FUNGAL ASSOCIATES OF KNOWN AND PUTATIVE VECTORS OF LAUREL WILT AND NUTRITIONAL ASPECTS OF THE LAUREL WILT PATHOGEN *RAFFAELEA LAURICOLA*

By

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To the Creator, my parents Javier Saucedo Zuñiga † and Teresa Carabez Barragan and those who are persistent and follow their dreams

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Laurel wilt is a lethal disease caused by *Raffaelea lauricola*, the primary symbiont of an Asian ambrosia beetle, *Xyleborus glabratus*. *Xyleborus glabratus* recovered from laurel wilt-affected silkbay trees, and putative, alternative vectors of *R. lauricola* from avocado (*X. affinis, X. bispinatus, X. volvulus*, *Xyleborinus saxesenii* and *Xylosandrus crassiusculus*) were assayed for the presence of the pathogen, as well as other culturable fungi. Distinct populations of fungi were isolated from each of the assayed beetle species. *Raffaelea* species were the predominant symbionts of *Xyleborus* species and *R. lauricola* was isolated from all species except *X. saxesenii* and *X. crassiusculus*. *Xyleborus bispinatus* carried significant amounts of the pathogen and was recovered more frequently from it than other species of ambrosia beetle from avocado. These results support the hypothesis that *X. bispinatus* plays an important role in the avocado pathosystem.

No-choice assays were conducted to assess the response of newly eclosed, unfertilized females of *X. bispinatus* to artificial diets of different ambrosia fungi. *Xyleborus bispinatus* developed successfully on *R. lauricola*, *R. arxii*, *R. subalba* and *R. subfusca*, but not on a symbiont of *X. crassiusculus*, *Ambrosiella roeperi*. Reproduction, fecundity and survival of

foundress females were positively impacted by the *Raffaelea* symbionts, but negatively impacted on *A. roeperi*- and no-symbiont diets.

To examine why different symbionts were utilized or not by *Xyleborus bispinatus*, mineral content of various fungi that colonize the xylem of avocado were examined; mycelium from liquid cultures of three different categories of fungi were tested: ambrosial fungi, plant-pathogenic endophytes and non-pathogenic endophytes. Few differences were found among the different fungal taxa, which confirmed our hypothesis that mineral content would not differ between symbionts and non-symbionts; other traits, such as olfactory, organoleptic or gustatory features, are presumably more important than mineral content in these interactions. Ambrosia beetle symbionts did not colonize avocado wood faster than fungal endophytes.

In summary, this research provided insights about potential laurel wilt vectors in avocado orchards, their interactions with *R. lauricola* and other ambrosia beetle symbionts, and nutritional attributes of different fungi that reside in avocado xylem.

CHAPTER 1
INTRODUCTION

Xyleborine ambrosia beetles (Curculionidae: Scolytinae) have intimate nutritional relationships with symbiotic fungi (Farrell et al. 2001, Mueller et al. 2005). Foundress females disseminate their fungal partners to, and propagate them in, natal galleries that they bore in host tree xylem. Gardens of these fungi serve as their and their progeny's primary food, and enable them to utilize a protected, but nutrient-poor environment for reproduction, the xylem (Bleiker et al. 2009). Although these interactions were initially conceived as relationships between a given beetle and a single, primary symbiont, the "true ambrosial fungus" (Batra 1963, Baker 1963, Francke-Grosmann 1956, 1963; Leach et al. 1940), the association with more than one species of fungus has been recognized as typical for these insects (Baker and Norris 1968). That some of the symbiotic fungi can be horizontally transferred among beetle species (are not uniquely associated with a single species) is a poorly understood phenomenon.

Ambrosial fungi are usually saprobes that do not cause significant damage. In rare cases, they are phytopathogens that cause moderate to serious damage on host trees (Ploetz et al. 2013). In an extreme case, *Raffaelea lauricola*, the nutritional symbiont of *Xyleborus glabratus*, has killed millions of trees in the Lauraceae family that are native to the southeastern USA. *Raffaelea lauricola* and *X. glabratus* are natives of Asia that were introduced to Georgia in the early 2000s (Hughes et al. 2017). To date, *R. lauricola* has been found in nine other ambrosia beetles, all of which were present in the USA prior to the introduction of *X. glabratus* (Ploetz et al. 2017c, b). Little is known about the movement of this destructive pathogen to additional, potential vector species.

In intensive field surveys, *X. glabratus* has not been found among laurel wilt affected-avocado groves (0 of 79,025 ambrosia beetles in Miami-Dade County and only 11 of 4,181 from

Brevard County) (Carrillo et al. 2012, unpublished). In 2014, ambrosia beetles other than *X. glabratus* were able to transmit *R. lauricola* to potted avocado and swamp bay trees under no-choice conditions (Carrillo et al. 2014). From reared individuals, *R. lauricola* was recovered most frequently from species of *Xyleborus* compared to those in *Xyleborinus* and *Xylosandrus* (Ploetz et al. 2017a). However, it is not known which species play important roles in the avocado pathosystem or what role *R. lauricola* plays in the biology of ambrosia beetles from which the pathogen has been isolated.

Given the rarity of *X. glabratus* in avocado, the persistent association of *R. lauricola* with other ambrosia beetles in laurel wilt-affected avocado trees, the poor understanding of the roles of other ambrosia beetles in the avocado pathosystem, and unknown attributes of ambrosial symbionts other than *R. lauricola* in avocado xylem, the following research objectives were addressed:

1. Improve the understanding of the epidemiology of laurel wilt in avocado, as well as the biology of the alternative vectors with their fungal symbionts that inhabit their mycangia.

Adult females of *X. affinis*, *X. bispinatus*, *X. volvulus*, *X. crassiusculus* and *X. saxesenii* were evaluated for the presence of *R. lauricola* and fungal associates from six avocado orchards in Miami-Dade County, as well as individuals of *X. glabratus* from a stand of silk bay trees in Highlands County, Florida.

Hypothesis: Ambrosia beetles other than *Xyleborus glabratus* carry significant amounts of *R. lauricola* and play an important role in the epidemiology of laurel wilt of laurel wilt of avocado.

2. Examine responses of a putative, alternative vector of *Raffaelea lauricola*, *Xyleborus bispinatus*, to artificial diets of different ambrosia fungi, including *R. lauricola* on artificial, avocado sawdust-based media.

Newly eclosed, unfertilized females of *X. bispinatus* were reared in no-choice assays and one of five different symbionts or no symbiont. Interactions were observed between these fungi and the foundress females and her male progeny.

Hypothesis: Different species of ambrosia fungi impact the reproductive success of *Xyleborus bispinatus*.

3. Investigate the nutritional composition of different fungal taxa that are found in avocado xylem and different species of ambrosia beetles.

The mineral composition of three different groups of fungi were analyzed: ambrosial symbionts, plant pathogenic endophytes and non-pathogenic endophytes. Fungi were grown on artificial substrates.

Hypothesis: There are no differences between the ambrosial and non-ambrosial fungi in mineral content.

CHAPTER 2
REVIEW OF LITERATURE

Avocado

Avocado (*Persea americana* Miller) is a native of MesoAmerica (William 1977). It is an evergreen in the Lauraceae family, which contains 50 genera (Marais 2004), and is the most economically important species in the family (Litz et al. 2007). The highly nutritional fruit of avocado contain carbohydrates, dietary fiber, vitamins (A, B1, B2 B6, C, D, E, K_1), fatty acids (monounsaturated and polyunsaturated), minerals (calcium, phosphorus, iron, sodium, potassium, magnesium), phytochemicals (xanthophyll carotenoids, phenolic compounds, phytosterols), proteins and amino acids (glutamic acid, cysteine and glycine) (Knight 2002, Dreher and Davenort 2013).

Avocado is divided into three different botanical races. The Mexican race (*Persea americana* var. *drymifolia* Blake) originated in Central Mexico, and is adapted to the tropical highlands. The Guatemalan race (*Persea americana* var. *guatemalensis* Williams) originated in areas of Guatemala, is adapted to medium elevations. The West Indian or Antillean race (*Persea americana* var. *americana* Miller) originated in lowland areas in the Pacific coast of Central America, is adapted to low altitude and the humid tropics (Popenoe 1941, Scora and Bergh 1992, Newett et al. 2002, Schnell et al. 2003).

Mexico produces 31% of the world's avocados, followed by the Dominican Republic, Colombia, Peru and Indonesia (8.2%, 6.4%, 6.1% and 5.8, respectively) (FAOSTAT 2016). Avocado was introduced to Florida, in 1833 (Popenoe 1920, Nakasone and Paull 1998), probably from Cuba (Fairchild 1945). In Florida, 3,000 hectares were in production in 2014 (USDA 2014). Commercial production worth $54 million per year occurs primarily in South Florida (98%), where West Indian and West Indian-Guatemalan hybrids predominate (Crane et al.

2007). Florida is the second largest avocado producer in United States, behind California. In Florida, avocado is the third most important fruit behind citrus and blueberry (Evans et al. 2010); 85% of production is sold outside the state (Degner et al. 2002, Evans et al. 2010).

Laurel Wilt

Distribution

In 2002, an exotic ambrosia beetle, *Xyleborus glabratus*, along with its nutritional symbiont, the lethal pathogen *Raffaelea lauricola*, were introduced from Asia to Port Wentworth, Georgia, probably via infested wood packing material (Fraedrich et al. 2008, Harrington et al. 2008, Hughes et al. 2016). In 2003, laurel wilt began to kill stands of redbay (*Persea borbonia*) and other native species in the Lauraceae family in the southeastern United States (Kendra et al. 2013). By 2005, the disease had spread to northern Florida, and in 2007 it killed the first avocado tree in Jacksonville, FL (Mayfield et al. 2008, Reid et al. 2009). In 2011, the disease spread to Florida's avocado production region, Miami Dade-County (Ploetz et al. 2011a). Laurel wilt continues to spread to other states, including Georgia, South Carolina, Florida, Mississippi, Alabama, North Carolina, Louisiana, Arkansas and Texas (Barton et al. 2017). Single, clonal introductions of *R. lauricola* and *X. glabratus* are probably responsible for the laurel wilt epidemic in the USA (Hughes et al. 2017).

Symptoms

Laurel wilt kills avocado trees more rapidly than any other disease of avocado. A single inoculation with only 100 spores of *R. lauricola*, can induce symptom development and eventually kill both avocado and swampbay (Hughes et al. 2015a). Internally, xylem dysfunction is apparent as soon as 3 days after inoculation. Gums and tyloses occlude xylem vessels and impede water movement (Inch et al. 2012, Inch and Ploetz 2012). Externally, symptoms begin a few weeks after inoculation as sectorial wilting of terminal leaves that change from dark olive-

green to reddish-brown color and progresses to the rest of the canopy (Ploetz et al. 2012). Trees of native *Persea* spp. may retain leaves for a year or longer, but avocado usually defoliates within the first months of symptom onset (Ploetz et al. 2012). Internally, infected sapwood becomes discolored with reddish-brown and grayish-black streaks. Depending on the host species and environmental conditions, symptom development and death of the tree can occur within a few weeks or a few months (Hughes et al. 2015b, Ploetz et al. 2017a).

Disease Cycle

The disease cycle of laurel wilt and the life cycle of its primary vector, *X. glabratus*, in forest species has been thoroughly investigated; however, little is known about the identity of the vectors that initiate the disease cycle in avocado. The disease cycle in forest species begins with females of *X. glabratus* flying in the early evening in search of a suitable host for breeding (Kendra et al. 2012). Volatile emissions of α-copaene, α-cubebene, α-humulene, α-calamenene from healthy hosts (Kendra et al. 2014), as well as volatiles emitted by its symbiotic fungi *R. lauricola*, attract females of *X. glabratus* (Hulcr et al. 2011). In addition, volatiles of infected redbay trees are more attractive to *X. glabratus* females than volatiles from its fungal partner or host plant alone (Kuhns et al. 2014). Furthermore, *X. glabratus* females prefer trees with larger stems (Fraedrich et al. 2008, Mayfield and Brownie 2013). Although boring attempts are localized in the stem of these species at low height, 35 to 100 cm above the ground (Brar et al. 2012), another ambrosia beetle associated with laurel wilt-affected avocado trees, *Xyleborus volvulus*, has been trapped as high as 6 m in avocado orchards (Menocal, unpublished).

Once females of *X. glabratus* find a potential host, these pioneer females land and inoculate *R. lauricola* as they try to bore into healthy trees. Interestingly, these females abandon trees during entrance cavities construction and *X. glabratus* are not found in the host (Fraedrich

et al. 2008). As *R. lauricola* infection and tree decline progresses, new females of *X. glabratus* are able to colonize the tree and initiate reproduction (Martini et al. 2017).

Subsequently, adult females of *X. glabratus* establish gardens of *R. lauricola*, which are carried in specialized structures in their bodies, also known as pre-oral mycangia. These type of mycangia are present in adult females of *Xyleborus* species but absent in males and early stages of females (larvae and pupae) (Six and Paine 1998). Pre-oral mycangia are small sacs located in the mandibles and are only present in some taxa, whereas elytral mycangia, cavities at the base of the pronotum adjacent to the scutellum, and mesothoracic mycangia, membranous invaginations in the mesonotum, are found in species of *Xyleborinus* and *Xylosandrus*, respectively (Hulcr and Stelinski 2017).

After *R. lauricola* infects a susceptible host, root-graft transmission can play an important role in disseminating the pathogen via interconnected roots. Root-graft transmission is common in avocado orchards and does not depend on beetle vectors. When infected trees and their root systems are not removed promptly, the pathogen can spread to adjacent healthy trees thereby enlarging the disease focus (Ploetz et al. 2016a). Although root-graft transmission of *R. lauricola* apparently plays a major role in tree-to-tree spread, the vectors that carry *R. lauricola* and initiate laurel wilt outbreaks in avocado orchards are unclear (Ploetz et al. 2017b).

Host Range of Laurel Wilt

X. glabratus and *R. lauricola* have been recovered infrequently from hosts outside the Lauraceae (e.g. Fagaceae, Fabaceae, Dipterocarpaceae, Pinaceae and Theaceae) (Rabaglia et al. 2006, Hulcr and Lou 2013). In the southeastern USA, laurel wilt has been reported in 14 native and non-native Lauraceae hosts, nine under natural conditions and five after artificial inoculation with *R. lauricola*. (Hughes et al. 2015b).

Among the naturally affected hosts are redbay (*Persea borbonia*), an aromatic evergreen tree that has been heavily impacted (Fraedrich et al. 2007, Fraedrich et al. 2008), swamp bay (*Persea palustris* Sarg), which has an important cultural significance for the Seminole tribe in Florida (Snow and Stans 2001) and plays an important role in south Florida ecosystems (Fraedrich et al. 2008, Rodgers et al. 2014), and silkbay (*Persea humilis* Nash), a Florida endemic that plays an important role in the scrub habitats of Central and South Florida (Hughes et al. 2012). The only other reported natural host in *Persea* is avocado.

Other naturally affected hosts include sassafras (*Sassafras albidum* Nees), a deciduous tree that is distributed throughout the US and into southern Canada (Fraedrich et al. 2008, Smith et al. 2009a), pondspice (*Litsea aestivalis* Fernald) an endangered shrub of wetland areas in Florida (Fraedrich et al. 2008, Surdick and Jenkins 2009, Hughes et al. 2011), pondberry (*Lindera melissifolia* Blume), another endangered/threatened shrub (Fraedrich et al. 2011, Hughes et al. 2015b), bay laurel (*Laurus nobilis*), an economically important host from western Asia that has ornamental and culinary uses (Hughes et al. 2014), and another non-native, camphortree (*Cinnamomum camphora* Presl), that is an Asian native that is used primarily as an ornamental tree in warmer regions of southeastern USA and California. Colonization of camphortree by *R. lauricola* is not as rapid and extensive as it is in redbay and avocado (Fraedrich et al. 2015). Camphortree is a poor reproductive host for *X. glabratus*, and multiple aborted attacks are needed for this host to succumb to laurel wilt disease (Hughes et al. 2015b, Fraedrich et al. 2015, Campbell et al. 2016a).

Five trees have been reported as hosts after artificial inoculation. Under laboratory conditions, California laurel (*Umbellularia californica* Nuttall) is prominent within western forest ecosystems (Fraedrich et al. 2008); since it attracted, and was a suitable host for

reproduction of, *X. glabratus*, it could facilitate the spread of *X. glabratus* along the Pacific Coast and in areas in which avocado is produced in California. Northern spicebush (*Lindera benzoin* Blume) is a deciduous shrub in alluvial forests of the southeastern (Fraedrich et al. 2016), gulf licaria (*Licaria triandra* Kosterm) is a federally threatened and endangered species in Florida (Ploetz and Konkol 2013), lancewood (*Nectandra coriaceae* Grisebach) is another native in southern Florida (Hughes and Ploetz, unpublished), and Viñátigo (*Persea indica* Sprengel) is from Spain (Hughes et al. 2015b).

Disease Management

Given its wide establishment in the southeastern United States, eradicating laurel wilt is not possible (Hughes et al. 2015b). However, multiple management strategies have been implemented to reduce the impact and spread of laurel wilt into new regions. State regulations have been established to discourage long-distance movement of vector- and pathogen-infested wood (Florida Department of Agriculture 2010). Sanitation is the first line of defense in order to manage laurel wilt disease in avocado. Rapid detection and destruction of symptomatic trees (Ploetz et al. 2016a), chipping the infested logs and spraying them with contact insecticides reduce vector populations and the viability of *R. lauricola* propagules (Spence et al. 2013). Elimination of only symptomatic branches is not an effective strategy for managing laurel wilt.

Intensive efforts in chemical control have been investigated against laurel wilt. Several fungicides have been tested for *in vitro* assays against *R. lauricola* (Ploetz et al. 2011b), including demethylation inhibitors, quinone inside and outside inhibitors, nutritional and systemic acquired resistance (SAR) treatments, but triazole fungicides have been most effective in field-grown avocado trees (Ploetz et al. 2016a). Macroinfusions with propiconazole fungicides can protect healthy trees and inhibit pathogen movement via root-graft transmission. Nevertheless, applying large volumes of fungicides to the xylem is an ongoing challenge, since it

is expensive and labor-intensive. Moreover, the efficacy of these fungicides is temporary and lasts only 10-11 months. Treated trees should be retreated every year. Thus, effective chemical management strategies (macroinfusion) are usually used to protect high-value trees and germplasm collections rather than entire commercial production areas (Ploetz et al. 2016a, Ploetz et al. 2017a).

Several insecticides are effective against *X. glabratus* (Peña et al. 2011). However, drench applications with systemic insecticides are inefficient, except for Emamectin Benzoate which partially controls *X. glabratus*, and contact insecticides, such as Z-Cypermethrin+Bifenthrin and Lambda-Cyhalothrin+Thiamethoxam, reduce populations of *X. glabratus*, but no do not stop boring activity (Carrillo et al. 2013). Chemical control strategies are needed for the primary vector of laurel wilt in the avocado system. More importantly, the efficacy of insecticide applications for managing this disease should be demonstrated.

Entomopathogenic fungi have been used as biological control of *X. glabratus*. Both fungi, *Beauveria bassiana* (GHA) and *Isaria fumosorosea* (Ifr3581 and PFR) caused mortality of the ambrosia beetle, however, none of the strains prevented boring attempts into avocado logs (Carrillo et al. 2015), which allows the inoculation of healthy trees with *R. lauricola*. Therefore, effective management strategies against the vectors to impede laurel wilt transmission into avocado trees has not been determined. Likewise, although recent research on biological control with 18 endophytic fungi recovered from avocado trees demonstrated inhibitory activity *in vitro* against *R. lauricola*, endophytes neither inhibited the movement of *R. lauricola* nor significantly reduced the development of laurel wilt development in inoculated avocado trees (Pérez et al. 2018). In summary, biological control agents have not mitigated the impact of laurel wilt disease.

Host resistance has been investigated in surviving redbay trees in areas with high disease pressure (Hughes et al. 2013). Similarly, commercial avocado cultivars from the three botanical races (Guatemalan, Mexican, and West Indian) have been screened for their responses to laurel wilt (Ploetz et al. 2012). West Indian cultivars, which comprise most of the commercial cultivars in Florida, are the most susceptible, whereas Guatemalan and Mexican cultivars were more resistant to laurel wilt.

The mechanisms by which an avocado cultivar may have a resistance response against laurel wilt have been associated with xylem anatomy. These xylem features have also been analyzed with avocado cultivars from different origins. Preliminary reports suggest that West Indian cultivars have larger vessels than Guatemalan and Mexican cultivars (Blanchette and Ploetz, unpublished). Although latter cultivars have shown a better response against laurel wilt development, more research is needed to elucidate the role of the xylem and the range of responses against laurel wilt.

Ongoing research investigates the influence of different rootstock/scion combinations on laurel wilt development. Ploetz et al. (2015) hypothesized that clonal avocado rootstocks might impact the susceptibility of a given scion to laurel wilt disease. Previous reports support this hypothesis that rootstocks influence the grafted scion in xylem function and susceptibility to vascular wilt diseases (Fassio et al. 2009, Venturas et al. 2014). Although Guatemalan and Mexican cultivars would have less susceptibility to laurel wilt, developing new scion genotypes resistant to laurel wilt is a long-term alternative strategy.

Raffaelea lauricola

Most ambrosia fungal symbionts are saprophytes that colonize dead wood. Whereas *R. lauricola* systemically colonizes host xylem and causes vascular wilt (Ploetz et al. 2017b), other phytopathogenic fungal symbionts, such as *R. quercus-mongolicae*, *R. quercivora* and *Fusarium*

euwallaceae, cause localized wilting (are not systemic) (Kim et al. 2009b, Matsuda et al. 2010, Ploetz et al. 2013).

The genetic uniformity of *R. lauricola* and *X. glabratus* indicate that both symbiotic organisms were introduced in the United States as clonal populations in a single event. In comparison to the US population, greater genetic diversity was found in isolates of *R. lauricola* from its native region, Southeast Asia (Hughes et al. 2016, Wuest et al. 2017). Although the genetic uniformity of populations of *R. lauricola* from different regions differ, the pathogenicity of Asian and US populations has been confirmed with avocado and swampbay (Ploetz et al. 2016b). The fact that only 100 conidia may kill a susceptible avocado tree demonstrate that *R. lauricola* is a highly virulent pathogen (Hughes et al. 2015a).

Raffaelea lauricola is a dimorphic fungus with a yeast phase (budding spores) within the mycangia and a filamentous (mycelia) phase within the host, gallery walls and in culture media. It produces hyaline conidiophores that are usually aseptate and unbranched with variable lengths (8.5) 13–60 (120) × (l.0) 2.0 (2.5) μm wide, bearing variably shaped conidia, that is primarily oblong to ovoid with a length of (3.0) 3.5–4.5 (8.0) × (1.0) 1.5–2.0 (3.5) μm (Harrington et al. 2008). Colonies of *R. lauricola* are initially mucilaginous but eventually become filamentous along the colony edge, and develops pale light-brown pigmentation after 2 weeks.

Volatiles released by *R. lauricola* attract its primary vector, *X. glabratus*, whereas *Xylosandrus crassiusculus* and *Xyleborinus saxesenii* are repelled by the fungus, and *X. ferrugineus* was neither attracted nor repelled by the pathogen (Hulcr et al. 2011). Although *R. lauricola* is the predominant fungal symbiont of *X. glabratus*, additional *Raffaelea* species have been isolated from *X. glabratus*, albeit with fewer colony-forming units than *R. lauricola*

(Harrington and Fraedrich 2010, Campbell et al. 2016b). The other *Raffaelea* species do not induce disease (Dreaden et al. 2016) but their roles in beetle ecology are unknown.

Nutritional Symbiosis of Ambrosia Fungi

Ambrosia symbionts are asexual fungi in the Ophiostomatales, Microascales and Hypocreales orders within the Ascomycota (Ploetz et al. 2013) and, rarely, the Polyporales in the Basidiomycota (You et al. 2015, Kasson et al. 2016). They are the major source of nutrients for ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) that, in turn, disperse the fungi to suitable hosts (Martin 1992). This fungiculture has evolved independently multiple times (Mueller et al. 2005) in the most advanced and largest group of ambrosia beetles, Xyleborini (Farrell et al. 2001). Nonetheless, only females within this group are able to cultivate the fungal symbiont (Mueller et al. 2005). Females do not lay eggs within the galleries until the fungal symbiont is actively growing, which thus ensures that subsequent larvae and pupae have food (Biedermann et al. 2009).

Ambrosia beetles depend on their fungal partners for essential vitamins, amino acids, and sterols (De Fine Licht and Biedermann 2012). Fungal symbionts absorb the nutrients from nutrient impoverished xylem tissue and provide a higher quality source of food for the beetles, which can be assimilated by adult females and their progeny (Beaver 1989). Unlike bacteria, fungal symbionts provide the major important source of food for ambrosia beetles, ergosterol, an important constituent of mycelia and spores (Weete 1973). This vital nutrient is produced by most fungi as a component of their membranes and is essential for normal growth and reproduction of ambrosia beetles; brood pupation fails in the absence of this vital nutrient (Norris et al. 1969, Kok et al. 1970, Kok and Norris 1973, Morales et al. 2000). However, little is known about the nutritional content of ambrosia fungal symbionts and their effect on their ambrosia beetle partners.

Xyleborus glabratus and Vectors of *Raffaelea lauricola*

The *Xyleborini* group has a haplodiploid reproduction system, also known as arrhenotoky, in which a mother has precise control over the sex of her offspring, developing diploid daughters from fertilized eggs and haploid sons from unfertilized ones (Hamilton 1967). Males are wingless, do not disperse or emigrate (inbreeding system), and they only mate with their sisters (Hamilton 1967, Maner et al. 2013).

Sclerotized females of *X. glabratus* are dark-brown to black in color with a glabrous surface on the elytra. They have a slender shape with a convex declivity, a posterolateral margin and multiple deep punctures on the declivity. Their length varies from 2.1-2.4 mm. Males of *X. glabratus* are smaller, about 1.8 mm long, have a reddish pronotum and flattened spines over the head capsule. Elytra are dark brown and longer than the pronotum (Rabaglia et al. 2006).

Although some *Xyleborus* species are attracted to ethanol (Miller and Rabaglia 2009), *X. glabratus* is attracted to sesquiterpenes from host wood (Hanula and Sullivan 2008, Kendra et al. 2011, Niogret et al. 2011), specifically to volatiles of α-copaene, α-cubebene, α-humulene and calamenene from Lauraceae species, which has been confirmed with olfactory chemoreception via electroantennography (Kendra et al. 2014). Additional field lures with sesquiterpenes have been tested to attract *X. glabratus*. These were based on Manuka oil from *Leptospermum scoparium* (Hanula and Sullivan 2008) and cubeb oil from *Piper cubeba* (Hanula et al. 2013, Kendra et al. 2015). Currently, the most effective lure to attract *X. glabratus* are essential oils with α-copaene content (Kendra et al. 2016). Although females of *X. glabratus* have a preference for hosts with large stem diameters (Fraedrich et al. 2008), trees with small diameters are also affected in laurel wilt epidemics since *X. glabratus* populations may persist in low populations within those hosts (Maner et al. 2014).

After females inoculate their galleries with *R. lauricola*, they lay eggs on the colonized gallery walls (Hughes et al. 2015b). In summer months (June, July and August), new diploid females emerge after 40 days in search of new suitable hosts, although overlapping generations may occur within the same tree (Hanula et al. 2008). Their life cycle is longer in colder months (November, December and January) (Brar et al. 2012, Maner et al. 2013). In Florida, host-seeking flight occurs in the late afternoon or early evening, several hours earlier than other *Xyleborus* species (Kendra et al. 2012). Peaks of flight activity have been reported during the winter months in Georgia and South Carolina (Hanula et al. 2008, 2011), whereas these peaks have been detected in early spring in Florida (Brar et al. 2012).

Brar et al. (2013) demonstrated that avocado is a poor reproductive host for *X. glabratus*, and although progeny was produced in it, more were produced in redbay and swampbay. *X. glabratus* is rarely found in avocado trees despite intensive surveys within laurel wilt affected-avocado groves (0 of ~79,000 from trapped or reared beetles in Miami-Dade County and only 11 of 4181 in Brevard County) (Carrillo et al. 2012, unpublished). Instead, other ambrosia beetles have been associated with laurel wilt transmission in avocado (Carrillo et al. 2014, Ploetz et al. 2017c). In reared or trapped individuals from 10 of these other beeetle species, *R. lauricola* has been recovered in 246 of 726 (34%) from North-American native *Persea* spp and only in 58 of 931 (6%) from avocado with a prevalence in *Xyleborus* species (Ploetz et al. 2017a, c).

In olfactometer assays, females of *X. glabratus* were highly attracted to *R. lauricola*, whereas *X. saxesenii* and *X. crassiusculus* were repelled the pathogen. Another beetle associated with *R. lauricola*, *X. ferrugineus*, responded in a neutral fashion (Hulcr et al. 2011). *X. glabratus* carried the highest concentration of the fungi (an average of 2,800 CFUs per beetle), but nine other beetles had less frequent associations with the pathogen (Ploetz et al. 2017 b, c). Despite

the lower inoculum loads they carried, some individuals carried enough propagules of *R. lauricola* to kill an avocado tree (Hughes et al. 2015a).

R. lauricola is more prevalent and abundant in *Xyleborus* species than other genera of ambrosia beetles (Ploetz et al. 2017c). *Xyleborus* species have paired pre-oral mycangia which seem to be less selective and have promiscuous associations with different *Raffaelea* species. Previous reports suggest that a single species of *Raffaelea* might be carried by different species of ambrosia beetles and that multiple species of *Raffaelea* might be found in a single species of *Xyleborus* (Harrington et al. 2010, Campbell et al. 2016b). However other genera with large invaginations within mesonotal mycangia, such as *Xylosandrus* spp., can be highly infested with greater quantities of the pathogen (Ploetz et al. 2017c). The acquisition of *R. lauricola* by these ambrosia beetles requires more research (Carrillo et al. 2014, Hughes et al. 2015b, Ploetz et al. 2017a, b, c).

Tree Diseases Associated with Bark and Ambrosia Beetles

Fungal symbionts of bark and ambrosia beetles cause destructive tree diseases. International trade and travel play an important role as the principal methods by which exotic pathogens and pests are introduced into new locations where they have not coevolved with native hosts (Haack 2006, Ploetz 2007). Recent alarming disease outbreaks have resulted from the introduction of exotic beetle vectors carrying foreign plant pathogens (Wingfield et al. 2008). Naïve hosts, which do not benefit from a long history of coevolution, have unpredictable responses to these exotic threats, and in rare circumstances these impacts are catastrophic (Ploetz et al. 2013, Wingfield et al. 2016). The best known example is Dutch Elm Disease caused by *Ophiostoma ulmi* and *Ophiostoma novo-ulmi*, which affects *Ulmus* spp. in Europe and the United States (Holmes and Heybroek 1990). Although the pathogen is primarily transmitted by the native elm bark beetle *Scolytus scolytus* in Europe and *Hylurgopinus rufipes* in North America,

ten additional bark beetles have also been indicated as vectors (Webber 1990, 2004). This vascular wilt pathogen is also transmitted via root grafting (Vega 2005). Both modes of transmission are similar to laurel wilt disease transmission in avocado.

Seca and sudden decline are destructive diseases of mango, and have decimated large mango-producing areas in Brazil and Oman (Ploetz et al. 2013). Seca of mango was initially reported in Brazil in 1930 and has been associated with *Ceratocystis mangicola* and *C. mangivora* (Viegas 1960, van Wyk et al. 2011), whereas in 1998 *C. manginecans* was associated with sudden decline in Oman and Pakistan (van Wyk et al. 2007). The original hosts of these pathogens are unclear (Ploetz et al. 2013).

Thousand canker disease affects eastern black walnut (*Juglans nigra*) and several species of wingnut trees (*Pterocarya* spp.). Massive attacks by the bark beetle vector, *Pityophthorus juglandis*, are needed to kill trees since its fungal symbiont, *Geosmithia morbida,* is not a systemic pathogen (Kolařík et al. 2011). The role of *Fusarium solani*, which has been recovered from symptomatic trees but only caused small, localized lesions, is unclear (Tisserat et al. 2009). *P. juglandis* was firstly reported in 1928 in New Mexico, but thousand canker disease is responsible for widespread mortality of *J. nigra* in 16 states of United States and it was recently introduced in Europe (Faccoli et al. 2016). The native original host of *P. juglandis* is *Juglans major* (Arizona walnut), which is less susceptible to the beetle attacks and the symptoms of mortality and dieback do not occur (Kolařík et al. 2011). Unexpectedly, hosts outside of its native range have been affected, and this lethal disease is becoming an international threat to the *Juglans* species (Tisserat et al. 2009, Daniels et al. 2016). Several species of *Geosmithia* have been associated as fungal associates of bark and ambrosia beetles, but *G. morbida* is the only fungal symbiont for which the pathogenicity has been reported (Tisserat et al. 2009).

Japanese oak wilt is a destructive disease caused by *Raffaelea quercivora*, which is vectored by *Platypus quercivorus*. Mass mortalities has occurred of trees in the Fagaceae family (Kinuura and Kobayashi 2006). Its geographic range has expanded to northern regions of Japan where it is spreading to new host species (Kamata et al. 2002). *R. quercivora* is a weak pathogen, and it does not move systemically through infected trees, and multiple beetle attacks are needed to cause trees to wilt and die (Murata et al. 2009).

Korean oak wilt is similar to Japanese oak wilt. It is caused by *Raffaelea quercus-mongolicae,* and is transmitted by *Platypus koryoensis* (Kim et al. 2009b). The pathogen is not systemic, and symptoms develop after many beetle attacks (Lee et al. 2011). Only trees with high densities of beetle attacks develop severe symptoms. An aggregation pheromone produced by unmated males plays an important role in the mass attack by the beetles (Kim et al. 2009a). Similar to the laurel wilt system, the vector is guided by visual cues to locate hosts with large stem diameters (Fraedrich et al. 2008, Lee et al. 2011).

Avocado dieback is a localized, nonlethal disease associated with mildly pathogenic *Fusarium* spp. in the Ambrosia *Fusarium* Clade that are transmitted by cryptic *Euwallacea* spp. This disease was recently reported in Israel and California (O'Donnell et al. 2015). The first description of *Euwallacea fornicatus* was in Sri Lanka in 1868 with the name of 'the tea shot hole borer', but its *Fusarium* symbiont was not tested as the primary factor to affect the plant host (Kasson et al. 2013). Recently, *Fusarium euwallaceae* from *Euwallacea* nr. *fornicatus* recovered from avocado in California were able to induce disease in sufficient density (Eskalen et al. 2012, Mendel et al. 2012). This ambrosia beetle is morphologically similar but genetically different than *Euwallacea fornicatus* in Sri Lanka; it affects a broad host range, thus, its common

name, polyphagous shothole borer (Stouthamer et al. 2013). The disease usually causes portions of the canopy to die.

Similar to the Dutch Elm disease and unlike the other diseases described above, laurel wilt is systemic (Ploetz et al. 2017a). As mentioned above, species other than *X. glabratus* may transmit the pathogen in the avocado pathosystem (Carrillo et al. 2014, Ploetz et al. 2017a). Laurel wilt remains a serious threat in unaffected regions of avocado commercial production as well as unaffected regions with other susceptible hosts, such as *Umbellularia californica* and *Persea indica* in Mediterranean areas (Ploetz et al. 2017b).

Ambrosia Beetles Associated with Avocado

Avocado is not a suitable host for *X. glabratus,* the primary vector of *R. lauricola* (Brar et al. 2013), so other ambrosia beetles may be involved with laurel wilt transmission to avocado (Carrillo et al. 2014). Although 21 species of ambrosia beetle were found in avocado (Carrillo et al. 2012, Atkinson et al. 2013), only *Ambrosiodmus lecontei*, *Xyleborinus andrewesi*, *X. gracilis, X. saxesenii, Xyleborus affinis, X. bispinatus, X. ferrugineus, X. volvulus, X. glabratus,* and *Xylosandrus crassiusculus* carried *R. lauricola*. The greatest frequency and abundance of *R. lauricola* propagules occurs in *Xyleborus* species, which may play an important role in the laurel wilt epidemic (Ploetz et al. 2017c). Importantly, some of these ambrosia beetles are globally distributed and occur where Lauraceae hosts are not affected by laurel wilt (Gohli et al. 2016). For example, several *Xyleborus* species (*X. bispinatus, X. ferrugineus, X. affinis, X. intrusus*) were recently found in the main avocado production areas in Michoacan, Mexico (Ochoa, 2014). Also, bark beetles that occur in avocado orchards in South Florida (Johnson et al. 2016) have been scarcely examined as potential vectors of *R. lauricola* (Ploetz et al. 2017c).

Host Responses to *Raffaelea lauricola*

A single inoculation with only 100 spores of *R. lauricola* can kill a susceptible avocado tree (Hughes et al. 2015a). Colonization of susceptible host trees by *R. lauricola* occurs in the xylem and triggers induced defenses such as delayed production of tyloses that block water conduction and lead to wilt and tree death (Inch et al. 2012, Campbell et al. 2016a). These responses have been investigated on different Lauraceae trees and the range of responses vary depending on the host.

In avocado, laurel wilt symptoms develop rapidly and wilting of terminal leaves appear two weeks after inoculation. These symptoms usually appear in sectorial branches. Internally, vascular discoloration develops after four weeks of inoculation. Latter symptoms have been associated with the delayed production of tyloses (Inch et al. 2012).

Conversely, Camphortree (*Cinnamomum camphora*), which is endemic to Asia and more tolerant to laurel wilt, requires multiple inoculations to induce branch dieback (Fraedrich et al. 2015). Localized wilt is developed in infected branches and inoculated trees appear to recover and remain relatively unaffected (Smith et al. 2009b). The tolerance response of this host to *R. lauricola* infection has been attributed to low colonization of the xylem (Campbell et al. 2016a). Whether the tylose development play an important role in host defense response of camphortree remains unclear. However, the abundance and rapid production of tyloses might be important determinants in laurel wilt tolerance (Campbell et al. 2016a).

Plant Defenses Responses to Vascular Wilt Diseases

Vascular wilt diseases induce severe symptoms of chlorosis, sudden wilt, vascular discoloration and loss of turgor. Furthermore, physiological and histological changes occur within the host such as increases in respiratory rates, low hydraulic function, xylem malfunction, hypertrophy, permeability of the cells and different changes in the metabolism of the host plant

(Beckman 1964, Dimond 1970, Yadeta and Thomma 2013). Species in only a limited number of fungal genera, *Fusarium*, *Verticillium*, *Ceratocystis*, *Ophiostoma* and *Raffaelea*, are able to colonize the nutritionally poor habitat of the xylem (de Sain and Rep 2015, Ploetz et al. 2017b).

Plants develop two types of defenses against vascular wilt pathogens: pre-existing defenses that provide physical-chemical barriers to inhibit fungal invasion and inducible responses (Freeman and Beattie 2008, Six and Wingfield 2011, Yadeta and Thomma 2013). Phenolics, alkaloids and terpenoids comprise the chemical defenses against the vascular wilt pathogens. These metabolites are substances produced by the plant cell with antimicrobial activity, and help to eliminate fungal pathogens and insects as well (Prusky et al. 1991, Freeman and Beattie 2008, Yadeta and Thomma 2013, Li et al. 2014).

Induced defense responses are activated when successful pathogens overcome these pre-existing defenses. Tyloses and gels are common responses, as well as embolism (Rioux et al. 1998). Tyloses outgrowths are formed by the parenchyma cells and contribute to the xylem occlusion reducing the hydraulic conductivity of the xylem and blocking the spread of the pathogen (Rahman et al. 1999). In vascular wilts, resistant plants develop tyloses faster and more abundantly than susceptible plants. Nonetheless, blocking all of the xylem vessels might induce drought stress, or when severe, kill the plant (Kozlowski and Pallardy 1996, Fradin and Thomma 2006). Gels and gums result from host tissue rather than the pathogen itself, and contain pectins and fatty substances that accumulate around tyloses to block off the xylem structure (Powers 1954, Rahman et al. 1999). In resistant hosts, the gels and gums are persistent, whereas in susceptible hosts the responses become fluid after two or three days of infection (Beckman 1964).

Hypersensitive response is a rapid programmed cellular death to limit pathogen growth by sacrificing some cells to save the entire organism (Freeman and Beattie 2008). This response is more pathogen-specific, and it occurs when plant receptors recognize Pathogen-Associated Molecular Patterns introduced into the host and plant immunity is triggered (PTI). Pathogens capable of avoiding PAMP-triggered resistance, deliver effectors that provide virulence to the pathogen, resulting in effector trigger susceptibility (ETS). Subsquently, intracellular plant receptors recognize effectors inside the host cells and activate effector-triggered immunity (ETI). Consequently, failure of the host plant to detect pathogen effectors, or new effectors after the acquisition of the pathogen, will suppress ETI, and plant receptors capable of recognizing effectors and ETI can be triggered again, leading to hypersensitive responses and race classification (Jones and Dangl 2006). Systemic acquired resistance is another defense mechanism induced against a broad spectrum of plant pathogens. This defense response is induced by signaling molecules of jasmonic and salicylic acid systemically and locally activated by the expression of pathogenesis related-genes (Durrant and Dong 2004).

CHAPTER 3
PARTNERSHIPS BETWEEN AMBROSIA BEETLES AND FUNGI: LINEAGE-SPECIFIC PROMISCUITY AMONG VECTORS OF THE LAUREL WILT PATHOGEN, *RAFFAELEA LAURICOLA*

Nutritional mutualisms that ambrosia beetles have with fungi are poorly understood. Although these interactions were initially thought to be specific associations with a primary symbiont, there is increasing evidence that some of these fungi are associated with, and move among, multiple beetle partners. We examined culturable fungi recovered from mycangia of ambrosia beetles associated with trees of *Persea humilis* (silk bay, one site) and *P. americana* (avocado, six commercial orchards) that were affected by laurel wilt, an invasive disease caused by a symbiont, *Raffaelea lauricola,* of an Asian ambrosia beetle, *Xyleborus glabratus*. Fungi were isolated from 20 adult females of *X. glabratus* from silk bay and 70 each of *Xyleborus affinis*, *Xyleborus bispinatus*, *Xyleborus volvulus*, *Xyleborinus saxesenii* and *Xylosandrus crassiusculus* from avocado. Using partial sequences of ribosomal (LSU and SSU) and the nuclear (β-tubulin) genes, one to several operational taxonomic units (OTUs) of fungi were identified in assayed individuals. Distinct populations of fungi were recovered from each of the examined beetle species. *Raffaelea lauricola* was present in all beetles except *X. saxesenii* and *X. crassiusculus*, and *Raffaelea* spp. predominated in *Xyleborus* spp. *Raffaelea arxii*, *R. subalba* and *R. subfusca* were present in more than a single species of *Xyleborus,* and *R. arxii* was the most abundant symbiont in both *X. affinis* and *X. volvulus*. *Raffaelea aguacate* was detected for the first time in an ambrosia beetle (*X. bispinatus*). Yeasts (Ascomycota, Saccharomycotina) were found consistently in the mycangia of the examined beetles, and distinct, putatively co-adapted populations of these fungi were associated with each beetle species. Greater understanding is needed to determine how mycangia in ambrosia beetles interact with fungi, including yeasts which play currently under-researched roles in these insects.

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Background

Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae) have obligate nutritional relationships with symbiotic fungi (Farrell et al. 2001, Mueller et al. 2005). These ancient, coevolved associations represent the earliest known examples of fungus farming by insects (Vanderpool et al. 2017). Most of the nutritional mutualists are ascomycetes in the Ophiostomatales, and, to lesser extents, in the Microascales and Hypocreales (Farrell et al. 2001, Hulcr and Stelinski, 2017, Mueller et al. 2005, O'Donnell et al. 2015).

Mycangia are specialized structures adapted for the transport and protection of fungal symbionts in ambrosia beetles (Six 2003). Diverse types of mycangia have evolved in different lineages of these insects (Hulcr and Stelinski 2017). For example, in the tribe *Xyleborini*, *Xylosandrus* spp. have large, complex invaginated sacs in the elytra, mesonotal mycangia, *Xyleborinus* spp. have small elytral mycangia, and *Xyleborus* spp. have small cavities inside the frontal portions of the head, pre-oral or mandibular mycangia.

Ambrosial mutualisms were originally thought to be species-specific interactions between a fungus and beetle species (Batra 1966, Gebhardt et al. 2004, Kinuura 1995). Although recent studies have confirmed that specific associations occur in some beetle lineages, non-specific interactions have been revealed in others (Hulcr and Stelinski 2017, Mayers et al. 2015, Ploetz et al. 2017a). Recently, the degree of specificity in these relationships was correlated with mycangium type (Hulcr and Stelinski 2017, Kostovcik et al. 2015, Mayers et al. 2015). Using an environmental sequencing approach, Kostovcik et al. (2015) reported that multiple fungal associates with similar abundances were detected in mycangia of *Xyleborus affinis* (Eichhoff) and *Xyleborus ferrugineus* (Fabricius) (each with pre-oral mycangia), whereas communities in *Xylosandrus crassiusculus* (Motschulsky) (mesonotal mycangium) were dominated by a single symbiont, *Ambrosiella* sp. Similarly, Mayers et al. (2015) found that

single symbionts predominated in ambrosia beetle species with large, complex mycangia. Although the available data indicate that large, complex mycangia are more selective environments than pre-oral mycangia, further study is needed.

Relatively little is known about the specific roles many fungal symbionts play in the lifecycles of their beetle associates. In discussing the fungal nutrition of *X. saxesenii*, De Fine Licht and Biedermann (2012) suggested that different fungi might serve as food sources for adults and larvae of *X. saxesenii*. Similarly, Biedermann et al. (2013) proposed that auxiliary fungi might provide nutrients for the ambrosia beetle partners that supplement those provided by the primary nutritional symbiont.

Xyleborus glabratus Eichhoff (Coleoptera: Curculionidae: Scolytinae) originated in Southeast Asia, it was detected in the Western Hemisphere in 2002 in Port Wentworth, Georgia, USA (Haack 2006, Rabaglia et al. 2006). Shortly afterwards, laurel wilt, a destructive disease caused by the primary nutritional symbiont of *X. glabratus*, *Raffaelea lauricola* T.C. Harr., Fraedrich and Aghayeva (Ophiostomatales), began killing trees of native redbay (*Persea borbonia* Spreng) in the surrounding area (Fraedrich et al. 2008). At least 14 tree species in the Lauraceae plant family are now known to be susceptible to the disease, including an important commercial fruit crop, avocado (*P. americana* Miller) (Hughes et al. 2015a).

Anthropogenic movement of infested wood has facilitated the long-distance spread of laurel wilt, but *X. glabratus*-assisted spread has played a more important role in its local and mid-range movement (Hughes et al. 2015b). Primary factors involved in the spread of the disease within and among avocado orchards are less clear (Ploetz et al. 2017a, b). Although root-graft transmission of *R. lauricola* apparently plays a major role in tree-to-tree spread, the vectors that initiate laurel wilt outbreaks in avocado orchards are unclear (Ploetz et al. 2017b).

Based on data obtained from studying the life cycle of *X. glabratus*, Brar et al. (2013) indicated that avocado was not a good reproductive host for the beetle. This may explain the uncommon association of *X. glabratus* with this host tree in south Florida. In 2012, *X. glabratus* was not trapped or reared from field-collected bolts of avocado in Miami-Dade County (0 of 79,000 individuals of ambrosia beetles) and was identified only 11 times among 4,181 individuals examined from Brevard County (Carrillo et al. unpublished). In 2015, *X. glabratus* was not recovered out of 176,252 individuals collected in Miami-Dade County (Carrillo et al. unpublished). The role that alternative species of ambrosia beetles may play in the epidemiology of this disease is a topic of current research (Carrillo et al. 2012). Previously, *R. lauricola* was recovered from 6% (58 of 928) of individuals of eight ambrosia beetle species associated with avocado; 35% (n=79) of the beetle species, *X. bispinatus*, carried the pathogen (Ploetz et al. 2017c).

Recent results support the hypothesis that *X. bispinatus* plays an important role in the epidemiology of laurel wilt on avocado. Artificial inoculations of avocado plants in no-choice conditions demonstrated its probable role as a vector of *R. lauricola* (Carrillo et al. unpublished). Furthermore, laboratory rearing experiments demonstrated that the beetle could complete its life cycle when fed solely on a *R. lauricola*-based artificial medium (Saucedo et al. 2017).

The present research had two main objectives. First, to improve the understanding of the epidemiology of laurel wilt in avocado, we evaluated the presence of *R. lauricola* in *X. affinis*, X. *bispinatus*, *X. volvulus*, *X. crassiusculus* and *Xyleborinus saxesenii* Ratzeburg, each of which were prevalent in commercial orchards affected by the disease. Second, to shed light on the biology of these interactions, we determined the identity and abundance of other fungi inhabiting the mycangia of the targeted beetle species. We report associations of several described and

undescribed fungi with the ambrosia beetles targeted in this study. We also report distinct internal communities of yeasts (Ascomycota, Saccharomycotina) in ambrosia beetles. We propose that these are adapted relationships that coevolved with the different beetle species. This study provides new insights into the associations between mycangial fungi and members of the *Xyleborini*, in particular those in the genus with most species, *Xyleborus* tribe.

Materials and Methods

Beetle sampling

Ambrosia beetles were reared from trees affected by laurel wilt. Logs were taken from six avocado orchards (three to four trees from each orchard) in Miami-Dade County, Florida, and three trees of silkbay (*Persea humilis* Nash) from Highlands County (Table 3-1, Fig. 3-1), and placed in opaque plastic containers affixed with transparent glass jars lined with moist paper towels, as described previously (Carrillo et al. 2012). Beetles that emerged from bolts were attracted to light and collected in the jars, and assayed while still active. A total of 70 adult females each of *X. affinis*, *X. bispinatus*, *X. volvulus*, *X. crassiusculus* and *X. saxesenii* were recovered from avocado, and 20 females of *X. glabratus* were reared from silkbay. Beetles were identified by their morphological characteristics.

Symbiont Isolation

Live females were surface-disinfested in 70% ethanol for 15 s followed by three rinses in sterile deionized water. Heads of species with pre-oral mycangia (*X. affinis*, *X. bispinatus*, *X. glabratus* and *X. volvulus*) and entire bodies of those with mesonotal (*X. crassiusculus*) or pronotal mycangia (*X. saxesenii*) were then ground in 200 μL of sterile deionized water with a glass pestle tissue grinder (Corning, Inc 77241) for 1 min. Serial dilutions of the macerated beetle parts (1:1, 1:10 and 1:100) were streaked on individual Petri dishes containing a semi-selective medium for Ophiostomatales that contained cyclohexamide, streptomycin, malt agar,

ampicillin and rifampycin (CSMA+) (Harrington 1981, Ploetz et al. 2013). In addition, 1:10 and 1:100 dilutions were streaked on half-strength potato dextrose agar plus streptomycin (PDA+), which enabled the isolation of a wider suite of fungi. After 1 week at 23°C, colony forming units (CFUs) with different phenotypes were counted and representatives were subcultured, single spored, and stored at -80ºC prior to molecular identification.

DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from 1- to 3-week-old cultures of four representatives of each cultural phenotype, as described by Justesen et al. (2002). Partial sequences of the ribosomal large subunit 28S (LSU), small subunit 18S (SSU) and beta-tubulin (β-tubulin) regions were amplified with primer pairs LROR/LR5, NSU1/NSU4 and Bt2a/Bt2b, respectively (Glass and Donaldson 1995, Rehner and Samuels 1994, Vilgalys and Hester 1990, White et al. 1990). PCR and thermocycler conditions were as described by Dreaden et al. 2014a.

PCR amplicons were Sanger sequenced by Eurofins (Eurofins MWG Operon, Louisville, KY, USA), and sequences were edited and assembled into contigs using Geneious® 9.1.5 software (https://www.geneious.com, Kearse et al. 2012). We assigned isolates to taxa, using a threshold of 99-100 % similarity, with the Basic Local Alignment Search Tool (BLAST), and the nucleotide database of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/BLAST), under default settings. The identity of a subset of isolates of *R. lauricola* was also confirmed with taxon-specific microsatellite markers, CHK and IFW (Dreaden et al. 2014b). Voucher specimens of notable symbionts were deposited at the Westerdijk Fungal Biodiversity Institute (formerly Centralbureau von Schimmelcultures, CBS) (Utrecht, The Netherlands) (Table 3-2).

Phylogenetic Analyses

Datasets were built with partial LSU, SSU and β-tubulin sequences of the different phenotypes, and from types or vouchered strains retrieved from GenBank (Table 3-2). Datasets of individual genes were aligned with MAFFT 7 (Katoh and Standley 2013) under the E-INS-I method, and trimmed to a similar length with MEGA 7 software (Kumar et al. 2016). Although we included as many closely related species as possible, taxon sampling was dependent on sequence availability in public nucleotide databases. Taxon and outgroup selection were as reported by Dreaden et al. 2014a for the Ophiostomatales, Harrington et al. 2014 for the Microascales, and Am-In et al. 2011, Endoh et al. 2008 and Hong Yun et al. 2015 for the Saccharomycetales. For the Ophiostomatales, 23 of the 34 described species of *Raffaelea* were included, whereas four of 18 species of *Ambrosiella* were included in the Microascales phylogeny. For these orders, sequences from the three regions were concatenated using SeaView 4 software (Galtier et al. 1996). Since β-tubulin sequences could not be generated for *R. arxii* and *R. sulphurea*, they were treated as missing data. For the Saccharomycetales, only the LSU region was used because SSU and β-tubulin sequences were not available for type strains in most of the lineages we detected.

Phylogenies were generated under Maximum Likelihood (ML) using RAxMLGUI 1.5 software with the GTR+GAMMA model and 1000 bootstrap replicates (Stamatakis, 2014). Prior to analysis, concatenated datasets were partitioned to calculate different parameters for LSU-SSU ribosomal and β-tubulin gene regions, no phylogenetic conflict was detected between the regions (Dreaden et al. 2014a, Gazis et al. 2014). Trees were visualized with FigTree 1.4.3 software and edited in Adobe Illustrator 19.2.1 (Golding 2010). Partial sequences of the fungal symbionts obtained in this study were deposited in GenBank. (Table 3-2).

Statistical Analysis

CFUs of *R. lauricola* recovered from different species of ambrosia beetles were compared using non-parametric statistical methods with JMP Pro12 software (SAS Institute Inc., Cary, North Carolina). To determine whether CFUs of *R. lauricola* differed among individual locations and whether CFUs recovered for each symbiont differed in a given beetle species, a Kruskal-Wallis rank sum test was used. Significant differences in groups were determined with a *Post-hoc* Dunn test and *p*-values adjusted with the Benjamin-Hochberg method (Dunn 1964).

Results

Culturable fungi were isolated from the mycangia of all 370 beetles analyzed. All trees in this study were affected by laurel wilt and, thus, infected by *R. lauricola*. A total of 18 Operational Taxonomic Units (OTUs) were recovered from the different species of ambrosia beetles assayed in this study, nine, eight and one from Saccharomycetales, Ophiostomatales and Microascales, respectively. Multigene and LSU phylogenies confirmed that phenotype grouping was accurate and corresponded with a given OUT.

Isolation of *R. lauricola* from Ambrosia Beetles

Raffaelea lauricola was recovered from all beetles examined except *X. saxesenii* and *X. crassiusculus* (Table 3-3). The CFUs (mean 6,970 ± 585.8, range 3,600-11,600) of *R. lauricola* isolated from *X. glabratus* recovered from silk bay were significantly higher than those obtained from other beetle species recovered from avocado (Kruskal-Wallis chi-square=118.95, *P*=<0.0001, df=3) (Table 3-3). Among beetle species recovered from avocado, *X. bispinatus* had significantly higher CFUs of the pathogen (mean 127 ± 32.7) than *X. affinis* (4.6 ± 2.4) and *X. volvulus* (0.69 ± 0.4) (*P*=<0.0001, df=3), as well as higher maximum CFUs (respectively, 1,200,

140 and 20). *Raffaelea lauricola* was also recovered more frequently from individuals of *X. bispinatus* (49% of the assayed individuals), than *X. affinis* (11%) and *X. volvulus* (6%).

Mycangial isolations of *R. lauricola* differed for beetles reared from silk bay vs. avocado (*P*=<0.0001, df=6). Among avocado orchards, isolations of *R. lauricola* differed significantly only between orchard 4 and orchard 6 (Table 3-3). There were no significant differences among orchards in the CFUs of *R. lauricola* isolated from *X. bispinatus* (*P*=0.8771, df=3), *X. affinis* (*P*=0.1794, df=5) and *X. volvulus* (*P*=0.1787, df=5).

Communities of Microbial Symbionts

Distinct populations of fungi were recovered from the examined beetle species, although some symbionts were found in several beetle species (Table 3-4; Figs. 3-2 – 3-4). Only two OTUs were recovered from *X. glabratus* reared from silk bay, but significantly more CFUs (mean of 6,970) of its primary symbiont, *R. lauricola*, were isolated compared to the second symbiont *R. subalba* (747) (*P*=<0.0001, df=1). Both fungi were recovered from 100% (20 of 20) of the assayed individuals.

In contrast, nine OTUs were isolated from mycangia of *X. bispinatus* (Table 3-4; Figs. 3-2 – 3-4). Significantly more CFUs of a yeast, *Ambrosiozyma* sp. (isolates JR45-48, JR193-194, JR207-208), were isolated compared to other symbionts (766, *P*=<0.0001, df=8), and it was isolated from 74% (52 of 70) of the assayed individuals. Five species of *Raffaelea* were found in *X. bispinatus*, including *R. lauricola* (mean CFUs of 127, 49% of assayed individuals), *R. subfusca* (226, 44%), *R. subalba* (207, 33%), *R. arxii* (189, 20%), and *R. aguacate* (63, 9%). In addition, three other yeast species were isolated, a *Lipomyces* lineage closely related to *L. oligophaga* (184, 30%, isolates JR191 and 192), a *Candida* lineage closely related to *C. nemodendra* (242, 21%, isolates JR201 and 202), and an unidentified lineage labelled as "unidentified yeast" in Fig. 4 (5, 7%, isolates JR41-44).

Although we observed fewer OTUs in *X. affinis*, *Raffaelea* species also predominated in its mycangia. *Raffaelea arxii* was most the abundant (CFU mean of 785) and prevalent (86% of the assayed individuals), followed by significantly lower levels of *R. subfusca* (50, 4%) and *R. lauricola* (5, 11%) (*P*=<0.0001, df=4). We also isolated two yeasts from *X. affinis*, *Ambrosiozyma cicatricosa* (22, 4%, isolates JR116 and 117 and JR173 and 174) and a species of *Candida* related to *C. berthetii* (119, 21%, isolates JR187-189).

Raffaelea arxii was also the most significant symbiont in *X. volvulus* (663, 79%) (*P*=<0.0001, df=4). In *X. volvulus*, this species was represented by different morphotypes that could not be distinguished molecularly (Figs. 3-2 and 3-5). In addition, we recovered an unknown *Raffaelea* lineage (516, 40%, isolates JR156 and 157), *R. lauricola* (0.7, 6%), an unknown *Candida* lineage (105, 24%, isolates JR49-52), and *A. cicatricosa* (72, 16%, isolates JR69-72) from this beetle species.

Raffaelea sulphurea was the most significant symbiont in *X. saxesenii* (1,383, 86%) compared to an undescribed, but previously reported, lineage closely related to *R. canadensis* (*Raffaelea* sp. PL1001, Simmons et al., 2016) (30, 13%) and a yeast, *Ogataea allantospora*, (117, 9%) (*P*=<0.0001, df=2). Only two OTUs were recovered from *X. crassiusculus; Ambrosiella roeperi* (4,016, 97%) was the most significant symbiont of compared to the yeast *Cyberlindnera xylebori* (1,375, 27%) (*P*=<0.0001, df=1).

Discussion

In ambrosial symbioses, the predominant nutritional mutualist is usually deemed the "primary" or "principle" symbiont (Batra 1966, 1967, Scott and Du Toit 1970). However, the methods that are used to isolate and identify the most prevalent species and how and which portions of a beetle are assayed for its isolation can have profound effects on its identity. Bateman et al. (2016) indicated that "imprecise symbiont identification, and disregard for

anatomical complexity" of these insects has confused descriptions of many of these relationships. In the present study, we used nucleotide sequences from one protein coding and two ribosomal RNA-encoding regions to identify known or previously undescribed taxa in the Ophiostomatales (*Raffaelea* spp.) and Microascales (*Ambrosiella roeperi*) (Figs. 3-2 and 3-3); the mycangial associations and the widely recognized nutritional role these taxa play in the *Xyleborini* suggest that these fungi function as primary or secondary symbionts in the studied ambrosia beetles.

We isolated distinct populations of fungi from the beetle species examined. Since the pre-oral mycangia of *Xyleborus* spp. are found in the heads of females (Hulcr and Stelinski 2017), most, if not all, of the fungi we isolated from surface-disinfested heads of *X. affinis*, *X. bispinatus*, *X. glabratus* and *X. volvulus* originated in the mycangia of these beetles. *Xylosandrus crassiusculus* has a mesonotal mycangium, which occur in the insects' elytra (Hulcr and Stelinski 2017). Although surface-disinfested bodies of this species may have contained fungi associated with other internal structures, such as the beetle's gut, we note that few fungi were isolated from it (Table 3-4) and that the nutritional mutualism previously associated with the species, *A. roeperi*, predominated in our assays (Harrington et al. 2014). Thus, we assume that the fungi recovered from the above species were mycangial associates. In contrast, *X. saxesenii* has a pronotal mycangium located in its elytra (Hulcr and Stelinski 2017). Although we presume that the fungi recovered from *X. saxesenii* were also mycangial associates, we note that the beetle's primary symbiont, *R. sulphurea* has also found in its gut (Biedermann et al. 2013). Thus, we can only be sure that the three fungi recovered from *X. saxesenii* resided internally (were not surface contaminants).

As reported previously (Harrington et al. 2010, Campbell et al. 2016b, Ploetz et al. 2017b), we detected *R. lauricola* in *X. affinis*, *X. bispinatus*, *X. glabratus* and *X. volvulus*. Our results also corroborate the presence of *R. arxii* in *X. affinis*; *R. arxii* and *R. subalba* in *X. bispinatus*; *R. subalba* in *X. glabratus*; and *R. sulphurea* in *X. saxesenii* (Harrington and Fraedrich 2010, Biedermann et al. 2013, Campbell et al. 2016b). We report for the first time *R. subfusca* in *X. affinis* and *X. bispinatus*. Furthermore, we identified potentially novel species of *Raffaelea* in *X. volvulus* (isolates JR156 and 157) and *X. saxesenii* (isolates JR144 and 145) (Fig. 3-2).

As more ambrosial partnerships are studied, promiscuous associations are becoming increasingly evident, especially in those involving *Raffaelea* symbionts (Harrington et al. 2010, Kostovcik et al. 2015, Campbell et al. 2016b, Ploetz et al. 2017b). Horizontal and vertical movement of the latter nutritional mutualists is apparently common, especially among *Xyleborus* congeners (Ploetz et al. 2017c). Although it appears that the pre-oral mycangium in this genus has an important influence on symbiont diversity, fundamental insight is lacking for the relatively non-selective nature of this organ (Kostovcik et al. 2015). Additional research is needed on the exceptional flexibility that is apparent in *Raffaelea* - *Xyleborus* symbioses, as it has probably facilitated the emergence of secondary vectors of *R. lauricola*.

The isolation of *R. aguacate* from *X. bispinatus* represents the first time this fungus has been detected in an ambrosia beetle. *Raffaelea aguacate* is a close, nonpathogenic relative of *R. lauricola* (Fig. 3-2) that had previously been known only from an avocado tree that was erroneously diagnosed with laurel wilt due to a misidentification with a single locus (Simmons et al., 2016). Since we isolated *R. aguacate* infrequently and at low CFU numbers we assume it

plays a secondary role in the life cycle of *X. bispinatus*, and the primary beetle partner of this fungus remains unknown.

Previously, *X. bispinatus* was shown to successfully complete its lifecycle on artificial diets of *R. arxii*, *R. lauricola*, *R. subalba* and *R. subfusca* (Saucedo et al. 2017). In the present study, additional flexibility in these symbioses is suggested by the presence of *R. arxii* in *X. affinis*, *X. bispinatus* and *X. volvulus*. The predominance of *R. arxii* in *X. affinis* and *X. volvulus* also suggests that this fungus is the primary mutualist in these beetle species. *Raffaelea arxii* had been reported as the "principle" symbiont of *X. volvulus* (formerly *X. torquatus*) in South Africa, although this was based on isolations from natal galleries of the beetle (no assays of the beetle itself were reported) (Scott and Du Toit 1970). This is the first report of a fungal symbiont playing primary roles in different ambrosia beetle species.

Raffaelea sulphurea, which was the most prevalent symbiont in our assays of *X. saxesenii* (86%, 60 of the 70 assayed individuals and mean CFUs of 1,383), had been reported previously as the predominant symbiont of *X. saxesenii* (Francke-Grosmann 1956, Batra 1967, Biederman et al. 2013). *Raffaelea* sp. PL 1001, which was recovered from 13% (9 of 70) of the individuals of *X. saxesenii* that we assayed, may be the primary symbiont of another species in the genus, *Xyleborinus andrewesi*, as Bateman et al. (2015) isolated it from 100% of the individuals assayed with an average of 5,205 CFUs. Thus, it appears that horizontal transfer of a *Raffaelea* symbiont has also occurred between species with a pronotal mycangium.

In the LSU-based phylogeny used to infer the taxonomic position of yeasts (Ascomycota, Saccharomycotina) we isolated, several taxa did not group with known reference species. We included all curated reference LSU sequences available in our analysis. Although the prevalence of undescribed taxa may reflect incomplete taxon sampling (i.e. poor coverage for yeasts in the

LSU dataset), it is also possible that these lineages represent new species. Yeasts associated with scolytid bark beetles play roles that range from detoxifying host tree wood to beetle attraction or repellency (Davis 2015) but much less is known about yeasts that are associated with ambrosia beetles (Blackwell 2017a, b, Endoh et al. 2011, Suh et al. 2008).

Much of the scant information that is available on yeasts in ambrosia beetles is related to their gut mycoflora (Blackwell 2017a, b, Suh et al 2008). Although gut content was not assessed in the *Xyleborus* spp. we studied, it may have been detected in *X. saxesenii* and *X. crassiusculus*. However, regardless of where they were located we note that the yeast taxa in the present study were more specifically aligned with their beetle partners than were other symbionts. Of nine yeast OTUs, only *Ambrosiozyma cicatricosa* was found in more than a single beetle species (*X. affinis* and *X. volvulus*). In contrast, *Ogataea allantospora* was only recovered from *X. saxesenii*, *Cyberlindnera xylebori* from *X. crassiusculus*, and diverse undescribed lineages in other beetle species; an undescribed yeast (JR 41-44) were recovered from *X. bispinatus*, *Candida* sp. 2 was recovered from *X. affinis*, and *Candida* sp.3 was recovered from *X. volvulus* (Fig. 3-4).

Ambrosiozyma was erected by Van der Walt (1972) for filamentous yeasts with septal pore bodies that had a primary association with ambrosia beetles, they recovered *A. cicatricosa*, from "the tunnel linings" of *X. volvulus*. In our study, *A. cicatricosa* was found infrequently in the mycangia of *X. affinis* and *X. volvulus*, whereas an unidentified lineage in the genus (isolates JR45-48, 193-194 and 207-208) was the most significant symbiont in *X. bispinatus*. This is the first report of *A. cicatricosa* in *X. affinis*, and the first time a yeast was shown to be the most consistent and significant fungus in the mycangium of an ambrosia beetle.

Undescribed lineages of *Candida* were each found in *X. affinis*, *X. bispinatus* and *X. volvulus*. Although members of this genus are well-known gut inhabitants of wood-boring

beetles (Blackwell 2017a, b, Suh et al. 2008) our isolations indicate specific mycangial partnerships with taxa in this genus. From *X. bispinatus,* an undescribed lineage of *Lipomyces* was also isolated (isolates JR191-192). Although this yeast genus is infrequently associated with ambrosia beetles (Van Der Walt et al. 1987, Kurtzman et al. 2007), the undescribed lineage was found in 30% of the individuals we assayed, with CFUs as high as 2,200 per individual.

As indicated above, single yeast taxa were recovered from *X. saxesenii* (*Ogataea allantospora*) and *X. crassiusculus* (*Cyberlindnera xylebori*). Other *Ogataea* species have been recovered from galleries of ambrosia beetles in Japan (Nakase et al. 2008) and *C. xylebori* has been isolated from mycangia of *X. crassiusculus* recovered from redbay trees in Georgia, USA and from galleries in *Acer rufinerve* in Japan (Hong et al. 2015, Ninomiya et al. 2013).

The association of specific yeast communities with the ambrosia beetles that we recovered from avocado suggests that they are vertically transmitted (generation to generation within a species). Unlike previous reports that detected yeasts in external beetle habitats such as gallery linings/fungal gardens (Kurtzman et al. 2007, Nakase et al. 2008, Ninomiya et al. 2013, Van Der Walt et al. 1987, Van der Walt 1972), we associated them as intimate, internal associates. The consistent and often specific associations we detected between different yeast taxa and different beetle species suggests that co-adapted and stable relationships may occur between these fungi and their beetle partners. Future work should consider whether these fungi perform ancillary functions that influence the nutrition of their beetle associates, as proposed by Biedermann et al. (2013). Likewise, their potential effects on beetle behavior and the insect x host tree interaction warrant attention.

In summary, additional research is needed to identify factors that influence the range and specificity of fungi that are associated with ambrosia beetles. Mycangium type appears to play an

important role in the species that depend on these structures for mutualism transport, but little is known about other factors that are correlated with the suites of fungi that these insects carry. We note that host specificity and environmental parameters that affect bark beetles and their symbionts (Hulcr et al. 2007, Six and Bentz 2007) are understudied factors in ambrosia beetle biology. Whether differences in brood galleries (*Xyleborus* spp.) create relatively narrow natal tunnels (Saucedo et al. 2017) compared to large cave-like galleries produced by *Xyleborinus* and *Xylosandrus* species (Atkinson et al. 2000, Biedermann 2010), feeding behavior (*Xyleborus* spp. consume only fungi whereas *X. crassiusculus* and *X. saxesenii* consume their respective symbionts plus fungus-infested wood) (Biedermann 2007), or other attributes of the species we studied influence the spectrum of fungi they carry is not clear but worthy of study.

Table 3-1. Geographic location of different sampling sites in this study.

Orchards	Cultivar	Age (years)	County
Forest	Silkbay	15-20	Highlands
Avocado 1	'Simmonds'	20-25	Miami-Dade
Avocado 2	Mix of West Indian cultivars	20-25	Miami-Dade
Avocado 3	'Lula'	25-30	Miami-Dade
Avocado 4	'Simmonds'	25-30	Miami-Dade
Avocado 5	'Simmonds'	20-25	Miami-Dade
Avocado 6	'Lula'	30-35	Miami-Dade

Table 3-2. Reference sequences from GenBank and fungal symbionts sequenced and used in the phylogenetic analyses.

Fungal species	Accession numbers			
	Voucher	GenBank		
		LSU	SSU	β-tubulin
Alloscaidea africana	Y-6762	JQ689066	-	-
Ambrosiozyma ambrosiae	CBS6003	KY106091	-	-
Ambrosiozyma angophorae	CBS5823	KY106094	-	-
Ambrosiozyma cicatricosa	CBS6157	KY106095	-	-
Ambrosiozyma cicatricosa JR69		MG674013	-	-
Ambrosiozyma cicatricosa JR70		MG674012	-	-
Ambrosiozyma cicatricosa JR71		MG674011	-	-
Ambrosiozyma cicatricosa JR72		MG674010	-	-
Ambrosiozyma cicatricosa JR116		MG674005	-	-
Ambrosiozyma cicatricosa JR117		MG674004	-	-
Ambrosiozyma cicatricosa JR173		MG673998	-	-
Ambrosiozyma cicatricosa JR174	CBS15269	MG673997	-	-
Ambrosiozyma kamigamensis	CBS10899	KY106096	-	-
Ambrosiozyma kashinagacola	Y-63631	NG055218	-	-
Ambrosiozyma llanquihuensis	CBS8182	KY106097	-	-
Ambrosiozyma maleeae	Y-63635	NG055219	-	-
Ambrosiozyma monospora	CBS2554	KY106099	-	-
Ambrosiozyma neoplatypodis	CBS10900	KY106102	-	-
Ambrosiozyma oregonensis	CBS5560	KY106103	-	-
Ambrosiozyma philentoma	CBS6276	KY106104	-	-
Ambrosiozyma platypodis	CBS4111	KY106108	-	-
Ambrosiozyma pseudovanderkliftii	CBS10904	KY106109	-	-
Ambrosiozyma sp. JR45		MG674021		
Ambrosiozyma sp. JR46		MG674020		
Ambrosiozyma sp. JR47		MG674019		
Ambrosiozyma sp. JR48		MG674018		
Ambrosiozyma sp. JR49		MG673991		
Ambrosiozyma sp. JR194	CBS15266	MG673990		
Ambrosiozyma sp. JR207		MG673987		
Ambrosiozyma sp. JR208		MG673986		
Ambrosiozyma vanderkliftii	CBS10905	KY106110	-	-
Ascoidea hylecoeti		U76198	-	-
Candida aaseri	CBS1913	KY106268	-	-
Candida andamanensis	CBS10859	KY106293	-	-
Candida berthetii	CBS5452	KY106320	-	-
Candida conglobata	CBS2019	KY106399	-	-
Candida dendronema	CBS6271	KY106411	-	-
Candida krabiensis	CBS10097	KY106540	-	-
Candida maris	CBS5151	KY106557	-	-
Candida multigemmis	CBS6524	KY106588	-	-
Candida naeodendra	CBS6032	KY106592	-	-
Candida nemodendra	CBS6280	KY106597	-	-
Candida sp. 1 JR201		MG673989		
Candida sp. 1 JR202	CBS15265	MG673988		
Candida sp. 2 JR187	CBS15268	MG673996		

Fungal species	Accession numbers			
	Voucher	GenBank		
		LSU	SSU	β-tubulin
Candida sp. 2 JR188		MG673995		
Candida sp. 2 JR189		MG673994		
Candida sp. 3 JR49		MG674017		
Candida sp. 3 JR50	CBS15271	MG674016		
Candida sp. 3 JR51		MG674015		
Candida sp. 3 JR52		MG674014		
Candida trypodendroni	CBS8505	KY106852	-	-
Cyberlindnera amylophila	CBS7020	KY107350	-	-
Cyberlindnera euphorbiae	CBS8083	KY107351	-	-
Cyberlindnera japonica	CBS7209	KY107369	-	-
Cyberlindnera meyerae	CBS7076	KY107374	-	-
Cyberlindnera misumaiensis	CBS8062	KY107377	-	-
Cyberlindnera petersonii	CBS5555	KY107385	-	-
Cyberlindnera xylebori	CBS21287	KY107422	-	-
Cyberlindnera xylebori JR128		MG674003	-	-
Cyberlindnera xylebori JR129		MG674002	-	-
Cyberlindnera xylebori JR130	CBS15278	MG674001	-	-
Cyberlindnera xylebori JR138		MG674000	-	-
Cyberlindnera xylebori JR139		MG673999	-	-
Lipomyces kononenkoae	CBS8113	KY108299	-	-
Lipomyces oligophaga	CBS7107	KY108304	-	-
Lipomyces sp. JR191		MG673993	-	-
Lipomyces sp. JR192	CBS15267	MG673992	-	-
Lipomyces tetrasporus	CBS5910	KY108316	-	-
Lipomyces yamadae	CBS7532	KY108327	-	-
Lipomyces yarrowii	CBS7785	KY108328	-	-
Ogataea allantospora	CBS10576	KY108668	-	-
Ogataea allantospora JR87		MG674009		
Ogataea allantospora JR88		MG674008		
Ogataea allantospora JR89		MG674007		
Ogataea allantospora JR90	CBS15270	MG674006		
Ogataea dorogensis	Y27599	NG055143	-	-
Ogataea kodamae	CBS7081	KY108676	-	-
Ogataea minuta	Y-411	NG055141	-	-
Ogataea paradorogensis	CBS10978	KY108688	-	-
Ogataea polymorpha	CBS5032	KY108711	-	-
Ogataea siamensis	CBS10095	KY108716	-	-
Pichia mexicana	CBS7066	KY110154	-	-
Schizosaccharomyces pombe	Y-12796	NG042649	-	-
Unidentified yeast JR41		MG674025		
Unidentified yeast JR42		MG674024		
Unidentified yeast JR43		MG674023		
Unidentified yeast JR44		MG674022		
Ambrosiella beaveri	CBS121750	EU825650	KR673882	EU825656
Ambrosiella ferruginea	CBS408.68	EU984285	EU984254	EU977461
Ambrosiella hartigii	CBS404.82	EU984288	EU984256	EU977463
Ambrosiella xylebori	CBS110.61	EU984294	AY858659	EU977469

Fungal species	Accession numbers			
	Voucher	GenBank		
		LSU	SSU	β-tubulin
Ambrosiella roeperi	C2448	KF646767	KR673886	-
Ambrosiella roeperi JR134	CBS142880	MF138153	MF138158	MF138161
Ambrosiella roeperi JR135		MG673985	MG674051	MG674069
Ambrosiella roeperi JR136		MG673984	MG674050	MG674068
Ceratocystis adiposa	CBS600.74	EU984304	EU984263	EU977479
Ceratocystis coerulescens	C13-12	AY214000	EU984264	AY140945
Ceratocystis eucalypti	C457	AF222482	KC305134	FJ411353
Ceratocystis fagacearum	C1305	AF222483	AF222520	FJ411370
Ceratocystis ficicola	CMW38543	KM495342	AB576865	KY68577
Ceratocystis fimbriata	IFO30501	AF222484	AF222521	EF070442
Ceratocystis moniliformis	CBS155.62	EU984305	EU984265	EU977480
Ceratocystis paradoxa	CBS601.70	AF275498	AF222529	JX518363
Ceratocystis platani	CBS115162	KF646771	KC493161	EF070425
Ceratocystis radicicola	CBS114.47	AF275513	KF953932	KF953931
Ceratocystis virescens	CBS128997	AF222489	AF222530	EF070441
Colletotrichum gloeosporioides	CBS79672	AY705727	KX463049	DQ084508
Thielaviopsis basicola	CBS414.52	AF222459	KX925307	KC691496
Thielaviopsis thielavioides	CBS130.39	AF222480	AF222518	KC691499
Raffaelea albimanens	CBS271.70	EU984296	EU984259	EU977471
Raffaelea aguacate	CMW38067	KJ909296	KF026302	KJ909297
Raffaelea aguacate JR213		MG673961	MG674027	MG674053
Raffaelea aguacate JR214	CBS144257	MG673960	MG674026	MG674052
Raffaelea amasae	CBS116694	EU984295	AY858660	EU977470
Raffaelea ambrosiae	CBS185.64	EU984297	AY497518	EU977472
Raffaelea arxii	CBS273.70	EU984298	AY497519	-
Raffaelea arxii JR25	CBS142878	MF138151	MF138156	-
Raffaelea arxii JR26		MG673978	MG674044	-
Raffaelea arxii JR159		MG673969	MG674035	-
Raffaelea arxii JR160		MG673968	MG674034	-
Raffaelea arxii JR178		MG673966	MG674032	-
Raffaelea arxii JR179		MG673965	MG674031	-
Raffaelea brunnea	CBS378.68	EU984284	AY858654	EU977460
Raffaelea canadensis	CBS168.66	EU984299	AY858665	EU977473
Raffaelea ellipticospora	C2550	HQ688663	KJ909299	KJ909298
Raffaelea fusca	C2394	EU177449	KJ909300	KJ909301
Raffaelea fusca 87p2	CBS139934	KR018424	KR018398	KR018437
Raffaelea fusca 90p2	CBS139935	KR018415	KR018399	KR018441
Raffaelea gnathotrichi	CBS379.68	EU177460	AY858655	-
Raffaelea lauricola	PL159	KJ909303	EU257806	KJ909302
Raffaelea lauricola JR9		MG73981	MG674047	MG674065
Raffaelea lauricola JR10		MG673980	MG674046	MG674064
Raffaelea lauricola JR33	CBS142879	MF138152	MF138157	MF138160
Raffaelea lauricola JR34		MG673977	MG674043	MG674062
Raffaelea lauricola JR177		MG673967	MG674033	MG674057
Raffaelea lauricola JR190		MG673964	MG674030	MG674056
Raffaelea montetyi	CBS451.94	EU984301	AY497520	EU977475
Raffaelea quercivora	MAFF41091	AB496454	AB496428	GQ225691

Fungal species	Accession numbers			
	Voucher	GenBank		
		LSU	SSU	β-tubulin
Raffaelea quercus-mongolicae		KF513155	GQ225703	GQ225688
Raffaelea santoroi	CBS399.67	EU984302	EU984261	EU977476
Raffaelea subalba	C2401	EU177443	KJ909304	KJ909305
Raffaelea subalba JR1		MG673983	MG674049	MG674067
Raffaelea subalba JR2		MG673982	MG674048	MG674066
Raffaelea subalba JR17	CBS142877	MF138150	MF138155	MF138163
Raffaelea subalba JR18		MG673979	MG674045	MG674063
Raffaelea subfusca	C2335	EU177450	KJ909306	KJ909307
Raffaelea subfusca JR195		MG673963	MG674029	MG674055
Raffaelea subfusca JR196	CBS144258	MG673962	MG674028	MG674054
Raffaelea sulcati	CBS805.70	EU984291	AY858666	EU977477
Raffaelea sulphurea	C593	EU984292	EU170272	EU977467
Raffaelea sulphurea JR140		MG673976	MG674042	-
Raffaelea sulphurea JR141		MG673975	MG674041	-
Raffaelea sulphurea JR142		MG673974	MG674040	-
Raffaelea tritirachium	CBS726.69	EU984303	EU984262	EU977478
Raffaelea sp. PL1001	CMW38062	KJ909293	KJ909294	KJ909295
Raffaelea sp. PL1001 JR144		MG673973	MG674039	MG674061
Raffaelea sp. PL1001 JR145		MG673972	MG674038	MG674060
Raffaelea sp. JR156		MG673971	MG674037	MG674059
Raffaelea sp. JR157		MG673970	MG674036	MG674058
Ophiostoma ulmi	CBS298.87	DQ368627	M83261	EU977489
Ophiostoma ips		AY172022	AY172021	GU170412

[a] Accession numbers are for voucher specimens in Y (NRRL, ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA), CBS (Centralbureau von Schimmelcultures, known currently as the Westerdijk Fungal Biodiversity Institute Utrecht, Netherlands), C (TC Harrington Iowa State University), CMW (Collection Mike Wingfield, FABI, University of Pretoria, South Africa), KACC (Korean Agricultural Culture Collection), MAFF (Japanese Collection of Microorganisms, National Institute of Agrobiological Resources, Tsukuba, Japan), PL (Dreaden et al. 2014a), JR (this study).

Table 3-3. Presence and prevalence of *Raffaelea lauricola* from different species of ambrosia beetles recovered from different avocado orchards[a].

	Species of ambrosia beetles																
Orchard	*Xyleborus bispinatus*			*Xyleborus affinis*			*Xyleborus volvulus*			*Xyleborinus saxesenii*			*Xylosandrus crassiusculus*			Totals	
	n	Mean	Range	N	Mean	Range	n	Mean	Range	n	Mean	Range	n	Mean	Range	Mean [b]	Range
1	7	77.1	0 - 400	17	0.2	0 – 4	17	0	0	17	0	0	17	0	0	7.3 ab	0 - 400
2	7	40.9	0 - 220	17	7.2	0 – 80	17	0.2	0 - 4	17	0	0	17	0	0	5.6 ab	0 - 220
3	6	20.7	0 - 80	6	26.7	0 - 140	6	0	0	6	0	0	6	0	0	8.9 ab	0 - 140
4	0	np	np	10	0	0	10	0	0	10	0	0	10	0	0	0 b	0
5	0	np	np	10	4	0 – 20	10	0	0	10	0	0	10	0	0	1.0 ab	0 - 20
6	50	158.8	0 - 1,200	10	0	0	10	2	0 - 20	10		0	10	0	0	88.4 a	0 - 1,200
Totals[c]	70	127 a	0 - 1,200	70	4.6 b	0 - 140	70	0.7 b	0 - 20	70	0 b	0	70	0 b	0		
n*Rl*[d]	34 a			8 ab			4 ab			0 b			0 b				

[a] n = number of female adults of given ambrosia beetle species from a given avocado orchard that were assayed for Raffaelea lauricola on CSMA+ (see text). Means and ranges are colony-forming units (CFUs) of R. lauricola isolated. CFUs are not significantly different (α=0.05) within columns/among avocado orchards for a given species of ambrosia beetle based on Dunn's Kruskal-Wallis test with p-values adjusted using the Benjamin-Hochberg method. np = not present.

[b] Within the column, means followed by the same letter do not have significantly different CFUs (α=0.05) based on Dunn's Kruskal-Wallis test with p-values adjusted using the Benjamin-Hochberg method.

[c] Within the row, means followed by the same letter do not have significantly different CFUs (α=0.05) based on Dunn's Kruskal-Wallis test with p-values adjusted using the Benjamin-Hochberg method.

[d] nRl = number of individuals from which Raffaelea lauricola was isolated; numbers followed by the same letter are not significantly different (α=0.05), based on Dunn's Kruskal-Wallis test.

Table 3-4. Isolation of fungal symbionts/operational taxonomic units (OTUs) from different beetle species recovered from trees affected by laurel wilt[a].

Beetle species	Host tree	Symbiont/operational taxonomic unit (OTU)	n[b]	Number of individuals with symbiont (%)	CFU mean[c]	CFU range
Xyleborus glabratus	Silk bay	***Raffaelea lauricola***	20	100	6,970 a	3600-11600
		Raffaelea subalba	20	100	747 b	140-1400
		Ambrosiozyma sp.	52	74	766 a	0-3800
Xyleborus bispinatus	Avocado	*Raffaelea lauricola* [d]	34	49	127 b	0-1200
		Raffaelea subfusca [d]	31	44	226 b	0-2000
		Raffaelea subalba [d]	23	33	207 b	0-2140
		Lipomyces sp.	21	30	184 b	0-2200
		Candida sp. 1	15	21	242 b	0-4600
		Raffaelea arxii [d]	14	20	189 b	0-2480
		Raffaelea aguacate	6	9	63 b	0-1400
		Unidentified yeast	5	7	5 b	0-200
Xyleborus affinis	Avocado	*Raffaelea arxii*	60	86	785 a	0-4400
		Candida sp. 2	15	21	119 b	0-1200
		Raffaelea lauricola	8	11	5 b	0-140
		Raffaelea subfusca	3	4	50 b	0-1700
		Ambrosiozyma cicatricosa	3	4	22 b	0-1000
Xyleborus volvulus	Avocado	***Raffaelea arxii***	55	79	663 a	0-4400
		Raffaelea sp.	28	40	516 b	0-4060
		Candida sp. 3	17	24	105 c	0-1020
		Ambrosiozyma cicatricosa	11	16	72 dc	0-1400
		Raffaelea lauricola	4	6	0.67 d	0-20
Xyleborinus saxesenii	Avocado	***Raffaelea sulfurea***	60	86	1383 a	0-6400
		Raffaelea sp. PL1001	9	13	30 b	0-800
		Ogataea allantaspora	6	9	117 b	0-4200
Xylosandrus crassiusculus	Avocado	***Ambrosiella roeperi***	68	97	4016 a	0-17200
		Cyberlindnera xylebori	19	27	1375 b	0-17800

[a] For a given ambrosia beetle species, means followed by different letters have significantly different CFUs (α=0.05) based on Dunn's Kruskal-Wallis test with *p*-values adjusted using Benjamin-Hochberg method. Bold-faced symbionts are the presumed primary nutritional mutualist of *X. glabratus* (Harrington et al. 2008), *X. volvulus* (Scott and Du Toit 1970),
X. saxesenii (Biedermann et al. 2013) and *X. crassiusculus* (Harrington et al. 2014). Primary mutualists have not been reported for *X. affinis* and *X. bispinatus*.

[b] n = number of individuals from which a given symbiont was isolated. A total of 20 individuals of *X. glabratus*, and 70 of the each of the remaining species were assayed on CSMA+ and ½ strength PDA.

[c] Within the column, means of colony-forming units (CFUs) for a given species that are followed by the same letter are not significantly different (α=0.05) based on Dunn's Kruskal-Wallis test.

[d] These symbionts have supported reproduction of *X. bispinatus* in artificial gnotobiotic systems (Saucedo et al. 2017).

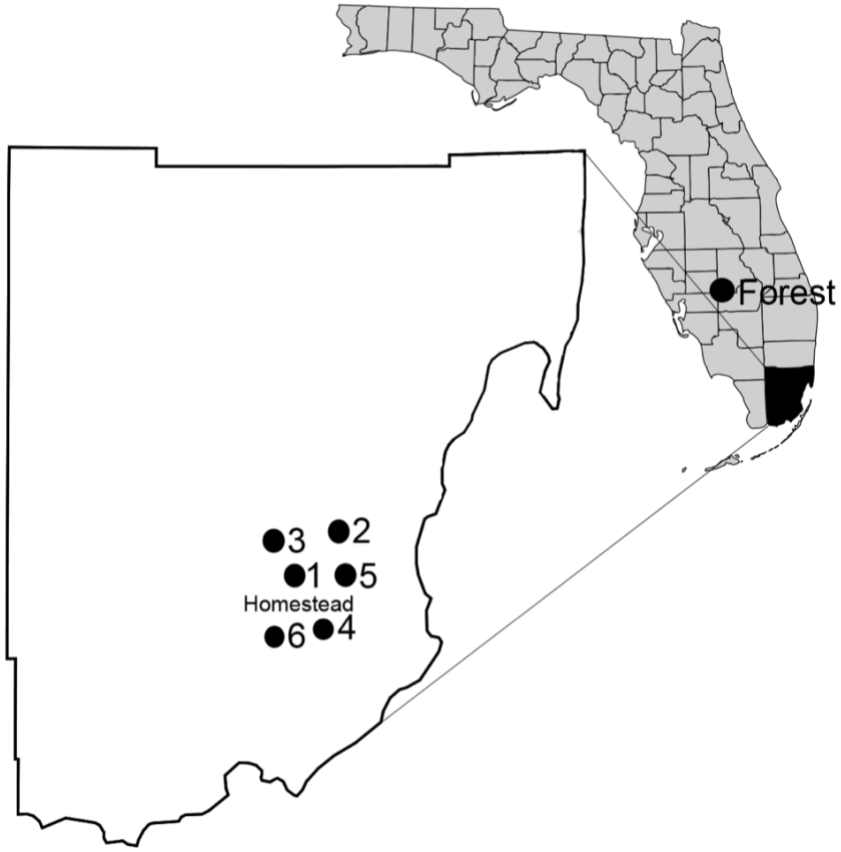

Figure 3-1. Map of Florida indicating geographic locations of the different sample sites described in Table 3-1.

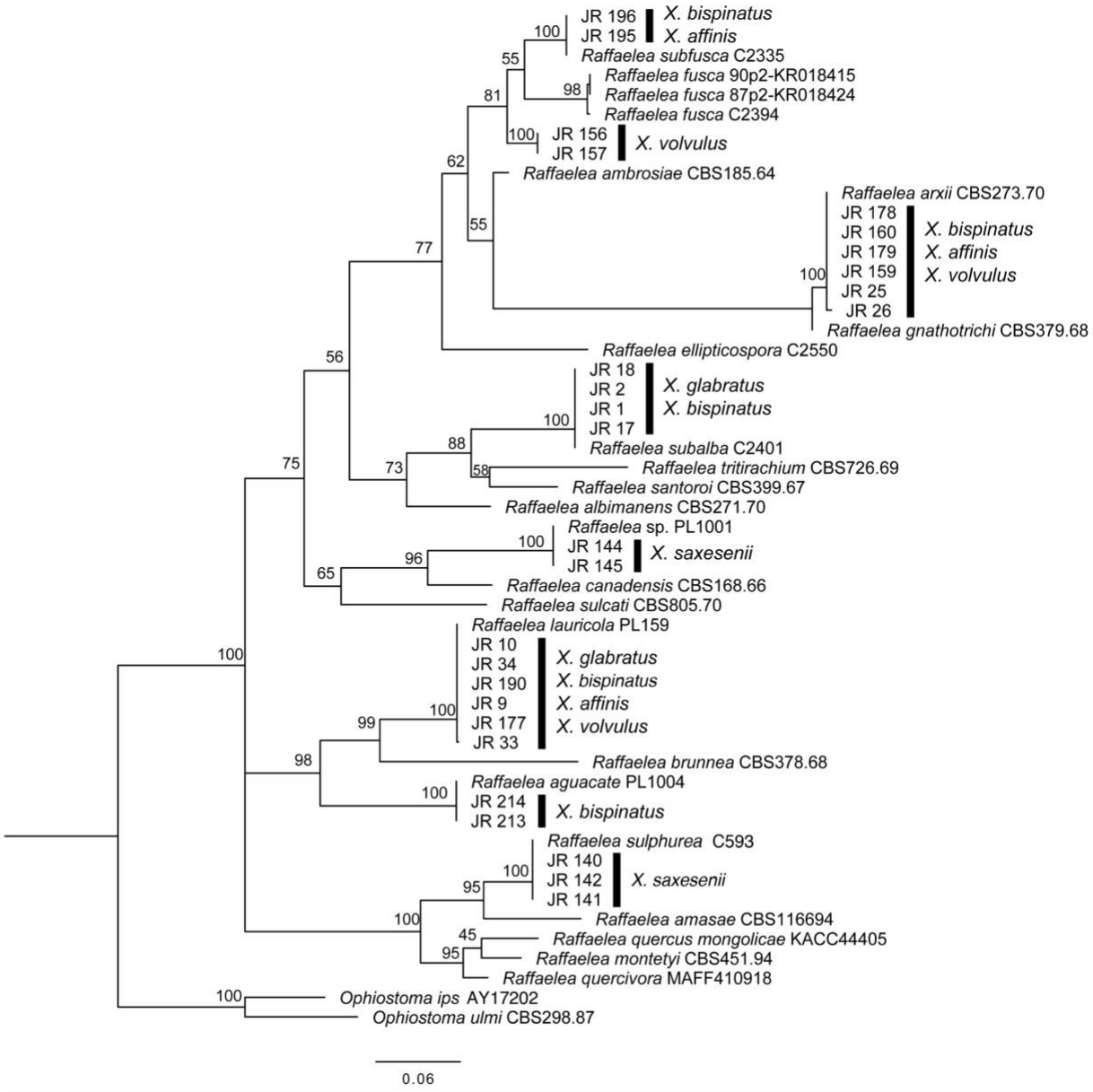

Figure 3-2. Maximum Likelihood phylogenetic tree of *Raffaelea* species generated with partial sequences of LSU, SSU and β-tubulin. *Ophiostoma ips* and *Ophiostoma ulmi* were used as outgroup taxa. Vertical bars opposite different species/OTUs indicate ambrosia beetle species in which the symbiont was found.

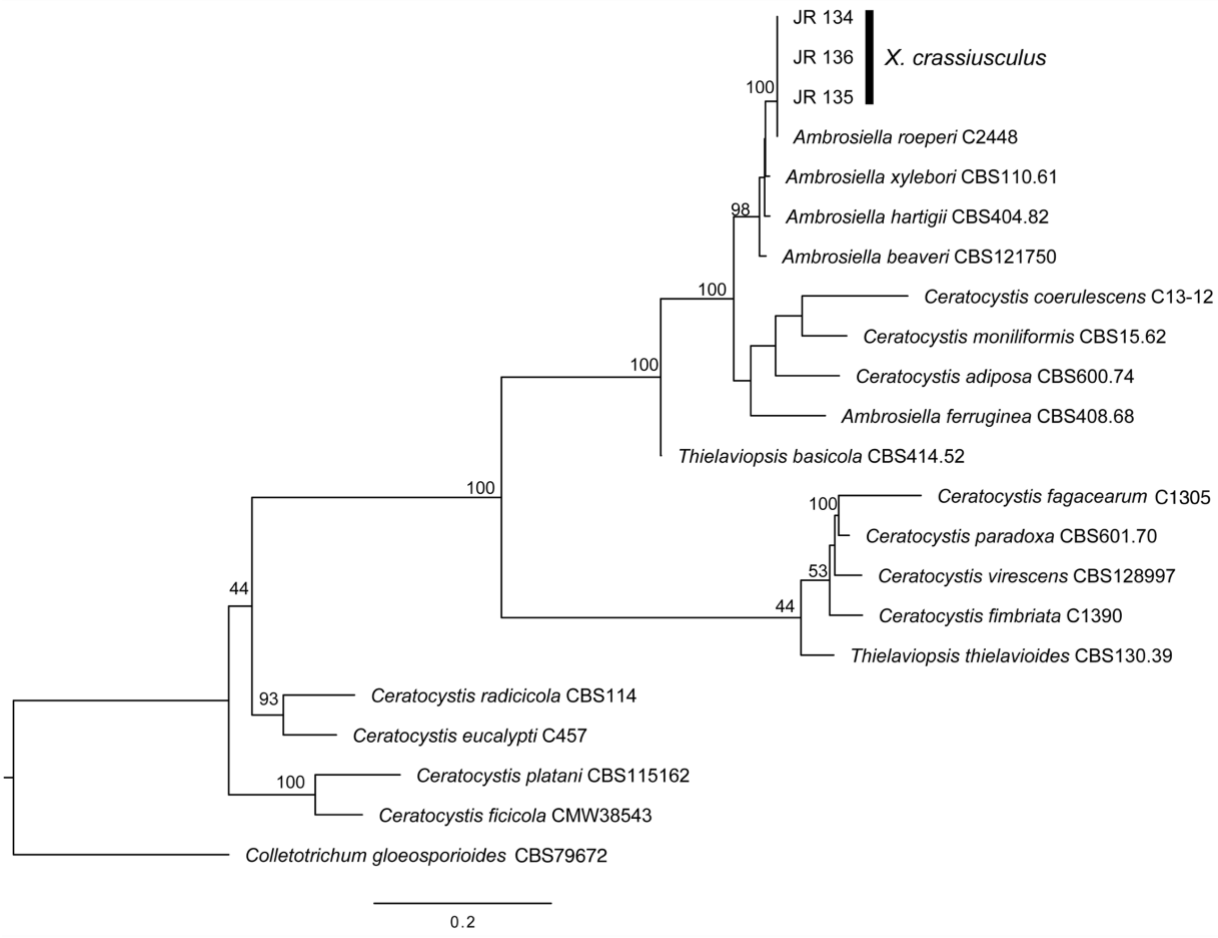

Figure 3-3. Maximum Likelihood phylogenetic tree of Microascales with partial sequences of LSU, SSU and β-tubulin. The vertical bar indicates the beetle species in which the symbiont was found.

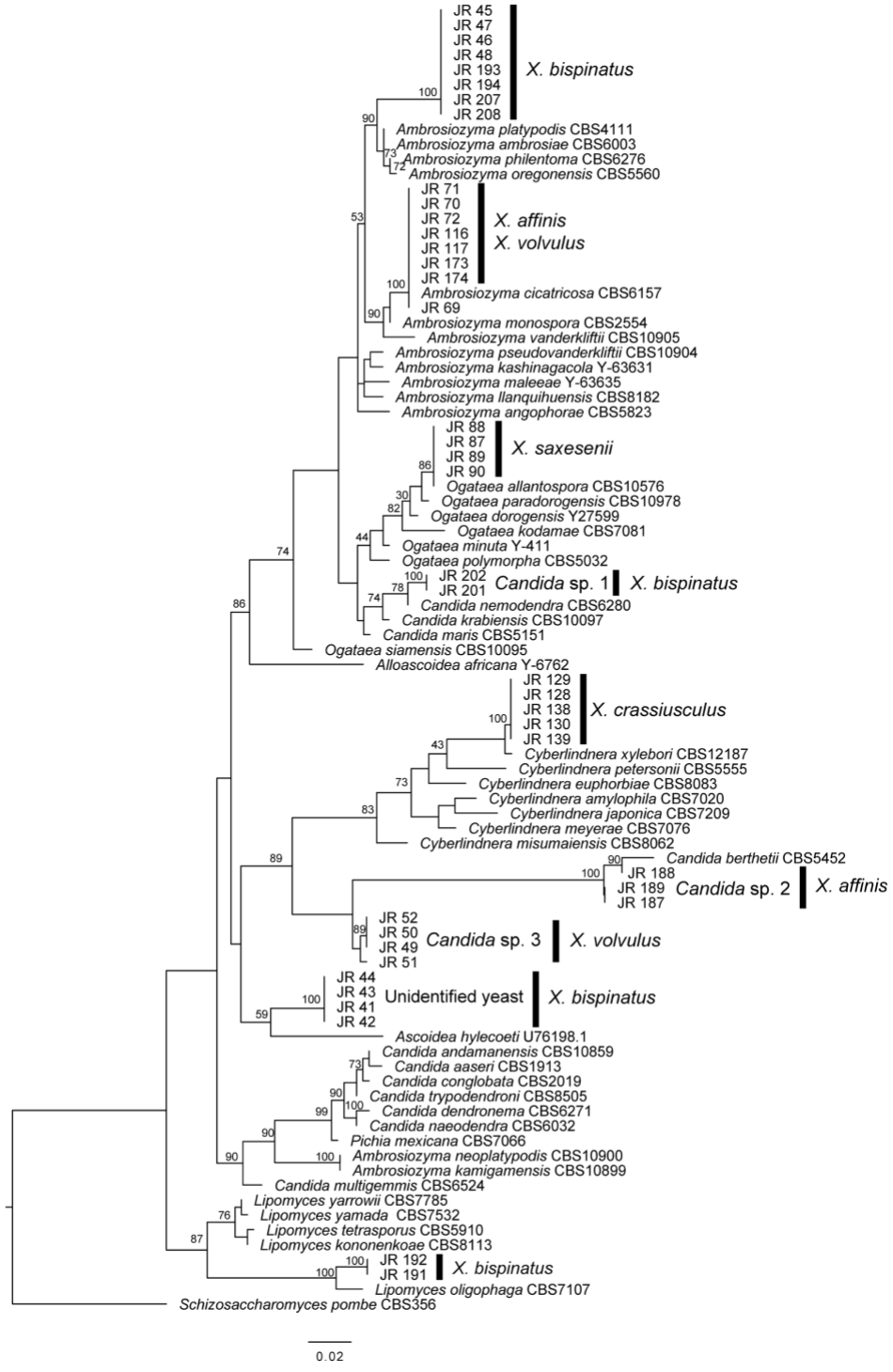

Figure 3-4. Maximum Likelihood phylogenetic tree of Saccharomycetales with partial sequences of LSU region. Vertical bars opposite different species/OTUs indicate ambrosia beetle species in which the symbiont was found.

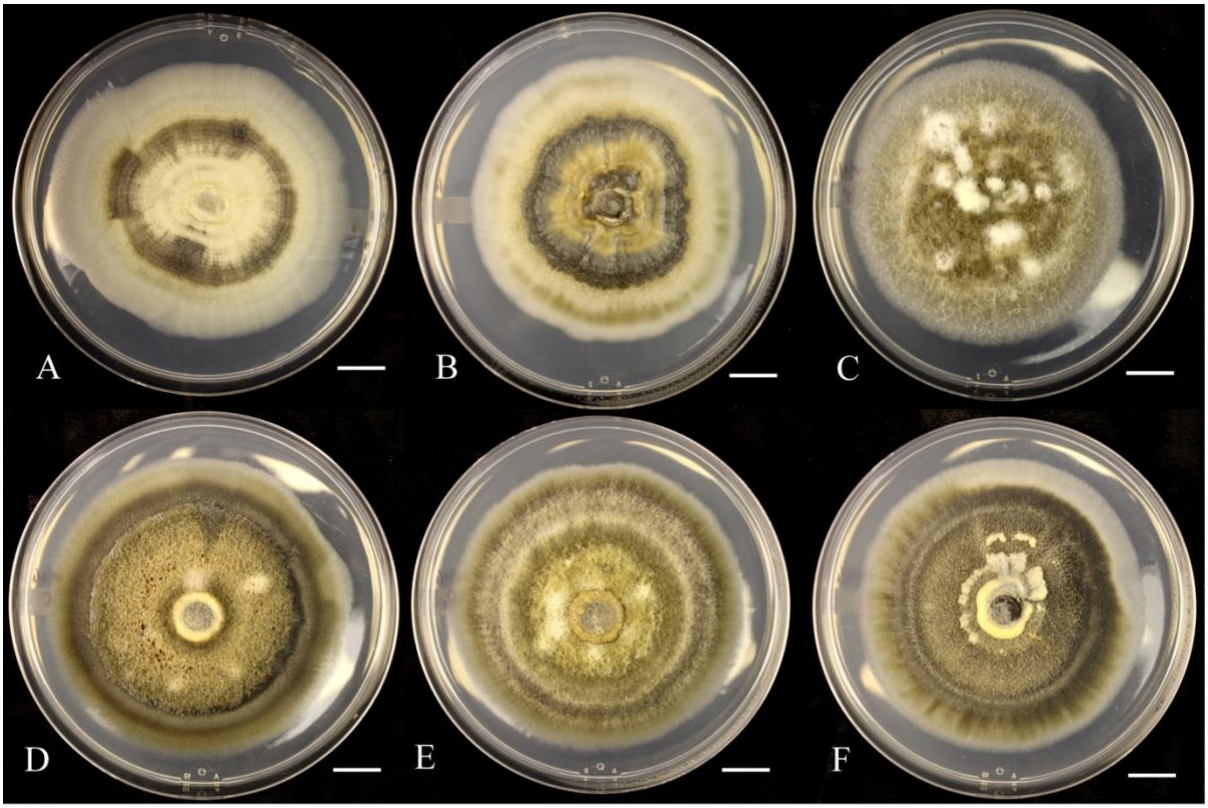

Figure 3-5. Different morphotypes of *R. arxii* isolates recovered from *X. affinis*, *X. bispinatus* and *X. volvulus*. Isolates JR25 (A) and 26 (B) were recovered from *X. bispinatus*, JR159 (C) and 160 (D) from *X. volvulus* and JR178 (E) and 179 (F) from *X. affinis*. Colonies are on malt extract agar, are 4 weeks old, and each isolate appears in the Maximum Likelihood tree in Fig. 3-2. Bars=1 cm.

CHAPTER 4
NUTRITIONAL SYMBIONTS OF A PUTATIVE VECTOR, *XYLEBORUS BISPINATUS*, OF THE LAUREL WILT PATHOGEN OF AVOCADO, *RAFFAELEA LAURICOLA*

Ambrosia beetles subsist on fungal symbionts that they carry to, and cultivate in, their natal galleries. These symbionts are usually saprobes, but some are phytopathogens. Very few ambrosial symbioses have been studied closely, and little is known about roles that phytopathogenic symbionts play in the life cycles of these beetles. One of the latter symbionts, *Raffaelea lauricola*, causes laurel wilt of avocado, *Persea americana*, but its original ambrosia beetle partner, *Xyleborus glabratus*, plays an uncertain role in this pathosystem. We examined the response of a putative, alternative vector of *R. lauricola*, *Xyleborus bispinatus*, to artificial diets of *R. lauricola* and other ambrosia fungi. Newly eclosed, unfertilized females of *X. bispinatus* were reared in no-choice assays on one of five different symbionts or no symbiont. *Xyleborus bispinatus* developed successfully on *R. lauricola*, *R. arxii*, *R. subalba* and *R. subfusca*, all of which had been previously recovered from field-collected females of *X. bispinatus*. However, no development was observed in the absence of a symbiont or on another symbiont, *Ambrosiella roeperi*, recovered from another ambrosia beetle, *Xylosandrus crassiusculus*. In the no-choice assays, mycangia of foundress females of *X. bispinatus* harbored significant colony-forming units of, and natal galleries that they produced were colonized with, the respective *Raffaelea* symbionts; with each of these fungi, reproduction, fecundity and survival of the beetle were positively impacted. However, no fungus was recovered from, and reproduction did not occur on, the *A. roeperi* and no symbiont diets. These results highlight the flexible nature of the ambrosial symbiosis, which for *X. bispinatus* includes a fungus with which it has no evolutionary history. Although the "primary" symbiont of the neotropical *X. bispinatus* is unclear, it is not the Asian *R. lauricola*.

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Background

Xyleborine ambrosia beetles (Curculionidae: Scolytinae) have symbiotic nutritional relationships with fungi (Farrell et al 2001, Mueller et al. 2005). Foundress females disseminate their fungal partners to, and propagate them in, natal galleries that they bore in host tree xylem. Gardens of these fungi serve as their and their progeny's primary food, and enable them to utilize a protected, but nutrient-poor environment for reproduction, the xylem (Bleiker et al. 2009).

Xyleborine ambrosia beetles have a haplodiploid reproduction system, also known as arrhenotoky, in which diploid females develop from fertilized and haploid males from unfertilized eggs (Cognato et al. 2011, Farrell et al. 2001, Norris 1979, Normark 2003). Males are flightless, and are generally restricted to their natal gallery in which they mate with their mother and sisters. In contrast, mature females disperse from the natal colony and are responsible for establishing broods in new sites, which depend upon the ambrosial gardens that they establish and manage.

Regardless of the female's mating status, her reproductive success and the numbers of offspring that she produces depends on the availability of nutritional symbionts for the developing colony. Females carry their nutritional fungi (the so-called "ambrosia") in specialized structures in their bodies called mycangia or mycetangia (Six 2003). Mycangia are absent in male xyleborines, and females of a given species possess a single type. Pre-oral mycangia, which are small sacs in the mandibles, are found in some taxa, whereas elytral mycangia, small cavities at the base of pronotum adjacent to the scutellum, and mesothoracic mycangia, large invaginations between the meso- and metanotum, are found in others (Francke-Grosmann 1967, Hulcr and Stelinski 2017).

Understandings of ambrosial symbioses have evolved as our appreciation of their complexity and the tools with which they could be studied have become increasingly

sophisticated (Bateman et al. 2016). These interactions were initially conceived as relationships between a given beetle and a single, primary symbiont, the "true ambrosial fungus" (Batra 1963, Francke-Grosmann 1956, 1963, Leach et al. 1940), even when multiple species of fungi were consistently associated with a given beetle species (Baker 1963). Batra (1966) later recognized that ambrosia beetles "eat one or more auxiliary ambrosia fungi". The association with more than one species of fungus was soon recognized as typical for these insects (Baker and Norris 1968).

Subsequent work has sought better information on the identity of these fungi. DNA analyses (initially partial sequences of single genes, but increasingly multi-gene geneologies) have enabled accurate censuses of the fungi (and bacteria) that are associated with these insects (Harrington and Fraedrich 2010, Hulcr et al. 2012, Kostovcik et al. 2015). A recent meta-analysis provided what is, to date, the most complete pictures of the diverse fungal communities that populate the mycangia of these insects. In that study, Kostovcik et al. (2015) inferred with ITS2 pyrosequencing Operational Taxonomic Units (OTUs) of fungi in the mycangia of *Xyleborus affinis* Eichhoff, *Xyleborus ferrugineus* Fabricius and *Xylosandrus crassiusculus* Motschulsky; each of the communities that were detected was dominated by a core group, that contained eight (48.2% of the OTUs and 78% of the reads), six (42.3% and 64%), and 11 (62.4% and 86%) of the OTUs that were observed in the respective species. In mycangial assays of culturable fungi, core suites of symbionts have also been evident. For example, three species were the predominate associates of *Euwallacea* nr. *fornicatus* (Freeman et al. 2016; Lynch et al. 2016), and three or fewer fungi were usually present in individuals of *Xyleborus glabratus* Eichhoff (Campbell et al. 2016b).

Although powerful insight into community structure and composition has been obtained from such studies, comparatively few have investigated the function of the detected taxa in these

systems. The vertical transmission of these fungi to new natal galleries and the obligate dependence of the beetles on the nutritional symbionts would be expected to promote fidelity in these systems and, thus, a prevalence of nutritional associates, as was presumed by many early workers (Batra 1966, Baker and Norris 1968). However, common associates could be adapted to survive or multiply in or on the insect or in their natal galleries; it should not be assumed that associates serve nutritional roles. For example, Freeman et al. (2016) reported that of the three species that predominated in the *Euwallacea* nr. *fornicatus* system they studied, larvae completed their development when fed on only two of the fungi, *Fusarium euwallaceae* and *Graphium euwallaceae*; they could not discern a role for a third prevalent fungus, *Paracremonium pembeum* (*Acremonium pembeum*).

Ambrosial fungi are typically saprobes that colonize the inner walls of the natal gallery. In rare cases, the fungal symbionts are pathogens that cause moderate to serious damage on host trees (Ploetz et al. 2013). In an extreme case, *Raffaelea lauricola*, a symbiont of an Asian ambrosia beetle, *Xyleborus glabratus*, is a lethal, vascular pathogen of trees in the southeastern USA, all of which are in the Lauraceae (Fraedrich et al. 2008).

We became interested in the nutritional function of *R. lauricola* after the fungus was isolated from ambrosia beetle species other than *X. glabratus*, soon after the beetle was introduced to the USA (Harrington and Fraedrich 2010). To date, *R. lauricola* has been found in nine other ambrosia beetles, all of which were present in the USA prior to the introduction of *X. glabratus* (Ploetz et al. 2017a, c). The horizontal transfer of symbionts among species of ambrosia beetle had been documented previously (Batra 1966, Harrington 2005, Gebhardt et al. 2004), but is poorly understood. Why and under what circumstances fungal symbionts establish

in new ambrosia beetle species warrants further study, especially when it involves the movement of a destructive pathogen to additional, potential vector species (Ploetz et al. 2017b, c).

In recent surveys, *X. glabratus* was rarely trapped or reared in laurel wilt-affected trees of avocado (*Persea americana* Miller) in Florida (0 of 79,025 ambrosia beetles in Miami- Dade County and only 11 of 4181 in Brevard County) (Carrillo et al. 2012, Kendra 2017). Rather, other ambrosia beetle species were associated with these trees. Several of these species harbor *R. lauricola* (Ploetz et al. 2017c), and some of them transmitted the pathogen to avocado in no-choice tests (Carrillo et al. 2014). We describe experiments with one of these beetles.

Xyleborus bispinatus (Fig. 1), a close relative of *X. glabratus* (Cognato et al. 2011), is established in South Florida (Atkinson et al. 2013), breeds in laurel wilt-affected avocado trees, and carries *R. lauricola* frequently (Ploetz et al. 2017c). In prior work with laurel wilt-affected avocado trees, the pathogen was isolated from 35% of 69 individuals of *X. bispinatus* (in four experiments, a range of 15% to 100% of the individuals that were assayed), compared to 60% of the assayed *X. glabratus* (Ploetz et al. 2017c).

Ambrosia beetles depend upon their fungal symbionts for development, sexual maturation and reproduction (Norris 1972, 1979, Kingsolver and Norris 1977). However, little is known about the role phytopathogenic symbionts play in the life cycles of these insects. Although some of those that predominate in a given beetle (vector) must play a primary, nutritional role, others might increase the fitness of a given beetle by increasing the suitability of a susceptible host tree by reducing host defenses (inducing disease development), thereby facilitating colonization by, and brood development of, the beetle partner. As suggested above, additional, nonpathogenic and non-nutritional reasons for the prevalence of a given associate are also possible.

In the present study, no-choice assays were conducted to assess the response of *X. bispinatus* to different ambrosia fungi, including *R. lauricola*. Artificial diets were used to determine the *in vitro* interaction between these fungi and foundress females and her progeny.

Materials and Methods

Symbiont Recovery and Identification

Fungal symbionts were isolated from beetles reared from laurel wilt-affected logs of avocado harvested in Miami-Dade County, FL USA. *Raffaelea lauricola, R. arxii* and *R. subalba* were obtained from pre-oral mycangia of *Xyleborus bispinatus*, and *Ambrosiella roeperi* from mesothoracic mycangia of *Xylosandrus crassiusculus*. Symbionts were identified with partial sequences of the large sub unit (LSU) and small sub unit (SSU) of ribosomal DNA, and, with the exception of *R. arxii*, β-tubulin regions. In addition, the identity of *R. lauricola* was confirmed with taxon-specific microsatellite markers described by Dreaden et al. 2014b. Voucher specimens of the symbionts that were used in this study have been deposited at the Westerdijk Fungal Biodiversity Institute (formerly Centralbureau von Schimmelcultures, CBS) (Utrecht, The Netherlands).

Artificial Media and Inoculation with Fungal Symbionts

A modified artificial medium, described by Menocal et al. (2017), consisted of 240 g of avocado sawdust, 36 g agar, 6 g sucrose, 6 g starch, 6 g yeast extract, 12 g casein, 1.5 g Wesson's salt mixture, 0.421 g tetracycline, 3 mL germ oil, 3 mL peanut oil, 6 mL ethanol and 1600 mL distilled water. Sterile medium (20 mL in 50 mL plastic centrifuge tubes CentriStarTM, Corning) was inoculated with a conidial suspension with 500 µl of 8×10^6 conidia/mL of a given symbiont. Media were colonized for 10 days prior to use, and sterile deionized H_2O was used as a control treatment. Each treatment was replicated with 24 single tube experimental units.

Feeding of Unfertilized Females and Colony Initiation

Female pupae with non-developed mycangia (Six and Paine 1998) were obtained from laboratory stock cultures of *X. bispinatus* maintained for 11 generations. Pupae were rinsed in sterile water for 4–5 seconds to remove phoretic contaminants, and were placed (fed) on 2-week-old potato dextrose agar (PDA) cultures of a given symbiont for 2 days. Control pupae were exposed solely to PDA and transferred to water- treated medium. After pupae become sclerotized adults, females were surface-disinfested with 70% ethanol for 10 s, dried with sterile filter paper, and individuals were introduced to 24 pre-colonized tubes of each of the above symbionts or noncolonized, control tubes (one female per tube). Four entry holes, 1 cm deep and 2 mm wide, were made on the medium surface to encourage gallery formation. Tubes were capped with lids fitted with a 1-cm metallic screen opening for airflow (Fig. 4-2), and maintained in darkness under laboratory conditions at 25 °C and 75 % relative humidity. Observations of behavior (grooming, gallery construction) and colony development were recorded biweekly until colonies were dissected.

Data Collection

Since females used to establish these colonies were unfertilized, all progeny were males. Each tube (experimental unit) was evaluated for brood production, survival of foundress females, male progeny, female fecundity, fungal content of mycangia and galleries, number of entry holes and gallery length.

Forty-five days after the introduction of foundress females, all colonies were dissected and eggs, larvae, pupae, dead and live males, total brood (all developmental stages included), and female survival were recorded (Fig. 1). Fungi growing on top of the media were removed and the numbers of entry holes, other than the initial artificial holes, were counted. From the primary gallery, the length of all galleries along and within the artificial media plug was

measured with a plastic flexible ruler (Fig. 4-2). The location of the brood chamber, and whether it was located in single or multiple galleries, was recorded (Fig. 4-2).

Serial dilutions (1:1, 1:10, 1:100) of macerated mycangia and 2-mm^2-samples of the gallery surface were used to calculate colony-forming units (CFUs) of the different symbionts. To recover *Raffaelea* spp., dilutions were plated on malt-extract agar plus cycloheximide and streptomycin (CSMA) (Harrington 1981), amended with rifampicin and ampicillin and for *A. roeperi* PDA amended with streptomycin and ampicillin. Tunes that contained fungi recovered from each experimental unit were counted and identified and only tubes with the inoculated symbiont were included in a given symbiont treatment.

Statistical Analyses

A non-parametric statistical analysis was performed with the statistical software R v. 3.3.2 (R Development Core Team 2016) to determine whether the fungal symbionts affected the reproductive behavior of *X. bispinatus*. Tubes in which the inoculated symbiont was recovered from mycangia and/or galleries were considered as single experimental units of a given treatment. Despite the precautions that were taken to ensure that foundress females were exposed to only one fungus or water, some tubes did not contain the desired treatment. For example, of the 24 original tubes only 17 *R. lauricola*, 16 *R. arxii*, 13 *R. subalba* and 18 *A. roeperi* diets were colonized with the desired fungus and not contaminated; only the latter tubes were considered in these analyses (Table 4-1). Several units of *Raffaelea* treatments, from which no symbiont was recovered from both the galleries and foundress mycangia, were added as units of the no symbiont treatment. Unexpectedly, *R. subfusca*, which was also recovered in several of the contaminated tubes mentioned above, was the predominant symbiont in 15 tubes of the water control treatment; it was considered as an additional treatment below. *Raffaelea subfusca* had

been recovered previously from *X. bispinatus* (Saucedo et al. 2018), and we presume it was introduced via phoretic contamination of foundress females.

Mean egg, larva and pupa production, the number of dead and live males, total brood, foundress survival, female fecundity, gallery length and the number of entry holes were compared with the Kruskal-Wallis rank sum test. This test can be applied to one-way data with non-normal distribution with more than two groups, and the *p* value (0.05) is divided by the number of comparisons made (15); thus, values <0.003 were significantly different. The Kruskal-Wallis rank sum test was also used to assess CFU differences for fungal symbionts in mycangia and galleries, as well as to compare the frequencies of brood chamber locations. Results were adjusted with the Benjamin-Hochberg method and a *post-hoc* Dunn test (Dunn 1964) was performed to classify which diets significantly impacted the reproductive behavior and survival of the beetles. Simple linear regressions between the total brood and gallery lengths produced by *X. bispinatus* females on the different diets were performed with the statistical software R v. 3.3.2.

Results

Symbiont Identification

LSU, SSU, and β-tubulin sequences for isolates of *A. roeperi* (accession CBS142880 in the Westerdijk Fungal Biodiversity Institute), *R. lauricola* (CBS142879), *R. subalba* (CBS142877) and *R. subfusca* (CBS142881) in this study were 99–100% matches for the consensus sequences of these species in GenBank; LSU and SSU sequences for the isolate of *R. arxii* were also 99–100% matches (GenBank accession numbers for *A. roeperi*: LSU-MF138153, SSU-MF138158, β-tubulin- MF138161; *R. lauricola*: LSU-MF138152, SSU-MF138157, β-tubulin-MF138160; *R. subalba*: LSU-MF138150, SSU- MF138155, β-tubulin-MF138163; *R. subfusca*: LSU- MF138154, SSU-MF138159, β-tubulin-MF138162; and *R. arxii*: LSU-

MF138151, SSU-MF138156). We were unable to amplify the β-tubulin region for *R. arxii*, which may be typical for this species (Dreaden et al. 2014a). In addition, the taxon- specific microsatellite markers of Dreaden et al. (2014b) were amplified for the isolate of *R. lauricola*.

Colony Initiation

Egg production was observed until week 4 on all diets, except that based on *R. subalba* (week 3) (Table 4-1). Larvae were observed from weeks 1 to 5 on *R. lauricola* diet, whereas on *R. arxii* and *R. subalba* diets lasted until week 4, and on *R. subfusca* diet lasted from weeks 2 to 4. Pupae were produced from weeks 3 to 5 on *R. lauricola*, *R. arxii*, *R. subalba* diets, and from weeks 4 to 5 on the *R. subfusca* diet. Similarly, male adults were observed from weeks 4 to 5 on *R. lauricola*, *R. arxii* and *R. subalba* diets, but only during week 5 on *R. subfusca* diet (Table 4-1). None of the assayed individuals on *A. roeperi* and no-symbiont treatments were able to initiate a colony.

Dissection of Artificial Diets

Although there were no significant differences among the mean total broods produced by *X. bispinatus* females on the different *Raffaelea* diets (Table 4-2), differences between these treatments and the *A. roeperi* and no- symbiont diets (on which no progeny were produced) were significant (Kruskal-Wallis test=57.243, *P*=4.507e-11, df=5). Similarly, egg (*P*=0.002, df=5), larva (*P*=6.914e-09, df=5) and pupa (*P*=1.909e-06, df=5) production were significantly different on the *A. roeperi* and no-symbiont diets compared to the *Raffaelea* diets, but did not differ among the *Raffaelea* diets.

The mean survival rate for female foundresses was significantly higher on the *Raffaelea* diets than on the *A. roeperi* and no-symbiont diets (*P*=5.068e-09, df=5). Female survival rates were 100 % on the *R. arxii* diet, 94 % on the *R. lauricola* diet, 93 % on the *R. subfusca* diet, and 77 % on the *R. subalba* diet, whereas only 39 % of the females survived on the *A. roeperi* diet,

and 13 % on the no-symbiont diet. Male survival was significantly different on *Raffaelea* diets compared with *A. roeperi* and no-symbiont diets (P=1.432e-08, df=5), but no significant differences were observed among the *Raffaelea* diets (Table 4-2).

Mean female fecundity was significantly higher on *Raffaelea* diets than on *A. roeperi* and no-symbiont diets (P=3.043e-13, df=5), and fecundity rates mirrored those for foundress survival, as 100 % of the females on *R. arxii* diet produced progeny, vs 94 % of those on *R. lauricola*, 93 % of those on *R. subalba* and 67 % of those on *R. subfusca* diets. There were no significant differences in fecundity among the different *Raffaelea* diets.

Although mean gallery length was longer on the *Raffaelea* diets than on the A. roeperi (5.3 cm) and no-symbiont diets (3.8 cm), only those on the *R. lauricola* (7.54 cm) and *R. arxii* (8.44 cm) diets were significantly greater (P=0.003, df=5) (Table 4-2). With the exception of the significant difference between the *A. roeperi* (0.67) and *R. subfusca* diets (0.07), the average number of entry holes per tube did not differ among the other treatments (P=0.02, df=5) (Table 4-2). The simple linear regression model did not correlate gallery length and total broods that were produced with different diets with the exception of that based on *R. arxii* (R^2=0.36576, P=0.013) (Fig. 4-3).

Mycangia and Gallery Content and Location of Brood Chamber

Mean CFUs of the inoculated (presented) fungi within the mycangia of females on *Raffaelea* diets were significantly different than those on *A. roeperi* and no-symbiont diets (P=2.718e-10, df=5), as *A. roeperi* was neither recovered from mycangia nor galleries of that treatment (although it did colonize the medium surface). Mean CFUs recovered from mycangia of females on *R. lauricola* diet, 1228, were higher than on other *Raffaelea* diets, but with no significant differences compared to the other *Raffaelea* diets (mean CFUs ranging from 579 to 649). The frequency with which a given species was recovered from mycangia (frequency of

recovery) also did not differ among the *Raffaelea* species, but it was significantly different compared to the *A. roeperi* and no- symbiont diets (*P*=1.359e-11, df=5). Although the inoculated symbiont was recovered from 100 % of the galleries in the different *Raffaelea* diets, only *R. lauricola* was recovered from 100 % of the assayed mycangia; *R. arxii* was recovered in 75 % of these assays, *R. subalba* 77 %, and *R. subfusca* 67 % (Table 4-3). Although there were significant differences in CFU numbers in the galleries of *Raffaelea* diets compared to the *A. roeperi* and no-symbiont diets (*P*=8.32e-14, df=5), there were no significant differences among *Raffaelea* diets.

There was a tendency for females on all *Raffaelea* diets to create most of their brood chambers in the second gallery, followed by the primary gallery (Table 4-4, Fig. 4-2). Although the number of brood chambers in primary galleries was significantly greater on *Raffaelea* than on *A. roeperi* and no- symbiont diets (on which no progeny were produced), there were no significant differences among the *Raffaelea* diets. Likewise, there were significant differences on *Raffaelea* diets compared with *A. roeperi* and no-symbiont diets for use of the secondary gallery as a brood chamber (*P*=1.987e-05, df=5), but no significant differences among the *Raffaelea* diets. Very few females established broods in the tertiary gallery, and there were no significant differences among the different diets (*P*=0.3768, df=5). Interestingly, three females on the *R. lauricola* diet laid eggs in both secondary and tertiary galleries; females did so on no other diet (*P*=0.01, df=5).

Discussion

A primary symbiont for *X. bispinatus* has not been reported. However, since *Raffaelea* species were recognized previously as the predominant symbionts of *Xyleborus* species (Harrington and Fraedrich 2010, Campbell et al. 2016b, Ploetz et al. 2017c), the reproductive success of *X. bispinatus* on four different species of *Raffaelea* was not unexpected. Nevertheless,

nutritional flexibility displayed by *X. bispinatus* was apparently limited, in that *A. roeperi*, the primary symbiont of an ambrosia beetle in another genus, *Xylosandrus crassiusculus*, did not support *X. bispinatus* development.

Little is known about the nutritional impacts fungi have on their ambrosia beetle associates. Previously, larvae of *Euwallaceae* nr. *fornicatus* fed on *Paracremonium pembeum* did not complete their life cycle, whereas those reared on *Fusarium euwallaceae* and *Graphium euwallaceae* completed development (Freeman et al. 2016). Similarly, the fungus *Paecilomyces variotii* had a negative impact on larvae and adults of *Xyleborinus saxesenii* Ratzeburg, but the beetle's primary nutritional symbiont, *Raffaelea sulfurea*, had positive effects (Biedermann et al. 2013). In addition, a detrimental impact had been shown previously with another nutritional symbiont, as Ott (2007) demonstrated that the fitness of and number of progeny produced by *X. crassiusculus* was decreased on a *R. lauricola* diet. Nonetheless, the present study appears to be the first to report the nutritional impact of several different symbionts on a given ambrosia beetle species. Better understandings are needed for these interactions and why some of these fungi are used as food sources by these insects.

Nutrients provided by different fungal diets are key determinants of the fitness and fecundity of ambrosia beetles. *X. bispinatus* females are synovigenic (produce eggs throughout their life cycle) and, thus, must continuously ingest nutrients to support egg production (Gottlieb et al. 2014). Despite these requirements, four species of *Raffaelea*, including the new encounter phytopathogen *R. lauricola*, enabled high rates of fecundity and significant brood production in *X. bispinatus* (Table 4-2).

Recent research suggests that mated females of *X. bispinatus* increase their reproductive potential in the presence of *R. lauricola* (Menocal et al. 2017). Thus, regardless of the mating

status of *X. bispinatus* females, *R. lauricola* can positively influence their reproduction. Conversely, the reproduction of *X. volvulus* Fabricius, another ambrosia beetle associated with the transmission of laurel wilt (Carrillo et al. 2014), was negatively impacted on *R. lauricola* diet, as females from field-collected bolts, which were colonized with other symbionts, had higher total broods on uncolonized diet (11.1) than on *R. lauricola* diet (3.8) (Menocal et al. 2017).

In the present study, higher numbers of CFUs of *R. lauricola* were recovered from mycangia of *X. bispinatus* (individual range: 200–2800) than were previously reported in field-collected females from native *Persea* spp. (0–53) and avocado (0–320) that were affected by laurel wilt (Ploetz et al. 2017c). Although the no-choice conditions that were used in the present study might be expected to increase propagule numbers in mycangia of the assayed females, we note that the numbers of no-choice CFUs of *R. lauricola* in *X. bispinatus* resemble those found in *X. glabratus* (0– 11,600 CFUs in *Persea* spp. and 0–1480 CFUs in avocado) (Ploetz et al. 2017c). These results highlight the ability of *X. bispinatus* to acquire and carry large amounts of *R. lauricola*, more than enough to kill susceptible avocado trees (Hughes et al. 2015a).

Gallery construction is an important element of ambrosia beetle reproduction. *Xyleborus bispinatus* females on *Raffaelea* diets produced tunnels of similar lengths, and those on *R. lauricola* and *R. arxii* diets were significantly longer than those on *A. roeperi* and no symbiont diets. Longer galleries of *Xyleborus pfeili* Ratzeburg resulted in greater brood production of that species (Mizuno and Kajimura 2009). Although our results indicate that longer galleries of *X. bispinatus* were generally more productive, gallery length on only *R. arxii* diet was significantly related to brood production (Fig. 4-3).

The number of entry holes has been used to estimate population size and describe the phenology of another ambrosia beetle, *Euwallacea* nr. *fornicatus* (Cooperband et al. 2016). However, it was not a good predictor of the reproductive behavior of *X. bispinatus*.

Xyleborus species lay eggs in multiple branches of excavated galleries, unlike *Xyleborinus* species that propagate in large cave-like galleries (Biedermann 2010). Regardless of which *Raffaelea* species they were fed, *X. bispinatus* females primarily laid eggs in secondary galleries in the present study.

Ambrosia beetles have obligate symbioses with their nutritional partners. Our results indicate that *X. bispinatus* can produce at least one generation on four different species of *Raffaelea*, including the laurel wilt pathogen *R. lauricola*. Results from the present study support prior speculation that *X. bispinatus* may be an important alternative vector in the avocado pathosystem (Ploetz et al. 2017c).

Gnotobiotic systems provide invaluable tools for examining interactions between an insect host and its symbionts (Douglas 2011). Ambrosia beetles acquire symbionts in their mycangia and/or on their exoskeletons via feeding in natal galleries. In the present study, gnotobiotic *X. bispinatus* were obtained by surface disinfesting lab-reared pupae and new adults that were later fed with selected symbionts. Most gnotobiotic *X. bispinatus* offered sterile media did not produce progeny confirming that *X. bispinatus*, as other ambrosia beetles, has an obligate mutualism with fungal symbionts. However, some surface-disinfested beetles in this study established symbioses with *R. subfusca*, which may have persisted on the pupal cuticle and new adult exoskeleton. Alternatively, the fungus may have been carried internally in a structure other than the mycangia, which are not present in the pupal stage (Six and Paine 1998). With the exception of the *R. subfusca* experimental units, the majority of new adults in our study

selectively interacted with, and established natal colonies of, the presented fungus. These results may indicate that the initial feeding period is key in determining which symbiont is used by a developing ambrosia beetle colony. Moreover, unknown attributes of a symbiont apparently determine the food value a fungus provides for a given ambrosia beetle partner, as only the *Raffaelea* symbionts in this study supported reproduction. Research is needed to understand these temporal and nutritional interactions.

Table 4-1. Timeline for developmental stages of *Xyleborus bispinatus* reared on different *Raffaelea* spp.

R. lauricola (17)	Eggs Larvae		Pupae	Males	
R. arxii (16)	Eggs Larvae		Pupae	Males	
R. subalba (13)	Eggs Larvae		Pupae	Males	
R. subfusca (15)	Eggs	Larvae		Pupae	Males
	Week 1	Week 2	Week 3	Week 4	Week 5

Table 4-2. Colony statistics (Mean±SE) for *Xyleborus bispinatus* reared on different ambrosial symbionts.

Fungal symbiont	Eggs	Larvae	Pupae	Dead males	Live males	Total brood	Female survival (%)	Female fecundity (%)	Gallery length (cm)	Entry holes
R. lauricola (*n*=17)	1.65±0.52 a	3.12±0.39 a	1.35±0.22 a	0.65±0.13 a	2.12±0.35 a	8.76±0.96 a	94±0.08 a	100 a	7.54±0.65 a	0.53±0.14 ab
R. arxii (*n*=16)	0.88±0.43 a	2.06±0.41 a	1±0.23 a	0.38±0.13 ab	1.94±0.36 a	6.31±0.99 a	100 a	94±0.06 a	8.44±0.67 a	0.44±0.14 ab
R. subalba (*n*=13)	0.23±0.17 a	1.46±0.45 a	1.15±0.25 a	0.3±0.15 ab	1.38±0.39 a	4.54±1.1 a	77±0.09 a	77±0.07 a	7.04±0.75 ab	0.23±0.16 ab
R. subfusca (*n*=15)	2.13±0.99 a	1.4±0.42 a	0.73±0.24 a	0.07±0.14 ab	1.1±0.37 a	5.4±1.02 a	93±0.09 a	67±0.07 a	6.24±0.7 ab	0.07±0.15 b
A. roeperi (*n*=18)	0 b	0 b	0 b	0 b	0 b	0 b	39 b	0 b	5.3±0.63 b	0.67±0.14 a
No symbiont (*n*=16)	0 b	0 b	0 b	0 b	0 b	0 b	13 b	0 b	3.8±0.67 b	0.19±0.14 ab

Data within a column that are followed by different letters are significantly different based on Dunn test (α=0.003). SE=Standard Error.

Table 4-3. Recovery of symbionts from mycangia and natal galleries of *Xyleborus bispinatus*.

Fungal symbiont	Mycangia			Gallery (2 mm^2)		
	Frequency of recovery (%)	CFU mean±SE	CFU range	Frequency of recovery (%)	CFU mean±SE	CFU range
R. lauricola (*n*=17)	100 a	1,228±157 a	200-2800	100	108,564±14,492 a	6000-280000
R. arxii (*n*=16)	75 a	621±162 a	0-1600	100	91,243±14,938 a	5500-244000
R. subalba (*n*=13)	77 a	579±179 a	0-1600	100	83,276±16,572 a	200-210400
R. subfusca (*n*=15)	67 a	649±167 a	0-3600	100	151,386±15,428 a	800-320000
A. roeperi (*n*=18)	0 b	0 b	0	0	0 b	0
No symbiont (*n*=16)	0 b	0 b	0	0	0 b	0

Data within columns followed by different letters are significantly different based on Dunn test (α=0.003). SE=Standard Error.

Table 4-4. Number (Mean±SE) of brood chambers in each location (gallery level).

Fungal symbiont	Primary	Secondary	Tertiary	Secondary and Tertiary
R. lauricola	6 (0.35±0.08) a	7 (0.41±0.09) a	1	3
R. arxii	2 (0.13±0.08) a	11 (0.69±0.1) a	2	0
R. subalba	4 (0.31±0.09) a	5 (0.38±0.11) a	1	0
R. subfusca	2 (0.13±0.08) a	8 (0.53±0.1) a	0	0
A. roeperi	0 b	0 b	0	0
No symbiont	0 b	0 b	0	0

Data within columns followed by different letters are significantly different based on Dunn test (α=0.003). SE=Standard Error.

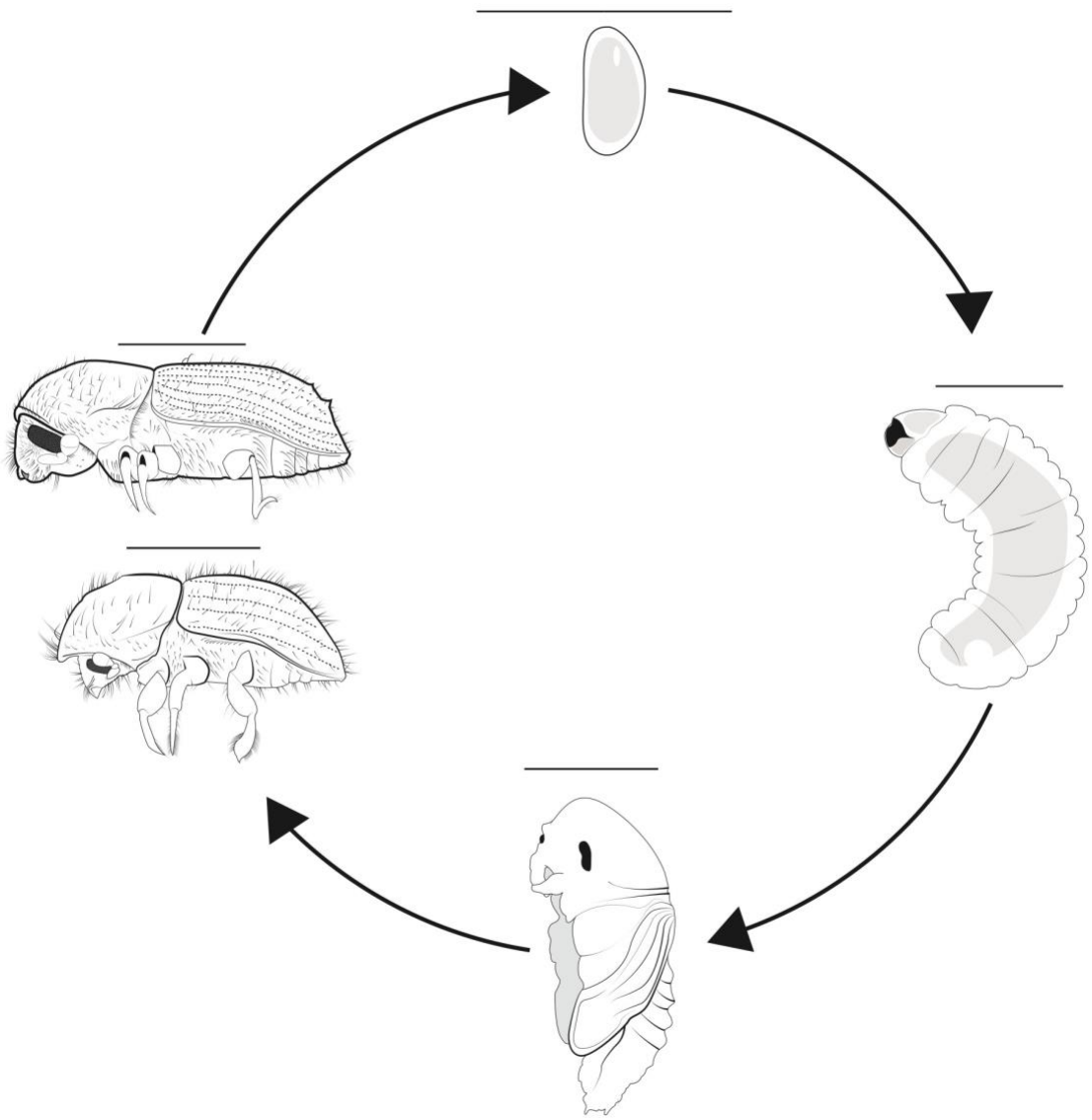


Figure 4-1. Life cycle of *Xyleborus bispinatus*. In clockwise order, an egg (top image) is followed by a larva, pupa, adult male (botton) and adult female (top). Scales associated with the different life stages are 1 mm.

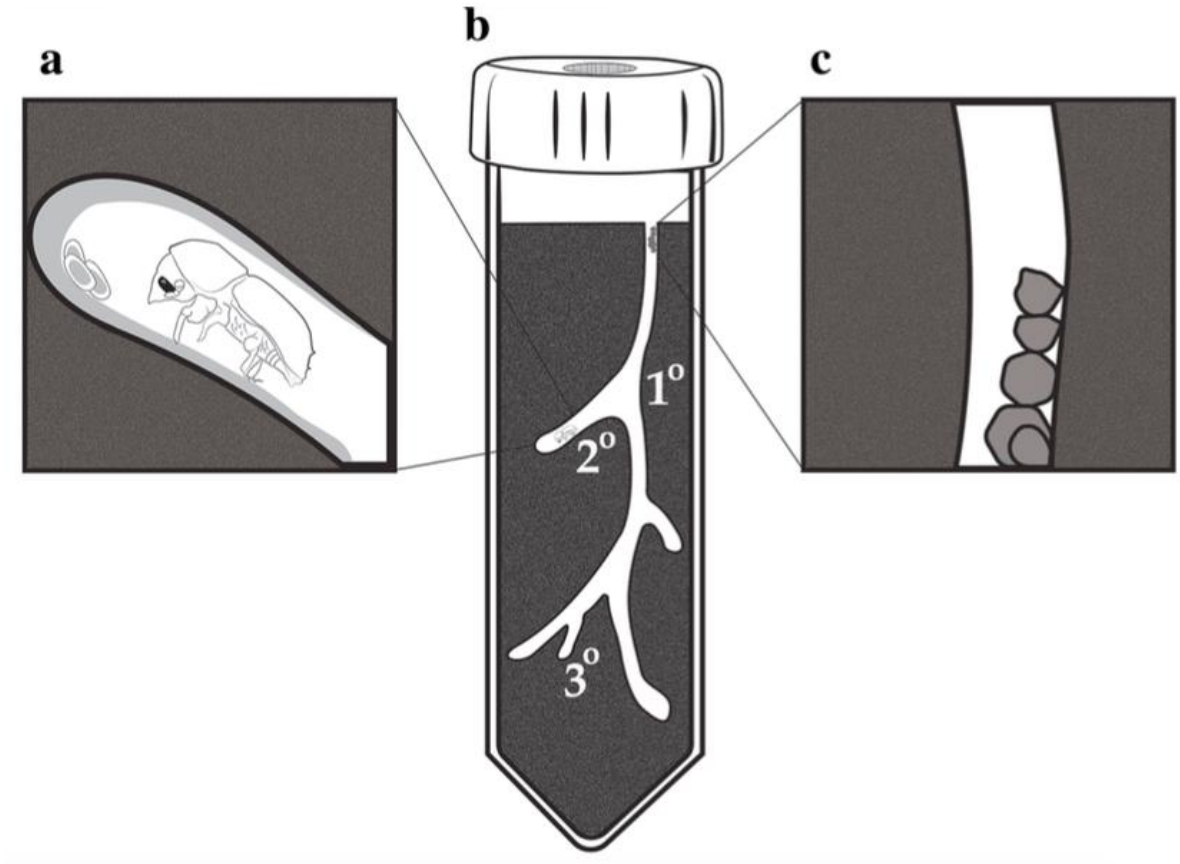


Figure 4-2. Schematic illustration of the artificial rearing system used in this study. **a**) Adult females and eggs of *Xyleborus bispinatus* surrounded with a fungal layer in a secondary (**2º**) gallery. **b**) Tube in which primary (**1º**), secondary (**2º**) and tertiary (**3º**) galleries have been produced in the artificial medium. Note female foundress and eggs in the indicated secondary gallery, and the screen opening on the tube cap. **c**) Frass/refuse generated by foundress female.

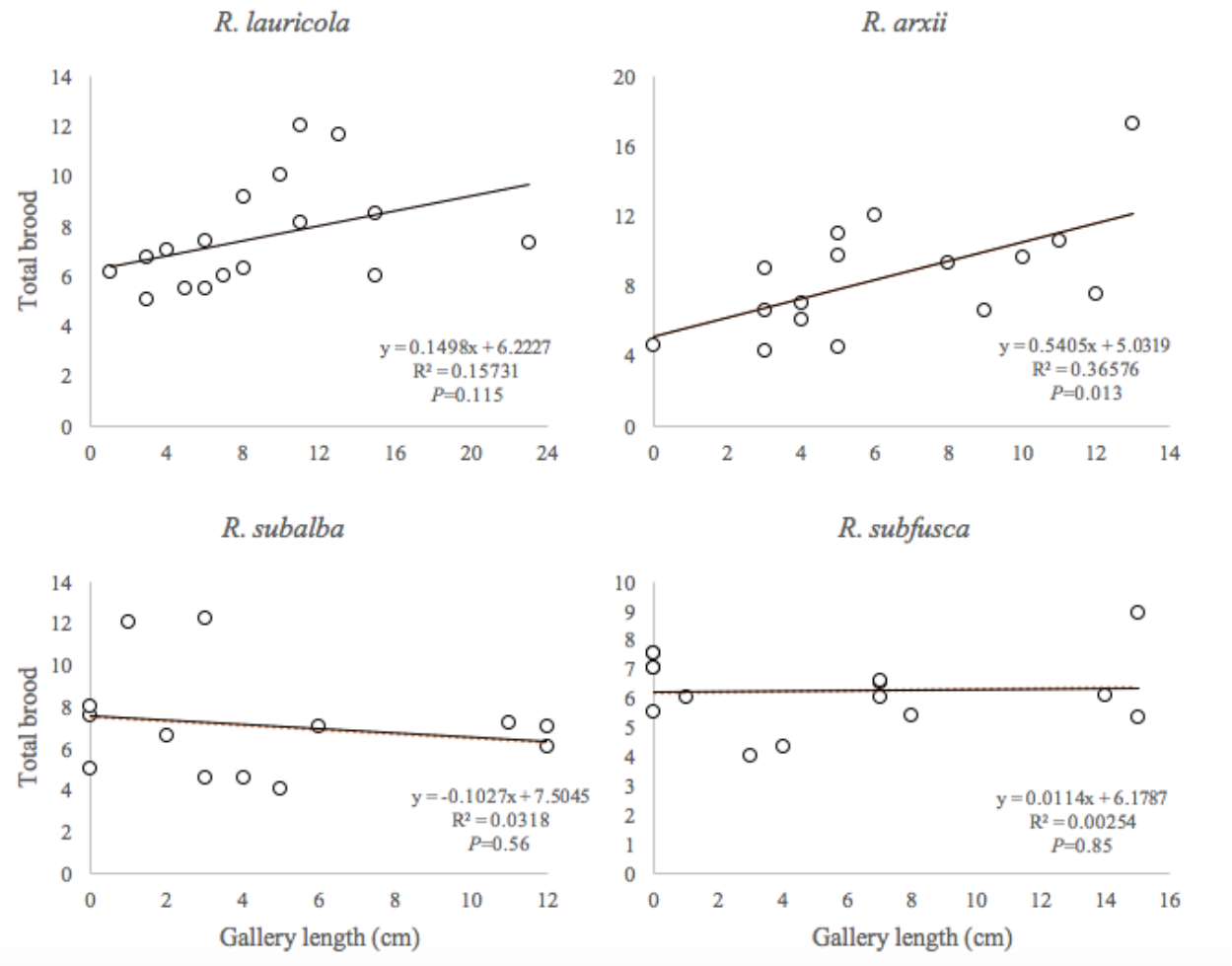


Figure 4-3. Simple linear regressions of total broods produced by *Xyleborus bispinatus* on different *Raffaelea* diets plotted against total gallery length.

CHAPTER 5
MINERAL CONTENT OF AMBROSIA FUNGI, XYLEM ENDOPHYTES FROM AVOCADO AND HOST AND NON-HOST XYLEM TISSUE

Nutritional symbiotic fungi are essential for the survival and reproduction of ambrosia beetles. Fungal symbionts enable ambrosia beetles to thrive and reproduce in the impoverished xylem tissue. These fungal symbionts are commonly saprobes and concentrate nutrients from dead wood, but in unusual cases some are phytopathogenic and kill healthy trees. For instance, *Raffaelea lauricola*, nutritional partner of *Xyleborus glabratus*, causes laurel wilt of avocado. Since the introduction of *R. lauricola* to South Florida, the pathogen has been recovered from other native beetles in the region, but it is not known whether these beetles have adopted it as a nutritional symbiont or how it compares nutritionally to its other established symbiotic fungi. In this study, we investigated the mineral profiles of fungal symbionts as well as endophytes and sawdust from hosts that they colonize. The different fungi were grown on malt-extract liquid media and avocado wood to measure the colonization rates and compare the nutritional profiles for each substrate. Our results indicate that endophytes colonize avocado wood faster than fungal symbionts, and among fungal symbionts, *R. sulphurea*, *A. roeperi* and *R. lauricola* were the fastest colonizers of avocado wood. Few differences of mineral content were found among the different fungal taxa, and there were slight differences between avocado, silkbay and mango sawdust. Our results confirm that *R. lauricola* does not possess extraordinary nutritional attributes, and that other important traits such as olfactory, organoleptic or gustatory features are more important than nutritional content.

Background

Fungi are essential dietary sources for insects in the orders Hymenoptera (ants), Isoptera (termintes) and Coleoptera (ambrosia beetles) (Mueller et al. 2005). Mutualistic symbioses with fungi are critical for the reproduction and survival of ambrosia beetles (Curculionidae:

Scolytinae). Ambrosia beetles colonize xylem tissue in host plants, where nutrients are scarce, and have developed an obligatory dependence on fungi (Mueller et al. 2005, Jordal and Cognato 2012). Ambrosial fungi serve as the sole source of food for adult females and progeny of these insects, and the foundress females cultivate gardens of fungi within their natal galleries where new generations of mature females acquire and disperse the fungi to new hosts (Beaver 1989).

Although host plant tissue may provide important elements to supplement the dietary requirements of mycophagous beetles, these nutrients are scarce and are not usually found in a digestible form (Clayton 1964, Martin 1979). In contrast, fungal symbionts contain essential nutrients such as proteins, lipids, carbohydrates, sterols, amino acids and vitamins that these insects cannot synthesize and that the host does not provide in adequate amounts. Although nutrients provided by the ambrosial fungi are important, the nutritional composition of these fungi is poorly understood.

Ambrosial symbionts have pleomorphic growth, and occur in a yeast-like mucilaginous phase within specialized structures of ambrosia beetles called mycangia, as well as in a filamentous mycelial phase within the host that is consumed by the beetles (Beaver 1989). Although the hyphae of ambrosial fungi usually do not colonize further than the lining of natal gallery, the capacity of ambrosial fungi to colonize and concentrate nutrients from xylem tissue are key determinants in the fitness and reproduction of ambrosia beetles (Francke-Grosman 1956, Beaver 1989). The rate of growth of ambrosial fungi determines the success and the developmental rate of females' progeny in ambrosia beetles as well as the capacity to colonize the natal galleries constructed in the host (Kaneko 1965). Little is known about the colonization of natural substrates by ambrosial fungi.

While the mineral composition of macrofungi is well known (Cochrane 1958, Mallikarjuna et al. 2013), little is known about the mineral content of ambrosia fungi. In insect nutrition, phosphorous, potassium, calcium, magnesium, sodium, chloride, iron, zinc and manganese play important roles (Behmer 2008). Nitrogen consumption by insects has been positively correlated in larger size of individuals compared with species that have a diet low in nitrogen (Ayres et al. 2000). Nitrogen content of fungal tissue is highly variable and depends upon the fungi's growth medium. Aside from carbon, which comprises 40-50 % of fungal tissue (Lilly 1965), phosphorous and potassium are the most abundant elements in fungi (Cochrane 1958, Garraway and Evans 1984, Mallikarjuna et al. 2013).

Although most ambrosia fungi are saprophytic, there are unusual cases in which the fungi are pathogenic and kill susceptible hosts (Ploetz et al. 2013). For instance, *Raffaelea lauricola*, the primary nutritional symbiont of the Asian ambrosia beetle, *Xyleborus glabratus*, is a vascular wilt pathogen of host trees in the Lauraceae family (Fraedrich et al. 2008).

After *X. glabratus* was introduced into the US, *R. lauricola* was recovered from nine other ambrosia beetle species (Ploetz et al. 2017c). Saucedo et al. (2017) demonstrated that one of these species, *Xyleborus bispinatus* Eichhoff, could reproduce exclusively on a diet of *R. lauricola*, *R. arxii*, *R. subalba*, and *R. subfusca* whereas *Ambrosiella roeperi*, primary symbiont of *X. crassiusculus*, did not support reproduction. Although there appears to be a relationship between the type of mycangium a beetle possesses and its propensity to carry diverse symbionts (Saucedo et al. 2018), there are many unknowns regarding these symbioses, including whether nutrients that the ambrosia fungi provide are responsible for flexibility that is evident in some of these interactions.

The reasons why some ambrosia beetles carry different symbionts in their mycangia and utilize them as nutritional resources are not clear. However, better understandings of the nutritional attributes of the fungal symbionts could provide insight into the flexible or non-flexible nature of these interactions, and why *R. lauricola* has moved among some native ambrosia beetles in the US. We examined the mineral content of three different groups of fungi that colonize the xylem of avocado: ambrosial fungi, plant-pathogenic endophytes (fungi that may become pathogenic) and non-pathogenic endophytes. We hypothesized that ambrosial fungi significant differences in mineral content compared to other fungi that occur in avocado xylem. Furthermore, we assessed the growth of the different fungi on avocado wood to evaluate whether different colonization rates were evident among the different categories of fungi.

Materials and Methods

Source of Samples

Ambrosia fungi were recovered from adult females of ambrosia beetles reared from laurel wilt affected-avocado trees in Miami-Dade County, Florida. *Raffaelea* species were recovered from pre-oral mycangia of *Xyleborus bispinatus*, with the exception of *R. sulphurea,* which was recovered from pronotal mycangia of *Xyleborinus saxesenii* Ratzeburg, and *A. roeperi,* from the mesonotal mycangia of *Xylosandrus crassiusculus* Motschulsky (Saucedo et al. 2017). Pathogenic and non-pathogenic endophytes were recovered from the xylem of healthy avocado trees (Pérez et al. 2018) (Table 5-1). Sawdust was collected from healthy trees of avocado, silkbay (*Persea humilis* Nash) and mango (non-host) (*Mangifera indica* L) as described previously (Menocal et al. 2017).

Identification of Fungi

Genomic DNA of fungal endophytes was amplified with primer pairs ITS1 and ITS4 of the partial region of the internal transcribed spacer (ITS) (White et al. 1990). Partial regions of

the large subunit (LSU), small subunit (SSU) and β-tubulin DNA of fungal symbionts were amplified with primer pairs LROR/LR5, NSU1/NSU4 and Bt2a/Bt2b, respectively (White et al. 1990, Glass and Donaldson 1995), with the exception of the β-tubulin region of *R. arxii* and *R. suphurea*. Blast queries of the nucleotide database of the National Center for Biotechnology Information (NCBI) and Maximum Likelihood phylogenetic analyses described by Saucedo et al. (2018) and Pérez et al. (2018) were used to identify fungi, and the identity of *R. lauricola* was corroborated with taxon-specific microsatellite markers, CHK and IFW (Dreaden et al. 2014b).

Fungal Biomass Production and Colonization Assays

Mycelium was produced on malt-extract broth (MEB) (Difco™ Malt Extract Broth BD 211320). Erlenmeyer flasks (125 ml) containing 50 ml of MEB were inoculated with 9-mm mycelial plugs and placed in a gyratory shaker (200 rpm). Two weeks later, mycelium was collected, rinsed with sterile deionized water, air-dried for 48 hours at 24 ºC, and stored at -80 ºC until processing. Each fungus was replicated 10 times and completely randomized. Mycelial biomass production was performed twice. Sawdust of avocado, silkbay and mango was obtained using a sander to generate a fine powder texture from dry xylem tissue of debarked logs.

Avocado wood pieces (5 cm^2) were sterilized in an autoclave on the liquid cycle for 60 min and inoculated with 9 mm mycelial plugs from single-spore isolates. Mycelial plugs were placed upside down on the center of the wood piece and radial growth was measured in the same direction every 12 hours. Avocado pieces were placed in petri dishes (15 cm diameter) until mycelia colonized the edge of the wood piece. Each fungus was replicated four times and completely randomized.

Mineral Content

Total nitrogen, carbon and sulfur content of mycelium and sawdust samples were determined by combustion (Vario Max Elemental CNS Analyzer, Elementar Analysensysteme Gmbh), whereas potassium, calcium, iron, zinc, magnesium, manganese and sodium were determined with inductively coupled plasma-mass spectrometry (ICP-MS, Perkin Elmer Elan DRCe, Perkin-Elmer, Wellesley, MA).

Statistical Analysis

Means for mineral content were analyzed using a one-way ANOVA with the Tukey HSD test using JMP Pro 13.2.0 software (SAS Institute Inc., Cary, North Carolina). The area under the growth progress curve (AUGPC) was used to quantitate colonization rates for fungi; it was calculated numerically using the trapezoidal rule, in which the average value of two data readings was multiplied by the time interval (the number of hours from the first data reading to the second one) (Campbell and Madden 1990). A Kruskal-Wallis rank sum test was performed to determine whether the AUGPC for each fungus differed. We adjusted the *p*-values using the Benjamini-Hochberg method and a *post-hoc* Dunn test (Dunn 1964) was performed to statistically separate groups at $p<0.0001$.

Results

Colonization of Avocado Wood

All fungi were able to colonize sterilized avocado wood. Colonization rate of the pathogenic endophyte, *Lasiodiplodia theobromae* (AUGPC mean of 7034), was significantly greater than that for other fungi with the exception of *Nigrospora oryzae* (6930) (Kruskal-Wallis chi-square=47.80, *P*=<0.001, df=12) (Figure 5-1). *Fusarium solani* (6100), grew faster than the other fungi with the exception of the three ambrosial fungi, *R. sulphurea* (6030), *A. roeperi* (5625) and *R. lauricola* (5391). *Raffaelea arxii* (2799) colonized avocado wood more slowly

than the other fungi, but the growth rate was not significantly less than *R. subfusca* (3660), *R. subalba* (3560) and *Hypoxylon monticulossum* (3438). *Purpureocillium lilacinum* (5084) and *Chaetomium globossum* (4456) grew at an intermediate rate significantly different from the other endophytes as well as three ambrosial fungi (*R. sulphurea*, *R. subalba*, and *R. arxii*).

Mineral Content

Nitrogen content (% of dry weight) in mycelium of *F. solani* (4.33 %) and *H. monticulossum* (4.28 %) was significantly greater than that in *R. aguacate* (3.14 %), *R. subfusca* (2.96 %), *R. lauricola* (2.95 %), *P. lilacinum* (2.59 %), and *L. theobromae* (3.12 %). Nitrogen content in sawdust from avocado (1.41 %), silkbay (0.3 %) and mango (0.2 %), as well as malt-extract powder (1.41 %), was significantly lower than that in all of the fungi that were tested (p=0.05) (Figure 5-2). *Purpureocillium lilacinum* (52.93 %) had the greatest amount of carbon and had significantly more than mango sawdust (42.42 %) and malt-extract powder (40.29 %), but no significantly different than other non-pathogenic endophyte *C. globossum* (45.09 %), a pathogenic endophyte, *H. monticulossum* (46.15 %), two ambrosial fungi, *A. roeperi* (49.56 %) and *R. sulphurea* (47.36 %), and avocado (45.62 %) and silkbay (45.53 %) sawdust. *Ambrosiella roeperi* and *R. sulphurea* were the ambrosial fungi with the highest amount of carbon compared with the other *Raffaelea* species (p=0.05). No significant differences were found among sulfur content in all the samples (p=0.05).

Avocado sawdust (652.5 mg/kg) had significantly more calcium (mg/kg) than the rest of the samples. In a lesser degree, mango sawdust (337 mg/kg) also had significantly higher amounts of calcium with the exception of *A. roeperi* (259 mg/kg) which was the ambrosial fungi with the greatest amount of calcium. With the exception of *R. lauricola* (143 mg/kg) compared with *R. arxii* (45 mg/kg), there were no significant differences among the other *Raffaelea* species. In contrast, malt-extract powder (9.7 mg/kg) had the lowest calcium concentration, but

not significantly less than most ambrosial fungi excluding *A. roeperi* and *R. lauricola*, nor significantly less than *P. lilacinum* (101 mg/kg) and *L. theobromae* (96 mg/kg) (p=0.05) (Figure 5-2).

Silkbay sawdust (95 mg/kg) had the significantly highest amount of iron (mg/kg), whereas *Nigrospora oryzae* (48.5 mg/kg) and *H. monticulossum* (45 mg/kg) were significantly different than two non-pathogenic endophytes, *P. lilacinum* (15.8 mg/kg) and *C. globossum* (14.4 mg/kg), two pathogenic endophytes, *F. solani* (21 mg/kg) and *L. theobromae* (20 mg/kg), three ambrosial fungi, *R. subfusca* (22.6 mg/kg), *R. sulphurea* (19.65 mg/kg) and *R. lauricola* (19.45 mg/kg), as well as malt-extract powder (7 mg/kg). Additionally, *R. arxii* (39 mg/kg) was the only ambrosial fungi with significant differences compared with *P. lilacinum* and *C. globossum* and with malt-extract powder (p=0.05).

Among ambrosial fungi, *R. arxii* (15,350 mg/kg), *R. subfusca* (14,650 mg/kg), *R. subalba* (14,250 mg/kg) and *R. lauricola* (13,850 mg/kg) had the highest amount of potassium (mg/kg), which was only statistically greater than the pathogenic endophyte *H. monticulossum* (6,760 mg/kg), malt-extract powder (4,625 mg/kg) and both silkbay (3,265 mg/kg) and mango (2,320 mg/kg) sawdust. Likewise, avocado sawdust (12,790 mg/kg), a non-pathogenic endophyte *N. oryzae* (12,700 mg/kg) and two pathogenic enodphytes *L. theobromae* (11,060 mg/kg) and *F. solani* (10,630 mg/kg) were significantly higher than malt-extract powder, silkbay and mango sawdust (p=0.05).

Ambrosiella roeperi (3,155 mg/kg) was the ambrosial fungi with the most magnesium (mg/kg) and along with *C. globossum* (2,790 mg/kg), *F. solani* (2,555 mg/kg), *H. monticulossum* (2,325 mg/kg) and four additional ambrosial fungi, *R. subalba* (2,565 mg/kg), *R. arxii* (2,510 mg/kg), *R. lauricola* (2,330 mg/kg), *R. subfusca* (2,290 mg/kg) and *R. sulphurea* (2,035 mg/kg),

it had statistically larger quantities than *P. lilacinum* (1,155 mg/kg), mango (1,195 mg/kg), avocado (1,085 mg/kg) and silkbay (1,017 mg/kg) sawdust and malt-extract powder (698.5 mg/kg) (p=0.05).

Only avocado sawdust (37.5 mg/kg) had significantly more manganese (mg/kg) compared with the other sawdust from the other hosts, malt-extract powder and the fungi. There were no significant differences among the sawdust types or other fungi with the exception of *C. globossum* (19.65 mg/kg) when compared with *P. lilacinum* (8.22 mg/kg), three ambrosial fungi, *R. sulphurea* (7.28 mg/kg), *R. subalba* (7.25 mg/kg), and *R. lauricola* (6.66 mg/kg), and malt-extract powder (6.71 mg/kg) (p=0.05).

R. lauricola (2,390 mg/kg) had the most significant amount of sodium (mg/kg) among all the members of the different groups of fungi, but not significantly greater than malt-extract powder (2,190 mg/kg). Also, another ambrosial fungus, *A. roeperi* (2,010 mg/kg) and a pathogenic endophyte, *L. theobromae* (1,970 mg/kg), were not significantly different than malt-extract powder. Similarly, *C. globossum* (1,470 mg/kg) had less sodium but no significantly lower than *R. arxii* (1,295 mg/kg), *H. monticulossum* (1,205 mg/kg) and *N. oryzae* (1,190 mg/kg). In contrast, avocado (293 mg/kg) and silkbay (281.5 mg/kg) had the significant lowest amounts of sodium compared with mango (857 mg/kg) sawdust and the other fungi from the different groups (p=0.05).

Significantly more zinc (mg/kg) was found in *L. theobromae* (27.7 mg/kg) compared with four different species of *Raffaelea*, *R. aguacate* (15.7 mg/kg), *R. subfusca* (14.35 mg/kg), *R. lauricola* (13.45 mg/kg) and *R. sulphurea* (10.54 mg/kg) including *F. solani* (8.51 mg/kg), *P. lilacinum* (6.31 mg/kg), malt-extract powder (2.85 mg/kg) as well as silkbay (6.69 mg/kg) and mango (1.18 mg/kg) sawdust. Nonetheless, zinc content of *L. theobromae* was not significantly

higher than three ambrosial fungi, *A. roeperi* (25.75 mg/kg), *R. subalba* (20.2 mg/kg), *R. arxii* (16.55 mg/kg), two non-pathogenic endophytes, *C. globossum* (24.3 mg/kg), *N. oryzae* (20.9 mg/kg), a pathogenic endophyte, *H. monticulossum* (22.75 mg/kg) and avocado (24.15 mg/kg) sawdust (p=0.05).

Discussion

We report that fungal symbionts and endophytes differ in their mineral content and rates at which they colonized host wood. Although *R. lauricola* is the only fungus that was tested that colonizes avocado xylem systemically (Ploetz et al. 2013, Dreaden et al. 2016, Pérez et al. 2018), all fungi in the present study grew on sterile avocado wood. Kaneko (1965) indicated that "the rate of growth of the ambrosial fungi influences the rate of growth of the insect and its offspring" based on a study with *X. germanus*. In the present study, *R. sulphurea*, *A. roeperi* and *R. lauricola* colonized avocado wood more rapidly than *R. arxii*, *R. subalba* and *R. subfusca*. Nonetheless, *R. lauricola* and the three latter species all supported the development of *X. bispinatus* when grown on avocado sawdust medium (Saucedo et al. 2018). Except for *H. monticulossum*, the non-ambrosial fungi colonized avocado wood faster than ambrosial fungi.

Nitrogen, which is known to be low in wood substrata (Cowling and Merrill 1966), plays an important role in ambrosia beetle nutrition. Batra and Downing (1963) indicated that ambrosial fungi provide a highly nitrogenous diet for these insects, and Abrahamson (1969) stated that ambrosia beetles have an obligatory reliance on nitrogen provided by their fungal symbionts. In fungi, nitrogen content generally ranges from 1-7 % (dry weight), depending upon the species, type of tissue, type of substrate, and age of cultures (Cochrane 1958, Cowling and Merrill 1966, Martin 1979). These authors report that nitrogen content differ among different tissues of the wood-rotting *Fomes laricis*, *F. fomentarius* and *Polyporus versicolor*, the fruiting bodies ranged from 0.71 to 1.13 %, the mycelia from 0.23 to 3.27 % and the spores 3 % and that

fungi grown in culture range from 2.27 to 7.6 %. They also noted that nitrogen content is higher in young developing tissue and decreased with age. In the fungal mycelia we harvested at the same age, nitrogen content ranged from 2.59 to 4.33 %, and there were no significant differences among ambrosial fungi (Figure 5-2). Conversely, sawdust from different trees contained much lower levels of nitrogen (range of 0.2-0.37 %). Although the ambrosia fungi we examined increased nitrogen to 10 times the levels found in wood substrates, it was also clear that other fungi that could grow on avocado wood also accumulated nitrogen.

Carbon is an essential element for the synthesis of compounds that form cell wall materials, proteins and nucleic acids (Cochrane 1958). Usually, carbon is the major component of fungal tissue and accounts for 40-50 % of its dry weight and is not affected by age or growth conditions (Lilly 1965). These results coincide with the carbon content found in all the samples that we tested, in which *R. subfusca* contained the lowest amount (36.78 %) and *P. lilacinum* the highest (52.93 %). While the carbon content in most of the fungal symbionts was not higher than the sawdust host samples, it is an important element found in the fungal symbionts (French 1972).

Calcium is not considered a mineral for fungal growth, but it is important for the membrane structure and a major nutrient required by plants (Griffin 1981). In comparison with the calcium content reported from some members of members of Basidiomycota (842-1,687 mg/kg) (*Amanita* species) (Vetter 2005), the calcium levels found in the Ascomycota fungi (45-259 mg/kg) that we analyzed were lower. The calcium content of the sawdust from avocado (652.5 mg/kg) and mango (337 mg/kg) were the greatest, whereas the fungal samples had consistently lower quantities, with *A. roeperi* (259 mg/kg) having the most and *R. arxii* (45 mg/kg) having the least. We found no significant differences in potassium levels (7,975-15,350

mg/kg) in fungal samples with exception of *H. monticulossum* (6760 mg/kg). These results were much lower in comparison with edible basidiocarps reported by Vetter (2005) (4,700-55,600) and species of *Pleurotus* (24,720-36,340 mg/kg) (Mallikarjuna et al. 2013), *Agaricus* (26,400-60,590 mg/kg) and *Termitomyces* (34,240 mg/kg) (Sudheep and Sridhar 2014).

Although considerable levels of calcium, iron, magnesium, manganese, potassium, sodium and zinc are essential dietary sources necessary for enzymatic reactions in most insects (Medici and Taylor 1967, Dadd 1973, Behmer 2003), the concentrations of these minerals was distinct in all the members of the different groups of fungi or sawdust that we analyzed. The greatest iron content was found in silkbay sawdust (95 mg/kg). For the fungal samples, iron concentration (14.4-48.5 mg/kg) were much lower compared with Agaricales (63.50-112.33 mg/kg) and Polyporales (54-84.33 mg/kg) reported by Kalyoncu (2010) as well as Agaricales (52.3 mg/kg), Boletales (96.3 mg/kg), Polyporales (137 mg/kg) and Pezizales-Ascomycota (36.5-117 mg/kg) described by Lalotra et al. (2016). In contrast, the magnesium content of the Ascomycota fungi assayed (1,155-3,155 mg/kg) was higher than the content in Basidiomycota described by Mallikarjuna et al. (2013) (210-407 mg/kg) and Vetter (2005) (979-1,561 mg/kg) but similar to the Saccharomycetales-Ascomycota (1,450-3,100 mg/kg) described by Okorokov et al. (1975). Sulfur has been detected in different groups of fungi from and may function as a component for amino acid and vitamin synthesis (Garraway and Evans 1984), however, none of the samples that we analyzed contained significant amounts of sulfur.

The concentration of manganese in fungi varies considerably and is required for the activation of enzymes (Cochrane 1958). The manganese levels in our samples were all similar with the exception of avocado sawdust, which had the highest amount (37.5 mg/kg), and *R. lauricola*, which had the lowest amount (6.66 mg/kg). The fungal sample results were similar to

those reported by Mallikarjuna (2013) (5.4-11.2 mg/kg) but lower than the range described by Vetter (2005) (8.6- 48.1 mg/kg) and Lalotra et al. (2016) (33.6-54.4 mg/kg). In comparison with studies of sodium content found in Basidiomycetes made by Surinrut et al. (1987) (260-870 mg/kg), Kalyoncu (2010) (66.5-117.33 mg/kg) and Vetter (2005) (222-2755 mg/kg), the Ascomycota fungi in our study contained higher levels of sodium (716.5-2,390 mg/kg). Although this content might have been influenced by the high levels of sodium found in the malt-extract powder (2,190 mg/kg) to culture the fungi, Sudheep and Sridhar (2014) reported similar levels of sodium in the Agaricales-Basidiomycota collected in forestry soils (1,713-1,447 mg/kg). Conversely, our zinc levels were lower than the data indicated by Surinrut et al. (1987) (68-131 mg/kg), Vetter (2005) (41.9-176 mg/kg), Kalyoncu et al. (2010) (47.4-87.4 mg/kg), Mallikarjuna et al. (2013) (15.8-94.4 mg/kg) and Lalotra et al. (2016) (52.3-137 mg/kg). The lowest (6.31 mg/kg) and the highest (27.7 mg/kg) average sodium content was found in *P. lilacinum*-Hypocreales and *L. theobromae*-Botryosphaeriales, respectively.

Bridges and Norris (1977) indicated that fungal symbionts provide a "rich meal" of essential nutrients for the reproduction of *X. ferrugineus*. Our results suggest that both fungal symbionts and endophytes accumulate elements that are otherwise scarce or inaccessible in wood. However, none of the fungi we studied accumulated exceptional amounts of any mineral we evaluated.

Due to the unexceptional mineral content of ambrosial fungi, we assume that other gustatory or organoleptic features of these foods are more important than the elements we assessed. These results support our hypothesis that *R. lauricola* does not possess an exceptional mineral nutrient when compared to other fungal symbionts or endophytes. Additional work is warranted to understand the nutritional value of symbionts in the diets of ambrosia beetles.

Table 5-1. Source and taxonomy of tested fungi.

Fungi[a]	Taxonomic order	Source[b]	Category
Raffaelea lauricola	Ophiostomatales	*Xyleborus bispinatus*	Ambrosial fungi
Raffaelea arxii	Ophiostomatales	*Xyleborus bispinatus*	Ambrosial fungi
Raffaelea subalba	Ophiostomatales	*Xyleborus bispinatus*	Ambrosial fungi
Raffaelea subfusca	Ophiostomatales	*Xyleborus bispinatus*	Ambrosial fungi
Raffaelea aguacate	Ophiostomatales	*Xyleborus bispinatus*	Ambrosial fungi
Raffaelea sulphurea	Ophiostomatales	*Xyleborinus saxesenii*	Ambrosial fungi
Ambrosiella roeperi	Microascales	*Xylosandrus crassiusculus*	Ambrosial fungi
Chaetomium globossum	Sordariales	Avocado xylem	Non-pathogenic endophytes
Nigrospora oryzae	Trichosphaeriales	Avocado xylem	Non-pathogenic endophytes
Purpureocillium lilacinum	Hypocreales	Avocado xylem	Non-pathogenic endophytes
Fusarium solani	Hypocreales	Avocado xylem	Pathogenic endophytes
Hypoxylon monticulossum	Xylariales	Avocado xylem	Pathogenic endophytes
Lasiodiplodia theobromae	Botryosphaeriales	Avocado xylem	Pathogenic endophytes

[a]Ambrosia fungi were identified with partial sequences of LSU, SSU ribosomal genes and β-tubulin genes with the exception of *R. arxii* and *R. sulphurea*. Non-pathogenic and pathogenic endophytes were identified with partial sequences of the partial region of the ITS.

[b]Taxonomic order of Ophiostomatales and Microascales were assigned based on Saucedo et al. 2018, whereas Sordariales, Trichosphaeriales, Hypocreales, Xylariales and Botryosphaeriales on Pérez et al. 2018.

[c]*Raffaelea* species were recovered from pre-oral mycangia of *X. bispinatus* with the exception of *R. sulphurea* recovered from elytral mycangia of *X. saxesenii* and *A. roeperi* from mesonotal mycangia of *X. crassiusculus*. Endophytes were recovered from healthy trees of avocado xylem.

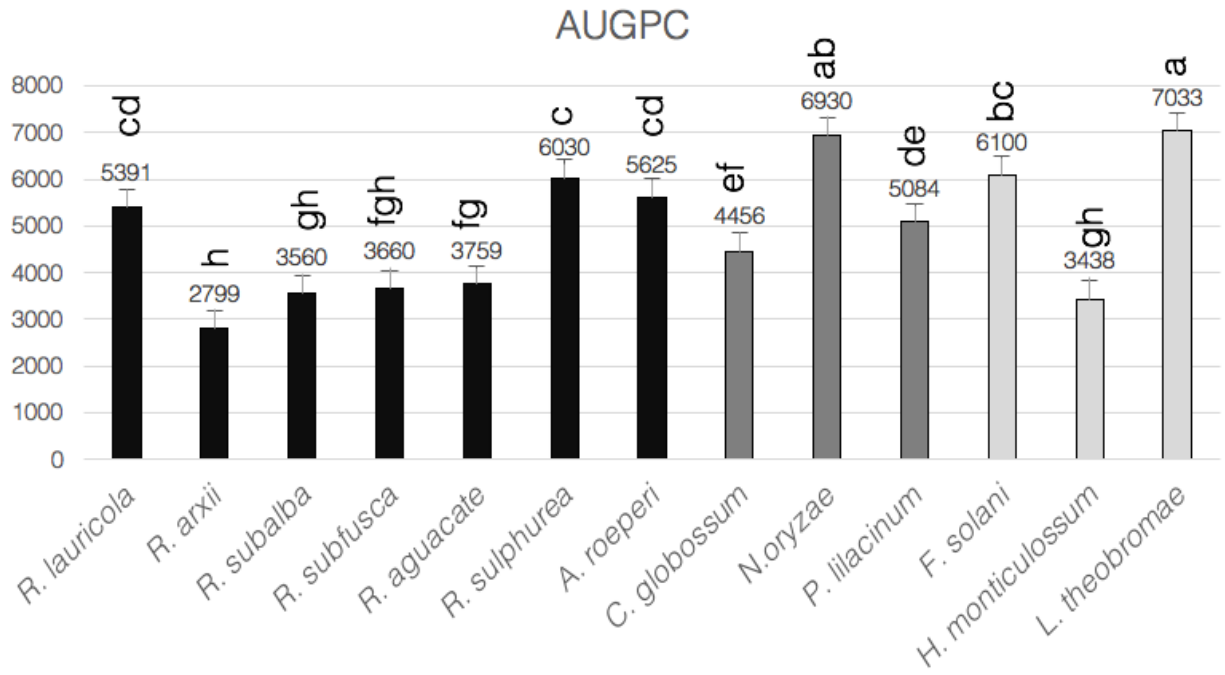


Figure 5-1. Area under growth progress curve (AUGPC) of colonization rates of fungal symbionts and endophytes. Fungal species not connected by the same letter are significantly different based on one-way ANOVA with the Tukey HSD at $p<0.05$. Bars colored in black represent the ambrosia fungi (*Raffaelea* and *Ambrosiella*), dark gray the non-pathogenic fungi (*Chatetomium*, *Nigrospora*, *Purpureocillium*) and light gray the pathogenic fungi (*Fusarium*, *Hypoxylon*, *Lasiodiplodia*).

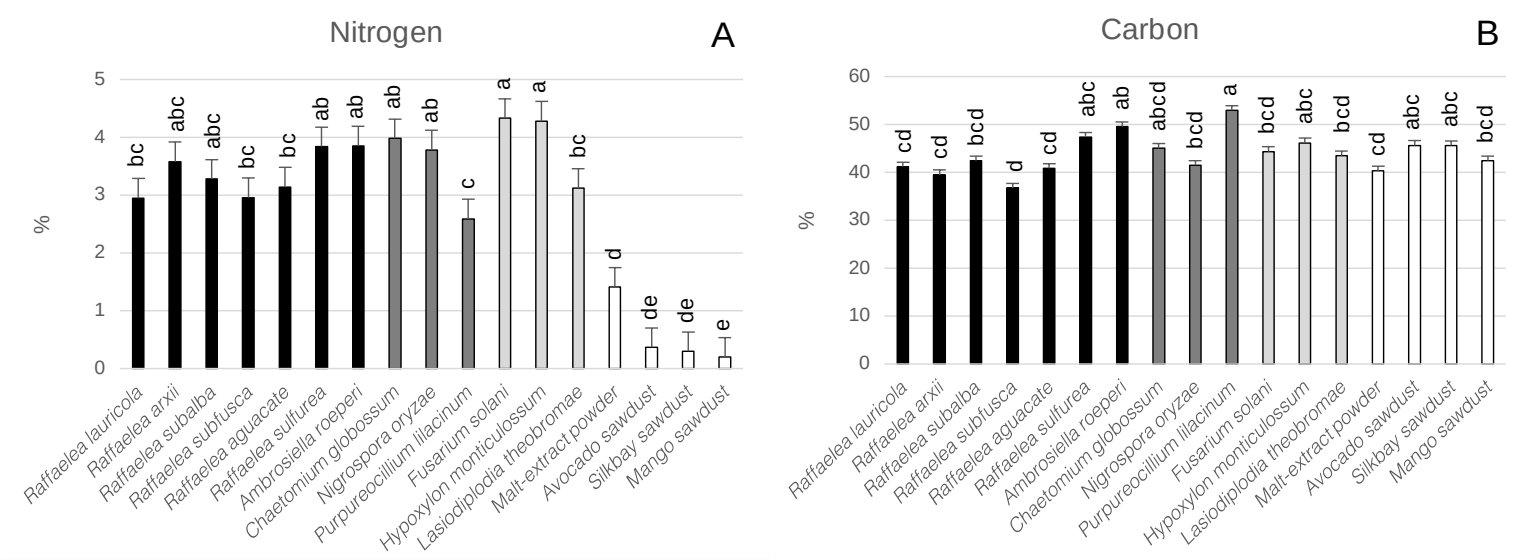
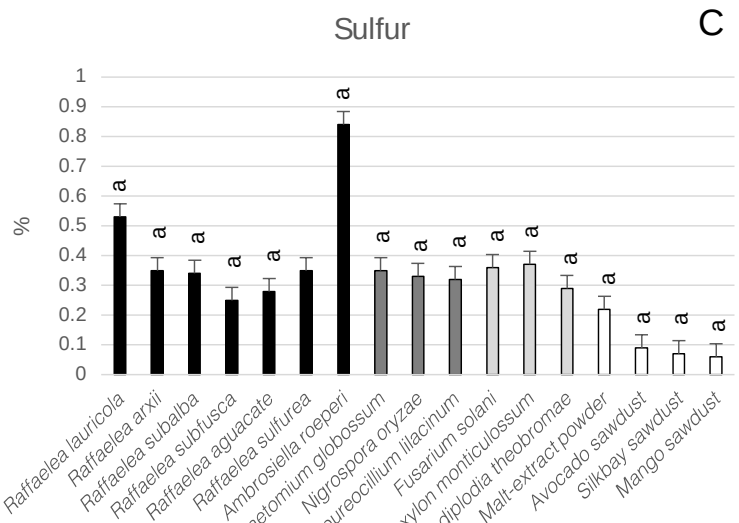


Figure 5-2. Mineral composition of fungi grown on malt-extract liquid media and sawdust samples. Bars separated by different letters are significantly different based on one-way ANOVA with the Tukey HSD at $p<0.05$. Nitrogen (A), Carbon (B) and Sulfur (C) are expressed as % of dry weight, whereas Calcium (D), Iron (E), Potassium (F), Magnesium (G), Manganese (H), Sodium (I), Zinc (J) are expressed as mg/kg. Bars colored in black represent the ambrosial fungi, dark gray the non-pathogenic fungi, light gray the pathogenic fungi and white the sawdust samples and malt-extract powder. Figure 5-2 continued on the following pages.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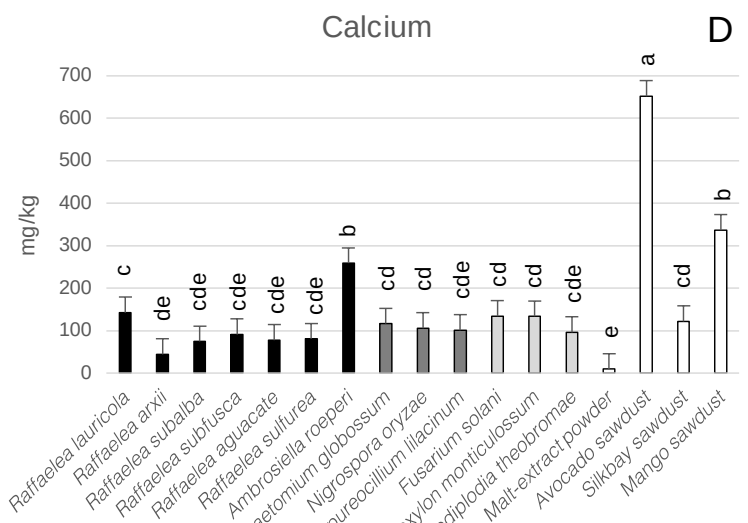
Raffaelea lauricola
Raffaelea arxii
Raffaelea subalba
Raffaelea subfusca
Raffaelea aguacate
Raffaelea sulfurea
Ambrosiella roeperi
Chaetomium globossum
Nigrospora oryzae
Purpureocillium lilacinum
Fusarium solani
Hypoxylon monticulossum
Lasiodiplodia theobromae
Malt-extract powder
Avocado sawdust
Silkbay sawdust
Mango sawdust


Calcium
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mg/kg
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600
500
400
300
200
100
0
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Raffaelea lauricola
Raffaelea arxii
Raffaelea subalba
Raffaelea subfusca
Raffaelea aguacate
Raffaelea sulfurea
Ambrosiella roeperi
Chaetomium globossum
Nigrospora oryzae
Purpureocillium lilacinum
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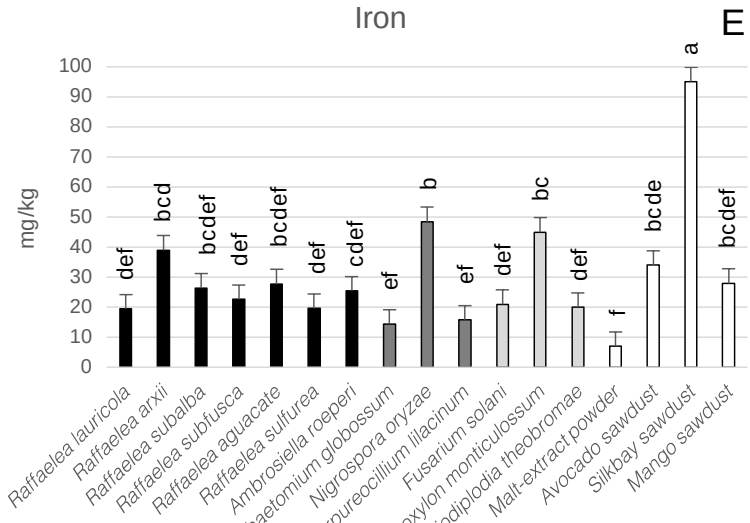
Malt-extract powder
Avocado sawdust
Silkbay sawdust
Mango sawdust


Iron
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mg/kg
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40
30
20
10
0
def bcd bcdef def bcdef def cdef ef b ef def bc def f bcde a bcdef
Raffaelea lauricola
Raffaelea arxii
Raffaelea subalba
Raffaelea subfusca
Raffaelea aguacate
Raffaelea sulfurea
Ambrosiella roeperi
Chaetomium globossum
Nigrospora oryzae
Purpureocillium lilacinum
Fusarium solani
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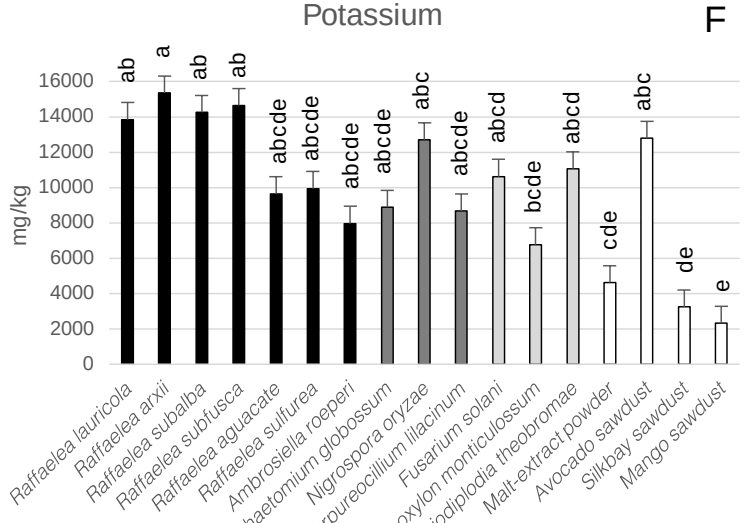
Lasiodiplodia theobromae
Malt-extract powder
Avocado sawdust
Silkbay sawdust
Mango sawdust


Potassium
F
mg/kg
16000
14000
12000
10000
8000
6000
4000
2000
0
ab a ab ab abcde abcde abcde abcde abc abcde abcd bcde abcd cde abc de e
Raffaelea lauricola
Raffaelea arxii
Raffaelea subalba
Raffaelea subfusca
Raffaelea aguacate
Raffaelea sulfurea
Ambrosiella roeperi
Chaetomium globossum
Nigrospora oryzae
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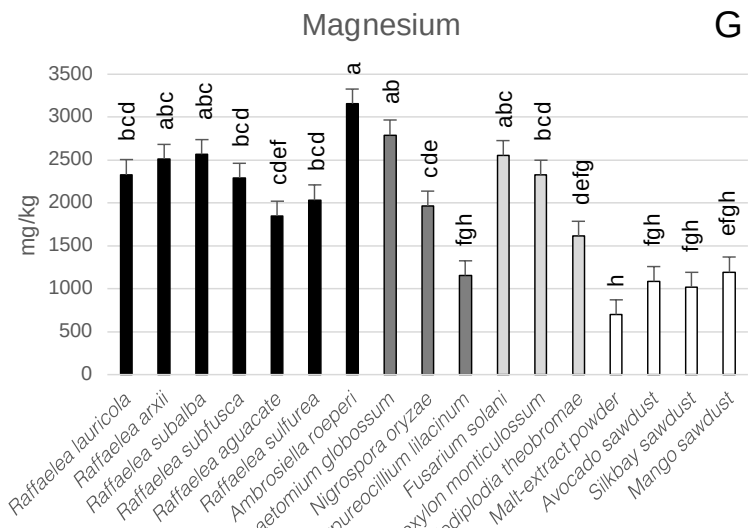
Malt-extract powder
Avocado sawdust
Silkbay sawdust
Mango sawdust


Magnesium
G
mg/kg
3500
3000
2500
2000
1500
1000
500
0
bcd
abc
abc
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fgh
abc
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defg
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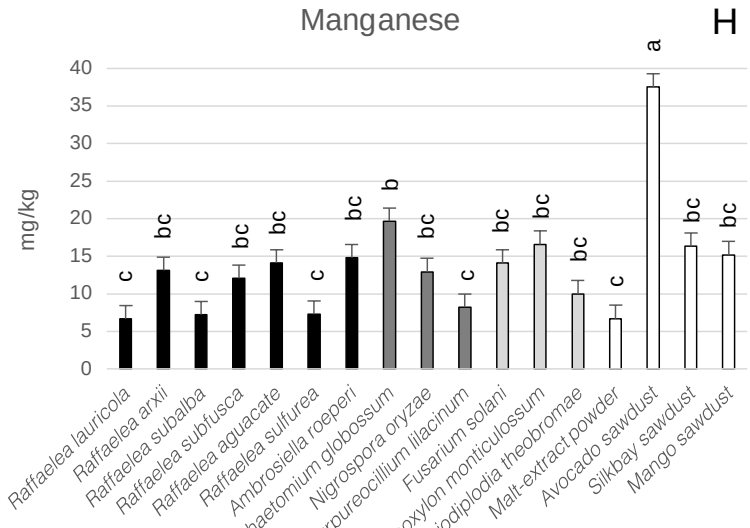
fgh
fgh
efgh
Raffaelea lauricola
Raffaelea arxii
Raffaelea subalba
Raffaelea subfusca
Raffaelea aguacate
Raffaelea sulfurea
Ambrosiella roeperi
Chaetomium globossum
Nigrospora oryzae
Purpureocillium lilacinum
Fusarium solani
Hypoxylon monticulossum
Lasiodiplodia theobromae
Malt-extract powder
Avocado sawdust
Silkbay sawdust
Mango sawdust


Manganese
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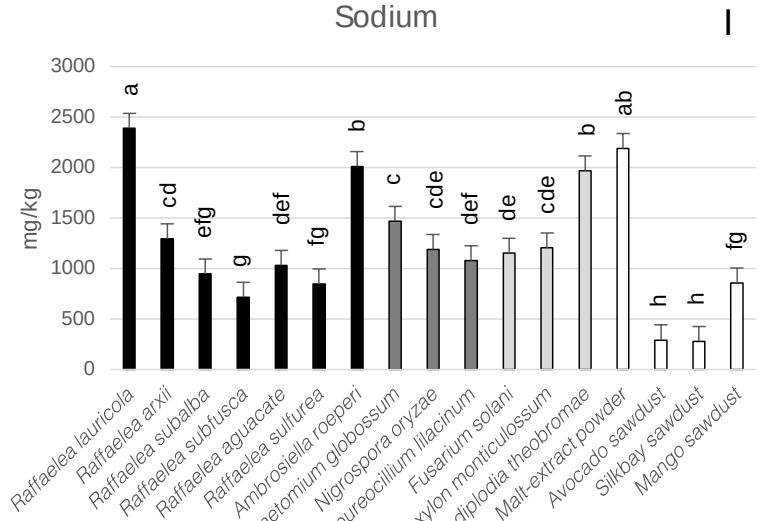
Raffaelea lauricola
Raffaelea arxii
Raffaelea subalba
Raffaelea subfusca
Raffaelea aguacate
Raffaelea sulfurea
Ambrosiella roeperi
Chaetomium globossum
Nigrospora oryzae
Purpureocillium lilacinum
Fusarium solani
Hypoxylon monticulossum
Lasiodiplodia theobromae
Malt-extract powder
Avocado sawdust
Silkbay sawdust
Mango sawdust


Sodium
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Raffaelea lauricola
Raffaelea arxii
Raffaelea subalba
Raffaelea subfusca
Raffaelea aguacate
Raffaelea sulfurea
Ambrosiella roeperi
Chaetomium globossum
Nigrospora oryzae
Purpureocillium lilacinum
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Malt-extract powder
Avocado sawdust
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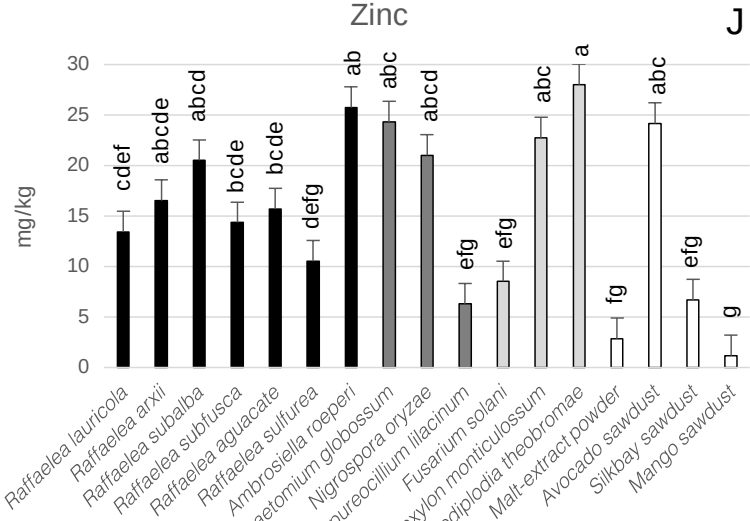
Zinc
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Raffaelea lauricola
Raffaelea arxii
Raffaelea subalba
Raffaelea subfusca
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Raffaelea sulfurea
Ambrosiella roeperi
Chaetomium globossum
Nigrospora oryzae
Purpureocillium lilacinum
Fusarium solani
Hypoxylon monticulossum
Lasiodiplodia theobromae
Malt-extract powder
Avocado sawdust
Silkbay sawdust
Mango sawdust

CHAPTER 6
CONCLUSIONS

This research investigated the epidemiology of laurel wilt in avocado and examined the response of a probable alternative vector of *R. lauricola*, *Xyleborus bispinatus*, to symbionts recovered from it and another ambrosia beetle, *Xylosandrus crassiusculus.* Symbiont communities were assessed in ambrosia beetles commonly recovered from laurel wilt-affected avocado, and the mineral content of these and other fungi recovered from avocado xylem was determined.

Our results provide insight into the associations of several described and undescribed fungi with members of the *Xyleborini* and suggest an exceptional flexibility in *Raffaelea – Xyleborus* symbioses. Distinct fungal populations were recovered from pre-oral mycangia of *Xyleborus* species, and *Raffaelea* symbionts were their predominant partners (*R. arxii*, *R. lauricola*, *R. subalba*, *R. subfusca*, *R. sulphurea* and undescribed species). The abundant and persistent association of *R. arxii* with *X. affinis* and *X. volvulus*, suggest that this species may be the primary symbiont of two different beetle species. However, it is unlikely that *X. affinis* and *X. volvulus* are important vectors of *R. lauricola* due to the infrequent association and the scarce amounts of the pathogen found in both beetle species. Fungal communities associated with elytral and mesonotal mycangia of *X. saxesenii* and *X. crassiusculus* were less diverse and *R. lauricola* was not recovered. *Xyleborus bispinatus* had indiscriminate associations with multiple *Raffaelea* species and yeasts and its primary symbiont(s) is(are) unclear. *Xyleborus glabratus* had the greatest and most consistent association with *R. lauricola* in silkbay trees and *X. bispinatus* in avocado, which supports the hypothesis that *X. bispinatus* may be an important alternative vector of *R. lauricola* in the avocado pathosystem. This study also indicates that specific yeast communities are adapted as internal associates of the beetle species in this study.

In vitro interactions between *X. bispinatus* and different *Raffaelea* species were flexible, as *R. arxii, R. lauricola, R. subalba* and *R. subfusca* supported reproduction of the beetle. However, no development was observed in the absence of a symbiont or on a symbiont of *X. crassiusculus*, *Ambrosiella roeperi*. In this study, high number of colony-forming units of *R. lauricola* were recovered from mycangia and natal galleries of *X. bispinatus*. These results highlight the ability of *X. bispinatus* to acquire and carry significant amounts of *R. lauricola*, more than enough to kill a susceptible avocado tree. This research also indicates that the initial feeding period of gnotobiotic *X. bispinatus* is crucial to determine which symbiont is used to develop an ambrosia beetle colony.

The last chapter of this thesis described mineral profiles of different categories of fungi that colonize the xylem of avocado. These results suggest that both fungal symbionts and endophytes accumulate elements (magnesium, nitrogen, potassium, sodium and zinc) that are otherwise scarce or inaccessible in wood. However, none of the ambrosial fungi accumulated exceptional amounts of any mineral we evaluated, compared with fungal endophytes. Moreover, our results indicate that fungal symbionts are not exceptional colonizers of avocado wood. Based on these results, we confirm that the mineral content of *R. lauricola* is not different from that of other fungal symbionts or endophytes; other gustatory or organoleptic features are apparently more important determinants of these interactions.

The results of this research provide important information about laurel wilt transmission in avocado orchards. In the present study, we confirm that *X. bispinatus* may play an important role in the epidemiology of laurel wilt in avocado. *Xyleborus bispinatus* carried higher numbers of CFUs of *R. lauricola* in field and laboratory conditions than all but *X. glabratus*, more than enough to initiate a lethal infection of a susceptible avocado tree. *Raffaelea lauricola* had a

positive impact on the reproduction of *X. bispinatus*. However, mineral content of *R. lauricola* did not differ from that in other ambrosia fungi and fungal endophytes. The research also sheds light on the influence of fungal symbionts, including an exotic plant pathogen, on a putative ambrosia beetle vector.

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BIOGRAPHICAL SKETCH

José Ramón Saucedo Carabez was born in Culiacan, Sinaloa, México but he grew up in Uruapan, Michoacan, México. The youngest of three children, José Ramón pursued his father's career as agronomist in the sugarcane fields in Michoacan. In 2010, he received his B.Sc. degree in Agronomy from the Facultad de Agrobiología "Presidente Juárez"-Universidad Michoacana de San Nicolás de Hidalgo in Uruapan, Michoacan. From 2008-2012, he worked as an agronomist on crops such as avocado, blackberry, mango, sugarcane and tomato. In 2013, he received his M.Sc. degree in Plant Pathology from Colegio de Postgraduados in Texcoco, Estado de México. Afterwards, he transitioned to work with blueberry, blackberry, raspberry and strawberry at Driscoll's in the Department of Applied Research in Central Mexico (Michoacán and Jalisco). In 2014, he received his Ph.D. degree from the University of Florida in the Plant Pathology Department in the summer of 2018. José Ramón is happily married and has always been very hardworking and dedicated to achieve his personal and academic goals. He enjoys doing sport outdoors and specially playing basketball in the street courts. Although he has enjoyed the experience of being at the University of Florida as a graduate student under the direction of Randy Ploetz, he looks forward to returning to his family in Michoacan and establishing the blueberry orchard he planned with his father and family.