Volatiles from the symbiotic fungus *Raffaelea lauricola* are synergistic with Manuka lures for increased capture of the Redbay ambrosia beetle *Xyleborus glabratus*

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Abstract

1 Redbay ambrosia beetle *Xyleborus glabratus* is an invasive wood boring beetle that has become established in the southeastern U.S.A. and transmits a fungus *Raffaelea lauricola* that causes lethal laurel wilt. Among susceptible Lauraceae hosts are redbay *Persea borbonia* and avocado *Persea americana*.

2 There is a crucial need for detection of this pest as it moves into new areas. Consequently, our goal was to create a better lure for the monitoring and control of redbay ambrosia beetle.

3 We analyzed volatile emissions of *R. lauricola*, created a synthetic odour blend based on this analysis and tested this odour blend as a potential attractant in a redbay forest infested with *X. glabratus*. The synthetic *Raffaelea* odour blend was not attractive to the beetles by itself. However, it synergistically increased attraction to host-mimic volatiles.

4 We tested four commercial release devices for dispensing *Raffaelea* odour at various release rates. Two prototypes with the highest release rate, when paired with commercial manuka oil lures, captured more beetles than manuka oil lures alone. These results indicate that a synthetic blend of volatiles based on the odour of the symbiotic fungus of *X. glabratus* may be useful for the development of more sensitive monitoring lures for this invasive pathogen vector.

Keywords Avocado, isoamyl acetate, isoamyl alcohol, laurel wilt, *Persea americana*, *Persea borbonia*, Scolytinae.

Introduction

The exotic redbay ambrosia beetle *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae) has recently become established in the southeastern U.S.A. (Rabaglia et al., 2006; Fraedrich et al., 2008). Typically, ambrosia beetles use dead or dying trees as hosts in which they inoculate and cultivate a symbiotic fungus for food. The physical holes in the tree combined with inoculation of these fungi plays a valuable role in biomass turnover in forest ecosystems (Edmonds & Eglitis, 1989). However, *X. glabratus* appears to attack healthy trees in the U.S. (Fraedrich et al., 2008) and introduces the fungus *Raffaelea lauricola* that is lethal to trees in the family Lauraceae (Harrington et al., 2008; Harrington & Fraedrich, 2010). This disease, called laurel wilt, results in similar symptoms to other diseases transmitted by wood boring beetles, such as Dutch elm and oak wilt (Fraedrich et al., 2008; Mayfield et al., 2008).

Although redbay *Persea borbonia* (L.) Spreng. and the related species swampbay *Persea palustris* (Raf.) Sarg. are considered the primary hosts, several other Lauraceae are acceptable hosts, including avocado *Persea americana* Mill., a major fruit crop in southern Florida and California, and two threatened or endangered tree species: pondspice *Litsea aestivalis* (L.) Fernald and pondberry *Lindera melissifolia* (Walter) (Fraedrich et al., 2011). The U.S. Avocado industry is valued at $US 30 million per year in Florida alone; therefore, this disease has the potential to negatively impact local farmers and economies, as well as create shortages of a favorite food.
crop (Evans et al., 2010). In addition, the destruction of redbay trees is devastating to forests and could alter ecosystem composition, leading to abnormal forest succession (Shields et al., 2011), as shown in other systems (Coleman et al., 2008; Spaulding & Rieske, 2010). Because of the imminent threat to the avocado industry in southern Florida, there is a critical need for more efficient and less expensive traps to monitor and control this insect pest.

To date, R. lauricola has been detected in dead avocado trees in southern Florida but has not yet been proven as the cause of tree death. Furthermore, the insect vector X. glabratus has seldom been detected within those groves. There are some factors that may explain the presence of the fungus and the virtual absence of the vector. First, avocado is not preferred over redbay or swampbay (Persea palustris) (Mayfield & Hanula, 2012) and it is possible that the beetle is present, although the population is below the detection limit of current trapping methods. Second, until recently, manuka oil (essential oil from the leaves of Leptospermum scoparium J. R. Forst et G. Forst that contains similar chemicals to redbay) lures have been the only commercially available lure for monitoring X. glabratus (Hanula & Sullivan, 2008; Kendra et al., 2012); however, the effectiveness of manuka oil lure for trapping X. glabratus has been variable and short lived (Kendra et al., 2012; Hanula et al., 2013). Third, it is possible that the avocado trees have succumbed to other causes, and R. lauricola has been introduced into them by other secondary ambrosia beetle species (Carrillo et al., 2012). It is critical to distinguish why the fungus is detected ‘ahead’ of the vector to enable prediction of the likely movement of the vector through the area in the immediate future. This is another example of why a more efficient monitoring approach is needed for detection of the vector at low population densities.

Xyleborus glabratus is attracted to volatiles produced by cultures of their fungal symbiont in laboratory bioassays (Hulcr et al., 2011). Therefore, we tested the hypothesis that the attractiveness of manuka oil lures could be enhanced by adding the odour of the symbiotic fungus, thereby more closely imitating the odour of a host tree infected with R. lauricola. We first analyzed the volatiles from R. lauricola cultures of their fungal symbiont in laboratory bioassays (Hulcr et al., 2011). Therefore, we tested the hypothesis that the attractiveness of manuka oil lures could be enhanced by adding the odour of the symbiotic fungus, thereby more closely imitating the odour of a host tree infected with R. lauricola. We first analyzed the volatiles from R. lauricola and two other common ambrosia fungi and identified the major components of the odour blends via gas chromatography-mass spectrometry (GC-MS). Subsequently, we tested bioactivity of a synthetic reconstitution of this blend and its field efficiency in prototype, commercial release devices using sticky traps.

**Materials and methods**

**Chemicals and fungal cultures**

Isoamyl acetate (≥ 97% purity; #W205532), isoamyl alcohol (≥ 98% purity; #W205710) and ethyl acetate (≥ 99.7% purity; #34972) were purchased from Sigma Aldrich (St Louis, Missouri). Ethanol (200 proof; #20701) was purchased from Decon Laboratories (King of Prussia, PA). Pure cultures of ambrosia beetle symbionts R. lauricola, Ambrosiomyza sp. and Ambrosiella sp. were prepared as described by Hulcr et al. (2011) and Kolarik and Hulcr (2009). The cultures were maintained on potato dextrose agar (PDA) and were subcultured every 2–3 weeks. Manuka oil lures (#3083) were purchased from Synergy Semiochemicals Corp. (Canada). Raffaelea lauricola odour blend (ROB) release devices A, B, C and D and ethanol release devices were purchased from Alpha Scents (West Linn, Oregon).

**GC-MS sample preparation**

We examined the headspace volatiles of the symbiotic fungus of X. glabratus and R. lauricola, as well as Ambrosiella sp. and Ambrosiomyza sp., which are two other ambrosia fungi that are not the primary symbionts of X. glabratus (Hulcr et al., 2011). Fungi were grown on slants of PDA for the sampling. Fifteen microlitres each of PDA medium was autoclaved in 40-mL glass vials with tin foil as a lid. The media were allowed to solidify at 7°C for approximately 75–100% of the surface area was covered with fungus. A triphase 50/30 μm DVB/Carboxen/PDMS StableFlex™ solid-phase microextraction (SPME) for volatiles and semivolatiles with molecular weight between 40 and 275 (Supelco, Bellefonte, Pennsylvania) fibre was inserted through the tin foil lid and exposed to the fungal odours for 5 min. The SPME fibre was desorbed for 5 min at 240°C under splitless conditions and the odour constituents were separated over 40 min on a Stabilwax (Restek, Bellefonte, Pennsylvania) capillary column (60 m × 0.25 mm inner diameter; 0.5 μm film thickness) using a temperature gradient from 40 to 240°C at 7°C/min. Helium was used as a carrier gas at 2 mL/min. Identification of the compounds was performed using a Clarus 500 quadrupole mass spectrometer and Turbo Mass software (Perkin Elmer, Shelton, Connecticut). Linear retention times of authentic standards, when available, and mass spectra from the NIST database were used to identify compounds.

**Field sites**

Field sites were selected based on monitoring of X. glabratus populations in the autumn of 2010, 2011 and 2012 at Lake Kissimmee State Park (LKSP), in Polk County, FL. In the autumn of 2012, monitoring for X. glabratus was conducted at Wekiwa Springs State Park (WSSP), Orange County, Florida. To measure X. glabratus abundance, full size elm bark beetle traps (Great Lakes IPM, Vestaburg, Michigan) were attached to trees in five locations (between 27°55′51.64″N, 81°22′25.38″W and 27°55′16.98″N, 81°22′39.12″W) in LKSP in 2010, 2011 and 2012 and five locations along the green horse trail in WSSP in 2012 (between 28°43′36.35″N, 81°28′47.72″W and 28°43′35.76″N, 81°29′2.46″W). The trapping locations at both parks were within or along wet flatwood habitats containing declining redbay trees. Each location had one trap baited with a manuka oil lure and one trap with no lure as a control. Beetles were collected from the traps weekly and manuka oil lures were replaced every 2 weeks. The number of X. glabratus was observed using a stereomicroscope and recorded for each trap for each week. Based on this monitoring, we chose to conduct...
our field trials with ROB lures at WSSP because of the larger proportion of beetles captured (see Results).

Field experiments

Elm bark beetle sticky panel traps were cut in half and one half was stapled to wooden posts (height 1.5 m). The posts were randomly placed in replicate blocks in WSSP with at least 6 m between each trap. The minimum distance between replicate blocks was approximately 60 m. Three field experiments were conducted to test: (i) the attractiveness of ROB and (ii) prototype commercial release devices containing this same odour blend, as well as (iii) a different prototype commercial device characterized by a much higher release rate of the _Raffaelea_ odour blend. The intent of these analyses was to determine whether beetle captures with any of the potentially proprietary release devices were sufficiently high to justify further commercial development.

The _Raffaelea_ odour blend tested (ROB) was a mixture of 36.5 : 29 : 22 : 12.5 of ethyl acetate : ethanol : isoamyl alcohol : isoamyl acetate by volume and was based on GC-MS analysis of _R. lauricola_ odour. In the first field experiment, 1 mL of ROB was pipetted into 7-mL polyethylene Beem vials (Thermo Fisher Scientific, Waltham, Massachusetts) for release of odour. The lids were sealed with hot glue (#BSS6-4; Arrow Fastener Co., LLC, Saddle Brook, New Jersey) to prevent the lid from opening in the field. We refer to this initial lure design as device 0. In subsequent field experiments, the same odour blend was presented with other release devices manufactured by Alpha Scents. For each experiment, the lure placement was re-randomized.

In the first experiment, traps were baited with one of four treatments: blank (control), manuka oil lure, manuka oil with device 0, or device 0 alone. The total number of _X. glabratus_ was recorded per trap for the 2-week trapping period. Three trials of this experiment were conducted: 25 October to 8 November 2012 (n = 5); 8 November to 22 November 2012 (n = 5); and 6 December to 20 December 2012 (n = 12). The temperature during trapping demonstrated a high of 24.5 ± 3.3 °C and a low of 15.1 ± 3.4 °C. In addition, the number of beetles from other species of Scolytinae was recorded for the third trial.

In a second field trapping experiment from 6 January to 20 January 2013, three proprietary release devices (Alpha Scents) containing ROB were tested. The lures differed in the release device used; devices A and C were polyethylene vessels differing in volume and wall thickness. Device A had a smaller internal volume compared with device C and device 0. Device C had a higher internal volume than device 0. The third (device B) was a mylar packet containing a cellulose disc and sealed with a layer of polyethylene. The temperature during trapping was a high of 24.3 ± 4.7 °C and a low of 12.7 ± 4.8 °C. Six replicate blocks of traps were baited with combinations of lures: manuka oil (positive control), blank (negative control), manuka oil with device 0, manuka oil with device A, manuka oil with device B, and manuka oil with device C. The number of _X. glabratus_ was observed using a stereomicroscope and recorded for each trap for 2 weeks of trapping.

In a third field trapping experiment from 1 March to 15 March 2013, a new prototype, device D, was evaluated. The purpose of this experiment was to evaluate a higher release of the ROB and to test the effect of a higher release of ethanol on the capture of _X. glabratus_. Additional ethanol release devices were investigated because polyethylene (i.e. the material used for release devices of ROB) is not very permeable to alcohols. Device D comprised a polyethylene bag (10 × 15 cm) enclosing a foam matrix and the synthetic odour blend. In this experiment, six replicates of nine lure combinations were tested: blank control, manuka oil, ethanol, manuka oil with ethanol, device C with manuka oil, device C with manuka oil and ethanol, device D with manuka oil, device D with manuka oil and ethanol, and device 0 with manuka oil. The temperature during trapping was a high of 21.6 ± 4.0 °C and a low of 8.4 ± 3.1 °C.

Release rate

The release rate (mg/day) was measured gravimetrically for each type of device. The devices were hung outdoors at Lake Alfred, Florida, in March 2013. Devices were weighed on subsequent days to determine the weight released during each 24-h period for approximately 30 days. The mean high temperature during this period of time was 22.8 °C and the mean low temperature was 9.4 °C. Four replicates of each device were tested, except for device A and device B where three replicates were tested. Gravimetric release rates were calculated based on the reduction in weight between subsequent days. The mean release for each lure was calculated for days 1–3 and 3–7. The data were also fitted with an exponential decay curve. Release of individual components of ROB was not measured for the devices.

Statistical analysis

All statistical analyses were performed using the statistical software package R, version 2.15.3 (http://www.r-project.org). For all trapping experiments of _X. glabratus_, the data were not normally distributed; therefore, we performed a generalized linear model (GLM) with a log link function for Poisson distribution. The data also showed over-dispersion, meaning that, in contrast to a Poisson distribution where the mean is equal to the variance, the variance of the data in the present study was larger than the mean. Consequently, we corrected the standard errors using a quasi-GLM model where the variance is given by ϕ × μ and where ϕ is the dispersion parameter and μ is the mean. The standard errors are corrected by multiplying them with the square root of ϕ (Zuur _et al._, 2009). Trapping data for nontarget scolytine beetles were not overdispersed, and therefore we used a Poisson distribution in our GLM. We started with a model that included lure treatment, block number and trial date as fixed variables: Beetles ~ Lure + Trial + Block. When trial date or block number did not show a significant effect (α > 0.05), they were removed from the model to obtain a minimal adequate model (Crawley, 2009). When a significant effect of the lure treatment was found, a post-hoc Tukey’s test (function ‘glht’, package ‘multcomp’) was performed to
determine which treatments (lure combinations) differed with respect to their attraction of beetles.

Results

GC-MS identification of fungal odours

_Raffaelea lauricola_ headspace contained ethyl acetate, ethyl alcohol, isobutyl alcohol, isovaleric acid and isoamyl alcohol. _Ambrosiozyma_ sp. headspace volatiles contained primarily ethyl acetate and isovaleric acid. _Ambrosiella_ sp. headspace contained ethanol, isobutyl alcohol and isoamyl alcohol (Fig. 1). The control vial containing only potato dextrose agar and no fungi had only trace amounts of ethyl acetate (data not shown). The relative abundance of the major constituents of _R. lauricola_ headspace was used to create a synthetic odour blend for field trapping (ROB).

Monitoring of beetles in two Florida state parks

In LKSP, 57 _X. glabratus_ were captured during 57 days of trapping, and the redbay trees appeared healthy and abundant. However, when the park was re-sampled in 2011, the manuka oil baited traps captured substantially more _X. glabratus_ than in the previous year. We captured 4667 _X. glabratus_ during 57 days of trapping. In 2011, many trees were dead or dying with visible symptoms of laurel wilt and most trees examined showed evidence of ambrosia beetle boring in the trunks. In 2012, we re-sampled at LKSP and sampled for the first time at WSSP. In 19 days of trapping, 191 _X. glabratus_ were captured at LKSP; however, at WSSP, we captured 3985 _X. glabratus_.

Field trials of _Raffaelea_ devices

We tested device 0 by itself and in conjunction with manuka oil lures (Fig. 2A) in three trials. We found a significant difference in trap captures among the four treatments (\( \chi^2 = 15.37, F_{3,70} = 82.68, P < 0.001 \)). There was no significant difference between mean capture of _X. glabratus_ on traps baited with device 0 alone compared with blank traps (\( P = 0.524 \)) (Fig. 2A). Traps baited with device 0 paired with manuka oil captured more _X. glabratus_ on average than traps baited with manuka oil alone (\( P = 0.003 \)) (Fig. 2A).

We also examined the number of other nontarget Scolytinae beetles of various species present on the traps during the December trapping period (Fig. 2B). Catch of nontarget Scolytinae was significantly different among trap types (\( F_{3,44} = 14.59, \)
Raffaelea odours synergize with Manuka oil lures

Captures of Xyleborus glabratus (A) or nontarget scolytines (B) over 2 weeks on unbaited traps (negative control) or traps baited with manuka lure, device 0, or manuka with device 0. Bars labeled by the same lower case letter are not significantly different.

Figure 2 Captures of Xyleborus glabratus per 2 weeks with three prototype commercial Raffaelea-odour devices: A, B and C. The number of X. glabratus captured per 2 weeks with device C was compared with device 0 paired with manuka and the pooled data for the three device types that captured the fewest beetles (n = 5). Bars labeled by the same lower case letter are not significantly different.

Release rates

Dispensers differed greatly in their release rates. Device D had the highest release rate, followed by device C then B, and lure A had the lowest release rate. The release rates were modelled by exponential decay. The decay constants, as well as mean release for days 1–4 and 4–7 are reported in Table 1. The release rates for each lure type over 32 days are shown in Fig. 5(A–D).

Discussion

Our main hypothesis was based on previous data indicating that the volatiles produced by cultured R. lauricola were attractants for X. glabratus (Hulcr et al., 2011). The hypothesis was supported in field tests. Volatiles from R. lauricola synergistically increased the attractiveness of tree volatiles to X. glabratus. Previous research suggested that the odours of the fungus were not attractive based on trapping with wounded healthy and infected trees (Hanula et al., 2008); however, the release of volatiles from wounding those trees may have overridden the underlying odours of the fungus. Based on our data using synthetic fungus and host-mimic odours, we conclude that the vector beetle incorporates stimuli from both the host
Figure 4 Captures of *Xyleborus glabratus* per 2-week interval with prototype commercial *Raffaelea*-odour device C and D with ethanol against various other lures (*n* = 5). Bars labeled by the same lower case letter are not significantly different.

Table 1 Gravimetric release rate analysis of odor lures tested for attraction of *Xyleborus glabratus*

<table>
<thead>
<tr>
<th>Lure/device</th>
<th>Mean release rate (mg/day)</th>
<th>Exponential decay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days 1–3</td>
<td>Days 3–7</td>
</tr>
<tr>
<td>Device 0</td>
<td>51.3 ± 8.3</td>
<td>30.7 ± 2.6</td>
</tr>
<tr>
<td>Device A</td>
<td>3.3 ± 0.6</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Device B</td>
<td>117.4 ± 17.7</td>
<td>45.5 ± 8.5</td>
</tr>
<tr>
<td>Device C</td>
<td>31.3 ± 3.4</td>
<td>17.4 ± 0.4</td>
</tr>
<tr>
<td>Device D</td>
<td>220.8 ± 42.8</td>
<td>520.8 ± 46.2</td>
</tr>
<tr>
<td>Manuka oil lure</td>
<td>98.2 ± 25.1</td>
<td>35.2 ± 6.5</td>
</tr>
</tbody>
</table>

The ROB blend that we tested is based on GC-MS analysis as sampled by SPME. SPME has limitations when used to quantify volatile release from a source because not all compounds adhere to the solid phase with equal efficiency. In addition, the differing internal volume and wall thickness of our release devices likely affected the permeability of various compounds as our release rate analyses suggest. Device 0 was not completely sealed; therefore, it is possible that ethanol was released from this device at a higher rate compared with the completely heat-sealed proprietary devices. We chose to increase the release rate of ethanol in a subsequent experiment. In this field trapping experiment, we evaluated device 0 and device C paired with manuka oil, as well as device C with manuka oil and ethanol against a new high release prototype, device D. Traps baited with device D with manuka oil and device D with manuka oil and ethanol captured the most *X. glabratus*. Ethanol did not appear to impact catch of *X. glabratus*. When we performed pairwise comparisons between lure combinations with and without ethanol, there were no significant differences. This is congruent with previous studies suggesting that ethanol does not attract *X. glabratus* (Hanula & Sullivan, 2008; Hulcr et al., 2011; Kendra et al., 2012). Future studies may want to examine the role of ethanol for the catch of this species when released with other odours at the appropriate release rate and blend.

Device 0 by itself captured more nontarget Scolytinae than the unbaited control traps, although the catch of these nontarget species was low compared with the catch of *X. glabratus*. The similarity of odours released by various ambrosia fungi may explain some of these nontarget captures; ethanol may be a main driver for the attraction of nontarget beetles because many are known to be attracted to this compound. As a tree declines from *X. glabratus* infestation, it becomes more attractive to other generalist Scotylinae as a result of the release of odours of wood decay. Our lures may be perceived as an indicator of availability of a generalists’ host. Therefore, it may not be surprising that, when ROB was present, traps captured more nontarget species of Scolytinae than those without ROB.

Our trapping experiments evaluated the efficacy of devices during the first 14 days of deployment. Gravimetric release rate analysis revealed considerable variability in release between device types during the initial few days of deployment. The best performing ROB lure for capture of *X. glabratus* was device D and this lure also exhibited steady gravimetric release, suggesting that this lure may be effective for long-term, unsupervised deployment.
Based on our findings, it appears that a more effective lure for *X. glabratus* may be possible by combining manuka oil (or other relevant plant odours) with *Raffaelea* associated odours into a single release device or at least when used in combination. The components used in ROB are commercially available and relatively inexpensive because they are common compounds used in the flavor industry. Based on the captures of *X. glabratus* recorded with an initial prototype of a commercial lure, it appears that such a release device should be specifically tuned for the ideal release rate and the blend of the odour constituents to optimize monitoring of this beetle. This combination of fungal and plant odourants may improve monitoring of *X. glabratus* in locations where beetle densities are low and below current detection thresholds, particularly in areas where the beetle is not yet known to be established.
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References


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