Life history, rearing, and habits of the redbay ambrosia beetle (Coleoptera: Curculionidae: Scolytinae) in the field and laboratory

By

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(Under the Direction of S. Kristine Braman and James L. Hanula)

Abstract

The goal of this work was to gain a better understanding of the biology of the invasive pest *Xyleborus glabratus*, the redbay ambrosia beetle (RAB). The development of RAB in the field was monitored by attaching emergence traps over individual beetle galleries on mature redbay (*Persea borbonia*). Galleries were active throughout the year and could potentially be active for greater than one year and produce over 100 adults. Fine mesh screen was used to protect lower boles of mature redbay trees from RAB attack, but RAB were found to attack at heights greater than 10m so this was ineffective to protect trees from laurel wilt. RAB were reared on semi-artificial diet *in vitro* and success rates were achieved similar to those achieved in the field. The ability of the laurel wilt pathogen, *Raffaelea lauricola*, to grow on various wood species was tested and found to be variable depending on species.

INDEX WORDS: Redbay ambrosia beetle, *Xyleborus glabratus*, Laurel wilt, *Raffaelea lauricola*, invasive, rearing, ambrosia fungus, redbay, *Persea borbonia*, ambrosia beetles

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The redbay ambrosia beetle (RAB), *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), and its associated fungus *Raffaelea lauricola* (T.C. Harr., Fraedrich & Aghayeva) are recent introductions to the southeastern United States and have been responsible for rapid and widespread mortality of mature redbay (*Persea borbonia* (L.) Spreng) trees. Introduction of nonnative species can result in devastating losses both ecologically and economically. International trade is the principle method by which exotic species are introduced to a region, and the volume of goods and number of trading partners has increased the rate of introductions in recent years. Marini et al. (2011) found that intensity of trade measured by the value of imports is a strong predictor of the richness of exotic bark and ambrosia beetle species in a region. There are now over 2000 species of nonnative insects established in the United States, and 92 % of interceptions at port monitoring sites are from the Coleoptera (Haack, 2001; 2006). Wooden packing materials such as crates and pallets are especially important in the transportation of bark and wood boring insects. Crates made from recently cut trees that have not been chemically or heat treated, and that contain bark are more likely to harbor boring insects (Haack, 2001; 2006).

Biology of Scolytinae and RAB. Beetles in the subfamily Scolytinae include the bark and ambrosia beetles and are among the most common and most damaging introduced insects. As of 2002, at least 50 species of exotic scolytines are known to be established in USA (Haack, 2001; 2006), and 39 of these are ambrosia beetles (Rabaglia et al., 2006). Scolytines commonly live under bark or within wood, but can also inhabit food products such as seeds and nuts. Ambrosia

beetles, including X. glabratus, tunnel and breed within sapwood of dead or dying trees and feed exclusively on cultivated fungi known as ambrosia. Ambrosia beetles represent a polyphyletic group with at least seven separate evolutionary derivations from the bark beetles which feed and reproduce in the nutrient rich phloem of trees (Harrington et al., 2010). The tribe Xyleborina, for which Xyleborus is the type genus, accounts for the greatest abundance of exotic bark beetles in North America (Rabaglia et al., 2006). *Xyleborus glabratus* is distinguished from other Xyleborus of North America by its slender shape, convex declivity with numerous punctures, and the nearly subquadrate postereolateral margin. Females are 2.1-2.4mm in length and dark brown to black in color while males are smaller, about 1.8mm in length, and are flightless (Rabaglia et al, 2006). Females greatly outnumber males in colonies (Kirkendall, 1983). Specialized paired sacs called mycangia are located at the base of the mandibles of adult *X. glabratus* females, as well as many other members of Xyleborus, are used to transport budding spores of the symbiotic fungi. Mycangia can take other forms in different species of bark beetles including pouches at the base of the elytra or sacs between pronotal and mesonotal segments. (Harrington, 2005) Upon boring into new trees, spores from the beetles' mycangia inoculate the sapwood with the ambrosia fungi. Although most bark and ambrosia beetles infest only dead or dying trees, RAB attack live trees of the family Lauraceae in North America . Initial attacks on healthy trees are usually in small numbers and do not often result in successful gallery formation (Fraedrich et al., 2008). Beetles will bore a short distance into a healthy tree and abort the gallery, but this action is often sufficient to infect susceptible trees with the pathogen. As the fungus spreads and the tree begins to die, greater numbers of RAB are attracted to the tree (Mayfield et al., 2009). Once RAB females successfully bore into the sapwood, they establish galleries in which eggs are laid. Larvae feed on the ambrosia fungus growing on the gallery

walls and develop into adults within the galleries. At some point the host becomes uninhabitable or galleries become overcrowded causing adults to leave the gallery and either locate a new host tree or bore into a new location on the same host. Females are generally mated before leaving a gallery, but it is possible for initially unmated females to lay unfertilized eggs that develop into haploid males (Biedermann, 2010). It has traditionally been thought that these males then mate with the mother to produce diploid female offspring, but Biedermann (2010) did not see this behavior when studying male behavior in *Xyleborinus saxesenii* Ratzeburg. This type of reproduction is known as haplodiploidy and greatly increases the invasiveness of a species by making it possible for a single female to establish a population. Another ambrosia beetle that is common on redbay and can cause damage that superficially resembles the initial stages of laurel wilt is the black twig borer, *Xylosandrus compactus* (Eichoff). These beetles bore into the underside of small diameter branches and develop brood chambers within the pith where their ambrosial fungi are cultivated. This behavior will cause wilting and death of small branches, but very rarely leads to large branch or whole tree death. (Mayfield et al., 2009)

Raffaelea spp. Ambrosial fungi grow as asexual anamorphs of *Ophiostoma* and produce conidia which are grazed upon by larval and adult ambrosia beetles. The genus *Raffaelea* is composed entirely of species which grow only within woody plants along the walls of ambrosia beetle galleries (Batra, 1967). *Raffaelea spp.*are difficult to differentiate morphologically because conidia and conidiophores lack pigmentation and are mostly simple with few distinguishing features (Gebhard and Oberwinkler, 2005). The laurel wilt disease is caused by the vascular wilt fungus *Raffaelea lauricola* which is routinely isolated from the mycangia of the redbay ambrosia beetle and from infected trees (Fraedrich et al., 2008). Isolations from *X. glabratus* collected in Japan, Taiwan, and Georgia, USA revealed the presence of *R. lauricola* at high frequencies,

indicating that the pathogen was carried to the USA from Asia by *X. glabratus*. (Harrington et al., 2011). The fungus causes black discoloration of wood and induces wilting and tree death by stopping the flow of water through a host tree (Fraedrich et al. 2008; Harrington et al., 2008) The fungus itself does not block water-carrying xylem vessels, but rather induces the formation of tyloses (outgrowths of parenchyma cells of xylem vessels) and phenolic-, pectin-, and lipid-containing gels which are strongly correlated with the appearance of wilting symptoms (Inch et al., 2012). Full crown wilt of a mature redbay takes as little as 2-3 weeks or up to 3 months after initial attack (Mayfield et al., 2009). Inch and Ploetz (2011) found that xylem function and hydraulic conductivity in stems of avocado were significantly impaired within three days after inoculation with *R. lauricola,* but visible wilting of foliage did not occur until 14 days after inoculation. The fungus rapidly spreads through the main stem and all branches, and seven days after inoculation *R. lauricola* was recovered throughout the stem of 1-1.5m tall potted avocado plants (Inch et al., 2012). *Raffaelea lauricola* was not recovered, however, from the avocado fruit of infected trees (Ploetz, 2012). Following full crown wilt epicormic shoots often grow from the root collar of otherwise dead trees.

At least five other *Raffaelea* species have been isolated from *X. glabratus* as well, contrary to the accepted idea that one or few fungal species are associated with a single beetle species (Harrington et al., 2010). At least three other *Raffaelea* species are known to be plant pathogens. *Raffaelea quercivora* and *R. quercus-mongolicae* are pathogenic symbionts of Platypodine ambrosia beetles and are responsible for Japanese oak wilt and Korean oak wilt, respectively. *Raffaelea canadensis* has recently been seen to cause symptoms on avocado in California that included crown wilt and discolored sapwood, but this did not result in tree death.

(Inch and Ploetz, 2011) *Raffaelea lauricola* is unique among these in that it is the only species capable of systemic dissemination leading to vascular wilt and death of a host tree.

Lauraceae. RAB and laurel wilt in North America have so far been restricted to members of the family Lauraceae. The family is composed of 54 genera containing 2000-2500 species and has a worldwide distribution in tropical and subtropical climates, especially Southeast Asia and South America. Almost all members of the family are evergreen trees or shrubs and have aromatic oils which have often been isolated for cultural, medicinal, and culinary uses (Watson and Dallwitz, 1999)

RAB are highly attracted to redbay, *Persea borbonia*, and this species has suffered the greatest losses from laurel wilt in North America (Hanula et al. 2008). Redbay is an aromatic evergreen tree which, while not very valuable commercially, is ecologically very important. The dense shading of a redbay canopy results in a substantial decrease in forest floor temperature under the canopy. The fall fruits of the tree are an important food source for songbirds, turkey, deer, and black bear (Brendemuehl et al., 1990). Several species of swallowtail butterfly, especially the Palamedes swallowtail , *Papilio palamedes* Drury, are highly dependent on and specific to redbay for their life cycles (Lederhouse et al., 1992). The native range of redbay spans throughout the southeastern costal plain from Delaware to Texas and also the Bahamas (Brendemuehl et al., 1990). The most economically important member of the Lauraceae family in North America is the non-native avocado, *Persea americana* Mill. As of February, laurel wilt had spread to Miami-Dade County in southern Florida, just 14.5km from the main avocado producing area in the Southeastern US with approximately 7400 acres of avocado orchard (Ploetz et al., 2011). Potential economic losses due to laurel wilt on avocado in Florida have been estimated at $27million-$54million (Evans et al., 2010). California and Mexico are even

bigger avocado producers with annual crops worth $415 million and $645 million, respectively (Kendra et al., 2011). In addition to the avocado industry, if RAB and laurel wilt reach the west coast they could potentially impact native populations of California bay laurel (*Umbellularia californica*, Nutt), which has also been shown to be susceptible to laurel wilt (Fraedrich, 2008).

The mostly widely distributed member of the Lauraceae in the US is *Sassafras albidium* (Nutt.) Nees, whose native range spans from central Florida all the way into southern Ontario, Canada (Koch and Smith, 2008). Two other Lauraceae species of special concern are federally endangered pondberry (*Lindera messifolia* (Walter) Blume) and pondspice (*Litsea aestivalis (*L. Fernald)) which is listed as threatened in GA and endangered in FL and MD (Hughs et al., 2011). *Raffaelea lauricola* has been isolated from wilted specimens of both of these species (Fraedrich, et al., 2008).

Artificial inoculations with *R. lauricola* have been used to test the susceptibility to laurel wilt of a variety of other plants that have not been found as hosts in the field. Those showing some susceptibility to the disease include northern spicebush (*Lindera benzoin* (L.) Blume), lancewood (*Ocotea coriaceae* (Sw.) Britton), and eastern sweetshrub (*Calycanthus floridus* L.) (Mayfield et al., 2009). These plants may not be at risk, however, if the RAB is not attracted to them or unable to utilize them for breeding.

Lauraceae is very prominent and diverse in Central and South America, and it is unclear what impact laurel wilt may have if it ever reaches these regions. The aromatic oils of Lauraceous species are unique and their potential uses are largely unknown. For example, it was recently shown that essential oil extracts from pondberry can repel ticks and mosquitoes (Oh et al., 2012). Oils from other Lauraceae species are known to have antioxidant and antimicrobial properties (Joshi et al., 2010).

Fungicide trials. Mayfield (2008) demonstrated that the fungicide propiconizole (Alamo ®; Syngenta Crop Protection Inc., Greenville, NC) at concentrations greater than one part per million completely suppresses growth of *R. lauricola* in vitro. Injections of the fungicide into the root-flares of 10 mature redbay trees prior to inoculation with *R. lauricola* prevented crown wilt in all trees for at least 30 weeks compared with 9 of 10 untreated trees that developed crown wilt. Propiconizole was detected in the trunks of trees for at least 7.5 months after injection, but was less detectable in smaller branches. While fungicide injections could be used to protect high value trees, applications are intensive and costly, and it is not fully clear how long trees will are protected. Also, Alamo® is not registered for use on Avocado at this time. (Mayfield, 2008).

Impact and range of RAB. *Xyleborus glabratus* is native to regions of southeast Asia including Japan, Taiwan, Bangladesh, Myanmar, and India (Rabaglia, 2006). In its native region, a range of hosts is known for the beetle including species from the families Dipterocarpaceae, Fagaceae, Fabaceae, and Lauraceae. The beetle is not a known pest in its native range and likely attacks only stressed or dying trees, as is common for most ambrosia beetles (Fraedrich et al., 2008). However, in the U.S., all known hosts of *X. glabratus* belong to the family Lauraceae, and individuals will bore into living healthy trees (Koch and Smith, 2008). Mature redbay trees die quickly when challenged with *Raffaelea lauricola*. Experiments by Fraedrich et. al. (2008) showed that challenging redbay seedlings with *X. glabratus* females resulted in 96% of beetles boring into plants and 70% tree mortality. Mortality of mature redbay trees was found to increase from 10% to 92% in just 15 months at one study site in Duval County, Florida (Mayfield, 2007). Once laurel wilt reaches an area, large trees are more rapidly affected than seedlings, probably because RAB are less likely to attack these smaller trees. Bark beetles often combine visual cues with olfactory cues as well as random landings to find hosts

trees, all of which reduce the likelihood of a beetle landing on smaller trees (Campbell et al., 2006). Mayfield (personal communication) found that sticky traps similarly baited with essential oils and attached to either artificial silhouettes or non-attractive standing pines of varying diameter intercepted more RAB as silhouette or stem diameter increased. This explains why areas infested with RAB and laurel wilt lose all or nearly all mature redbay following infestation, and only the smallest, usually less than 2.5cm diameter, trees remain surviving (Fraedrich et al., 2008).

The potential range of RAB in North America is uncertain, and depends on several factors. One is the ability of RAB to successfully reproduce in and spread through host trees other than redbay such as sassafras. Also, climate outside of the southeastern coastal region may be a limiting factor for the spread of the beetle. This is largely because most of the U.S. receives less rainfall than even the driest parts of the native range of *X. glabratus*, especially in the summer months (Koch and Smith, 2008).

Spread of RAB and laurel wilt. *Xyleborus glabratus* was first detected in North America in 2002 at a survey trap at Port Wentworth, GA, and had likely been established for some time (Rabaglia et al., 2006). The beetle was likely introduced through infested wood shipping materials. *Xyleborus glabratus* and its associated pathogen are steadily spreading throughout the range of redbay. By 2003 substantial redbay death due to laurel wilt was seen in Georgia and South Carolina, and by 2005 the disease had reached Florida. By 2004 an estimated 80% of redbay trees on Hilton Head Island, SC were dead (Fraedrich et al., 2008). Wilting and tree death of sassafras have been reported in GA, SC, and FL since 2005. Unlike redbay trees which can retain leaves for up to one year or longer after wilting, sassafras leaves rapidly drop off once trees become infected quickly resulting in tree death (Smith et al., 2009). Because sassafras

reproduces clonally, it is possible that infection can spread through connected root systems. Rapid movement of laurel wilt through a clump of sassafras without evidence of RAB attack was noted by Cameron (2008), suggesting this possibility. When laurel wilt was first detected on Jekyll Island, GA in 2006, a team of workers with chainsaws cut down and burned over 400 trees displaying symptoms in an effort to sanitize the disease. Despite this valiant effort, laurel wilt had spread throughout the island by 2007, indicating that merely removing trees displaying wilt symptoms is not sufficient to stop the disease spread (Mayfield et al., 2009). It is likely that some infested trees yet to show symptoms were not cut, and also stumps could have provided sufficient wood for brood to develop and continue the spread of the pathogen.

A residentially planted avocado tree in Jacksonville, FL was reported in September, 2007 to be infected with laurel wilt (Mayfield et al., 2008). Also since 2007, wilted branches of camphor trees have been reported in parts of Georgia and Florida, but these trees seem to be less vulnerable to the laurel wilt pathogen, often showing wilting only on smaller branches and sometimes being able to recover (Smith et al., 2009). Laurel wilt of redbay was first reported in southern Mississippi in 2009, 10-15 years earlier than models had estimated for natural spread (Riggins et al., 2010). The disease has now spread as far south as Miami-Dade County in Florida (Ploetz et al., 20011), and its northern spread has reached North Carolina. Populations in MS and Miami-Dade County, FL are far removed from the main area of infection and therefore likely to have been initiated by human movement of RAB, possibly through infected firewood.

Population dynamics of RAB. In 2006 and 2007 Hanula et al. (2008) investigated the seasonal flight activity of this beetle, its host associations, and population levels at eight locations ranging from old infested to newly infested areas. Traps were attached to initially healthy, artificially wounded trees and showed that RAB were active throughout the year but

very few were caught from March to late May 2006 and from mid-November 2006 through March 2007. Peak adult activity occurred in early September. Males are flightless but a few males were found in every sample from 3 June 2006 to 30 January 2007. Based on these male emergence data Hanula et al. (2008) suggested brood development took 50-60 days. Uninfested redbay wood was attractive to *X. glabratus* females and it remained attractive for up to 70 days. Wood infested with beetles and containing the *Raffaelea* sp. fungus were similar in attraction to uninfested redbay wood but both uninfested and infested wood were more attractive than non-host species. Sassafras was not attractive to *X. glabratus* and very few beetle entrance holes were found in sassafras wood compared to redbay. Conversely, avocado was as attractive to females as redbay and both were more attractive than the non-host red maple (*Acer rubrum* L.). However, avocado had relatively few entrance holes in the wood so it is unclear whether it is a good host for brood development.

In 2007 Hanula et al. (2008) trapped in areas where all large redbay had been lost to laurel wilt and compared them to areas where the infestation was very active, areas newly infested and an area outside the known infestation. Both trap catch and numbers of entrance holes in trap bolts of redbay were significantly correlated with the number of dead trees with leaves attached. Areas where the beetles had eliminated mature host trees had very low population levels ranging from 0.04 – 0.12 beetles/trap/day compared to the very active infested areas that had catches of 4-7 beetles/trap/day. Those results showed that populations of *X. glabratus* decrease dramatically after suitable host material is gone and provide hope that management strategies can be developed to restore redbay trees.

Attraction of RAB to manuka oil. In 2006, Hanula and Sullivan (2008) used a Clevenger extractor to collect essential oils from redbay, and Poropak Q to collect volatile emissions.

GC/MS analyses of these extracts showed which chemicals were contained in redbay and eluted from redbay wood. Individual compounds were tested for attractiveness to RAB in 2007. However, several compounds were not available in large enough quantities to test so manuka oil was tested since it contained all three of the compounds needed (Porter and Wilkins, 1999). After these trials it was clear manuka oil was attractive so several followup studies were performed in 2007 that demonstrated it was as attractive to RAB as redbay wood and that white sticky traps captured beetles as well as flight intercept traps constructed from Plexiglas panels. Late in the 2007 trapping season phoebe oil, an essential oil of *Phoebe porosa* Mez., (a commercially important tree species in Brazil) that contains high quantities of α-copaene was tested (Weyerstahl et al., 2006). Although phoebe oil caught more beetles than manuka oil and slightly more than redbay wood it was not significantly more attractive. A manuka oil fraction that has a much higher composition of α-copaene and several other sesquiterpenes was provided by Coast Biologicals Limited (New Zealand), the sole manuka oil producer. It was not more attractive than whole manuka oil. Likewise, studies of release rate showed that lures releasing 5 mg/day were as attractive as lures releasing 200 mg/day (Hanula and Sullivan, 2008). The use of this commercially available lure has helped agencies monitor the spread of RAB.

Research area. The site for all field based portions of these studies was located in Emanuel County, Georgia about 26km due south of Swainsboro, GA (32° 39′ 07″ N, 82° 28′ 04″ W). The site is privately owned by Larry Jordan with Georgia GJ Properties Inc. The site was originally intended to be developed into a residential neighborhood but has remained undeveloped. There are at least three rather distinct plant communities at the site. The upland sites included a planted pine plantation with various herbs, woody shrubs, and small trees in the understory. An upland sand hill community consisted of small to medium size turkey oak

(*Quercus laevis* Walter) and bluejack oak (*Q. incana* Bartram) and occasional longleaf pine (*Pinus palustris* Mill.) in the overstory and various herbaceous species including prickly pear (*Opuntia compressa* Raf.) and gopher apple(*Licania michauxii* Prance) sparsely populating the understory. A very sharp ecotone defined by about 1m elevation separates these two communities from a bay forest dominated by loblolly bay (*Gordonia lasianthus* (L.) Ellis) and redbay with an understory of *Itea, Smilax,* and *Lyonia* species. This bay forest was the site of these experiments.

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CHAPTER 2

GALLERY PRODUCTIVITY, EMERGENCE, AND FLIGHT ACTIVITY OF THE REDBAY AMBROSIA BEETLE, *XYLEBORUS GLABRATUS*

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ABSTRACT Flight and emergence activity of the redbay ambrosia beetle (RAB), *Xyleborus glabratus*, were monitored during from March 2011 through August 2012 using Lindgren funnel traps baited with manuka oil and emergence traps attached directly over individual beetle galleries on infested redbay, *Persea borbonia*, trees. At the beginning of the study only a few trees showed signs of laurel wilt, but by the end all large redbay in the study site were infested and killed. Gallery success rates and time until adult emergence were highly variable depending on month of gallery formation. Host tree dbh significantly influenced the number of beetles produced in a gallery, time until initial brood emergence, total time a gallery was active, and likelihood a gallery would be successful. The maximum time a single RAB gallery was active was 497 days, and the maximum productivity of a gallery was 316 adult beetles. Lindgren trap captures reflected emergence trap collections but with a delay of about one month. Peaks of activity occurred in fall 2011 and spring 2012, but at least some adult beetles were collected using both methods in every month of the year.

KEY WORDS *Persea borbonia*, exotic, invasive, laurel wilt, *Raffaelea lauricola*

Introduction

The redbay ambrosia beetle (RAB), *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), and its associated fungus *Raffaelea lauricola* (T.C. Harr., Fraedrich & Aghayeva) are recent introductions to the southeastern United States and are causing extensive mortality to redbay (*Persea borbonia* (L.) Spreng). Ambrosia beetles, including *X. glabratus*, tunnel and breed within sapwood of dead or dying trees and feed exclusively on cultivated fungi known as ambrosia. *Xyleborus glabratus* is not a known pest in its native range of Southeast Asia and

likely attacks only stressed or dying trees, as is common for most ambrosia beetles. However, in the United States *X. glabratus* bores into living healthy trees of the family Lauraceae which includes redbay. Mature redbay trees die quickly when challenged with *Raffaelea lauricola*. Beetles will initially bore a short distance into a healthy tree and usually abort the gallery, but this is often sufficient to infect susceptible trees with the pathogen. (Fraedrich et al. 2008). As the fungus spreads and the tree begins to die, greater numbers of RAB are attracted to the tree (Mayfield et al. 2008). Once RAB females successfully bore into the sapwood, they establish galleries in which eggs are laid. Larvae feed on the ambrosia fungus growing on the gallery walls and develop into adults within the galleries. At some point the daughters disperse, although dispersal can come at a high cost (Biedermann et al. 2009, 2011), and either locate a new host tree or bore into a new location on the same host. Females are generally mated before leaving a gallery, but it is possible for initially unmated females to lay unfertilized eggs that develop into haploid males which can then mate with the mother to produce diploid female offspring (Biedermann et al., 2010). Brood eventually emerge from the original entrance hole in contrast to most phloem-feeding bark beetle species in which each individual produces its own exit hole upon emergence (McClelland et al. 1978). The behavior of ambrosia beetles simplifies monitoring their emergence and allows for careful observation of a single gallery throughout its active period.

Emergence rates can be inferred from baited trap catch, but there are problems with this approach. Bentz (2006) found that pheromone baited flight-intercept traps caught specimens of *Dendroctonus ponderosae* Hopkins over a period of 130 days, but emergence traps showed an emergence period of only 30 days. Much work has looked at the emergence of bark beetles (e.g., Bentz, 2006, Lessard et al. 1990, McClellend et al. 1978), but less has examined the emergence

of ambrosia beetles. Norris et al. (1968) looked at the emergence of *Xyleborus ferrugineus* Fabricus and *Xyleborus posticus* Eichhoff and found that both showed diurnal patterns of emergence. Gagne and Kearby (1979) found that host black hickory trees (*Carya texana* Buckl.) could support *Xyleborus celcus* Eichhoff for up to two years and peak emergence activity coincided with the beetle's two yearly attack periods. Knowing peak periods of activity and emergence can be very important for control strategies. We examined emergence of *X. glabratus* from individual galleries that were initiated over a one year period from18 March 2011 to 6 March 2012 and how it related to timing of gallery initiation, size of the tree attacked, and adult flight activity.

Materials and Methods

The study area was a riparian forest composed of primarily of redbay and loblolly bay (*Gordonia lasianthus* (L.) Ellis) with scattered remnant loblolly pine (*Pinus taeda* L.). At the time the study was initiated very few redbay trees were dead or exhibiting wilt symptoms, but by the end of the study in August 2012 nearly all large redbay trees in the area were dead.

Between 18 March 2011 and 6 March 2012 a total of 432 emergence traps (Fig. 1) were attached to mature *Persea borbonia* trees infected with laurel wilt. Traps were made by soldering a two-hole pipe strap to a 1.3cm (0.5 inch) diameter copper street elbow. The tabs of the straps were then cut approximately three quarters through so they could be bent to provide attachment points to the tree. Traps were attached to trees with screws and sealed with silicone caulk to create a water tight seal. A 1.3 cm (0.5 inch) diameter hole was drilled into the lid of a 20mL scintillation vial so the lid could be slipped over the end of the copper elbow. The lid was

sealed to the copper elbow using Goop brand plumbing adhesive. The scintillation vials could then be screwed onto and off of the lid for examination of the vial's contents.

A total of eleven trees ranging in size from 10.4-26.1cm dbh (diameter at breast height or 1.4m) were selected for the study. Some trees selected were recently naturally infected with laurel wilt but were in the very early stages so very few beetle attacks were present. Others were healthy trees on which sections of outer bark were scraped smooth to encourage RAB attacks and make those attacks easy to find. For the first four trees the outer bark was scraped from ground level to approximately 2m high. Scraping some small areas of phloem and xylem were exposed which were attractive to RAB (Hanula et al. 2008), and these areas were usually quickly attacked. On trees selected later in the study, small sections (about 100-200cm^2) were scraped each week. Once a section of bark was scraped, it was carefully examined for any beetle entry points which were marked with either pencil or a map pin. The following week these sections were again examined for attacks, and emergence traps were placed only over attacks known to have occurred since the last visit. Scraping small sections weekly allowed for emergence traps to be attached to trees every visit for up to three months, whereas scraping the entire lower bole in one week resulted in trees becoming so heavily attacked that new attacks were not easily discerned after a only a few weeks. After installation, contents of each trap were checked weekly from 18 March through 14 November, 2011 then either weekly or biweekly from 28 November, 2011 until 30 August, 2012. Vials containing beetles were capped and transported back to the lab for examination under a dissecting microscope. Male and female *X. glabratus* along with any other ambrosia beetles collected were identified and counted.

In addition to the emergence traps, four 8-funnel Lindgren traps were hung within the periphery of the study site. Traps were baited with manuka oil lures (half lures, Synergy

Semiochemical Corp., Burnaby, BC) which are as attractive as redbay wood to RAB (Hanula and Sullivan 2008). Lures were changed every other month from May, 2011 until April, 2012 then every month until August 2012 because lure longevity changed (Kendra et al., 2011; Hanula unpubl. data). From May 2011 until July 2011 all four traps were hung at a height of about 1m from ground level. In July 2011, two of the four traps were raised to a height of about 3m above ground level, and they remained at this height until the end of the experiment. This was done because the study site had extensive underbrush, and it was thought that RAB may have had difficulty following the odor plume near the ground.

Statistical Analysis. Differences in time until emergence by month of gallery initiation were analyzed using general linear models procedure (Proc GLM, SAS institute 1985), and the Ryan-Einot-Gabriel-Welsch (REGWQ) multiple comparison test (Day and Quinn 1989) was used to separate the mean time until first emergence for each month. Simple linear regression analysis was used to examine the relationships between gallery height from ground level and host tree size (measured as dbh of the tree) to time until first emergence, length of time a gallery was active, and productivity of a gallery (Proc Corr; SAS institute 1985). Productivity was a measure of the total number of both male and female RAB collected from an individual gallery. Length of time a gallery was active was calculated as the number of days between cage installation and the final date on which adult beetles were collected in that cage. A pooled Satterthwaite T-test (proc Ttest, SAS institute 1985) was used to analyze the difference in productivity among galleries from which males did and did not emerge. A T-test was also used to compare the number of RAB collected in Lindgren funnel traps hung at two different heights. Rates of emergence were calculated by dividing the total number of adult RAB collected on a date by the number of emergence traps containing adult RAB on that date.

Results

Of the 432 gallery entrances covered with emergence traps, 235 (54.4%) successfully produced at least two RAB adults (table 1). Galleries that produced only one adult were not included in analyses since the recovered beetle was likely the foundress abandoning the gallery for some reason. On average, successful galleries produced 23.40±2.50 ($\bar{x}$±SE) adult RAB but one had 316 adults emerge from it. Galleries were active for an average of 231.93±6.13 days, one gallery produced beetles for 497 days, and five were active for over one year. A total of 5345 female and 196 male RAB were collected during the study resulting in a sex ratio of about 27:1 female:male. Ambrosia beetles other than *X. glabratus* were recovered from a total of 18 traps or approximately 4% of the galleries covered. Other Scolytinae species recovered were: *Xylesandrus germanus* Blandford (N=2), *Xyleborinus saxeseni* Ratzeburg (N=9), *Xyleborus affinins* Eichhoff (N=4), and *Xylesandrus crassiusculus* Motschulsky (N=3). Some bark beetles outside of Scolytinae were also recovered including *Silvanus* sp. (N=3 traps), *Tenebroides* sp. (N=3 traps), and *Colydium* sp. (N=1 trap), but these likely entered the traps from the outside by bypassing the silicone sealant. One reason some entry points were misidentified was that entry holes were gauged by eye alone since the standard way of gauging RAB entrance holes by using a medium size paper clip or similar diameter map pin inserted into the hole (Hanula et al. 2008) could kill the foundress. All male broods occurred in six galleries but only one of these contained multiple (4) males. Galleries from which only females emerged were common (N=127). Successful galleries from which both males and females emerged produced more (p=.0208) adults (29.56 ±4.23, N=108) than galleries from which no males emerged (17.80±2.75, N=127).

Success was highest in the summer months of June, July and August, while only about one in four females that initiated galleries during September and October successfully produced

offspring (table 1). In addition to having the lowest success rate, traps installed in October took the most time before emergence began. Emergence from those traps took an average of 207.0±11.0 days, significantly longer than from all other months except September and November (fig.2). Galleries initiated in spring through early summer were more successful and produced brood more quickly. Success rates were highly variable between trees and ranged from 90.2% down to 16.7%. Traps were placed at heights from ground level up to 2.1m, and within this range height of a trap was not correlated with either number of beetles produced ($p=.1081$), time until initial emergence ($p=.1351$), or length of time a gallery was productive ($p=.9334$). However, dbh of the host tree was correlated with number of beetles produced time until initial emergence and total time a gallery was productive (figure 3). Success rates of galleries (those that produced brood) were also higher on larger trees ($p<.0001$) (fig. 3a).

The rates of emergence relative to adult flight activity are closely related, but with changes in flight activity (indicated by Lindgren trap captures) seen about one month later than those in emergence trap collections (fig.4). Adults were collected in every month emerging from galleries and in attractant baited traps, but numbers were by far the lowest in the winter months from December to February. Both emergence and flight activity peaked sharply in March 2012. There were no significant differences ($p=.0800$) in trap catch between Lindgren traps hung at 1m and 3m heights (1m $\bar{x}$ =85.53±27.11, 3m $\bar{x}$ =34.25 ±8.78). We grouped emergence by month that the galleries were initiated, and found that peaks in emergence were similarly timed across cohorts (fig.5).

Discussion

Emergence traps designed to be somewhat similar to those introduced by Nord and Lewis (1970) to monitor the emergence of the Columbian timber beetle (*Corthylus columbianus* Hopkins) except they used a strait piece of metal or plastic tubing which resulted in a horizontal collection chamber, whereas those here had a vertical and removable collection chamber. The vertical chamber prevented beetles that dropped into a vial from returning to the tree to bore back in. Another common way of monitoring the emergence of wood boring insects is to attach a section or sleeve of screen over an infested tree and determine the emergence per unit area (McClellend et al. 1978, Wagner et al. 1984, Bentz 2006). However, the individual emergence traps used here had the advantages of deterring an emerging beetle from reinfesting the host tree and isolating individual beetle galleries to obtain more detailed information on each.

During the 17 months of this study the redbay population changed dramatically. At the beginning very few redbay trees showed signs of laurel wilt, but by the end every large redbay (>5 cm dbh) in the stand was infested and killed. This time period, therefore, covered the bulk of the RAB's infestation cycle. This is consistent with results of Shields et al. (2011) and Fraedrich et al. (2008) who reported 100% mortality of all redbay over 10.3cm dbh within two years of initial laurel wilt detection at study sites at Etoniah Creek State Forest, Putnam Co., FL and Fort George Island, Duval Co., FL, respectively.

Multiple adult beetles were collected from a single gallery in as little as 29 days after emergence trap installation. Since traps were installed on attack points that were no more than one week old, some RAB were therefore able to complete their lifecycle in 36 days or less. Completing the life cycle within this period was uncommon, however, and multiple adults were

collected within 40 days after trap installation from only 8 galleries. This development time is similar to that reported for *Xyleborus celsus* (Gagne and Kearby 1979). Both time to complete development as well as likelihood that a gallery would be successful were strongly influenced by the time of year a gallery was initiated. Rates of successful brood production varied greatly between individual trees, and it is unclear exactly what factors contributed to this variation. Two of the three trees with lowest success rates were also the smallest trees in the study each with a dbh of about 10cm. Also, traps on the two least successful trees had average installation dates in September, at time of year when initiated galleries were the least successful; so, time of gallery initiation could be a major factor contributing to this variation.

Beetles were active throughout the year and at least some adults were collected in both Lindgren funnel traps and emergence traps every month. Sections of trees where bark was freshly scraped were also readily attacked throughout the year. This is in contrast to most other ambrosia beetles which are inactive during the winter and begin flight in early spring (Chapman and Kinghorn 1958, Webber and McPherson 1983, Webber and McPherson 1991, Coyle et al. 2005). Peaks of emergence and flight activity in late summer to early fall of 2011 reported for RAB in this study are consistent with previous reports (Hanula et al. 2008, 2011), however the spike in activity during the following spring and subsequent decline throughout the summer was unusual. This difference could be attributed to the timing of exhaustion of viable host material. Ambrosia beetles can gain both direct and indirect fitness benefits from remaining in their natal gallery after maturation (Peer and Taborsky 2007, Biedermann et al. 2011), and because *X. glabratus* is active throughout the year it is likely that brood would remain in the natal gallery until host condition deteriorates. Diameter of the host tree was important to gallery productivity, and in larger trees an individual gallery was more likely to be successful, more brood were

produced, brood emerged sooner, and galleries were active over a greater period of time. However, there was a downward trend in size (dbh) of selected test trees over time, and this resulted in many of the smaller trees in the test being selected in the fall when gallery success and productivity was lower (table 1, fig.5). Therefore time of year galleries were founded may have contributed to the variation seen in fig.3.

Judging by the data indicated on figure 5, time of emergence seems to be more closely correlated to date or time of year than to time spent within a tree. For example, traps installed as disparately as April and August 2011 show a drop in emergence on 20 September, 2011 followed shortly by a peak on 4 October, 2011. A possible explanation for this is that beetles emerge at times when the weather is favorable for flight. This idea is supported by Chapman and Kinghorn (1958) who observed that flight activity of the ambrosia beetle *Trypodendron lineatum* Oliver increased dramatically over a one day period coinciding with increase in temperature. Adult RAB did not begin to emerge from galleries initiated in fall 2011 until spring 2012. Galleries initiated between October 2011 and March 2012 show relatively brief periods of adult emergence. For these galleries emergence began in March-April 2012 and had declined nearly to zero by August 2012. This is very different than galleries initiated in April of 2011 which were still productive a full year later in April 2012.

The sex ratio of adults emerging from galleries in the field was approximately 27females:1male. This contrasts with the results of rearing RAB in vitro which showed a sex ratio of about 8 females: 1male. This discrepancy could be explained by a failure of some male beetles to emerge from galleries in trees in contrast to culture tubes from which all beetles were extracted. Regardless, females greatly outnumbered males in this study. Because males are flightless they have generally been thought to rarely emerge from galleries (Biedermann 2010),

but Peer and Taborsky (2004) reported a male emergence rate of 68% for *Xylesandrus germanus*. Biedermann (2010) found that male *Xyleborinus saxesenii* will usually only emerge from galleries after all offspring had matured and no more eggs were laid. In this study, males emerged from slightly less than half of the successful galleries, but those from which they did were nearly twice as productive on average.

Redbay ambrosia beetle galleries can be very productive over a long period of time. Beetles were active throughout the year; so, it would be difficult to target control strategies based on timing of peak flight and emergence.

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Table 2.1. Number of emergence traps installed and number successful by month. Galleries were considered successful if more than two adult RAB were recovered in a single emergence trap over the length of the experiment.

Month	Total no. of traps	No. produced brood	% Success
March	23	17	73.9%
April	19	8	42.1%
May	8	4	50.0%
June	31	24	77.4
July	47	42	89.4%
August	70	47	67.1%
September	82	26	37.7%
October	72	20	27.8%
November	44	28	63.6%
December	14	10	71.4%
Feb/March 2012	22	9	40.9%
Overall	432	235	54.4%

Fig 2.1. Emergence trap constructed from a 1.3cm diameter copper street elbow and pipe strap that was soldered to the elbow then the strap was cut part way through so the ends could be bent down to form attachment points to hold the trap to the tree.

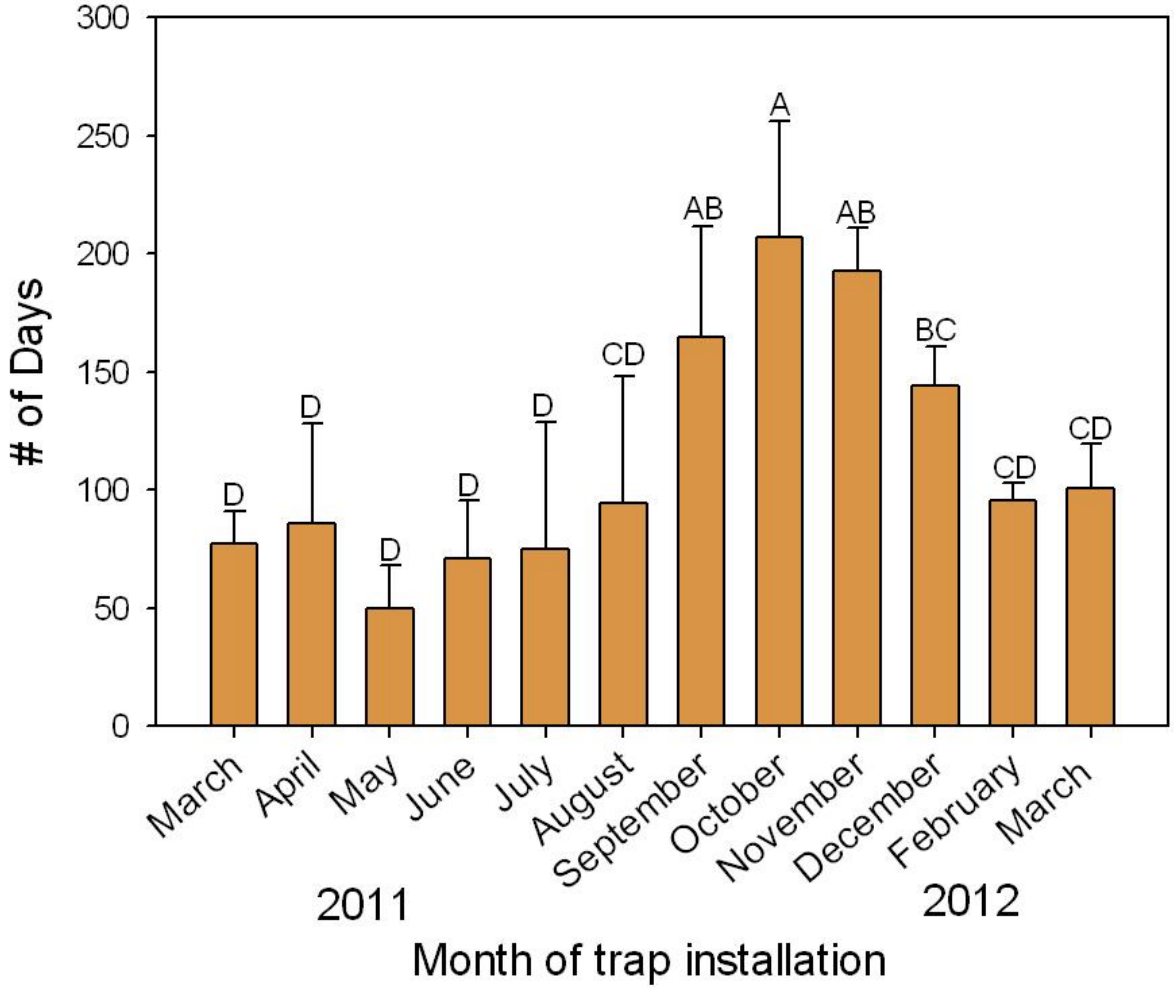

Fig 2.2. Number of days ($\bar{x}$ ± SE) until first adult emergence by month of gallery initiaition. Columns with the same letters are not significantly different (**α**=.05).

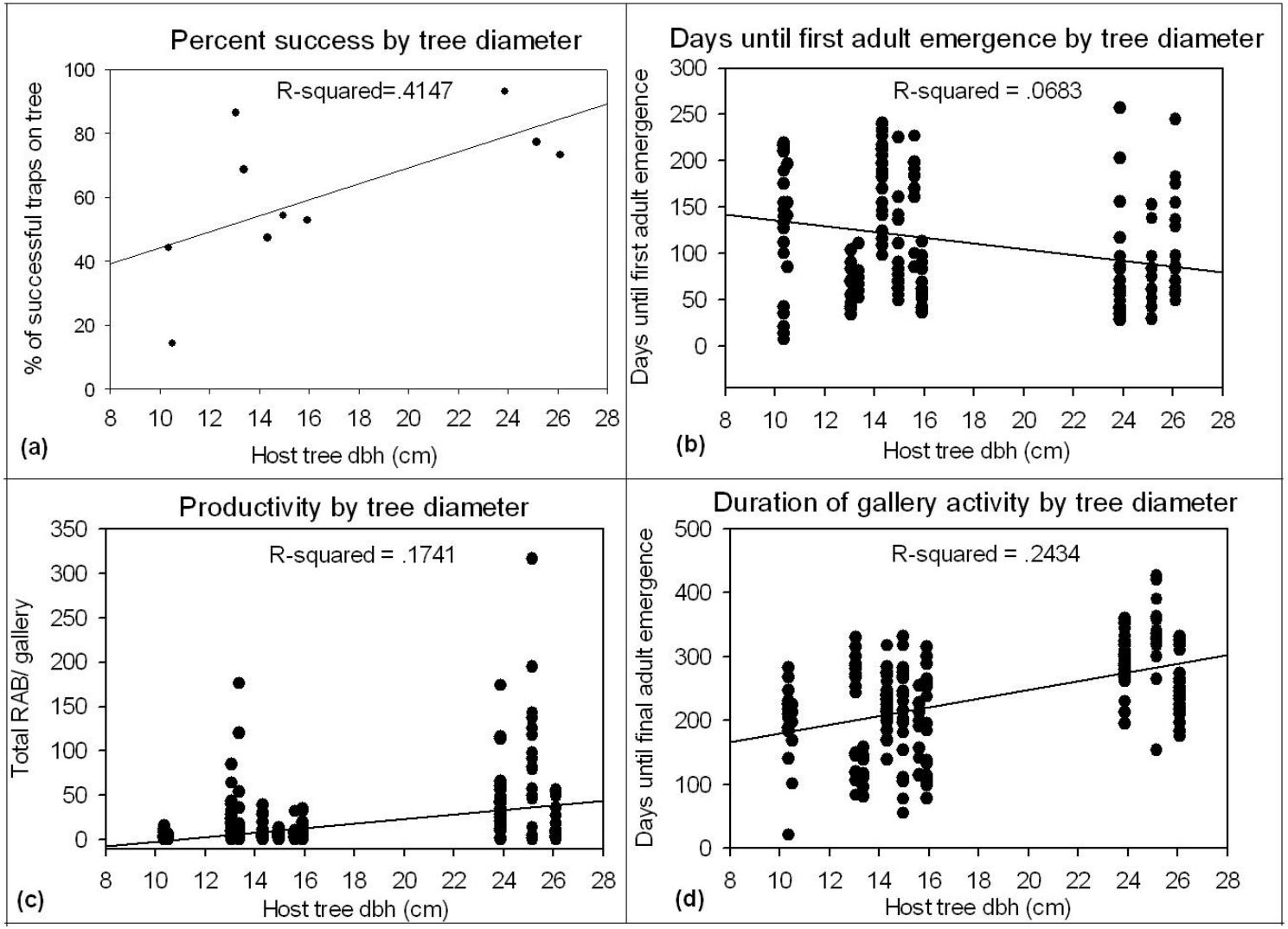

Fig. 2.3. Effect of host tree dbh on: (a) percent of gallery success, (b) time until first adult emergence, (c) gallery productivity (total number of adults emerged), and (d) amount of time galleries were active. All relationships were significant at $p<.0001$.

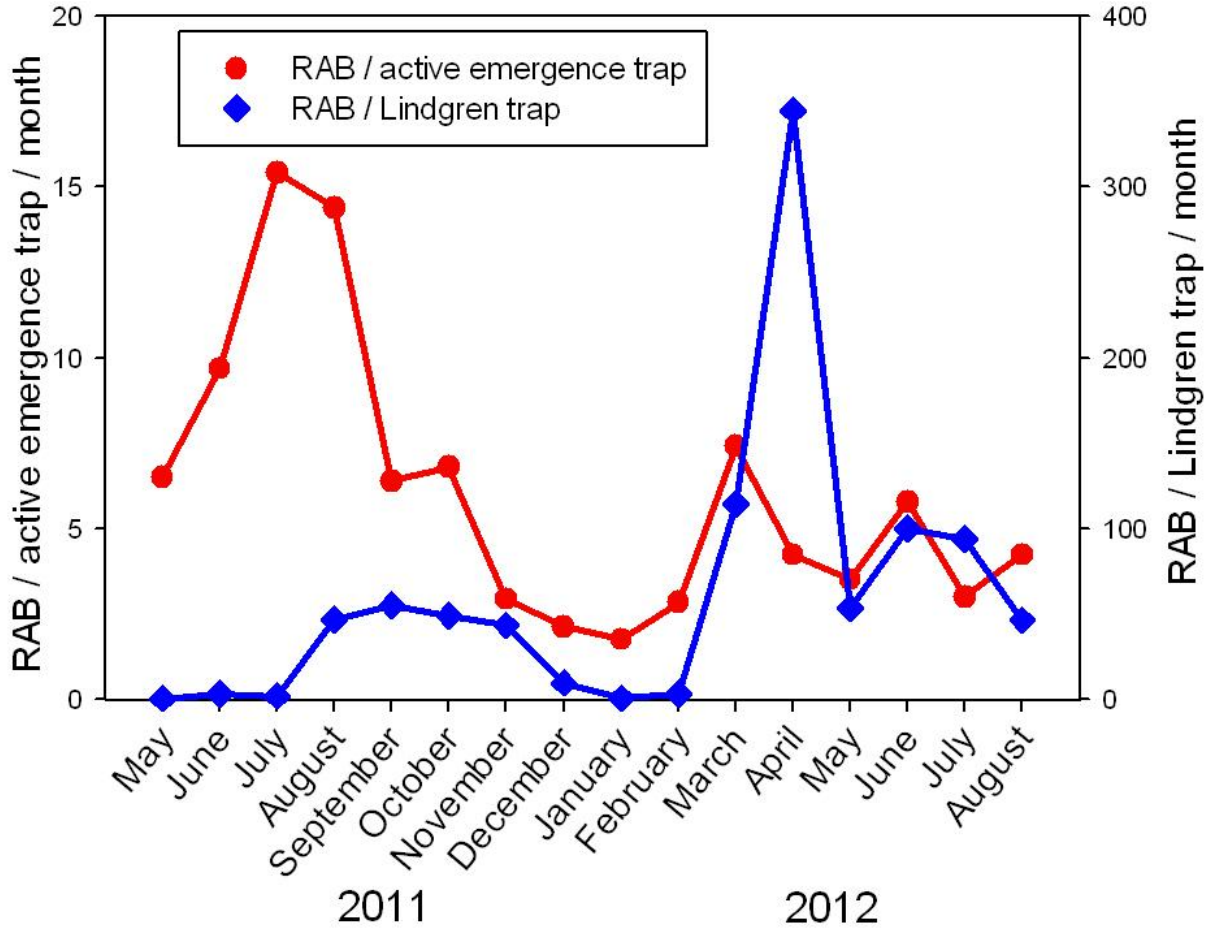

Fig. 2.4. RAB flight and emergence activity from May 2011 through August 2012. Lindgren trap collection values represent the average of four traps. Lindgren collection data for October 2011 were lost, so and average of September and November was substituted. Rate of emergence was calculated for each month by dividing the total number of adult RAB collected in a month by the number of emergence traps which contained beetles during that month.

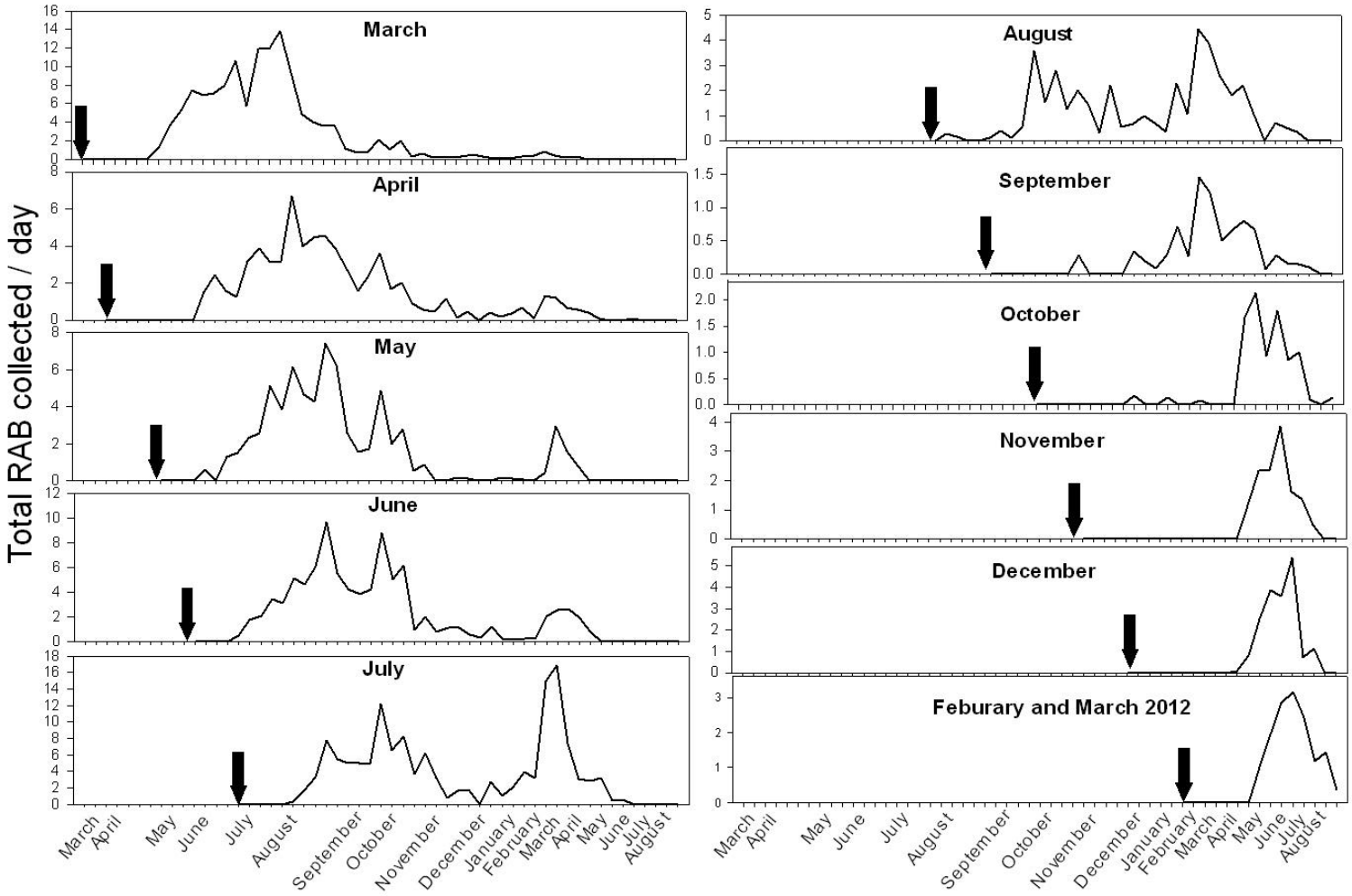

Fig. 2.5 Adult RAB emergence by month of gallery initiation. Number of galleries per month is given in table 1. Arrows indicate approximate time of gallery initiation. Values represent the total number of RAB adults collected per cohort per collection date.

CHAPTER 3

PHYSICAL PROTECTION OF MATURE REDBAY TREES FROM ATTACK BY *XYLEBORUS GLABRATUS* USING FINE MESH SCREEN AND EFFECTS OF MOISTURE CONTENT, STEM DIAMETER, AND HEIGHT ON *X. GLABRATUS* ATTACK RATES

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ABSTRACT Fine mesh screen was used to create a physical barrier to prevent redbay ambrosia beetles, *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), from accessing various parts of the boles of redbay, *Persea borbonia* (L.) Spreng, trees and infecting them with the laurel wilt fungus, *Raffaelea lauricola* (T.C. Harr., Fraedrich & Aghayeva). Screen barriers prevented beetles from attacking boles of mature redbay trees from the ground to 1 or 3 m and from 1-3 m above ground. No significant difference in the number of days until tree death was seen between treated and control trees. A total of 45 trees were involved in the test, all of which died within 243 days from the beginning of observation with an average of 165.87 ± 6.94 days until tree death. Initial attack points of *X. glabratus* were found to vary from ground level to heights of at least 6.62m. Moisture content was measured throughout the height of test trees, but no correlation was found between moisture content and the number of attacks at a particular height. Height and stem diameter were correlated with RAB attack rates. Trees were seen to show wilting symptoms characteristic of laurel wilt with as few as two *X. glabratus* entry points. Physical protection using these methods is not an effective technique for control of laurel wilt on redbay, and these results suggest that insecticides will have to be highly effective and cover most of the tree to prevent infection.

KEY WORDS laurel wilt, *Raffaelea lauricola*, *Persea borbonia*,

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The redbay ambrosia beetle (RAB), *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), and its associated fungus *Raffaelea lauricola* (T.C. Harr., Fraedrich & Aghayeva) are recent introductions to the southeastern United States and are causing extensive mortality to redbay (*Persea borbonia* (L.) Spreng). Ambrosia beetles, including *X. glabratus*, tunnel and

breed within sapwood of dead or dying trees and feed exclusively on cultivated fungi known as ambrosia. *X. glabratus* is not a known pest in its native range of Southeast Asia and likely attacks only stressed or dying trees, as is common for most ambrosia beetles. However, in the United States *X. glabratus* will bore into living healthy trees of the family Lauraceae which includes redbay. Mature redbay trees die quickly when challenged with *R. lauricola*. Beetles will initially bore a short distance into a healthy tree and usually abort the gallery, but this is often sufficient to infect susceptible trees with the pathogen. (Fraedrich et al. 2008). As the fungus spreads and the tree begins to die, greater numbers of RAB are attracted to the tree (Mayfield et al. 2008).

It is known that many bark and ambrosia beetles exhibit specific attack patterns and distributions within host trees and that different species will prefer different parts of a tree. In a test that set ethanol-baited traps at heights of .5m, 1.7m, and 3m, Reding et al. (2010) found that traps placed at a height of .5m accounted for nearly 80% of the trap catch for *Xylesandrus germanus* Blanford. The same test showed a more even distribution for *Xylesandrus crassiusculus* Motschulsky, although the traps at .5m and 1.7m did catch significantly more than those at 3m. For both of these species, the average attack point on a tree has been shown to be <30cm from the ground. Not all bark and ambrosia beetles first attack at the base of a tree however. For example, the western pine beetle, *Dendroctonus brevicomis* LeConte, is known to first attack on the upper mid-bole of a tree with subsequent attacks further down the stem (Miller and Keen, 1960). *Xyleborus glabratus* have been shown to generally stay low to the ground. In varied-height trapping trials Hanula et al. (2011) found that traps hung at or below a height of 1.5m caught about 85% of the beetles collected, and Brar et al., (2010) also trapped the greatest number of RAB from 35-100cm above ground level. Based on this and observations by

Fraedrich (personal communication) that the lower bole frequently seems to be the first part of a tree to be attacked, it was hypothesized that protecting the lower bole of a redbay tree may result in whole tree protection from RAB and laurel wilt. Previous studies have successfully used wire mesh screen to exclude beetles, and this was one of the earliest methods used attempting to prevent bark beetle attack. As early as 1926, wire mesh screen was being successfully deployed to protect lodgepole pines from mountain pine beetle in Crater Lake National Park. Covering the lower 25" (7.6m) was sufficient to protect lodgepole pines (Miller and Keen 1960). Moeck et al. (1981) found that wire mesh screen wrapped around the boles of ponderosa pine effectively excluded *Dendroctonus brevicomis* (LeConte) and *D. ponderosae* (Hopkins), but not the smaller *Gnathotrichus retusus* (LeConte) or *Ips latidens* (LeConte). Covering trees with screen is rather labor intensive; therefore, even if effective it may be impractical to protect forest trees. However, it might give an indication of how high a physical or insecticidal barrier must go to prevent attack and might be useful for protecting high value trees near homes or in avocado (*Persea americana* Mill) orchards, which are also susceptible to laurel wilt and attractive to RAB (Fraedrich 2008, Hanula et al. 2008, Mayfield et al. 2008). Effective physical protection using screen also has the advantage of potentially lasting over a period of several years compared to pesticides which must be reapplied with much greater frequency.

To test whether protecting the lower bole of redbay trees might be able to prevent the trees from being infected with laurel wilt, sections of mature redbay were wrapped in fine mesh screen to physically block beetles from boring into the trees. We examined barrier height and where initial attacks occurred when no barrier was present. In addition, we examined the moisture content of the wood along the boles of trees to see if it was correlated with the initial area of attack.

Materials and Methods

This study took place in Emanuel County, Georgia about 26km due south of Swainsboro, GA (32° 39′ 07″ N, 82° 28′ 04″ W). The site was privately owned by Larry Jordan with Georgia GJ Properties Inc., and was originally intended to be developed into a residential neighborhood but has remained undeveloped. A very sharp ecotone defined by about 1m elevation change separated an upland sand hill community from a bay forest dominated by loblolly bay (*Gordonia lasianthus* (L.) Ellis) and redbay with an understory of *Itea, Smilax,* and *Lyonia* species. This bay forest was the site of the experiment.

A total of 45 mature redbay, *P. borbonia,* trees were selected in February 2011. Trees ranged in size from 8.6cm–15.7cm dbh, and all appeared healthy at the beginning of the experiment. Trees were randomly assigned to one of four treatments that consisted of: 1) unwrapped control trees, 2) screen from ground level to approximately 1 m, 3) screen from 1-3 m, or 4)screen from ground level to 3 m. Each treatment was replicated 10 times but an additional 5 control trees were selected for studies to determine the initial point of attack. Boles of treated trees were wrapped with fine mesh (.68mm aperture) fiberglass screen that covered the appropriate parts of the boles. Screen was wrapped around the tree bole, and the seams were folded over and then tightly stapled to prevent beetles from entering through them. These vertical seams were further sealed with 100% silicone caulk, and wire was twisted tightly around the tops and bottoms of the screened sections. Upper and lower edges of the screen were further sealed to the bole with Great Stuff ™ foam insulation. For those trees covered to ground level, a $1m^2$ section of fine stainless steel screen (.35mm aperture) was cut so that it could be wrapped around the base of the tree but lie flat on the ground (fig.1). This was done to protect the roots and intercept any beetles that fell to the ground after impact with the tree. The fiberglass screen was

stapled to the stainless steel screen and the seam was sealed further with silicone caulk and the seam of the steel screen where it was cut to fit around the tree was stapled to a piece of wood and sealed with foam insulation. Care was taken throughout the process to avoid wounding the tree boles or exposed root flares.

Trees were monitored for signs of laurel wilt on a monthly basis for the first five months, then on a weekly basis until the end of the experiment. Trees were monitored for discoloration, wilting in the crown, or apparent RAB entry points and frass. Trees with no obvious wilt were marked as healthy, those with some discoloration but not enough to confidently declare infection were noted, and those with obvious crown wilt were marked as dead. Dead trees were cut down and all bark was scraped from the trunk up to a height of about 9 m so that beetle attacks could be found easily. Since we were interested in determining the initial point of attack, trees were cut as soon as possible after infection was noticed, usually the same or following week. Attacks of *X. glabratus* were identified by gauging the diameter of gallery entrances using a standard map pin, approximately 1mm in diameter. Attacks were counted, and heights of all attacks were measured from ground level. Cross sections of the tree were then cut at approximately 60cm intervals throughout the length of the trunk until major branching occurred, usually around 8-10m. These sections were labeled and stored in plastic garbage bags during transport to the laboratory where diameter and wet weight of each section were determined. The sections were then placed into a drying oven for at least four days at 40^{o}C. After drying, sections were again weighed and percent moisture content was calculated by: (wet weight-dry weight/wet weight) x 100. Trees were wrapped on 17 February 2011, and the last one was felled on 18 October 2011.

Statistical Analysis. Differences in time until tree death by treatment for all trees and for the subset of trees on which screens were effective were analyzed using the general linear models

procedure (PROC GLM, SAS institute 1985). A pooled t-test was used to compare whether screen failure affected time until tree death. Among unwrapped control trees relationships between moisture content, height, stem diameter, and number of RAB attacks were analyzed using PROC GLM (SAS institute 1985). For these analyses each tree was broken into 60cm sections and values were expressed as attacks per section. Relationships between number of attacks by stem diameter and height were determined using the dynamic fit wizard of SigmaPlot 10.0 (2006).

Results

All trees in the experiment died within 243 days after the beginning of the experiment with an average of 165.87 ± 6.94 days until tree death. There was no significant difference ($p=.0768$) in the number of days until tree death by treatment (fig. 2). Attacks were not counted and measured for one tree (treatment no. 3; wrapped from 1m-3m) because there was a very active yellow jacket's nest in the ground near the tree. Data for attacks on one other tree (treatment no. 4; wrapped from ground level-3m) were missing. Time of death for both of these trees was known. Screens were not totally effective, and at least 15 of the 30 trees wrapped with screen had at least one attack underneath the screen (table 1), but whether or not the screen was fully effective did not impact the number of days until tree death ($p=.2689$, PROC GLM, SAS). Also, among the trees where the screen did fully exclude beetles, there was no difference ($p=.3892$, PROC GLM, SAS) in days until tree death between treatments. Greatest tree mortality was seen in early August 2011 when nine trees, at least one from each treatment, died within one week.

Analysis relating to moisture content and attack height was done only on the 15 control trees because attack height was affected by screened sections on treated trees. Moisture content steadily decreased as height increased, and percent moisture content was strongly correlated with height (Fig. 3). However, there was no correlation ($p=.4396$) between the moisture content of a tree at a particular height and the number of RAB entry points at that height. The number of attacks was correlated with both stem diameter (Fig. 4a) and height (Fig. 4b). The lowest attack point on the control trees ranged from 0 to 6.62m with an average of 2.38 ± .74m. Over half (435/739) of the recorded attacks on control trees were above 2m, and the distribution of attacks on control trees peaks at about 1m (fig. 4b). The highest attack point recorded in the experiment was at 10.6m, but the branches of trees were not closely examined; so, some attacks could have occurred at greater heights than this especially further into infestation once the lower portions of a host tree had been heavily colonized.

Discussion

Physical protection of trees using these methods is not a viable control option for *X. glabratus*. This is mainly because the beetles can attack at any height on a tree. Also, the beetles are able to pass through fiberglass screen material, despite the mesh size being smaller than the diameter of an adult RAB. Beetles may have either pushed apart the screen fibers or chewed directly through the screen. This was not anticipated, but concurrent *in vitro* rearing experiments demonstrated that RAB are able to bore through the walls of plastic petri dishes (unpublished data). It is therefore not surprising that they may also bore through fiberglass screen. However, the finer mesh stainless steel screen used to protect the roots of trees was too heavy and difficult to work with to allow use on the bole without risking damage to the bark and thus attracting beetles to the wound sites. Because of the very small size and distribution of at least a few flying

beetles to heights of up to14 m (Hanula et al. 2011), any solution which protects only part of a host tree will likely prove ineffective for control of this pest. A larger number of RAB attacks were seen above 2m, and this contrasts with earlier studies which had shown that 85% of RAB trap catch occurred below 2m (Hanula et al., 2011). RAB attacked at the highest rate near ground level, but attack rates remained fairly high up to about 8m (fig. 4b).

Obvious wilting symptoms were seen on trees with as little as two attacks found. This strongly supports the idea that very few beetles or even a single beetle can infect and kill trees susceptible to laurel wilt. This makes potential control even more difficult because, at least on redbay, control must be 100% effective in order to protect a tree from laurel wilt.

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Table 3.1. Failure rate of screens by treatment. Screens were considered to have failed if one or more RAB gallery entrance holes were found beneath the wrapped area.

Treatment	# Screens successful	# Screens failed
Ground-1m	3	7
1-3m*	5	4
Ground-3m*	5	4
Total	13	15

*Attack counts and heights for one tree of each of these treatments were unknown.

Fig. 3.1. Lower bole of redbay tree wrapped in fine mesh fiberglass screen with flare of stainless steel screen at base. Top is wrapped with wire and sealed with Great Stuff^{TM} foam insulation.

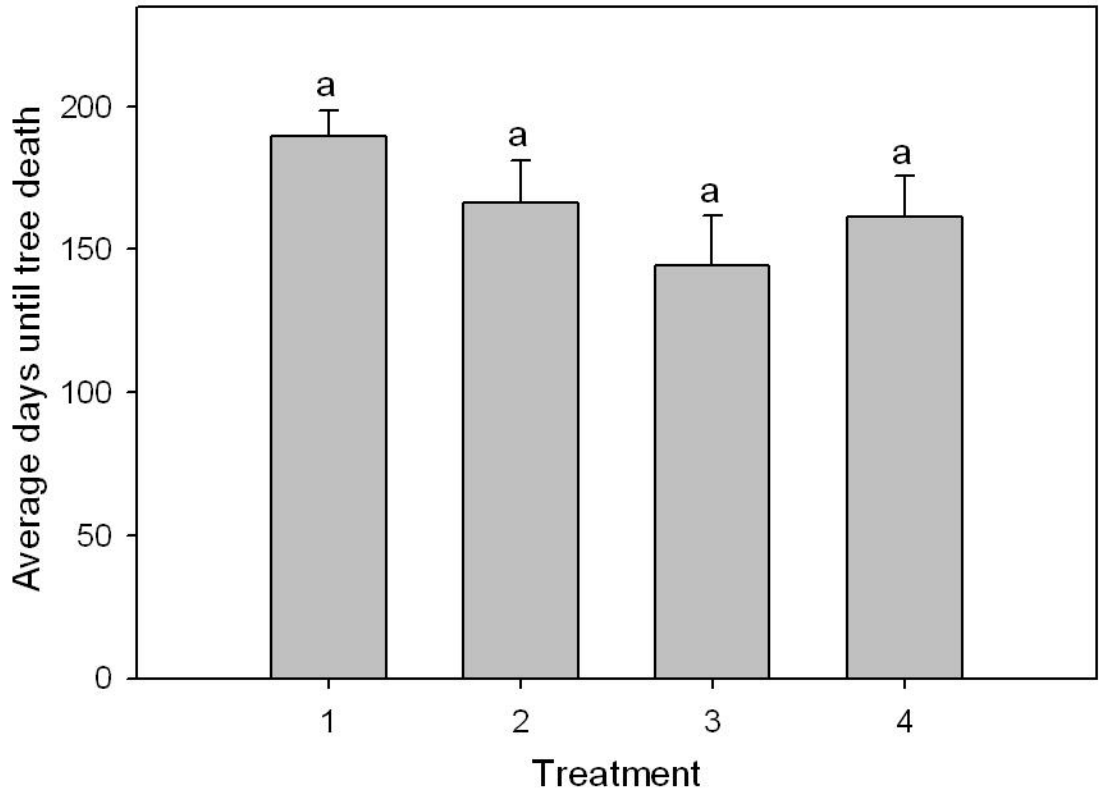

Fig. 3.2. Length of time redbay trees survived following treatment with screen to prevent redbay ambrosia beetle from attacking different areas of tree boles. Treatments were: 1) unwrapped control trees, 2) screen from ground level to approximately 1m, 3) screen from 1-3m, and 4)screen from ground level to 3m. Columns with same letter are not significantly different (a =0.05, GLM).

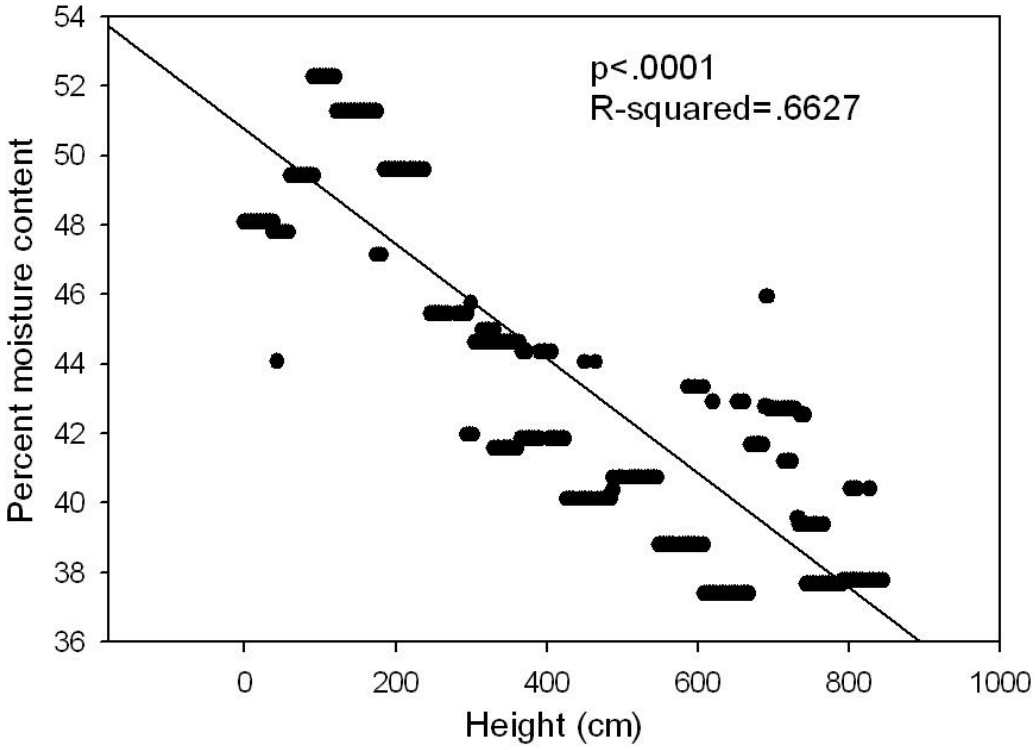

Fig. 3.3. Relationship between height and moisture content on unwrapped control trees that were in the early stages of laurel wilt disease. Each point represents a single RAB attack point (N=726).

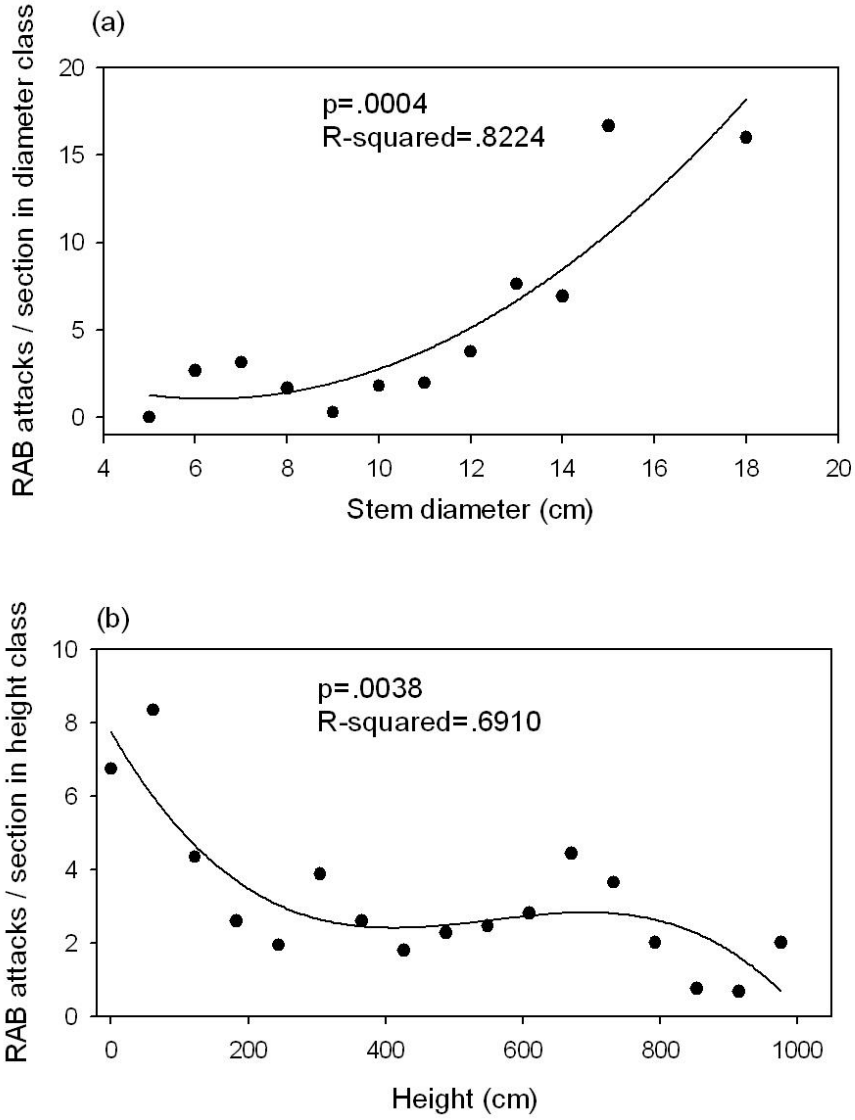

Fig. 3.4. (a) Relationship between rates of RAB attack and stem diameter. Values represent total number of attacks within a 1cm stem diameter range divided by the number of sections that fell within that size range ($y=6.00-1.57x+0.13x^2$). **(b)** Relationship between rates of RAB attack and height. Values represent total number of attacks within a 60cm height range divided by the number of sections that fell within that height range ($y=117.34-0.51x-6.66E-007x^3$).

CHAPTER 4

REARING OF THE REDBAY AMBROSIA BEETLE, *XYLEBORUS GLABRATUS*, ON SEMI-ARTIFICIAL MEDIA

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ABSTRACT Various methods of rearing *Xlyborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae) *in vitro* using semi-artificial media were tested. Comparison of two types, modified (MM) and standard (SM) media, adapted from Bidermann et al. (2009) showed that the more solid consistency of the MM resulted in greater rates of successful brood production in cultures. A two-layered media structure with a nutrient rich lower layer and a nutrient poor upper layer proved to be superior to a single-layered structure. Using a two-layered structure, success rates were achieved that were similar to or greater than those seen for *X. glabratus* in field conditions. Most media recipes used wood from redbay, but some success was seen using wood from loblolly pine (*Pinus taeda*), pondberry (*Lindera melissifolia*), and California bay laurel (*Umbellularia californica*). A two-layered structure with nutrient levels slightly higher than those in the MM presented by Biedermann et al. (2009) is recommended for the *in vitro* rearing of *X. glabratus*.

KEY WORDS laurel wilt, *Raffaelea lauricola*, redbay, *Persea borbonia*

Like other ambrosia beetles in the subtribe Xyleborina, *Xyleborus glabratus* Eichoff (Coleoptera: Curculionidae: Scolytinae) , lives within the sapwood of stressed or dying trees and feeds only on mutualistic fungi. (Kirkendall et al. 1997). In the case of *X. glabratus* the primary mutualistic fungus is *Raffaelea lauricola*, the laurel wilt pathogen (Fraedrich et al., 2008). This lifestyle makes observation of the beetle in its natural habitat very difficult and also poses an interesting challenge for culturing ambrosia beetles because culture conditions must be suitable for both the beetles and their symbiotic fungi. The first ambrosia beetle to be successfully reared in vitro was *Xyleborus ferrugineus* Fabricus (Knoke and Saunders 1967) which was reared using sawdust and

agar based media in glass culture tubes. Since that time, a variety of ambrosia beetle species have been reared with some success including: *Xyleborus pfeili* Ratzberg (Mizuno and Kajimura 1997, 2009), *Xyleborus dispar* Fabricus (French and Roeper 1972), *Xyleborus affinis* Eichhoff, *Xyleborinus saxesenii* Ratzeburg, and *Xylesandrus germanus* Blandford (Biedermann et al. 2009). While working with *X. saxesenii*, Biedermann et al. (2009) found this particular species exceptionally difficult to rear in the laboratory using previously established media recipes, having a success rate of only 7.2%. By modifying the media with a much greater concentration of wood dust, they were able to bring this success rate up to 23.9% which is similar to the rate of successful gallery formation for *X. saxesenii* in field conditions. One of the major issues limiting success in laboratory cultures of Xyleborina beetles is contamination, usually by undesirable fungi or bacteria (Biedermann 2009). Fungi become established at the mouth of a culture tube and can rapidly spread down through the media, overwhelming symbiotic ambrosial species. In response to this Mizuno and Kajimura (1997, 2009) tested a multi-layered media structure to slow or prevent the spread of undesirable microorganisms. They initially used a three-layered structure which contained progressively more nutritional material, namely yeast and starch, as depth into a culture tube increased (Mizuno and Kajimura 1997) and later they found that a two-layered structure was just as effective as three layers for inhibiting the spread of undesirable microorganisms (Mizuno and Kajimura 2009). By using a two- layer instead of a single-layer media structure, they were able to increase the success rate of rearing *X. pfeili* from 60% to 90%.

Laboratory cultures of RAB are useful because little is understood about the biology of this invasive pest, and much more needs to be known in order to possibly slow or contain its spread. Examples of concepts that could be researched utilizing an artificial rearing technique include life cycle details, host compatibility, climatic interactions, and mutualistic fungi

relationships. Lab cultures also have advantage over field-based methods of study in that they are able to provide homogenous and consistent conditions. Here we describe a series of trials to develop an effective rearing technique for RAB.

Materials and Methods

Trial 1. Two types of artificial media adapted from Biedermann et. al. (2009) were tested. The standard medium (SM) contained 0.35g streptomycin, 1g Wesson's salt mixture, 5g brewer's yeast, 5g casein, 5g corn starch, 10g sucrose, 20g agar, 2.5mL wheat germ oil, 5mL 95% ethanol, 500mL deionized water, and 75g redbay sawdust. Sawdust was prepared by first processing debarked redbay sections through a wood chipper and then grinding the wood chips in a Wiley lab mill fitted with a 1mm final screen. All ingredients were mixed thoroughly, and the media was autoclaved at 121^{o} C for 30 minutes. Immediately after autoclaving, the SM was transferred to a sterile bench and poured into 18 x 150mm culture tubes which were then fitted with plastic caps.

The modified medium (MM) contained 0.35g streptomycin, 1.25g Wesson's salt mixture, 10g casein, 5g corn starch, 5g sucrose, 30g agar, 2.5mL wheat germ oil, 2.5mL peanut oil, 5mL deionized water, and 200g of redbay sawdust. The more solid consistency of the MM required that it be packed into culture tubes before autoclaving. In order to prevent MM from expanding out of the tubes while autoclaving, cotton plugs were placed into the mouth of the filled tubes and heavy glass plates were set on top of the upright tubes so the plate held the plugs in place. After autoclaving the tubes were transferred to a sterile bench where the cotton plugs were removed, and the tubes were immediately covered with plastic caps. This first trial consisted of 33 tubes of MM and 35 tubes of SM.

All beetles used for this trial were reared from naturally infested logs in the laboratory. Rearing bins were made from black plastic file boxes (38cm x 30cm x 25cm). A 6cm diameter hole was cut into the bottom of each bin and into the lids of clear plastic collection jars. The lids were attached to the bottom of the boxes with small screws so that the holes in the lids and bins were aligned. Moist cotton was placed into each jar, and the jars were fitted onto the lids to collect beetles as they emerged. A single LED bulb was fixed below each plastic jar, and the bins were otherwise kept in dark conditions. Boxes were filled with several short sections of heavily infested redbay wood, and beetles were collected from the plastic jars 3-5 times per week. Emergence of *X. glabratus* from logs stored in this fashion can continue for up to approximately four months. After collection, beetles were stored in a refrigerator at 4^{o}C for no more than two weeks until introduction into culture tubes.

Both SM and MM were allowed to dry for at least four days before introducing beetles. Immediately prior to introduction, beetles were surface sterilized with 95% ethanol for five to ten seconds and then rinsed with sterile deionized water. Rapid transfer of beetles from refrigeration to ethanol seemed to result in reduced mortality compared with beetles ethanol rinsed at room temperature. A small hole was scratched into the media surface in each tube to facilitate boring, and a single adult *X. glabratus* female was introduced head first into this hole. Only actively moving beetles were selected for use in the experiment. Tubes were wrapped in paper to exclude light and stored in a dark incubator at 24^{o}C. Introductions of beetles into tubes took place between 28 November, 2010 and 3 February, 2011. This extended time period was due to initially low live beetle availability. Full examination of tubes was performed by cutting off the closed end of culture tubes using a Dremel rotary tool fitted with an abrasive cutting disc. Media was then pushed out of the tube using a wooden dowel rod. The media, which maintained the

form of the culture tubes (Fig. 1), was dissected, and the number of live adult females, dead adult females, males, pupae, larvae, and eggs was recorded. Subsets of tubes were opened in this manner at two, three, and four month intervals. Tube dissections were performed between 2 February, 2011 and 13 May, 2011. Maximum tunnel or gallery length was also measured. This measure did not account for multiple branches of the tunnels and therefore gives only a rough estimate of gallery size. A successful gallery was defined as one in which more than one mature adult was recovered and, gallery productivity was defined as the total number of adults, pupae, and larvae produced per culture tube. The number of eggs is not included in this measurement because it is possible that some of these were missed during gallery dissection due to the small size and lack of movement of the eggs..

Trial 2. The second trial compared beetles reared from naturally infested logs in the laboratory as described above to those reared on artificial media and recovered from culture tubes. All tubes used in this trial contained MM. Beetles reared from logs were inoculated into 39 tubes and beetles reared from semi-artificial media were inoculated into 38 tubes. Beetles were introduced into tubes on 22 April, 2011 and 3 May, 2011. Subsets of tubes were opened as described above every two weeks beginning at one month post introduction. Otherwise all methods were the same as trial 1.

Trial 3. Successful establishment of the symbiotic fungi is a critical component of *in vitro* ambrosia beetle cultures. Therefore, pre-inoculation of media with *R. lauricola* could potentially result in higher gallery success rates. A total of 37 tubes of MM were prepared as described above. Of these, twelve were inoculated with 0.5mL of *R. lauricola* spore mixture at a concentration of $1.1x10^6$ spores/mL. The spore mixture was prepared from *R. lauricola* cultures grown in 100 x 15mm plastic petri dishes containing malt extract agar (MEA) media for six

weeks. Sterile deionized water was poured into the *R. lauricola* culture plate and agitated using a scoopula, and the resulting mixture was filtered through cheese cloth to remove any pieces of media. Concentration of spores in the mixture was then determined using a hemocytometer. Four days after this inoculation, single adult RAB females were introduced into each tube in the manner described above. Beetles selected were reared either from infested logs or media cultures. Media was prepared on 3 June, 2011; *R. lauricola* inoculations were performed on 9 June, 2011; and beetles were introduced on 16 June, 2011.

Trial 4. A total of 37 tubes of MM were prepared as described above except that dust of loblolly pine, *Pinus taeda* L., was substituted for redbay wood dust. Of the 37tubes, 16 were inoculated with 0.5mL of *R. lauricola* spore mixture at a concentration of $2.9x10^6$ spores/mL. An additional 32 tubes of MM were prepared substituting sweetgum, *Liquidambar styraciflua* L., dust for redbay dust. Also, 32 tubes of MM with redbay dust and 24 tubes of media containing only 30g agar, 200g redbay wood dust, and 580mL deionized water were prepared. The later were prepared to see if the other nutrients in the media were necessary since the fungus can grow on redbay wood alone. Tubes were prepared between 23 June, 2011 and 30 June, 2011.

Trial 5. Twenty tubes of redbay based MM were prepared, and 0.5mL of manuka oil was added into the mouth of each tube after autoclaving. Manuka oil attracts *X. glabratus* (Hanula and Sullivan 2008), so it was added to see if increased attractants might facilitate beetle boring into the tubes and subsequent success. Tubes were prepared on 22 July, 2011 then allowed to dry for four days before introducing single adult RAB females on 26 July, 2011.

Trial 6. Five media recipes varying in structure and quantity of ingredients were prepared. Media Type A was the same as the MM type used in previous trials. Types B, D, and E were

made with greater quantities of starch and yeast as indicated in (Table 3). Types C, D, and E incorporated a two-layered structure. In these the lower 12cm of each tube was filled with media as indicated in table 1, and a 1.5cm layer of depleted media was added above this. The depleted layer consisted only of 75g redbay dust, 220mL di H_2O, 3g sucrose, and 11g agar. In addition to cotton plugs in the mouth of each tube, the plastic lids were also placed onto the tubes prior to autoclaving, and heavy glass plates were set on top of the rack of tubes. Greater concentrations of starch and yeast cause greater expansion during autoclaving; so, the lids and extra weight helped to prevent the media from expanding out of the tubes. After autoclaving, cotton plugs were immediately removed. Several days later individual RAB females were introduced into each tube. In 20 of the type C tubes, a single drop of manuka oil was added between the two layers prior to autoclaving. The trial began in September 2011, and tubes were dissected 40 days after introduction of beetles. The incubators used in previous trials were contaminated with mites so all tubes in this trial were stored in a dark cabinet at room temperature.

Trial 7. Another trial was performed to test the effect of two-layered media structure and increased quantities of starch and yeast on beetle production. Two other wood types in the family Lauraceae, California bay laurel (*Umbellularia californica* (Hook. & Arn.) Nutt.) and pondberry (*Lindera melissifolia* (Walter) Blume), were also tested as substitutes for redbay. Type A is the same as type MM used in initial trials, and other types are based on this recipe. All tubes were prepared with sawdust from freshly harvested wood except for type E-dry which used redbay dust prepared several months prior. Media was prepared using the same methods as in the previous trial. In 16 of the 32 type A tubes, three adult female RAB were simultaneously introduced into each tube. In all other tubes a single RAB female was introduced as before. Pondberry based tubes were made 21 October 2011, and all other types were made on either 11

or 15 December, 2012. Moisture content of fresh redbay, dry redbay, and spicebush samples were measured by cutting a small sample of each type of wood from bolts before they were processed into sawdust. Samples were weighed then dried in an oven at 40^{o}C for 48 hours then weighed again. Moisture content of the samples was: 39 % for fresh redbay, 9.1% for dry redbay, and 41.5% for spicebush. All tubes in this trial were stored in a dark cabinet at room temperature.

Trial 8. Another trial was performed to test the effect of two-layered media structure and increased quantities of starch and yeast on gallery success and beetle production. Media was prepared using the same methods as in the previous trial. Type A was identical to type MM used in initial trials, and other types differed only as indicated in table 3. After beetles were introduced into tubes, all 240 tubes were stored at room temperature in a 48 quart Igloo brand ice chest sterilized with bleach (5.95% NaClO) prior to beginning the experiment to reduce contamination from mites and other fungi. Media was made on 23 May 2012, 24 May 2012, 30 May 2012, and 6 June 2012. Beetles were introduced into tubes on 30 May 2012, 5 June 2012, and 11 June 2012. Half of the tubes of each type were dissected 41-44 days after beetle introduction, another ten of each type were dissected from 68-69 days after beetle introduction, and the remaining tubes were dissected from 77-91 days after introduction. Upon dissection cultures were recorded as contaminated if bacteria or undesirable fungi were present in the galleries in amounts that seemed sufficient to negatively alter the quality of the culture.

Some disadvantages of rearing ambrosia beetles in test tubes and destructively sampling at designated times included the difficulty of extracting beetles from the media and the loss of information about the productivity of the tubes over longer time periods. Near the end of the trial four tubes were set up to collect beetles as they emerged from the media without destructively sampling the tubes. All selected tubes contained multiple observable adult RAB and little or no

observable fungal contamination. For each tube, the top was cut from the cap so that an open tube 18mm inner diameter was created. An emergence trap similar to those used to monitor emergence of *X. glabratus* from trees (Maner et al. in review) was inserted into the cut end of the cap (Fig. 2). At a sterile bench, all frass was scraped from the mouth of each of the four culture tubes and examined for any beetles that might be in the frass. The cap and emergence trap were then replaced onto the tubes, and sealed with packing tape. Contents of the emergence vials were then monitored for one week.

Statistical Analysis. For the first two trials Fischer's exact test was used to evaluate differences in success rates between media type and foundress collection method, respectively. T-tests were used to examine differences in productivity (total adults + pupae + larvae / tube) and tunnel length. Trials 3, 4, and 5 failed; so, no analysis was done. For trials 6, 7, and 8 differences in success rates were analyzed with a Chi-squared test. For analyses of trial 7 in which more than one female was added to some tubes the total number of adults was divided by three in the 16 tubes which received 3 beetles. Differences in productivity between media types were analyzed with the general linear model (PROC GLM) and means were separated with the Ryan-Einot-Gabriel-Welsch (REGWQ) multiple comparison test. For trial 8 a separate analysis of productivity using the GLM procedure and the REGWQ means separation was performed for the subset of tubes which successfully produced brood.

Results

Trial 1. Only 3 of 34 introduced females produced adult brood in the SM media, equating to a success rate of 8.8% which was significantly lower ($p=0.0121$) than the modified media in which , 33.3% of females successfully produced offspring. Average tunnel length was also

lower in the SM ($P < 0.0001$) with a mean of 18.5mm compared with 60.9mm in the MM. (Table 1). Due to the low success rate of SM no further tests of this media type were conducted. The maximum productivity from a single female on the MM was 34 mature females, 3 pupae, 9 larvae, and 5 eggs at the time of dissection while the maximum from a female on the SM was 10 mature females, 1 larva and 7 eggs.

Trial 2. There was no difference in the success rate ($p=0.4508$), productivity ($p=0.3325$,), or tunnel length ($p=0.4436$, Satterthwaite T-test) of MM tubes initially started with beetles collected from naturally infested logs and those started with beetles collected from *in vitro* culture tubes. (Table 2)

Trial 3. All tubes in this trial were discarded without dissection on 26 August, 2011. Most had mites and extensive fungal contamination. No successful galleries were observed. The mites were identified by Dr. John Moser as *Histiogaster rotundus* Woodring, a general feeder whose natural diet consists primarily of fungi, yeasts, and bacteria growing in beetle galleries (Woodring, 1969).

Trial 4. Almost all of the tubes in this trial were contaminated by mites and various fungi. At approximately three weeks, several of the pine based tubes contained observable eggs and larvae, but before full dissections were performed all galleries were overwhelmed with mites and fungi so they were discarded. After this trial use of the incubator was discontinued due to contamination by mites which were able to access tubes by crawling under the plastic caps. Also, some of the beetles reared from naturally infested wood may have had mites attached before introduction into a tube. Once a gallery was contaminated with mites undesirable fungi rapidly spreadand hundreds of miteswere seen in some tubes.

Trial 5. All beetles introduced to tubes containing manuka oil died within minutes of introduction. In previous trials beetles failed to bore into the cultures about 25% of the time so manuka oil was added to encourage beetles to tunnel into the media, but it was apparently toxic to the beetles at the levels tested.

Trial 6. Very few tubes, only 7 of 183, successfully produced beetles, and there were no significant differences in success of media types tested (p=0.1387).

Trial 7. Media C had the highest female productivity, but it was not significantly higher than types A or E (Fig. 3). In media C every foundress that bored more than 10mm into the tube produced brood while females on type E had the highest success rate of 51.6%. Some successful galleries were seen in media made with both of the alternate wood types, pondberry and California bay laurel.

Trial 8. Overall, 111 of the 236 tubes in this trial successfully produced adult beetles, and there were significant (p=.0002) differences in success rates between treatments. The two recipes with the highest rate of successful females were B and D with success rates of 72.5% and 60.5%, respectively. Differences in total productivity (total adults + pupae + larvae) were not significant (p=.0604),. However, when two-layered media types were compared to single-layered media, females in two-layered media produced more adult brood than single-layered treatments (p=.0454, Proc Ttest, SAS 1985). When only tubes in which brood had been successfully produced were considered, type F had the greatest productivity but not significantly higher than that on type E (Fig.5). Successful females on media type E produced more brood than those on type A but not the other media types. Although successful females on media types E and F were generally more productive, these media types had relatively low success rates (fewer foundresses

produced brood) (figs.4 and 5). Also, based on visual observations the two-layered media were less likely to have visible or high levels of contamination at the time of dissection than single-layered ($p<0.0001$, fig.6). Of the 60 most productive tubes, 42 were two-layered cultures, and these were evenly distributed between the three two-layer types (B, D, and F) and the two-layered recipes always had higher success rates than their single-layered counterparts. The greatest number of adults recovered from any tube was 63 (all females) from a tube containing media type D. The single most productive foundress overall was on media type F and she produced 51 adult females, one adult male, 26 pupae, 63 larvae, and 28 eggs (169 total progeny) at the time of dissection. All male broods occurred in four tubes, and 4-5 adult male beetles were found in each of these. All female broods were common, and 48 of the 111 successful galleries had no males. A total of 1155 adult females and 129 adult males were recovered from the culture tubes in trial 8. In addition to the beetles recovered from individual culture tubes, 241 females and 48 males had escaped from the tubes and were found lose within the ice chest. The total number of adults therefore was 1396 females and 177 males. This indicates a sex ratio of about 8:1 (F:M). Adult beetles were collected in each of the four tubes to which emergence traps were attached, and in one week a total of 10 females and 2 males were recovered.

Discussion

Female tunneling activity often occurred along the walls of the culture tubes making observation of the colony possible without disturbing the culture media (fig.1). Eggs are usually laid individually or in small clusters near the end of a short tunnel branching from one of the primary tunnels, or simply within a primary tunnel. Clusters of up to 10 eggs were occasionally seen. Galleries excavated larger than the tunnel diameter were rare. Galleries consisting of

simple tunnels all the same diameter are common to *Xyleborus sp*. but contrasts with the disc-shaped brood chambers often created by other Xyleborini genera (Biedermann et. al. 2009).

Mature adults were produced within 30 days following introduction of a female in some tubes, and all adults found at this time excepting the foundress were teneral adults. Live adults along with eggs and larvae were found within tubes opened as late as 106 days after initiation. In some cases the introduced females were found alive and boring throughout the media for as long as 90 days without depositing any eggs and, in these cases, very little fungal growth was seen within the tunnels. This is consistent with the behavior of other ambrosia beetles that will not oviposit until a successful ambrosia garden has been established (Peer and Taborsky 2007). Females of many other ambrosia beetle species are known to guard the entry point of a gallery with their bodies (Biedermann et al, 2009; personal observations), but this behavior was not seen in *X. glabratus* in culture.

Biedermann (2010) found that occurrence of *X. saxesenii* males in galleries was not random but there was an overrepresentation of galleries containing one or two males and fewer with zero males than a Poisson distribution of random occurrences would predict. Castrillo et al. (2012) found both sexes present in over 90% of successful *in vitro* galleries of *X. germanus*. This was not the case with *X. glabratus* and galleries with no males were very common but, because 48 males had dispersed from their tubes, calculations for the occurrence of males could not be performed. The low number of all male broods (4 of 111 successful galleries) indicates either that unfertilized females are rare or that they are less successful at producing brood. This possibility is supported by Biedermann (2010) who found that none of daughters recovered from all female *in vitro* galleries of *X. saxesenii* were able to successfully produce brood. The

proportion of all male galleries found in this study was similar to that seen for *X. saxesenii* (Biedermann, 2010) and *X. germanus* (Peer and Taborsky, 2004).

The initial trial testing SM versus MM showed that the more solid MM gave significantly better results. This could be because the MM more closely resembles actual wood and therefore better simulates the beetle's natural habit. Beetles reared *in vitro* were just as effective at producing brood as those collected from naturally infested wood. The effect of rearing multiple generations *in vitro* was not tested, and this could potentially result in some altered fitness for the beetles. Keeping cultures free of contamination proved to be a critical part of successfully rearing *X. glabratus*. Mites in particular resulted in many culture failures. Since the mites are able to spread from tube to tube, entire experiments were quickly ruined. Another factor that caused some difficulty was keeping the media from overflowing from the tubes during the autoclave cycle. The solid consistency of the MM prevented it from being poured into tubes; so, it had to be packed in by hand prior to autoclaving. The media expanded upon heating, and greater concentrations of starch and yeast seemed to result in greater expansion. However, tubes could be autoclaved while capped without cracking which was an important aid in keeping the expansion of the media under control. The ability of beetles to escape underneath the tube caps was a problem. Caps could be sealed with parafilm, but beetles would likely chew through it. Cork, rubber, or cotton stoppers could be used instead of caps, and this has been done in other studies (Saunders and Knoke 1967, Mizuno and Kajimura 1997, 2009). This behavior of the beetles can be taken advantage of however, as was demonstrated by attaching emergence cages to the tubes and collecting beetles as they emerge from the tubes. Previous researchers (Saunders and Knoke, 1967) collected beetles from culture tubes by inverting the tubes *en masse* over a

collection tray, but this method might lead to contamination and requires the tubes to be aligned in an unnatural vertical position.

Trial 8 resulted in successful brood production rates of 47% among all media types with a maximum of 72.5% for media type B (table 5, Fig. 4). This is similar to or possibly better than the roughly 50% successful gallery formation rate seen for RAB in the field (Maner et al., in review). Biedermann et al. (2009) achieved *in vitro* success rates for *X. saxesenii* of about 20% which is similar to the rate of successful gallery formation for that species in the field. Both Weber and McPherson (1983) and Biedermann et al. (2009) found success rates for *X. germanus* to be about 30%. Castrillo et al. (2012) also reared *X. germanus* on artificial media based on wood from several different host trees and achieved success rates of about 70%. They found gallery productivity but not success rates varied with wood type. *X. dispar* was reared by French and Roeper (1971) who reported 47% of introduced beetles successfully produced progeny on a media that used alpha-cellulose in place of wood dust. They also tested preinoculation of the media with the beetle's mutualistic fungus *Ambrosiella hartigii* Batra, but success rates were lower overall for preinoculated tubes. *X. ferrugines* and *X. affinis* have both been reared with success rates of up to 90% (Saunders and Knoke, 1967; Biedermann et al., 2009). Therefore the results achieved here for *X. glabratus* are well within the range of success rates reported for in-vitro production of ambrosia beetles in other studies, however, there may be ways to improve the success and productivity of *X. glabratus* cultures. Preinoculation of media with *R. lauricola* might improve the success rate by allowing the ambrosial fungi to outcompete other undesirable species. Although an attempt was made to test this, contamination by mites ruined the experiments.

Using a two-layered media structure as introduced by Mizuno and Kajimura (1997, 2009) increased *X. glabratus* success rates and productivity of galleries while decreasing contamination rates. Increasing nutrient levels did increase productivity in trial 8, but two of the media with higher nutrient levels also had the lower rates of success (Fig. 5) probably because the higher nutrient levels make the media more habitable to undesirable microorganisms. Therefore, for future rearing of *X. glabratus* an appropriate media recipe would be similar to either type B or D from trial 8 of these experiments. Type B has the advantage of being less expansive during autoclave cycles; so, this may be the preferred choice. Levels of starch and yeast intermediate of these two recipes could be ideal to maximize productivity. Based on these results a recommended recipe for rearing *X. glabratus* in vitro is given in table 6.

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Table 4.1. Results from trial 1 comparing the standard media (SM) and modified media (MM) of Biedermann et. al. (2009) for rearing *X. glabratus*. Success rate, productivity, and tunnel length were all significantly less ($a<.05$, Fischer's exact test) in the standard media.

Media type	**No. tubes**	**No. successful**	**Total productivity per successful tube mean ± SE**	**Tunnel length (mm) mean ± SE**
MM	33	11	20.82±4.63	60.91±8.60
SM	35	3	9.00±1.53	18.51±4.86

Table 4.2. Results from trial 2 comparing the success rate of *X. glabratus* females reared from naturally infested logs and those reared on artificial media. Collection method had no effect on success rate, productivity, or tunnel length (a<.05, Satterthwaite T-test).

Foundress beetle collection method	**No. tubes**	**No. successful**	**Total productivity per successful tube mean ± SE**	**Tunnel length (mm) mean ± SE**
From infested redbay bolts	39	9	13.33±2.56	43.72±5.40
From *in vitro* culture tubes	38	12	14.58±2.13	50.52±6.99

Table 4.3. Components of various media types tested in trial 6. Type A is identical to the modified media (MM) from Biederman et. al. (2009) used in initial trials and other types vary from MM as indicated. In two-layered types the lower 12cm of each tube was filled with media as indicated and a 1.5cm layer of depleted media that consisted of only of 75g redbay dust, 220mL di H_2O, 3g sucrose, and 11g agar was added above.

Media type	**Single or two-layered**	**Amount of yeast**	**Amount of starch**	N
A	Single	1x	1x	39
B	Single	5x	8x	40
C	Two	1x	1x	41
D	Two	1x	8x	38
E	Two	3x	6x	38

Table 4.4. Components of the different media types tested in trial 7. Type A is identical to the modified media (MM) of Biederman et. al. (2009) used in initial trials and other types vary from MM as indicated. In two-layered types the lower 12cm of each tube was filled with media as indicated and a 1.5cm layer of depleted media that consisted of only of 75g redbay dust, 220mL di H_2O, 3g sucrose, and 11g agar was added above.

Media type	Media base material	Single or two-layered	Amount of yeast	Amount of starch	N
A	Fresh *P. borbonia* dust	Single	1x	1x	32
B	Fresh *P. borbonia* dust	Single	3x	6x	32
C	Fresh *P. borbonia* dust	Two	1x	1x	32
E	Fresh *P. borbonia* dust	Two	3x	6x	32
E-dry	Dry *P. borbonia* dust	Two	3x	6x	17
Calif. Bay	Fresh *U. californica* dust	Two	3x	6x	37
Pondberry	Fresh *L. mellisafolia* dust	Two	3x	6x	32

Table 4.5. Media types in trial 8. Type A is identical to the modified media (MM) of Biederman et. al. (2009) used in initial trials and other types vary from MM as indicated. In two-layered types the lower 12cm of each tube was filled with media as indicated and a 1.5cm layer of depleted media that consisted of only of 75g redbay dust, 220mL di H_2O, 3g sucrose, and 11g agar was added above.

Media type	Single or Two- layered	Amount of Yeast	Amount of Starch	Amount of Sucrose	N
A	Single	1x	1x	1x	39
B	Two	1x	1x	1x	40
C	Single	2x	2x	1x	41
D	Two	2x	2x	1x	38
E	Single	4x	4x	3x	38
F	Two	4x	4x	3x	40

Table 4.6. Recommended media recipe for rearing the redbay ambrosia beetle, *X. glabratus*. The recipe produces enough media to fill approximately 36 culture tubes (18x150mL).

Ingredient	Lower layer (12cm)	Depleted upper layer (1.5cm)
Streptomycin	0.35g	0g
Wesson's salt mixture	1.25g	0g
Yeast	8g	0g
Starch	8g	0g
Casein	10g	0g
Agar	30g	5.5g
Sucrose	5g	1g
Wheat germ oil	2.5mL	0g
Peanut oil	2.5mL	0g
95% ethanol	5mL	0g
Redbay sawdust	200g	38g
Di H_2O	580mL	110mL

Figure 4.1. Example of *in vitro* media with RAB galleries after removal from the glass culture tube. Several adult *X, glabratus* are observable in the galleries.

Figure 4.2. Four active RAB culture tubes set up with emergence traps to collect emerging RAB adults as way of simplifying extraction of adults and to prevent them from escaping by crawling under the culture tube cap.

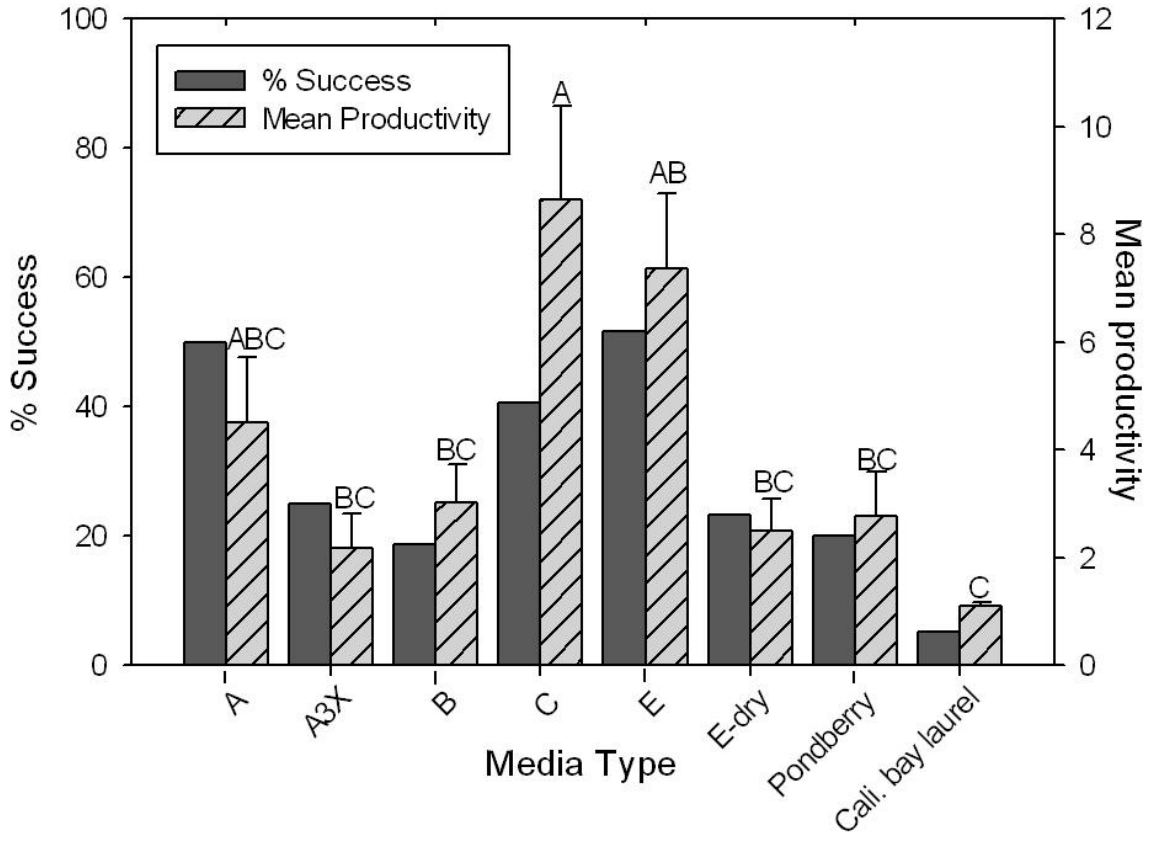

Figure 4.3. Results for trial 7 comparing various media. Media details are provided in Table 4. Solid bars are the percent of tubes that produced brood by media type, and striped bars are mean productivity (adults + pupae + larvae) per tube by media type. Columns with the same letters are not significantly different (**α**=.05, REGWQ multiple comparison).

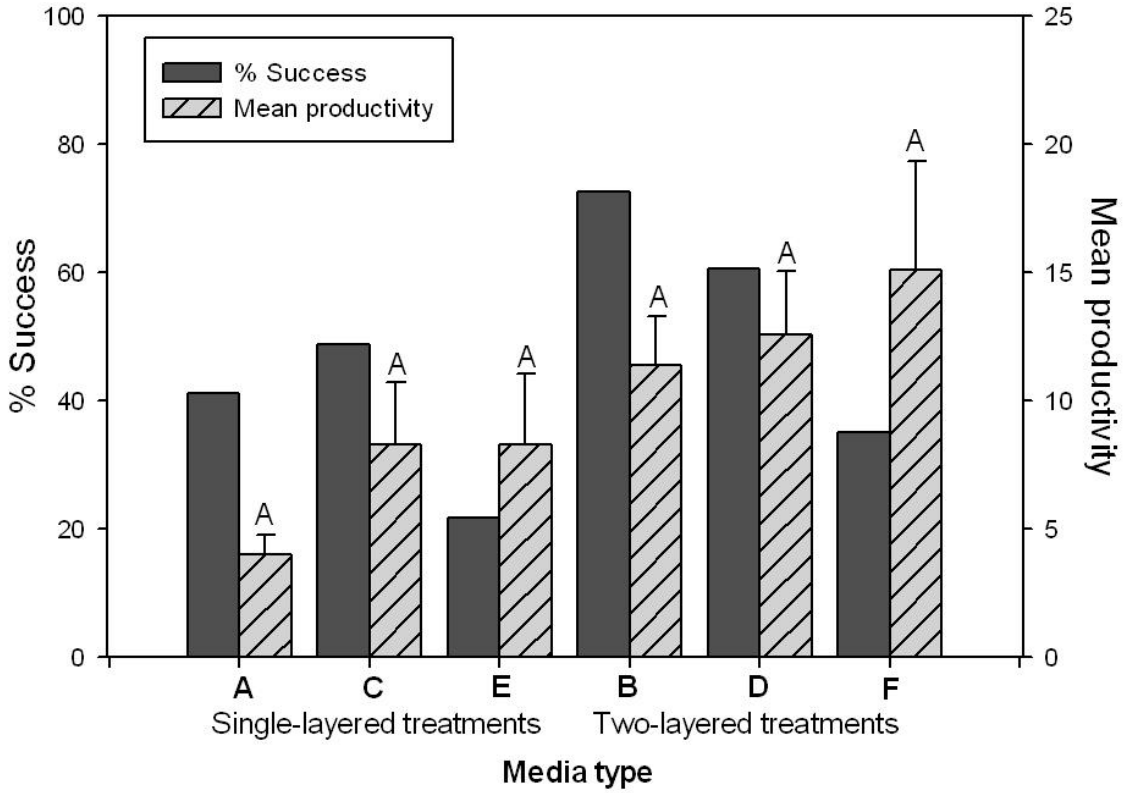

Figure 4.4. Results for trial 8 testing various media types detailed in Table 5. Solid bars are the percent of tubes that produced brood by media type, and striped bars are mean productivity (adults + pupae + larvae) per tube by media type. Columns with the same letters are not significantly different (**α**=.05, REGWQ multiple comparison).

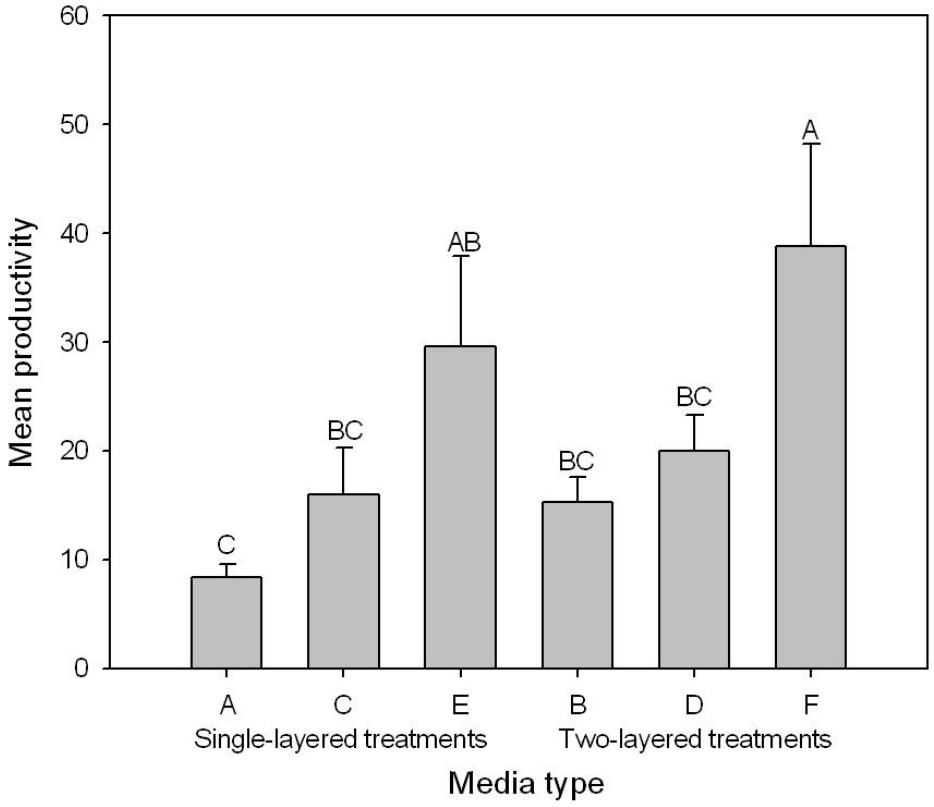

Figure 4.5. Results for trial 8 comparing various media types detailed in Table 5. Productivity was calculated only from galleries that successfully produced brood. Bars with the same letters are not significantly different (**α**=.05, REGWQ multiple comparison).

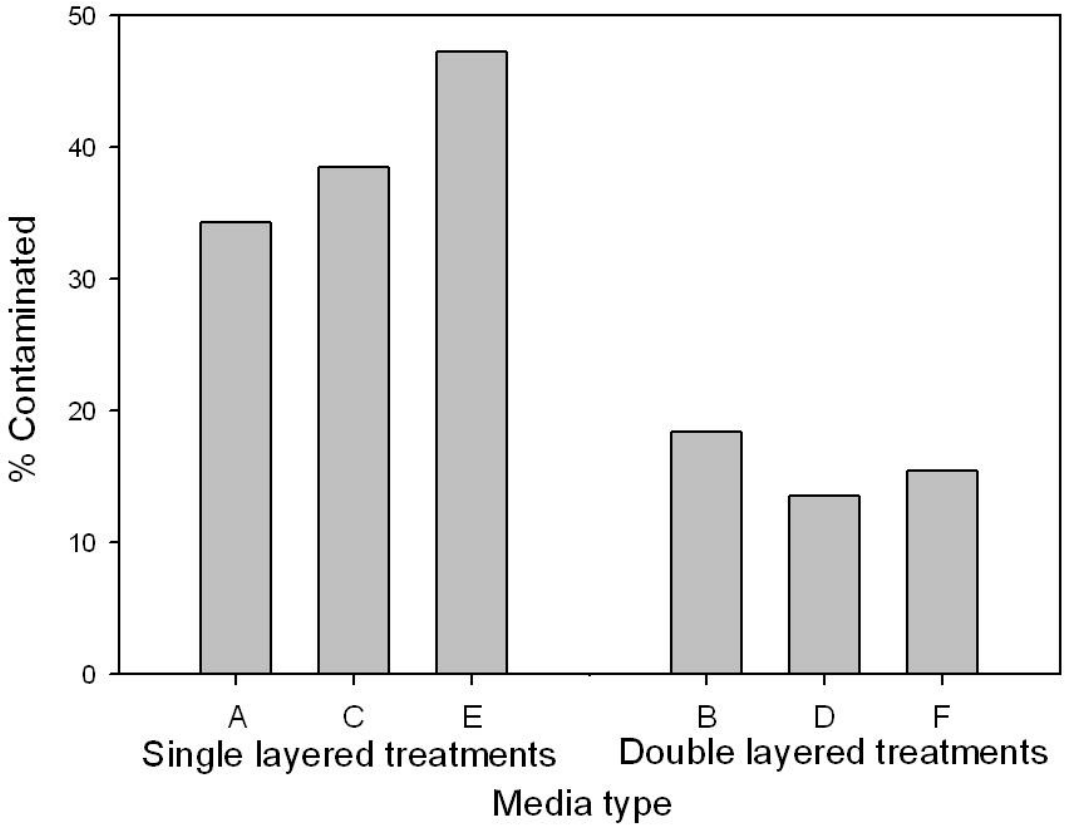

Figure 4.6. Rates of visually observable contamination of culture tubes by media type for trial 8. Media details are provided in Table 5.

CHAPTER 5

GROWTH OF THE LAUREL WILT PATHOGEN *RAFFAELEA LAURICOLA* ON WOOD OF VARIOUS TREE SPECIES

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ABSTRACT The ability of fungal symbionts of ambrosia beetles to utilize wood of various species ultimately determines the success of the beetle to survive and reproduce in that tree species. To test the ability of the laurel wilt pathogen, *Raffaelea lauricola*, to grow on a variety of wood species, 1.5cm^3 blocks cut from freshly felled trees were inoculated with solutions of *R. lauricola* spores and allowed to incubate. At the end of the incubation period spores were collected from the surface of blocks and counted. *R. lauricola* grew significantly better on some species than others. Greatest growth occurred on known redbay and other known host species as well as non-host loblolly pine (*Pinus taeda*) and white mulberry (*Morus alba*). Other species such as green ash and sweetgum were not favorable for growth of R. lauricola. The factors that favor and inhibit fungal growth on host and non-host species are unknown and require further investigation.

KEY WORDS Invasive, exotic, *Xyleborus glabratus*, *Raffaelea lauricola*, ambrosia fungus

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Raffaelea lauricola is a vascular wilt pathogen associated with the recently introduced redbay ambrosia beetle (RAB), *Xyleborus glabratus*, and the laurel wilt disease it causes has resulted in extensive mortality of redbay, *Persea borbonia* throughout the southeastern coastal region of the US (Fraedrich et al. 2008, Hanula et al., 2008, Harrington,et al. 2008). It is known that in their native range RAB utilize trees from a wider host range including species from the families Lauraceae, Dipterocarpaceae, Fagaceae, and Fabaceae, but the beetle is not a known pest in its native range (Fraedrich et al., 2008). Laurel wilt in the US has only been seen to infect members of the family Lauraceae, and it is largely unknown if RAB and *R.lauricola* are able to successfully spread and cause damage to tree species outside of the Lauraceae. One possible

explanation for this restricted distribution is that RAB prefer to bore into trees of this family. If this is the case, host preference by the beetle could be the major limiting factor in determining susceptible species. Another explanation may be that *R. lauricola* will only grow or only be pathogenic in wood of the Lauraceae. If this is true then host exclusivity of the fungus limits the host range of the beetle. If not, the disease could be spread to other tree species if RAB either alters its host preferences, does test probing of trees to determine their suitability, or another organism begins to vector the pathogen. Thus far there is no evidence that this occurs and the laurel wilt fungus has never been recovered from a species outside of the Lauraceae in the US. Other ambrosia beetles, however, that utilize dead redbay trees can be contaminated with the laurel wilt fungus but have not been shown to transmit the fungus to other trees (R. Ploetz, personal communication). While it is widely known that many fungi are host-exclusive (Zhou and Hyde, 2001), much less is understood about the mechanisms regulating which species a fungus may utilize. Roets *et al.* (2011) found that *Ophiostoma* and *Gondwanamyces* species generally grew better on media amended with tissue from a natural host *Protea* species compared with other nonhost species of *Protea*, although they did not offer a mechanism to explain this finding. They also saw that host temperature could partially explain which fungal species may colonize a particular *Protea* species, but this would likely not be as important among trees as it is in *Protea* infructescences (seed organs). When fungi are moved from host to host by an insect, and the insect is dependent on the fungus for food as is the case for ambrosia beetles, then even less is known about factors determining how this complex interaction functions.

Redbay ambrosia beetle shows a strong attraction to members of the Lauraceae (Hanula et al 2008, Mayfield and Hanula 2012) but essential oils extracted from manuka (*Leptospermium scoparium*) in the family Myrtaceae are attractive (Hanula and Sullivan, 2008) as is lychee wood

(*Litchi chinensis*) in the family Sapindaceae (Kendra et al., 2011). This suggests that a variety of plants may contain the attractive compound(s) for RAB but they may not be able to utilize them as hosts because the wood is unsuitable for its ambrosia fungus. Therefore, we tested woods from a variety of species within and outside of the Lauraceae to determine if they could support fungal growth and possibly serve as alternative hosts for this mutualistic relationship.

Materials and Methods

Trial 1. Forty 1.5cm^3 blocks of redbay wood were cut from freshly harvested bolts gathered at a study site in Emanuel County, GA. To simulate a RAB gallery tunnel, holes 1.6mm in diameter were drilled along the grain of the wood into half of the blocks. A spore mixture (concentration undetermined) was prepared from an isolate of *R. lauricola* grown in 100 x 15mm plastic petri dishes containing malt extract agar (MEA) media for six weeks. The culture (HH5) was originally collected in November, 2004 from laurel wilt contaminated redbay on Hilton Head Island, South Carolina by S. Fraedrich. Sterile deionized water was poured into the *R. lauricola* culture dish and agitated using a scoopula, and the resulting mixture was filtered through cheese cloth to remove any pieces of media. All blocks were surface sterilized twice with 95% ethanol and flamed then placed into sterile glass petri dishes. Half of the blocks (10 with holes and 10 without) were inoculated with 1mL of a *Raffaelea lauricola* spore mixture, and half (10 with holes and 10 without) were inoculated with 1mL sterile deionized water. Petri dishes were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI) and placed in a dark incubator at 24^{o}C. Blocks were allowed to incubate for three weeks then examined. Thin sections of some blocks were cut with a straight-edged razor for microscopic examination. The trial began on 29 October, 2010, and final observations were made on 19 November, 2010.

Trial 2. Twelve 1.5cm^3 blocks were cut from each of four different material types, and 1.6mm holes were drilled into all blocks. Blocks were cut from freshly harvested redbay, water oak (*Quercus nigra*), sweetgum (*Liquidambar styraciflua*), and PVC "wood" trim (AZEK Building Products, Inc., Scranton, PA 18505). After surface sterilization as above, four blocks of each type were placed into sterile glass petri dishes with filter paper and inoculated with 1mL of *R. lauricola* spore mixture, four were placed into dishes without filter paper and inoculated with 1mL of *R. lauricola* spore mixture, two were placed into dishes with paper and inoculated with 1mL of sterile deionized water, and two were placed into dishes without paper and inoculated with 1mL of sterile deionized water. All Petri dishes were sealed with Parafilm and placed in a dark incubator at 24^{o}C. Blocks were allowed to incubate for three weeks then examined. At the end of three weeks, a subset of two blocks per type was transferred individually to 125mL Erlenmeyer flasks each containing 75mL of sterile deionized water. The flasks were placed on an orbital flask shaker and shaken at 200RPM for 20 minutes. The contents were then vigorously shaken by hand and poured through cheesecloth into another flask, and the concentration of spores in each sample was counted using a hemocytometer. After two more weeks, another subset was sampled in the same manner except that after being shaken the contents of the flasks were not transferred or filtered through cheesecloth. The trial began on 3 December, 2010 and spore counts were made on 5 January, 2011 and 21 January, 2011.

Trial 3. Ten blocks (1.5cm^3) were cut from freshly harvested bolts for each of 15 tree species, and holes were drilled through all blocks. The blocks were placed into 15 x 60mm plastic petri dishes without filter paper. Species were: redbay, red maple (*Acer rubrum*), white mulberry (*Morus alba*), green ash (*Fraxinus pennsylvanica*), loblolly pine (*Pinus taeda*), Chinese elm (*Ulmis parvifolia*), sycamore (*Platanus occidentalis*), tulip poplar (*Liriodendron tulipifera*),

Carolina cherry (*Prunus caroliniana*), water oak (*Quercus nigra*), sweetgum (*Liquidambar styraciflua*), red cedar (*Juniperus virginiana*), hickory (*Carya sp.*), yellow birch (*Betula allegahniensis*), and *Sassafras albidum*. Redbay samples were collected from the research site in Emanuel Co., GA. All other species were collected near Athens, GA except sassafras and yellow birch which were collected near Helen, GA For each wood type, 10 blocks were inoculated with 1mL of *R. lauricola* spore mixture and 3 were inoculated with 1mL of sterile deionized water. The concentration of the spore mixture used was $2.55x10^6$ spores/mL for all but sassafras and yellow birch which received $2.24x10^6$ spores/mL. All blocks were inoculated on either 15 or 16 February, 2011 except for sassafras and yellow birch which were started on 3 March, 2011. All samples were incubated in the dark for six weeks at 24°C. Each block was then placed in a 125mL Erlenmeyer flask containing 80mL sterile deionized water and shaken on an orbital flask shaker at 210RPM for 20 minutes. Spore concentrations of each sample were then determined using a hemocytometer.

Trial 4. Growth of *R. lauricola* on camphor (*Cinnamomum camphora*) as well as fresh-cut and dry redbay was tested using the same methods as above except that blocks were incubated for only three weeks before counting spore concentrations. The trial began on 26 May, 2011. Fresh redbay was harvested from the Emanuel Co., GA site on 24 May, 2011 while the dry redbay had been harvested from the same site at least two months prior and held a room temperature until blocks were cut for the experiment. Concentration of the spore mixture used to inoculate blocks was $3.36x10^6$ spores/mL.

Trial 5. Growth of *R. lauricola* on sassafras, sweetgum, camphor, and loblolly pine wood was tested using the same methods as previous trials. Ten blocks were inoculated with spores for sassafras and loblolly pine, and five blocks of sweetgum and camphor were inoculated. An

additional two (sweetgum and camphor) or three (sassafras and pine) blocks were inoculated with sterile H_2O as a control. Blocks were inoculated with 1mL spore mixture ($2.91x10^6$ spores/mL) and were again incubated for three weeks after inoculation. Blocks were shaken in Erlenmeyer flasks containing 50mL of sterile deionized H_2O instead of 80mL as before. The final concentration of spores in flasks was determined with a hemocytometer as previously described. The trial began on 17 June, 2011 and spore concentrations were counted on 6-8 July, 2011.

Trial 6. Growth of *R. lauricola* on pondberry (*Lindera melissifolia*), California bay laurel (*Umbellularia californica*), and redbay was tested using the same methods as previous trials. California bay laurel was acquired from California as part of a study on attraction and utilization of bay laurel by the redbay ambrosia beetle (Mayfield personal communication) so it was cut approximately 7 days before the start of the trial. Blocks were inoculated with 1 ml of solution containing 6.51×10^6 spores/mL. The test began on 2 September, 2011. After three weeks incubation blocks were shaken in 80mL sterile H_2O on an orbital shaker at 210RPM for 20 minutes, and spores recovered from the blocks were counted.

Trial 7. We were interested in determining why *R. lauricola* grew on some species of wood and not others. One possibility is that aromatic oils in the wood support fungal growth. To test this theory, blocks of nonhost sweetgum wood were treated with essential oil (91.7 ±2.0% safrole, MysticWays Essential Oils, Dallas TX) extracted from *Sassafras albidum*, a known host of the laurel wilt pathogen. Forty $1.5cm^3$ blocks of sweetgum and 10 blocks of redbay were prepared from freshly harvested wood. Before being inoculated with *R. lauricola* spore mixture, sweetgum blocks underwent one of four treatments. Ten blocks were soaked in pure sassafras essential oil for five hours then inoculated with *R. lauricola* spore solution. Ten were soaked in

pure sassafras oil for five hours then dried in a fume hood for twelve hours before inoculation. Ten were treated with two drops of sassafras oil immediately prior to inoculation, and as a control ten were inoculated with *R. lauricola* spore mixture without being treated with sassafras oil. All blocks were placed in 60x15mm plastic petri dishes and inoculated with 1mL of *R. lauricola* spore mixture at a concentration of $2.23x10^6$ spores/mL. An additional ten blocks of redbay were inoculated with the spore mixture but not treated with sassafras oil. All blocks were placed in a Styrofoam ice chest at room temperature for a period of forty days. After this time blocks were placed in Erlenmeyer flasks with 80mL diH_2O and shaken for 20 minutes at 210 RPM. Each flask was then shaken vigorously by hand before counting the concentration of the mixture with a hemocytometer.

Statistical Analysis. Variation in the number of conidia of *R. lauricola* recovered from the various wood treatment was analyzed using the general linear models procedure (Proc GLM, SAS institute 1985), and the Ryan-Einot-Gabriel-Welsch (REGWQ) multiple comparison test (Day and Quinn 1989) was used to separate the means between wood types. A significance level of 0.05 was used in all comparisons.

Results

Trial 1. Following the three week incubation period, hyphae of *R. lauricola* were seen on the surface of all 20 blocks inoculated with the spore solution. Hyphae were not observed on blocks inoculated with sterile water. Blocks with drill holes had hyphae growing through the hole and over the most of the block's surface (fig1.a &b). Hyphae were also found growing through vessels in the wood druing microscopic examinations of thinly sliced cross-sections (fig1.c&d).

Trial 2. This trial compared the growth of *R. lauricola* on blocks of redbay, sweetgum, water oak, and PVC, and examined whether the presence of filter paper aided fungal growth. Two weeks after inoculation, some hyphal growth was seen on 7 of the 8 blocks of both redbay and white oak inoculated with spores. The growth on white oak, however, was sparse and covered much less area. No hyphal growth was seen on either sweetgum or PVC. Spore counts revealed that spore concentrations on blocks of redbay were more than 15 times that of any other wood type, but because of the small sample size, these results were not significant ($p=.2130$, table 1). No differences in hyphal density or spore counts were seen between samples grown with and without filter paper. Also, samples of PVC on filter paper which were inoculated with spore mixture had spore counts of zero suggesting that the fungus is unable to grow on filter paper alone.

Trial 3. Wood from fifteen different tree species was inoculated with *R. lauricola*, and after two weeks of incubation, lush hyphal growth suspected to be *R. lauricola* was observed on samples of loblolly pine and white mulberry. Very minimal growth was observed on redbay and sassafras samples. By six weeks at least some hyphal growth appearing to be *R. lauricola* was also observed on Chinese elm and sycamore. Spore concentrations recovered from wood blocks following washing differed among wood species, ($p<0.0001$) with loblolly pine and white mulberry having higher spore concentrations than any other species (Fig. 2). Very few spores were recovered from redbay or sassafras in this trial, and these two species were not significantly different from any other species except loblolly pine and white mulberry which had more spores.

Trial 4. Inoculations of *R. lauricola* on camphor as well as fresh-cut and dried redbay revealed that fresh-cut redbay samples grew significantly more spores than either camphor or air-dried redbay samples. The average concentration of recovered spores in mixtures from fresh samples of redbay was approximately 20 times higher than that from air-dry samples ($p=.0145$, Fig.3a).

Trial 5. After three weeks incubation, hyphal growth appearing to be *R. lauricola* was obvious on six of ten sassafras blocks and four of ten loblolly pine blocks, but very little was seen on camphor or sweetgum. After washing and shaking the blocks, significant differences ($p=.0494$, PROC GLM, SAS) in the concentration of resulting spore mixtures were seen between wood types, but separation of means failed to revealed significantly differences among treatments. (Fig. 3b)

Trial 6. *Raffaelea lauricola* grew well on pondberry, California bay laurel, and redbay, and fungus appearing to be *R. lauricola* was seen growing on almost every block in this trial except those inoculated with sterile H_2O. After shaking, high concentrations of spores were found among all wood species, but there were no significant differences in spore concentration ($p=.1338$, Fig.3c).

Trial 7. Addition of essential oils from sassafras had no effect on growth of *R. lauricola* (fig. 4). No hyphal growth was seen on any blocks of sweetgum regardless of treatment, and almost no spores were found in the post-shake mixtures from them. Only redbay blocks had more spores than the controls and spore concentrations recovered from redbay wood in this trial were similar to the other trials except trial 3 (fig.5).

Discussion

Except for camphor which has shown some resistance to the laurel wilt disease (S. Fraedrich, unpublished data), *R. lauricola* grew well on all lauraceous species tested and also on white mulberry and loblolly pine. *R. lauricola* grew well on sassafras and redbay for all trials in which these were tested except for trial 3. This lack of growth was unexpected and cannot be explained by the freshness of the samples because in both cases fresh wood was used. It is unknown what accounts for the variable growth of *R. lauricola* on different species of wood, and further testing is needed to determine whether structural, chemical, or other features of the wood contribute to the growth of the fungus. Wood structure of redbay and loblolly pine are very dissimilar; so, it is unlikely that wood structure alone accounts for the difference. Members of the family Lauraceae are generally very aromatic, and the fact that RAB are highly attracted to these species suggests the possibility that some of these aromatic compounds may facilitate the growth of *R. lauricola*. Robinson et al. (2011) examined the preference of various fungi including one *Ophiostoma* species for different wood types and found that particular fungi were better colonizers of some wood species based on the decay type of the fungus. Sugar content, but not nitrogen availability, was found to be a significant factor determining how suitable host wood was to fungus colonization. Conversely, Chen and Johns (1993) found that nitrogen availability significantly impacted growth of *Monascus purpureus*, and Abraham et al. (1993) found that nitrogen source significantly impacted the growth of *Ophistoma piceae*. Variations in sugar or nitrogen content could have contributed to the varied growth of *R. lauricola* on the wood types tested here but were not analyzed in these trials. Concentrations of other chemicals could also enhance or reduce the ability of fungi to grow on a particular wood type. For example, Venalainen (2003) found that the greater concentrations of two stilbenoid compounds in scots pines (*Pinus sylvestris*)

caused blocks cut from the heartwood to be more resistant to decay by a brown-rot fungus, *Coniophora puteana*, than blocks cut from the sapwood.

In further trials a standard inoculum concentration and incubation time might be helpful. Three weeks seems to be sufficient for hyphal growth and sporulation on wood blocks, and longer incubation times pose risk of desiccation. A spore concentration of $2.0x10^6$ spores/mL is adequate, and it is usually possible to make 50-100mL of mixture at this concentration from a single *R. lauricola* culture plate. Nevertheless, concentration of inoculum did not seem to have much effect on final spore recovery. The inoculum used for trial 6 was over two times more concentrated than that in other trials, but the amount of spores recovered from redbay blocks in this trial was on the lower end of all trials and only redbay blocks in trial 3 had lower spore production (fig5).

Clearly the laurel wilt pathogen can grow on wood of species outside of the family Lauraceae. This combined with the results showing that other species of ambrosia beetle can be contaminated with *R. lauricola* and possible move it to other tree species (R. Ploetz, personal communication) suggests that over time *R. lauricola* may become more widely distributed in, but not necessarily pathogenic to, other tree species. What limits redbay ambrosia beetle to the Lauraceae is unclear since *R. lauricola,* its primary mycangial fungus (Harrington et al., 2011), does not appear to be limited to that family. A variety of factors including host preferences of the vector and host tree defenses could be responsible for limiting the disease, and further studies should be done to assess the potential for laurel wilt to spread outside of Lauraceae.

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Table 5.1. Results for trial 2 examining the effects of filter paper on *R. lauricola* growth on wood of three tree species and PVC blocks the same size.

Wood type	Filter paper	Spore count (mean±SE) [a]
Redbay	No	6.04±4.37
	Yes	15.00±12.25
Sweetgum	No	0±0
	Yes	0.94±0.19
Water oak	No	0.73±0.40
	Yes	0±0
PVC	No	0.13±0.13
	Yes	0±0

[a] Means were not significantly different at $p<0.05$.

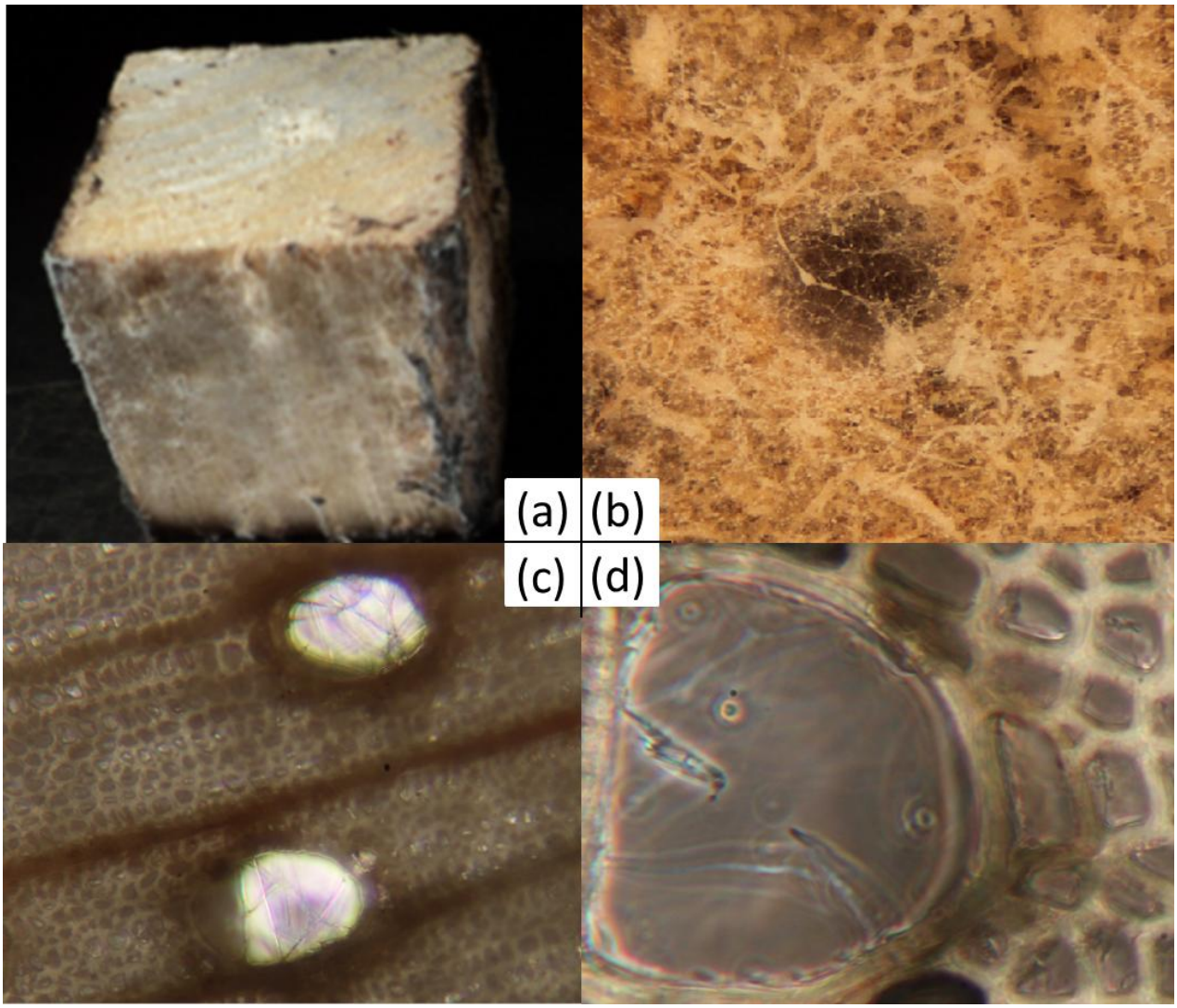

Fig. 5.1. Growth of *R. lauricola* on redbay and sassafras wood. (a) Block of sassafras wood covered in hyphae; (b) Hyphae growing through 1.6mm hole drilled through block of redbay wood; (c & d) fungus growing through the vessels of inoculated redbay wood.

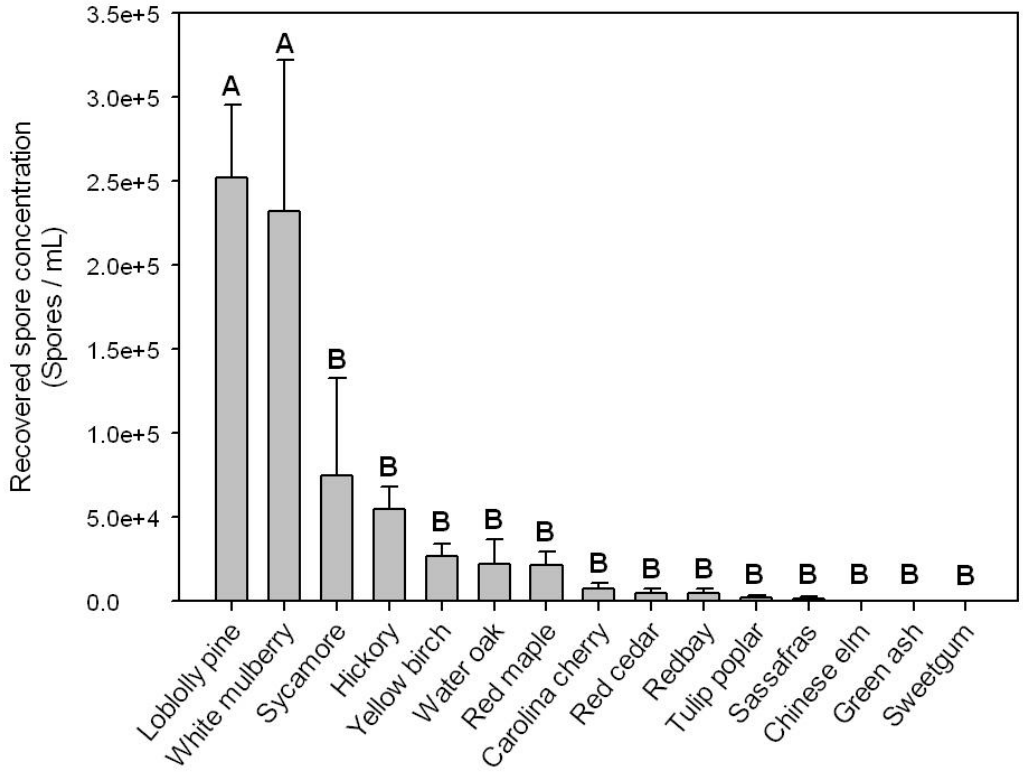

Fig. 5.2. Results for trial 3 comparing growth of *R. lauricola* on various types of wood cut from freshly felled trees. Bars with the same letters are not significantly different (α=0.05).

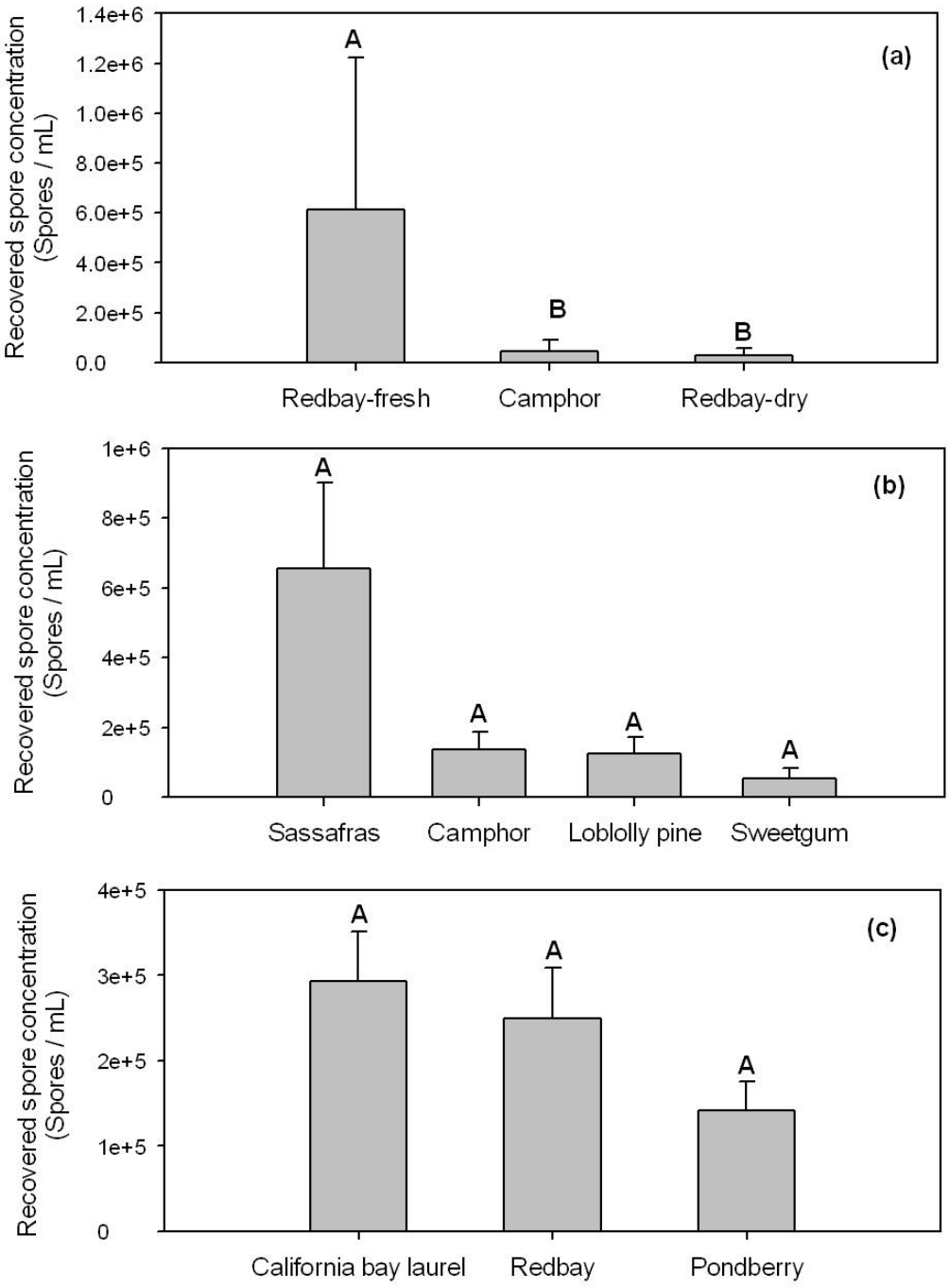

Fig. 5.3. Results for trials 4 (a), 5 (b), and 6 (c) comparing growth of R. lauricola on redbay and other species of wood in petri dishes. Within graphs, bars with the same letters are not significantly different. Significant differences were found between wood types in trials 4 (a) and 5 (b) ($p=.0145$ and $p=.0494$, respectively), but post-hoc analysis revealed no significantly different pairs for trial 5 (b).

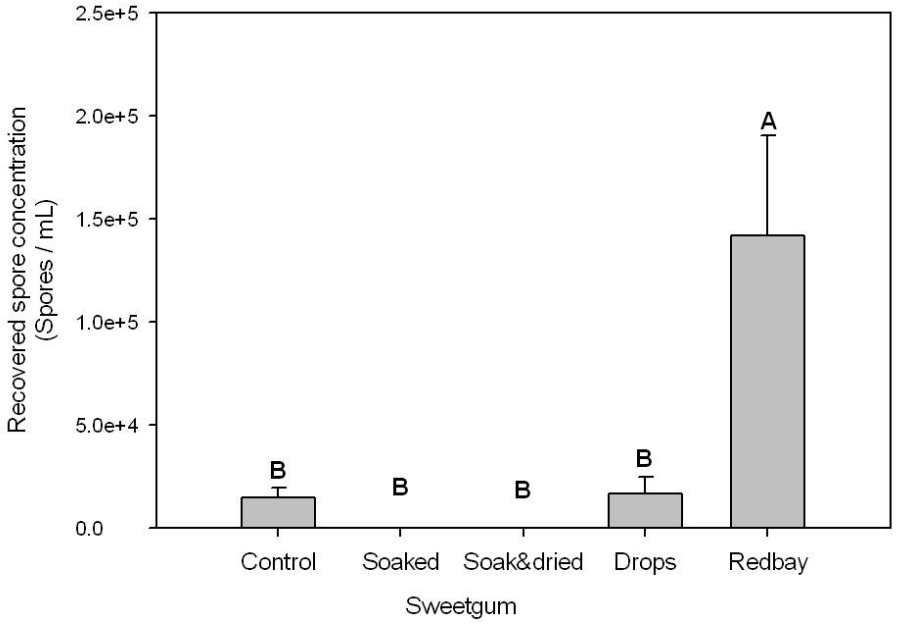

Fig. 5.4. Results from trial 7 in which *R. lauricola* growth was compared on fresh redbay wood and fresh sweetgum wood that was either soaked in essential oils extracted from sassafras, soaked and air dried or on which two drops of oil were added. Bars with the same letter are not significantly different.

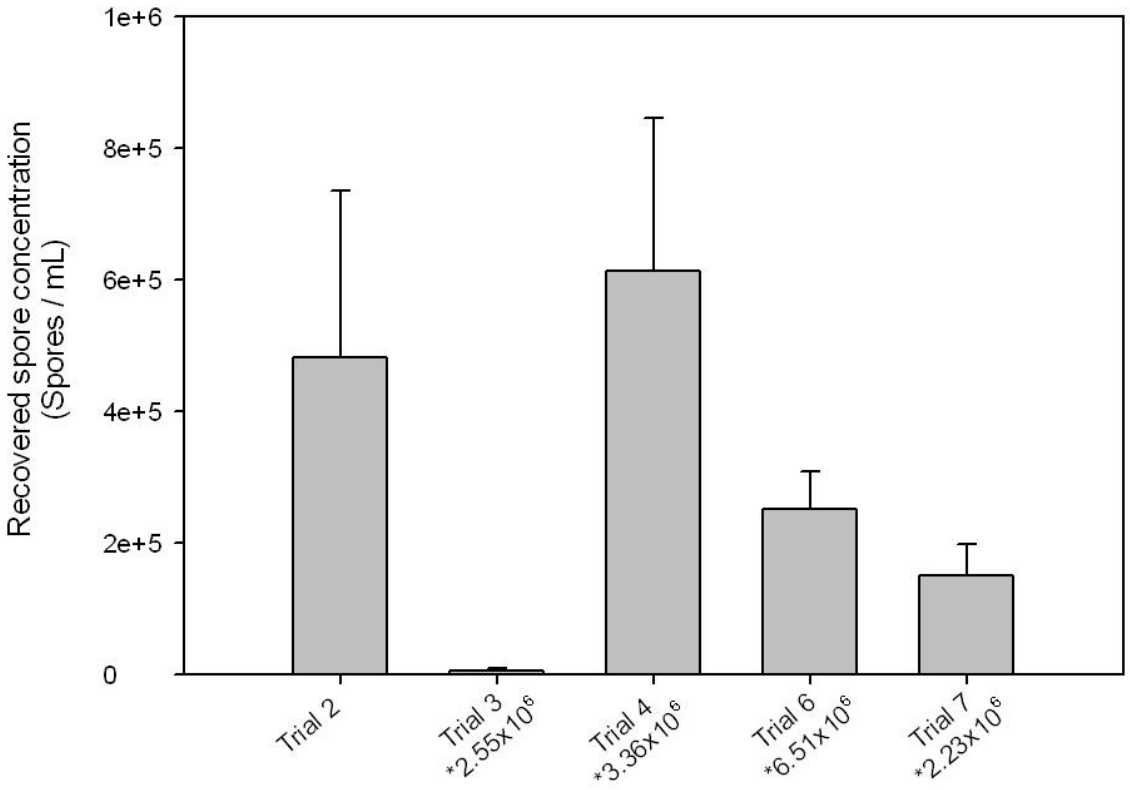

Fig. 5.5. Comparison of recovered spore concentrations from fresh redbay wood between trials in which the initial inoculum spore concentration varied.*Concentration (spores/mL) of initial inoculum.