Environmental Entomology, XX(X), 2018, 1–10
doi: 10.1093/ee/nvx194
Research

Chemical Ecology

Behavioral Responses of *Pityophthorus juglandis* (Coleoptera: Curculionidae: Scolytinae) to Volatiles of Black Walnut and *Geosmithia morbida* (Ascomycota: Hypocreales: Bionectriaceae), the Causal Agent of Thousand Cankers Disease

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Subject Editor: Lukasz Stelinski

Received 11 September 2017; Editorial decision 15 November 2017

Abstract

Thousand cankers disease (TCD) is a pest complex formed by the association of the walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), with the fungal pathogen *Geosmithia morbida* Kolařík, Freeland, Utley and Tisserat (Ascomycota: Hypocreales: Bionectriaceae). Current monitoring and detection efforts for WTB rely on a pheromone lure that is effective over a limited distance while plant- and fungal-derived volatiles that may facilitate host location remain poorly understood. In this study, we test the hypothesis that adult beetles are attracted to volatiles of black walnut, *Juglans nigra* L. (*Juglandaceae*), and the pathogen, *G. morbida*. We measured the response of beetles to head-space volatiles collected from leaves and stems of 12 genotypes of black walnut and found genotypic variation in the attractiveness of host trees to adult WTB. Volatile profiles of the most attractive genotypes contained more α-pinene and β-pinene, and adult beetles were attracted to both of these compounds in olfactometer bioassays. In bioassays, we also demonstrated that adult WTB are attracted to volatiles of *G. morbida*. These findings suggest that, in addition to the aggregation pheromone, dispersing WTB potentially use host plant and fungal volatiles to locate suitable larval hosts. Finally, we conducted a field experiment to determine the extent to which ethanol, a common attractant for bark beetles, and limonene, a known bark beetle repellent, influence the behavior of adult WTB to pheromone-baited traps. Although ethanol did not increase trap capture, WTB were repelled by limonene, suggesting that this compound could be used to manipulate and manage WTB populations.

Key words: *Juglans nigra*, volatile organic compound, walnut twig beetle

Thousand cankers disease (TCD) is a pest complex caused by a fungus, *Geosmithia morbida* M. Kolařík, E. Freeland, C. Utley, and N. Tisserat (Ascomycota: Hypocreales: Bionectriaceae), that is vectored by a bark beetle, the walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae) (Blackman 1928, Tisserat et al. 2009, Kolařík et al. 2011). The disease has led to the widespread death of walnut trees (*Juglans* spp.) throughout the western United States (Cranshaw and Tisserat 2008; Tisserat et al. 2009, 2011; Utley et al. 2013), and was found in Knoxville, Tennessee in July 2010—the first discovery of the pest complex in the eastern United States (Grant et al. 2011). Since that time, the disease or pathogen have been found in six additional eastern U.S. states including Indiana, Maryland, North Carolina, Ohio, Pennsylvania, and Virginia (Fisher et al. 2013, Hadžiabdić et al. 2014, Juzwik et al. 2015), and threatens black walnut (*Juglans nigra* L.) throughout its native range (Grant et al. 2011, Seybold et al. 2012a, Tisserat and Cranshaw 2012, Utley et al. 2013, Wiggins et al. 2014). In 2013, TCD was also detected within walnut plantations in Italy (Montecchio and Faccoli 2014).

Geosmithia morbida is a dry-spored anamorphic fungus and the first reported phytopathogenic member of the genus (Kolařík et al. 2011). WTB is the primary vector of the plant pathogen and is capable of

colonizing all North American *Juglans* and *Pterocarya* (Juglandaceae) species (Grant et al. 2011, Utley et al. 2013, Hishinuma et al. 2016). An area of dead tissue called a canker is created under the bark when the phloem of the walnut tree is infected with *G. morbida* (Tisserat et al. 2009). In the early stages of the disease, small cankers develop around galleries of colonizing beetles, and there are few outward signs of infection apart from small beetle entrance holes in the bark. At this stage of the disease, the fungus is often restricted to the phloem, but cankers will expand into the cambium as the disease progresses (Utley et al. 2013). In the more advanced stages, cankers become more diffuse, causing tissues to become dark-colored and macerated. A high population density of WTB is necessary to vector enough *G. morbida* to affect tree health, and an affected tree often succumbs to the disease only after having been colonized by thousands of beetles (Utley et al. 2013).

Black walnut is among the most valuable hardwoods in the North Central Hardwoods region (USFS 2002), and is the most susceptible walnut to TCD (Utley et al. 2013). The long-term protection of the walnut resource at risk to TCD requires the integration of multiple tactics, including more efficient and effective monitoring techniques that target WTB host location behavior. Host colonization of many bark beetles involves four distinct phases: dispersal, host selection, concentration, and establishment (Wood 1982). Beetles may locate a host based on undirected flight and, upon landing, short-range olfactory or gustatory cues may act as behavioral arrestants and mediate the acceptance of a suitable host (Raffa and Berryman 1982, 1983). Pioneering beetles may also respond to long-range olfactory cues such as plant volatiles to locate a potential host tree (Wood 1982, Borden 1997). However, beetles attempt to colonize a tree only if it is of appropriate species, susceptible to attack, and not occupied by other woodborers or conspecifics at high densities (see Borden 1997). If these conditions are met, a beetle will likely colonize the host and release an attractant pheromone (concentration phase) and ultimately mate and oviposit (establishment). Adult WTB may experience a very similar decision matrix while colonizing walnut hosts. Each of these decision points which ultimately lead to host acceptance and colonization by WTB may be mediated by semiochemicals that act as attractants, repellents, or behavioral arrestants (Smith 2005), and could potentially be exploited to reduce their impact on black walnut.

Insect host selection and reproduction also relies heavily on the ability to perceive and respond to olfactory cues released by host plants (Dicke and Baldwin 2010). For many plants, genetic factors influence their suitability and resistance to insect herbivores, and insects can discriminate between resistant and susceptible host plants based on olfactory cues (e.g., Gaum et al. 1994, Storer and van Emden 1995, da Costa et al. 2011). Moreover, forest trees display significant genotypic variation in their susceptibility to colonization by a variety of insects, including bark beetles (see Wagner 1991). Genetic variation among trees comprising half-sibling cohorts can influence their susceptibility to ambrosia beetle pests (Ott et al. 2011, Utley et al. 2013). Volatile organic compounds (VOCs) released by susceptible tree genotypes can be different from those released by tree types that are more resistant, and differences influence herbivore attraction to these trees (Li et al. 2002, Rull and Prokopy 2004). For example, twig and bark extracts of one clone of field elm (*Ulmus minor* Mill., Ulmaceae) were less preferred by *Scolytus scolytus* (F.) (Coleoptera: Curculionidae), a vector of Dutch elm disease, compared with those of four other clones tested (Pajares et al. 2004), suggesting that plant compounds play a role in host discrimination.

Fungal associates play important roles in the life histories of conifer bark beetles which often form symbiotic relationships with specific fungi (Kandasamy et al. 2016). For example, fungal associates of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae), increase available nitrogen in the phloem and produce ergosterol, a critical sterol necessary for beetle development and reproduction (Bleiker and Six 2007). A similar mutualistic relationship may exist between WTB and *G. morbida*, but the extent to which WTB gains fitness advantages from fungal associates is unknown (Luna et al. 2014). Bark beetles may also rely on fungal volatiles for host location especially when the fungus is closely associated with the host plant resource (Kandasamy et al. 2016). Through choice test experiments, Luna et al. (2014) demonstrated that although WTB larvae are attracted to volatiles of common tree fungi, including *G. morbida* and *Fusarium solani* (Mart.) Sacc. (Ascomycota: Hypocreales: Nectriaceae), there remains no evidence that larvae feed on fungal conidia. However, in our preliminary experiments, all WTB life stages, as well as frass, were tested and yielded positive detections for the presence of *G. morbida* using species-specific microsatellite loci (D. Hadžiabdić, unpublished data). Nevertheless, the extent to which adult WTB is attracted to volatiles of *G. morbida* remains unclear.

Current monitoring efforts for TCD rely on visual surveys for symptomatic trees and multi-funnel traps baited with aggregation pheromone to detect adult WTB (Seybold et al. 2012c, 2013). A volatile male-produced pheromone that unites the sexes has been identified and is commercially available as a monitoring tool for WTB (Seybold et al. 2012b, 2013). Nevertheless, WTB likely locate suitable hosts by orienting to plant volatiles (Ciesla 2011), and adult beetles are preferentially attracted to stressed and girdled host trees (Bray et al. 2012). Despite the important role host compounds may play in the colonization behavior of WTB, volatiles that mediate host location remain poorly understood. There remains a critical need to identify semiochemicals that either attract or repel WTB as potentially effective intervention agents against TCD. For example, ethanol is a general attractant for scolytine beetles and enhances the attraction of many woodborers to semiochemical-baited traps and trap trees (Montgomery and Wargo 1982, Miller and Rabaglia 2009). Moreover, compounds such as limonene, a common repellent for other beetle species (see Miller 2007), may be used in combination with attractants to manipulate WTB beetle populations in a push-pull tactic to protect high value plantings (Cook et al. 2007).

Here, we describe the results from performing a series of behavioral experiments to test the hypothesis that volatiles of black walnut and *G. morbida* act as semiochemicals for adult WTB. Specifically, we predicted that: i) genotypes of black walnut will vary in volatile profiles and influence the attraction of adult WTB; ii) those compounds that are highly represented in volatile profiles of the most attractive genotypes will be attractive to adult beetles; and iii) adult beetles will be attracted to volatiles produced by *G. morbida*. Finally, we report the results of a field experiment designed to determine the extent to which adult beetles are attracted by ethanol and repelled by limonene.

Materials and Methods

Source of Beetles Used in Experiments

Insects used in bioassays were reared from naturally infested material collected in Knox and Blount counties in Tennessee, USA adjacent to the Great Smoky Mountains National Park. Adults emerged from infested walnut limbs in a greenhouse at the University of Tennessee-Knoxville according to Browne (1972) and were sorted by sex and stored at 4°C until they were used in experiments.

Response of Adult WTB to Volatiles of Black Walnut Genotypes

Volatile Collection of Black Walnut Genotypes

Headspace volatiles were collected from the twigs and leaves in the canopy of 12 genotypes of black walnut growing in a plantation at Martell Forest (Tippecanoe Co., IN, USA)—accession nos. 13, 44, 48, 49, 55, 68, 109, 119, 130, 132, 288, and 295. By sampling from similarly sized trees grown in plantations, variation among factors associated with age, site index, and environmental conditions that may influence volatile emission were minimized. Sampled trees varied in age from 30 to 40 yr and had a diameter at breast height of 20–30 cm. A boomlift (AT-37G, Altec Inc., Birmingham, AL, USA) was used to gain access to the canopy of each tree and ~10 grams of intact stems and leaves from a branch terminal were enclosed within an inert plastic bag (see Stewart-Jones and Poppy 2006; Reynolds Consumer Products LLC, Lake Forest, IL, USA). Volatiles were collected onto a filter containing 200 mg of 80/100 mesh HayeSep-Q (Ohio Valley Specialty, Marietta, OH, USA) attached to the bag. Charcoal-purified air was pushed and pulled through the system at a rate of ~1 liter/min by a portable pump (no. 268137, Maxon Precision Motors, Inc., Sachselm, Switzerland) powered by a small deep-cycle battery (Matrix Battery Co., Shenzhen, China). Volatile collections occurred from 1 August to 7 September 2011 for 4 hr each and collections were made at least twice from three or more ramets of each clone. After each collection period, volatiles were eluted from the filters with 2 ml of $MeCl_2$ and stored separately in individual glass vials at 4°C.

Olfactometry Bioassay Measuring Attraction of WTB to Volatiles of Black Walnut Genotypes

The attraction of adult beetles to volatiles collected from the 12 genotypes of black walnut was tested by measuring the walking response of adult WTB in a glass straight-tube olfactometer (30 × 3 cm diam.). One end of the olfactometer was connected to a small glass chamber containing the odor source with air pulled through the system by a laboratory vacuum supply (~1 liter/min). Treatments of 150 µl of the crude extract from each clone or 150 µl of $MeCl_2$ as a control were pipetted onto a piece of filter paper and placed within the glass chamber. A similar assay has been previously used to assess the colonization behavior of the scolytine *Phloeotribus scarabaeoides* (Bern.) (Coleoptera: Curculionidae) (Peña et al. 1992, Szauman-Szumski et al. 1998).

Newly emerged adults (n = 5 of either male or female beetles) were placed at the downwind end of the olfactometer and allowed to respond to the odor source. In response to an attractive odor, beetles moved upwind in the olfactometer toward the odor source. If a beetle walked more than 10 cm from the entry point in 30 min, it was marked as a response; alternatively, if a beetle did not walk further than 10 cm, that beetle was marked as not responding, or no response. All bioassays were completed under red light at University of Tennessee-Knoxville. Male and female WTB were tested separately and individuals were only used once. Preliminary bioassays revealed that the behavior of individual beetles was not influenced by the presence of conspecifics in the olfactometer, so five beetles were used per trial and each bioassay was replicated until six trials were conducted per treatment (n = 30). Olfactometers and glass chambers were washed with detergent and dried in a laboratory oven after each treatment was tested. These bioassays were conducted 14–18 November and 5–9 December 2011.

The responses of WTB to volatile extracts were compared with those of $MeCl_2$ controls with a non-parametric Kruskal–Wallis ANOVA on ranks, followed by a post-hoc Dunn's test. Attraction did not differ between the sexes ($P > 0.05$); therefore, the responses of males and females were combined for the analysis. Analyses were conducted in statistical package JMP 13 (SAS Institute Inc. 2016).

Identification of Walnut VOCs

Volatile extracts of the 12 black walnut genotypes that were used in bioassays above were analyzed by coupled gas chromatography–mass spectrometry (GC–MS) with electron impact ionization (EI, 70 eV) using an Agilent 6890N GC (Agilent Technologies, Santa Clara, CA, USA) equipped with a HP-INNOWax capillary column (30 m × 0.25 µm × 0.25 µm film; J&W Scientific, Folsom, CA, USA) in splitless mode, interfaced to an Agilent 5975B mass selective detector, with helium carrier gas. One microliter of extract was injected into the heated inlet (250°C) and the oven temperature was programmed from 40°C/2 min, then increased 5°C/min to 150°C, hold time 5 min, and then ramped at 10°C/min to 230°C with a 10 min hold time. Spectra were acquired in full scan mode and quantitative data presented in Table 1 were produced by these analyses.

Individual compounds were identified by their mass spectra, in comparison with those in the National Institute of Standards and Technology (NIST) mass spectral library (ca. 120,000 spectra; ChemStation Version D.05.01; Hewlett Packard Corp., Palo Alto, CA, USA), and by matching their retention times and mass spectra to those of authentic standards for the following: camphene, β-caryophyllene, cymene, D-limonene, (±)-α-pinene, (–)-β-pinene, and sabinene (Sigma-Aldrich, Milwaukee, WI, USA). The abundance of each compound in extracts was calculated as a percentage of the total corrected peak area of all identified compounds present in the total ion chromatograms.

Table 1. Mean (±1 SE) relative abundances of volatile chemicals in headspace collections of the most and least attractive genotypes of black walnut

Compound	Most attractive (N = 9) Mean ± SE	Least attractive (N = 3) Mean ± SE
α-Thujene	1.15 ± 0.63	1.16 ± 0.44
α-Pinene	56.27 ± 6.09	34.71 ± 13.92
Camphene	0.21 ± 0.13	n.d.
β-Pinene	12.64 ± 0.80	10.19 ± 0.61
Sabinene	8.74 ± 5.28	28.95 ± 14.37
β-Myrcene	1.72 ± 0.95	1.57 ± 0.60
α-Terpinene	0.24 ± 0.21	0.02 ± 0.02
D-Limonene	3.08 ± 1.0	1.25 ± 0.25
β-Phellandrene	0.54 ± 0.14	0.67 ± 0.22
γ-Terpinene	0.76 ± 0.32	2.12 ± 1.14
β-Ocimene	1.44 ± 1.11	0.45 ± 0.29
Cymene	0.88 ± 0.44	2.34 ± 1.04
3-Hexenyl acetate	0.50 ± 0.21	0.63 ± 0.43
α-Cubebene	0.61 ± 0.42	0.46 ± 0.22
2-Cyclohexen-1-ol	0.23 ± 0.12	3.73 ± 1.70
α-Copaene	0.56 ± 0.32	0.87 ± 0.36
Decanal	2.41 ± 1.78	0.08 ± 0.04
β-Bourbonene	0.06 ± 0.04	0.16 ± 0.11
Bergamotene	0.14 ± 0.14	0.22 ± 0.18
β-Cubebene	0.94 ± 0.35	n.d.
β-Caryophyllene	3.12 ± 0.83	8.09 ± 0.82
Terpinen-4-ol	0.07 ± 0.05	0.43 ± 0.22
α-Humulene	1.59 ± 1.49	0.07 ± 0.07
Germacrene D	1.59 ± 0.69	1.68 ± 0.93
α-Muurolene	0.26 ± 0.24	n.d.
Δ-Cadinene	0.25 ± 0.07	0.14 ± 0.08

n.d. = compound not detected.

We evaluated the influence of genetic variation by comparing the constitutive composite volatile profiles of the most attractive genotypes (nos. 13, 44, 48, 49, 68, 109, 132, 288, and 295) with the least attractive (nos. 55, 119, 130) using permutational multivariate analysis of variance (PERMANOVA; Anderson 2001), employing Euclidian measurements of dissimilarity and 10,000 permutations. Individual extracts from all volatile collections from each genotype used in assays were included in this analysis. We illustrate composite volatile profiles by principal components ordination analysis using Euclidian distances. We used normalized data for PERMANOVA, principal components ordination, and similarity percentages (SIMPER) analyses. For ease of interpretation, we present the mean individual volatile compounds produced in the SIMPER analysis as raw values in Table 2.

Olfactometer Bioassay Measuring Attraction to Synthetic Walnut VOCs

Among the compounds that were more highly represented in the most attractive genotypes, α-pinene and β-pinene contributed most to the overall dissimilarity between the most and least attractive genotypes (see results, Table 2), so the attraction of WTB to these compounds was measured in straight-tube olfactometer bioassays (described earlier). Individual compounds were purchased from Sigma Aldrich (St. Louis, MO, USA) and their capacity to attract WTB was measured. The bioactivity of (±)-α-pinene (98% pure) and (−)-β-pinene (99% pure) was first tested through serial dilutions in $MeCl_2$ with $MeCl_2$ as the control from 8 to 11 January 2013. Optimized dilutions were determined for α-pinene (1/10 dilution in $MeCl_2$) and β-pinene (1/1000 dilution in $MeCl_2$), after which 150 µl of the solution was then pipetted directly onto filter paper and placed in the odor chamber of the olfactometer. Bioassays were conducted as previously described.

The responses of WTB to authentic standards were compared with the $MeCl_2$ control with a non-parametric Kruskal–Wallis ANOVA on ranks, followed by a post-hoc Dunn's test. Analyses were conducted in statistical package JMP 13 (SAS Institute Inc. 2016).

Attraction of WTB to Volatiles of *G. morbida*

Culture of *G. morbida* Isolates

G. morbida was collected from branch samples of TCD-affected trees growing in Blount Co., TN in 2010 (isolate no. 10; Lat. 35.74214; Long. -83.97303). The following year, an additional isolate was collected from a symptomatic tree in Blount Co., TN (isolate no. 17; Lat. 35.71088; Long. -83.88363). In 2013, an isolate was also obtained from a TCD-symptomatic black walnut growing near Logan, UT (isolate UT-95-02).

To collect the isolates, branch samples from affected trees were double bagged in zipper-seal plastic bags, sealed in a 19-liter plastic bucket, and transported to the University of Tennessee-Knoxville (Permit no. P526P-15-03500; P526P-14-04158). Outer bark was removed from the samples with a drawknife and small, elliptical, necrotic cankers were observed. Wood chips (3–4 mm^2) from cankers were excised and placed on 1/10 strength potato dextrose agar (PDA) amended with 30 mg/liter streptomycin sulfate (Fisher

Table 2. Mean contribution of individual volatile compounds to total volatile production in the most and least attractive genotypes of black walnut

Compound	Most attractive	Least attractive	Individual contribution (%)	Cumulative contribution (%)
α-Pinene	56.27	34.71	32.45	32.45
Sabinene	8.74	28.95	29.88	62.33
β-Caryophyllene	3.12	8.09	5.81	68.14
2-Cyclohexen-1-ol	0.23	3.73	3.96	72.10
β-Pinene	12.64	10.19	3.18	75.28
Decanal	2.41	0.08	2.72	78.00
Cymene	0.88	2.34	2.25	80.25
γ-Terpinene	0.76	2.12	2.11	82.36
D-Limonene	3.08	1.25	2.10	84.46
β-Myrcene	1.72	1.57	2.09	86.55
Germacrene D	1.59	1.68	2.06	88.61
β-Ocimene	1.44	0.45	1.83	90.44
α-Humulene	1.59	0.07	1.82	92.26
α-Thujene	1.15	1.16	1.51	93.77
β-Cubebene	0.94	0.00	1.06	94.83
α-Cubebene	0.61	0.46	0.97	95.80
α-Copaene	0.56	0.87	0.95	96.75
3-Hexenyl acetate	0.50	0.63	0.73	97.48
β-Phellandrene	0.54	0.67	0.48	97.96
Terpinen-4-ol	0.07	0.43	0.46	98.42
Bergamotene	0.14	0.22	0.35	98.77
α-Muurolene	0.26	0.00	0.30	99.07
α-Terpinene	0.24	0.02	0.29	99.36
Camphene	0.21	0.00	0.23	99.59
Δ-Cadinene	0.25	0.14	0.23	99.82
β-Bourbonene	0.06	0.16	0.18	100.00

Data are ranked in order of the largest contribution (%) of individual volatile compounds to the overall dissimilarity between volatile profiles, as determined by SIMPER analyses. Mean individual volatile compounds are presented as raw values, but normalized data were used in SIMPER to determine contributions to dissimilarities in volatile profiles. Compounds appearing above dashed and solid lines indicate those which explain >70% and >90%, respectively, of the dissimilarity between the volatile profiles.

Scientific, Fair Lawn, NJ, USA) and 30 mg/liter chlortetracycline HCl (Sigma) [= 1/10 PDA+] and incubated on a 12:12 (L:D) h cycle at 22°C for 5–7 d. Fungal isolates were identified as *G. morbida* by using culture morphology and characteristics of conidiophores and conidia, as well as using molecular tools [both the internal transcribed spacer (ITS) region of the ribosomal RNA operon—ITS1 and ITS4 (White et al. 1990) and species-specific microsatellite loci (Hadžiabdić et al. 2012)].

Collection of Fungal Volatiles

Headspace VOCs were collected from *G. morbida* grown individually on ½ strength PDA and a control consisting of uncolonized PDA. Four 100-mm colonized or uncolonized PDA plates were enclosed within an inert bag and volatiles were collected on a filter containing 100 mg of 80/100 mesh HayeSep-Q (Ohio Valley Specialty, Marietta, OH, USA) attached to the bag. Charcoal-purified air was pushed and pulled through the system for 4 hr at a rate of ~1 liter/min by a portable pump (no. 268137, Maxon Precision Motors, Inc., Sachselm, Switzerland) powered by a small deep-cycle battery (Matrix Battery Co., Shenzhen, China). Volatile collections of *G. morbida* isolates occurred from 7 October to 26 November 2013 and filters were eluted with 2 ml of $MeCl_2$ and stored at 4°C in individual glass vials.

Straight-tube Olfactometer Bioassay Measuring Attraction

The walking response of adult WTB to volatile extracts of *G. morbida* was tested in the straight-tube olfactometer bioassay as described previously. Following the same procedure, the activity of volatile extracts from *G. morbida* colonized ½ strength PDA (isolates Gm 10, Gm 17, and Gm UT-95-02), uncolonized ½ strength PDA, and $MeCl_2$ were tested to determine the extent to which WTB responds to *G. morbida* volatiles. Bioassays were conducted 25–27 November 2013 at University of Tennessee-Knoxville. Responses of WTB to extracts of fungal volatiles and those from uncolonized ½ strength PDA were compared with the $MeCl_2$ control with a non-parametric Kruskal–Wallis ANOVA on ranks, followed by a post-hoc Dunn's test. Analyses were conducted in statistical package JMP 13 (SAS Institute Inc. 2016).

Ethanol and Limonene Field Experiment

A field experiment was conducted to assess the capacity of ethanol and limonene to attract and repel WTB, respectively, to pheromone-baited traps. Treatments were randomly assigned to four-unit Lindgren multi-funnel traps (Contech Enterprises, Victoria, BC Inc., Canada) as follows:

1. No lure (blank);
2. WTB pheromone lure (WTB [+]);
3. Ethanol (EtOH);
4. (*R*)-+-Limonene (>85% purity);
5. Ethanol plus WTB lure (EtOH+); and
6. Limonene plus WTB lure (Limonene+).

Lures consisted of low release ethanol pouches (90% [aq]) (Synergy Semiochemicals Corp., Burnaby, BC, Canada), limonene in a bubble pouch (Synergy Semiochemicals Corp.), and a commercially available WTB pheromone lure (3-methyl-2-buten-1-ol; Contech Enterprises, Inc., Victoria, BC Canada, prod. no. 300000736; Seybold et al. 2013). Transects were erected along the leading edge of the WTB infestation in Blount and Knox Co., TN (Table 3). Three transects were maintained from 12 September to 14 November 2013 and traps were checked weekly.

Table 3. Study sites for ethanol and limonene field experiment in 2013

County, State	Location	Site Coordinates
Blount, TN	Maryville-Alcoa Greenway	35°44′09.2″N 83°58′49.1″W
Knox, TN	Campbell Station Park	35°53′16.0″N 84°09′59.3″W
Knox, TN	Lakeshore Park	35°55′30.7″N 83°59′29.0″W

Four-unit Lindgren multi-funnel traps were suspended from three meter frames constructed of aluminum conduit pipe along linear transects. All traps were coated with Fluon PTFE (AGC Chemicals Americas, Exton, PA, USA) to enhance trapping efficiency (see Graham et al. 2010). Collection cups were filled with marine & RV antifreeze (i.e., propylene glycol) (SPLASH Products, St. Paul, MN, USA) to kill and preserve captured insects.

All transects contained one trap of each treatment (10 m apart, position assigned randomly). Each week, the position of treatments was indexed within transects and lures were examined and replenished when visibly depleted. All captured beetles were returned to the Ginzel laboratory at Purdue University for identification (Permit no. 15-IN-18-012).

Differences between treatment means were tested with the non-parametric Friedman's Test (PROC FREQ, option CMH; SAS Institute Inc. 2010). Any collection date with traps that yielded less than one beetle was removed from the analysis to account for days in which beetles were not flying. Differences between pairs of means were tested with the Ryan–Einot–Gabriel–Welsch Multiple Range Q Test (REGWQ), a means-separation test, which controls for maximum experiment-wise error rates (PROC GLM; SAS Institute Inc. 2010).

Results

Response of Adult WTB to Volatiles of Black Walnut

Straight-tube Olfactometer Bioassay: Black Walnut Genotypes

Adult WTB were more attracted to volatile collections of black walnut genotypes than the $MeCl_2$ control (Fig. 1; $H_{(12)} = 51.86$; $P < 0.001$). Additionally, some genotypes were more attractive than others, with accession nos. 55, 119, and 130 being the least attractive and all other genotypes being more attractive (Dunn's $P = 0.042$).

Identification of Walnut VOCs

Twenty-five VOCs were characterized from twigs and leaves of 12 known clonal genotypes of black walnut (Table 1). All of the compounds identified are monoterpene and sesquiterpene hydrocarbons with the exception of decanal and (*Z*)-3-hexenyl acetate, which are aliphatics.

There were quantitative differences in the profiles of constitutive volatiles released from the most and least attractive genotypes (PERMANOVA Pseudo-$F = 30.8$, Pseudo-$P = 0.007$). Two hydrocarbon monoterpenes (α-pinene and β-pinene) were abundant volatiles among the profiles of the most attractive genotypes, accounting for almost 69% of the total and more highly represented in the profiles of the most attractive genotypes when compared with those that were least attractive (Table 2). In fact, α-pinene contributed over 32% to the total dissimilarity between the most and least attractive genotype. Sabinene was most abundant in the volatile profiles of the less attractive genotypes, making up almost 29% of the total volatile emission, and contributed almost 30% of the difference between the two categories (Table 2). Also, 2-cyclohexen-1-ol and

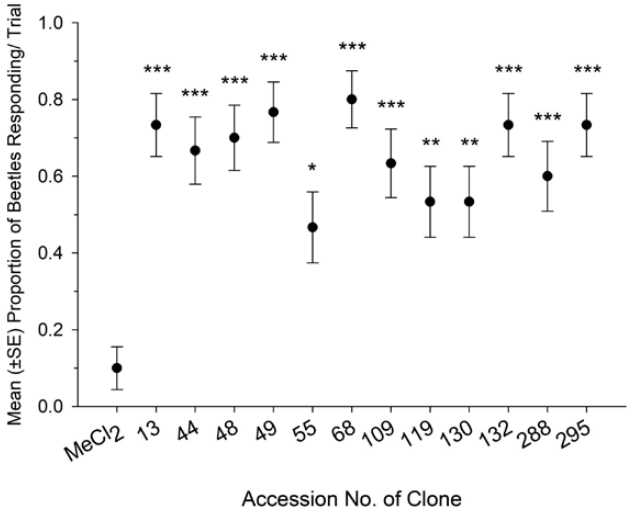

Fig. 1. Attraction of adult WTB to volatiles of black walnut genotypes over that of a $MeCl_2$ control. Error bars display standard error. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

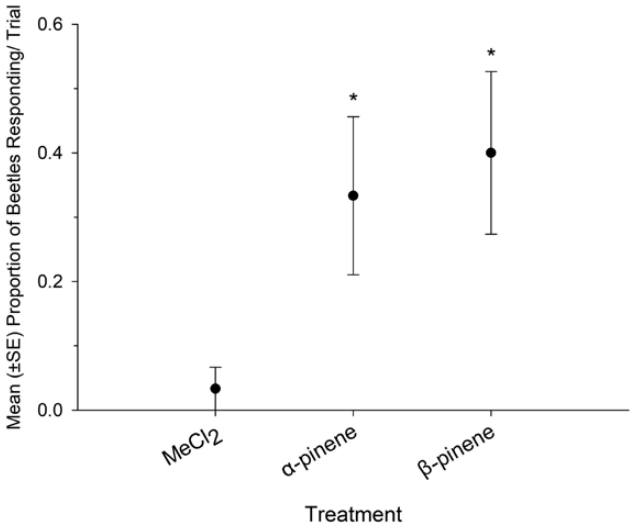

Fig. 2. Attraction of adult WTB to a $MeCl_2$ control, α-pinene, and β-pinene. *$P < 0.05$.

β-caryophyllene together contributed almost 10% of the difference between the most and the least attractive genotypes and were more highly represented in the less attractive genotypes (Table 2).

Straight-tube Olfactometer Bioassays: Synthetic Walnut VOCs

Adult beetles were more attracted to α-pinene and β-pinene than to the $MeCl_2$ control (Fig. 2; $H_{(2)} = 11.89$; $P = 0.0026$). There were no differences in WTB responses to α-pinene and β-pinene and attraction did not differ between the sexes for either assay ($P > 0.05$).

Attraction of WTB to Volatiles of *G. morbida*

More WTB were attracted to the volatile extracts of *G. morbida* than to the $MeCl_2$ control (Fig. 3; $H_{(4)} = 27.36$; $P < 0.001$) and the attraction was strongest to isolates from TN. There was no difference between WTB response to the $MeCl_2$ control and volatiles collected from uncolonized PDA plates (Dunn's $P > 0.05$).

Ethanol and Limonene Field Experiment

No treatment was more attractive to WTB than the pheromone lure alone (Fig. 4; Friedman's $Q_{17,132} = 6.04$, $P < 0.001$, $N = 25$; REGWQ $P < 0.05$). Ethanol did not increase attraction to the pheromone lure but this combination was the only treatment as attractive as the pheromone lure alone. Three times fewer beetles were captured in traps baited with the combination of limonene and WTB pheromone than the pheromone lure alone. Traps without the pheromone lure (blank, ethanol, and limonene) all captured very few beetles.

Discussion

The results of our olfactometer bioassays suggest that adult WTB are not only attracted to the volatiles of black walnut but also to those of *G. morbida*. This is the first record of adult WTB being attracted to fungal volatiles. Moreover, in the field experiment, adult WTB were not attracted to ethanol but were repelled by limonene, suggesting that this compound could be used to manipulate and manage WTB populations.

The discrimination of walnut genotypes by WTB based on volatile profiles, suggests that genotype may influence the host selection behavior of this insect. Resistance of plants to insect herbivory strongly depends on those characteristics that affect herbivore performance or preference, and plant genotypes may influence the expression of these traits. For example, the mass, emergence time, and infestation rates of the weevil, *Anthonomus pomorum* (L.), (Coleoptera: Curculionidae) from flower buds differs significantly between cultivars of apple (Mody et al. 2015). Similarly, adult WTB colonized black walnut at differing densities in laboratory trials when given the choice between the maternal 'Sparrow' parent and the paternal 'Schessler' parent (Hefty et al. 2016). Differences in colonization densities between black walnut cultivars, in addition to the influence of host genotype on the attraction of WTB in bioassays, suggest that it may be possible to propagate black walnut cultivars that are less attractive to dispersing WTB.

Every compound, or an isomer of each, identified in our volatile collections has been previously isolated from black walnut (Farag 2008, Fojtová et al. 2010) with the exception of α-muurolene, which has been extracted from leaves of the closely related *J. regia* (Bou Abdallah et al. 2016). High levels of hydrocarbon terpenes were emitted from the leaves and stems of trees in our study, with α-pinene, β-pinene, and sabinene dominating the volatile profiles. Our results generally support previous research that found these three compounds accounted for over 80% of the total emissions from black walnut peels (Buchbauer et al. 1993). The volatile profiles from branches of *J. regia* bearing leaves and fruits were similar to those we collected from *J. nigra* (Casado et al. 2008). Notably, β-pinene was the most abundant volatile from *J. regia* and together with (*Z*)-3-hexenyl acetate, (*E*)-(β)-ocimene, limonene, germacrene D, 1,8-cineole, sabinene, (*E*)-β-farnesene, (*E*)-β-caryophyllene, β-myrcene, and β-phellandrene constituted as much as 90.5% of the total volatile emissions (Casado et al. 2008).

Isoprenoids comprise key components of essential oils, floral odors, and defensive resins and may act as semiochemicals for insects (Mahmoud and Croteau 2002). One such compound, α-pinene, is emitted from a variety of trees and attracts an array of scolytine beetles (Miller and Rabaglia 2009). This monoterpene not only synergizes the attraction of conifer bark beetles to traps baited with pheromone lures (e.g., Kinzer et al. 1969), but may also inhibit attraction or act as a behavioral arrestant in other bark and ambrosia beetle systems (Renwick and Vité 1970, Payne et al. 1973, Miller and Rabaglia 2009, Ranger et al. 2011). Among invasive scolytine beetles with deciduous

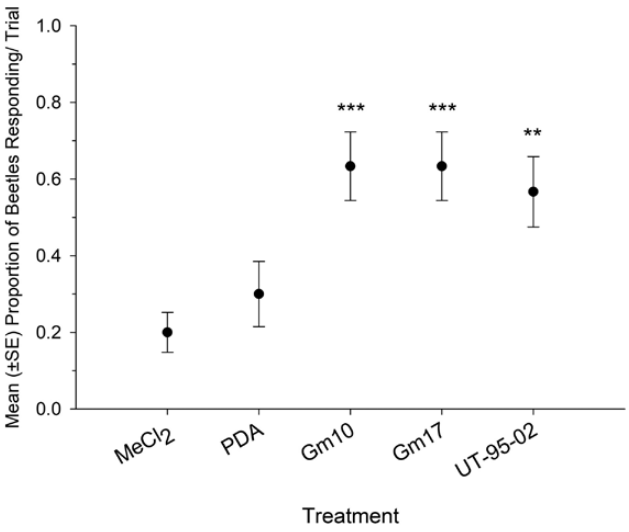

Fig. 3. Attraction of adult WTB to three isolates of *G. morbida* (isolates Gm 10, Gm 17, and Gm UT-95-02) grown on ½ strength PDA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

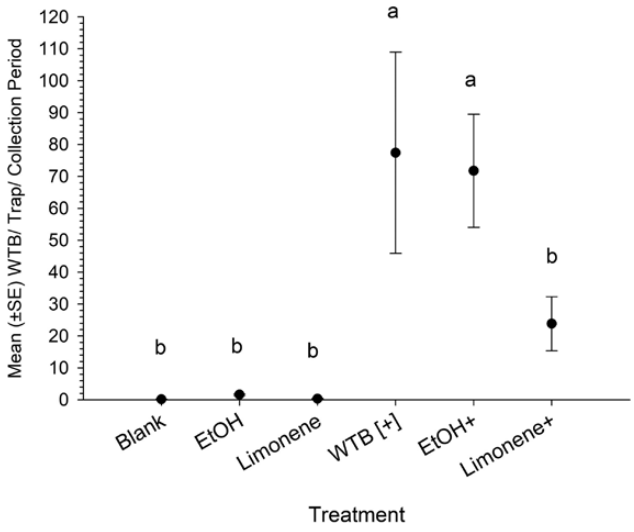

Fig. 4. Mean (± 1 SE) number of adult WTB captured in traps in the ethanol and limonene field experiment in fall 2013. A 'plus' sign signifies the lure was paired with a WTB pheromone lure. Bars marked with the same letter are not significantly different (REGWQ $P < 0.05$).

hosts, α-pinene increases the attraction of *Xylosandrus germanus* (Blandford) and *Xylosandrus crassiusculus* (Motschulsky) (Coleoptera: Curculionidae) to ethanol-baited traps in some cases (Miller and Rabaglia 2009, Ranger et al. 2011, Miller et al. 2015). Moreover, *Xyleborinus saxesenii* (Ratzeburg) (Coleoptera: Curculionidae), whose host range includes nearly all North American genera of deciduous trees (Bright 1968), is attracted to traps baited with ethanol and β-pinene over those baited with either the combination of α-pinene and ethanol or an exotic bark beetle lure containing ipsdienol, *cis*-verbenol, and 2-methyl-3-buten-2-ol (Petrice et al. 2004). Although adult WTB were attracted to α- and β-pinene in our olfactometer experiments, pioneering beetles likely respond to a more complete blend of host volatiles to locate suitable black walnuts. Once on a host tree, adults soon colonize branches and, thus, profiling the volatiles of bark may also reveal compounds that act as attractants or arrestants.

In the present study, we demonstrated for the first time that adult WTB are attracted to volatiles of *G. morbida*. The population structure of *G. morbida* suggests that the fungus is genetically complex, highly diverse, and evolved in association with *Juglans* spp. and WTB (Kolarik et al. 2011, Hadžiabdić et al. 2014, Zerillo et al. 2014). Interestingly, beetles used in our bioassays originated from TN populations and were more strongly attracted to volatiles from TN isolates of *G. morbida* over those from UT isolates, suggesting there may be some preference for co-evolved isolates. Although there are few examples of fungal volatiles increasing attraction of bark beetles to hardwood trees, WTB attraction to *G. morbida* volatiles suggests fungal volatiles play a role in the host location behavior of WTB. Similarly, semiochemicals produced by trees infected with Dutch elm disease attracts the insect vector *Hylurgopinus rufipes* (Eichh.) (Coleoptera: Curculionidae) in both Y-tube choice bioassays and field experiments (McLeod et al. 2005). Moreover, the redbay ambrosia beetle, *Xyleborus glabratus* Eichh. (Coleoptera: Curculionidae), is attracted to volatiles of its fungal associate, *Raffaelea lauricola* T.C. Harr., Freadrich & Aghayeva (Ascomycota: Ophiostomatales) (Hulcr et al. 2011). A synthetic odor blend of *R. lauricola* volatiles enhanced the attraction of adult beetles to traps baited with host plant volatiles, and *X. glabratus* is not attracted to volatiles of non-associated fungi (Kuhns et al. 2014). Fungi associated with bark beetles produce a range of volatile compounds either de novo or from precursors present in their environment, and these compounds or blends can be highly specific in their chemistry (Brand and Barras 1977, Brand et al. 1977, Leufven et al. 1988, Hanssen 1993). Therefore, to establish the extent to which this relationship is mutualistic, field experiments should be conducted to compare the attraction of WTB to walnut bolts inoculated with *G. morbida* compared with uncolonized host material.

We exercise caution when relating the results of our laboratory-based attractant assays to the behavior of naturally dispersing beetles; however, several studies have demonstrated that bark beetles behave in a manner consistent with those observed in olfactometer bioassays and semiochemical-baited traps in the field. For example, in olfactometer bioassays both sexes of *Dendroctonus brevicomis* LeConte (Coleoptera: Curculionidae) are increasingly attracted to aggregation pheromone components and avoid the pheromone source in the presence of verbenone, an anti-aggregation pheromone (Byers and Wood 1980, Byers et al. 1984). Similarly, *Tomicus piniperda* (L.) (Coleoptera: Curculionidae) are attracted to host monoterpenes both in the olfactometer and when used as lures with sticky traps in the field (Byers et al. 1985). Nevertheless, the extent to which natural populations of WTB respond to specific host-plant and fungal volatiles and their capacity to enhance attraction to pheromone-baited traps in the field requires further investigation. Although, WTB may orient to green leaf volatiles, like those tested in this experiment, the beetles attack the branches and trunk of walnut trees. Profiling the volatiles of bark may be more advantageous to identify those compounds attractive to adult WTB.

In our field experiment, adult WTB were not attracted to ethanol-baited traps alone, and ethanol also did not enhance the attraction of WTB to pheromone-baited traps. This is an unexpected result given that ethanol is one of the most common semiochemicals used to detect scolytine beetles, especially those that have a relatively broad host range and opportunistically attack weakened trees (Oliver and Mannion 2001, Ranger et al. 2010, Reding et al. 2011, Miller et al. 2015). Similar to WTB, however, other species that are primary colonizers of healthy trees (e.g., *X. glabratus*) are also not attracted to ethanol (Johnson et al. 2014). Nevertheless, limonene repelled adult WTB from pheromone-baited traps, suggesting that

there is the potential to utilize this compound as a means to manage beetle populations and protect trees from TCD. In fact, the addition of limonene to the pheromone lure decreased trap capture of WTB three-fold compared with the pheromone lure alone (Fig. 4). In other systems, limonene can elicit such a strong inhibitory response that a mixture of 5% limonene added to the attractant, α-pinene, results in almost complete inhibition of attraction to two pine weevils (Norlander 1990).

One drawback to the use of repellents for manipulating beetle populations is that they might only divert beetles from a local area without reducing the total number of beetles and affected trees in a stand. One way to overcome this hurdle in high-value plantings is to pair the repellent with an attractant in a 'push–pull' tactic. In such a strategy, a repellent ('push') stimulus is located within the commodity planting and acts to repel pest species toward an attractive stimulus ('pull') from where the pest is later removed (Cook et al. 2007). Push–pull systems have been used to manage bark beetles in a manner such that anti-aggregation pheromone dispensers are placed on valuable, healthy trees and traps baited with aggregation pheromone are used to draw beetles into a stand of dead trees (Borden et al. 2006, Gillette et al. 2012, Gitau et al. 2013). These strategies require optimization to integrate the technique into systems that will facilitate tree protection (Ranger et al. 2016). Although such a strategy of using limonene as a repellent and the commercially available pheromone lure and/or host and fungal volatiles as an attractant could be effective in manipulating WTB populations, the operational capacity of this strategy has yet to be evaluated in the field.

Acknowledgments

We thank Emily DeFries, Gary Frazier, Gabriel Hughes, Tyler Stewart, and Nikki VanDerLaan from the Department of Entomology at Purdue University for their technical assistance. Thanks to Phil Flanagan (University of Tennessee-Knoxville) for help with the bioassays conducted at UTK. We thank Brian Beheler and Jim McKenna for help with the aerial lift at Martell Forest and the Department of Forestry and Natural Resources at Purdue University and the Hardwood Tree Improvement and Regeneration Center for access to their walnut plantation. We also thank David Wakarchuk (Synergy Semiochemicals Corp.) for supplying ethanol and limonene lures. This research was conducted in partial fulfillment of a MS degree for B.L.B. from Purdue University and was funded through USDA-NIFA, North Central Regional IPM Grant (2012-34103-19756) and with support from the USDA National Institute of Food and Agriculture Hatch project #1009630; TEN00495.

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