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Semiochemical attractants for the beech leaf-mining weevil, *Orchestes fagi*

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Abstract

The beech leaf-mining weevil, *Orchestes fagi* L. (Curculionidae: Curculioninae: Rhamphini), a pest of European beech, *Fagus sylvatica* L. (Fagaceae), was recently discovered infesting American beech, *Fagus grandifolia* Ehrh., in Nova Scotia, Canada. Adult *O. fagi* feed on both young and mature leaves of beech as well as on other species (e.g., raspberry, *Rubus* spp.), but oviposition and larval feeding are restricted to beech. Females oviposit in young developing beech leaves at the time of bud burst. We characterized volatiles emitted from buds, leaves, and sapwood of American beech and examined their potential as attractants alone or when combined with other weevil pheromones for *O. fagi*. We predicted that adults would be attracted to volatiles emitted from beech leaves, especially those emitted from bursting beech buds. Gas chromatography/mass spectrometry (GC/MS) analyses of volatiles collected from buds at pre- and post-budburst identified two diterpene hydrocarbons, 9-geranyl-*p*-cymene (**1**) and 9-geranyl-α-terpinene (**2a**), that were emitted in large amounts at the time of bud burst. Compound **1** significantly increased mean catch of males and total *O. fagi* (but not females) on sticky traps compared with unbaited controls. Y-tube bioassays confirmed attraction of male *O. fagi* to bursting beech buds and compound **1**. Attraction of male *O. fagi* to **1**, emitted in large quantities from American beech, is likely adaptive because both oviposition and mating of *O. fagi* coincide with budburst. Our data suggest that traps baited with **1** may be useful for monitoring the spread of *O. fagi* in North America.

Introduction

The beech leaf-mining weevil, *Orchestes fagi* L. (syn. *Rhynchaenus fagi*) (Coleoptera: Curculionidae: Curculioninae: Ramphini), also known as the beech flea weevil, is a common, univoltine pest of beech trees, *Fagus sylvatica* L. (Fagaceae), in Europe (Alford, 1995). The adults overwinter in a variety of substrates such as bark crevices and leaf litter (Nielsen, 1970; Morrison et al., 2017) and emerge in spring. Males are sexually mature before overwintering but females spend the winter in reproductive diapause which is broken in spring by a period of lengthening photoperiod (Bale, 1979). Adults have a distinct preference for young, developing beech leaves in spring (Bale & Luff, 1978; Moise et al., 2015), and mating and egg development occur only after females have fed; thus, mating and oviposition coincide with beech budburst (Bale, 1979). Oviposition starts within 48 h of feeding, and eggs are laid directly in the central vein on the underside of young developing leaves (Bale, 1984). Larvae mine the leaves toward the leaf margin and undergo three instars before pupating at the end of the mine (Nielsen, 1966; Bale, 1984). In Europe, the new generation of adults emerges in late June and prefers to feed on leaves of raspberry (*Rubus* spp.) and other species instead of older beech leaves (Bale & Luff, 1978) before seeking overwintering sites and entering diapause (Bale, 1979), but in North America, Moise et al. (2015) found that new-generation adults did little to no feeding on any plant, including

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beech. During heavy weevil infestations, the tips of beech leaves turn yellow or brown and wilt (Alford, 1995; Bignucolo & Körner, 2010).

Orchestes fagi was discovered infesting American beech in Nova Scotia, Canada, in 2012, but based on anecdotal reports of typical beech weevil damage, it was likely present in Halifax for at least 5 years before its discovery. American beech is distributed throughout most of eastern North America, has several uses such as flooring, furniture, and fuelwood, and beech nuts are an important food source for many species of birds and mammals (Tubbs & Houston, 1990). American beech has already been devastated by beech bark disease but many beech survive in the 'aftermath' forest, and some appear to be resistant (Houston & O'Brien, 1983; McCullough et al., 2005). Several consecutive years of damage by the beech leaf mining weevil may progressively weaken trees and increase their susceptibility to attack by root disease fungi, *Armillaria* spec. (Tubbs & Houston, 1990), and mortality of weevil-infested American beech trees has been observed in greater Halifax beginning in 2015 (JD Sweeney, R Johns, C Hughes and A Morrison, unpubl.). Risk of *O. fagi* spread via human-assisted movement of firewood and logs is high because of its habit of overwintering on the boles of American beech as well as red spruce, *Picea rubens* Sarg., and red maple, *Acer rubrum* L., growing in the same vicinity (Morrison et al., 2017). Effective tools for survey and detection of *O. fagi* would be beneficial for tracking its spread and managing its impact on American beech.

Chemically and behaviorally, weevils are difficult to study (Bartelt, 1999). They respond to pheromones with short, hopping flights and walking instead of upwind flights typical of moths, and laboratory bioassays of weevil pheromones are often subtle and activity elusive. Many weevil pheromones consist of blends of components that act synergistically, and the loss of a key component during work-up can make the remaining components behaviorally inactive. In addition, activity of the pheromone can be difficult to observe without admixture of appropriate host volatiles. Most published weevil pheromones are emitted by males and attract both sexes and are either terpenoid, polyketide, or fatty acid in origin (Bartelt, 1999).

As part of an initial study into the chemical ecology of *O. fagi* and the development of detection and survey tools, we screened potential pheromone components as well as volatiles from the wood, leaves, and bursting buds of American beech. We were particularly interested in volatiles that might be emitted from freshly opening beech buds because they are targeted for oviposition by the female weevil (Bale, 1984), Grimm (1990) reported that *O. fagi* were attracted to bursting buds of European beech, and we have frequently observed mating pairs of *O. fagi* inside freshly burst and expanding buds of American beech. Synchronization between *O. fagi* oviposition and budburst is adaptive because sclerification of beech leaves starts as soon as 1 week after budburst; first instars are less able to mine older, tougher leaves, and their mortality increases (Nielsen, 1966, 1968). We hypothesized that both sexes of *O. fagi* would be attracted to bursting beech buds as well as to predominant volatiles emitted by bursting beech buds.

Methods and materials

Host volatile collections

American beech wood and leaves were collected from six haphazardly selected trees at the Acadia Research Forest (45.992712°N, 66.362238°W) in New Brunswick, Canada, on 27 June 2012. One mature leaf and one small piece of sapwood (about 2 × 2 × 1 cm) were collected from each tree (on the bole at breast height); the leaves and sapwood samples were pooled separately to make two composite samples, one of foliage and the other of sapwood. Solid phase micro-extraction (SPME) was used to collect volatile compounds using a manual SPME sampler with a red 100-μm polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA). Materials to be sampled were placed in a glass collection chamber (4 cm diameter, 11 cm high, with one end narrowed to 0.5 cm) with the large end sealed with Parafilm. The SPME fiber was then inserted and exposed through the small end for 1 h and then exposed for 4 min in the injection port of an Agilent 7890A gas chromatograph (GC) coupled to a 5975C series mass spectrometer (MS) with a Zebron ZB-5HT Inferno column (30 m × 0.25 μm film × 0.25 mm; Phenomenex, Torrance, CA, USA). The injection port was kept at 250 °C and had a 4 mm i.d. glass liner with helium as carrier gas. The temperature program was as follows: 35 °C held for 3 min, ramped to 200 °C at 10 °C per min, and held for 10.5 min. Blanks were analyzed to check for contamination from the sampling apparatus. Identification of compounds was by comparison to published spectra (NIST, Agilent 2014), retention time, and electron ionization (IE) mass spectral fragmentation patterns compared with authentic synthetic materials previously synthesized and characterized in our laboratory (Mayo et al., 2016) or from commercial sources.

Beech branches were collected from the Halifax area in May 2014, and the freshly cut ends placed in water in the lab at 20–22 °C to allow bud development. Every 2 days until budburst, we cut 2–3 branch sections, each 10–15 cm long and collected volatiles for 2 h using SPME fibers as described above. The SPME fibers were then exposed for 4 min in the injection port of an Agilent 7890A GC coupled to a 5975C series MS with the Zebron

ZB-5HT Inferno column with helium as carrier gas. The injection port was kept at 250 °C and had a 4 mm i.d. glass liner. The temperature program was as follows: 70 °C held for 3 min, ramped to 245 °C at 15 °C per min, and held for 50 min.

Chemical synthesis

The synthesis of two diterpenes, 9-geranyl-*p*-cymene (**1**) [(*E*)-2,6-dimethyl-10-(*p*-tolyl)-2,6-undecadiene] and 9-geranyl-α-terpinene (**2a**) [(*E*)-2,6-dimethyl-10-(4′-methyl-1′,3′-cyclohexadienyl)-2,6-undecadiene], and a third compound, the cyclohexylidene 1,1-dimethyl-3-methylene-2-vinylcyclohexane (**3**), and all spectral data have been reported elsewhere (Mayo et al., 2016); both synthetic diterpenes are racemates (Figure 1). Retrosynthetically, **1** was the simpler of the two diterpenes to synthesize because the *p*-tolyl moiety was readily obtained from the Grignard reagent *p*-tolylmagnesium bromide, and the 13-C chain was constructed from geranylacetone or fuscumol (Silk et al., 2007; Mayo et al., 2016). (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was synthesized by selective oxidation of geraniol by a H_2O_2–Pt black system under solvent and halide-free conditions (Kon et al., 2008), followed by Wittig methylenation; DMNT was purified using Kugelrohr distillation. Grandisol (1-methyl-2-(1-methylethenyl)-cyclobutaneethanol; racemic), 4-methyl-5-nonanol, and 4-methyl-5-nonanone were obtained from Bedoukian Research (Danbury, CT, USA); grandisal was obtained by PCC (pyridinium chlorochromate) oxidation of grandisol. All other compounds were obtained through commercial sources (Sigma-Aldrich, Oakville, ON, Canada) and were used as received.

Insects

Overwintered adult *O. fagi* were collected near Ashburn Golf Club (AGC), NS, Canada (44.6453°N, −63.6361°W) in May 2014 and 2015, by beating branches of American beech after buds had burst and leaves were expanding. Because mating coincides with adult feeding on young beech leaves at the time of budburst (Bale, 1984), we assumed that most adults that we collected had been mated. Adults were used in bioassays within 2 weeks of collection and were otherwise maintained in the dark in a chamber inside a refrigerator at 5–10 °C and 80% r.h., and provided sugar water ad libitum. Sexes were separated, based on the shape of the fifth abdominal sternite, which is distinctly concave on the lateral regions in males but has a smooth, convex shape throughout in females (S Pawlowski, unpubl.). Beetles were acclimatized to ambient temperature for 24 h before use in bioassays.

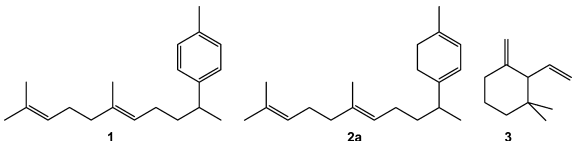

Figure 1 Structures of host attractants. (**1**) 9-geranyl-*p*-cymene [(*E*)-2,6-dimethyl-10-(*p*-tolyl)-2,6-undecadiene], (**2a**) 9-geranyl-α-terpinene [(*E*)-2,6-dimethyl-10-(4′-methyl-1′, 3′-cyclohexadienyl)-2,6-undecadiene], and (**3**) the dimethylcyclohexane homoterpene, 1,1-dimethyl-3-methylene-2-vinylcyclohexane.

Laboratory behavioral bioassays

Behavioral responses of adult male and female *O. fagi* to host compounds and plant material were assayed separately in a Y-tube olfactometer (1 cm diameter) using methods adapted from De Silva et al. (2013). Incoming air was charcoal filtered and passed through a two-channel air delivery system (Analytical Research Systems, Micanopy, FL, USA) to be subsequently split and humidified in two adjoining distilled water bubblers. Air flow entered each arm of the Y-tube at a rate of 1 l per min. Light was monitored using a Traceable light meter (Control Company, Webster, TX, USA) to ensure a difference of <20 lux between arms. The entire Y-tube apparatus was positioned vertically to assist in the natural upward movement of *O. fagi* (Grimm, 1990).

A single trial involved release of a single adult *O. fagi* placed at the exodus of the olfactometer and allowed 10 min to climb the central column and select a stimulus. A stimulus was deemed 'selected' when the beetle passed 7 cm into a stimulus arm. Beetles that failed to select a stimulus were recorded as non-responders (NR). Bioassays were done with one sex at a time and each was deemed complete when 20 adult *O. fagi* of a given sex had selected a stimulus according to the above criteria. Stimuli selected to assess behavioral response included: (1) bursting beech bud, (2) bursting beech bud with one adult *O. fagi* male, (3) bursting beech bud with one adult *O. fagi* female, (4) fully flushed beech leaves, (5) beech wood slivers, (6) 10 µl (1 mg ml^{-1} in hexane) 9-geranyl-*p*-cymene applied to a 15 × 5 mm strip of filter paper. All bioassays conducted using plant and conspecific materials were run opposite a clean air blank; the 9-geranyl-*p*-cymene bioassay was conducted opposite a 1 µl hexane blank on filter paper.

Between each exposure of an individual weevil to a pair of stimuli in the olfactometer, stimulus-free air was passed through the olfactometer for 5 min to minimize lingering odors from the passage of the preceding weevil and stimuli

in each arm were exchanged to minimize directional bias. In 2014 and 2015, bursting buds and beech foliage were collected for bioassays from trees located at the Harriet Irving Botanical Garden on the Acadia University campus (45.0875°N, −64.3684°W), or from AGC. Use of different sampling areas permitted collection of both bursting buds and mature leaves as plants developed more rapidly at the Acadia University site. Plant material and conspecifics were monitored for wilting and inactivity, respectively, and were replaced accordingly; 9-geranyl-*p*-cymene was replaced every 30 min to account for loss of volatility. A new *O. fagi* individual was used in each trial. Before and after each different pair of stimuli were tested, the olfactometer was washed with soap and water, scrubbed with a test-tube brush, rinsed with acetone, and allowed to dry in a fume hood for >30 min to allow complete evaporation of the acetone.

Field trapping bioassays

We conducted three trapping experiments from 2013 to 2016, testing various combinations of beech volatiles and known components of the aggregation pheromones of other weevil species. The field sites were located in or near Halifax, NS, Canada, near AGC, and Sandy Lake (SL) (44.7433°N, −63.6671°W). The beech trees at AGC were located either on the golf course or within a 5- to 7-m-wide strip of beech and red maple (*Acer rubrum* L.) along a residential street that bordered the golf course. The SL site was a natural mixed deciduous–coniferous forest containing mature and immature American beech, with red spruce, *Picea rubens* Sarg., eastern hemlock, *Tsuga canadensis* (L.), balsam fir, *Abies balsamea* (L.), yellow birch, *Betula alleghaniensis* Britt, and red maple as the dominant species.

With the exception of (3*Z*)-hexenol lures which were purchased commercially (Synergy Semiochemical, Burnaby, BC, Canada) compounds were loaded on red rubber septa (Wheaton Science Products, Millville, NJ, USA) in amounts that ranged from 50 to 2000 μg per septum and with release rates ranging from ca. 1.2–50 μg day^{-1} (determined by weight loss at 25 °C; Table 1). Lures were attached to traps using color-coded paperclips. We used yellow double-sided sticky cards (Smart et al., 1997; Szendrei et al., 2011) that measured 7.5 cm × 13 cm (Contech Enterprises, Victoria, BC, Canada) in 2013–2015 and 10 cm × 25 cm (Bug-Scan; Biobest, Westerlo, Belgium) in 2016. We also tested light green, non-sticky boll-weevil traps (Boll Weevil Eradication Foundation Traps, Great Lakes IPM, Vestaburg, MI, USA) in bioassay 1 only. Treatments were replicated 8× in randomized complete blocks with 20–30 m spacing between traps and blocks. The traps were suspended about 1.5 m above the ground from lower branches of American beech and either checked weekly to count the adult *O. fagi* captured (2013–2015) or collected at the end of the trapping period and stored at −18 °C until processed (2016). Although trapping efficacy will have decreased with cumulative insect catch, the traps were not saturated and were still sticky to touch when collected at the end of the 8-week trapping period in 2016. All adult *O. fagi* on both sides of each sticky trap and within boll weevil traps were identified using keys (Sweeney et al., 2012), and reference and voucher specimens were deposited at the Atlantic Forestry Centre, Fredericton, NB.

Field bioassay 1 was a 2 × 4 factorial experiment that tested the efficacy of two trap designs (yellow sticky cards vs. light green non-sticky boll weevil traps) and four lure treatments: (1) grandisol, (2) grandisal, (3) (3*Z*)-hexenyl acetate, and (4) an unbaited control for catching adult

Table 1 Purity, load, and release rate of semiochemical lures used in field trapping bioassays for capture of *Orchestes fagi* near Halifax, NS, Canada, in 2013–2016. Release rates from septa were estimated by weight loss at 25 °C

Chemical	Lure type	% purity	Load per lure	Release rate (μg day^{-1})	Source
(3*Z*)-hexenol	Pouch	98	8 g	60000[1]	Synergy Semiochemicals
(3*Z*)-hexenyl acetate	Septa	98	50 μg	ca. 1.2	Sigma-Aldrich
Grandisol	Septa	95	50 μg	ca. 1.2	Bedoukian Research
Grandisal	Septa	95	50 μg	ca. 1.2	Atlantic Forestry Centre
4-methyl-5-nonanol	Septa	98	50 μg	ca. 1.2	Bedoukian Research
4-methyl-5-nonanone	Septa	98	50 μg	ca. 1.2	Bedoukian Research
9-geranyl-*p*-cymene[2] (**1**)	Septa	95	2.0 mg	ca. 20	Atlantic Forestry Centre
9-geranyl-α-terpinene[2] (**2a**)	Septa	95	0.2 mg	ca. 2	Atlantic Forestry Centre
1-dimethyl-3-methylene-2-vinylcyclohexane (**3**)	Septa	95	2.0 mg	ca. 50	Atlantic Forestry Centre

[1]Release rate supplied by manufacturer.
[2]Racemic.

O. fagi. Traps were set up from 29 May–5 June 2013 at AGC with eight replicates per treatment. Grandisol and grandisal were tested because they had been identified previously as sex/aggregation pheromone components in other weevil species (Tumlinson et al., 1969; Hedin et al., 1979; Booth et al., 1983; Phillips et al., 1984; Bartelt, 1999), and (3*Z*)-hexenyl acetate was identified in volatiles from leaves of American beech (see results).

Bioassay 2 tested the attraction of the leaf volatile, (3*Z*)-hexenol, on its own, or combined with one of three compounds previously identified in pheromones of other weevils (Bartelt, 1999). The 2 × 4 factorial experiment [(3*Z*)-hexenol present vs. absent crossed with (1) grandisal, (2) 4-methyl-5-nonanol, (3) 4-methyl-5-nonanone, or (4) blank] was set up 18–31 July 2013 with four replicates at AGC and four replicates at SL.

Bioassay 3 tested four lure treatments: 9-geranyl-p-cymene (**1**), 9-geranyl-α-terpinene (**2a**), and 1,1-dimethyl-3-methylene-2-vinylcyclohexane (**3**), plus a blank control. Compounds were loaded on red rubber septa at 2.0 mg per septum for **1** and **3** and 0.2 mg per septum for **2a** (we lacked sufficient quantities of **2a** to permit a 2 mg per septum load). Lures containing **1** (ca. 20 μg day^{-1}) and **2a** (2 μg day^{-1}) were estimated to last for at least 14 weeks and **3** (ca. 50 μg day^{-1}) for ca. 4 weeks in the field, based on release rates at 25 °C (Table 1) and mean daily temperatures in Halifax of 14–19 °C in June–August (Environment Canada website: climate.weather.gc.ca). Treatments were replicated 8× with four blocks at AGC and four blocks at SL, 8 June–2 August 2016. A subsample of about 55% of total *O. fagi* adults were collected from all traps and were sexed to compare sex ratios among lure treatments.

Data analysis

The numbers of responding adults that selected stimulus 1 vs. 2 in the Y-tube olfactometer bioassays were compared with an expected 1:1 ratio using χ^2 tests. For field trapping bioassays, total *O. fagi* catch on each trap was summed over the entire trapping period of each experiment and analyzed with generalized linear mixed models (Proc GLIMMIX) using SAS/STAT v.9.2 software (SAS System for Windows 2002–2008). If a trap was found disturbed, e.g., a lure was missing during weekly checks, the data for all treatments in the affected block for that week were omitted from analysis. Lure treatments were considered fixed effects, and blocks were random effects. Data were modeled with both Poisson and negative binomial distributions (log link). We report results for the model-distribution with the lowest value of Akaike's information criterion corrected for small sample sizes (AIC_c). The Laplace method of estimating model parameters was used because count means were sometimes less than 5 (Bolker et al., 2008). Post hoc comparison of means was done with the Tukey-Kramer test on least square means adjusted for multiple comparisons ($\alpha = 0.05$). For field bioassay 3 only, we also tested for the effects of lure treatment on catch of males and females separately, based on a subsample of adults from all traps. For bioassay 5, the effect of lure on sex ratio of *O. fagi* was determined using a 4 × 2 χ^2 contingency table.

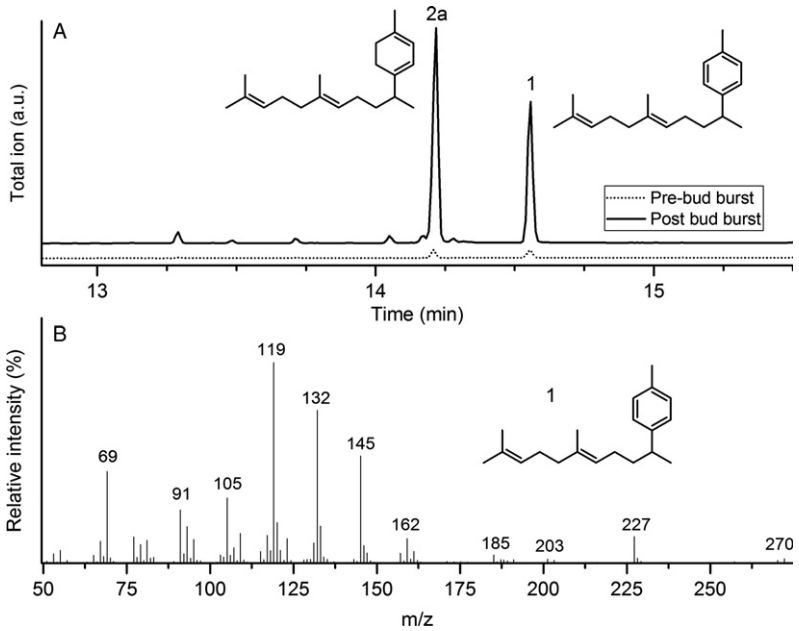

Figure 2 Volatiles from beech buds based on solid phase micro-extraction (SPME) analysis before and after bud burst. (A) SPME/GC-MS analysis, (B) EI mass spectrum of **1** (9-geranyl-*p*-cymene); 70 eV. a.u., arbitrary unit.

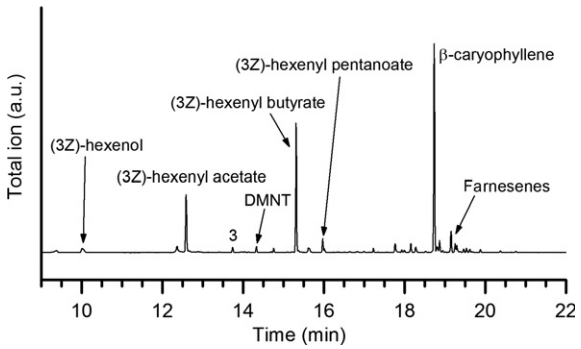

Figure 3 Volatiles from leaves of *Fagus grandifolia*. DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene. a.u., arbitrary unit.

Results

Analysis of volatiles from American beech leaves, sapwood, and buds

GC-MS analyses of beech buds from American beech showed low levels of several peaks of apparent terpenoid structure, but as the bud began to flush, two very large peaks appeared in the chromatogram at the 1–2 μg per bud level (Figure 2). These two compounds were identified as the diterpenes 9-geranyl-*p*-cymene [(*E*)-2,6-dimethyl-10-(*p*-tolyl)-2,6-undecadiene] (**1**; mass spectrum in Figure 2B) and 9-geranyl-α-terpinene [(*E*)-2,6-dimethyl-10-(4′-methyl-1′,3′-cyclohexadienyl)-2,6-undecadiene] (**2a**). Both matched the retention times and mass spectra of authentic and characterized synthetic compounds. A third compound, the dimethylcyclohexane homoterpene 1,1-dimethyl-3-methylene-2-vinylcyclohexane **3**, an additional putative attractant of *O. fagi*, was tentatively identified in both beech leaf volatiles at very low levels (Figure 3) and in beech sapwood (Figure 4; EI mass spectrum in Figure 4B). Compounds **3** and DMNT matched retention times and mass spectral characteristics of authentic materials (Mayo et al., 2016). Compound **3** also bears a curious structural resemblance to published cyclohexylidene curculionid terpenoid pheromones (Bartelt, 1999; Szendrei et al., 2011). Sapwood emitted α-cubebene, a series of isomeric farnesenes, high levels of (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and trace levels of the dimethylcyclohexane homoterpene **3** (Figure 4).

Beech leaves from American beech also produced many other volatile compounds under our sampling conditions, including green leaf volatiles, a series of (3*Z*)-hexenol alkanoic esters, β-caryophyllene, and low levels of other sesquiterpenes of unknown structure (Figure 3).

Behavioral bioassays

Male *O. fagi* responded positively to 9-geranyl-*p*-cymene compared with a hexane blank in Y-tube olfactometer bioassays (Figure 5). Males also responded positively to bursting beech buds, but negatively when a conspecific male was present on the bursting bud (Figure 5). Female presence on the bud did not enhance male attraction. Of all stimuli presented, adult females were significantly attracted only to mature (i.e., fully flushed) beech leaves (Figure 5).

Field trapping bioassays

In bioassay 1, which tested the effect of trap type and lure on trap catch, yellow sticky cards captured more adult *O. fagi* than the non-sticky boll weevil traps

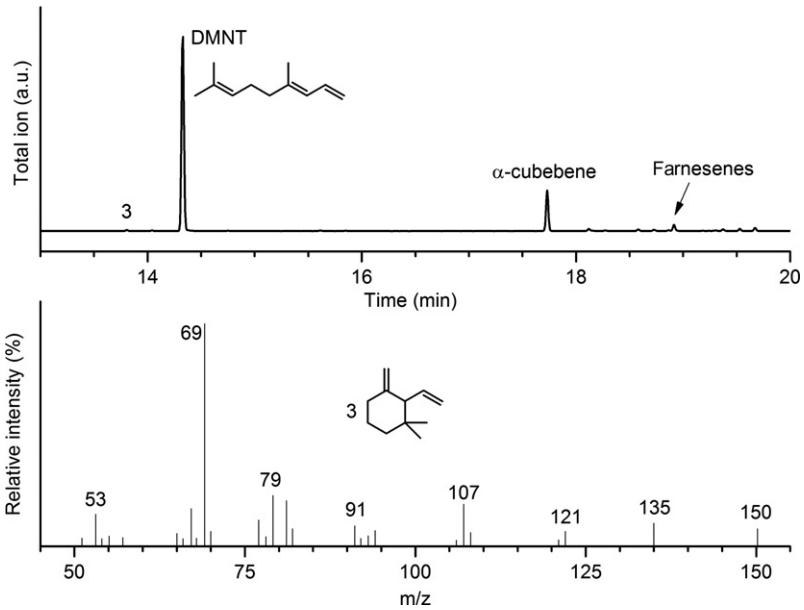

Figure 4 Volatiles from sapwood of *Fagus grandifolia*. DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene. a.u., arbitrary unit.

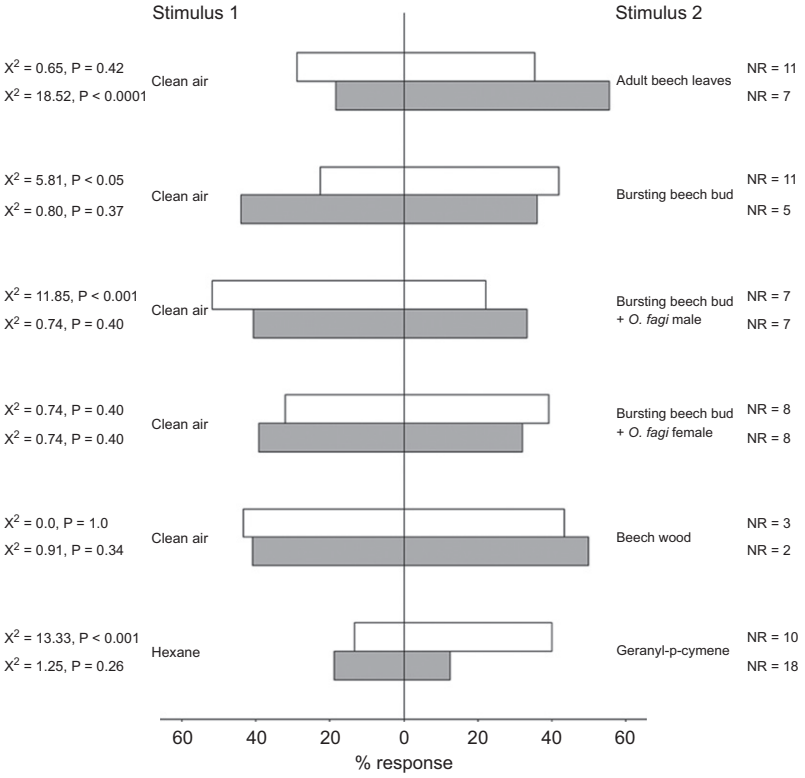

Figure 5 *Orchestes fagi* adult male (white bars) and female (gray bars) preference (% response) to stimuli presented in a Y-tube olfactometer bioassay. NR, number of non-responders.

($F_{1,56}$ = 122, P<0.0001), but lure treatment ($F_{3,56}$ = 0.31, P = 0.82) and lure*trap type interaction ($F_{3,56}$ = 1.29, P = 0.29) were not significant (Figure 6). In bioassay 2, testing the response of *O. fagi* to traps baited with pheromones previously identified in other weevil species (grandisal, 4-methyl-5-nonanal, 4-methyl-5-nonanone, and unbaited control), with or without (3*Z*)-hexenol, mean catch was not affected by any of the pheromones ($F_{3,56}$ = 2.09, P = 0.11) but was increased, albeit slightly, by (3*Z*)-hexenol ($F_{1,56}$ = 4.20, P = 0.05) (Figure 7). In bioassay 3, in which we tested the attraction of compounds identified in bursting beech buds (**1**, **2a**) and sapwood (**3**), lure treatment affected catch of males ($F_{3,24}$ = 3.65, P = 0.03) and total *O. fagi* ($F_{3,24}$ = 3.90, P = 0.02), with greater catches on traps baited with 9-geranyl-*p*-cymene (diterpene **1**) than on unbaited controls; there was no effect on catch of females ($F_{3,24}$ = 1.06, P = 0.38) (Figure 8). Lure treatment affected the sex ratio of *O. fagi* (χ^2 = 7.90, d.f. = 3, P = 0.048) with a male bias apparent on baited traps compared with unbaited controls.

Discussion

The diterpene, 9-geranyl-*p*-cymene (**1**), emitted in large quantities from American beech buds at the time of budburst, was significantly attractive to male *O. fagi* but not females in both laboratory and field bioassays. To the best of our knowledge this is the first diterpene weevil host attractant identified and is the first effective trap attractant reported for *O. fagi*. We have not yet investigated the chirality of **1** and **2a** and its possible effects on *O. fagi* behavior, but this should be addressed in subsequent studies.

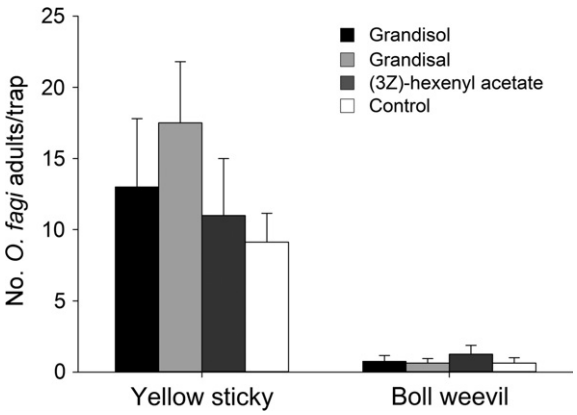

Figure 6 Effect of trap type (yellow sticky cards vs. non-sticky boll weevil traps) and lure treatment on mean (+ SE; n = 8) catch of *Orchestes fagi* adults in Halifax, NS, Canada, 29 May–5 June 2013.

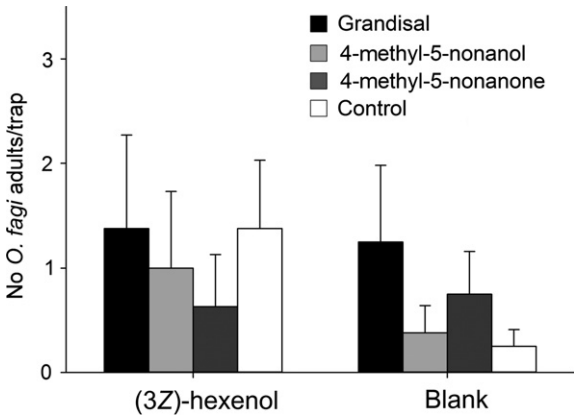

Figure 7 Effect of (3*Z*)-hexenol and three pheromone compounds known from other weevil species on mean (+ SE; n = 8) catch of *Orchestes fagi* adults in Halifax and Bedford, NS, Canada, 18–31 July 2013.

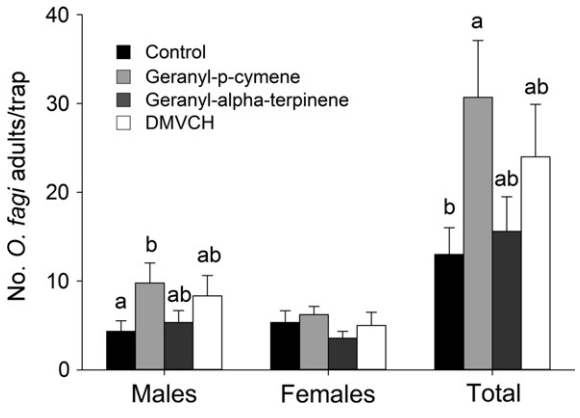

Figure 8 Mean (+ SE; n = 9) catches of male, female, and total *Orchestes fagi* adults (sexes pooled) on yellow sticky cards baited with plant volatiles (DMVCH, 1,1-dimethyl-3-methylene-2-vinylcyclohexane) or unbaited (control) in Halifax and Bedford, NS, Canada, 2016. Means within males and within total (i.e., pooled sexes) capped with different letters are significantly different (Tukey–Kramer test: $P<0.05$). Note: mean and SE values for males and females were calculated from a subsample of 431 of the total 749 weevils caught on all traps.

Previous studies have demonstrated that chirality of plant volatiles and curculionid pheromones can have critical effects on insect behavior, with antipodes of the active enantiomer having either inert or antagonistic effects on attraction of target species (Bartelt, 1999; Zarbin et al., 2007).

Of the green leaf volatiles we tested, we observed slight but significant attraction to (3*Z*)-hexenol but no response to (3*Z*)-hexenyl acetate, similar to the results of Pawlowski (2014). However, we cannot conclude that (3*Z*)-hexenol is relatively more attractive to the weevil than (3*Z*)-hexenyl acetate because the release rate of the alcohol was several orders of magnitude greater than that of the acetate. Furthermore, to increase the chances of catching *O. fagi*, we suspended our sticky traps directly from beech branches, and it is possible that response to the lures, especially the (3*Z*)-hexenyl acetate septa that emitted only ca. 1.2 μg day^{-1}, was influenced or masked by background concentrations of volatiles emitted from the beech foliage. Further testing of these and other beech volatiles is warranted at a range of release rates because some may act as synergists with an as yet unidentified pheromone of *O. fagi*. In the boll weevil *Anthonomus grandis* Boheman, host compounds such as (2*E*)-hexenol, (3*Z*)-hexenol, 1-hexanol, and various terpenes enhanced the activity of the pheromone in bioassays (Hedin et al., 1979; Dickens, 1989; Dickens et al., 1990). The pattern of synergism between pheromone and host volatiles has been found to occur throughout the genus and with many weevils (Jaffé et al., 1993; Bartelt, 1999) and other Coleoptera (Silk et al., 2007; Hanks & Millar, 2013).

These diterpenes are not unprecedented in the plant world. Compounds **1** and **2a** have been detected in the oils of various plants: compound **1** in *Sideritis trojana* Bornm. (Kirimer et al., 2008), *Sideritis cilicica* Boiss. & Bal. (Iscan et al., 2005), *Anthemis dipsacea* Bornm. (Kurtulmus et al., 2009), *Anthemis rosea* Sm. ssp. *carnea* (Kirimer et al., 2014), *Calamintha pamphylica* Boiss. & Heldr. ssp. *pamphylica*, *davisii*, and *alanyense* (Alan et al., 2011), *Artemisia absinthium* L. from Tajikistan (Sharopov et al., 2012), and *Cydonia oblonga* Miller (Gonenc et al., 2012). Both compounds **1** and **2a** have been found together in the same plant, for instance, in *A. absinthium* (Judzentiene et al., 2009), *Sideritis dichotoma* Huter (Kirimer et al., 2004), and *Helichrysum* species (Bohlmann et al., 1978). It is plausible that compounds **1** and **2a** are generated through the mevalonate pathway via geranylpyrophosphate (Mayo et al., 2012). DMNT [(*E*)-4,8-dimethyl-1,3,7-nonatriene] is a plant stress compound related to insect herbivory (Turlings & Tumlinson, 1992; Paré & Tumlinson, 1999).

The cyclohexylidene, compound **3**, did not significantly increase mean trap catches compared with unbaited control, but this experiment bears repeating because these lures were probably exhausted after 4 weeks, whereas the trapping period lasted 8 weeks. Compound **3** has been reported before as a plant-derived natural product. The crepe myrtle *Lagerstroemia caudata* Chun & FC How ex SK Lee & LF Lau and *Lagerstroemia indica* L., chiefly known for their colorful and long-lasting flowers, have had their flower volatiles analyzed using SPME and GC-MS. The main volatile ingredients and their relative

contents are 1,1-dimethyl-3-methylene-2-vinylcyclohexane (**3**) (50.3%) as the main ingredient and *iso*-geraniol (26.2%), (3*E*,6*E*)-3,7,11-trimethyl-1,3,6,10-dodecatetraene (11.4%), 2-ethenyl-1,3,3-trimethyl-cyclohexene (14.7%), and bergamotene as minor. There is a report of **3** being a constituent of the volatiles of the plant *Melinis minutiflora* P. Beauv. (Harraca et al., 2011).

Attraction of males to bursting beech buds and to volatiles emitted at the time of budburst, such as 9-geranyl-*p*-cymene, was expected and is likely adaptive. Both oviposition and mating of *O. fagi* coincide with budburst, and beech leaves begin to toughen as soon as 1 week after budburst, which reduces the ability of first instars to mine (Nielsen, 1966, 1968). Lack of attraction of females to bursting beech buds or to 9-geranyl-*p*-cymene in the olfactometer was unexpected and suggests that female *O. fagi* may require the stimulus of a male sex pheromone (Bartelt, 1999) in addition to budburst volatiles as positive olfactory cues when seeking oviposition sites as well as mates. Female *O. fagi* are in reproductive diapause until mid-March, and mating, egg maturation, and oviposition all occur within a short window during budburst when females feed on the young developing leaves (Bale, 1979). However, the lack of response of females to our treatment of a male on a bursting bud in the behavioral bioassay does not support this argument. It is possible that conditions in the laboratory bioassays or of the males themselves were not conducive for pheromone emission. For example, pheromone production by male plum curculio, *Conotrachelus nenuphar* Herbst, was increased under conditions of high humidity, and positive response of females to males vs. blank controls in olfactometer bioassays varied with age (mature>immature), mating status (virgin males>mated males), and number of males present (two males>one male) (Hock et al., 2014). Although all male *O. fagi* used in our bioassays were mature, we did not know their mating status and did not compare response of females to one male vs. >1 male on a bud. However, the significant negative response of *O. fagi* males to the stimulus of a male on a bursting bud suggests that the males on the buds were either emitting a pheromone or that they were feeding in the buds and altering the plant volatiles being emitted.

Our results clearly indicate that 9-geranyl-*p*-cymene, emitted in high concentrations from bursting beech buds, is attractive to male *O. fagi* and would be a useful attractant for use in trapping surveys to monitor spread of this invasive species in North America. If *O. fagi* males emit an aggregation pheromone, as observed in other weevil species (Bartelt, 1999), we predict that 9-geranyl-*p*-cymene and possibly additional beech volatiles identified in this study may act as synergists and would therefore be useful in the pheromone identification process. Further research is required to elucidate host and mate location processes in this species and to improve tools for survey of *O. fagi*, including isolation and identification of a male-produced pheromone, investigation of additional beech volatiles as potential synergists, and effect of trap color on catches.

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