Evaluation of repellents for the redbay ambrosia beetle, Xyleborus glabratus , vector of the laurel wilt pathogen

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ORIGINAL CONTRIBUTION

Evaluation of repellents for the redbay ambrosia beetle, *Xyleborus glabratus*, vector of the laurel wilt pathogen

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Abstract

The redbay ambrosia beetle, *Xyleborus glabratus*, is the vector of the laurel wilt disease fungal pathogen, *Raffaelea lauricola*. Since the vector's initial detection in the USA in the early 2000s, laurel wilt has killed millions of redbay, *Persea borbonia*, trees and other members of the plant family Lauraceae. To protect host trees from beetle attack and laurel wilt infection, we tested the efficacy of host- and non-host-derived and commercial compounds as *X. glabratus* repellents in field experiments. In our first trial, the major constituents of the non-host tree, longleaf pine, *Pinus palustris*, and SPLAT Verb (verbenone 10%) were paired with manuka oil attractants and beetle captures were counted. Verbenone and a 1 : 1 blend of myrcene and camphene were intermediate to both the manuka positive and blank negative controls. Subsequently, we tested different blends of methyl salicylate (MeSA), a host defence and signalling compound, and verbenone in SPLAT dispensers using freshly cut redbay bolts as an attractant. All treatments reduced *X. glabratus* captures and boring holes as compared to the redbay (-) repellent positive control; however, SPLAT Verb and SPLAT MeSA-Verb (5% each) achieved the highest repellency, with results comparable to that of the non-host (laurel oak). These trials establish that host-derived and commercially available repellent compounds can reduce *X. glabratus* attacks and therefore have potential as part of an integrated management strategy against laurel wilt and its vector.

Introduction

Since establishment by European settlers, over 450 invasive insects and several serious tree pathogens have colonized the urban and rural forests of the United States (Aukema et al. 2010). Of these accidental introductions, some species have become important forest pests, causing widespread tree mortality and severe ecological disturbances (Wingfield et al. 2016). Wood borers, in particular, can be an especially damaging group, with introduced insects such as the emerald ash borer (*Agrilus planipennis* Fairmaire) and the native mountain pine beetle (*Dendroctonus ponderosae* Hopkins) killing billions of susceptible host trees (Romme et al. 1986; Herms and McCullough 2014). The term 'ambrosia beetle' is an ecological classification that combines different phylogenetic groups of minute wood-boring beetles belonging to the Curculionidae (subfamilies Scolytinae and Platypodinae). Unlike bark beetles, which feed on host phloem, ambrosia beetles are completely reliant on their symbiotic fungi for nutrition (Batra 1985). Ambrosia beetles commonly attack and colonize stressed and wounded trees and are, therefore, usually considered benign or beneficial to ecosystem health as they accelerate forest nutrient recycling (Rudinsky 1962). However, there are exceptions; non-native ambrosia beetle species may attack susceptible healthy trees and become detrimental pests in forestry, agriculture and the nursery trade (Hulcr and Dunn 2011; Ploetz et al. 2013).

Among the ambrosia beetles that attack live and apparently healthy trees, the redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff, was first detected near Savannah, Georgia, in monitoring traps maintained by the US Forest Service in 2002 (Haack 2006). Native to Asia (Rabaglia et al. 2006), *X. glabratus* was likely introduced to the USA through untreated wood packing material (e.g. crates and pallets). Soon after initial detection, *X. glabratus* and its pathogenic fungal symbiont, *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva, were associated with attacks of healthy redbay (*Persea borbonia* [L.] Spreng.) trees and the newly described, lethal vascular disease named laurel wilt (Fraedrich et al. 2008; Harrington et al. 2008). Laurel wilt disease affects most USA native and some non-native members of the Lauraceae family (Kendra et al. 2014; Hughes et al. 2015a). Laurel mortality has been most severe in forests of the south-eastern Atlantic and Gulf coastal plains where, in total, millions of mature redbay, swamp bay (*Persea palustris* [Raf.] Sarg.) and silk bay (*P. humilis* Nash) trees have been attacked by the beetle, infected and killed (Fraedrich et al. 2008; Shields et al. 2011; Kendra et al. 2012a; Spiegel and Leege 2013; Cameron et al. 2015). *Xyleborus glabratus* attacks apparently healthy Lauraceae, introducing the laurel wilt pathogen into the xylem, disrupting vascular function and causing the leaves to wilt in a matter of weeks to a few months (Fraedrich et al. 2008; Hughes et al. 2015a,b).

Due to the wide distribution of Lauraceae in the Americas, laurel wilt threatens a diversity of habitats, including the central and western forests, where sassafras (*Sassafras albidum* [Nutt.] Nees) and California laurel (*Umbellularia californica* [Hook. & Arn.] Nutt.) are native, respectively (Fraedrich 2008; Fraedrich et al. 2015). In 2012, laurel wilt was found in another Lauraceae member, avocado (*P. americana* Mill.), damaging commercial production (Mosquera et al. 2015). However, in south Florida, other ambrosia beetle species may also be involved in *R. lauricola* transmission in avocado (Carrillo et al. 2012, 2014), requiring unique considerations for the management of the disease in that system (Ploetz et al. 2011; Crane et al. 2015). Given its rapid spread, laurel wilt represents a threat to the commercial avocado growers of California and, to the world's largest producer, Mexico (Pisani et al. 2015).

Current laurel wilt management relies on rapid sanitation and chemical treatments of susceptible host trees (Mayfield et al. 2008; Spence et al. 2013; Hughes et al. 2015a). As with the Dutch elm and oak wilt diseases in America, proper sanitation consists of tree felling and covering logs or chips in plastic sheets to vector visitation, development and/or escape (Bruhn and Heyd 1992; Haugen 2001; Spence et al. 2013). Although effective in reducing vector populations, this treatment is only appropriate for diseased trees and may not be practical across a large area. A prophylactic application of the fungicide propiconazole can offer protection (Mayfield et al. 2008); however, the longevity of the treatment is variable, likely requiring frequent re-applications. Propiconazole applications, although cost prohibitive in forest settings, is an option currently used by avocado growers to limit the spread of laurel wilt within a grove. Topical and systemic insecticides are relatively ineffective in *X. glabratus* suppression due to their limited duration of efficacy in host trees (Peña et al. 2011; Carrillo et al. 2013). Entomopathogenic fungi have been described that infect *X. glabratus*, but they kill the beetles after they bore into the wood, not preventing the inoculation with *R. lauricola* (Carrillo et al. 2015). For avocado, pre-symptomatic aerial detection of laurel wilt diseased plants using spectral imagery is in development (Sankaran et al. 2012; de Castro et al. 2015), but not yet operational. Overall, there is no reliable tool available to prevent *R. lauricola* infection; consequently, there is a crucial need for management tools to prevent *X. glabratus* attack and to slow the spread of laurel wilt disease.

The host selection process of *X. glabratus* is largely driven by their chemical ecology, which is also important to the epidemiology of laurel wilt. *Xyleborus glabratus* does not respond to ethanol, a chemical indicator of plant stress and a common lure component for most ambrosia beetle species investigated to date (Hanula and Sullivan 2008; Johnson et al. 2014), but instead responds to other host volatiles (Niogret et al. 2011; Kendra et al. 2012a,b, 2014). Wood, leaf and fungal odours, in addition to visual cues such as stem silhouette, can direct *X. glabratus* attack, suggesting that a complex combination of cues may be utilized by the beetle for host selection (Hulcr et al. 2011; Mayfield and Brownie 2013; Kuhns et al. 2014a,b; Martini et al. 2015). Additionally, lure development has focused on the sourcing and enrichment of the attractant α-copaene (Kendra et al. 2016a,b), a plant-derived sesquiterpene common in laurel wilt hosts (Hanula and Sullivan 2008; Kendra et al. 2011; Niogret et al. 2011).

The study of insect repellents for tree protection, specifically against *X. glabratus* and laurel wilt, is just starting; however, trials with other ambrosia beetles show the potential of the use of repellents as part of an integrated pest management programme (Borden et al. 2001; Burbano et al. 2012; Ranger et al. 2013). To expand laurel wilt management options, we investigated the efficacy of a commercial repellent formulation and of additional host and non-host volatiles to deter visitation and attack by *X. glabratus* and other ambrosia beetle species on traps and host bolts in field experiments.

Methods and Materials

Experimental locations

Field sites were selected based on the presence of laurel wilt and a mixture of diseased and asymptomatic redbays still onsite. Experiments were conducted at Wekiwa Springs State Park (WSSP, Apopka, FL – 28°42′40.93″N, 81°27′45.85″W) in 2012 and in the Historic Haile Homestead (HHH, Gainesville, FL – 29°35′40″N 82°26′7″W) and Ichetucknee Springs State Park (ISSP, Fort White, FL – 29°58′2.47″N 82°46′33.82″W) in 2015. WSSP was a pine flatwoods forest dominated by redbay, slash pine (*Pinus elliottii* Engelm.) and red maple (*Acer rubrum* L.). HHH consisted of a mixed pine–hardwood forest dominated by live oak (*Quercus virginiana* Mill.), redbay, southern magnolia (*Magnolia grandiflora* L.), cabbage palmetto (*Sabal palmetto* [Walter] Lodd. ex Schult. & Schult. f.) and slash pine. ISSP's vegetation consisted of an oak–hickory overstory forest dominated by laurel oak (*Quercus laurifolia* Michx.), scrub hickory (*Carya floridana* Sarg.), redbay, southern sugar maple (*Acer floridanum* [Chapm.] Pax) and cabbage palmetto.

Chemicals and formulations

Manuka oil lures were purchased from Synergy Semiochemicals Corp. (Burnaby, BC, Canada), and the chemicals eucalyptol (C80601), myrcene (M100005), limonene (62122), α-pinene (147524), β-pinene (W290300) and ocimene (W353901) were purchased from Sigma-Aldrich (St. Louis, MO). SPLAT® (ISCA Technologies, Riverside, CA) is an inert, wax-based matrix that allows for the infusion and subsequent slow release of volatile compounds. ISCA Technologies supplied SPLAT Verb (active ingredient verbenone at 10%) (Mafra-Neto et al. 2014a; Fettig et al. 2015) and formulated SPLAT MeSA (methyl salicylate at 10%) and a SPLAT MeSA/Verb combination (5% each). Verbenone is a ketonic, anti-aggregation semiochemical produced by the mycangial fungi of certain bark beetles (Brand et al. 1976) and is commonly deployed as spatial repellent (Amman et al. 1989, 1991; Gibson et al. 1991; Miller et al. 1995; Mafra-Neto et al. 2014b; Fettig et al. 2015; Perkins et al. 2015). Methyl salicylate is a plant-derived volatile derivative of salicylic acid (Loake and Grant 2007) and has been found to be repellent against *X. glabratus* in laboratory experiments (X. Martini and M. A. Hughes, unpublished).

Non-host plant volatile collection and analysis

The needles of the non-host tree, longleaf pine (*Pinus palustris* Mill.), were found to be significantly repellent to *X. glabratus* in laboratory olfactometer bioassays (data not shown). Therefore, the major chemical constituents of longleaf pine were analysed by gas chromatography–mass spectrometry (GC-MS) as described below, and combinations of volatile components were used for repellency in field trials. Longleaf pine needle samples of approximately 1.0–1.5 g were cut into small pieces and were placed in a clean 40-ml glass vial and sealed with a lid and septa for volatile equilibration. After equilibrating for at least 15 min at 21°C, a triphase 50/30 μm DVB/Carboxen/PDMS StableFlex™ solid page microextraction (SPME) fibre (Supelco, Bellefonte, PA) was inserted through the septum and exposed to the leaf odours for 30 s. The SPME was desorbed onto a GC-MS wax column, and the odour constituents were separated over 40 min on a Restek Stabilwax capillary column using a temperature gradient from 40 to 240° at 7°/min. Identification of the compounds was performed using a PerkinElmer Clarus 500 quadrupole mass spectrometer and Turbo Mass software (GC-MS). Linear retention times of authentic standards, when available, and matching mass spectra to the NIST database were used to identify components. The per cent abundance of major constituents is provided in table 1.

Treatment repellents and experimental design

Experiment 1: Test of repellents with manuka oil as attractant
The goal of this field trial was to provide an initial screening of non-host volatiles and verbenone for potential repellency to *X. glabratus*. At WSSP, Elm Bark Beetle Sticky Traps (Great Lakes IPM, Vestaburg, MI) were prepared as two sticky cards (46 × 32 cm) that were affixed to a wooden post at 1 m in height (see Kuhns et al. 2014a). Treatments consisted of the pairing of two separate components: a manuka oil lure (attractant) (Hanula and Sullivan 2008) and either a non-host synthetic volatile blend or SPLAT Verb (potential repellents). Manuka lures were

Table 1 GC-MS analysis of volatiles emitted from longleaf pine (*Pinus palustris*) needles and the synthetic volatile blends used in field repellent experiment 1. Mixtures were made by volume

Linear retention time	Identification	Relative abundance (%) Longleaf Pine	Synthetic blends for field testing (% by volume)		
			Pine 1	Pine 2	Pine 3
1038	α-Pinene	23.1	67	–	–
1097	Camphene	1.1	–	50	–
1131	β-Pinene	51.8	33	–	–
1135	Sabinene	2.2	–	–	–
1189	Myrcene	1.8	–	50	–
1220	Limonene	1.6	–	–	50
1248	Ocimene	2.0	–	–	50
	Total	83.6	100	100	100

constructed from an internal matrix soaked in manuka oil, enclosed in a plastic pouch (Synergy Semiochemicals Corp.). The putative *X. glabratus* repellents consisted of the following synthetic blends: 'pine 1' was a 2 : 1 mixture of α-pinene and β-pinene by volume, 'Pine 2' was a 1 : 1 mixture of myrcene and camphene by volume, 'Pine 3' was a 1 : 1 mixture of limonene and ocimene by volume (table 1), and SPLAT Verb contained 10% verbenone by volume. Synthetic liquid blends (5 ml) of each artificial plant odour were injected directly into the manuka lure using a 21-gauge hypodermic needle and glass syringe. The hole was covered with a small piece of cellophane tape. Control manuka lures were also punctured and patched in a similar manner, but nothing was injected into the lure. For the SPLAT Verb treatment, one dollop (17.5 g deployed as 1 cm diameter sphere) of SPLAT was wrapped in medical gauze and attached with a paper clip to the sticky trap in close proximity to the manuka lure. Traps baited with manuka oil only served as positive controls, and non-baited traps served as negative controls. Traps were arranged in a randomized complete block design, consisting of four blocks of six beetle traps (n = 24). Within-block traps were placed no less than 6 m apart, with blocks no less than 60 m apart. Sticky cards were collected weekly, and *X. glabratus* captured were identified and counted. The mean temperature was 25.5 ± 0.2°C and relative humidity 78.0% ± 1.3%.

Experiment 2: Test of repellents with cut logs as attractant

The goal of this field trial was to test the repellency of verbenone and MeSA, the only volatiles that showed repellency against *X. glabratus* in the initial field experiment (see Results section) and in laboratory tests (X. Martini and M. A. Hughes, unpublished) as compared with natural tree host tissue. Field trials were conducted at HHH and ISSP in 2015. To increase the number of *X. glabratus* captured on controls, we utilized freshly cut redbay bolts as the attractant (positive control) instead of manuka lures, and non-host, laurel oak bolts as a negative control. To obtain the fresh wood tissue, a single uninfected redbay and laurel oak were cut down and the main stem sectioned into segments (≈30 cm length, 10 cm diam.). A large construction nail was hammered into the bottom of a cut end, and the bolt was then placed into the top of a hollow metal pole (3 cm dia.) that was previously driven into the ground ≈1.5 m in height, an appropriate location for capture of *X. glabratus* (Brar et al. 2012). Two (28 × 23 cm) sticky cards (Wing trap sticky liner – Scentry Biologicals Inc., Billings, MT) were stapled to the lower portion of the treatment bolt on opposite sides (Figure S1). Repellent treatments consisted of the redbay bolts plus SPLAT MeSA, SPLAT Verb or a MeSA/Verb mixture (1 : 1). Dollops (17.5 g) of SPLAT compounds were applied directly to the bolts with a pre-calibrated caulking gun and allowed to cure for 24 h before field deployment (Figure S1). Positive and negative control bolts were left untreated.

Traps were arranged in a randomized complete block design. A block consisted of a linear transect with treatments spaced at 10 m and blocks 30 m apart. Treatments were randomly positioned within blocks and were rotated to the next position every trapping period to minimize positional effects. To further reduce the potential of a positional bias, no traps were placed within 3 m of symptomatic redbays. Traps were inspected and rotated every 14 days for HHH and ISSP. At each trapping period, two circular wounds (2 cm dia.) were made with a cork borer to expose fresh redbay xylem and thus refresh the bolts' attractiveness. At the end of every trapping period, sticky cards were replaced and brought to the laboratory where beetles were identified and counted under a dissection microscope. The trial was terminated once each treatment completed a full positional rotation cycle along the linear transect (five rotations/10 weeks). At the end of the trial, trap bolts were transported to the laboratory (University of Florida, Gainesville), where they were debarked and *X. glabratus* entrance holes were counted as described in Hanula et al. (2008). A subsample of bolts with beetle entrance holes was further dissected using a band saw and examined for the presence of *X. glabratus* and the extent of gallery formation. The mean temperature was 22.9 ± 0.3°C, and relative humidity, 87.0% ± 0.7%.

Statistical analysis

The reported means ± SE and sums were calculated in Microsoft Excel (2010). Statistical analyses of beetle captures and *X. glabratus* entrance holes were calculated using generalized linear mixed models with a negative binomial distribution using the GLIMMIX procedure on SAS 9.4 (SAS Institute, Cary, NC). For boring activity analysis, laurel oak data were omitted due to the lack of *X. glabratus* entrance holes on this host. Weekly capture data were analysed using a linear mixed model where WSSP was square root-transformed to meet the assumptions of the model, while counts at HHH and ISSP were not transformed. At WSSP, the violation of the model assumptions was probably due to low beetle counts. Block and site locations were treated as random effects in all models. Type III tests were used to determine significance of the fixed effects. Multiple comparisons of least squares means were analysed and adjusted for using Tukey's multiple comparison procedure. Standard errors were calculated as [std dev/$\sqrt{n}$], where n = number of replicates. Figures were prepared using GraphPad Prism v. 7.0 (GraphPad Software, La Jolla, CA) and edited in Adobe Illustrator CS6 (Adobe System Inc., San Jose, CA). All mean temperature and relative humidity data were collected and analysed from the Florida Automated Weather Network (FAWN) provided by the University of Florida.

Results

Non-host plant synthetic blends

Volatile analysis of longleaf pine needles revealed the predominant constituents as α and β pinene; additionally, myrcene, camphene, limonene and ocimene comprised the less abundant components of the volatile profile (table 1). All of these components except ocimene are present in redbay wood odours (Kuhns et al. 2014a; Martini et al. 2015); however, long leaf pine does not contain eucalyptol, a known attractant of redbay ambrosia beetle (Kuhns et al. 2014a). Combinations of components were selected for field testing based on their relative abundance in longleaf pine and are shown in table 1.

Experiment 1: Test of repellents with manuka oil as attractant

Manuka oil attracted significantly more *X. glabratus* beetles than the non-baited control, with most of the treatment repellents showing intermediate trap catches (fig. 1). Captures decreased for all treatments after 4 weeks (fig. 1). Mean weekly *X. glabratus* captures were statistically higher ($P = 0.007$) for manuka + pine 1 (4.4 ± 1.0), manuka + pine 3 (5.1 ± 1.2) and manuka alone (5.6 ± 1.0) than the negative control treatment (0.3 ± 0.1) (table 2). Mean beetle captures with manuka + pine 2 (2.9 ± 1.0), and manuka + SPLAT Verb (2.1 ± 0.5) did not differ significantly to all other treatments (table 2). The manuka positive control captured the most total *X. glabratus* throughout the duration of the experiment (90), and SPLAT Verb was the repellent with the fewest (34) total captures (table 2). Non-*X. glabratus* ambrosia beetle captures were low and statistically similar ($P = 0.42$) among all treatments, with means ranging from 0.9 to 1.9 weekly catches and total sums 14–30 beetles (table 2). Non-target ambrosia beetles comprised 17%–53% of all total captures per treatment (table 2) and included members of the genera *Xyleborus* and *Xylosandrus* (data not shown).

Experiment 2: Test of repellents with cut logs as attractant

The effects of block and site location (ISSP and HHH) were not statistically significant (at $P > 0.05$, data not shown); thus, data from both locations were combined. For *X. glabratus*, all repellent treatments and the laurel oak negative control captured significantly fewer beetles than the redbay positive control in our 14-day trapping periods ($P = 0.0001$) (fig. 2, table 3). Both the SPLAT MeSA-Verb (3.5 ± 0.6) and SPLAT Verb (2.2 ± 0.6) repelled the most beetles, and mean trap captures were statistically similar to the non-host laurel oak (5.8 ± 0.8 beetles) (table 3). Initially, SPLAT MeSA had a relatively high level of repellency, similar to the other best performing potential repellents; however, after four weeks, the effectiveness of this repellent treatment decreased, resulting in more mean captures in SPLAT MeSA (14.6 ± 2.4 beetles/biweekly) than the treatments containing verbenone (fig. 2, table 3). The redbay bolts without SPLAT captured the most beetles per trapping period (39.8 ± 3.9) (table 3). A total of 1992 *X. glabratus* beetles were captured with the redbay positive controls during the experiment. SPLAT MeSA-Verb and SPLAT Verb treatments captured fewer beetles than the laurel oak control (290 total *X. glabratus*), with a total of 177 and 108, respectively (table 3). Compared to the positive control, SPLAT MeSA-Verb reduced total captures by 91% and SPLAT Verb by 94.5%, indicating strong levels of repellency. Captures of

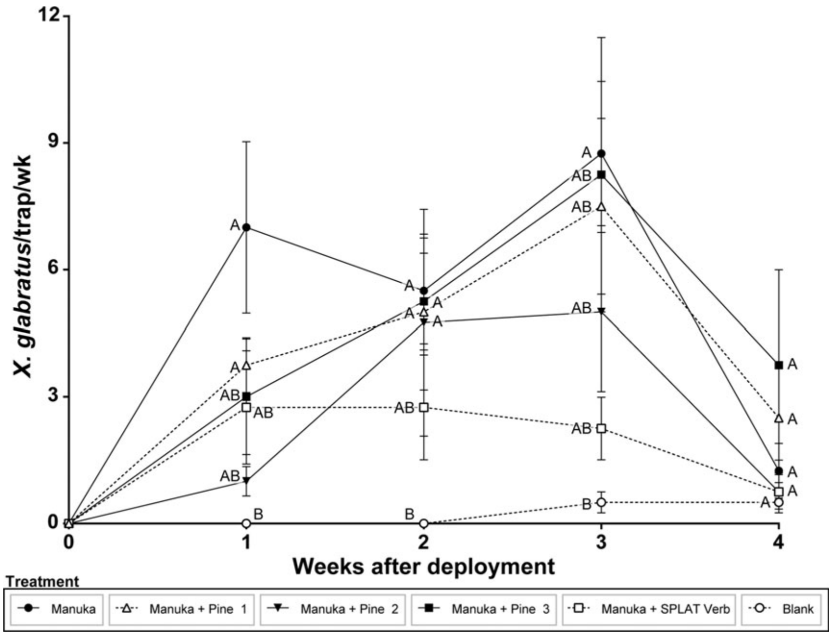

Fig. 1 Trapping experiment at Wekiwa Springs State Park (WSSP) in Florida. Mean *Xyleborus glabratus* captures ± SE over four weeks. Traps consisted of sticky panels (46 × 32 cm) affixed to wooden posts baited with manuka oil. Putative repellent synthetic blends were labelled as pine 1–3 (see table 1) and SPLAT Verb. Traps baited with only manuka oil were positive controls, and negative controls were unbaited traps. Separations of means are listed at weekly intervals. Columns denoted with different letters are statistically different at $\alpha = 0.05$ according to Tukey's multiple comparisons procedure of treatment least squares means. n = 24.

Table 2 Summary results of *X. glabratus* repellency experiment at Wekiwa Springs State Park (WSSP)

	Xyleborus glabratus		Non-target ambrosia beetle spp.		
Treatment*	Mean weekly trap captures†	Sum of trap captures	Mean weekly trap captures†	Sum of trap captures	Per cent of total captures
Manuka (pos. control)	5.6 ± 1.0 A	90	1.2 ± 0.4 A	19	17.4
Manuka + pine 1	4.4 ± 1.0 A	75	1.9 ± 0.4 A	30	28.6
Manuka + pine 2	2.9 ± 1.0 AB	46	0.9 ± 0.3 A	14	23.3
Manuka + pine 3	5.1 ± 1.2 A	81	0.9 ± 0.2 A	14	14.7
Manuka + SPLAT Verb	2.1 ± 0.5 AB	34	1.2 ± 0.2 A	19	35.8
Blank (neg. control)	0.3 ± 0.1 B	16	1.2 ± 0.4 A	18	52.9
P value	0.007		0.42		

Weekly means + SE and total sums of *X. glabratus* and non-target ambrosia beetles captured during four week experiment (four replicates per treatment, n = 24).

*Treatments consisted of manuka lures (attractant) paired with a test repellent. Positive and negative controls were manuka lure only and non-baited traps, respectively. See table 1 for constituents of synthetic blends.

†Columns denoted with different letters are statistically different at $\alpha = 0.05$ according to Tukey's multiple comparisons procedure of treatment least squares means.

other bark and ambrosia beetles were low (0.4–1.6 beetles/biweekly, 22–79 total sum) and seemed not to be affected by treatment (P = 0.48) (table 3). Non-target ambrosia beetles comprised 3%–27% of all total captures per treatment (table 3) and included members of the genera *Xyleborus*, *Xylosandrus*, *Hypothenemus* and *Ambrosiodmus* (data not shown).

Xyleborus glabratus entrance-hole counts were higher for the redbay positive control with a mean of 16.6 ± 3.7 per bolt (table 3). Significantly fewer mean boring holes occurred on redbay bolts treated with SPLAT MeSA (4.3 ± 1.0) as compared with the positive control. SPLAT Verb was the most effective repellent with 1.2 boring holes per bolt at experimental conclusion. SPLAT MeSA-Verb (3.3 ± 1.1 boring holes) was statistically similar to the treatments containing methyl salicylate and verbenone alone. SPLAT MeSA-Verb and SPLAT Verb suppressed *X. glabratus* bolt boring by over 80% and 92%, respectively, compared to the control with untreated redbay bolts (table 3). No beetle boring was detected on the non-host laurel oak. To explore the extent of *X. glabratus*

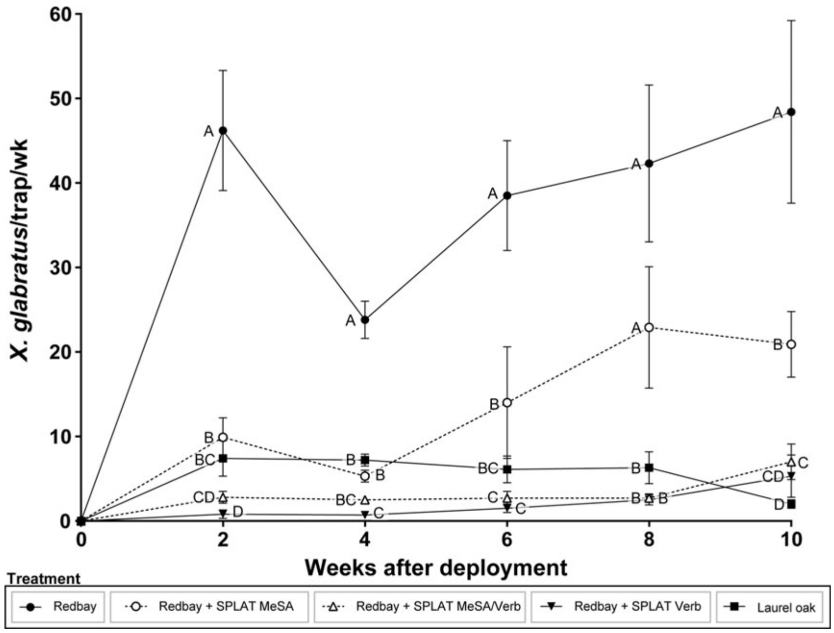

Fig. 2 Trapping of combined experiments at Historic Haile Homestead (HHH) and Ichetucknee Springs State Park (ISSP) in Florida. Mean *Xyleborus glabratus* captures ± SE over 10 weeks. Traps consisted of a redbay bolt with two sticky cards attached. Repellents consisted of SPLAT + methyl salicylate (MeSA), SPLAT Verb and a blend of SPLAT Verb + MeSA. Traps baited only with redbay bolts were positive controls and with laurel oak (non-host) bolts were negative controls. Separations of means are listed at 14-day intervals. Columns denoted with different letters are statistically different at $\alpha = 0.05$ according to Tukey's multiple comparisons procedure of treatment least squares means. n = 50.

colonization, a subset of bolts were further dissected by sectioning them into thin slices with a band saw. Most boring activity consisted of shallow tunnels extending only ≈ 1 cm into the sapwood, lacking signs of significant gallery formation or beetle reproduction (e.g. eggs or callow beetles).

Discussion

Here, we evaluated volatile compounds for their potential repellency against *X. glabratus*, the primary vector of the laurel wilt pathogen (*Raffaelea lauricola*) that has been causing high levels of mortality in native Lauraceae in the eastern USA. The volatile blends chosen were based on chemical analyses of non-host volatiles, and damage-induced volatiles known to repel this species, as well as known repellents for other bark beetle species. In our first experiment, we paired manuka lures with three synthetic volatile blends and SPLAT Verb (verbenone 10%). A 50 : 50 blend of camphene and myrcene, as well as a commercially available formulation of verbenone (SPLAT Verb), was intermediate to all treatments. Further testing with fresh host bolts or improved lures would likely results in the higher capture rates needed to truly discriminate between these treatments.

In our subsequent experiments, we abandoned the use of manuka oil lures as an attractant to investigate repellency in the field and instead used fresh redbay wood due to its superiority as an attractant (Kendra et al. 2011) and relevance to our overall goal of protecting live host trees. We selected verbenone due to the relatively low number of beetles captured when it was included in the initial experiment and methyl salicylate (MeSA) for its repellency to *X. glabratus* under laboratory conditions (X. Martini and M. A. Hughes, unpublished). Methyl salicylate is a plant-derived volatile ester released following stress events, such as herbivory, pathogen infection and associated disease-resistance pathways (Shulaev et al. 1997). The compound is normally absent in healthy plant tissue; however, upon herbivore and pathogen attack, MeSA acts as a volatile signalling molecule that induces host systemic acquired resistance (SAR) and salicylic acid defence response pathways to other areas of the plant and neighbouring plants (Shulaev et al. 1997; Loake and Grant 2007). Increased MeSA levels induced by insect feeding have been linked to repellency in certain herbivores (Hardie et al. 1994; Losel et al. 1996) and the attraction of natural enemies (Shimoda et al. 2002; James 2003; Mallinger et al. 2011), suggesting that this volatile has a complex and variable role in host signalling and defence. Borden et al. (2001) found that MeSA reduced striped ambrosia beetle (*Trypodendron lineatum* Oliver) densities in traps baited with an aggregation pheromone in British Columbia. MeSA (10%) alone reduced captures and boring holes of *X. glabratus* for the duration of the experiment; however, longevity of effectiveness was lower as compared with verbenone from the release devices tested here. It is possible that optimizing the release rate and duration of effective release of

Table 3 Summary results of *X. glabratus* repellency experiment at Historic Haile Homestead (HHH) and Ichetucknee Springs State Park (ISSP) combined.

	Xyleborus glabratus			Non-target ambrosia beetle spp.		
Treatment*	Mean biweekly trap captures†	Sum of trap captures	Mean boring holes per bolt†	Mean biweekly trap captures†	Sum of trap captures	Per cent of total captures
Redbay (pos. control)	39.8 ± 3.9 A	1992	16.6 ± 3.7 A	1.4 ± 0.2 A	70	3.4
Redbay + SPLAT MeSA	14.6 ± 2.4 B	724	4.3 ± 1.0 B	0.9 ± 0.2 A	44	5.7
Redbay + SPLAT MeSA-Verb	3.5 ± 0.6 CD	177	3.3 ± 1.1 BC	0.4 ± 0.1 A	22	11.1
Redbay + SPLAT Verb	2.2 ± 0.6 D	108	1.2 ± 0.6 C	0.8 ± 0.2 A	39	26.5
Laurel oak (neg. control)‡	5.8 ± 0.8 CD	290	0 ± 0.0	1.6 ± 0.3 A	79	21.4
P value	<0.0001		<0.0001	0.48		

Biweekly (14 days) means + SE and total sums of *X. glabratus* and non-target bark and ambrosia beetle species captured during 10-week experiment. n = 50.

*Treatments consisted of a cut redbay bolt attractant with a 17.5 g dollop of SPLAT test repellent. Positive and negative controls were untreated redbay and a laurel oak bolts, respectively.

†Columns denoted with different letters are statistically different at $\alpha = 0.05$ according to Tukey's multiple comparisons procedure of treatment least squares means.

‡Due to the lack of boring holes in the non-host laurel oak, these data were omitted from statistical analysis.

MeSA from SPLAT or other semiochemical dispensers may further improve the effectiveness of MeSA as a repellent against *X. glabratus*.

In our experiments, *X. glabratus* was repelled most effectively by the commercial SPLAT Verb formulation of verbenone (10%) alone and SPLAT formulated with a 1 : 1 mixture of verbenone and MeSA (5% each). These two repellent treatments reduced capture of *X. glabratus* to levels observed with the laurel oak negative control. Boring by *X. glabratus* was reduced most effectively by the SPLAT MeSA-Verb and SPLAT Verb treatments over 10 weeks, decreasing entrance holes by fivefold and over 10-fold, respectively, compared to the untreated redbay bolts. Interestingly, beyond the initial entrance cavities (Brar et al. 2013), no galleries, eggs or larva were observed in any redbay transverse sections, regardless of treatment. This observation is congruent with Fraedrich et al. (2008) who suggest that redbay host trees are not suitable for *X. glabratus* colonization and reproduction until the advanced stages of laurel wilt and mortality induced by *R. lauricola*.

Verbenone has been widely tested as an anti-aggregant to protect pine trees in the USA against several *Dendroctonus* bark beetle species, including the mountain (*D. ponderosae* Hopkins) (Lindgren et al. 1989; Shea et al. 1992; Fettig et al. 2015), southern (*D. frontalis* Zimmerman) (Payne and Billings 1989) and western (*D. brevicomis*) pine beetles (Fettig et al. 2008). When examined with ambrosia beetles, verbenone reduced captures of *Xylosandrus compactus* Eichhoff, *X. crassiusculus* Motschulsky and *Xyleborinus saxesenii* Ratzeburg in Hawaii (Burbano et al. 2012) and *Euwallacea validus* Eichoff, *Xylosandrus germanus* Blandford and two other taxa in Ohio in ethanol-baited traps (Ranger et al. 2013). Collectively, these data suggest that verbenone-based compounds should have potential as an effective *X. glabratus* repellent and management tool, especially in conjunction with additional components of integrated management (i.e. sanitation and fungicides).

Although these results are promising, further testing is required to assess some of the unique difficulties of protecting host trees against *X. glabratus* and laurel wilt. Most notable is the extreme virulence of the fungal pathogen, *R. lauricola,* to host Lauraceae. Unlike other pestiferous bark and ambrosia beetles that require a high density of attacks in a very short time to overwhelm and kill their hosts (Lee et al. 2011; Tarno et al. 2011; Gitau et al. 2013), even very few attacks of *X. glabratus* and a low load of fungal propagules are sufficient to inoculate and kill healthy redbay trees (Fraedrich et al. 2008; Hughes et al. 2013, 2015b). As our tests utilized cut bolts as attractants, which likely differed in terms of volatile release profile from non-wounded intact stems, further tests on healthy, standing forest and landscape trees are required to fully evaluate the practical utility of these compounds. Bolts treated with verbenone or the MeSA-verbenone mixture had very few successful boring attacks, indicating that larger-scale testing is warranted. The verbenone alone and 1 : 1 MeSA and verbenone blend treatments were similar in effectiveness. MeSA is less expensive than verbenone; the

1 : 1 blend of MeSA and Verbenone is ca. 45% less expensive than verbenone alone. Further research is needed to determine whether this blend could allow for a more cost-effective tool to manage *X. glabratus* in residential landscapes, forests or avocado groves than verbenone alone in SPLAT.

This initial investigation did not address dosage and environmental factors affecting the longevity of dispenser effectiveness. It will also be important to determine whether these repellents affect other *Xyleborus* species in Florida. *Xyleborus volvulus* Fabricius and *Xyleborus ferrugineus* Fabricius also transmit *R. lauricola* to avocado in no-choice experimental tests (Carrillo et al. 2014). If verbenone and/or MeSA act as general repellents against *Xyleborus* species, it is possible these repellents could be used against multiple ambrosia species currently affecting south Florida avocado groves.

Furthermore, a 'push–pull' system (Cook et al. 2007; Gillette et al. 2012) combining the best repellents identified in this study to 'push' the vector away from susceptible hosts, in combination with the best available attractant (i.e. 50% α-copaene lure; Kendra et al. 2016a,b) to 'pull' them to areas of non-hosts, may increase the effectiveness of these semiochemicals than if used alone. Also, combining repellents with application of fungicides may improve management. For example, well-timed application of repellents in concert with application of propiconazole could extend the duration of tree protection. Integration of several tactics will be necessary for management of *X. glabratus* and laurel wilt in the south-eastern USA, and the application of behaviour modifying chemicals for the vector warrants further investigation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Trap design utilized in *X. glabratus* repellency experiments at Historic Haile Homestead (HHH) and Ichetucknee Springs State Park (ISSP).

Supporting Information

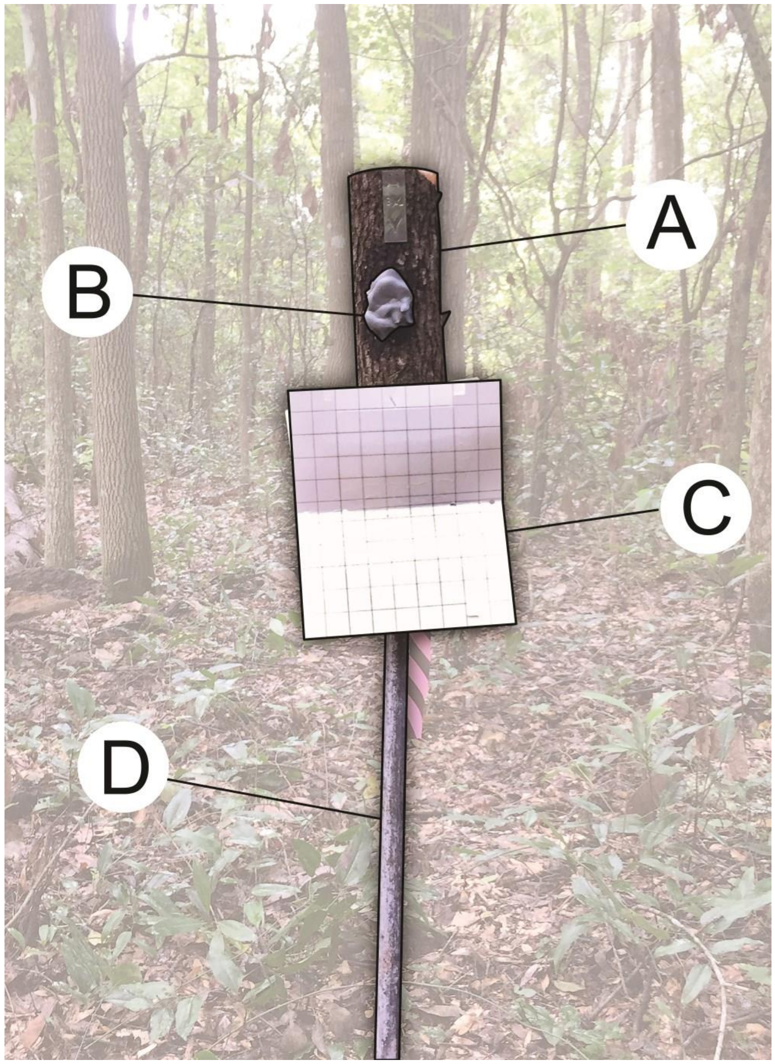

Figure S1. Trap design utilized in *X. glabratus* repellency experiments at Historic Haile Homestead (HHH) and Ichetucknee Springs State Park (ISSP). A) healthy redbay bolt (attractant), B) SPLAT repellent, C) sticky cards (on bolt front and back) and D) metal support pole.