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# The Fungus *Raffaelea lauricola* Modifies Behavior of Its Symbiont and Vector, the Redbay Ambrosia Beetle (*Xyleborus Glabratus*), by Altering Host Plant Volatile Production

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**Abstract** The redbay ambrosia beetle *Xyleborus glabratus* is the vector of the symbiotic fungus, *Raffaelea lauricola* that causes laurel wilt, a highly lethal disease to members of the Lauraceae family. Pioneer *X. glabratus* beetles infect live trees with *R. lauricola*, and only when tree health starts declining more *X. glabratus* are attracted to the infected tree. Until now this sequence of events was not well understood. In this study, we investigated the temporal patterns of host volatiles and phytohormone production and vector attraction in relation to laurel wilt symptomology. Following inoculations with *R. lauricola*, volatile collections and behavioral tests were performed at different time points. Three days after infection (DAI), we found significant repellency of *X. glabratus* by leaf odors of infected swamp bay *Persea palustris* as compared with controls. However, at 10 and 20 DAI, *X. glabratus* were more attracted to leaf odors from infected than non-infected host plants. GC-MS analysis revealed an increase in methyl salicylate (MeSA) 3 DAI, whereas an increase of sesquiterpenes and leaf aldehydes was observed 10 and 20 DAI in leaf volatiles. MeSA was the only behaviorally active repellent of *X. glabratus* in laboratory bioassays. In contrast, *X. glabratus* did not prefer infected wood over healthy wood, and there was no associated significant difference in their volatile profiles. Analyses of phytohormone profiles revealed an initial increase in the amount of salicylic acid (SA) in leaf tissues following fungal infection, suggesting that the SA pathway was activated by *R. lauricola* infection, and this activation caused increased release of MeSA. Overall, our findings provide a better understanding of *X. glabratus* ecology and underline chemical interactions with its symbiotic fungus. Our work also demonstrates how the laurel wilt pathosystem alters host defenses to impact vector behavior and suggests manipulation of host odor by the fungus that attract more vectors.

**Keywords** Bark beetles · Salicylic acid · Pathogen-induced volatiles · Laurel wilt

Xavier Martini and Marc A. Hughes have equal contribution to this study

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## Introduction

Plant pathogens modify the physiological status of their host plant. In addition to causing potential changes to plant phloem, xylem, phytohormones or micro-element ratios (Berger et al. 2007), plant pathogens can also modify volatiles emitted by their hosts qualitatively and quantitatively (Davis et al. 2012; Mann et al. 2012; Mayer et al. 2008; McLeod et al. 2005). In the case of plant pathogens vectored by insects, these changes in host odors

following the infection may influence vector behavior (Eigenbrode et al. 2002; Mauck et al. 2010, 2016).

Several studies have reported that headspace volatiles of pathogen-infected plants can be more attractive than those of their uninfected counterparts. The manipulation mechanisms involved in these host-vector-pathogen interactions have been described in several plant diseases of economic importance such as potato leafroll virus (Eigenbrode et al. 2002), cucumber mosaic virus (Mauck et al. 2010), citrus greening (Mann et al. 2012), or zebra chip disease of potato (Davis et al. 2012). McLeod et al. (2005) reported that the bark beetle, *Hylurgopinus rufipes* Eichhoff, is preferentially attracted to elm trees infected with the Dutch elm disease pathogen (*Ophiostoma novo-ulmi*) compared with uninfected elms. Increased attraction of beetles to infected hosts could lead to increased disease transmission and thereby an abundance of infective vectors (Martini et al. 2016). However, most previous experimental protocols have compared uninfected plants to pathogen-infected plants without distinction between the different stages of disease symptomology. In one case, the preference of the aphid *Myzus persicae* between potato plants infected with potato leafroll virus and uninfected potato plant were examined over time (Werner et al. 2009). The authors found that preference of aphids for infected plants only occurred 4 and 6 weeks after virus infection, indicating that release of attractive pathogen-induced volatiles might not be constant over time. In the current study, we report how behavioral responses of *Xyleborus glabratus* Eichhoff (Coleoptera: Curculinoidae: Scolytinae) to host plant volatiles are modulated during different stages of infection by the laurel wilt pathogen, *Raffaelea lauricola.*

The redbay ambrosia beetle, *X. glabratus*, and fungal pathogen, *R. lauricola*, are native to Asia (Harrington et al. 2011; Rabaglia et al. 2006) and likely entered the United States simultaneously in the early 2000s in solid wood material (Haack 2003; Rabaglia et al. 2006). *Xyleborus glabratus* is attracted to most native Lauraceae tree and shrub species in the United States (Hughes et al. 2015b). Upon attack, ambrosia beetles inoculate host sapwood with *R. lauricola*, a mycangial symbiotic fungus that leads to complete crown wilt and death (Fraedrich et al. 2008). Eleven Lauraceae species susceptible to laurel wilt have been described thus far (Kendra et al. 2013). Among the trees most affected by laurel wilt are swamp bay (*Persea palustris* [Raf.] Sarg.) and redbay (*P. borbonia* [L.] Spreng), which are both important components of forests in the southeastern United States (Brendemuehl 1990; Fraedrich et al. 2008; Hughes et al. 2015b; Kendra et al. 2013) and significant food sources for wildlife (Fraedrich et al. 2007). The spread of laurel wilt in forests of the southeast United States altered the tree community composition (Spiegel and Leege 2013). Such changes could lead to soil erosion (Gao et al. 2015), disturbance of forest hydrology (Ford and Vose 2007), and alteration of water quality (Jenkins et al. 1999; Yorks et al. 2003). Additionally, recent infection of avocado (*P. americana* Mill.) groves by laurel wilt in south Florida puts the health of economically important tree crops in danger (Crane et al. 2013; Ploetz et al. 2011).

Contrary to most ambrosia beetle species that prefer to attack stressed and weakened trees (Hulcr and Dunn 2011), *X. glabratus* readily attacks healthy and undamaged host trees (Fraedrich et al. 2008; Mayfield et al. 2008; Mayfield and Brownie 2013). The initial inoculation with *R. lauricola* of trees by pioneer beetles is followed by an incubation phase during which *X. glabratus* are not present in the tree interior of the newly infected tree. This phase is succeeded by wilting of individual branches that quickly progresses throughout the entire canopy ultimately resulting in tree death. During decline, there is an important increased arrival of other *X. glabratus* females and other ambrosia beetle species (Fraedrich et al. 2008; Kendra et al. 2013). To explain this sequence of events, we investigated how infection with the fungus changes the volatile profile emitted by the host plant and how these changes affect response of *X. glabratus* to odors produced by infected trees.

Several studies have investigated the chemical ecology of *X. glabratus* since its arrival in North America. It was quickly established that contrary to other bark and ambrosia beetle species, *X. glabratus* is not attracted by ethanol, a semiochemical associated with tree decay (Hanula et al. 2008; Johnson et al. 2014). Extensive research has shown that *X. glabratus* is particularly attracted to sesquiterpenes present in the wood and cambium of host Lauraceae, particularly $\alpha$-copaene (Hanula and Sullivan 2008; Hanula et al. 2008; Niogret et al. 2013) and has led to the development of new and effective lures emitting $\alpha$-copaene (Kendra et al. 2015a). To a lesser degree, *X. glabratus* is also attracted to the volatiles of its symbiotic fungus, *R. lauricola* (Hulcr et al. 2011; Kuhns et al. 2014b), but also to host plant leaf volatiles (Martini et al. 2015). This attraction toward redbay tree volatiles may explain attacks occurring on apparently undamaged redbay trees. This attraction is mediated by monoterpenes found in both leaf and wood volatiles of redbay trees.

The effect of *R. lauricola* infection on volatile release from host Lauraceae has not been previously investigated. Hanula et al. (2008) demonstrated no discrimination between fungus-infected and healthy host logs by *X. glabratus* during field-trapping experiments. However, since it has been demonstrated that *X. glabratus* are also attracted by leaf volatiles (Martini et al. 2015), which can be altered by pathogen infection (Jansen et al. 2011), we hypothesized that changes in headspace volatile release from Lauraceae following infection by *R. lauricola* may influence the behavior of *X. glabratus*. We present a series of experiments investigating how infestation changes swamp bay leaf and wood volatiles as well as phytohormones in leaf tissues. We investigated if these changes

induced by *R. lauricola* infection modified the host preference of the vector *X. glabratus*. Our next objectives were to measure quantitative and qualitative changes induced by *R. lauricola* infestation and test isolated compounds in bioassays to reproduce the behavior of the vector.

## Materials and Methods

**Insects, Plants and Fungus** Beetles were reared from infested swamp bay and redbay logs collected in Kanapaha Botanical Garden (29°36′41.0″N, 82°24′35.8″W) in Gainesville, Florida. The logs were stored at 23 °C in large plastic containers with holes connected to mason jars filled with humidified Kimwipe® tissues (Kimberly-Clark, Roswell, GA) to attract and collect *X. glabratus*. Beetles were collected and observed under a dissection microscope to ensure mobility (no missing legs and ability to walk) 1–2 hr prior to olfactometer assays. Plant material was purchased from a northern Florida nursery and consisted of swamp bay saplings with a single dominant stem of 1.5 cm diameter at 10 cm above the soil line and were 1.5 m tall.

The *R. lauricola* isolate PL571 (GenBank JQ861956.1) was selected for its consistent morphology in culture and previous use in inoculation studies (Hughes et al. 2015a). Fungal isolates were grown on malt extract agar (MEA) at room temperature and after 7–14 days, conidia were collected and inoculum was prepared as in Hughes et al. (2015a).

Dichloromethane (99.8% purity), nonyl acetate (99%), methyl salicylate (> 99%), *cis*-3-hexen-1-ol (98%), *trans*-3-hexen-1-ol (97%), 2-hexenal (95%), β-caryophyllene (80%),(−)-β-pinene (99%), (±)-camphor, p-cymene (99%), (+)-α-pinene (98%), d-limonene (90%), (+)-sabinene (75%), eucalyptol (99%), (−)-borneol, (−)-4-Terpineol (95%), (±)-α-terpineol (96%), (−)-bornyl acetate (98%), (±)-α-terpinyl acetate (90%), *(E)* -2-undecenal, and mixture of α-and β-farnesene isomers [including *(E)*-β-farnesene, *(Z)*-α-farnesene, *(E-)*-α-farnesene] were purchased from Sigma-Aldrich (St. Louis, MO). (*E*)-4,8-Dimethyl-1,3,7,-nonatriene (DMNT) (99%) was provided by Dr. Alan Knight (USDA-ARS, Wapato, WA).

**Olfactometer System** A four-choice olfactometer modified from Vet et al. (1983) (Analytical Research System, Gainesville, FL) was used to evaluate the behavioral response of *X. glabratus* to natural and synthetic odors. A detailed description of the olfactometer and protocol are found in Martini et al. (2015) who demonstrated attraction of *X. glabratus* to leaf volatiles from redbay and swamp bay. Briefly, the olfactometer consisted of four arms departing from a 30 cm × 30 cm polytetrafluoroethylene (PTFE) square arena. Four independent odor fields were created in the chamber by a constant airflow of 0.35 l/min pushed through each arm of the olfactometer and by pulling air (0.50 l/min) out through the floor's central air evacuation hole. The olfactometer floor and arms were covered with filter paper (25 cm diameter laboratory filter paper, Curtin Matheson Scientific, Houston, TX) to improve beetle movement.

The filter paper was changed between each bioassay and the olfactometer was washed with acetone and Sparkleen® detergent (Fisherbrand, Pittsburgh, PA). Each arm of the olfactometer was connected to a 350 ml glass vial that served as a collection trap for beetles and then to a custom-made air delivery system (ARS, Gainesville, FL). The ambient air supply was purified through a charcoal filtration system. Airflow was measured with a flowmeter (Varian, Walnut Creek, CA) to ensure equivalent airflow within each arm. The olfactometer was positioned under a 150 W high-pressure sodium grow light (Hydrofarm, Petaluma, CA). Twenty-five *X. glabratus* females were released into the center of the olfactometer, which was covered with a Plexiglas sheet and black filter paper so that only the glass traps were illuminated. Beetles were introduced into the olfactometer between 15:00 and 17:00 hr and the number of beetles that entered the arms and fell into each trap was counted the following morning. Abiotic conditions were maintained at 23 ± 1 C°, 49% RH and a L14:D10 photoperiod. Beetles that did not leave the central olfactometer arena were designated as non-responders (NR).

**GC-MS Analysis of Leaf and Wood Volatiles** A volatile collection system was used to identify the profile of swamp bay leaf volatile odors. It consisted of four parallel glass cylinders (38 cm height, 23 cm ID) with an outlet for incoming air at the cylinder's top and another connected to a vacuum at the bottom. Each cylinder was equipped with a PTFE guillotine (Analytical Research System, Gainesville, FL) so that each plant was separated into two parts in terms of headspace collection. A volatile collection trap with 30 mg of HayeSep Q adsorbent (Volatile Assay Systems, Rensselaer, NY) was connected to the bottom outlet with a PTFE fitting. Volatiles emitted from the upper portion of each plant enclosed within each glass chamber were swept downward by the incoming humidified and charcoa1 filter purified air at a rate of 1.0 l/min. Volatiles were forced to the bottom of the chamber by pulling air at 0.6 l/min through volatile collection traps with a controlled vacuum from the automated volatile collection system. Swamp bay wood volatiles were collected by rasping 1.0 g of stem tissue (bark, cambium and sapwood) and placing this material within an 18 cm glass tube. Air was pushed at a rate of 1.0 l/min and pulled at 0.6 l/min through volatile collection traps for a 1-hr collection period. We chose to rasp wood to increase the release of volatiles, given that we were unable to collect volatiles in the past directly from undamaged bark (Martini et al. 2015). Wood rasping has been conducted in previous analysis of wood volatiles from Lauraceae trees (Niogret et al. 2011).

Volatiles were extracted from the collection traps by washing with 150 μl of dichloromethane. Nonyl acetate (1 μg) was added to the extracts as an internal standard. One μl of each sample was manually injected into a Clarus 500 gas chromatograph-mass spectrometer (GC-MS) (PerkinElmer, Shelton, CT). The GC was equipped with a column capillary injector system and flame ionization detector. Data collection, storage, and subsequent analyses were performed on Perkin Elmer chromatographic data system TurboMass™. Helium was used as the carrier gas at a linear flow velocity of 2 ml/min. All samples were analyzed on a fused silica RTX-5 capillary column (Restek Corporation, Bellefonte, PA), 60 m × 0.25 mm ID. The temperature of the column oven was maintained at 40 °C for 1 min and then increased at a rate of 7 °C/m to a final temperature of 300 °C and maintained at 300 °C for 6 min. The injector temperature was set at 270 °C with the detector set at 200 °C. Quantitation was based on GC-MS profiles and were assigned by comparing peak areas of nonyl acetate with the peak areas of compounds from the leaf and wood extracts. Constituents of the plant volatile emissions were identified by comparison of mass spectra with spectra and linear retention indices in the National Institute of Standards and Technology database, and the spectra and retention times obtained from authentic reference compounds on the RTX-5, when available.

**Do Leaf Volatiles Modify *X. glabratus* Behavior Following Infection with the Fungus, *R. lauricola?*** To inoculate healthy swamp bay saplings, two 2-mm diameter holes (15 mm deep) were drilled on opposite sides of the main stem at approximately 45° angle to the vertical. Fifty μl of an *R. lauricola* spore suspension ($1.0 \times 10^6$ spores/ml) were pipetted into the open wounds, with negative control plants receiving distilled water. All plant wounds were sealed in Parafilm after inoculation and maintained in greenhouse in Lake Alfred, FL. Leaf volatiles were collected the day before inoculation, and then 3, 10 and 20 days after inoculation (DAI). Leaf volatiles were analyzed with GC-MS as described above and an olfactometer bioassay was implemented to determine if *X. glabratus* were attracted to leaf volatiles of *R. lauricola* infected swamp bay.

*Xyleborus glabratus* beetles were tested in the four-choice olfactometer as described above. For practical reasons, due to space available in the olfactometer room, we could test only the odor of two undamaged whole swamp bay trees simultaneously. The odors of a *R. lauricola* infected and uninfected plants were used in two opposite arms of the olfactometer for each treatment and therefore only two treatments were compared simultaneously. The two remaining arms delivered filtered, humidified air only (blanks). Odor sources consisted of an approximately equal volume of foliage from whole infected and non-infected swamp bay plants (leaves were not excised), placed within a two-port glass cylinder (38 cm height, 14.4 cm ID). To separate and seal the upper portion of the plant within the dome, two interlocking PTFE boards with a single middle-hole (1 cm diameter) were enclosed around main stem. The response of *X. glabratus* to swamp bay leaf volatiles was tested before infection with *R. lauricola* and 3, 10 and 20 days after infection. Each leaf volatile treatment was tested with five to six replicate trees and up to 25 beetles per assay (112 to 147 beetles tested in total). Non-responder beetles, and beetles that did not choose any of the leaf odor source were excluded from the analysis.

**Does *R. lauricola* Infection Alter Swamp Bay Wood Volatiles and Affect *X. glabratus* Behavior?** Trees were infected with *R. lauricola* and maintained in greenhouse in Lake Alfred, FL as described above. They were subsequently investigated in the four-choice olfactometer as described above. Odor sources consisted of 1.5 g of freshly rasped swamp bay cambium and wood, which were directly placed into the glass trap of each olfactometer arm. Identical odor sources were randomly assigned to two opposite arms for each treatment and therefore only two treatments were compared simultaneously. Initially, we performed a control test to determine whether beetles are attracted to healthy wood volatiles vs clean air in our olfactometer. The responses of *X. glabratus* to swamp bay wood infected with *R. lauricola* and to water-inoculated swamp bay wood controls were then tested 3, 10 and 20 days after inoculation and wood volatiles were collected and analyzed with GC-MS. Each wood volatile treatment was tested with three to four replicate trees and up to 25 beetles per assay (73 to 100 beetles tested in total).

**Behavioral and Antennal Responses of *X. glabratus* to Natural and Synthetic Volatiles** The behavioral response of *X. glabratus* to synthetic volatiles was tested based on the above-described inoculations trials and GC-MS analyses (see results). Odorants with more than 3 fold increased release rates after fungus inoculation were selected. Eucalyptol and α-copaene were not selected given that these two compounds are already known attractants of *X. glabratus* (Hanula and Sullivan 2008; Kendra et al. 2011, 2014; Kuhns et al. 2014a). Test compounds were dissolved in 100 μl of dichloromethane at both 0.1 and 1.0 μg/μl dosage rates and pipetted onto 2 cm Richmond cotton wicks (Petty John Packaging, Inc. Concord, NC). These release devices were placed into two opposite glass olfactometer traps, as described above. The response of *X. glabratus* was tested in the four-choice olfactometer system. The six paired test stimuli were: (1) DMNT vs. solvent (dichloromethane), (2) methyl salicylate (MeSA) vs solvent, (3) d-limonene vs solvent, (4) farnesene (mixture of $\alpha$ and $\beta$ isomers) vs solvent, (5) 3-hexen-1-ol vs solvent, (6) 2-hexenal vs solvent. Three replicate bioassays of up to 25 beetles (total of ≈75 beetles) were performed for each treatment.

Test substrates for electroantennogram (EAG) recordings consisted of dichloromethane (negative control), and extracts from uninfected swamp bay leaf volatiles (the same extracts collected and analyzed with GC-MS), leaf volatiles from *R. lauricola* infected swamp bay (3 DAI), uninfected wood volatiles, a serial dilution of MeSA (1 mg/ml, 100 μg/ml, 10 μg/ml), Manuka oil solution (1%), d-limonene (1 mg/ml), and a mixture of $\alpha$- and $\beta$-farnesene isomers (1 mg/ml). The standard reference compound was ethanol (99.5%; ACROS organics, Fisher Scientific, USA), which has been shown previously to elicit strong EAG responses from *X. glabratus* antennae (Kendra et al. 2012). Electroantennogram recordings from *X. glabratus* antennae were performed with a modified Syntech KOMBI-probe (adapted from Kendra et al. 2012), which utilized a gold-plated two-pronged antennal holder modified with thin gold wire to accommodate the minute antennae of *X. glabratus*. Each preparation consisted of a single-excised antenna (mean length 0.37 ± 0.01 mm) mounted between the ground electrode and gold wire using salt-free conductive gel (Spectra 360, Parker Laboratories, Fairfield, NJ, USA) (Kendra et al. 2012) (Supplement 1). A filtered and humidified airstream passed over the antenna at 30 cm/s. The headspace of pure odorants were puffed into the airstream through a hole in the glass tube, 10 cm upstream from the insect antenna. The resulting voltage in the electroantennogram (EAG) recording in response to volatile odorants was recorded. The signal from the amplifier was conditioned using a Syntech IDAC-4 interface (Syntech, Kirchzarten, Germany). Signals acquired from the IDAC-4 were displayed and stored on a computer running Syntech software. The antenna was first presented with a negative control, consisting of an injection of clean air equal in volume to the sample injections (150 μl). This was followed by injection of the ethanol standard as a positive control. Thereafter, test samples were injected in random order, with an injection of ethanol between the tests samples, and a final injection of ethanol to ensure that the mounted antenna was still active. There was a 1-min interval (clean air flush) between sample injections to prevent antennal adaptation. EAG responses to test substrates were measured initially in millivolts (peak height of depolarization) and then normalized to percentages relative to the average response obtained with the three injections of ethanol (Kendra et al. 2012).

**Phytohormone Changes in *R. lauricola* Infected Trees** Five swamp bay trees were inoculated with *R. lauricola* and distilled water as described above. In this case, swamp bay were maintained outdoors in Gainesville, FL. Five mature leaves were removed from each tree at 0 (before), and 3, 10, 20 and 30 DAI. These samples were immediately stored at −80 °C until analysis. Leaf samples were processed for phytohormone analysis via GC-MS following the protocol of Nehela et al. (2016). Leaf tissues were ground in liquid nitrogen and then 750 μl of the extraction solvent were added, vortexed, and centrifuged. Samples were extracted and the supernatants were combined and concentrated under a nitrogen stream and stored in a freezer (−80 °C) until analysis. Acidic phytohormones, salicylic acid (SA), jasmonic acid (JA) and abscissic acid (ABA), were derivatized with methylchloroformate as described by (Hijaz and Killiny 2014). For the GC-MS analysis of the phytohormones, we used a Clarus 680 GC with SQ8-T MS system (Perkin Elmer, Waltham, MA, USA) fitted with an Elite-5MS capillary column (low bleed, 30 m × 0.25 mm × 0.025 _m film thickness; Perkin Elmer, Waltham, MA, USA). Helium was the carrier gas with flow rate 1 ml $min^{-1}$. The temperature was as follows; the column was held at 50 °C for three min, and then increased to 200 °C at a rate of 4 °C $min^{-1}$, held for 5 min. TurboMass software version 6.1 (Perkin Elmer, Waltham, MA, USA) was used to analyze chromatograms. Identification of all phytohormones was performed by comparing their retention times, linear retention indices (Acompora Zellner et al. 2008), and the selected ions with those of authentic standards.

**Statistical Analysis** All statistical analyses were performed with the statistical software R v 3.3.1 (R core team, Vienna, Austria) and SigmaPlot 13.0 (Systat Software, San Jose, CA, USA). Behavioral responses of *X. glabratus* in the olfactometer were analyzed by first checking if the data were homogenous (Zar 2009). Thereafter, data for each treatment were pooled and tested with a chi-square test. Regarding volatiles, for each compound the differences in leaf emission were assessed with a generalized linear model (GLM) with Poisson distribution. The independent variables were the infection status and days after infection (DAI). Changes between control and infected trees were considered significant, when the interaction DAI x Treatment was significant at $\alpha = 0.05$.

EAG responses were analyzed by using the corrected normalized EAG values. Analysis of variance with repeated measure was performed followed by a pairwise multiple comparison procedure (Holm-Sidak method), with 6 replicate females per treatment. Finally, dosage of phytohormones between *R. lauricola*-infected and control plants were compared with t-test at each data point.

## Results

**Do Leaf Volatiles Modify *X. glabratus* Behavior Following Infection with the Fungus, *R. lauricola*?** There was no difference in the attraction of *X. glabratus* towards leaf volatiles of intact plants between the different treatments groups prior to inoculation ($\chi = 0.44$, *d.f.* = 1, $P = 0.508$). Three days after infection, *X. glabratus* significantly avoided *R. lauricola* infected plants ($\chi = 18.31$, *d.f.* = 1,

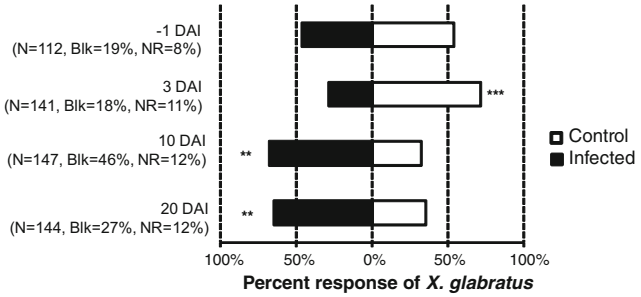


**Fig. 1** Percentage of *Xyleborus glabratus* responding to natural odors of swamp bay leaf volatiles within a four-choice olfactometer following infection with the fungus *Raffaelea lauricola*. Trees were asymptomatic 3 days after inoculation (DAI) and started showing wilting symptoms 10 DAI. N = total number of *X. glabratus* used during the experiments, *NR* Percent of non-responders, *blk* percent of *X. glabratus* that chose arms with clean air. Asterisks indicate significant differences between the two treatments (** = $P < 0.01$; *** = $P < 0.001$)

$P < 0.001$); whereas, they were significantly attracted to infected plants at 10 ($\chi = 7.81$, *d.f.* = 1, $P = 0.005$) and 20 DAI ($\chi = 7.68$, *d.f.* = 1, $P = 0.006$) (Fig. 1).

Major compounds in uninfected swamp bay leaf volatiles were $\beta$-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), nonanal, (*E, E*)-$\alpha$-farnesene, and sabinene (Table 1). Following infection with *R. lauricola*, there was a significant increase of MeSA (interaction DAI x treatment: $F_{3,27} = 2.82$, $P = 0.038$) and (*E, E*)-$\alpha$-farnesene (interaction DAI x treatment: $F_{3,27} = 5.49$, $P < 0.001$) release (Fig. 2, Table 1); however, the release of these two compounds decreased significantly at 10 and 20 DAI (Fig. 2, Table 1). Additionally, the amount of aldehydes (2-hexenal and (*Z*)-3-hexen-1-ol) and sesquiterpenes ($\alpha$-copaene and (*E*)-$\beta$-farnesene) in leaf volatiles increased significantly 10 and 20 DAI (Table 1). Twenty days after infection, the amount of DMNT (interaction DAI x treatment: $F_{3,27} = 19.99$, $P < 0.001$) and $\beta$-ocimene (interaction DAI x treatment: $F_{3,27} = 8.64$, $P < 0.001$) was significantly decreased, whereas abundance of eucalyptol (interaction DAI x treatment: $F_{3,27} = 5.72$, $P < 0.001$) and d-limonene (interaction DAI x treatment: $F_{3,27} = 3.79$, $P = 0.013$) significantly increased (Table 1).

**Do Wood Volatiles Alter *X. glabratus* Behavior Following Infection with the Fungus, *R. lauricola*?** Swamp bay wood volatiles were attractive to *X. glabratus* vs. clean air,

**Table 1** Compounds identified from swamp bay leaf volatiles released by control and *Raffaelea lauricola* infected plants over time

| Name of the compound | RT (min) | Uninfected trees[a] | 3 Days after infection | 10 Days after infection | 20 days after infection |
|---|---|---|---|---|---|
| 2-hexenal | 10.08 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.63 ± 0.41 |
| 3-hexen-1-ol | 10.14 | 0.00 ± 0.00 | 0.00 ± 0.00 | 2.39 ± 0.40 | 7.16 ± 1.78 |
| $\alpha$-pinene | 12.25 | 0.42 ± 0.20 | 0.22 ± 0.08 | 0.13 ± 0.08 | 0.44 ± 0.19 |
| sabinene | 13.21 | 1.10 ± 0.88 | 0.31 ± 0.14 | 0.25 ± 0.15 | 0.22 ± 0.10 |
| $\beta$-pinene | 13.40 | 0.24 ± 0.13 | 0.17 ± 0.04 | 0.12 ± 0.07 | 0.22 ± 0.06 |
| $\beta$-myrcene | 13.48 | 0.25 ± 0.14 | 0.19 ± 0.10 | 0.013 ± 0.01 | 0.16 ± 0.08 |
| octanal | 13.75 | 0.38 ± 0.14 | 0.44 ± 0.19 | 0.59 ± 0.17 | 0.7 ± 0.53 |
| D-limonene | 14.56 | 0.49 ± 0.13 | 0.38 ± 0.10 | 0.16 ± 0.08 | 2.38 ± 0.96 |
| eucalyptol | 14.69 | 1.04 ± 0.31 | 1.04 ± 0.39 | 0.27 ± 0.12 | 2.83 ± 1.10 |
| $\beta$-ocimene | 14.85 | 5.89 ± 1.95 | 5.85 ± 3.35 | 1.27 ± 0.58 | 0.58 ± 0.42 |
| linalool | 16.06 | 0.70 ± 0.28 | 0.45 ± 0.12 | 0.52 ± 0.12 | 0.14 ± 0.13 |
| nonanal | 16.16 | 1.13 ± 0.33 | 1.49 ± 0.40 | 2.51 ± 1.12 | 1.15 ± 0.49 |
| DMNT[b] | 16.41 | 5.17 ± 2.51 | 6.35 ± 1.55 | 4.62 ± 2.58 | 0.49 ± 0.44 |
| camphor | 17.50 | 0.53 ± 0.18 | 0.12 ± 0.07 | 0.17 ± 0.7 | 0.23 ± 0.14 |
| methyl salicylate | 18.52 | 1.06 ± 0.33 | 4.28 ± 3.36 | 0.60 ± 0.25 | 0.83 ± 0.67 |
| (*E*)-2-decenal[c] | 19.67 | 0.44 ± 0.17 | 0.25 ± 0.03 | 0.23 ± 0.08 | 0.54 ± 0.38 |
| 2-undecenal | 21.75 | 0.20 ± 0.12 | 0.05 ± 0.00 | 0.07 ± 0.04 | 0.32 ± 0.29 |
| $\alpha$-copaene[c] | 22.30 | 0.01 ± 0.00 | 0.00 ± 0.00 | 0.17 ± 0.00 | 0.30 ± 0.01 |
| (*E*)-$\beta$-farnesene | 23.59 | 0.00 ± 0.00 | 0.016 ± 0.00 | 0.025 ± 0.00 | 1.18 ± 0.31 |
| *(E,E)*-$\alpha$-farnesene | 24.37 | 1.34 ± 0.57 | 21.60 ± 19.39 | 1.35 ± 0.54 | 1.24 ± 1.03 |

Numbers presented are averaged ratios between compound peak areas and internal standard (nonyl acetate) peak areas

*RT* retention times

[a] Uninfected trees consist of the pool of control trees over the course of the experiment and the baseline of trees used for infection

[b] DMNT: (*E*)-4,8-dimethyl-1,3,7-nonatriene

[c] Determined with NIST database only

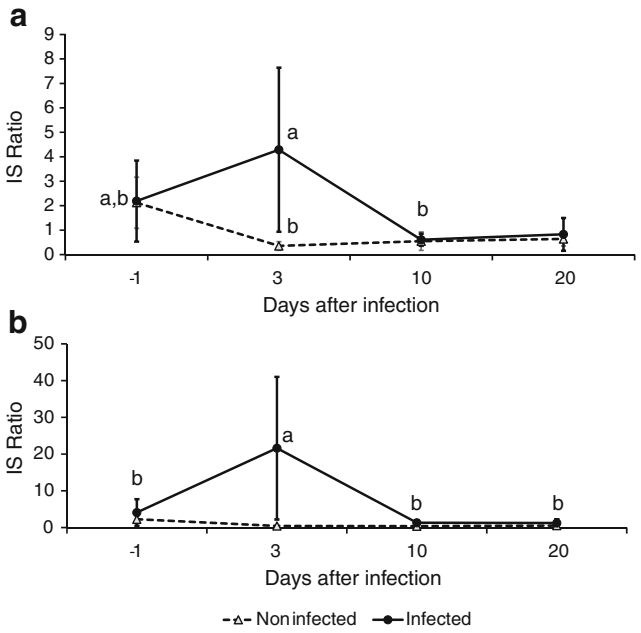


**Fig. 2** **a** Average (± SEM) release of methyl salicylate and **b** α-farnesene from swamp bay leaves infected with *Raffaela lauricola* (*dots*) or uninfected controls (*triangles*) over time. IS Ratio represents the averaged ratio between compound peak areas and internal standard peak areas

validating the effectiveness of the bioassay ($\chi = 15.211$, *d.f.* = 1, $P < 0.001$). There was no preference of *X. glabratus* between the wood volatiles of control and infected plants 3 DAI ($\chi = 0.23$, *d.f.* = 1, $P = 0.633$), 10 DAI ($\chi = 0.45$, *d.f.* = 1, $P = 0.502$), or 20 DAI ($\chi = 1.27$, *d.f.* = 1, $P = 0.259$) (Fig. 3).

Volatile profiles from rasped wood did not reveal any release of MeSA, DMNT or α-farnesene following infection, suggesting that changes observed in leaf volatiles 3 DAI did not occur in wood. Overall, the volatile blend of swamp bay wood following *R. lauricola* infection was particularly stable

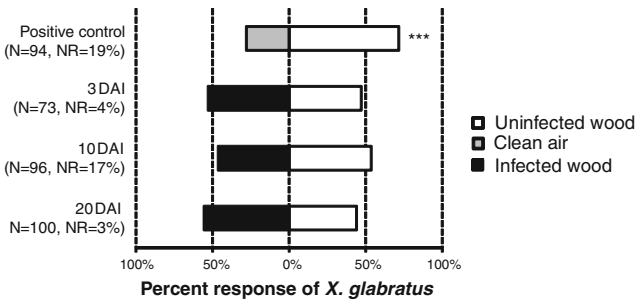


**Fig. 3** Percentage of *Xyleborus glabratus* responding to volatile odors of swamp bay wood within a four-choice olfactometer following infection with the fungus *Raffaelea lauricola*. We first established a positive control to ensure that our system worked properly with wood volatiles. Trees were asymptomatic 3 days after inoculation (DAI) and started showing wilting symptoms 10 DAI. *N* total number of *X. glabratus* used during the experiments, *NR* Percent of non-responders. *Asterisks* indicate significant differences between the two treatments (*** = $P < 0.001$)

and the amount of major sesquiterpenes did not increase significantly ($P > 0.05$) (Table 2).

**Behavioral and Antennal Responses of *X. glabratus* toward Natural and Synthetic Volatiles** To determine which volatiles were involved in repellence and attraction, we selected them based on the observation that repellence of *X. glabratus* toward infected trees was observed only 3 DAI. The abundance of two compounds [MeSA and (*E,E*)-α-farnesene] increased at 3 DAI and decreased subsequently as infected plants became more attractive in the olfactometer bioassay. In addition, the amount of DMNT significantly decreased at 20 DAI when volatiles from infected trees were more attractive than controls and thus, may also have repellent properties. Therefore, these three compounds were tested as potential repellents. Because there is no provider for isolated (*E*,*E*)-α-farnesene, we tested the behavioral response of *X. glabratus* to a mixture of α- and β-farnesene isomers.

The olfactometer bioassays revealed that MeSA was significantly repellent to *X. glabratus* when compared with the dichloromethane solvent control at a 1 μg/μl dosage, but not at 0.1 μg/μl. There was no statistical preference between DMNT and solvent at 1 μg/μl or 0.1 μg/μl dosages tested (Table 3). Similarly, the mixture of α- and β-farnesene isomers tested did not elicited any response in the olfactometer from *X. glabratus* at 1 μg/μl or 0.1 μg/μl. Regarding potential attractants, neither of the leaf aldehydes 2-hexenal or 3-hexen-1-ol, nor d-limonene showed any effect on *X. glabratus* behavior at the dosages tested (Table 3).

EAG responses of *X. glabratus* were significantly different between volatiles applied ($F_{10, 46} = 13.373$; $P < 0.001$) (Fig. 4a). Pairwise Multiple Comparison Procedures (Holm-Sidak method) revealed a significant increase ($P < 0.05$) in antennal response compared to solvent and clean air with infected (3 DAI) leaf volatile extracts, wood volatile extracts, and Manuka. In contrast, antennal response to leaf volatiles from uninfected plants, d-limonene or the mixture of α and β-farnesene isomers, did not differ significantly from solvent or clean air ($P < 0.05$) (Fig. 4a). Increasing the dosage of MeSA increased antennal response with 1 mg/μl and 100 mg/μl eliciting significantly higher EAG responses than solvent alone ($P < 0.05$) (Fig. 4b).

**Phytohormone Changes in *R. lauricola* Infected Trees** In contrast to the disease progression observed in the former experiments, symptom onset was slower in plants tested for phytohormone changes. Plants were asymptomatic at 3 and 10 DAI, and initial symptoms appeared 20 DAI. These plants significantly declined at 30 DAI. Overall, all phytohormones tested, with the exception of indole-3-propionic acid, increased in abundance in infected plants compared with control plants (Fig. 5). Salicylic acid was the first phytohormone to increase significantly at 10 DAI ($t = 5.502$, *d.f.* = 8, $P < 0.001$)

**Table 2** Compounds identified from swamp bay wood volatiles released by control and *Raffaelea lauricola* infected plants over time

| Name of the compound | RT (min) | Uninfected trees[a] | 3 Days after infection | 10 Days after infection | 20 days after infection |
|---|---|---|---|---|---|
| 3-hexen-1-ol | 10.14 | 0.49 ± 0.21 | 0.18 ± 0.18 | 0.16 ± 0.16 | 0.00 ± 0.00 |
| α-pinene | 12.25 | 0.92 ± 0.45 | 0.99 ± 0.30 | 0.80 ± 0.25 | 0.94 ± 0.39 |
| sabinene | 13.21 | 1.27 ± 1.01 | 0.57 ± 0.25 | 0.98 ± 0.43 | 1.81 ± 0.95 |
| β-pinene | 13.40 | 0.56 ± 0.29 | 0.44 ± 0.08 | 0.55 ± 0.26 | 0.52 ± 0.23 |
| β-myrcene | 13.48 | 0.26 ± 0.14 | 0.22 ± 0.02 | 0.23 ± 0.14 | 0.33 ± 0.14 |
| cymene | 14.48 | 0.40 ± 0.21 | 0.22 ± 0.07 | 0.35 ± 0.15 | 0.73 ± 0.37 |
| D-limonene | 14.56 | 0.78 ± 0.40 | 0.45 ± 0.09 | 0.62 ± 0.44 | 0.76 ± 0.35 |
| eucalyptol | 14.69 | 15.43 ± 12.00 | 4.26 ± 2.52 | 11.80 ± 4.96 | 10.13 ± 4.97 |
| linalool | 16.06 | 0.35 ± 0.18 | 0.83 ± 0.43 | 0.77 ± 0.44 | 0.36 ± 0.17 |
| camphor | 17.50 | 5.88 ± 4.71 | 2.58 ± 1.16 | 7.92 ± 7.35 | 2.06 ± 0.97 |
| ocimenol[b] | 17.76 | 0.01 ± 0.01 | 0.02 ± 0.02 | 0.06 ± 0.04 | 0.14 ± 0.09 |
| borneol | 17.85 | 2.38 ± 2.15 | 2.32 ± 2.32 | 0.65 ± 0.63 | 0.05 ± 0.03 |
| terpinen-4-ol | 18.03 | 0.40 ± 0.22 | 0.30 ± 0.03 | 0.40 ± 0.15 | 0.40 ± 0.20 |
| α-terpineol | 18.28 | 0.53 ± 0.25 | 0.49 ± 0.14 | 0.83 ± 0.38 | 1.82 ± 1.00 |
| α-cubebene[b] | 21.68 | 0.01 ± 0.00 | 0.11 ± 0.07 | 0.03 ± 0.03 | 0.02 ± 0.01 |
| α-copaene[b] | 22.28 | 0.23 ± 0.16 | 0.68 ± 0.42 | 0.27 ± 0.23 | 0.82 ± 0.35 |
| β-elemene | 22.48 | 0.02 ± 0.01 | 0.03 ± 0.03 | 0.05 ± 0.02 | 0.28 ± 0.19 |
| (*E*)-α-bergamotene[b] | 22.81 | 0.06 ± 0.04 | 0.00 ± 0.00 | 0.31 ± 0.30 | 0.36 ± 0.21 |
| β-caryophyllene | 23.20 | 0.57 ± 0.33 | 0.24 ± 0.21 | 0.94 ± 0.87 | 1.23 ± 0.59 |
| (*E*)-β-farnesene | 23.31 | 0.09 ± 0.05 | 0.01 ± 0.01 | 0.09 ± 0.06 | 0.40 ± 0.30 |
| guaia-6,9-diene[b] | 23.48 | 0.14 ± 0.07 | 0.03 ± 0.03 | 0.33 ± 0.20 | 0.81 ± 0.53 |
| α-humulene[b] | 23.83 | 0.05 ± 0.04 | 0.02 ± 0.02 | 0.07 ± 0.07 | 0.16 ± 0.08 |
| β-bisabolene[b] | 24.46 | 0.10 ± 0.06 | 0.01 ± 0.01 | 0.22 ± 0.15 | 0.81 ± 0.40 |
| δ-cadinene[b] | 24.90 | 0.05 ± 0.03 | 0.02 ± 0.02 | 0.05 ± 0.04 | 0.28 ± 0.15 |
| caryophyllene oxide[b] | 26.26 | 0.21 ± 0.11 | 0.00 ± 0.00 | 0.45 ± 0.44 | 1.08 ± 1.01 |

Numbers presented are averaged ratio between compound peak areas and internal standard peak areas

*RT* Retention time

[a] Uninfected trees consist of the pool of control trees over the course of the experiment

[b] Determined with NIST database only

**Table 3** Response of female *Xyleborus glabratus* to isolated compounds in olfactometer assays

| Treatment odor source | dosage | *n* | *NR* | Treated air(%)[a] | $\chi^2$ | *P*-Value |
|---|---|---|---|---|---|---|
| DMNT[b] | 1 μg/μl | 72 | 15 | 59.65 | 2.12 | 0.14 |
| DMNT | 0.1 μg/μl | 75 | 7 | 48.53 | 0.06 | 0.80 |
| methyl salicylate | 1 μg/μl | 72 | 10 | 24.19 | 16.52 | < 0.001 |
| methyl salicylate | 0.1 μg/μl | 73 | 7 | 48.48 | 0.06 | 0.81 |
| 3-hexen-1-ol | 1 μg/μl | 69 | 8 | 52.46 | 0.15 | 0.70 |
| 3-hexen-1-ol | 0.1 μg/μl | 75 | 14 | 44.26 | 0.37 | 0.80 |
| 2-hexenal | 1 μg/μl | 72 | 11 | 45.90 | 0.41 | 0.52 |
| 2-hexenal | 0.1 μg/μl | 75 | 11 | 45.31 | 0.56 | 0.45 |
| d-limonene | 1 μg/μl | 71 | 15 | 46.42 | 0.29 | 0.59 |
| d-limonene | 0.1 μg/μl | 75 | 4 | 52.31 | 0.19 | 0.71 |
| β- and α-farnesene isomers | 1 μg/μl | 73 | 14 | 59.32 | 2.05 | 0.15 |
| β- and α-farnesene isomers | 0.1 μg/μl | 72 | 10 | 59.68 | 2.32 | 0.13 |

*n* Sample size, *NR* Number of individual that did not respond

[a] Percentage of individuals (of those making a choice) choosing the treated air arm as compared with the control

[b] DMNT: (*E*)-4,8-dimethyl-1,3,7-nonatriene

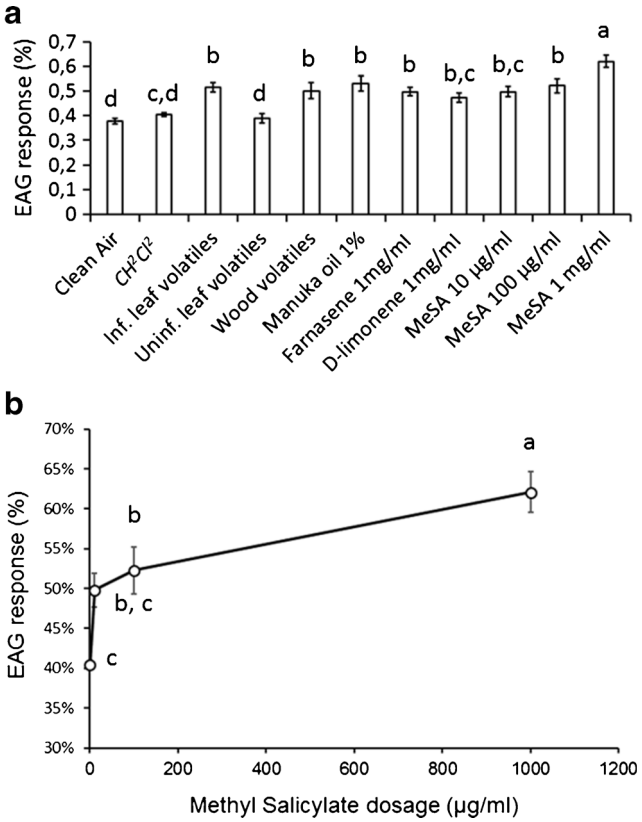


**Fig. 4** Average (±SEM) antennal response of *Xyleborus glabratus* to 150 µl samples injection of (**a**) various tree odorants and isolated compounds and (**b**) to a serial dilution of methyl salicylate. Leaf volatiles were collected over 24 hr, while wood volatiles extracted from 1.0 g of rasped stem tissues were collected over 1 hr. All volatiles were collected with volatile collection traps consisting of 30 mg of HayeSep Q adsorbent and eluted with 150 µl of dichlormethane, farnesene corresponds to a blend of $\alpha$- and $\beta$- farnesene

level (Fig. 5g). The increased amount of benzoic acid was below the $\alpha = 0.10$ level at 3 ($t = 1.891$, *d.f.* = 8, $P = 0.095$) and 10 DAI ($t = 2.073$, *d.f.* = 8, $P = 0.072$) and significant at the $\alpha = 0.05$ level at 20 DAI ($t = 3.044$, *d.f.* = 8, $P = 0.016$) (Fig. 5a). *(E)*-cinnamic acid ($t = 2.671$, *d.f.* = 8, $P = 0.028$), salicylic acid ($t = 4.504$, *d.f.* = 8, $P = 0.002$), and *(E)*-jasmonic acid ($t = 2.534$, *d.f.* = 8, $P = 0.035$) were all upregulated compared to controls at 20 DAI (Fig. 5b, d, g). At 30 DAI, only amounts of salicylic ($t = 3.692$, *d.f.* = 8, $P = 0.006$) and *(E)*-jasmonic acids ($t = 2.322$, *d.f.* = 8, $P = 0.049$) were significantly higher than the control (Fig. 5b, g).

## Discussion

Infection of swamp bay with the phytopathogenic fungus *R. lauricola*, induced profound changes, both quantitatively and qualitatively, in the odors emitted by the swamp bay leaves. These changes were accompanied by a significant shift in the response of the beetle vector to leaf volatiles. Three days after inoculation, beetles avoided infected trees and instead strongly preferred their uninfected counterparts. We initially hypothesized that the vector beetle would exhibit attraction to the symbiotic fungus rather than repellency following infection of the host plant. Repellence was associated with peaks of MeSA, DMNT and (*E, E*) $\alpha$-farnesene released 3 DAI. However, in the olfactometer bioassay, only MeSA appeared to repel beetles. It is possible though, that the repellency observed to MeSA may be further increased through synergistic interaction with other volatiles that are not active alone (Bruce and Pickett 2011). The antennal response of *X. glabratus* to MeSA was dosage-dependent, as measured electrophysiologically, and the results obtained were consistent with behavioral avoidance observed in olfactometer. The greater antennal responses of *X. glabratus* to leaf volatiles emitted by infected (3 DAI) than uninfected plants was also consistent with greater emission of MeSA measured quantitatively by GC-MS analysis, and with the avoidance of leaf volatiles from infected plants 3 DAI by the vector.

The $\alpha$- and $\beta$-farnesene mixture of isomers did not elicit a behavioral response from *X. glabratus* in the olfactometer, but this might be due to the stereoisomeric mixture of farnesene tested. Other changes in leaf volatiles were found after infection, such as a significant decrease in $\beta$-ocimene, an increase of eucalyptol release, and the appearance of sesquiterprenes, such as (*E*)-$\beta$-farnesene and $\alpha$-copaene at 10 and 20 DAI. Eucalyptol is a known attractant of *X. glabratus* (Kuhns et al. 2014a) and is usually present in Lauraceae leaf volatiles (Martini et al. 2015). Sesquiterpenes are typically absent in *Persea* bay leaf volatile emissions (Martini et al. 2015), but are instead released by woody portions of trees as was confirmed by our analyses of tree odors. These wood-derived volatiles, especially $\alpha$-copaene, are known attractants to *X. glabratus* (Kendra et al. 2015a). The appearance of sesquiterpenes in the volatile blend of leaves at the onset of wilt is likely associated with direct damage to leaves caused by wilting. This may explain the attraction of *X. glabratus* to infected swamp bay leaf volatiles 10 and 20 DAI and may cause the observed increased attack by *X. glabratus* to symptomatic and dying trees in nature (Fraedrich et al. 2008; Hughes et al. 2015b).

We found no significant changes in wood volatiles and *R. lauricola* infected wood did not induce a particular change in beetle behavior. These results confirm observations by Hanula et al. (2008), who found no difference during beetle trapping when using uninfected and *R. lauricola* infected redbay logs as baits. These results are surprising given that other studies found that volatiles released by the symbiotic fungus, *R. lauricola*, were attractive to *X. glabratus* (Hulcr et al. 2011; Kuhns et al. 2014b). The absence of *R. lauricola* fungal volatiles, such as ethyl acetate, ethanol, isobutyl alcohol, isoamyl acetate or isoamyl alcohol (Kuhns et al. 2014b) from the volatile profile of laurel wilt diseased wood, and the absence of *X. glabratus* preference between infected and

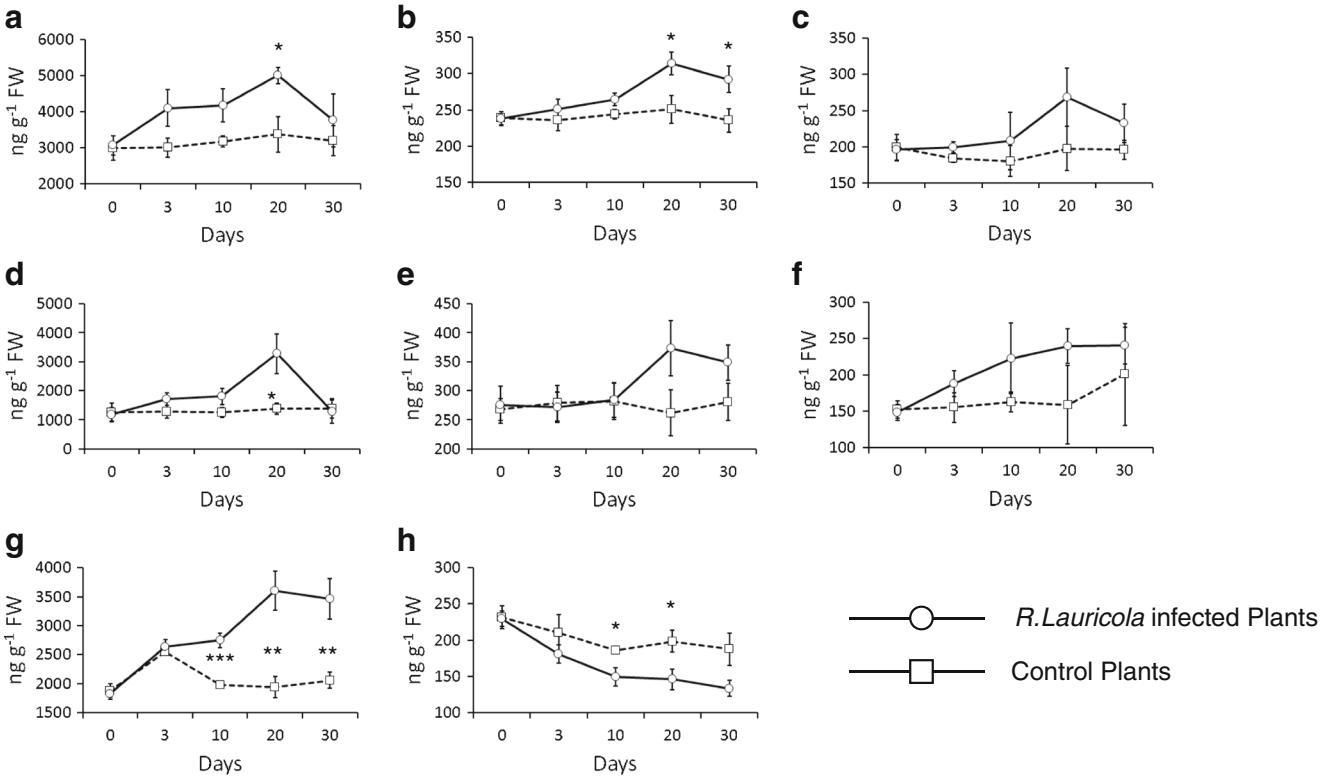


**Fig. 5** Average (±SEM) amount of phytohormones present in swamp bay leaves following artificial infection with the fungus *Raffaellea lauricola*. Trees were asymptomatic 3 and 10 days after inoculation (DAI) and started showing wilting symptoms 20 DAI. **a** benzoic acid, **b** *(E)*-jasmonic acid, **c** indole-3-butyric acid, **d** *(E)*-cinnamic acid, **e** indole-3-acetic acid, **f** abscisic acid, **g** salicylic acid, and **h** indole-3-propionic acid. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$

uninfected wood in our olfactometer bioassays suggest that *R. lauricola* fungal volatiles might be short distance attractants used within the trunk to attract and orient *X. glabratus* toward galleries built by conspecifics. Also, our experiments were conducted with small diameter trees. It is possible that the release and the change in volatiles would have been significantly different with more mature trees. It also possible that the deterioration of wood tissue by rasping changed the chemical content of wood volatile and explains the absence of discrimination between wood treatments.

The avoidance of MeSA by *X. glabratus* appears to be correlated with avoidance of potential hosts with induced defenses activated following fungal infection, as suggested by the increase of salicylic and benzoic acids following fungal infection of trees. Since the initial invasion of *X. glabratus* in the United States, it has been observed that pioneer beetles land and bore into healthy trees, inoculating the tree with *R. lauricola*. However, these pioneer beetles often abandon trees early during gallery construction (Fraedrich et al. 2008). Following *R. lauricola* inoculation, there is an incubation phase during which *X. glabratus* are not found in the tree (Fraedrich et al. 2008). It is only later when the tree begins to decline due to laurel wilt disease that new redbay ambrosia beetles are able to colonize the tree. Until now, this behavior and sequence of events remained unexplained. Our data provide the first plausible explanation of this behavioral response by *X. glabratus*. Following infection, MeSA increases in the leaf volatiles of the host. This increase in MeSA is associated with an increase of salicylate and benzoic acids, indicating activation of host defenses that potentially renders host trees sub-optimal for beetle infestation, and such trees are therefore avoided by *X. glabratus*. An increase in MeSA and salicylic acid is concomitant with a decrease in xylem functionality. As the disease progresses, hydraulic conductivity decreases halting water transport, thus causing leaves to wilt (Dreaden et al. 2017; Inch and Ploetz 2012) and MeSA release is coincidentally halted. The subsequent decrease of MeSA release during disease progression might be caused by manipulation of host odor by the fungus to suppress repellence of the vector. Laurel wilt damage and disappearance of MeSA are associated with the release of sesquiterpenes (including $\alpha$-copaene) in the leaf volatiles that normally are only released by wood. This increase of sesquiterpenes in leaf volatiles renders trees more attractive to *X. glabratus*, explaining the increased number of attacks observed on trees showing laurel wilt symptoms (Fraedrich et al. 2008).

Activation of tree defenses appeared with increased levels of phytohormones detected in leaves. Salicylate acid was the

first phytohormone to increase significantly, indicating that it is probably related to the earlier peak of MeSA detected in GC-MS (Kessler and Baldwin 2002; Loake and Grant 2007). Activation of the salicylate acid pathway is not surprising, as it generally mediates defenses against piercing-sucking insects and biotrophic pathogens (Loake and Grant 2007), while the jasmonic acid pathway is usually induced in association with chewing insect herbivores and necrotrophic pathogens (Spoel et al. 2007). Interestingly, as the disease propagated within trees and wilting appeared on leaves, most of the phytohormones, including jasmonic acid, increased but did not affect disease progression.

Several applications for management of the laurel wilt vector are possible based on our results. First, MeSA repellency against *X. glabratus* has been recently tested under field conditions. MeSA dispensers placed on redbay logs significantly reduced the arrival of *X. glabaratus* on these logs, as well as, the number of attacks. Additionally, a lure based on a 1:1 blend of MeSA and verbenone was found to be as effective as verbenone in repelling and decreasing attacks by *X. glabratus* (Hughes et al. 2017). Second, repellents could be used in combination with known attractants, such as cubeb oil or 50% enriched $\alpha$-copaene lures (Kendra et al. 2015b), to establish a pull-push system for commercially grown avocado or in Southeastern forests for managing the disease. A push-pull system consisting of attractive lures deployed strategically away from hosts in conjunction with a repellent deployed along borders of an area designated for protection (Cook et al. 2007; Eigenbrode et al. 2016) is envisooned. This may provide an alternative method for management of the pathosystem in areas where the introduced vector is threatening invaded ecosystems. Such techniques have been successfully developed and implemented against the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Borden et al. 2006; Gillette et al. 2012). Also, induced systemic acquired resistance of redbay or avocado defenses to decrease attack by invading beetles might be worth investigating. For instance, foliar application of MeSA to citrus trees elicits aboveground stimulation of the salicylic acid pathway, which increases the release of d-limonene in root volatiles that in turn attracts the entomopathogenic nematode, *Steinernema diaprepesi* Nguyen and Duncan (Filgueiras et al. 2016). It is not known if application of MeSA or other salicylate acid pathway stimulants could induce the release of methyl salicylate in uninfected redbay or swamp bay, and a first step would be to screen for compounds that activate the salicylic acid pathway. Identification of a salicylic acid pathway stimulant in Lauraceae to increase the release of MeSA in leaf volatiles could be an important breakthrough for a non-insecticide based management strategy for this pathosystem in exotically invaded areas. The concept is to 'prime' trees to become naturally repellent to *X. glabratus* by activating their defenses prior to initial fungal inoculation by *X. glabratus*. Such management methods require further investigation, and would consist of applying fundamental knowledge of pathogen-induced changes in plants that affect vector behavior for practical management of this lethal phytopathogen.

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