) for the diagnostic testing of the laurel wilt disease pathogen, *R. lauricola*, directly on-site. Thanks to the results of this study, forest managers will have the opportunity to implement containment strategies without delay, and limit this destructive disease from spreading further.

The second chapter of this thesis describes the design and optimization of a *R. lauricola* species-specific LAMP assay. The objectives of this study were to 1) develop and validate a *R. lauricola* species specific LAMP primers, 2) determine the earliest time after host infection that the assay can detect *R. lauricola* in host sapwood, and 3) test the assay's suitability for amplification of the target species directly within host plant tissue using both high quality and crude DNA extracts, and from *X. glabratus* tissue using crude DNA. We chose the β-tubulin gene of *R. lauricola* as target region for the reaction, and screened our primer set against the high quality DNA of 20 different *Raffaelea* species, three distantly related fungal species, one oomycetes species, and redbay DNA. As expected, amplification was observed only in those reactions that contained *R. lauricola* DNA. We also showed that the assay is quite sensitive, amplifying to detectable levels as low as 0.5 pg of DNA from pure culture mycelia, and as few as 50 *R. lauricola* conidia per reaction.

We then conducted a time course experiment with artificially inoculated redbay saplings to determine a limit of detection while testing directly from host plant tissue. Each sapling was inoculated with a spore suspension of *R. lauricola* or sterile water and then a subset of three plants per treatment was destructively sampled every two days. Testing directly from wood tissue at 50 cm above the inoculation point, we observed positive results from our assay as early as 10 and 12 days post inoculation using high quality DNA extracts or crude DNA extracts, respectively. Female *X. glabratus* beetles were also assayed using crude DNA and we observed amplification across all specimens. The results from this study show that in just a few hours after harvesting a sample, the LAMP assay can positively detect *R. lauricola* directly from host tissue.

The third chapter of this thesis applied our designed LAMP assay to naturally infected samples, both in our laboratory and in the field. Our objectives were to 1) test the suitability of the LAMP assay when using both high quality and crude DNA extracts of naturally infected redbay and sassafras wood samples in a laboratory setting with benchtop equipment and 2) determine the reliability of the LAMP assay when conducted directly in the field on portable equipment. Through experimentation, we discovered that reducing the volume of crude DNA template per reaction significantly reduced background noise and reaction inhibitors without diluting DNA concentrations to non-detectable levels. We collaborated with forest health professionals to collect symptomatic and asymptomatic redbay and sassafras wood tissues from the states of Arkansas, Georgia, Louisiana, North Carolina, South Carolina, and Texas. The assay detected *R. lauricola* within naturally infected symptomatic wood with both high quality and crude DNA, providing a rapid and accurate confirmation of samples with varying symptom presentation. Although we did not have any positive results when testing asymptomatic samples with crude DNA, each symptomatic sample that tested positive with LAMP also resulted in *R. lauricola* growth when plated, and vice versa.

To conduct in-field testing, we assembled a field kit that included a Genie® III portable LAMP device (Ebert, et al. 2010) in addition to a low-power electric kettle and a power inverter that could turn a vehicle into a mobile laboratory. With our portable testing kit, we conducted field testing of symptomatic redbay and sassafras specimens in the southern states of Georgia, Kentucky, South Carolina, North Carolina, and Tennessee. We were able to rapidly locate, process, and assay symptomatic wood tissue directly on-site to provide a definitive diagnosis of laurel wilt disease, which we then verified in our

laboratory with traditional plating and additional LAMP assays. The results from this study show that LAMP technology can accurately diagnose laurel wilt disease directly on site in minutes, with a true positive rate of 90.32%.

4.2 Future Research Directions

The results of our research have shown that LAMP technology can rapidly and accurately detect *R. lauricola* directly in-field. Implementation of this technology could inform forest health professionals so that they can immediately put into action Integrated Pest Management Strategies to contain the spread of laurel wilt disease. Moreover, the implications of these results could expand to other forest pathosystems as well. To facilitate the implementation of LAMP technology in forest systems, encourage its use by nontechnical personnel, and increase testing ease, we recommend the future work and research:

1) Our in-field study utilized a LAMP reaction mix which had been prepared the day before traveling to a field site to conduct testing. While the stability of our reaction mix was observed for at least 36 hours if kept under refrigeration, a lyophilized ready-to use reaction mix would eliminate the need for refrigeration. A lyophilized ready-to use reaction mix could be made commercially available and would also remove the technical expertise needed to assemble the reagents, significantly increasing the accessibility to LAMP technology.
2) Throughout our study, we extracted crude DNA by boiling samples with 5% Chelex 100. While adequate for our in-field analysis, an extraction protocol

that does not require a dedicated power supply would further reduce the amount of equipment needed to conduct LAMP assays in-field. Developing a "power-source free" method of DNA extraction as in Aglietti, et al. (2019), but for wood tissues, would enable personnel to conduct LAMP assays out of a backpack as opposed to being tethered to a vehicle.

3) LAMP technology is routinely used in agricultural and horticultural settings (e.g. King, et al. 2018, Congdon, et al. 2019). However, developing species-specific LAMP assays for other forest diseases would be extremely valuable, and improve the management of forest pests and pathogens by providing a quick and early detection procedure.
4) DNA technologies usually require a high degree of technical expertise to be implemented, but because of its features, LAMP can be performed by nontechnical users as well after minimal training (e.g. Tomlinson, et al. 2013, Thiessen, et al. 2018). Outreach efforts with state and federal forest health professionals should focus on imparting the knowledge to enable the wide scale use of LAMP technology in forest settings. The creation of simple, nontechnical training materials and the conducting of training sessions would facilitate the implementation of LAMP technology to a wider audience.

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