THE SURVIVAL AND LONGEVITY OF *Raffaelea lauricola* AND THE REDBAY AMBROSIA BEETLE (*Xyleborus glabratus*) IN CHIPPED AND INTACT WOOD

By

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To my family, thank you for all of your love and support

For life:

It's a dangerous business going out of your door, Frodo. You step into the road, and if you don't keep your feet, there is no telling where you might be swept off to.
- J. R. R. Tolkien, The Lord of the Rings, The Fellowship of the Ring

For Science:

One never notices what has been done; one can only see what remains to be done.
- Marie Curie

For Knowledge:

If I have one apple and you have one apple and we trade, we each have one apple. If you have an idea and I have an idea, and we trade, we now each have two ideas.
- George Bernard Shaw

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Laurel wilt is a relatively new tree disease in the southeastern U.S. that kills members of the Lauraceae plant family. This irreversible disease is caused by the exotic fungus *Raffaelea lauricola* and the exotic redbay ambrosia beetle, *Xyleborus glabratus*, which serves as a vector for the pathogen. *R. lauricola* colonizes the sapwood and can move into all portions of the tree. The presence of the fungus inside the tree causes it to wilt and die, within a few weeks during the summer. To date, the disease has killed millions of trees in this family and currently occurs from North Carolina, west to Mississippi and to south Florida. This study was designed to examine 1) the survival of the beetle and fungus in chipped redbay (*Persea borbonia*) trees that were killed by laurel wilt, 2) identify how long the fungal pathogen can persist in standing dead trees, 3) determine the thermal limit of the fungal pathogen, and 4) potential endophytic fungal competitors in redbay trees. Finally, we explored the effectiveness of protecting redbay trees with fungicide injections before they are attacked by the redbay ambrosia beetle.

We monitored the survival of *X. glabratus* and *R. lauricola* in wood chips that were generated using a standard commercial-grade chipper over seven months. After

two weeks, fourteen *X. glabratus* were found in the wood chips while 339 *X. glabratus* emerged from non-chipped bolts under netting during the same period. From the wood chips, *R. lauricola* was only recovered after two days post chipping indicating that the pathogen is not likely to survive outside its beetle vector or intact plant host or be moved from wood chips to other species. *R. lauricola* persisted in dead, standing trees for fourteen months and its optimum growth temperature was 28° C. Although this temperature is below the IPPC phytosanitary guidelines for treating wood pallets, it is similar to the maximum daily temperature that the fungus would be exposed to in temperate and subtropical areas. Finally, pre-treating asymptomatic trees with propiconazole protected over 70% of the study trees.

CHAPTER 1
INTRODUCTION TO THE LAUREL WILT PATHOSYSTEM

Laurel Wilt

Laurel wilt (LW) is a tree wilt disease that affects the Lauraceae plant family and caused by a non-native fungal pathogen (*Raffaelea lauricola* T. C. Harrin., Aghayeva, & Fraedrich) that is vectored by a non-native redbay ambrosia beetle, *Xyleborus glabratus*, (Eichhoff, Coleoptera: Curculionidae) (Hanula et al. 2008). *X. glabratus* was first detected near Port Wentworth, GA in 2002 and mortality to redbay trees (*Persea borbonia*) was first observed around that time (Rabaglia 2006, Fraedrich et al. 2008). To date, millions of trees in the Lauraceae have been killed. In addition to redbay, swampbay (*P. palustris*), scrubbay (*P. humilis*), avocado (*P. americana*), sassafras (*Sassafras albidum*), pondberry (*Lindera melissifolia*), northern spicebush (*L. benzoin*) and pondspice (*Litsea aestivalis*) have been found to be susceptible in natural areas (Fraedrich et al. 2006, Fraedrich et al. 2008, Hanula et al. 2008 and Hughes et al. 2011). Both pepperleaf (*Licaria triandra*) and spicebush (*L. latifolia*) were found to be susceptible when artificially inoculated. Diseased camphor (*Cinnamomum camphora*) has also been found infected in the field, however, entire trees are not dying, just portions are developing branch dieback (Smith et al. 2009).

R. lauricola is carried by ambrosia beetles inside their bodies near the mandibles, in sacs called mycangia (Beaver 1989). The fungal pathogen gains entry into trees through the boring activity of the beetle.

LW currently occurs from North Carolina (Forest Health Notes 2011), west to Mississippi (Riggins et al. 2010) and to south Florida (DPI 2012, USDA Laurel Wilt Distribution Map). The northern expansion of LW has not progressed as rapidly as the southern movement, although the disease has reached four southeastern counties of North Carolina. LW is likely to continue making long-distance jumps in its range due to

people transporting infested host material (Mayfield et al. 2009, Chemically Speaking 2009).

Symptoms of LW in redbay include the rapid wilting of the canopy, where the leaves may stay attached to the tree for over a year and dark blue to black staining of the sapwood, which is only visible when the bark is removed. During periods of heavy *X. glabratus* infestation, boring dust piles or boring dust tubes can be observed on trunks of trees; these are created by the boring activity of the beetle. The disease is irreversible and has caused the mortality of at least 90% of redbay greater than 1 inch diameter at Fort George Island, FL. (Fraedrich et al. 2008) and 100% of mature redbays in Etoniah Creek State Forest, FL over two years. (Shields et al. 2011).

In Chapter 2, I examine the effectiveness of chipping dead trees to eliminate both the fungus and beetle. The results clearly showed that both the beetle and fungus do not tolerate chipping, demonstrating that it is a very effective means of managing these exotic pests. Data from Chapter 3 indicates that *R. lauricola*'s optimum growth occurs a 28º C and that it can be killed at 47º C and that *R. lauricola* can persist on wood as a saprophyte. In Chapter 4, I explored how long *R. lauricola* can persist in a standing dead redbay tree and its distribution inside the stem of dead trees. I also identified sixty fungal endophytes from the sapwood of 25 trees that were growing in LW symptomatic and asymptomatic redbay trees in Washington Oaks Garden State Park, Flagler County, FL. Our 18-month study found that *R. lauricola* persisted for over 14 months and that it can colonize sapwood up to 7 cm below the vascular cambium. The final chapter of this dissertation presents data on the effectiveness of injecting trees with a systemic fungicide before they are infected with *R. lauricola*. Pre-injecting trees with a fungicide creates a barrier against the fungus in the tree's water conducting tissue. Overall, we found that injecting trees with

propiconazole before they are attacked by the redbay ambrosia beetle provides better than 70% chance that they will be temporally protected against the LW pathogen, *R. lauricola*.

Raffaelea lauricola

LW is caused by the fungus *R. lauricola*, an ophiostomatoid relative of the fungus that causes Dutch elm disease. *R. lauricola* is one of several fungal symbionts of *X. glabratus* (Harrington and Fraedrich 2010) and typical of ambrosia beetles, it is assumed that *X. glabratus* feeds on the fungal symbionts it carries with it in its mandibular mycangia (Beaver 1989, Hanula et al. 2008, Mayfield et al. 2009). Fungi that are symbionts of ambrosia beetles are asexual haploids that typically produce small conidia in tight clusters called sporodochia (Harrington and Fraedrich 2010). It is thought that adult beetles and larvae feed on the spores that develop inside the galleries created by female beetles (Batra 1967, Biedermann and Taborsky 2011). To some degree, *R. lauricola*, and possibly the other mycangia fungi, are capable of persisting as saprophytes inside dead or dying trees for many months.

The exact mechanism by which trees die is unknown. To date, no phytotoxic compounds have been identified (personal communication with the Forest Pathology Laboratory, University of Florida) and it does not seem as though the mycelia or spore production is so great as to overwhelm a tree's water conduction system (Inch and Ploetz 2011). When fungal tissue was observed with a scanning electron microscope, lipids and tyloses were present, which would block water flow, ultimately causing the trees to wilt. Since toxins and an overabundance of fungal tissue have not been found, it is possible that trees die due to an over stimulation of their defense system (Hulcr and Dunn 2012).

To explore how many spores *X. glabratus* may deposit inside a tree, Harrington and Fraedrich (2010) macerated beetles from Asia and the southeast USA and cultured their

mycangial contents. They found that *X. glabratus* may carry several species of fungi and that they carry between 10^3 to 10^6 spores of *R. lauricola* in their mycangia. A study at the University of Florida found that as few as 100 *R. lauricola* spores were enough kill healthy swampbay and avocado saplings (Hughes et al. unpublished). Hughes et al. (unpublished) and Dreaden et al. (2011) also explored the genetic variation of *R. lauricola* isolates from Asia and North American populations. When using AFLP profiles and microsatellites as genetic markers, they found that *R. lauricola* showed little to no genetic differences.

Since *R. lauricola* has been recovered from experimentally infected and naturally infected leaves, stems, roots and trunks of trees, it illustrates that the fungus is a systemic pathogen and quite mobile within trees, however, the fungus does not move into fruits (Inch and Ploetz 2011). It is common for redbay, and many other species, to produce green sprouts and suckers from the trunk and root system as a tree is dying. This is not a sign of resistance or recovery. These sprouts are a physiological response to stress by the tree. Of the millions of trees that have died from LW, there have been no reports of trunk or root sprouts persisting and developing into a new tree. In a host range study, 30 different plant taxa representing 6 families were challenged with *R. lauricola*. Fortunately in North America, only the Lauraceae were found to be susceptible to LW (Ploetz and Smith unpublished).

Xyleborus glabratus

Xyleborus glabratus Eichhoff (Coleoptera: Scolytidae) is a 2mm long wood-boring ambrosia beetle native to Asia. It is thought that *X. glabratus* and its fungal symbionts (including *R. lauricola*) entered the U.S. prior to 2003 and spread into the local forests (Rabaglia et al. 2006). The home range of *X. glabratus* is thought to be from Japan to

Taiwan (and likely other areas of southeast Asia) where it is not known to be a pest. In S.E. Asia, *X. glabratus* has been known to use members of the Dipterocarpaceae, Fagaceae, Fabaceae and Lauraceae (Rabaglia et al. 2006).

X. glabratus Males are flightless and haploid while females are diploid and act as the dispersal agents of the species. *X. glabratus* is most numerous in late summer and is most active just before sunset, and more often than not, beetles are captured in flight between 1 and 3 meters above the ground (Hanula et al. 2008). Only female *X. glabratus* beetles carry fungi, which reside in the beetle's mandibular mycangia (Hanula et al. 2008, Mayfield et al. 2008, and Beaver 1989). As females construct galleries in the sapwood of trees, fungal spores are released to develop into a fungal layer upon which the adults and larvae feed (Hanula et al. 2008, Rabaglia et al. 2006).*X. glabratus* has a haplo-diploidy reproductive strategy. Females have the ability to lay haploid eggs in the absence of males. The haploid eggs develop into males, which allows for the female to mate with male progeny to produce diploid eggs that will develop into females. Thus, one female can give rise to a new population of ambrosia beetles (Hamilton 1967 and Normark et al. 1999). The reproductive cycle of *X. glabratus* is thought to be between 50 – 60 days (Hanula et al. 2008) where females may lay between 1 and 8 eggs in the galleries she created (Brar unpublished).

In general, ambrosia beetles are typically associated with stressed, dying or dead trees. Ambrosia beetles use olfactory cues to identify stressed or dead trees which are then colonized by the beetles (Hulcr and Dunn 2011). One of the factors that makes LW unique is that *X. glabratus* attacks apparently healthy living trees in the Lauraceae. The cues directing *X. glabratus* to bore into living trees in the southeastern United States are unknown, but it may be due to an olfactory mismatch (Hulcr and Dunn, 2011). If this were

the case, then dead trees in Asia would have a similar olfactory signature as healthy Lauraceous trees in North America, but this has not been substantiated by research yet.

In newly infected trees, there is not an overwhelming massive attack by *X. glabratus* on one tree, possibly indicating that a single beetle has bored into the tree depositing enough *R. lauricola* spores to kill the tree.

Future Concerns of Laurel Wilt

The complex biology of bark and ambrosia beetles, not only in their native range but in novel areas as well, makes managing diseases vectored by them more difficult (Six and Wingfield 2011). In the case of LW, what the ultimate national and regional biodiversity implications are have yet to be realized (Mayfield et al. 2009), however, it is likely to be significant with the severe reduction of at least a couple of species within the Lauraceae and associated organisms, such as obligate arthropods (Goldberg and Heine 2009).

R. lauricola has been cultured out of the mycangia of ambrosia beetles other than *X. glabratus* (Ploetz et al. unpublished) and *R. lauricola* has been found in the sapwood of other dead tree species not known to be hosts of *X. glabratus* (Smith and Black, unpublished). Whether *R. lauricola* was the cause of the tree mortality is not known, but it should be a cautionary note that other beetle species are potentially moving *R. lauricola* into other tree species, which may serve as reservoirs for the fungus in the future.

X. glabratus surveys have been conducted in northern Florida and coastal Georgia since the initial detection in 2002. Researchers have found the beetle to still be present in low numbers (Hanula et al. 2008, Hughes et al. unpublished), which is a sign that *X. glabratus* is persisting on either the few remnant Lauraceous hosts or it has found an alternative host to complete its life cycle.

The Danger of Moving Exotic Wood Boring Insects

Wood boring beetles, both native and non-native, can be moved considerable distances within unfinished wood products, causing direct damage or serving as vectors of plant disease outbreaks in new locations. There have been many outbreaks of exotic species arriving in places where they did not exist before and they have affected all types of plants; agricultural monocots and dicots, ornamentals, and trees (Brasier 2008).

The movement of exotic organisms has increased dramatically over the last two hundred years (Liebold et al. 1995). Often, the impact is limited and does not pose a threat to food security and ecological integrity; however, there are many examples of new exotic pests that have led to significant ecological damage (Pimentel 1986, Liebhold et al. 1995). More specifically, wood-borne pests have threatened silviculture production (Pimentel 1986), urban trees (Dreistadt et al. 1990), and disrupted forest ecology (Liebhold et al. 1995). The primary means of movement of plant pests has been through the movement of ornamental plants from other countries (Perrings et al. 2005) and in untreated wood products (USDA 1992, USDA 1993, Bridges 1995). At U.S. ports of entry alone, 6788 individual exotic scolytid beetle interceptions were made between 1985 and 2000 at inspection stations (representing 67 species), one of which was *X. glabratus* in 2002. These beetles came from 49 different countries (Haack 2001).

A few of the important exotic insect pests of trees that have become established in the U.S. are: the Gypsy moth (*Lymantria dispar*), common pine shoot beetle (*Tomicus* sp.), sirex woodwasp (*Sirex noctilio*); and its associated pathogenic fungus, *Amylostereum areolatum*, the exotic beech scale (*Cryptococcus fagisuga*) that began transmitting a native fungus (*Nectria coccinea* var. *faginata*) which has led to a serious decline of American beech trees (*Fagus grandifolia*), the walnut twig borer (*Pityophthorus juglandis*), European

elm beetle (*Scolytus multistriatus*), the vector of *Ophiostoma novo-ulmi* - causal agent of Dutch elm disease (DED), Asian longhorned beetle (ALB) (*Anoplophora glabripennis*), emerald ash borer (EAB) (*Agrilus planipennis*) and the redbay ambrosia beetle (*Xyleborus glabratus*).

DED and EAB have decimated urban and forest elm and ash stands and ALB is threatening many species of hardwood across the N.E. US. Although keeping these pests from expanding their range through active management has not happened, it is important that federal, state, local governments and private landowners work to limit the spread of these diseases and pests through tree removal, proactive pruning and sanitation, public education outreach and prohibitions on moving untreated wood products.

In an attempt to combat this problem, wood sterilization treatments have been developed to kill wood-boring pests either before they depart or upon arrival at the port of entry. The main ways wood has been phytosanitized are by chipping the wood into small pieces, fumigation, or exposure to high temperatures (Denlinger and Yocum 1998, Burks et al. 2000, Wang et al. 2000, Cyr 2004, Simpson 2004, McCullough et al. 2007, Nzoko et al. 2008, Haack and Petrice 2009). International Standards for Phytosanitary Measures (CFR 2002, FAO 2009) require that wood products that are used in commercial shipping must be heat-treated for a minimum of 30 minutes to a core temperature of 56° C. Most nations honor this guideline and certify that their wood dunnage or unfinished wood products have been properly treated. However, even with phytosanitary rules in place, pests like the *X. glabratus* and other exotic wood boring beetles have, and will continue to arrive in the USA and damage urban and natural forests (Atkinson et al. 1991, Wang et al. 2000, Haack and Poland 2001, Evans and Oszako 2007, Nzoko et al. 2008, Haack and Petrice 2009).

LW highlights the danger of moving untreated wood products around the planet and that an innocuous organism from one place can lead to a major ecological catastrophe in another. Brasier (2008) has warned of a disease like this occurring for many years and he has argued for more stringent controls on the movement of non-treat wood, forest and landscape products. LW should be a sobering wake up call to anyone who cares about trees and maintaining a healthy forest system. As Dr. Whitney Crenshaw (University of Colorado) put it during the 2011 Nature Conservancy's Continental Dialogue on Non-native Forest Pests and Diseases Meeting (Boulder CO), "any tree pest is only one truck load of wood away from your community." In North America we functionally lost the American chestnut tree (*Castanea dentata*); a tree that once dominated eastern forest systems. And, it is happening again with the entire Lauraceae family in North America. The point is that massive tree mortality could happen to other culturally, ecologically and economically important species such as live oak (*Quercus virginiana*), bald cypress (*Taxodium distichum*), or giant redwood (*Sequoia sempervirens*) if the movement of untreated wood continues.

Endophytic fungi have been found in most trees species when they have been investigated. Any given tree species can potentially have several to a dozen endophytes present in its wood. With the movement of wood products around the globe, the potential for the introduction of fungi into new areas in untreated wood products is significant (Vannini et al. 2012). This has a major implication for phytosanitary concerns. With LW, one beetle and one species of fungus have killed tens of millions of trees in the southeastern U.S. It is possible then that the introduction of any fungus that is moved about by its evolutionary partner or by a native insect, has the potential to decimate any species at any time, without warning, and without the ability to predict the impending

catastrophe. The global community needs to recognize that shipping untreated wood products around the world poses a significant threat to urban and natural forests. The parties involved in global trade should make every effort to ensure that all wood products moved across any great distances are properly treated to kill hitch hiking insects and fungi.

Concluding Remarks

Laurel wilt is likely here to stay. From observations of *X. glabratus'* movement since 2002, it appears that mortality will continue to be high for species in the Lauraceae plant family (Mayfield et al. 2009 and Ploetz et al. 2011). As of yet, there is no demonstrated genetic resistance within the native North American Lauraceae. Genetic resistance in redbay and avocado is being tested (Ploetz et al. 2011 and Hughes unpublished) but more work is needed to verify and deploy resistant germplasm.

With so many invasive species present, there is a real concern that a threshold of biological stress might be reached that will either eliminate species outright or cause local extinctions. LW threatens the ecology of inland swamps, coastal hammocks, and the urban tree canopy. The loss of the tree will have a negative impact on food webs for organisms that depend on the tree. In particular, LW could cause the extinction of the Palamedes swallowtail butterfly (*Papilio palamedes*). This beautiful butterfly only lays eggs on *Persea* species (Minno et al. 1999, Hall and Butler 2005), meaning that without this genus of trees, the butterfly may be unable to reproduce. Its entire range of existence is from Virginia to Louisiana, the same range that LW may eventually occur.

Global trade will continue, therefore, the introduction of new pathogens will continue to occur here and across the globe. The knowledge gained by understanding the dynamics of this beetle vectored fungal disease may allow for better risk assessment and management of future invasions.

Even if all or most Lauraceous species in urban and forest settings are killed, active LW management programs, based on a combination of sanitation and pruning, could be instituted in avocado fields, which could aid in keeping this important agricultural crop from being decimated by LW. These practices may also be a useful tool when attempting to manage other wood-bound fungi and insects pests.

The work in this dissertation, and the work of dozens of other researchers, will hopefully aid in the identification of solutions to these pests. Here, I summarize my work demonstrating that chipping trees infested with *X. glabratus* and *R. lauricola* is an effective sanitation technique to significantly reduce the numbers of both pests. We identified the heat tolerance threshold of *R. lauricola* and its distribution inside trees and attempted to identify some of the fungal endophytes that may play a role in limiting the duration that *R. lauricola* can persists within a dead tree. Finally, fungicide injections were evaluated for their potential to protect a tree from this pathogenic fungus.

CHAPTER 2
ASSESSING THE SURVIVAL OF THE REDBAY AMBROSIA BEETLE AND LAUREL WILT PATHOGEN IN WOOD CHIPS

Introduction

Laurel wilt (LW) is a non-native vascular wilt disease that kills members of the Lauraceae plant family. The disease can kill a large tree within weeks of infection in the summer and is irreversible. The pathogen, *R. lauricola*, can be recovered from sapwood, leaves, and roots of trees. The disease has caused the mortality of over 90% of the redbays greater than 2.5 cm dbh at Fort George Island, FL (Fraedrich et al. 2008) and 100% of the mature redbays in Etoniah Creek State Forest, FL (Shields et al. 2011).

LW was first detected near Savannah, GA in 2002 (Rabaglia 2006) and currently occurs from North Carolina, west to Mississippi and south to southeastern Florida (Riggins et al. 2010, Forest Health Notes 2011, DPI 2012). LW has moved faster than was predicted (Koch and Smith 2008) through the natural dispersal of its beetle symbiont, *X. glabratus*. Large jumps in its distribution occurred within Florida and to Mississippi, North Carolina, and Alabama (Riggins et al. 2010, Forest Health Notes 2011) and anthropogenic movement of the LW vector and pathogen are responsible for a few of these jumps in distribution (Chemically Speaking 2009, Mayfield et al. 2009).

The movement of exotic organisms has increased dramatically over the last 200 years (Liebhold et al. 1995). Often, the impact is limited and does not pose a threat to food security and ecological integrity; however there are plenty of examples of new exotic pests that have led to significant ecological damage (Pimentel 1986, Liebhold et al. 1995). More specifically, wood-borne pests have damaged silviculture production (Pimentel 1986), urban trees (Dreistadt et al. 1990), and disrupted forest ecology (Liebhold et al. 1995). The primary means of movement of invasive plant pests has been through the

movement of ornamental plants from other countries (Perrings et al. 2005) and in untreated wood products (USDA 1992, USDA 1993, Bridges 1995). At U.S. ports of entry alone, 6788 individual exotic scolytid beetle interceptions were made between 1985 and 2000 at inspection stations (representing 67 species), one of which was the *X. glabratus* in 2002. These beetles came from 49 different countries (Haack 2001).

Over the past decade, the emerald ash borer (EAB) (Poland and McCullough 2006, McCullough et al. 2007) and Asian longhorn beetle (Wang et al. 2000), have become established in the N.E. U.S, leading to the destruction of millions of trees. Maybe the two most infamous exotic pathogens to become established in North America and have played a role in killing tens of millions of trees are *Ophiostoma novo-ulmi*, causal agent of Dutch elm disease (Dunn 2000), and *Cryphonectria parasitica* which causes chestnut blight and has functionally eliminated chestnut (*Castanea dentata*) from North America (Anagnostakis 1987).

Although exotic pests and pathogens have successfully colonized the USA, there are programs in place to monitor, manage and prevent the introduction of these pests. Since 1995, the U.S.A. has been a signatory on the international treaty that sets guidelines for the importation of solid wood packing material and followed international phytosanitary rules to reduce the potential for pests and pathogens to be transported (USDA 2000). To combat the introduction of pests, countries have developed a series of guidelines to minimize and kill pests before they leave the country of origin or once they arrive at a new location (CFR 2002 and IPPC 2009). Common phytosanitary techniques for eliminating wood-bound pests include heat sterilization (Denlinger and Yocum 1998, Nzoko et al., 2008), fumigation (Cyr 2004), and chipping of potentially infested material (McCullough et al. 2007, Wang 2000).

Since *R. lauricola* is vectored by a small beetle, chipping provides a potential option to eliminate the pest from dead trees. In a study by McCullough et al. (2007) on the management of the EAB, two types of wood processing machinery to kill the EAB were tested, chipping and grinding. Chipping was found to be more effective than grinding. In addition, heating emerald ash borer infested wood bolts between 60° to 65° C for 48 hours was sufficient to kill the beetle in a laboratory setting (Denlinger and Yocum 1998, McCullough et al. 2007). In a study to assess the survival of the Asian long horned beetle (*Anoplophora glabripennis* - ALB), surrogates (gypsy moth larva and plastic worms) that were chipped were essentially killed (Wang 2000). These results have led municipalities to recommend chipping as a means to dispose of dead and infested ALB trees, as well as systemic insecticide injections when appropriate for ALB management (MDAG 2012).

This study explores the effectiveness of chipping trees as a means of sanitation and evaluates the persistence of *R. lauricola* and *X. glabratus* in chipped wood. The objectives of the study were to: 1) evaluate the survival of *X. glabratus* after infested wood is chipped; 2) determine how long *R. lauricola* remains viable in wood chips following chipping; 3) determine if small wood chips can provide an adequate environment for *X. glabratus* development, and 4) determine if wood moisture and temperature are correlated with the occupation of intact bolts of wood by *X. glabratus* and *R. lauricola*.

Materials and Methods

This study was carried out at the University of Florida's Austin Cary Memorial Forest (ACMF), just north of Gainesville, FL during the summers of 2010 and 2011. Redbay (*Persea borbonia* (L.) Spreng.) trees used for this study had a dbh (diameter at breast height) of at least 7 cm and had recently died from LW. Each tree had completely wilted canopy, discolored sapwood and evidence of *X. glabratus* beetle boring activity.

Approximately 30 dead infested trees in 2010 and 20 dead infested trees in 2011 were harvested and chipped using a Vermeer model BC 935 wood chipper.

Emergence of *X. glabratus* from Wood Chips and Non-Chipped Bolts

During the summers of 2010 and 2011, 20 bolts of infested redbay wood, each 50 cm long, were used to evaluate the effectiveness of chipping to kill *X. glabratus*. The diameter and moisture for each bolt are listed in Table 2-1 and Table 2-2. Ten of the 20 bolts were randomly selected and individually chipped onto a clean tarp (wood chip average size was 1.3 cm^2). Wood chips were collected and placed in individual piles (4.5 x 4.5 x 1.8 dm); the remaining ten bolts were set on end. Wood chip piles and bolts were maintained in a non-temperature controlled structure at the Austin Cary Memorial Forest near Gainesville, FL. Both the wood chips and intact bolts were covered with a fine mesh (400 µm weave) where the mesh netting was stapled to the inside of a wood frame. A plastic trough that contained propylene glycol was placed around the perimeter of the wood chip piles and bolts. Beetles that emerged flew into the netting and would fall into the collection trough. Insects that emerged from the wood chips and bolts were collected every two weeks. At each collection event, the moisture of the wood chips and non-chipped bolts were recorded using a Protimeter® Timbermaster (General Electric Corporation, Shannon, Ireland). Insects that emerged were placed into separate containers and returned to the laboratory for identification.

In addition to the troughs, one yellow sticky card (Seabright Laboratories, Emeryville, CA) was stapled to the interior top portion of each netted structure and; it was also collected every two weeks to help assess the emergence of *X. glabratus*. Four sticky cards were also placed around the interior of the garage to survey for the presence of any *X. glabratus* that escaped from the netted structures.

Survival of *X. glabratus* in Wood Chips

Trees that were not used in the previous study were chipped onto a clean tarp and used to fill mesh bags, bins, and to create piles of wood chips. Eighteen 1 m^3 bins were filled with chips and ten 0.5 m^3 piles of wood chips were created to determine if *X. glabratus* and *R. lauricola* would persist in wood chips.

Nine of the bins were placed in full sun (bin-sun) while the other nine were placed in constant shade (bin-shade). For the piles of wood chips, a tarp was placed over all ten piles, where five were placed in full sun (tarp-sun) and five in constant shade (tarp-shade). Tarps were held down with cement blocks and completely covered the entire wood chip pile. Infested redbay wood chips were used to fill 352 mesh bags (150 cm^3) that were placed in the center and top of each pile and bin. A 1 m long string was tied to each bag to aid in their retrieval. The bags were filled within three hours of tree chipping (during the middle of the day). The material used for the mesh bags was the same as the material used for the netting over the wood chip piles and non-chipped bolts.

Mesh bags were extracted from the center and top of each bin and pile every other day after chipping for one month. There afterwards, bags were collected every two weeks for a total sampling period of ten weeks. For wood chip piles, mesh bags were placed on top of the chips but under the tarp. Underneath each bin and pile, a wood board was placed to isolate the wood chips from soil microorganisms.

A total of 352 mesh bags were extracted over a 10-week period. The mesh bags were taken back to the laboratory where the wood chips were placed on a white piece of paper to observe any moving beetles. Four wood chips from each mesh bag were set aside to test for the viability of *R. lauricola*. From those four wood chips, the moisture content was recorded, which were representative of the moisture at the center and top of

the bins and piles. The rest of the wood chips were placed into a 226 cm^3 insect rearing chamber to monitor for the emergence of the X. *glabratus*. Rearing chambers were kept at room temperature (24º C) and were exposed to approximately 12 hrs of light daily. Each collection jar was evaluated weekly for 90 days for the presence of *X. glabratus* and other wood boring insects. After 90 days, contents of each rearing chamber were evaluated using a dissecting microscope to determine if any *X. glabratus* remained inside the chambers. To test whether the rearing chambers provided an adequate environment for survival of *X. glabratus*, ten small LW infested redbay bolts (3 cm in diameter x 11 cm long) were placed into individual rearing chambers. The bolts were monitored every 14 days for six months.

***R. lauricola* Recovery from Chipped and Non-Chipped Wood**

At the beginning of the chipping study, wood plugs (approximately 13 x 1.5 mm) were taken from the non-chipped infested wood pile with an increment bore hammer (Haglöf, Sweden) to confirm the presence of *R. lauricola*. Wood cores were cut into smaller fragments (approximately 2 x 3 mm in size), surface sterilized for 60 seconds in 70% ethanol and rinsed in ddH_2O for 60 seconds. When dry, the wood sections were plated on cycloheximide-streptomycin malt agar (CSMA), a semi-selective medium for ophiostomatoid fungi (Harrington 1981), with antibacterial amendments. The amendments were the addition of 350 mg of ampicillin sodium salt and 500 µL of a 9 mg RifAmpin+1000 µL DMSO mixture (Ploetz et al. 2011). Hereafter, we refer to this medium as CSMA++. All plates were kept at 25˚ C in the dark. Plates were evaluated for the presence of *R. lauricola* every three days.

Confirmation of Suspect R*. lauricola* Isolates in Culture

R. lauricola confirmation for all wood plugs taken during the study were confirmed based on morphology. All isolates from wood chips that appeared to be *R. lauricola* were confirmed through genetic analysis.

Suspect isolates from wood chips were sub-cultured on CSMA++ for approximately 10 days and then DNA was extracted (Justesen et al. 2002) with the addition of a proteinase K digestion step. The small subunit (18s) rDNA was amplified via PCR using the primers NS1 (5'-GTAGTCATATGCTTGTCTC- 3') and NS4 (5' – CTTCCGTCAATTCCTTTAAG – 3') (White et al. 1990). A PCR master mix containing 12.5 µL of Amplitaq Gold (Applied BioSystems, Foster City, CA.), 9.5 µL of ddH2O, 1 µL of DNA, plus 1 µL of each primer was used. A MJ Research, PTC-200 Peltier thermocycler was used for PCR with the following cycling conditions: 95° C for 6 minutes then 40 cycles of 95° C for one min., 48° C for 30° sec. and 72° C for two minutes for DNA extension. ExoSAPit® (Affymetrix, Inc, USB Products, Santa Clara, CA) was used to purify the amplicon, which was then sent to the University of Florida's Interdisciplinary Center for Biotechnology Research for Sanger sequencing. The sequenced amplicons were compared to the GenBank (http://www.ncbi.nlm.nih.gov/) library, see Table 2-3**.**

Moisture and Temperature Measurements

Wood chip moisture content was taken from four randomly selected wood chips from each mesh bag from the inside and top of each bin or pile, Tables 2-4 through 2-7. For the non-chipped bolts, three moisture measurements were taken from the middle of the bolt, where readings were taken along the vertical axis of the bolt of wood (in the same direction of the wood grain), Tables 2-8 and 2-9. Four moistures readings were recorded from four wood chips from the chipped bolt piles, Tables 2-10 and 2-11.

Temperature probes (CAS Data Loggers, Chesterland, Ohio) were placed in the center of six bins and two piles for the sun and shade treatments. Temperature probes at the center of the piles recorded the temperature of the wood chips every hour. To determine the temperature on the tops of the bins we used a University of Florida weather station that was located 50 meters away.

Beetle Presence Near the Study Site

Two weeks prior to chipping, five, six-tier, manuka-oil baited multifunnel traps were suspended from trees 2 m above the ground around the study site to attract *X. glabratus* (Kendra et al. 2011). Traps were checked weekly from July through mid-December, every two weeks from mid-December through April, then weekly again through July 2011. The multifunnel traps were fitted with wet collection jars containing propylene glycol. Trap catches were collected and tabulated for scolytid catches only and the number of *X. glabratus* were tabulated for each collection. Both *X. affinis* and *X. volvulus* were common around the site but due to the time required to differentiate between the two, their numbers were combined.

Quantitative Analysis

A one-way ANOVA was used to identify differences between the emergence of *X. glabratus* from wood chips and non-chipped bolts as well as differences between non-chipped bolt and chipped bolt moisture and diameters used in 2010 and 2011 (Microsoft® Excel 2010). When significant differences were found ($\alpha = 0.05$) a Tukey-HSD comparison of means was used to determine the differences among samples. Linear regression analysis of *X. glabratus* emergence from non-chipped bolts vs. daily temperature, moisture and weeks since wood was chipped were conducted with Microsoft® Excel 2010.

Results

Emergence of *X. glabratus* from Wood Chips and Non-Chipped Bolts

The differences in moisture of felled trees that were used for the chipping study ranged from 100% to 27.7% with a mean of 60.4% and a standard deviation of 25%. A comparison the moisture of 50 randomly selected wood chips taken one hour after chipping ranged from 80% to 22% with a mean of 37.9% and a standard deviation of 11.9%.

When the data from all non-chipped bolts were combined for 2010 and 2011, there were a total of 2461 scolytid beetles that emerged over ten months. Over the first two weeks of both studies, 1200 scolytid beetles emerged, of which 339 were *X. glabratus*. Over the remaining six and a half months, 1261 scolytid beetles emerged, 1034 of which were *X. glabratus*, Figure 2-1. Over the same periods for both years, the emergence of *X. glabratus* from wood chips was statistically different, where only 14 *X. glabratus* emerged over seven months F (2,27) = 6.92, P = 0.0037.

A T-test comparing the moisture of bolts for both the non-chipped study was not significant F (1,18),= 0.18, P = 0.676. A T-test comparing non-chipped bolt diameters from 2010 and 2011 was also not significant F (1,18),= 0.018, P = 0.895. A T-test comparing the moisture of bolts used to make wood chips in both the 2010 and 2011 studies was not significant F (1,18),= 0.322, P = 0.577. However, a T-test comparing the diameters of bolts to be chipped in 2010 and 2011 was significant F (1,18),= 7.67, P = 0.0163 even though *X. glabratus* emergence was not statistically different F (1,18),= 0.343, P = 0.628.

In addition to discerning the effectiveness of chipping infested trees, potential correlations between the emergence of *X. glabratus* and non-chipped bolt moisture (Figure 2-2), daily outside temperature (Figure 2-3) and weeks since chipping (Figure 2-4) were

evaluated. Daily outside temperature and moisture were not well correlated with *X. glabratus* emergence (r^2 = 0.123 and r^2 = 0.064 respectively). The emergence of *X. glabratus* was correlated (r^2 = 0.698) to the length of time that the study ran, from July 2010 through January 2011.

Survival of *X. glabratus* in Wood Chips

Over the course of the study, only 3 of 352 insect rearing chambers yielded *X. glabratus*. Two *X. glabratus* emerged from wood chips, from the same rearing chamber, four days post chipping. Both beetles were female; one dark and one light yellow, indicating it likely just eclosed. After 90 days, the wood chips in the rearing chambers were examined for dead intact or fragments of *X. glabratus*. Two whole *X. glabratus* were found in two other rearing chambers when they were dissected, along with more than a dozen *X. glabratus* fragments. The two intact beetles came from wood chips in mesh bags that were extracted from the study piles at two days post chipping.

In total, fourteen scolytid beetles emerged from either wood chips in the garage piles or in the insect collection jars. No beetles were recovered more than two weeks post chipping. Other than *X. glabratus*, the species that were found in collection jars or by dissection were three *Xyleborus affinis*, five *Xylosandrus crassiusculus*, and one *Xyleborinus saxeseni*.

From the ten small bolts of redbay that were placed inside the rearing chambers to test suitability of the rearing chambers, 24 *X. glabratus* emerged over a six month period. No other species were found in the collection jars. These bolts were not dissected at the end of the evaluation.

This study was designed to run for ten weeks. However, the non-chipped bolts were left under netting for an additional eight weeks to observe *X. glabratus* activity.

Sixteen weeks after the start of the study, *X. glabratus* emergence from non-chipped bolts ceased. The remaining bolts were placed in an incubator at 30° C in an attempt to force out any remaining ambrosia beetles. Within one week, *X. glabratus* (and a few other scolytids) began emerging again. In total, an additional 241 *X. glabratus* emerged.

R. lauricola Recovery from Chipped and Non-Chipped Wood

Of the 20 wood cores extracted before chipping, all yielded *R. lauricola*, which were confirmed by morphology. Three weeks after chipping, ten wood cores were taken from the non-chipped bolts, eight of ten cores yielded *R. lauricola*, which were also confirmed by morphology.

Over the course of the chipping study, approximately 3600 wood pieces were plated from 352 mesh bags. *R. lauricola* grew from wood chips from only four mesh bags, two from the top of a bin-sun treatment and two from the top of a bin-shade treatment. These four mesh bags were collected two days post chipping. *R. lauricola* was never recovered beyond two days post chipping. Each of the four isolates produced a 99%, or 100% match to voucher isolates of *R. lauricola* in GenBank, Table 2-3. A few other fungi that were also isolated and identified by PCR from wood chips are listed Table 2-3.

Moisture and Temperature Effects

Wood and wood chip moisture had no effect on the emergence of beetles from non-chipped bolts or survival of *R. lauricola* in wood chips. In fact, since *R. lauricola* was only found after two days post chipping, moisture and temperature could not have been the reason for its disappearance. In Tables 2-4 through 2-11, the change in moisture of the wood chips for each treatment and bolts can be observed.

Temperature of the Bins and Piles

The maximum temperature of wood chips inside bin-sun, bin-shade, tarp-sun pile, and tarp-shade piles were 63.5, 60, 46, and 40º C respectively. The internal temperature data from each replicate for each treatment was averaged, Figure 2-5. Figure 2-6 illustrates that as moisture remained relatively constant, the temperature of the wood chip piles decreased over time.

To represent the temperature on the surface of the wood chip piles and bins, temperatures from the nearby weather station were used over the course of the study (May through July). The maximum temperature reached at the ACMF weather station was 35º C. The maximum daily temperature reached at Gainesville Regional Airport (approximately 9 km away) over the same period was 35.5º C. (http://www.wunderground.com/history/airport/KGNV/2011/5/31/DailyHistory.html?req_city=NA&req_state=NA&req_statename=NA).

Beetle Presence Near the Study Site

Scolytids collected from the five multifunnel traps monitored over a 12 month period were: 706 *X. glabratus*, 572 *Xylosandrus crassiusculus*, 125 *Xyloborinus saxeseni*, 814 *Xyleborus affinis*/*Xyleborus volvulus* (due to the similarity between these two species species level identification was not undertaken), 16 *Monarthrum mali*, 51 *Hypothenemus* sp. and 65 *Euplatypus* sp.

A T-test comparing two weeks of *X. glabratus* catches from five traps before and after chipping for both years showed that the means were not statistically different $F_{(1,18)} = 1.057$, $P = 0.152$.`

Discussion

Data collected during this study clearly shows that very few *X. glabratus* will survive the chipping process and that *R. lauricola* did not persist in wood chips longer than two days. Over 1,300 *X. glabratus* emerged from intact bolts while only 13 emerged from wood chips. All beetles that did emerge did so within two weeks of chipping. *R. lauricola* was only recovered two days post chipping (and only from 4 of 352 mesh bags) while the *X. glabratus* population was reduced by 99.5% compared to non-chipped bolts. Since the only beetles that survived chipping came from uncovered bins, it may be prudent to cover wood chips with a tarp for a week to ensure that any survivors are killed. Wood chips that came from inside the bins and from under the tarps were wet from condensation and colonized by *Aspergillis fumigatus, Syncephalastrum racemosum* and *Rhizopus microporus* along with other opportunistic saprophytic species. Thus, *R. lauricola* was no longer actively growing in the wood and may have been out-competed by these fungi.

Use of living, dead, dying and decaying trees by ambrosia beetles is driven by a complex set of variables, one of which is wood moisture (Graham 1925, Adams and Six 2007). However, this study did not show a correlation between a change in non-chipped bolt moisture and emergence of *X. glabratus*. *X. glabratus* emerged at a fairly constant rate for eight weeks from the non-chipped bolts while the other scolytid species emerged in mass over the first two weeks. Neither the quick emergence by the other scolytids nor the continued emergence of *X. glabratus* were positively correlated to non-chipped bolt moisture. Another factor of *X. glabratus* emergence that was analyzed was time in weeks since chipping. There was a correlation with time since chipping ($r^2 = 0.698$), however, the change in daylight hours due to the coming fall, generation time, the disappearance of *R.*

lauricola from the bolts or the arrival of an antagonistic fungal saprophyte could have played a role in that phenomenon.

Eight weeks into the study we removed Netted Log 1 (NL1) from the experiment to dissect it and look for *X. glabratus*. Due to the similar bore hole sizes between *X. glabratus* and *Xyleborinus saxeseni*, *Xyleborus affinis, X. ferrugineus* and *X. volvulus* we determined that any comparisons based on the number of bore holes would not be accurate. In addition, the small size of the beetles (2 mm in length) would also make it difficult to find all *X. glabratus* still inhabiting bolts of wood; therefore, no data are provided on the number of beetles that could potentially inhabit any given volume of wood.

For the bolts that were transferred to incubators after 18 weeks, it is interesting that the addition of sudden, intense heat caused *X. glabratus* to emerge. If the bolts were left at the study site, those 241 beetles may have emerged the following spring when the bolts would have warmed up naturally.

With tens of thousands of dead trees across Florida, there is the potential for wood processing facilities to convert these trees into mulch. This research also showed that *R. lauricola* would not be viable in wood chips for any length of time, meaning that there is virtually no chance for movement of *R. lauricola* from wood chips to healthy trees.

A study on the thermal tolerance of *R. lauricola* (Spence et al. unpublished) found that *R. lauricola* was killed in culture at 47º C. The center temperature of wood chips in the sun treatment reached 60º C, making it even more unlikely that *R. lauricola* could spread from a wood chip to anything else or be picked up by another beetle when wood chips are left for at least a few days in a pile. Although not directly tested, it is unlikely that *R. lauricola* poses a threat to healthy Lauraceous species when used as mulch.

Sanitation of fungal pathogens through heat treatments has been investigated for many crop pathogens and post-harvest saprophytes. Downer et al. (2008) tracked four fungal species for eight weeks inside unturned piles of green plant debris and compost. *Armillaria mellea* and *Trichoderma semipenetrans* did not survive more than two days in fresh green waste, while *Phytophthora cinnamomi* persisted for over 21 days in compost piles that reached 70° C. Another study examined of 38 fungal and oomycete pathogens in compost and found that 87% of them were reduced below detection levels at temperatures between 64–70° C after 21 days (Noble and Roberts 2004).

With bins of freshly chipped redbay trees, it is a bit surprising that multifunnel trap catches were not significantly different before and after chipping. There would have been fresh wood volatiles in the air from all the piles of wood chips which should have acted as an attractant to *X. glabratus*. This is in contrast to a study that found cut or rasped redbay bolts were more attractive to *X. glabratus* than bolts with intact bark (Niogret et al. 2011, Mayfield and Hanula 2012). One possible explanation is that the volatiles that are attractive to *X. glabratus* do not persist in wood chips comprised of a small volume of wood. *X. glabratus* activity over a twelve month period did not match the findings of Hanula et al. (2011). They found that *X. glabratus* was most active in September while our largest catches were in April.

These studies have demonstrated that chipping trees is an effective means of reducing both pests. In particular, the danger of moving the pathogen in wood chips is almost zero. This means that if dead trees are chipped for landscaping mulch, the pathogen will not persist and will not pose a threat to health of Lauraceous species. Even if all or most Lauraceous species in urban and forest settings are killed, active LW management programs could be instituted in avocado fields to prune out and chip infested

branches or whole trees. An active LW monitoring program that removes infested trees or branches as soon as LW is observed could aid in keeping this important crop from being decimated by LW. This process of chipping beetle and fungus infested wood may also be useful when attempting to manage other tree diseases.

Table 2-1. Features of non-chipped bolts placed under netting.

	Avg. dia.	Maximum dia.	Minimum dia.	Avg. moisture content
2010	13.43 cm	13.70 cm	12.80 cm	91.72 %
2011	13.50 cm	15.60 cm	10.80 cm	92.87 %

n = 20

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Table 2-2. Features of bolts that were chipped and placed under netting

	Avg. dia.	Maximum dia.	Minimum dia.	Avg. moisture content
2010	13.91 cm	15.80 cm	13.00 cm	92.74 %
2011	12.35 cm	15.20 cm	10.60 cm	91.32 %

n = 20

Table 2-3. Fungal species identified from wood chips in 2010.

Treatment	Species	% match	Primer pair	Accession #
Sun top 2	*R. lauricola*	100	NS1-4	EU257806.1
Sun top 1	*R. lauricola*	99	NS1-4	EU257806.1
Shade top 2	*R. lauricola*	100	NS1-4	JF797171.1
Shade top 1	*R. lauricola*	99	NS1-4	JF797172.1
Sun top 1	*Pichia caribbica*	100	ITS1-4	FN428931.1
Tarp sun top 5	*Aspergillis fumigatus*	99	ITS1-4	GU205082.1
Shade top 1	*Syncephalastrum racemosum*	88	ITS1-4	EU409811.1
Sun inside 5	*A. fumigatus*	99	ITS1-4	GU205082.1
Tarp sun inside 5	*Rhizopus microporus*	100	ITS1-4	FJ810505.1
Tarp sun inside 4	*Syncephalastrum racemosum*	92	ITS1-4	AB054045.1
Tarp shade inside 4	*Cunninghamella bertholletiae*	99	ITS1-4	DQ155288.1

Table 2-4. Average moisture of wood chips in full sun in days post chipping.

Treatment	2	4	6	8	10	12	14	29	40	56	70	84	Avg. Moisture	Std. Dev.
ST1	43.4	43.4	29.0	22.3	13.1	31.4	32.8	34.2	11.5	10.4	7.2	13.0	24.3	13.1
SM1	50.6	41.4	38.1	36.2	25.3	56.0	32.0	50.1	58.2	44.2	20.4	54.1	42.2	12.2
ST2	52.1	47.7	29.3	42.8	25.9	20.3	30.6	50.1	16.9	8.1	7.5	15.1	28.9	16.1
SM2	39.3	51.7	37.1	57.4	34.7	43.0	27.1	44.7	33.8	41.4	59.7	57.7	44.0	10.6
ST3	32.7	46.6	44.6	24.2	12.7	58.2	29.3	44.6	10.1	11.0	6.6	13.2	27.8	17.5
SM3	46.2	36.5	33.5	44.9	37.4	38.7	32.4	38.4	53.6	57.3	40.9	25.6	40.5	8.9
ST4	48.9	20.8	37.2	31.0	12.4	42.1	31.1	46.5	22.0	8.7	7.6	15.2	27.0	14.6
SM4	56.7	37.7	49.3	29.5	37.9	42.4	29.3	48.2	54.8	35.3	50.5	39.8	42.6	9.3
Avg. top moisture	44.3	39.6	35.0	30.1	16.0	38.0	31.0	43.9	15.1	9.6	7.2	14.1	27.0	13.7
Avg. mid moisture	48.2	41.8	39.5	42.0	33.8	45.0	30.2	45.4	50.1	44.6	42.9	44.3	42.3	5.6

ST = sun top, SM = sun middle of the pile, HT = shade top, HM = shade middle of the pile.

Table 2-5. Average moisture of wood chips in full shade in days post chipping.

Treatment	2			8	10	12	14	29	40	56	70	84	Avg. Moisture	Std. Dev.
HT1	53.4	59.4	38.0	62.4	54.1	47.2	26.0	23.7	32.3	22.1	7.9	14.5	36.8	18.4
HM1	49.3	46.3	37.9	48.8	46.2	28.4	25.8	32.7	25.6	24.8	27.6	22.0	34.6	10.5
HT2	55.3	36.9	38.2	39.7	42.9	26.7	23.2	21.8	20.6	16.4	7.9	12.9	28.5	14.0
HM2	55.7	43.5	36.1	41.4	45.2	41.0	24.5	25.9	30.7	28.2	26.6	25.3	35.3	10.0
HT3	57.3	33.5	35.5	47.8	34.6	32.4	27.6	21.9	17.0	13.4	8.2	13.0	28.5	14.7
HM3	40.1	40.8	37.1	49.0	36.8	39.6	25.9	25.1	19.9	29.0	26.6	24.2	32.8	8.8
HT4	58.5	45.0	38.2	29.0	22.0	32.4	18.0	18.6	23.4	12.8	7.8	14.2	26.7	14.8
HM4	41.6	49.8	40.2	26.2	52.6	41.6	27.5	27.6	24.9	22.2	25.4	24.5	33.7	10.8

HT = shade top, HM = shade middle of the pile.

Table 2-6. Average moisture of wood chips for tarp sun in days post chipping.

Treatment	14	29	40	56	70	84	Avg. Moisture	Std. Dev.
TST1	29.0	32.0	24.7	24.9	13.8	29.2	25.6	6.4
TSM1	27.1	29.1	22.4	27.6	31.3	49.5	31.2	9.5
TST2	14.0	18.8	16.3	18.9	13.4	25.8	17.9	4.5
TSM2	39.3	28.2	25.4	27.6	25.7	25.3	28.6	5.4
TST3	22.9	19.6	21.2	14.9	14.6	21.8	19.2	3.6
TSM3	27.9	26.3	34.7	19.6	22.6	24.5	25.9	5.2
TST4	37.7	20.6	42.6	33.5	40.1	42.3	36.1	8.3
TSM4	28.1	24.9	27.6	24.4	19.3	16.0	23.4	4.8

TST = tarp sun top, TSM = tarp sun middle of the pile

Table 2-7. Average moisture of wood chips for tarp shade in days post chipping.

Treatment	14	29	40	56	70	84	Avg. Moisture	Std. Dev.
THT1	26.7	35.8	37.1	40.6	51.7	46.3	39.7	8.7
THM1	24.9	25.5	26.3	28.5	29.9	27.2	27.1	1.9
THT2	23.5	22.7	29.7	50.5	39.8	42.9	34.9	11.3
THM2	25.1	32.6	32.8	26.6	21.1	26.1	27.4	4.5
THT3	27.6	36.7	47.2	42.6	76.3	66.8	49.5	18.5
THM3	24.8	33.7	29.1	27.6	26.2	25.6	27.8	3.3
THT4	26.1	27.3	54.3	50.7	71.8	64.0	49.0	18.8
THM4	29.7	33.1	28.4	22.5	24.8	23.7	27.0	4.1

THT = tarp shade top, THM = tarp shade middle of the pile

Table 2-8. Average moisture of non-chipped bolts in 2010 in days post chipping.

Treatment	14	29	40	56	70	84	98	112	127	140	Avg. Moisture	Std. Dev.
NL1	83.9	83.2	100.0	100.0							91.8	9.5
NL2	89.9	57.8	100.0	89.9	99.1	91.7	65.3	43.3	56.1	24.8	71.8	26.0
NL3	25.4	23.3	25.7	24.0	24.9	22.4	22.3	23.4	24.6	18.2	23.4	2.2
NL4	68.3	59.7	60.7	45.9	42.2	52.9	43.6	47.9	35.2	26.4	48.3	12.6
NL5	41.7	33.9	24.2	26.5	29.6	24.2	27.6	25.4	26.6	19.0	27.9	6.2
NL6	56	70.4	59.6	58.1	39.7	37.5	36.4	36.9	39.7	21.4	45.6	14.8
NL7	66.8	99.6	80.7	85.9	39.5	52.5	50.0	50.0	56.6	28.5	61.0	22.1
NL8	58.9	85.0	91.2	100.0	91.5	68.8	56.6	57.0	46.4	23.9	67.9	23.9
NL9	27.1	29.1	49.5	54.2	32.2	34.8	28.0	28.7	29.9	22.8	33.6	10.2
NL10	100	100.0	99.9	90.1	99.3	88.4	90.4	87.7	88.3	33.3	87.7	19.9

NL = netted log

Table 2-9. Average moisture of non-chipped bolts in 2011 in days post chipping.

Treatment	14	29	40	56	70	84	98	112	127	140	Avg. Moisture	Std. Dev.
	11	23									68.1	6.1
NL1	72.4	63.8									60.6	2.6
NL2	62.4	58.7									75.0	1.1
NL3	75.7	74.2									68.0	0.8
NL4	68.5	67.4									93.2	1.3
NL5	92.3	94.1									53.2	9.8
NL6	46.2	60.1									70.6	0.7
NL7	70.1	71.1									91.2	0.6
NL8	91.6	90.8									91.7	3.0
NL9	89.5	93.8									66.1	1.4
NL10	67.1	65.1									68.1	6.1

NL = netted log

Table 2-10. Average moisture of wood chips in 2010 in days post chipping – indoors under netting.

Treatment	14	29	40	56	70	84	Avg. Moisture	Std. Dev.
NM1	19.4	18.9	14.9	15.2	10.9	13.9	15.5	3.2
NM2	19.0	18.1	15.1	13.5	10.8	14.9	15.2	3.0
NM3	17.3	19.2	16.4	15.1	11.2	14.8	15.7	2.7
NM4	18.3	19.1	16.1	15.1	11.3	14.8	15.8	2.8
NM5	18.9	18.4	17.7	15.8	11.5	13.8	16.0	2.9
NM6	16.7	18.0	14.0	13.9	10.3	11.7	14.1	2.9
NM7	18.8	16.8	16.1	14.1	12.2	12.5	15.1	2.6
NM8	19.0	18.5	18.1	14.5	11.6	14.0	16.0	3.0
NM9	17.6	20.4	14.2	13.4	11.3	11.6	14.8	3.6
NM10	16.2	17.2	14.8	13.3	11.4	11.9	14.1	2.3

NM = netted wood chips.

Table 2-11. Average moisture of wood chips in 2011 in days post chipping – indoors under netting.

Treatment	14	29	Avg. Moisture	Std. Dev.
NM1	13.2	9.5	11.4	2.6
NM2	11.2	10.5	10.9	0.5
NM3	12.0	10.3	11.2	1.2
NM4	15.6	10.1	12.9	3.9
NM5	19.7	11.8	15.8	5.6
NM6	36.2	10.1	23.2	18.5
NM7	10.4	8.9	9.7	1.1
NM8	26.6	9.0	17.8	12.4
NM9	32.0	13.6	22.8	13.0
NM10	20.7	13.1	16.9	5.4

NM = netted wood chips

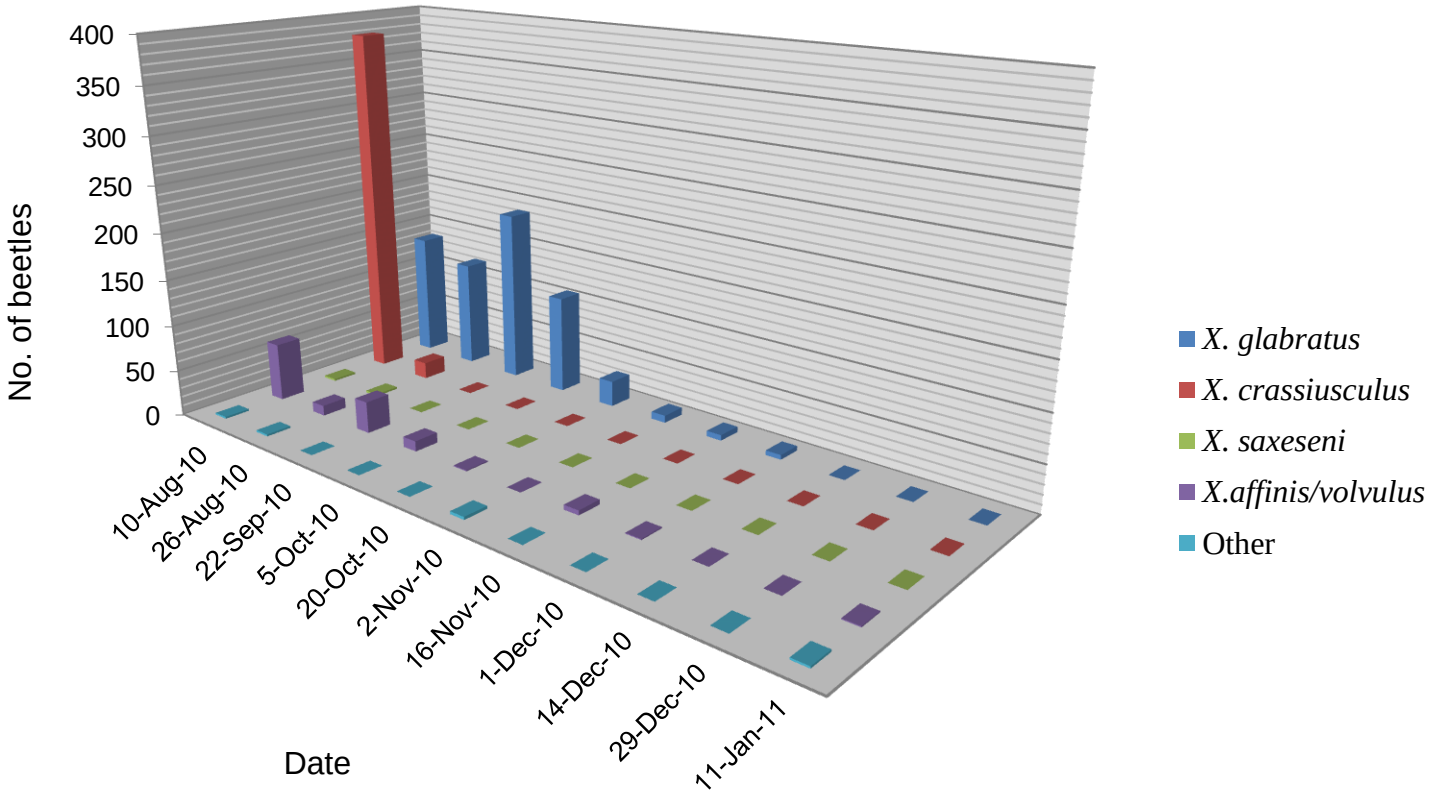

Figure 2-1. Beetle emergence from non-chipped bolts from August 2010 to January 2011. *X. affinis* and *X. volvulus* are species that are time consuming to differentiate therefore their numbers were combined.

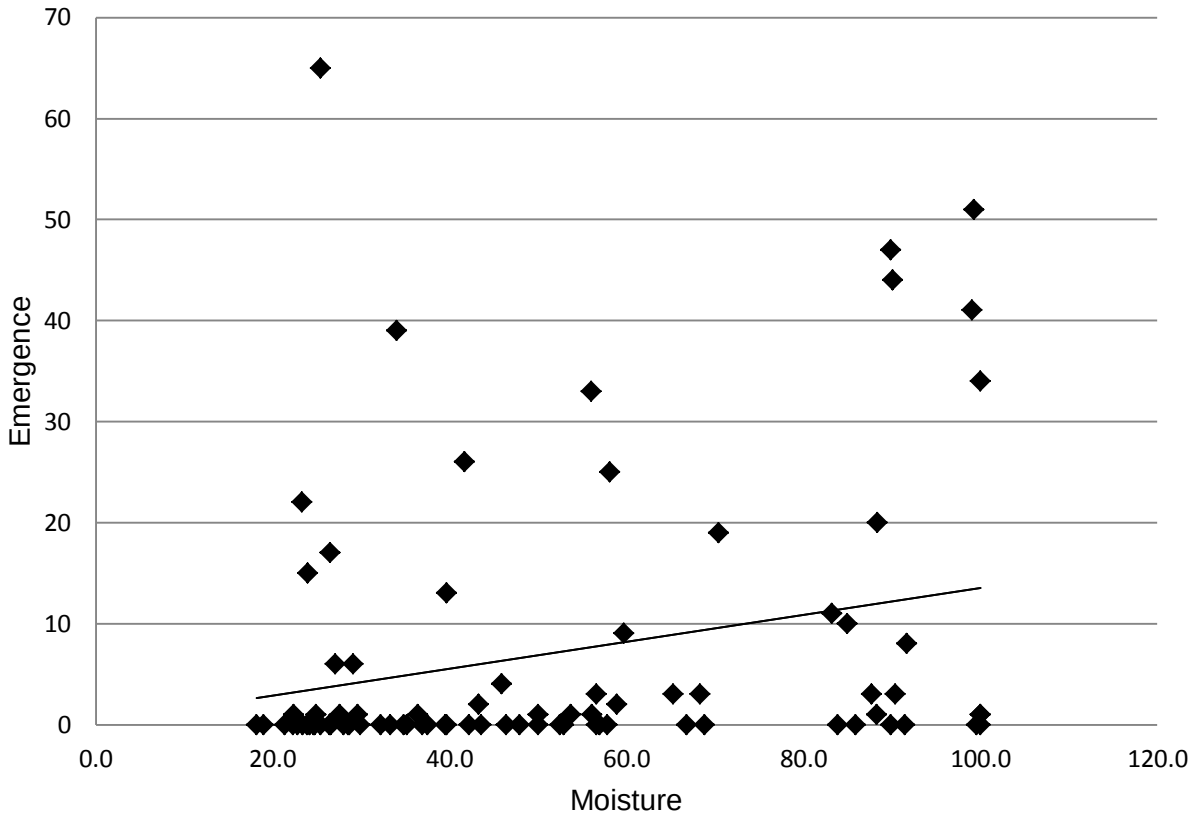

Figure 2-2. *Xyleborus glabratus* emergence and bolt moisture. Beetle emergence from non-chipped bolts was not correlated to bolt moisture, $y = 0.133x + 0.175$, $r^2 = 0.064$.

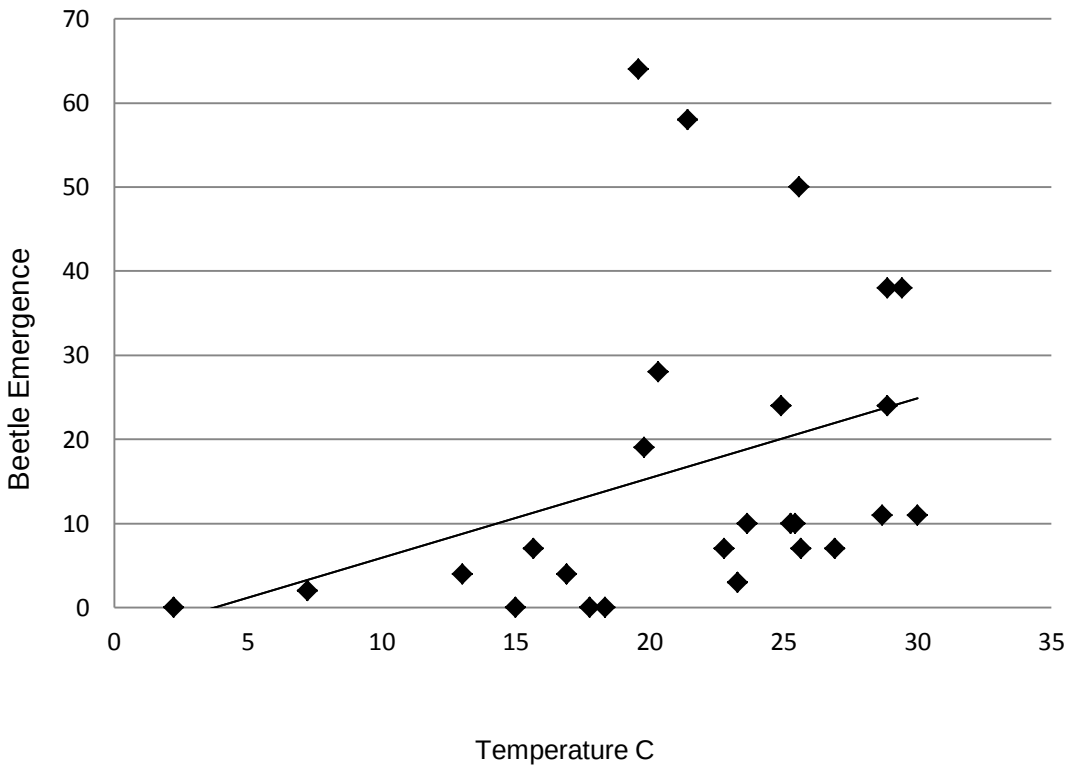

Figure 2-3. *Xyleborus glabratus* emergence and daily temperature. Beetle emergence from non-chipped bolts, $y = 0.9467x - 3.502$, $r^2 = 0.123$.

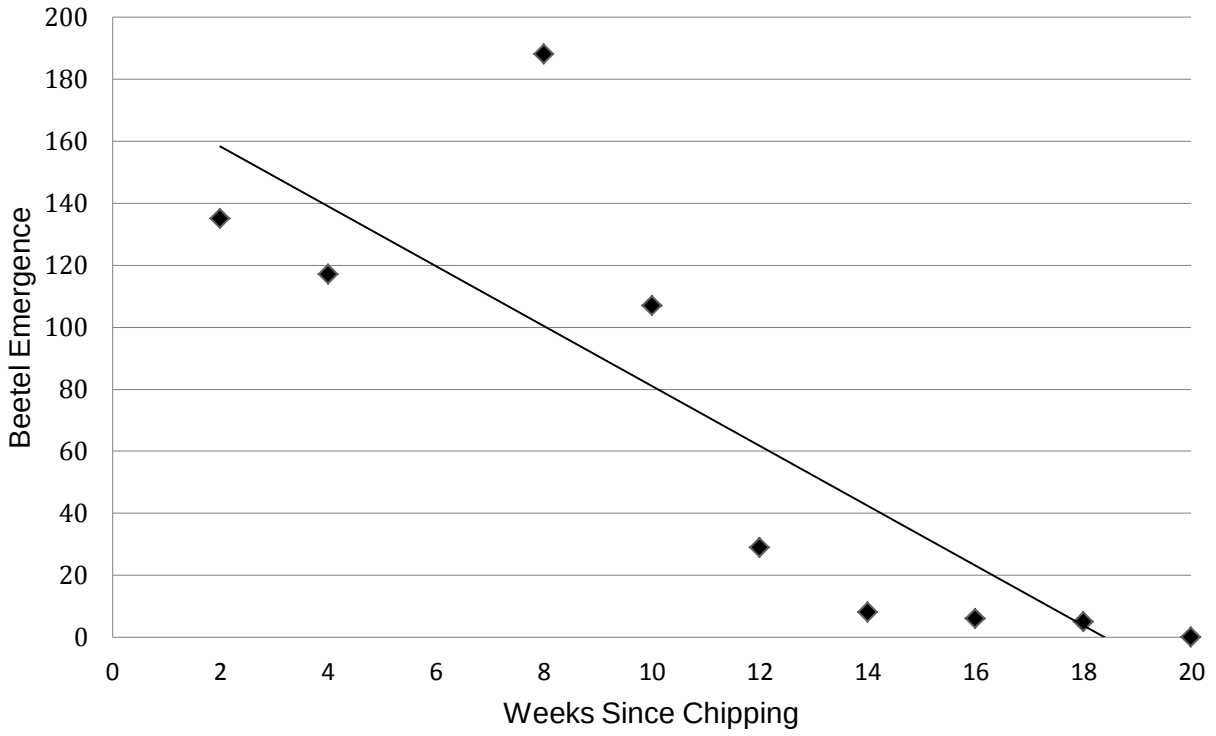

Figure 2-4. *Xyleborus glabratus* emergence from July 2010 through January 2011, y = -9.6537x + 177.66, r^2 = 0.698.

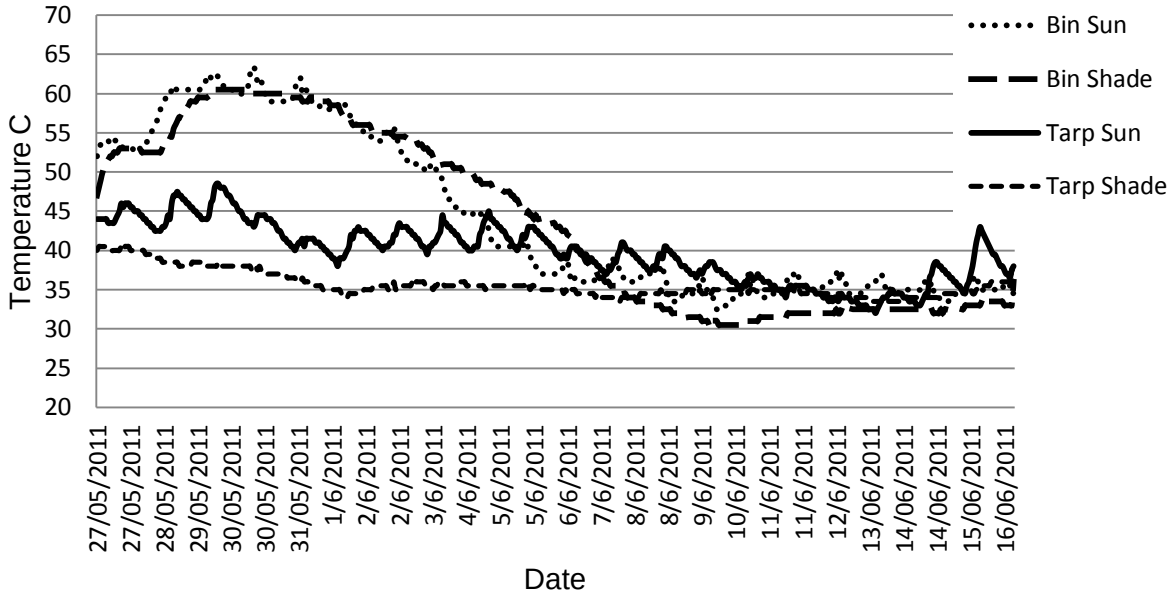

Figure 2-5. Temperatures of wood chip in piles and bins, in full sun, shade and under tarps. Temperature probes were placed in the center of each treatment.

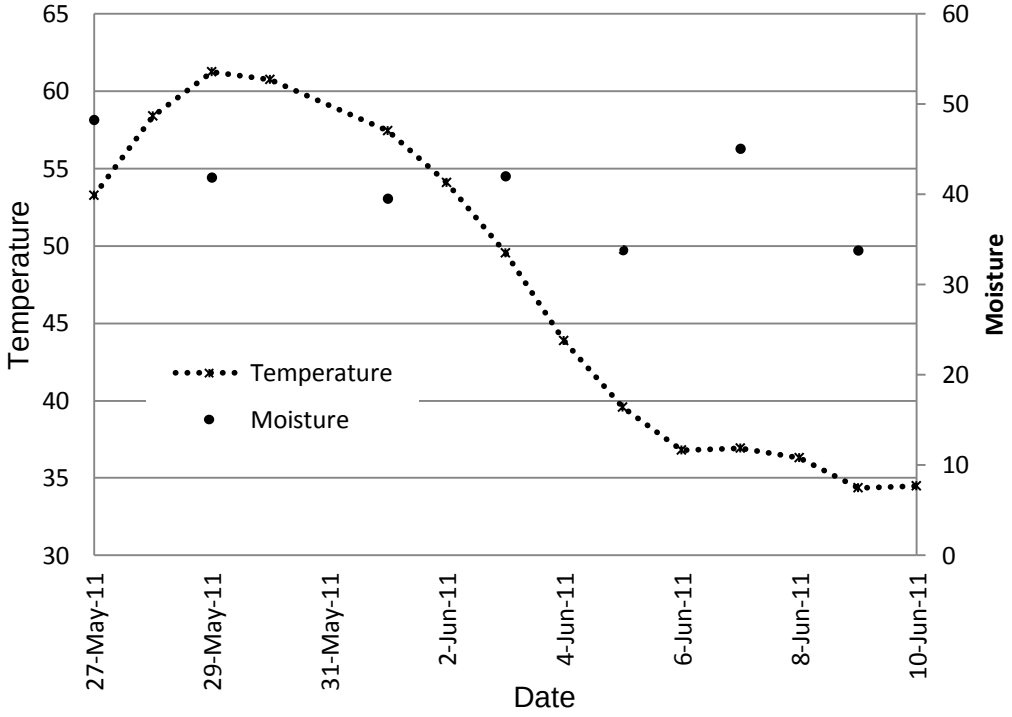

Figure 2-6. Temperature and moisture of wood chips (Inside bin measurements). This graph represents the change in wood chip moisture in relation to the temperature at the center of the wood chip piles.

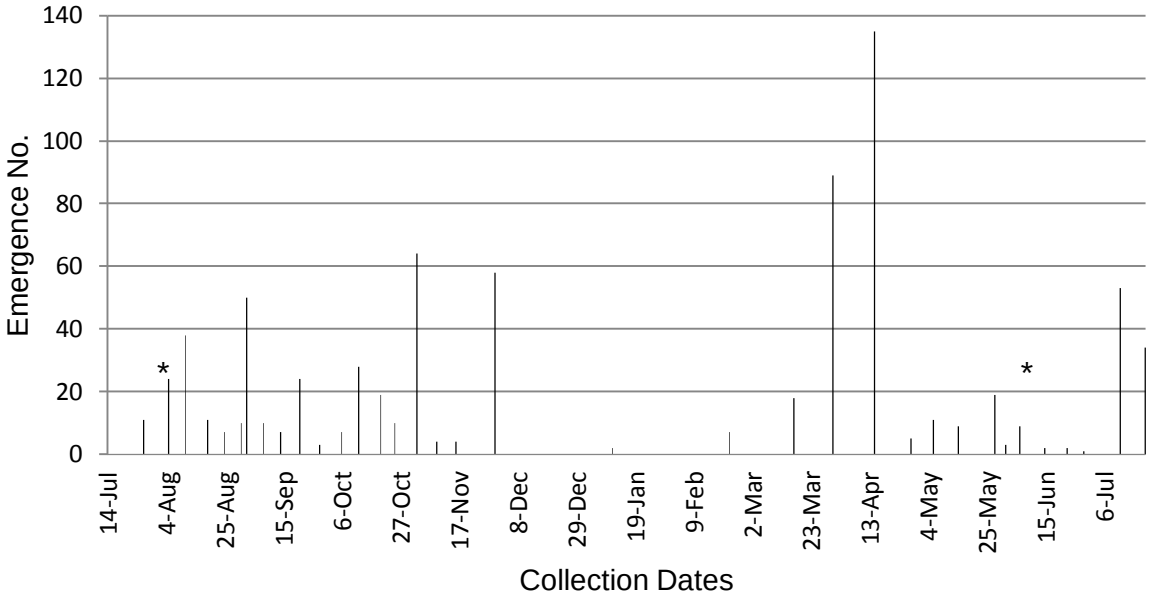

Figure 2-7. *Xyleborus glabratus* multifunel trap catches from 14 July 2010 – 21 July 2011. Beetles were captured in manuka-oil baited six-tier multifunnel traps at ACMF. Asterisks indicate when chipping occurred during both summers.

CHAPTER 3
EFFECT OF TEMPERATURE ON GROWTH AND VIABILITY OF *RAFFAELEA LAURICOLA*

Introduction

The Kingdom Fungi is estimated to contain 5.1 million species (Blackwell 2011) which occur in all ecosystems, from deep sea vents to the Antarctic (Ruisi et al. 2007, Le Calvez et al. 2009). Despite increasing knowledge of fungal diversity in surprising habitats, endophytes (fungal organisms living within plants) represent a potentially large pool of undescribed fungal species (Hawksworth 2001, Arnold et al. 2007). Endophytes can occur across broad regions or occur only inside individual host species and possibly for short periods of time (Arnold 2007, Pirttilä and Frank 2011). Since the 1960s, endopyhytes have been found occurring as hotspots of tropical biodiversity (Cooney and Emerson 1964, Arnold 2007), and have been investigated for their role in plant health and more recently, for their ecological (Clay and Holah 1999) and physiological roles (Scott 2001, Rudgers et al. 2006).

Endophytes occupy a niche within the leaves, roots, or wood of plants (Wilson 1995, Arnold et al. 2000, Hawksworth 2001). Endophytes that occur within woody tissue presumably exist in a fairly stable dark environment that does not differ greatly from the surrounding air temperature. Since plants can grow in extreme temperature conditions, from polar regions to deserts, it is likely that endophytes of plants in those environments have similar temperature tolerances. Not surprising then, yeasts have been documented to grow from 0º to 37º C (van Uden 1984) while optimum growth of *Neurospora crassa* is at 36º C and it remains viable from 3º to 44º C (Cooney and Emerson 1964).

Maximum photosynthetic activity for most plants occurs between 20 – 35º C and declines below and above those temperatures (Kozlowski and Pallardy 1997). The optimum temperature for vegetative growth may be similar for endophytic fungi, based on sapwood temperatures. When *Acacia* sp. trees were monitored for sap flow, it was found that air temperatures fluctuated between 15 – 29º C while sapwood temperatures were always higher, ranging from 18 – 31º C (Do and Rocheteau 2002). In a study in TN, two species of oak (*Quercus alba* and *Q. prinus*) and red maple (*Acer rubrum*) were monitored for sap flow, respiration and sapwood temperature. Their stem temperature data indicated that south sides of trees were hotter than north sides, while daily air temperatures ranged from 2 to 26º C and sapwood temperatures ranged from 5 and 30º C (Edwards and Hanson 1996).

As for the growth of pathogens and saprophytes in relation to temperature, *Aspergillus fumigatus* was grown for a period of two weeks between 4 and 30º C. Growth did not occur at 4º C, but the fungus grew more as heat increased with maximum growth at 30º C (Pasanen et al. 1991). In another study using *Aspergillus* and *Penicillium*, optimum growth was between 24 and 40º C while maximum observed heat tolerance was between 38 and 53º C (Ayerst 1969). The optimum growth of *Ophiostoma novo-ulmi*, causal agent of Dutch elm disease was between 20 and 22º C and the non-aggressive *O. ulmi* had its optimum growth between 27.5 and 30º C (Brasier et al. 1981). At 32º C, the causal agent of oak wilt (*Ceratocystis fagacerum*) was characterized by having reduced hyphal growth (Appel 1995).

The study reported here is part of a larger investigation into the fungal biology of *Raffaelea lauricola*, causal agent of laurel wilt (LW) and a native of southeast Asia,

which was positively identified in 2008 (Fraedrich et al. 2008). Epidemic levels of redbay tree mortality were found in coastal Georgia, likely indicating that LW has been in the southeastern USA since at least 2002. *R. lauricola* is carried by the redbay ambrosia beetle (*Xyleborus glabratus*) in their mandibular mycangia and used as the sole source of nutrition by the adults and larvae in the galleries the beetles create in trees (Fraedrich et al. 2008). In North America, *X. glabratus* vectors *R. lauricola* to trees, which kills them within a matter of weeks. *R. lauricola* has been recovered from dead trees in excess of 11 months (Spence unpublished). Unfortunately, humans are moving wood infested with *R. lauricola* and *X. glabratus* throughout the southeastern U.S. (Riggins et al. 2010) and it is likely that this disease will spread into other parts of the country where susceptible hosts exist, from the sub-tropical south to the northern edge of the temperate biome.

Since all native and some ornamental North American Lauraceae species are susceptible to *R. lauricola*, including the important economic agricultural crop of avocado (*Persea americana*) (Mayfield et al. 2009, Ploetz et al. 2011), limiting the spread of this disease is an important goal.

The objectives of this study were to 1) test growth of *R. lauricola* at a wide range of temperatures to identify its thermal metabolic limit, 2) evaluate viability of *R. lauricola* from infected wood following exposure to a range of temperatures, and 3) explore the ambient sapwood temperatures of living and dead redbay trees.

Materials and Methods

In Vitro Growth of *R. lauricola* at Different Temperatures

Pure cultures of *R. lauricola* recovered from sapwood of a LW-infected redbay tree (isolate PL57; GenBank Accession # JQ861956.1) were grown on cyclohexamide-

streptomycin malt agar (CSMA) (Harrington 1981) with antibacterial amendments. The CSMA amendments were the addition of 350 mg of ampicillin sodium salt and 500 µL of a 9 mg RifAmpin+1000 µL DMSO mixture, hereafter, we refer to this medium as CSMA++ (Ploetz et al. 2011). Once there was at least 4 cm of mycelia growth on the CSMA++ plates, single 5 mm agar and mycelium plugs were placed upside down on malt extract agar (MEA) (34 g of Difco malt extra agar and 4 g of agar/1 L of ddH_2O), sealed in individual growth chambers and placed in a dark incubator at varying temperatures. Growth was measured after 10 days of exposure to -7, 10, 15, 20, 24, 25, 28, 30, 33.5, 35 and 47º C. For each treatment there were ten sealed and separated replicates. Radial growth was measured in two directions, at right angles from each other, from the edge of the agar plug to the outer edge of mycelium.

After each temperature trial, plates were transferred to a dark incubator set at 25º C to determine the extent that *R. lauricola* can grow when returned to a more optimum temperature. Plates were left in the 25º C incubator for ten additional days; mycelial growth was measured as noted above.

Three incubators were used during these studies. All cultures that were assessed at 25° C were placed in a Fisher Scientific bench top incubator (Isotemp Model 637D). For cultures exposed to 10, 15 and 20º C a Conviron Adaptis (model A1000) was used and for 28, 30, 33.5, 35 and 47º C assays, a Precision Scientific Forced-Air Incubator (model 6M 31487) was used. All cultures were grown in the dark except for one 24º C trial that was left in continuous light. For this trial,20 agar plugs of *R. lauricola* were placed on MEA for 10 days where ten plates were left in 24 hrs. of light and ten kept in complete darkness at room temperature at 24º C. For the final

comparison, growth of *R. lauricola* on MEA was compared to the growth of *R. lauricola* on CSMA++ at 25º C for 10 days.

In addition to assessing the growth of *R. lauricola* on media at different temperatures, five sapwood cores (3 x 6 mm) from *R. lauricola*-colonized wood were taken from each of five bolts (10 x 7 cm) for two temperature treatments. Ten wood cores were extracted from all bolts and placed on CSMA++ to confirm the presence of the pathogen, which were kept at 25º C. Five bolts were then exposed to 33º and five to 35º C for ten days. After ten days, five more cores were taken from each of the bolts and placed on CSMA++ at 25º C to ascertain viability of *R. lauricola.*

Temperature of Infected and Healthy Redbay Trees

Data on the *in vitro* growth of *R. lauricola* may help determine the optimum growth conditions for the fungus within trees. However, growth on artificial medium may not approximate conditions in the tree and may allow for greater temperature tolerance. Thus, temperature profiles of redbay trees were also assessed *in vivo*.

Sapwood temperatures of living and dead redbay trees, which were killed by LW, were measured to identify the daily fluctuations that occur. Thermocouple data loggers (CAS Data Loggers, Chesterland, Ohio) were used to record sapwood temperatures of redbay trees on private property in South Daytona, FL. Each tree had a 3 mm hole drilled 10 cm deep into the trees at 1 meter above ground level. Each time a data logger was placed in a tree, the space around the probe was sealed with standard window caulk to keep out moisture and limit airflow around the probe tip. Data loggers were left in place for four days and for one week on three different occasions. All measurements were taken in March and April 2012.

For the first trial, probes were placed in three living trees and two dead trees. Outside air temperatures were not recorded. In the second trial, one probe was installed into the sapwood of living and dead trees while a partner probe was left outside the hole of each tree, exposed to the air around the tree. The probe recording air temperature was placed next to the bark on the north side of the tree to keep it in full shade during the day. This set up was used for two dead and one living trees. The final sapwood temperature measurement was taken from a single large redbay tree (53 cm DBH) where two probes were placed inside the tree on the northeast and northwest sides of the tree. Each probe had a companion probe to monitor the air temperature. The outside probes were placed against the trunk so that they were in constant shade.

Statistical Analyses

One-way ANOVAs were used to determine if the *in vitro* growth of *R. lauricola* was different at ten different temperatures and used to determine if the growth of *R. lauricola* from wood cores at to 33º and 35º C were statistically different from wood cores grown at 25º C. An alpha level of 0.05 and a post hoc means comparison was completed using the Tukey's HSD to evaluate differences between means.

Results

In Vitro Growth of *R. lauricola* at Different Temperatures

There was a significant effect of temperature on mean 10 day growth of *R. lauricola* on MEA ($F= 143.71$, $df= 23$, $P<0.001$), Figure 3-1. After ten days, growth of *R. lauricola* was greatest at 28º C (mean growth = 12.5 mm). *R. lauricola* also grew at 24º (dark), 25º, 30º and 32º C (mean growth of = 10.1. 11, 6.7 and 2.1 mm, respectively). *R. lauricola* showed no initial growth at 10, 15, 20, 33.5, 35 and 47º C.

After the initial exposures at –7,10, 15, 20, 28, 30, 32, 33.5, 35 and 47º C, all plates were transferred to 25° C for an additional 10 days. The regrowth of *R. lauricola* at 25° C occurred for all temperatures except 47º C, Figure 3-1. An interesting observation was that *R. lauricola* on CSMA++ grew statistically more than *R. lauricola* on MEA (F (1,18) = 168.35, $P < 0.0001$).

Growth of *R. lauricola* from LW infested bolts showed declining growth at 33 and 35º C when compared to growth at 25º C, Figure 3-2. For the three temperature comparisons, average growth of *R. lauricola* from wood plugs at 25º C was 21.9 mm. After ten days, wood plugs exposed to 33º C grew an average of 11.6 mm. After ten days, wood plugs exposed to 35º C grew an average of 4.5 mm at 25º C. The difference between growth at the tree temperatures was statistically significant F (2,27), = 175.3, $P < 0.0001$.

Temperature of Infected and Healthy Redbay Trees

For the first sapwood temperature measurement, three living and two dead redbay trees were monitored over the same eight days. The maximum temperature reached for the living trees was 29.5º C, while the maximum temperature for the dead trees was 30.5º C. The living tree generally lagged behind the dead tree in its warming and cooling cycle each day, Figure 3-3.

The second sapwood measurement taken was from one living and two dead trees. The probes were left in the sapwood for four days. Living trees become hotter (mean daily maximum = 26.9º C) than the surrounding air (mean daily maximum = 25.6º C) and are also hotter than dead trees (mean daily maximum = 25.1º C), Figure 3-4 . A

review of the temperatures showed no obvious differences between the living, dead, and air temperatures.

For the third sapwood temperature test, the average internal sapwood temperature (24.3º C) of the 53 cm diameter redbay tree was much more stable than the average outside temperature (23.9º C), however they did not differ significantly $F_{(1,826)} = 0.45$, $P = 0.50$, Figure 3-5. On April 4, the high temperature at the Daytona Beach, FL. airport was 33º C (wunderground.com), which was similar to the outside probe temperature, which was 35º C, on the same day.

Discussion

Except for 47° C, *R. lauricola* resumed growth after it was frozen and after it was heated in excess of 33.5° C. And, for the temperatures where it did grow, *R. lauricola's* growth was more vigorous 25° C, indicating that seasonal temperatures (hot or cold) will not reduce viability of this fungal pathogen. The only temperature tested where *R. lauricola* was killed was only at 47° C. This suggests that IPPC heat treatments of wood, or pallets, might be sufficient to kill this pathogen.

In an early review of the biology of *R. lauricola*, Harrington et al. (2008) measured the growth of the pathogen on malt extract agar (MEA) at three temperatures, 10, 25 and 30º C. At 25º C, the radius of the colonies averaged 30 mm while at 10º and 30º C, growth was less than 5 mm (Harrington et al. 2008). Their data for growth at 25 and 30º C produced similar results to our findings; however, the growth they observed at 10º C was more than we observed, which was zero.

Although seasonal fluctuations of sapwood temperatures were not fully evaluated here, the temperature profiles represent the period of time when *X. glabratus* is most

active (Hanula et al. 2008) and trees are expressing wilt symptoms and dying, often in less than a week. It is interesting that *R. lauricola's* optimum growth in this study occurred at 28° C, which is similar to the maximum average sapwood temperature of living and dead redbay trees, which was 24.7° C. It seems that when a redbay tree may be at its photosynthetic optimum, the fungus is also near a temperature that is optimal for its growth.

For the other two tree pathogens that are classified as wilt diseases, *R. lauricola* was not all that dissimilar. The optimum temperature for in vitro growth of *R. lauricola* was slightly higher than species of *Ophiostoma* which cause Dutch elm disease (Brasier et al. 1981). Although the European and North American strains of *O. novo-ulmi* had optimal *in vitro* growth at temperatures between 20 and 22º C. The thermal optimum of *R. lauricola* was more similar to the non-aggressive *O. ulmi*, which had its optimum growth between 27.5 and 30º C (Brasier et al. 1981). At 32º C, the causal agent of oak wilt (*Ceratocystis fagacearum*) exhibited reduced hyphal growth (Appel 1995). In another report on the tolerance of *C. fagacearum*, temperatures between 30 - 35°C reduced growth of the fungus *in vitro* (Lewis 1979, Tainter 1986).

From a phytosanitation perspective, the LW pathogen, *R. lauricola*, was killed at a temperature that was lower than temperatures that are recommended for wood treatment by the Food and Agriculture Organization of the United Nations (FAO 2008). The FAO oversaw the implementation of International Plant Protection Convention (IPPC) that developed the International Standards for Phytosanitary Measures ISPM 15, which were revised in 1997. The current phytosanitary regulations for the heat treatment of pallets and other wood products calls for the wood products to be heated

for a minimum of 30 minutes to reach a core temperature of 56° C (FAO 2008). Data from this study provides an initial assessment that *R. lauricola* might be eliminated from wood packing material if the wood products are heated to IPPC standards.

As for low temperature tolerance of *R. lauricola*, it appears that temperatures below freezing do not have a negative impact on the viability of *R. lauricola*. The implication of this finding is that logs or trees infected with *R. lauricola* in northern temperate areas could remain colonized and that cold temperatures alone may not eliminate this pathogen from the environment. The thermal tolerance of *X. glabratus* is unknown and what role it could play as vector in cooler temperate areas has not been tested.

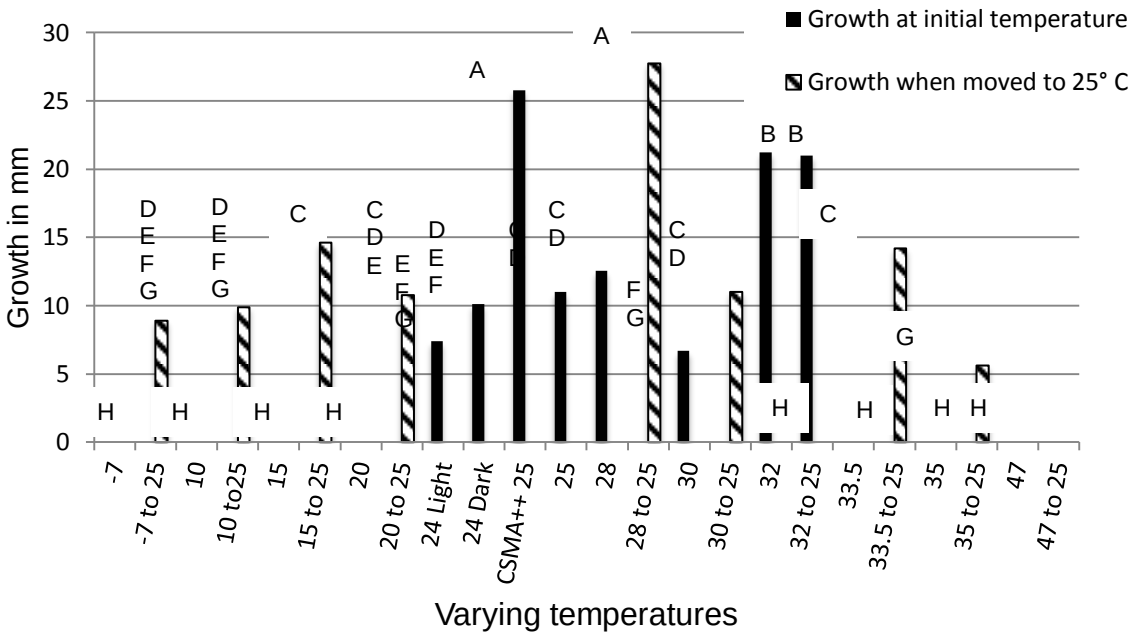

Figure 3-1. Growth of *R. lauricola* at varying temperatures. 10 sealed petri dishes of *R. lauricola* were exposed to each temperature for 10 days. Solid bars represent the average growth of *R. lauricola* at specific temperatures, hashed bars represent *R. lauricola* re-growth after being moved to 25º C measured after 10 days. Letters represent homogenous subgroups determined by a Tukey HSD ($F = 143.71$, df = 23, $P < 0.001$).

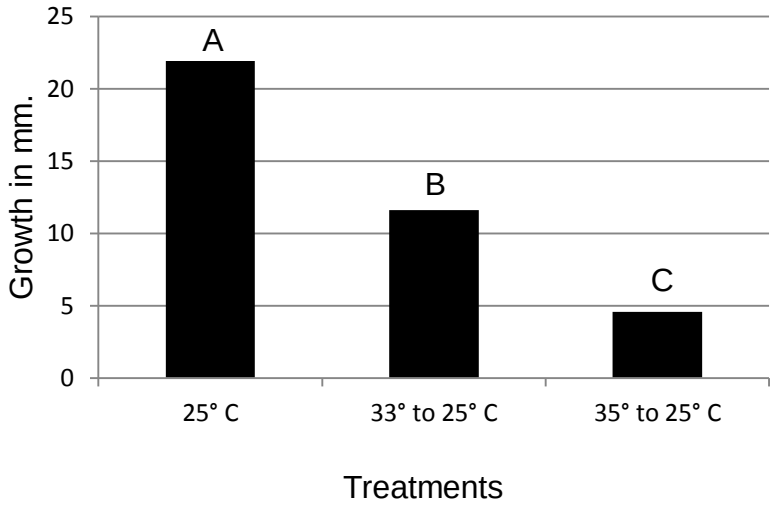

Figure 3-2. Growth of *R. lauricola* from wood plugs at varying temperatures. Five plugs from *R. lauricola*-infested wood were taken from five bolts for each treatment and plated on MEA to confirm the presence of the pathogen, noted as 25º C. Five bolts were exposed to 33 º and 35º C for ten days. After ten days, five more cores were taken from each of the five bolts and placed on MEA to ascertain viability of *R. lauricola* at 25º C, growth was statistically different $F_{(2,27)} = 175.32$. $P < 0.0001$, Letters represent homogenous subgroups determined by a Tukey HSD.

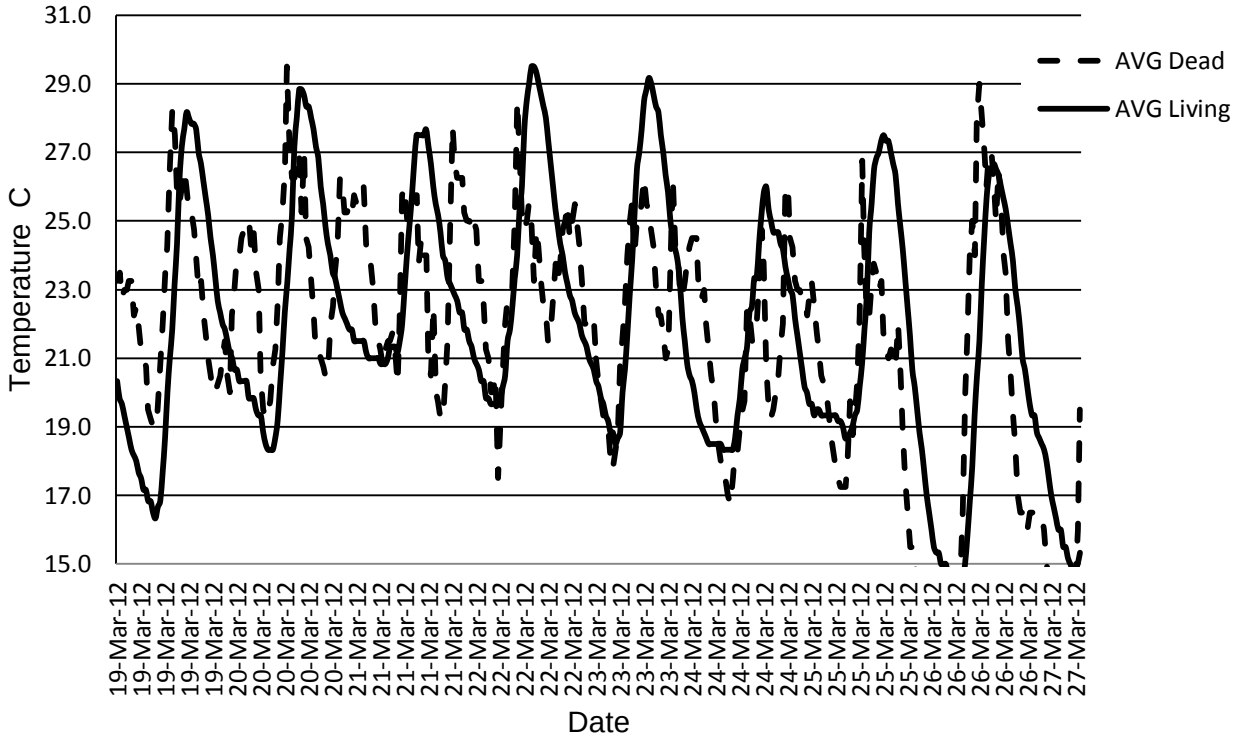

Figure 3-3. Internal temperatures of dead and living trees. Average internal sapwood temperatures measured from three living and teo dead redbay trees in Daytona Beach, FL. Each tree had a probe inserted approximately 10 cm into the sapwood where temperature was recorded once an hour for 8 days.

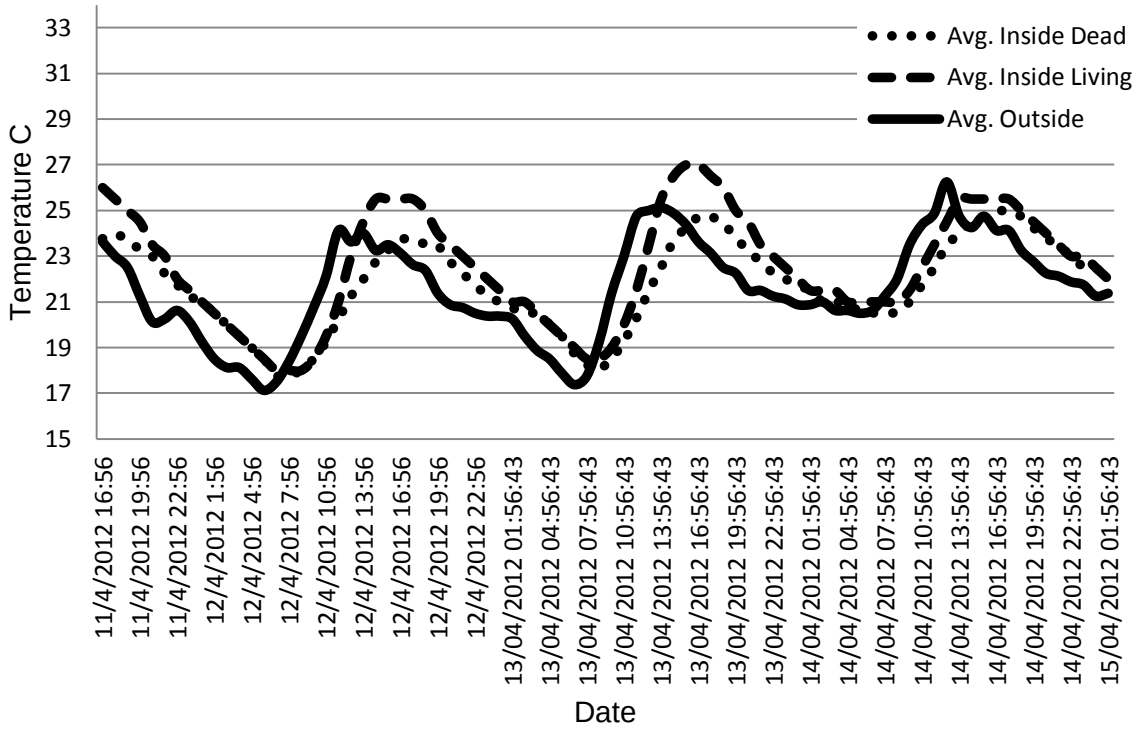

Figure 3-4. Internal sapwood temperatures of dead and living redbay trees vs. ambient air temperature. Average internal sapwood temperatures from one living and two dead trees and ambient air temperatures. Each tree had a probe inserted into the sapwood and a partner probe that was left exposed to shaded air on the north side of the tree.

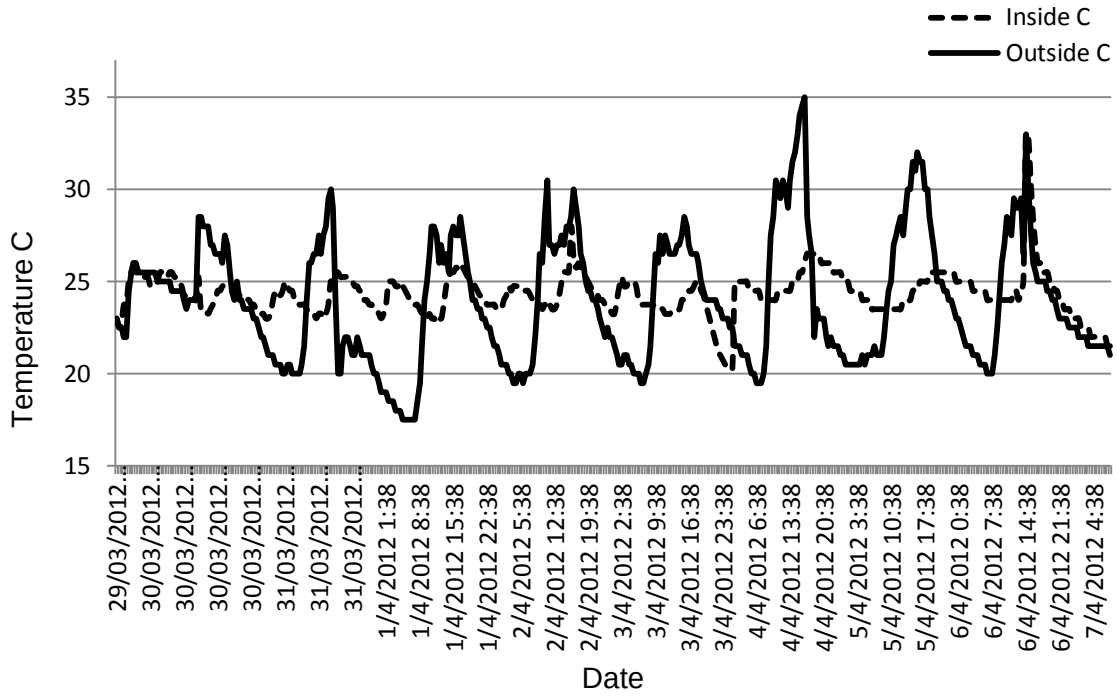

Figure 3-5. Internal and external temperatures from one large redbay tree. Two probes were inserted into the tree's sapwood, one on the northeast side and another in the northwest side. Each probe had a partner probe that was left just outside the bark and situated so it would not be exposed to full sun and record air temperature.

CHAPTER 4
PERSISTENCE AND DISTRIBUTION OF *RAFFAELEA LAURICOLA* IN DEAD, STANDING REDBAY TREES (*PERSEA BORBONIA*)

Introduction

Fungi occur in a wide variety of niches, on and in building material (Jellison et al. 1997, Schirra et al. 2000), as saprophytes (Schirra et al. 2000, Zuccaro et al. 2011), pathogens (Mendgen and Hahn 2002, Lowe and Howlett 2012), and as endophytes inside many species of plants (Arnold et al. 2003, Gennaro et al. 2003, Scott 2011), competing for space and nutrients (Glass and Kuldau 1992). Fungal succession and persistence are determined by many factors including nutrient availability, competition, and their interaction with insects (Paine et al. 1997, Hyde and Jones 2002, Suzuki 2002, Rollins et al. 2001, Hyde and Soytong 2008).

Fungal succession, like plant succession (Noble and Slayter 1980, van der Valk 1992), can occur in a linear replacement pattern, through broad seral steps, or through competition or interference (Frankland 1998, Boddy 2001, Fukasawa 2009). Fungi, like plants and animals, might also create an environment better suited for later successional species; ultimately displacing the pioneer (Jellison et al. 1997, Frankland 1998, Boddy 2001, Xu and Gordon 2003).

In part, this study explores the species present inside living and dead redbay trees (*Persea borbonia*), which are termed endophytes. Endophytes are fungi that can persist as latent pathogens or saprophytes, waiting for the right environmental conditions to grow and exploit the host. The biology of endophytes has not been precisely determined (Saikkonen 2007, Hyde and Soytong 2008), but it is known that some endophytes persist within an asymptomatic host for long periods of time and decline as the tree declines (ref). Others gain entry into in a tree, persist

asymptomatically but then actively grow as tree health declines, either through natural senescence or one mediated by disease (Oses et al. 2008, Parfitt et al. 2010).

Endophytes may also gain entry into the woody stems of a plant through horizontal transmission, where fungi may occur on bark and are then incorporated into the sapwood over time, or they could gain access through lenticels and other natural openings or wounds (Saikkonen 2007, Chaverri and Gazis 2011). Some endophytic fungi can enter trees with the help of bark or ambrosia beetles (Batra 1966, Goheen and Hansen 1993, Hulcr et al. 2007). Introduction of a fungus into a tree by an insect usually occurs when trees are in a state of decline or dead (Hulcr et al. 2007, Hulcr and Dunn 2011, Six and Wingfield 2011. In some cases, fungi are passively transmitted by insects while some, ambrosia beetles for example, have specialized sacs for carrying fungi, which are then deposited inside trees for the beetles to eat. In addition to being food for beetles, these fungi may be able to grow as saprophytes, aiding in the decomposition of the tree (Rollins et al. 2001, Henriques et al. 2006).

When ambrosia beetles occur within their native range they tend to go unnoticed without posing problems to healthy trees, when they do appear outside of their natural range they can significantly damage naive hosts. In the southeastern coastal plain of the USA, a new tree disease called laurel wilt (LW) has been identified. The disease is caused by the boring activity of the exotic redbay ambrosia beetle *(Xyleborus glabratus*) and the host response to the deposition of its fungal symbiont, *Raffaelea lauricola*. The fungus is causing significant mortality to trees in the Lauraceae (Fraedrich et al. 2007, Fraedrich et al. 2008, Hanula et al. 2008, Mayfield et al. 2008). Both the beetle and its

fungus are Asian in origin (Rabaglia 2008, Harrington et al. 2011). The beetle was first found in the Savannah GA area in 2003; its method of introduction is unknown.

Because of its ability to kill or facilitate the death of trees (Hulcr and Dunn 2011), *R. lauricola* is classified as a pathogen, however the mechanisms by which it causes hosts to wilt and die are apparently different from other wilt pathogens such as *Fusarium* and *Verticillium* (Agrios 2005). Due to a lack of direct evidence, it is thought that Lauraceous hosts are to over-reacting to the presence of *R. lauricola*, ultimately killing themselves in the process (Hulcr and Dunn 2012). Like many plant pathogens, *R. lauricola* must be able to persist as a saprophyte, to some degree, to acquire nutrients for it to survive while it exists as food for *X. glabratus*. To date, no studies have evaluated the persistence and distribution of *R. lauricola* within LW-killed redbay trees. The physical changes that take place in wood over time (i.e. moisture) and resource competition (fungal competitors) are two factors that may regulate the persistence of *R. lauricola* inside dead standing trees. Redbay is a ubiquitous species in coastal ecosystems in the southeast, however, there are no published papers regarding the endophytic constituents of these trees and no clue as to what role these endophytes might play in determining how long *R. lauricola* can persist inside a dead standing tree.

Understanding how long *R. lauricola* can persist as a saprophyte, how extensively it colonizes dead sapwood and an enumeration of fungal competitors would be useful for better understanding the epidemiology of LW and may help improve efforts to manage the disease through sanitation strategies. This study focused on the following four objectives: 1) evaluation of *R. lauricola* persistence inside dead standing

redbay trees, 2) identification of fungal endophytes from diseased and healthy trees, 3) assessment of *R. lauricola's* ability to grow as a saprophyte *in vitro*, and 4) determination of the spatial distribution of *R. lauricola* in sapwood of infected trees.

Materials and Methods

Twenty-six redbay trees (*Persea borbonia*) were evaluated from January 2011 to July 2012 in Washington Oaks Garden State Park, FL. The park covers 425 acres on a barrier island in Flagler County, FL. The study trees occurred in a typical maritime hammock (Spence et al. 1998) dominated by live oak (*Quercus virginiana*), pignut hickory (*Carya glabra*), southern magnolia (*Magnolia grandiflora*) and southern red cedar (*Juniperus virginiana*) with an understory of redbay (*Persea borbonia*) and yaupon holly (*Ilex vomitoria*). Study trees had a DBH (diameter at breast height, measured at 1.3 m above grade) that ranged from 6.4 to 24 cm. Trees were evaluated using a subjective disease rating scale at each visit until each tree died. The rating scale was based on crown wilt symptoms and ranged from 0 – 4, where 4 represented a dead tree and 0 was an asymptomatic tree. Of the 26 trees, ten were dissected and fourteen were regularly visited to determine how long *R. lauricola* would persist in wood tissue. Two trees were dropped over the course of the study, one because it never died and two because they fell over.

Persistence of *R. lauricola* in Standing Trees

Over the course of the 18-month study, eleven trunk cores were taken from each of the thirteen trees used for the *R. lauricola* persistence study. Sapwood cores (15 x 5 mm) were collected using an increment hammer (Haglöf, Sweden) to test for the presence of the pathogen at the start of the study. Two samples were collected at 2 m and two samples from 30 cm above natural grade, one from each side of the tree. The

head of the increment hammer was rinsed with 70% ethanol between samples for each tree. Samples were placed in separate vials and were kept in a cooler for transport back to the laboratory.

Sapwood core samples were cut into 4 or 5 smaller fragments (approximately 2 x 3 mm in size) and surface sterilized for 60 seconds in 70% ethanol and rinsed in ddH_2O for 60 seconds. Wood fragments were placed on cyclohexamide-streptomycin malt agar (CSMA), a semi selective medium for ophiostomatoid fungi (Harrington 1981), with antibacterial amendments. The CSMA amendments were the addition of 350 mg of ampicillin sodium salt and 500 µL of a 9 mg RifAmpin+1000 µL DMSO mixture, hereafter; we refer to this medium as CSMA++ (Ploetz et al. 2011). All plates were kept at 24˚ C under diurnal conditions. Samples were evaluated for the presence or absence of *R. lauricola* after ten days and determinations were made based on morphology.

Tree Sapwood Moisture

Over the course of the study, seven moisture readings were taken from each tree at 1.5 meters above grade. Moisture was recorded from three points around the stem, evenly spaced and taken along the vertical axis of the tree. On trees with thick outer bark, readings were taken between bark plates. The electrode needles were inserted approximately 1 cm into the sapwood. Moisture readings were taken with a Protimeter® Timbermaster (General Electric Corporation, Shannon, Ireland).

Potential *R. lauricola* Competitors

One of the upper and one lower sapwood cores that were used to track the persistence of *R. lauricola* was surface sterilized as noted above and plated on 2% malt extract agar (MEA). Fungi with unique morphological characteristics (morphotypes) were subcultured and stored on slants at -80º C until the DNA was extracted.

Twenty-four of the twenty-six trees were alive at the first sampling event in March of 2011, trees 24 and 25 died in February of 2011. The second set of wood cores were taken in mid April 2011 where eight additional trees had died, leaving twelve samples from asymptomatic trees.

To assess the taxonomic groups of fungi present in the wood cores, nuclear DNA was extracted from subcultures using the method Justesen et al. (2002) with the addition of proteinase K. The small subunit (18s) rDNA was amplified via PCR using the primers NS1 (5'-GTAGTCATATGCTTGTCTC- 3') and NS4 (5' – CTTCCGTCAATTCCTTTAAG – 3') and the internal transcribed spacer region rDNA (ITS) was amplified using primers ITS1 (3' GGCGTCCAAGTGGATCGCT) (White et al. 1990) and ITS4 (5' TCCTCCGCTTATTGATATGC) (Gardes and Bruns, 1993). A PCR master mix containing 12.5 µL of Amplitaq Gold (Applied BioSystems, Foster City, CA.), 9.5 µL of ddH2O, 1 µL of template DNA, plus 1 µL of each primer was used. A MJ Research, PTC-200 Peltier thermocycler was used for PCR with the following cycling conditions: 95° C for 6 minutes then 40 cycles of 95° C for one min., 48° C for 30° sec. and 72° C for two minutes for DNA extension. ExoSAPit (Affymetrix, Inc, USB Products, Santa Clara, CA) was used to purify the amplicon which was then sent to the University of Florida's Interdisciplinary Center for Biotechnology Research for Sanger sequencing.

Assessment of Saprophytic Capability of *R. lauricola*

To evaluate the ability of *R. lauricola* to grow as a saprophyte, agar plugs containing *R. lauricola* from a symptomatic redbay (strain PL1235; GenBank Accession # HM446155) were placed on 20 wood chips and 20 wood disks. The wood disks were approximately 5 cm in diameter and between 1 and 2 cm thick. Ten wood disks were

from a non-LW colonized tree and 10 were from a tree that died from LW within three months of this study; wood disks for both studies were collected from Washington Oaks Garden State Park, FL. 3 days before the study. Wood chips used for this study were from redbay trees that died from LW approximately one year prior to this study, *R. lauricola* was no longer alive in these wood chips. To make the wood chips, dead redbay trees were chipped were using a standard arborist wood chipper, (Vermeer model BC 935), average wood chip size was 2.2 x 1.7 cm.

Prior to the placement of the agar plugs on each of the treatments, 10 wood chips were soaked in ddH_2O for three days and ten were not. All twenty wood disks were also soaked in ddH_2O for three days. The wood chips and disks were soaked in ddH_2O in an attempt to make the moisture content of each treatment as even as possible, 10 wood chips were not soaked in water.

All wood chips and disks were autoclaved and cooled to room temperature before the agar plugs were placed on them. The moisture content of each wood sample was recorded after they were autoclaved but before agar plugs were placed on them. Single, 5 mm plugs of *R. lauricola* were placed on ten autoclaved non-LW infected disks, ten autoclaved LW infected disks, and on ten autoclaved and once LW infected wood chips (4 x 3 cm^2). The mycelia side of the agar plug was placed against the wood for all samples which were then placed in glass petri dishes and sealed with Parafilm. Plugs of *R. lauricola* were grown on 2% malt extract agar as a positive control. The growth of each culture was evaluated after two weeks for the presence mycelium or changes in wood color or moisture, which might indicate fungal penetration of wood tissue. All samples were kept at room temperature (24º C) under diurnal conditions.

To confirm the presence of *R. lauricola* after 10 days, agar plugs were removed from wood samples and sections of wood were excavated from below the area of the agar plug since this was the area where *R. lauricola* would have likely first penetrated into the wood. Excavated wood samples were placed on MEA for 10 days. Cultures that morphologically matched *R. lauricola* were then used for molecular identification. DNA was extracted from the cultures using the same procedures described previously. PCR with semi-specific LW primers (LWD3 5'-AACGCGTCAAAAGACAACAG-3'and LWD4 5'- TTCTAGGACCGCCGTAATG-3') was performed to amplify a partial sequence of SSU rDNA of *R. lauricola* (Dreaden et al. 2008). Five µL of PCR amplicons, 1 µL of loading buffer and 0.5 µL of SYBR Green per sample were visualized on a 1.5% agarose gel. The presence or absence of the amplicon was used as the criterion to determine if samples were positive for *R. lauricola*.

Distribution of *R. lauricola* in Standing Redbay Trees

Ten redbay trees were felled and dissected to identify which portions (trunk, branches, twigs, or leaves) of the tree colonized by *R. lauricola*. Samples were taken at 30 cm above grade and approximately every 1.5 - 2 m above the basal sample. Since redbay is not a dominant canopy tree, the trees were often leaning towards light or had multiple leaders towards the apex. Samples were collected as evenly as possible based on the branching architecture of the tree. In addition to trunk samples, samples were collected from scaffolding and lateral branches and from twigs and leaves at the outer edges of the canopy.

Samples were put in sealed bags and kept in a cool container and transported back to the Forest Pathology Laboratory at the University of Florida. Each trunk sample was dissected such that three groups of subsamples were collected from the outer,

inner and middle region of the sapwood, Figure 4-1. Wood samples were cut into small bits (1 x 1 cm) where the outer sapwood samples were collected from just below the vascular cambium and inner samples were collected from the center of the sample. Middle samples were collected half way between the inner and outer samples. For branch samples, just the outer sapwood below the cambium was used and the center of the branch for the inner sample. Wood samples were processed, stored, and evaluated as described above for *R. lauricola* persistence. For all samples, wood sections with beetle galleries were avoided.

The confirmation of *R. lauricola* was based on morphology, except for two trees. PCR using NS1/NS4 primers (procedure as noted above) was used to positively identify putative *R. lauricola* isolates from those two trees as well as several unique endophytes that were cultured at the same time.

Statistical Analysis

A one-way ANOVA was used to identify differences in moisture content in redbay trunks over the length of the study, differences in moisture content of wood samples used for the saprophytic ability of *R. lauricola* and the proportion of the occurrence of *R. lauricola* from different areas of the stem. When differences were found a Tukey-HSD comparison of means was used to determine which treatment groups differed from one another. An alpha level of 0.05 was used for all tests.

Results

Thirteen of the 26 redbay trees were tracked for the persistence of *R. lauricola* while ten trees were dissected to determine where *R. lauricola* occurs in the stem of the trees, three trees were dropped from the study, Table 4-1. Tree 5 in this study never

succumbed to the disease. After five months of taking samples, this tree was dropped from the study. Trees 10 and 19 had significant cavities which led to portions of the tree falling over; these trees were not dissected or tracked for the persistence of *R. lauricola* due to their deteriorated condition, however, wood cores were collected for the endophytic assay.

Persistence of *R. lauricola* in Standing Trees

Over the course of the 18-month study, *R. lauricola* disappeared in six of the thirteen trees. For three trees (12, 15, and 21) the fungus disappeared after 14 months, Table 4-1. Seven trees were found to have *R. lauricola* in the outer sapwood for eight months, where the fungus finally disappeared after 12 months (trees 1, 2, 6, 9, 11, 13 and 14). Tree 25 was the first tree to begin to wilt in February of 2011. This tree was cut down and dissected in August of 2011 since *R. lauricola* had not been detected in the outer sapwood since May of 2011. When this tree was dissected in August of 2011, *R. lauricola* was found to be present in wood that was dissected from inner sapwood. Due to time constraints, the study was ended before the ultimate longevity of *R. lauricola* in sapwood was determined for all the samples.

Tree sapwood moisture

Moisture fluctuated over time, likely tied to rainfall, which was not recorded. The overall trend showed that there was a decline in moisture over time; however, the decline was not linear since some measurements showed that dead trees can soak up water in high humidity or from rainfall, Figure 4-2.

The relationship between survey date, moisture and disease rating were significant yielding ANOVAs of F (4,120), = 8.93, $P < 0.001$ and F (4,120), = 3.40 , $P <$

0.011), respectfully. These data reaffirm the understanding that dead and dying trees will not have as much moisture in the outer sapwood as living trees.

Potential *R. lauricola* Competitors

Eighty-one fungal morphotypes were sub-cultured and grown as pure cultures. In total, 46 isolates were identified either using NS1-4 or ITS1-4 primers. Of the 46 isolates, 20 unique fungi were identified through sequence comparison to Genbank (http://www.ncbi.nlm.nih.gov/genbank/), Table 4-2. A number of studies suggest that endophytes of woody plants are rather loosely associated with their hosts, with a higher correlation between endophyte communities in a specific location rather than with a specific host in different locations (Arnold et al. 2003, Saikkonen 2007). The species identified in these redbay trees represent species that are present in divergent plant families and from distant places on the planet. DNA from twelve samples were not identified to the genera level and are reported as fungal endophyte or funal species based on the Genbank blast information. All but four of the 46 isolates were from the Ascomycota, where there were 14 representatives from the Sordariomycetes, 6 from the Eurotiomycetes and 5 from the Dothideomycetes. The four remaining isolates were from the Mucorales, representing two species, *Umbelopsis isabellina* and *Grongronella butleri*. Zone lines were observed in wood core indicating that species of Basidiomycetes were present inside the trees, however, this group of fungi did not grow out of our wood plugs allowing us to identify them.

Assessment of Saprophytic Capability of *R. lauricola*

Tests of the saprophytic ability of *R. lauricola* yielded mixed results. The pathogen was recovered from seven of the 10 wood chips that had been soaked in water. None of the non-soaked wood chips produced *R. lauricola*. Interestingly, the

agar plugs placed on the non-soaked wood chips shrived up and fell off, indicating that *R. lauricola* is not likely to colonize dry wood, like heat-treated pallets. *R. lauricola* did not grow from any of the samples from the LW infected or non-LW infected disks. However, all 10 of the non-LW disks had a white mycelia mat covering some portion of the disk. All attempts to grow this fungus on MEA failed. All of the positive controls yielded *R. lauricola* on MEA.

The mean moisture content of LW colonized disks was 42.8% (with a range of 40.4 to 50%); non-LW colonized disks was 32.7% (with a range of 26.8 to 36.2%), for the water-soaked wood chips it was 34.2% (with a range of 26.4 to 44.6) and for the non-water-soaked wood chips the mean moisture content was 9.45% (with a range of 7 to 13). The means of each treatment were statistically different from one another between the wood chips and LW colonized disks being similar but the LW colonized disks being different (F (3,36),= 142.05, $P < 0.001$), Figure 4-3.

Saprophytic fungi have the ability to degrade cellulose as a means of nutrient acquisition, and through the production of cellulolytic enzymes (Boddy 2001); fungi can persist in and on wood. Since *R. lauricola* exists inside trees, it seems that *R. lauricola* should have the ability to breakdown cellulose to survive. Wood chips and disks should provide a suitable substrate for *R. lauricola*. Standing dead trees do retain water for many months after they die (Figure 4-1). The average wood chip moisture from which *R. lauricola* was cultured from was 34%. In another test, 10 wood chips with a moisture content of 8% (with a range of 5 to 17%) were tested for the ability of *R. lauricola* to penetrate the wood chips. Here, *R. lauricola* did not grow on wood chips at this

moisture level at all; in fact, the dry wood absorbed the moisture in the agar plug, causing it to shrivel.

Distribution of *R. lauricola* in Standing Redbay Trees

R. lauricola was recovered from the branches and trunks of all trees in the study. Ten dead redbay trees were dissected to evaluate the distribution of *R. lauricola* in the sapwood. *R. lauricola* was consistently recovered from outer sapwood (2 mm below the bark) of tree trunks and branches for all 10 trees, Figure 4-4. *R. lauricola* was cultured from as deep as 7 cm below the active vascular cambium in tree 20, 6 cm in tree 8 and 6.5 cm in tree 16.

Forty seven percent of the samples produced *R. lauricola* from deeper than 2 cm in the stem and, 11% of the samples yielded *R. lauricola* from 5 cm or deeper in the stem. In contrast, *R. lauricola* was recovered from 92% of outer sapwood samples. Although *R. lauricola* has been recovered from roots, the pathogen was only cultured from the center (inner region) of one tree at 30 cm above grade (Tree 17). Table 4-4 lists the proportion of samples that produced *R. lauricola* for all nine trees for each of the three sections of the tree.

Location of recovery of *R. lauricola* in either the inner, middle or outer regions of the stems differed significantly (F (2,152), = 4.74 , P = 0.0101), Figure 4-5, meaning that *R. lauricola* was more commonly cultured from the outer region of the sapwood than inner regions. The pathogen was found equally in all portions of the tree, both vertically and horizontally (radius data) and the pathogen was recovered from all trees in the study. Due to the length of time between when the trees died and when samples were collected for dissection, none of the twigs or leaves yielded *R. lauricola*.

Discussion

Over the 18 months that samples were collected from the sapwood of redbay trees, *R. lauricola* was recovered from 100% of the trees for at least 6 months and for as long as 14 months. From the thirteen trees that that were followed for the persistence of *R. lauricola*, all but one (tree 26) continued to support *R. lauricola* in their tissues through April 2012. At no time during the study was *R. lauricola* recovered from leaves or twigs from these trees; however, it has been recovered from these tissues by the Forest Pathology Laboratory at the University of Florida. This was not surprising since the leaves dry out quickly due to the wilt produced by the fungus. It also highlights that if samples were going to be collected for a LW assay, leaves and twigs should be avoided.

These data make it clear that *R. lauricola* has the ability to persist for approximately one year inside dead redbay trees. If the persistence of a species of fungi inside trees is dependent on it symbiotic insect, than as long as those insects continue to protect and cultivate them, the fungi are likely to persist for long periods of time (Biedermann and Taborsky 2011). Since *R. lauricola* is persisting inside dead trees, it is likely that it is due to the activity of *X. glabratus.* And, *X. glabratus* is still being collected in low numbers in areas where the disease has killed most, if not all, suitable hosts (Hughes personal communication, Hanula et al. 2008). With *R. lauricola* and *X. glabratus* persisting in forest stands for many years, other wood boring beetles will likely come into contact with *R. lauricola*. These beetles may feed on the fungi or acquire it passively. In south FL, several species of xyleborini were found to be carrying *R. lauricola* in their mycangia (Ploetz unpublished).

If saprophytic endophytes do play a role in the ultimate exclusion of *R. lauricola*, it is not happening quickly. This study identified 46 unique fungal isolates that should be in completion with *R. lauricola*; however, it is not clear whether or not these organisms will play a role in the ultimate elimination of *R. lauricola* from trees.

Of the endophytes that were identified, several species, such as *Xylaria*, *Pestalotiopsis* and *Botryosphaeria* occurred in several trees. In addition to these commonly found fungal endophytes, several had broad distributions, occurring across broad geographic areas and across many divergent groups of plants. A few endophytes had a narrow range of plant associations; a Lecythophora sp. was found in only one tree and in Genbank (http://www.ncbi.nlm.nih.gov/nuccore/gu166485.1#feature_312192301) a publication noted it was associated with *Changnienia amoena*, an orchid species from China. *Byssochlamys nivea* was another redbay endophyte that appeared in one tree but it is also a fungus that is associated with food spoilage.

What factors will ultimately lead to the loss of *R. lauricola* from trees is unknown. Its disappearance could be due to lack of grooming or attention from its beetle symbiont or the fungus could have been overrun by other endophytes (fungal or bacterial). Whether the ultimate loss of *R. lauricola* is due to autogenic (within system-endophytic competition, displacement or interference) or allogenic processes (external force such as moisture or temperature) was not explored in this study.

It was demonstrated that *R. lauricola* has the ability to persist as a saprophyte on wood chips although it did not grow into the wood of the LW infected and non-LW disks. The only difference between the wood chips and the wood disks was age. The wood

disks were used within 5 days of being field collected while the wood chips were a year old and had been stored in a mesh bag. It is likely that fungi and or bacteria had already begun to break down the lignin and cellulose of the wood chips, making it easier for *R. lauricola* to colonize them. The exact role that moisture plays in the occupation of wood tissue by *R. lauricola* is not known. An attempt was made to control the moisture content of the wood chips, non-LW disks and LW disks at different levels. However, holding these wood samples at constant moisture after autoclaving was not achieved. Therefore, we only tested wood chips with wood moisture greater than 25% and below 15%. *R. lauricola* did not grow on wood with moisture below 15%.

Since *R. lauricola* has shown to be a fairly persistent resident inside dead redbay trees, it is likely that it is going to remain as a biotic component in south eastern forest systems for the foreseeable future. However, a host range study showed that only the Lauraceae are susceptible to LW in Florida (Ploetz and Smith unpublished) so if *R. lauricola* is transferred to other species of trees by other beetles species, it may not be as catastrophic as it has been for the Lauraceae.

Table 4-1. Arrival dates and persistence of *R. lauricola* inside standing dead redbay trees at Washington Oaks Garden State Park, Flagler County, FL. For each cell associated with a tree and a particular date, the percent of samples that produced a positive identification of *R. lauricola* are listed above the number of samples tested. The dates that trees were removed and dissected are also noted.

Tree No.	3 Jan 2011	27 Feb 2011	27 Mar 2011	18 Apr 2011	15 May 2011	15 Jun 2011	10 Jul 2011	9 Aug 2011	2 Oct 2012	22 Mar 2012	8 Jul 2012
1	0 (0/10)	0 (0/11)	0 (0/10)	0 (0/9)	0 (0/9)	0 (0/8)	20% (2/10)	75% (6/8)	0 (0/10)	57% (8/14)	0 (0/14)
2	0 (0/10)	0 (0/7)	0 (0/10)	0 (0/10)	0 (0/6)	0 (0/11)	50% (5/10)	50% (2/4)	36% (4/11)	46% (6/13)	25% (2/8)
3	0 (0/12)	0 (0/14)	0 (0/12)	0 (0/12)	0 (0/9)	0 (0/9)	67% (6/9)	71% (5/7)	90% (9/10)	Dissected on 14 Dec	
4	0 (0/10)	0 (0/11)	0 (0/10)	0 (0/10)	0 (0/8)	0 (0/10)	100% (9/9)	Tree dissected on 10 Aug 2011.			
5	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/11)	0 (0/8)	Tree never died, stopped taking cores.					
6	0 (0/9)	0 (0/12)	0 (0/13)	0 (0/11)	0 (0/7)	0 (1/11)	25% (2/8)	25% (1/5)	27% (3/11)	67% (6/9)	21% (3/14)
7	0 (0/9)	0 (0/11)	0 (0/9)	0 (0/11)	0 (0/10)	0.08% (1/12)	100% (8/8)	86% (6/7)	55% (6/11)	Dissected on 14 Dec	
8	0 (0/11)	0 (0/11)	0 (0/11)	0 (0/10)	0 (0/9)	10% (1/10)	75% (6/8)	Tree dissected on 10 Aug 2011.			
9	0 (0/10)	0 (0/13)	0 (0/10)	0 (0/13)	0 (0/10)	0 (0/9)	22% (2/9)	0 (0/7)	0 (0/11)	69% (9/13)	14% (2/14)
10	0 (0/10	0 (0/11)	75% (9/12)	The tree fell over and was dropped from the study							

Table 4-1. Continued

Tree No.	3 Jan 2011	27 Feb 2011	27 Mar 2011	18 Apr 2011	15 May 2011	15 Jun 2011	10 Jul 2011	9 Aug 2011	2 Oct 2012	22 Mar 2012	8 Jul 2012
11	0 (0/10)	0 (0/11)	0 (0/11)	0 (0/11)	0 (0/11)	0 (0/8)	10% (1/10)	63% (5/8)	36% (4/11)	36% (5/14)	15% (2/13)
12	0 (0/12)	0 (0/14)	0 (0/11)	45% (5/11)	50% (6/12)	64% (7/11)	33% (3/9)	38% (3/8)	58% (7/12)	58% (7/12)	0 (0/12)
13	0 (0/11)	0 (0/11)	0 (0/11)	0 (0/11)	0 (0/11)	0 (0/9)	50% (4/8)	63% (5/8)	73% (8/11)	21% (3/14)	0 (0/13)
14	0 (0/11)	0 (0/10)	0 (0/11)	0 (0/10)	0 (0/10)	0 (0/10)	33% (3/9)	86% (6/7)	36% (4/11)	18% (2/11)	0 (0/12)
15	0 (0/11)	0 (0/11)	0 (0/11)	79% (11/14)	42% (5/12)	42% (5/12)	50% (5/10)	100% (8/8)	42% (5/12)	67% (8/12)	14% (2/14)
16	0 (0/11)	0 (0/11)	0 (0/11)	77% (10/13)	17% (2/12)	Tree dissected on 8 June 2011.					
17	0 (0/10)	0 (0/14)	0 (0/10)	100% (13/13)	42% (5/12)	42% (5/12)	Tree dissected on 1 July 2011.				
18	0 (0/12)	0 (0/12)	0 (0/12)	100% (13/13)	100% (10/10)	90% (9/10)	Tree dissected on 1 July 2011.				
19	0 (0/11)	0 (0/11)	50% (6/12)	The tree fell over and was dropped from the study							
20	0 (0/10)	0 (0/10)	0 (0/10)	92% (12/13)	42% (5/12)	Tree dissected on 8 June 2011.					
21	0 (0/10)	0 (0/13)	0 (0/14)	100% (11/11)	29% (4/14)	40% (4/10)	70% (7/10)	50% (4/8)	20% (2/10)	0.06% (1/15)	0 (0/13)

Table 4-1. Continued

Tree No.	3 Jan 2011	27 Feb 2011	27 Mar 2011	18 Apr 2011	15 May 2011	15 Jun 2011	10 Jul 2011	9 Aug 2011	2 Oct 2012	22 Mar 2012	8 Jul 2012
22	0 (0/9)	0 (0/9)	0 (0/12)	0 (0/11)	0 (0/11)	0 (0/9)	0 (0/10)	56% (5/9)	0 (0/11)	21% (3/14)	58% (7/12)
23	0 (0/10)	0 (0/12)	0 (0/11)	64% (7/11)	50% (5/10)	18% (2/11)	25% (2/8)	71% (5/7)	36% (4/11)	37% (8/12)	27% (4/15)
24	0 (0/14)	40% (4/10)	45% (5/11)	58% (7/12)	17% (2/12)	0 (0/8)	0 (0/7)	Tree dissected on 10 Aug 2011.			
25	0 (0/10)	**50% (5/10**)	53% (8/15)	92% (12/13)	0 (0/12)	0 (0/11)	0 (0/9)	Tree dissected on 10 Aug 2011.			
26	0 (0/9)	0 (0/11)	0 (0/10	86% (12/14)	36% (5/14)	100% (11/11)	75% (6/8)	45% (5/11)	91% (10/11)	0 (0/14)	0 (0/13)

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Table 4-2. Endophytic fungi from redbay trees. Of the eighty-one morphologically unique endophytes identified in culture, forty six were successfully sequenced using the 18s ribosomal small subunit.

Tree #	Primers	Genbank Accession #	Species best match	Max identity
19Ua1	NS1-4	AY315416.1|	*Xylaria* sp.	407/407 (100%)
19Ua2	NS1-4	JN940801.1|	*Pestalotiopsis adusta*	659/659 (100%)
8La	NS1-4	JN941734.1|	*Elaphocordyceps ophioglossoides*	766/766 (100%)
12La5	NS1-4	EU593767.1|	*Xylaria* sp.	521/521 (100%)
3Ua1	NS1-4	JQ621878.1|	*Xylaria* sp.	511/511 (100%)
19Ua2	ITS1-4	GQ154594.1|	*Phaeomoniella prunicola*	394/404 (98%)
4Lb2	NS1-4	DQ195805.1|	*Pestalotiopsis disseminata*	750/750 (100%)
17La	NS1-4	FJ215705.1|	*Dothidotthia ramulicola*	809/809 (100%)
4Ua	ITS1-4	GQ154596.1|	*Phaeomoniella prunicola*	352/357 (99%)
13Ub2*	NS1-4	JN940801.1|	*Pestalotiopsis adusta*	677/677 (100%)
4La1	ITS1-4	HQ630365.1|	*Umbelopsis isabellina*	352/357 (99%)
19Ua2	ITS1-4	AB032070.1|	*Penicillium lagena*	571/571 (100%)
19La1	NS1-4	AY190271.1|	*Sordariomycete* sp.	737/745 (99%)
24Ua	ITS1-4	JF440625.1|	*Umbelopsis isabellina*	389/391 (99%)
6La2	NS1-4	JN938959.1|	*Penicillium corylophilum*	556/556 (100%)
7Ua	NS1-4	HQ878597.1|	*Jattaea discreta*	774/776 (99%)
4La	ITS1-4	GQ154596.1|	*Phaeomoniella prunicola*	423/437 (97%)
18Ua	NS1-4	DQ979498.1|	Fungal endophyte	566/568 (99%)
13Ua	NS1-4	AY190271.1|	*Sordariomycete* sp.	492/494 (99%)
25Lb	ITS1-4	GU187838.1|	Uncultured fungus	403/403 (100%)
12Ua3	NS1-4	AY190271.1|	*Sordariomycete* sp.	702/704 (99%)
5Ua	NS1-4	GU733368.1|	*Byssochlamys nivea*	540/540 (100%)
1Ua	ITS1-4	HQ630365.1|	*Umbelopsis isabellina*	393/393 (100%)

Table 4-2. Continued

Tree #	Primers	Genbank Accession #	Species best match	Max identity
12La1	ITS1-4	FJ042515.1|	*Botryosphaeria* sp.	535/546 (98%)
12La2	ITS1-4	GQ996147.1|	Fungal sp	576/583 (99%)
16Ua	ITS1-4	JN002164.1|	*Pestalotiopsis* sp.	489/495 (99%)
16Ub	ITS1-4	GU166485.1|	*Lecythophora* sp	429/437 (98%)
17Ub	ITS1-4	FJ042515.1|	*Botryosphaeria* sp.	534/534 (100%)
19La2	ITS1-4	GQ996133.1|	Fungal sp	463/465 (99%)
20Ub	ITS1-4	EU054426.1|	Fungal endophyte sp.	513/525 (98%)
22Lb	ITS1-4	FJ527869.1|	*Botryosphaeria* sp.	538/540 (99%)
24La	ITS1-4	GQ377490.1|	*Biscogniauxia mediterranea*	539/542 (99%)
2La2	ITS1-4	FJ527869.1|	*Botryosphaeria* sp	548/550 (99%)
3Ua	ITS1-4	GQ996147.1|	Fungal sp.	578/583 (99%)
5Lb	ITS1-4	EU686817.1|	Fungal endophyte	845/845 (100%)
11La1	ITS1-4	FJ613086.1|	Fungal sp.	574/574 (100%)
11La2	ITS1-4	FJ613086.1 -	Fungal sp.	1061/1061 (100%)
12La	ITS1-4	Q996147.1|	Fungal sp.	576/583 (99%)
13Ub1	ITS1-4	EU686817.1|	*Fungal endophyte*	840/841 (99%)
13Ub2	ITS1-4	EU326205.1|	*Chaetomium funicola*	533/535 (99%)
Tree 16, trunk	NS1-4	AY858654.1	*Ambrosiella brunnea* strain CBS 378.68	99
Tree 16, trunk	NS1-4	JF797171.1	*R. lauricola*	100
Tree 16, trunk	NS1-4	EU257806.1	*R. lauricola*	99
Tree 16, branch	NS1-4	EU257806.1	*R. lauricola*	100

Table 4-2. Continued

Tree #	Primers	Genbank Accession #	Species best match	Max identity
Tree 20, trunk	NS1-4	JQ040320.1	*Trichoderma asperellum* strain SHBS2013	92
Tree 20, trunk	NS1-4	HQ839775.1	*Gongronella butleri* *strain F13*	88
Tree 20, trunk	NS1-4	EU257806.1	*R. lauricola*	98
Tree 20, branch	NS1-4	JF797171.1	*R. lauricola*	99
Tree 20,	NS1-4	JF797171.1	*R. lauricola*	99
Tree 20	NS1-4	JN890295.1	Uncultured fungus	99

Table 4-3. Percent occurrence of *R. lauricola* in the trunks and braches of individual dead redbay trees that were cultured from the inside, middle and outer regions of each tree. For each tree, the height of sample collection and stem diameter are listed.

Sample location	Inner	Middle	Outer	Height in cm	Radius of stem in cm
Tree 3		DBH = 10.2 cm		Total height = 4.6 m	
trunk 1	0.1	0	0.75	6	30
trunk 2	0	0.8	0.9	4.5	213
trunk 3	0	0	0.5	3.5	412
branch 1	1		0.9	2	412
Tree 4		DBH = 5.8 cm		Total height = 3.4 m	
trunk 1	0.2	0.2	1	30	3.5
trunk 2	0	1	1	130	3
branch 1	0.2		0.9	152	1
branch 2	0		0	163	0.75
Tree 7		DBH = 13.2 cm		Total height = 4.6 m	
trunk 1	0	0.1	0.1	30	6.5
trunk 2	0.8	0.5	0.9	314	4.5
trunk 3	0.8	1	0.5	427	3.5
Tree 8		DBH = 15 cm		Total height = 7.9 m	
trunk 1	0	0	0.4	30	8.5
trunk 2	0.2	0	0.9	315	6
trunk 3	0	0	0.1	587	5
trunk 4	0	0.2	0.5	823	3.5
branch 1	0		0.5	152	3
branch 2	0		0.4	244	2
branch 3	0.7		0.6	597	3.5
branch 4	0.4		0.9	663	3
Tree 16		DBH = 13.5 cm		Total height = 8 m	
trunk 1	0.1	0.2	0.4	30	6.5
trunk 2	0	0.2	0.4	244	4
trunk 3	1	0.3	1	366	3.5
trunk 4	0	0.3	0.4	640	3.5
branch 1	0.6		0.6	315	2
branch 2	0.4		0.5	467	2
branch 3	0		0.9	640	2.5
branch 4	1		0.8	815	2.5

Table 4-3. Continued

Sample location	Inner	Middle	Outer	Height in cm	Radius of stem in cm
Tree 17		DBH = 10.0 cm		Total height = 10.7 m	
trunk 1	0.2	0.3	0.3	30	5.5
trunk 2	0	0.2	0.4	244	5
trunk 3	0.2	0.2	0.6	518	4.5
branch 1	0.3		1	315	4
branch 2	0.5		0.7	467	2.5
branch 3	0.3		0.9	640	1.4
branch 4	0.7		0.9	815	1.3
Tree 18		DBH = 11.2 cm		Total height = 12.5 m	
trunk 1	0	0.2	0.5	15	11
trunk 2	0	0	0.7	191	6
trunk 3	0.6	1	0.9	612	4
trunk 4	0.5	0.7	0.5	706	3
branch 1	0		0	315	1.5
branch 2	0		0	467	1.7
branch 3	0.9		1	640	3
branch 4	0		0	815	2.5
branch 5	0		0	732	1
branch 6	0		0	752	1
Tree 20		DBH = 24.1 cm		Total height = 16 m	
trunk 1	0	0	0.7	30	17
trunk 2	0	0	1	170	13
trunk 3	1	0.9	1	191	3
trunk 4	1	1	0.7	284	2.5
trunk 5	0.1	0.1	0.2	241	7
trunk 6	0.7	0.2	0.4	569	4
trunk 7	1	0.6	0.5	813	3
trunk 8	0.7	0.9	0.5	864	1.5
trunk 9	0	0	0	1046	2
branch 1	1		1	315	1.5
branch 2	0.3		0.3	467	2.5
branch 3	0		0	640	0.5
branch 4	0.3		0.3	815	6
Tree 24		DBH = 16.5 cm		Total height = 12 m	
trunk 1	0	0	0	33	9
trunk 2	0	0.9	1	305	6
trunk 3	0	1	1	607	3
branch 1	0		0	432	2

Table 4-3. Continued

Sample location	Inner	Middle	Outer	Height in cm	Radius of stem in cm
branch 2	0		0	686	1.5
branch 3	0		0	798	2
Tree 25		DBH = 16 cm		Total height = 8.7 m	
trunk 1	0	0	0	30	7.5
trunk 2	0	0	0.3	226	3.5
trunk 3	1	0	0	470	3.5
branch 1	0		1	470	2.5
branch 2	1		1	638	3
branch 3	0		0.2	726	2

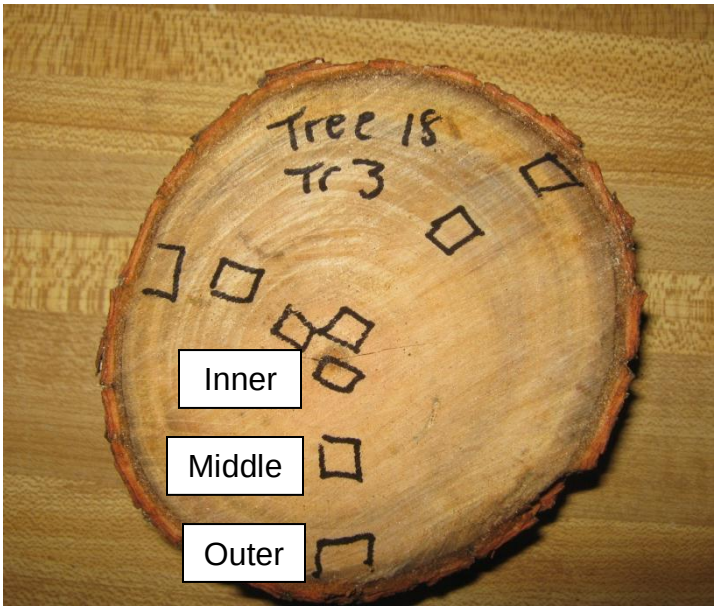

Figure 4-1. A typical section of redbay trunk indicating where samples were taken from to investigate the distribution of *R. lauricola* in the sapwood of standing trees. Photo by Don Spence.

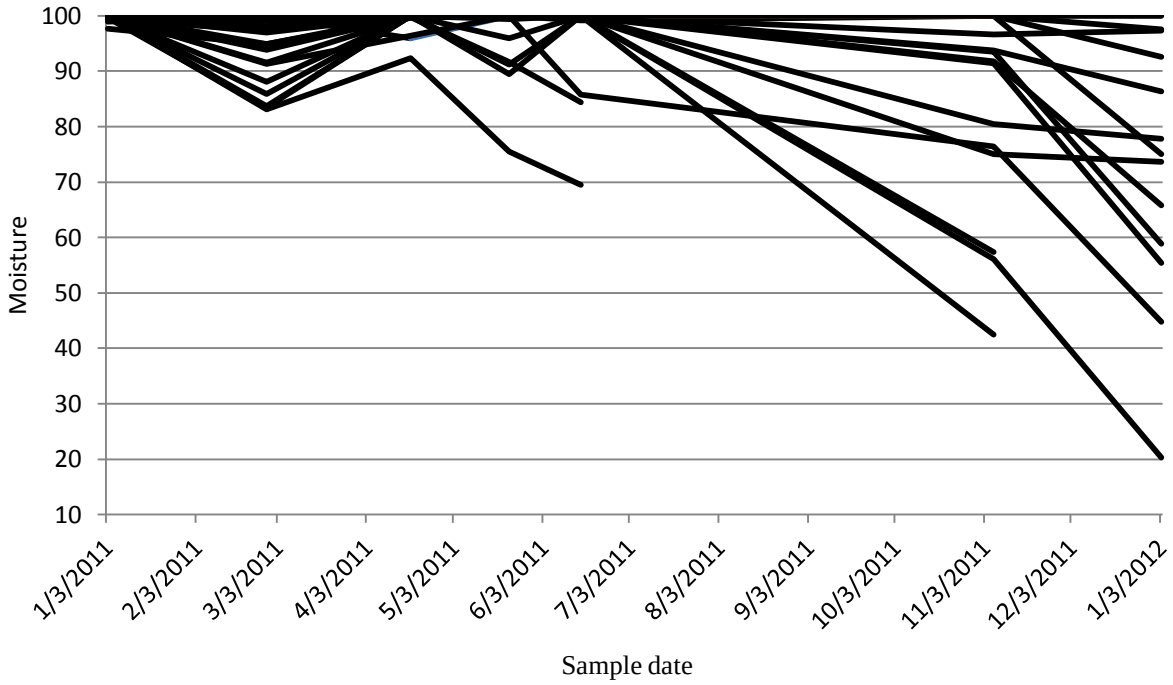

Figure 4-2. Moisture trend in dead redbay trees. The moisture for all trees (n = 23) over 12 months. The sharp drop in moisture during March 3011 corresponds to Florida's dry season.

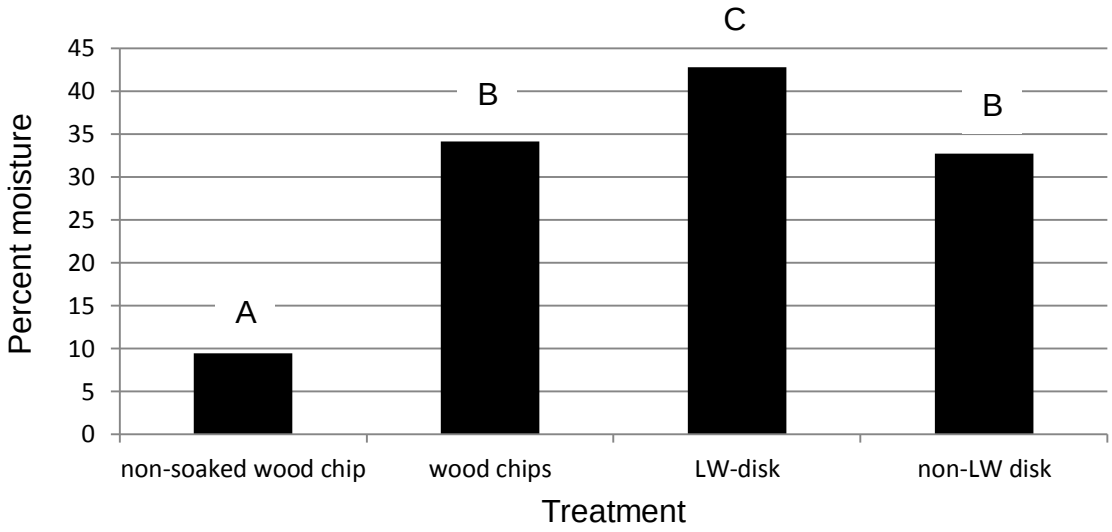

Figure 4-3. Moisture of wood chips. Each treatment had ten replicates, (F (3,36),= 142.05, P < 0.001, letters represent homogenous subgroups determined by a Tukey HSD.

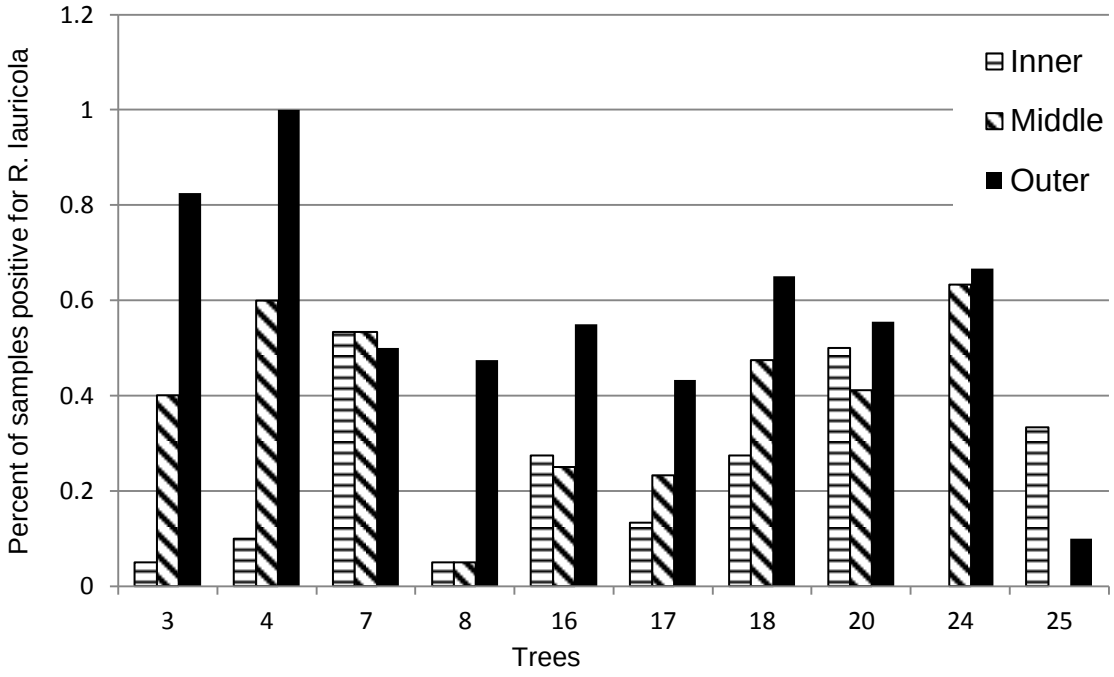

Figure 4-4. Percent of wood samples from each tree that were positive for *R. lauricola*. Inner samples came from the center of the stem. Outer samples were collected from 2 mm below the vascular cambium. Middle samples were collected half way between the inner and outer samples. In all cases, except tree 25, there was a greater recovery of *R. lauricola* from the outer sapwood than the inner. Tree 25 was the first tree to die and it appears that *R. lauricola* disappeared from the outer sapwood, but persisted further inside the tree.

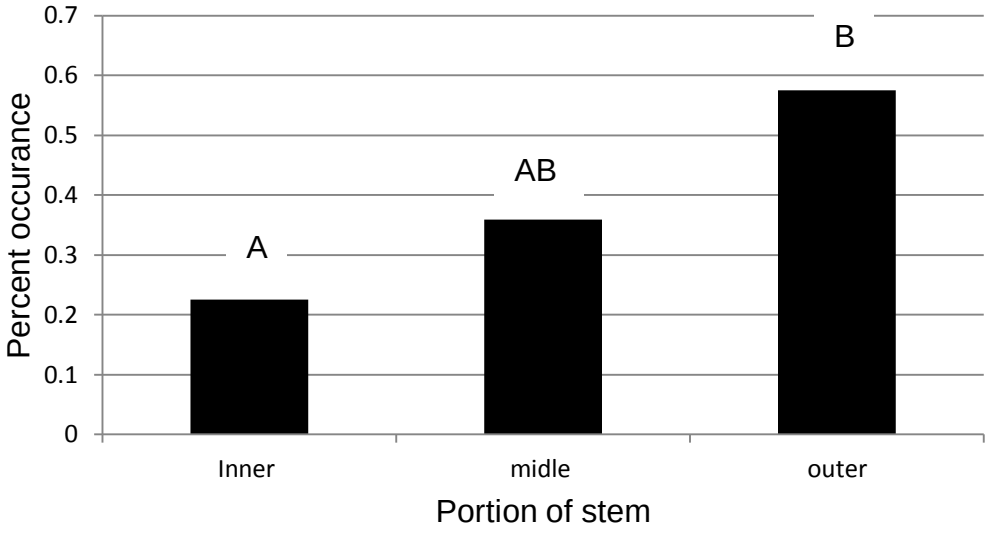

Figure 4-5. Proportion of growth of *R lauricola* from the inside, outside, and middle sections of sapwood. Differences in growth were statistically different, $F_{(2,152)} = 4.74$, $P = 0.0101$. Letters represent homogenous subgroups determined by a Tukey HSD.

CHAPTER 5
EVALUATING THE EFFECTIVENESS OF PRE-TREATING REDBAY TREES (*PERSEA BORBONIA*) WITH PROPICONAZOLE AND PINE-SOL TO PROTECT THEM AGAINST LAUREL WILT

Introduction

Laurel wilt (LW) is an exotic disease of the Lauraceae plant family (Fraedrich et al. 2007, Fraedrich et al. 2008, Hanula et al. 2008). The disease is caused by an exotic fungal pathogen*, Raffaelea lauricola*, which is vectored by the exotic redbay ambrosia beetle (*Xyleborus glabratus*) (Fraedrich et al. 2008). The fungus is carried in mandibular sacs (mycangia) inside the beetle's head. As beetles bore into trees, fungal spores are inoculated into the surrounding tree tissue (Beaver 1989).

The disease was first detected in 2002 near Savannah, GA. and has since spread to North Carolina and South Carolina, Mississippi, Alabama, and throughout most of Florida (Riggins et al. 2010, Forest Health Notes 2011, Laurel Wilt Working Group Meeting 30 March 2012). The native range of the beetle and fungus are the temperate areas of S.E. Asia, where laurel wilt has not been observed (Rabaglia et al. 2006 Harrington et al. 2011). Ambrosia beetles usually attack dead or dying trees; thus, the behavior of *X. glabratus* in North America is unusual. Female beetles excavate deep galleries into the sapwood of a tree where eggs are laid. As the female beetle bores into a tree, one or more symbiotic mycangial fungi (one of which is *R. lauricola*) may be deposited into the galleries on which adults and larvae will feed (Hanula et al. 2008 and Mayfield et al. 2008).

In the case of redbay (*Persea borbonia*) and other members of the Lauraceae in the S.E. USA, *X. glabratus* has been boring into healthy trees. Why the beetles are attacking healthy trees is unknown but one hypothesis is that healthy trees in the S.E.

USA are perceived as dead trees by the beetles (Hulcr and Dunn 2011). Whatever the reason, once attacked a healthy tree can die within in a few weeks. Avocado (*Persea americana*) is a member of the Lauraceae and is also susceptible to the disease, threatening this important food crop world-wide. An investigation into the physiological response of avocado to *R. lauricola* found that the trees produced deposits of lipid and gums in the water conducting cells of the trees in response to the presence of the fungus. However, the fungus was not present in overwhelming quantities that would lead to the occlusion of the water conducting tissue (Inch and Ploetz 2011). It has also been postulated that redbay trees, and other members of this plant family, inadvertently kill themselves due to an overreaction to the presence of the fungus (Hulcr and Dunn 2011). Once the small fungal spores enter the tree's water conducting tissue, they can be easily transported throughout the host's tissues. This is supported by the fact that the pathogen has been recovered from leaves, stems and roots (Hughes, unpublished). Since LW symptoms appears in the canopy first, it is xylem mobile. But since it has also been recovered from roots, it is likely that spores can be transported passively downward through non-functional xylem or through the phloem cells (which move photosynthates to the roots and to developing tissues).

Using fungicides to protect trees against pathogens has become commonplace, in particular for managing Dutch elm disease and oak wilt. Since the causal agent of Dutch elm disease (DED), *Ophiostoma novo-ulmi*, and *R. lauricola* are taxonomically related (Harrington et al. 2010), techniques used to manage DED have shortened the learning curve when it comes to pre-treating trees for LW. Individual elm trees (*Ulmus* sp.) have been kept alive for decades through macro-injections of propiconazole.

However, they require repeated treatments to maintain the protection against the fungus (Haugen and Stennes 1999, Dunn 2000).

In managing *Ceratocystis fagacearum*, the causal agent of oak wilt, macro injections of propiconazole have significantly reduced disease severity (Appel and Kurdyla 1992, Appel 2001). In this disease cycle, insects may be involved; however, the greatest disease spread comes from below ground root grafts between oak trees. In untreated trees, the pathogen easily moves from tree to tree through the vascular tissue, ultimately causing wilt and death. Trenching around trees has proven successful for limiting disease spread but so has the use of fungicides (Osterbauer and French 1992, Appel, 2001; Eggers et al., 2005, Ward et al. 2005).

In the only published study regarding fungicide efficacy against the LW pathogen in redbay, Mayfield et al. (2008) used Alamo® propiconazole in varying concentrations and found that it inhibited the growth of *R. lauricola* in culture. In field trials at Ft. Clinch State Park, FL., they treated 17 redbay trees with the label rate of propiconazole in 2007 and achieved measurable levels of success in protecting the trees against *R. lauricola*. Beyond this one study, no published data on the successful use of propiconazole exists for the treatment of LW in redbay.

Macro injections of Alamo® can be expensive. For trees that are too small or for homeowners who cannot afford the cost of the fungicide injections, cover sprays might be a potential option. Cover sprays of insecticides have been noted as a potential treatment for agricultural crop trees by Mayfield et al. (2008) but no direct information exists on its use for urban landscapes. In a review of the effectiveness of insecticides in an agricultural setting, Pena et al. (2011) observed mixed results of beetle attacks and

beetle emergence from insecticide treated bolts of avocado. Ultimately, they did not find a difference in beetle emergence or beetle attack between insecticide on treated and non-treated control bolts. Although the use of insecticides may prove at some point to be an effective treatment against *X. glabratus*, the broadcast spraying of insecticides would pose human health risks as well as risks to beneficial insects (Raupp et al. 2010). Pena et al. (2011) also noted, "contact insecticides that confer repellency may be a more effective strategy."

Based on this concept, a non-toxic repellant may be a more viable option. In dealing with beetles that attack pine trees, there is evidence that non-host volatiles (extracts from flowering plants) can disrupt the olfactory cues pine bark beetles use to locate host coniferous trees (Zhang and Schlyter 2004, Campbell and Borden 2006). To some degree, non-host volatiles and anti-aggregation pheromones dissuaded beetles from preferred hosts (Huber et al. 2000, Huber and Borden 2000, Fettig et al. 2005, Campbell and Borden 2006). To this end, the use of Pine-Sol® (Sodium petroleum sulfonate 1 - 5%, Isopropyl alcohol 1 - 5%, Alkyl alcohol ethoxylates 3 - 7%, and Pine oil 8 - 10%, The Chlorox Company, Oakland, California) might be a non-host volatile that could disrupt *X. glabratus'* ability to find Lauraceous hosts.

The objectives of this study were: 1) to evaluate the effectiveness of propiconazole (Alamo® fungicide) treatments to protect healthy redbay trees (*Persea borbonia* L. Spreng.) against *R. lauricola,* 2) test the efficacy of Pine-sol® as a repellent 3) test the effectiveness of cover spray treatments of Pine-Sol® at deterring *X. glabratus* from boring into healthy redbay trees and 4) attempt to identify how often redbay trees form root grafts.

Materials and Methods

Pre-treatment of Healthy Redbay Trees with Propiconazole and Pine-Sol Over Four Years

Over a 38-month period (February 2009 to May 2012), 226 healthy redbay trees in Putnam and Volusia Counties, FL., ranging from 2 to 203 cm (1 to 80 in) diameter at breast height (DBH), were either injected with Alamo®, a propiconazole fungicide (Syngenta Crop Protection Inc. Greensboro, NC) or given a cover spray of Pine-sol®. All trees were planted in residential areas, occurring in clusters or alone. Results of these treatments were analyzed in two sets; as a whole data set (all four years) and the first two years (2009 & 2010). Since the dynamics of host choice by *X. glabratus* are poorly understood, it is possible that some trees in areas in high disease incidence areas have not been attacked. By only looking at the data from 2009 and 2010 alone, we may gain a better understanding of the efficacy of propiconazole in relation to protecting trees from the LW pathogen.

For most trees the maximum label rate recommended for injections was used (Rainbow Scientific Macro-injection Manual 2005), which was 20 ml of propiconazole in 300 ml of water for each 2.54 cm DBH of tree. The appropriate solution mixture was placed in a pressurized tank. From the tank, two “T” connectors were used to distribute solution pressure evenly around the circumference of the tree and to each injector port. Tree injection ports were installed in healthy root flares and generally placed 10 to 30 cm apart but in some instances they were farther apart due to decay or lack of solid wood (Figure 5-1). A sharp 5.94 mm (15/64”) bit was used to drill into the root collar to a depth of 2.5 to 3 cm and injection tips were tapped into place with a rubber or wooden mallet. Once the tips and tubing were in place, 5 psi of pressure was applied to bleed

off any air in the tubing. Once air bubbles were removed, tank pressure was increased to 20 psi and then monitored for any leaks. When leaks were observed, the pressure valve was turned off and the set up modified to eliminate the leak. Due to the time involved with the uptake of the solution, the set up was often left overnight and checked the following day. If trees required more than 9 L of solution, the tank was reset as noted above to bring the total volume for the tree to the specifications noted in the macro-injection guide (Rainbow Tree Care Scientific Advancements 2005). In most cases the trees took up the full volume of solution; however, some trees did not. After 48 hours, any remaining solution was poured onto the root collar. Once the injection process was completed, plastic tubes and injection tips were soaked in water with a 10% bleach solution for several hours. All trees treated in 2009 and 2010 with propiconazole that had not died were revisited in May of 2012 and evaluated for LW symptoms.

At the beginning of the study, the label rate for Alamo® as reported by the chemical distributor, Rainbow Scientific Tree Care, was 10 ml per 1 L of water for each DBH 2.54 cm. The recommendations by the distributor changed in the fall of 2009 to the volume noted in the materials and methods. Only the first 11 injections were done at the lower rate, all other solutions were mixed as noted above. All fungicide injections and cover sprays were applied by individuals who retain current Florida Department of Agriculture Pesticide licenses.

Effectiveness of Pine-sol as a Deterrent to *X. glabratus*

To test the effectiveness of Pine-Sol® as a repellent, five 100 cm^2 areas of bark were chipped off the trunk of three redbay trees on 4 March 2012. Since exposed sapwood is more attractive to *X. glabratus* than bark (Niogret et al. 2011, Mayfield and

Hanula 2012) the bare areas should act as an effective attractant to any *X. glabratus* in the area. The bark and phelloderm were removed using a chisel and hammer, exposing the sapwood between 7 dm and 1.5 meters above grade. The distance between each exposed area was between 10 and 30 cm apart (Figure 5-2). Four treatments were used to test the repellant ability of Pine-sol®: (1) control, scraped bark with no treatment, (2) distilled water, (3) 10%, (4) 40% and (5) 80% Pine-Sol® in distilled water. Solutions were applied with a small disposable 3 cm wide paint brush. The volume applied was approximately 30 milliliters per treatment. For 14 weeks the trees were checked weekly for the presence of *X. glabratus* boring activity. The determining factor for a positive *X. glabratus* attack was the presence of an appropriately sized bore hole, which was approximately 1 mm. To assess the potential movement of *X. glabratus* at this study site, a six-tier multi funnel trap baited with manuka-oil bait was set up and also monitored weekly for 4 weeks.

The control for both the propiconazole and Pine-sol® treatments were considered to be redbay trees growing in the area near the treatment trees. A survey of adjacent trees was made at the time of treatment applications and trees were revisited in excess of 24 months later to determine their condition.

Effectiveness of Cover Spray Treatments of Pine-Sol

For foliar and bark applications were applied to 13 trees between 2010 and 2011. Pine-Sol® was mixed at a rate between 40 and 50% in tap water and applied at a volume sufficient to wet as much as the tree as possible. A typical tank sprayer was used to drench the trunk, branches, and canopy as much as possible. The treatments were re-applied between 2 to 8 weeks apart, depending on the client's wishes.

Occurrence of Root Grafts in Redbay

To ascertain how often or whether or not redbay trees form root grafts, a supersonic airknife X-LT (Alison Park, PA.) was used to expose the roots of twelve trees. The trees were located at the University of Florida's Austin Cary Memorial Forest in a pine flatwoods ecosystem. The airknife was used with an Ingersoll-Rand commercial air compressor that produced 185 cfm (cubic feet per minute) and 100 psi (pounds per square inch) of pressure.

Statistical Analysis

Chi square test was performed on both data sets (2009/2010 and 2011/2012). The response variable was tree survival, which was compared to thirteen variables: DBH, location of tree (town), location of tree (address), date of treatment, months dead after treatment, re-treatment date, volume of fungicide or Pine-sol®, hot spots of LW (6 or more LW symptomatic trees within 1 km), technician doing the application, presence of cavities or structural damage, uptake time, whether the tree was already showing LW symptoms, and whether the tree had multiple trunks or had a single trunk. The Pearson value was reported for all Chi square values along with the significant value, n, and degrees of freedom. In addition to the Chi square test, regression analysis was reported as an r-squared value. The parameter of tree size, DBH, was an important variable to consider. DBH measurement was transformed into its natural log, [ln] DBH, and then compared to survival and town of disease was cross with hot spots of disease and compared to tree mortality.

For the assessment of the Pine-Sol® applications, a one-way ANOVA would have been used; however after 14 weeks no beetle activity has been observed on any of the

treatments. The same has occurred with the Pine-Sol® cover sprays, no trees have died that were given this treatment.

Results

Propiconazole Treatment of Healthy Redbay Trees

Results of these treatments were analyzed in two sets; as a whole data set (all four years 2009 & 2012) and then just the first two years (2009 & 2010).

2009 – 2012 treatments, 4 years of data

To date, a total of 200 trees were injected with Alamo® propiconazole and 90% (179 of 200) were still alive in May of 2012. All 13 trees given a Pine-Sol® cover spray were also still alive in May 2012. Since the host choice dynamics of *X. glabratus* are poorly understood, enough time may not have elapsed to make any definitive statements about the trees treated between 2009 and 2012. Not included in the propaconazole survival number were 12 redbay trees that had LW symptoms or were immediately next to those trees. All 12 trees that had LW symptoms or were next to symptomatic trees at the beginning of the treatments died. Since these 12 trees did not accurately represent the effectiveness of the treatments they were not included in the percent survival analysis. This also indicates that propiconazole may not work as a therapeutic treatment.

Of the thirteen variables examined in the four-year data set, only three had a significant interaction with survival. Tree DBH data were ln-transformed to approximate normal distribution and were found to be significant ($X^2 = 3.82$, $P = 0.0488$, df = 1, n = 226). In addition to [ln]DBH, fungicide uptake time ($X^2 = 15.02$, $P = 0.005$, df = 4, n = 201, $r^2 = .05$) and trees that were already infected with *R. lauricola* ($X^2 = 64.29$, $P < 0.0001$, df = 2, n = 226, $r^2 = .27$) were significant. For both of those variables, the P

value was significant but the r^2 was not, indicating that those parameters did not fully explain tree survival.

2009 – 2010 treatments, 2 years of data

Data presented in this section will focus on the first two years of work (75 trees), from February 2009 through November 2010. Seventy-one trees were injected with propiconazole and four were given a cover spray of Pine-sol®. Of the 71 trees that were initially injected with propiconazole, 12 were LW symptomatic before fungicide injection or the trees were in close proximity to symptomatic redbay trees and had likely already been attacked and were infected with *R. lauricola* at the time of treatment. Each of these trees was given the label volume propiconazole; however, 100% of the trees that showed LW symptoms prior to fungicide injection died. Of the other 59 trees that did not exhibit any LW symptoms, survival as of May 2012 was 75% (44 of 59). The age classes of survival are listed in Figure 5-3. As can been seen in Figure 5-3, the survival was even for the most part with the size class of 61 – 80 cm having the highest survival rate.

Of the thirteen factors that were tested for correlation with redbay survival between 2009 and 2010, only three were significant, town of treatment+hot spots of disease, date of injection, and potentially pre LW-infected. Chi square analysis of town of treatment crossed with hot spots of disease ($X^2 = 15.88$, $P = 0.0262$ df = 7, n = 75, $r^2 = .16$), date of fungicide injection ($X^2 = 53.66$, $P = 0.0262$ df = 28, n = 75, $r^2 = .69$) and potentially pre-LW infected ($X^2 = 15.35$, $P = 0.0262$ df = 2, n = 75, $r^2 = .16$) all had a significant effect on tree survival.

Effectiveness of Pine-sol as a Deterrent to *X. glabratus*

Six weeks after the Pine-Sol® concentration study was initiated; no *X. glabratus* activity was observed on any of the treated stem sections. The applications were reapplied on 11 April 2012, six weeks after the initial application. After a total of 14 weeks, no beetle activity has been observed on any of the replicates. As of May 2012, five *X. glabratus* have been captured in the baited multifunnel trap.

Occurrence of Root Grafts Between Redbay Trees

None of the roots systems of the twelve trees produced any root grafts between any other redbay or other species.

Discussion

These data suggest that propiconazole can provide protection to redbay trees as long as they are asymptomatic. The first confirmed LW infected tree was identified in Daytona Beach, FL in January of 2009. And, this area of town has a significantly higher proportion of dead trees than the surrounding areas ($X^2 = 29.51$, $P < 0.0017$, df = 226) which indicates that the disease tends to spread to trees close to infected trees. This initial tree was over 50 miles from the next known LW infected area. However, two years after the initial diseased tree was identified, symptomatic LW trees were found over ten miles away from the initial diseased tree.

Trees treated by Mayfield et al. (2008) were relatively small (17 to 39 cm). Their success rate after two years was 65% (11 Of 17) and after three years with no additional treatments survival went down to 29% (5 of 17) (personal communication with Jeff Eickwort, co-author, FL Division of Plant Industry).

Eggers et al. (2005) found that protecting large trees against oak wilt was difficult. Data presented here did not show any difference between the survivability of

small vs. large trees when injected with propiconazole. A possible reason that Eggers et al. (2005) had a lower level of success of protecting larger trees may be due to the fact that a large diameter tree will have a large amount of functional sapwood that extends from the root collar to the top of the canopy. If any portion of a tree's water conducting tissue (xylem) is not infused with propiconazole, the pathogen may gain entry into the stem and become mobile in sapwood tissue behind the more functional secondary xylem. Additionally, oak trees create root grafts that allow the pathogen to move from tree to tree underground. Our investigation found no evidence that redbay trees form root grafts in the pine flatwoods ecosystem in north central Florida.

After three years of data, none of the trees that were given Pine-Sol® cover spray have died. However, definitive statements on successful protection of trees against the LW pathogen, whether the treatment was a cover spray or an injection, should be tempered with the caveat of time based on the disease severity in a given area. If a Lauraceous host is treated in an area where the disease does not exist than it is misleading to say that its continued survival is due to the fungicide injection.

Propiconazole fungicide injections have been successful in combating Dutch elm disease for decades (Stennes 2000) and since this disease is similar to LW in several ways, macro injections seem to provide the potential for similar levels of protection for years to come. In treatment of oak wilt, Wilson et al. (2005) observed some level of therapeutic success through the use of propiconazole. Therapeutic use was generally limited to the more resistant white oak group. Propaconizole injected after the pathogen had invaded the tree did not kill the fungus but it was able to keep red oaks alive longer

than non-treated controls. The therapeutic use of propiconazole against LW was always ineffective and trees always died.

Pruning out symptomatic limbs was observed to be a functional tool in the management of oak wilt (Eggers et. al. 2005). Three to four months after treating asymptomatic redbay trees, four trees began to wilt. The wilted areas of the canopy were pruned out approximately four months after the initial fungicide injection. Each of these trees is still alive as of May 2012. Although not part of this study, a tree on University of Florida's Gainesville, FL. campus was given a macro injection of propiconazole in the summer of 2010. In the fall of that year the end of one branch began to wilt. Three months later that wilted limb was pruned off; to date the tree is still alive.

LW can cause a redbay tree to wilt within a few days, indicating that the vertical movement of the pathogen in the xylem happens quickly. Since we have had success in keeping trees alive by pruning out wilted limbs, we suspect that the downward movement of the pathogen in non-functional xylem or through the phloem happens slower than the vertical movement.

There are several tragedies associated with the loss of redbay and other members of this family. From a biological perspective, the fruit produced by this plant is used by a variety of wildlife such as migratory birds and turkeys (Nelson 1994, Coder 2006, Mayfield 2007). However, the greatest loss associated with this disease may be lack of host plant material for the Palamedes swallowtail (*Papilio palamedes*) butterfly, a species that exclusively uses lauraceous species for its development (Hall 1994, Minno et al. 1999).

From a human perspective, the loss of this tree from coastal urban forests will be dramatic since in some communities it comprises upwards of 20% of the canopy (Spence 2008). A direct economic study has not been produced that outlines any negative impact on urban communities due to LW. However the cost incurred to remove trees will not be insignificant. Not only will property values decline with the loss of prominent shade trees (Anderson and Cordell 1988, CTLA 2000, Laverne and Winson-Geideman 2003) but a sense of community identity may also be lost to some degree. The strong connections people have towards trees (Ulrich 1986, Robbins 2012) have led some to use experimental treatments in attempts to protect their trees.

Data from the earliest treatments (2009 and 2010) have provided two years of observations where propiconazole protected 75% of asymptomatic trees. These data suggest that propiconazole can provide protection to redbay trees that are infused with the propiconazole before they are attacked by *X. glabratus*.

Figure 5-1. Typical setup of the macroinjection system. The root collar was excavated to observe root orientation and injection ports were spaced approximately 10 – 15 cm apart. Photo by Don Spence.

Figure 5-2. Three of the five treatments for the Pine-sol® experiment on tree 3. Each square is 100 cm^2 where the bark and phelloderm were scraped down to the sapwood. Photo by Don Spence.

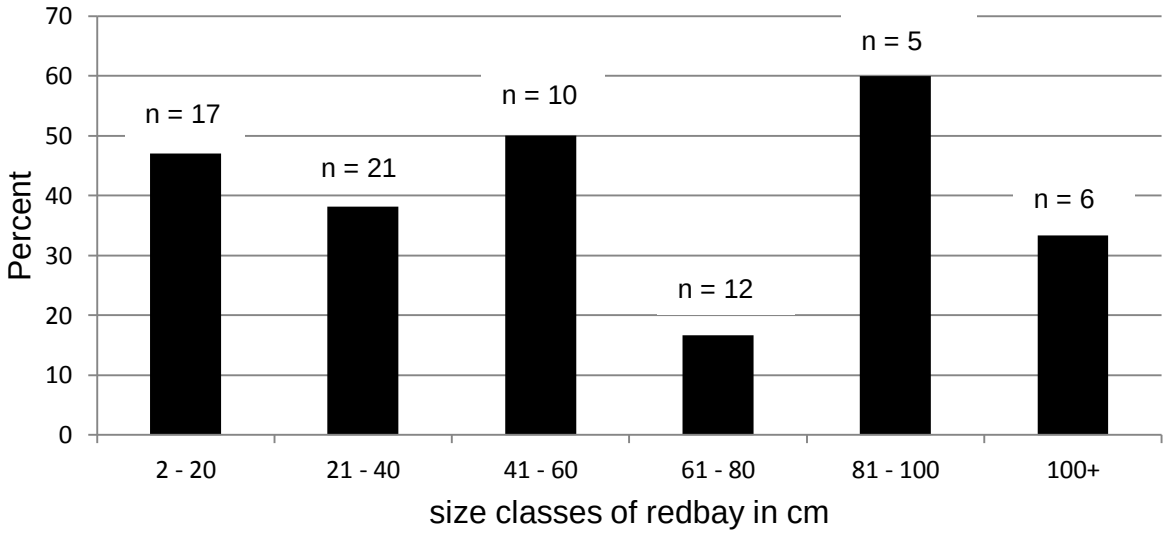

Figure 5-3. Survivability of redbay trees by size class (in cm) treated with propiconazole from 2009 & 2010. The number of trees in each size class that died is noted above each bar.

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BIOGRAPHICAL SKETCH

Donald John Spence was born in Daytona Beach, FL. in 1964. He graduated from Seabreeze High School, and although his academic achievements were limited, he was an accomplished surfer, swimmer and spring board diver. As a senior, Don won the 1 meter spring board competition for Volusia County, the regional competition and placed tenth at the State Championships. After high school, Don enlisted in the United States Coast Guard and served first on the law enforcement cutter USCGC Steadfast and aboard the Icebreaker, USCGC Polar Sea. His travels took him from the arctic to Antarctic, across the date line, timeline and around Cape Horn. In all, he circumnavigated North America, sailed six of the seven seas and visited five of the six continents as well the world's largest island. Don's final tour was as the Procurement Officer for LORAN Station L'Estartit in northeastern Spain.

Don returned to Daytona Beach to attend Daytona State College where he graduated with his AA in biology and was nominated for the Who's Who of Community Colleges. He was also inducted into the DBCC Hall of Fame for his extracurricular activities with student government and Amnesty International. In 1992, Don won the Vaughn-Jordan Scholarship at Stetson University to complete his Bachelor of Science degree in biology. Don graduated from Stetson University in 1994 and won the Service Award for the Biology Department. In 1995, Don started his consulting business, Botanical Systems, where he specialized in wetland identification, ecological restoration, plant surveys and plant identification. In 1996 Don matriculated into a graduate program at the University of Central Florida. Don's research project was the change in tree diversity over twenty years in nine maritime hammocks in East Central Florida. In 2001 Don changed his company's name to Native Florida Landscapes, LLC

and changed the focus of his business so that he could concentrate on tree surveys, protection, and health assessments. In the same year, Don also began teaching at Daytona State College as an adjunct professor of botany and biology. Due to a desire to work with and protect Volusia County's environment, in 2006 Don ran for and won and election for Seat 5 of the Volusia Soil and Water Conservation District. Don's real passion was in teaching. In an attempt to compete for a faculty position at the university level, Don entered the University of Florida's Doctor of Plant Medicine Program in 2009. After a year in the DPM program, Don had an opportunity to study an emerging tree disease (laurel wilt) and moved to a Ph.D. program in Plant Pathology. Don was accepted to work with Dr. Jason Smith in the Forest Pathology Laboratory where he studied features of the fungal pathogen that causes laurel wilt, along with its symbiont, the redbay ambrosia beetle. Don received his Ph.D. from the University of Florida in the summer of 2012 and he hopes to return to the beginning of his educational adventure and teach at Daytona State College again.