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Ability of stress-related volatiles to attract and induce attacks by *Xylosandrus germanus* and other ambrosia beetles

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Abstract

1 *Xylosandrus germanus* typically colonizes physiologically-stressed deciduous hosts but it is increasingly being recognized as a key pest of ornamental nursery stock. We tested the attractiveness of common plant stress-related volatiles to ambrosia beetles occupying the nursery agroecosystem, as well as their ability to induce attacks on selected trees. Experiments were conducted in Ohio, U.S.A.

2 Stress volatile attractiveness was first assessed by positioning traps baited with acetaldehyde, acetone, ethanol and methanol in ornamental nurseries. Cumulative trap counts confirmed that ethanol was the most attractive stress-related volatile to *X. germanus*. Methanol-baited traps were slightly attractive to *X. germanus*, whereas traps baited with acetaldehyde and acetone were not attractive to any ambrosia beetle.

3 A series of tree injection experiments were also conducted to determine the ability of these volatiles to induce attacks by ambrosia beetles under field conditions. Injection of ethanol into *Magnolia virginiana* induced the largest number of attacks, whereas injection of acetaldehyde induced more attacks than methanol or acetone. *Xylosandrus germanus* was the most predominant species emerging from *M. virginiana* injected with each of the stress-related volatiles. No attacks by wood-boring beetles were observed on water injected or uninjected control trees.

4 Solid-phase microextraction–gas chromatography–mass spectrometry confirmed the emission of acetaldehyde, acetone, ethanol and methanol after their injection into *M. virginiana*.

5 *Xylosandrus germanus* has an efficient olfactory-based mechanism for differentiating among host volatile cues. Injecting select trees with stress-related volatiles, particularly ethanol, shows promise as a trap tree strategy for *X. germanus* and other ambrosia beetles.

Keywords Ambrosia beetles, attractants, Coleoptera, Curculionidae, Scolytinae, trap tree.

Introduction

Over 50 exotic species of Scolytinae are established in North America (Haack, 2006). Several of these introductions are ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) that have become key pests in ornamental nurseries. *Xylosandrus germanus* (Blandford) is native to eastern Asia origin and is among the most economically important exotic ambrosia beetles in U.S. nurseries (Oliver & Mannion, 2001). Subsequent to first being detected in New York in 1932, *X. germanus* has become established in the northeastern, southern, midwestern and upper northwestern U.S.A. (Mudge *et al*., 2001; Rabaglia *et al*., 2006). The species has also been introduced to Europe, where it was first detected in Germany in 1951. It has subsequently spread to Austria, Belgium, France, Italy and Switzerland (Groschke, 1953; Fiorentino, 1985; Bruge, 1995; Fauna Europea), and has colonized large stands of beech *Fagus sylvatica* L. (Grégoire *et al*., 2001).

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Xylosandrus germanus overwinters as an adult in galleries within the wood of its host plants (Weber & McPherson, 1983a). Males are flightless, although flight activity of overwintering adult females can begin in the U.S.A. as early as March in North Carolina and Tennessee, and April in Ohio and Illinois (Weber & McPherson, 1983a; Oliver & Mannion, 2001). *Xylosandrus germanus* is a generalist with an extensive host range but deciduous hosts are preferred over coniferous species (Weber & McPherson, 1983b). Common hosts include *Magnolia* spp., red bud (*Cercis* spp.), dogwood (*Cornus* spp.), black walnut (*Juglans nigra* L.), beech (*Fagus* spp.), oak (*Quercus* spp.) and maple (*Acer* spp.) (Weber & McPherson, 1983b; C. M. Ranger, personal observation).

Attacks on apparently healthy trees by *X. germanus* have been suspected to occur in U.S. and European habitats (Weber, 1982; Kovach, 1986; Grégoire *et al*., 2001) but physiologically-stressed trees are usually targeted for colonization. Compared with healthy specimens, stressed trees produce greater concentrations of several volatile organic compounds, including acetaldehyde, acetone, ethane, ethanol, ethylene and methanol (Kimmerer & Kozlowski, 1982; Millar *et al*., 1986; Holzinger *et al*., 2000). A variety of stressors, including flooding, drought, mechanical damage, freezing and ozone exposure, can induce the production and emission of stress-related volatiles by plants (Moeck, 1970; Cossins, 1978; Kimmerer & Kozlowski, 1982; MacDonald & Fall, 1993; Von Dahl *et al*., 2006). For example, under anoxic conditions, the reduced availability of oxygen in plant roots leads to the production of acetaldehyde as an intermediate of fermentation to produce alcohol (Kreuzwieser *et al*., 1999). Acetaldehyde is subsequently converted into ethanol by alcohol dehydrogenase. Ethanol is then transported to leaf tissue via the xylem, where a proportion can be converted back into acetaldehyde and acetone by oxidative metabolism (Cossins, 1978; Kreuzwieser *et al*., 1999). Unlike ethanol and acetaldehyde, methanol is produced by healthy plants but wounding stress dramatically increases its emission (Nemecek-Marshall *et al*., 1995; Von Dahl *et al*., 2006). A variety of biochemical pathways can lead to the production of methanol, although the demethylation of pectin by pectin methylesterase is considered to be the most qualitatively important (Von Dahl *et al*., 2006).

An aggregation pheromone has not been identified for *X. germanus* but plant stress-related volatiles represent important host-location cues for this secondary ambrosia beetle. Ethanol is a primary attractant for *X. germanus* and a positive dose response has been documented (Klimetzek *et al*., 1986). Ethanol-baited traps are commonly used for monitoring ambrosia beetles in ornamental nurseries. Baiting healthy living trees with ethanol lures induces attacks by the pine shoot beetle *Tomicus piniperda* (L.) and other wood borers (Elliott *et al*., 1983; Schroeder & Eidmann, 1987), although this tactic has been unreliable at inducing attacks by *X. germanus* (C. M. Ranger, personal observation). In laboratory bioassays, Buchanan (1941) was able to induce heavy attacks by *X. germanus* after injecting elm (*Ulmus* sp.) bolts with 50% ethanol. However, the ability to induce attacks by *X. germanus* under field conditions by injecting trees with solutions of stress-related volatiles has not been reported. In an attempt to ultimately develop a trap tree strategy for *X. germanus*, the present study aimed to determine the attractiveness of plant-stress related volatiles and their ability to induce attacks by common nursery-infesting ambrosia beetles.

Materials and methods

Ambrosia beetle trapping

Stress-related volatiles were tested for their attractiveness to *X. germanus* and other ambrosia beetles: acetaldehyde (CH_3CHO, 99.5% purity; Acros Organics, Belgium), acetone (C_3H_6O, 99.5% purity; Acros Organics), ethanol (C_2H_5OH, 99.5% purity; Acros Organics) and methanol (CH_3OH, 99.5% purity; Fisher Chemical, Pittsburgh, Pennsylvania). Lures were prepared by filling low density polyethylene plastic bottles (15 mL in volume; PheroTech Inc., Canada) separately with 95% aqueous solutions of acetaldehyde, acetone, ethanol and methanol. Lures filled with deionized water served as blank controls.

Bottle traps were constructed using a Tornado Tube® (Steve Spangler Science, Englewood, Colorado) to connect the mouth of a 1-L plastic bottle to that of a 0.5-L plastic bottle. Three rectangular openings (length 12.5 cm, width 6 cm) were cut into the 1-L bottle to allow the entrance of ambrosia beetles. Lures were secured within the 1-L bottle and the traps were hung vertically 0.6 m above ground level by attaching the inverted end of the 1-L bottle to a metal rod. The 0.5-L collecting bottle was filled with propylene glycol (Sierra Antifreeze/Coolant; Old World Industries, Inc., Northbrook, Illinois) as a killing agent. On 9 April 2008, baited bottle traps were positioned 6 m apart along the edge of woodlots bordering five cooperating nursery sites in Lake County, Ohio, and one in Wayne County, Ohio. Traps were arranged in a completely randomized design at each of the six nurseries, resulting in six replicates for each trap treatment. Thus, one replicate for each trap was placed at each nursery. Traps were rotated weekly within each replicated block and weekly collections of ambrosia beetles were made until 17 July 2008.

Lures were refilled approximately every 3 weeks. The mean release rate of ethanol [molecular weight (MW) 46.1 atomic mass units (amu); boiling point (BP) = 78 °C] from the low release lure is 30 mg/day at 20 °C (PheroTech Inc). Release rates were not determined for acetaldehyde (MW 44.1 amu; BP = 21 °C), acetone (MW 58.1 amu; BP = 56 °C) and methanol (MW 32.0 amu; BP = 64.7 °C) (Merck Index, 1983) but they are expected to be similar to ethanol.

Injection of stress volatiles

The ability of stress-related volatiles to induce attacks by ambrosia beetles was assessed using an Arborjet® Tree I.V. Delivery System (Woburn, Massachusetts) to inject solvent solutions into multistem sweetbay magnolia *Magnolia virginiana* L. Trees were 4 years old and 1 m in height, and were growing in 13-L containers with a mixture of aged pine bark, peat and coarse sand (60:30:10, v/v). Injection sites were initiated by drilling a single 9.5-mm hole approximately 16 mm deep into the base of the *M. virginiana* trees, which had a base

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caliper of 40–50 mm. The hole was immediately plugged with an Arborjet® injection port (diameter 9.5 mm) and solutions of stress-related volatiles were injected through the port at a delivery pressure of 4.14 bar (60 psi). *Magnolia virginiana* was chosen for injection experiments because it is a common host for *X. germanus*, whereas it is also associated with a comparatively low cost to base caliper size ratio.

Experiment 1. The first tree injection experiment consisted of 125 mL of 50% ethanol injected into *M. virginiana* trees. Control trees were only drilled and plugged. Immediately after the tree injections were completed on 4 May 2008, eight pairs of ethanol-injected and control trees were positioned along the edge of a woodlot at Snyder Research Farm, Ohio Agricultural Research and Development Center, Wooster, Ohio. Paired trees were spaced 0.3 m from each other and each pairing was separated by 4.5 m. Trees were thoroughly examined for new galleries at 3, 7, 11, 18 and 25 days after injection. Gallery entrances were circled with a waterproof marker (Sharpie®; Sharpie, Oak Brook, Illinois) during each tree inspection, which allowed the determination of cumulative attacks by wood-boring beetles. The position of each experimental pairing along the edge of the woodlot was rotated weekly.

Experiment 2. The second tree injection experiment involved 75 mL of 50% acetaldehyde, acetone, ethanol and methanol solutions being injected into separate *M. virginiana* trees. Control trees were only drilled and plugged. Immediately after the tree injections were completed on 23 May 2008, experimental trees ($n = 5$ per treatment) were arranged in a randomized complete block design along the edge of a woodlot at a commercial nursery in Wayne Co., Ohio. Trees within each block were 1.5 m apart, with 6 m between each of the replicated blocks. Experimental trees within each replicated block were rotated weekly. Trees were thoroughly examined for new galleries at 4, 10, 17 and 24 days after injection.

Colonized *M. virginiana* branches were excised on 16 June 2008 (25 days after injection) and returned to our laboratory where their ends were sealed with paraffin wax (Royal Oak Enterprises, Roswell, Georgia) to reduce desiccation. Branch/trunk sections associated with each stress-volatile treatment were then confined to plastic containers with moistened sponges and held for 50 days under laboratory conditions. Ambrosia beetles emerging from the colonized branches associated with each treatment were collected and identified to species. Parent and brood adults were both collected but no distinction was made between the two.

Experiment 3. The third tree injection experiment consisted of 75 mL of 50% ethanol and 75 mL of deionized water being injected into separate *M. virginiana* trees. A water injection treatment was incorporated to assess whether the injection procedure induced attacks. Control trees were drilled and plugged but not injected. Immediately after the trunk injections were completed on 7 July 2008, experimental trees ($n = 6$ trees per treatment) were arranged in a randomized complete block design along the edge of a woodlot adjacent to a commercial nursery in Wayne Co., Ohio. Trees within each block were 1.5 m apart, with 6 m between each of the replicated blocks. Experimental trees within each replicated block were rotated weekly. Trees were thoroughly examined for new galleries at 1, 2, 3, 4, 7, 8 and 9 days after injection.

Volatile sampling and gas chomatography–mass spectroscopy (GC-MS) analysis

Sampling of volatiles was achieved by solid phase microextraction (SPME) using a fibre coated with Carboxen/polydimethyl siloxane (Carboxen/PDMS) at a film thickness of 75 μM (Supelco, Inc., Bellefonte, Pennsylvania). The Carboxen/PDMS coating is most appropriate for low MW C_1–C_8 compounds because larger MW compounds are strongly retained and potentially become difficult to desorb (Supelco, Inc.). The fibre was contained within a syringe-like manual sampling device. Fibres were conditioned at the beginning of each sampling day by holding the extruded fibre for 30 min at 280 °C within the injection port of a gas chromatograph (Varian CP-3800; Varian Inc., Palo Alto, California). The injection port was equipped with a Merlin Microseal septum system (Merlin Instrument Co., Half Moon Bay, California) and a deactivated glass liner with an inner diameter of 0.75 mm and a single tapered end (Varian, Inc.).

Static headspace sampling of volatiles by SPME was achieved by first positioning an intact *M. virginiana* branch containing leaf and bark tissue within a polyacetate bag (40.64 × 44.45 cm; Reynolds Kitchens, Richmond, Virginia). Prior to enclosing the bag, the SPME syringe holder was rested against a strip of an activated charcoal filter (length 7.6 cm, width 2.54 cm; Hunter Fan Company, Cordova, Tennessee), which was wrapped around the branch to prevent tissue damage and reduce the potential for passive influx of volatile contaminants. A zip-tie was then used to tighten down the open end of the polyacetate bag around the syringe holder, charcoal filter and underlying branch. The fibre was subsequently extruded and exposed to headspace volatiles contained within the polyacetate bag. The fibre was retracted into the syringe holder after 1 h of sampling. Volatiles were collected 24 h after *M. virginiana* plants were injected with 75 mL of 50% aqueous solutions of acetaldehyde, acetone, ethanol or methanol. Trees were injected according to the previously described technique and held in a greenhouse under natural light conditions. Control trees were drilled and plugged with the injection port but uninjected.

Volatiles were thermally desorbed by exposing the SPME fibre for 15 s in the previously-described GC injection port at 250 °C under splitless mode. A fused silica Rtx-1 column (length 30 m, inner diameter 0.25 mm) with a 0.25-μm coating of cross bonded 100% dimethyl polysiloxane (Restek Corp., Bellefonte, Pennsylvania) was used for analysis. The operating conditions of the oven were 35 °C for 2 min, ramping at 15 °C/min to 270 °C and then holding at the maximum for 5 min. The GC was equipped with a Varian 2200 mass spectral detector (GC-MS) operating in electron impact mode. System control and measurements of peak area were accomplished with Star Chromatography Workstation software (STAR TOOLBAR, version 6.8; Varian Inc.). Peak identifications were made by

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comparing mass spectra and retention times with those of authentic standards, along with mass spectral library searches. For the purposes of the present study, peak area values were only considered for acetaldehyde, acetone, ethanol and methanol.

Statistical analysis

Total cumulative counts of ambrosia beetles caught in bottle traps were analysed using one-way analysis of variance (ANOVA) (PROC GLM; SAS Institute, 2001). To help stabilize the variance, data were log $(x + 1)$ transformed but untransformed data are presented. Means were separated using Tukey's Studentized range [honestly significant difference (HSD)] test at $\alpha = 0.05$.

Repeated measures ANOVA (PROC GLM; SAS Institute, 2001) was used to test for the main effect of stress volatile injection on inducing attacks by ambrosia beetles, along with a treatment $\times$ time interaction effect. Repeated measures analysis was used because the number of attacks was cumulative and dependent, in part, on the previous number of recorded attacks. When the overall model was significant ($P < 0.05$), Tukey's HSD test at $\alpha = 0.05$ was used to compare the mean number of cumulative attacks at each sampling date.

Results

Ambrosia beetle trapping

Mean cumulative trap counts of *X. germanus* from 16 April to 17 July 2008 were significantly greater in ethanol-baited traps compared with acetaldehyde, acetone and methanol (Table 1). Methanol was the second most attractive volatile to *X. germanus*, whereas trap counts of acetaldehyde and acetone were not significantly different from the water-baited control (Table 1). Peak trap counts of *X. germanus* occurred on 21 May 2008 for both the ethanol and methanol-baited traps (data not shown).

Ethanol-baited traps also caught the significantly highest cumulative counts of *Hypothenemus dissimilis* (Zimmermann) compared with the remaining stress-related volatiles (Table 1). Significant differences in trap counts of *H. dissimilis* were not detected among acetaldehyde, acetone, methanol and the water-baited control. Peak trap counts for *H. dissimilis* in ethanol-baited traps occurred on 29 May 2008 (data not shown).

Several other species of ambrosia beetles were identified from the baited and control traps, namely, *Euwallacea validus* (Eichhoff), *Monarthrum fasciatum* (Say), *Xyleborinus saxeseni* (Ratzeburg), *Xyleborus sayi* (Hopkins) and *Xyloterinus politus* (Say) (Table 1). However, none of these species were significantly more attracted to any of the stress-related volatiles compared with the water-baited control.

Induction of attacks

Injection of living, potted *M. virginiana* trees with 125 mL of 50% ethanol induced attacks by wood-boring beetles under field conditions (Fig. 1). No attacks by wood-boring beetles occurred on any of the neighbouring uninjected controls throughout the duration of the experiment, even though they were positioned only 0.3 m from the ethanol-injected trees. As a result, a significant between-subjects main effect was detected in the number of tunnel entrances recorded on the ethanol-injected trees compared with the uninjected controls (repeated measures ANOVA; $F = 282.17$, d.f. $= 1,14$, $P < 0.0001$).

A mean $\pm$ SE of 6.4 ± 1.6 tunnel entrances were quantified 3 days after ethanol-injection of *M. virginiana*, which differed from the uninjected control trees ($F = 39.67$, d.f. $= 1,14$, $P < 0.0001$). A significant difference between the ethanol-injected and uninjected control trees was also detected 7 days ($F = 111.65$, d.f. $= 1,14$, $P < 0.0001$) and 11 days ($F = 235.08$, d.f. $= 1,14$, $P < 0.0001$) after treatment. A relatively low level of cumulative attacks (8.0 ± 1.4) by wood-boring beetles was detected 18 days after injection of *M. virginiana* trees with ethanol ($F = 359.89$, d.f. $= 1,14$, $P < 0.0001$). However, a sharp increase in the cumulative mean number of attacks on the ethanol-injected trees occurred between 19 and 25 days after treatment, which was confirmed by a significant within-subject time $\times$ treatment effect (Fig. 1; repeated measures ANOVA; $F = 192.10$, d.f. $= 4$, $P < 0.0001$). A cumulative mean $\pm$ SE of 49.4 ± 6.0 tunnel entrances were quantified on the ethanol-injected trees 25 days after treatment, which was significantly different from the uninjected control trees ($F = 1192.29$, d.f. $= 1,14$, $P < 0.0001$).

Injections of potted, living *M. virginiana* with 75 mL of 50% solutions of either acetaldehyde, acetone, ethanol or

Table 1 Mean $\pm$ SE cumulative number of ambrosia beetles caught in bottle traps baited with plant stress-related volatiles from 16 April to 17 July 2008.

	Ethanol	Methanol	Acetaldehyde	Acetone	Blank	*F*	*P*
Xylosandrus germanus	59.0 ± 27.6a	18.3 ± 14.6b	1.2 ± 0.5c	1.2 ± 0.5c	0.5 ± 0.2c	12.93	<0.0001
Hypothenemus dissimilis	7.2 ± 4.4a	0.2 ± 0.2b	0.5 ± 0.5b	0.0 ± 0.0b	0.0 ± 0.0b	11.71	<0.0001
Xyleborus sayi	3.3 ± 2.9a	0.2 ± 0.2a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	2.06	0.09
Euwallacea validus	1.0 ± 0.8a	0.7 ± 0.7a	0.7 ± 0.4a	0.0 ± 0.0a	0.2 ± 0.2a	0.66	0.73
Xyleborinus saxeseni	0.7 ± 0.7a	0.2 ± 0.2a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.82	0.66
Xyloterinus politus	0.7 ± 0.5a	0.8 ± 0.8a	0.3 ± 0.3a	1.8 ± 1.1a	0.2 ± 0.2a	1.20	0.30
Monarthrum mali	0.2 ± 0.2a	0.0 ± 0.0a	0.0 ± 0.0a	0.2 ± 0.2a	0.0 ± 0.0a	0.75	0.56

Means within rows followed by the same superscript letter are not significantly different (d.f. = 4,25 for each comparison; $\alpha = 0.05$; Tukey's honestly significant difference test; $n = 6$ traps/treatment).

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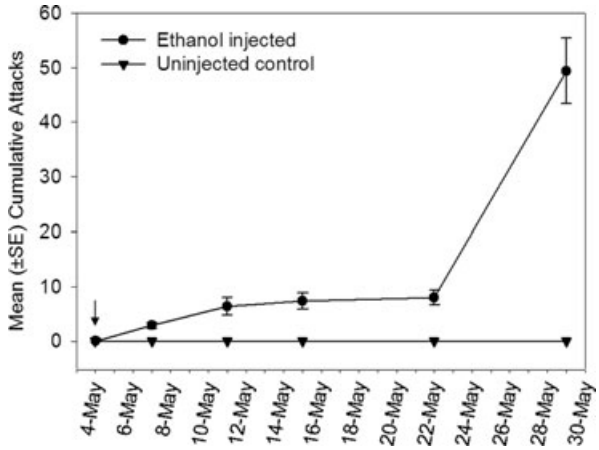

Figure 1 Mean ± SE cumulative attacks by ambrosia beetles in *Magnolia virginiana* injected with 125 mL of 50% ethanol versus an uninjected-control. Arrow indicates injection date. Cumulative attacks on the ethanol-injected trees were significantly greater than the uninjected-control trees on each sampling date, except 4 May 2008 ($n = 8$ trees per treatment; one-way analysis of variance ; $\alpha = 0.05$; Tukey's honestly significant difference test; for statistical values, see Results).

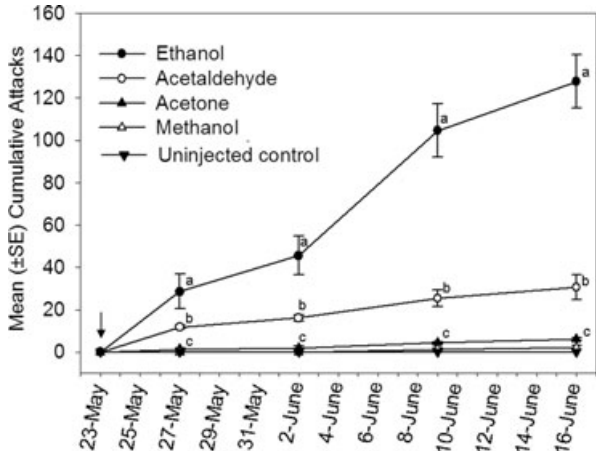

Figure 2 Mean ± SE cumulative attacks by ambrosia beetles in *Magnolia virginiana* injected with 75 mL of 50% solutions of either acetaldehyde, acetone, ethanol, methanol or an uninjected-control. Arrow indicates injection date. On each sampling date, means with the same letter are not significantly different ($n = 5$ trees per treatment; one-way analysis of variance; $\alpha = 0.05$; Tukey's honestly significant difference; for statistical values, see Results).

methanol induced variable levels of attacks by wood-boring beetles, as indicated by a significant between-subjects main effect (repeated measures ANOVA; $F = 15.45$, d.f. $= 4,7$, $P = 0.0014$; Fig. 2). A cumulative mean ± SE of 28.6 ± 8.2 tunnel entrances were detected on the ethanol-injected trees 4 days after injection, which was significantly greater than the mean number of attacks on trees injected with acetaldehyde (11.8 ± 0.9), methanol (1.4 ± 0.5) and acetone (0.0 ± 0.0) ($F = 99.28$, d.f. $= 4,20$, $P < 0.0001$). Ethanol-injected trees also had higher cumulative attacks compared with the other treatments at 10 days ($F = 118.33$, d.f. $= 4,20$, $P < 0.0001$), 17 days ($F = 124.52$, d.f. $= 4,20$, $P < 0.0001$) and 24 days ($F = 120.37$, d.f. $= 4,20$, $P < 0.0001$) after treatment. The cumulative mean ± SE number of tunnel entrances on ethanol-injected trees increased dramatically over a 24-day duration to 127.8 ± 12.6, resulting in a significant within-subject time × treatment effect ($F = 63.92$, d.f. $= 24$, $P < 0.0001$).

Although not as effective as ethanol, the injection of acetaldehyde into *M. virginiana* induced a significantly greater number of attacks than methanol or acetone 4, 10, 17 and 24 days after treatment ($F = 15.45$, d.f. $= 4, 7$, $P = 0.0014$; Fig. 2). No attempted or successful tunnel entrances were observed on any of the uninjected control trees.

Xylosandrus germanus was the predominant species of ambrosia beetle emerging from galleries created in *M. virginiana* trees after attacks were induced by injecting acetaldehyde, acetone, ethanol and methanol (Table 2). In particular, *X. germanus* represented 77.7% of the total ambrosia beetles emerging from ethanol-injected trees, followed by *M. fasciatum* (12.5%), *X. sayi* (6.7%), *X. saxeseni* (2.5%) and *Monarthrum mali* (0.4%). Similarly, *X. germanus* represented 85.1% of the specimens emerging from acetaldehyde-injected trees, followed by *M. fasciatum* (9%), *X. sayi* (4.5%) and *X. saxeseni* (1.5%). Only *X. germanus* emerged from acetone-injected and methanol-injected trees. In each case, parent and brood adults were both collected but no distinction was made between the two.

The injection of *M. virginiana* with 75 mL of 50% ethanol resulted in numerous attacks by wood-boring beetles but no attacks were observed on trees injected with 75 mL of deionized water or the uninjected controls (repeated measures ANOVA; $F = 157.08$, d.f. $= 2,15$, $P < 0.0001$; Fig. 3). A mean ± SE of 4.5 ± 1.2 tunnel entrances was detected 1 day after ethanol-injection, which differed significantly from the water-injected and uninjected control trees ($F = 43.20$, d.f. $= 2,15$, $P < 0.0001$). The cumulative number of attacks in ethanol-injected trees was also significantly greater than the water-injected and uninjected control trees 2 days ($F = 43.20$, d.f. $= 2,15$, $P < 0.0001$), 3 days ($F = 117.20$, d.f. $= 2,15$, $P < 0.0001$), 6 days ($F = 216.20$, d.f. $= 2,15$, $P < 0.0001$), 7 days ($F = 299.12$, d.f. $= 2,15$, $P < 0.0001$) and 8 days ($F = 360.77$, d.f. $= 2,15$, $P < 0.0001$) after treatment. Attacks on ethanol-injected trees steadily increased over an 8-day duration to a cumulative mean of 60.7 ± 11.6, resulting in a significant within-subject time × treatment effect ($F = 180.85$, d.f. $= 12$, $P < 0.0001$; Fig. 3).

Solvent injection was lethal to the experimental trees in all three tree injection experiments. By contrast, water-injection and drilling/plugging-only was not lethal to any of the trees, nor did it induce attacks.

Volatile profiles

Headspace SPME sampling and GC-MS were effectively used to detect and identify the emission of acetaldehyde, acetone, ethanol and methanol from *M. virginiana*. For the purposes of the present study, only these four stress-related volatiles were considered in the analysis. In addition to the trunk injections resulting in one of the four volatiles being

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Table 2 Species composition of ambrosia beetles emerging from galleries created in *Magnolia virginiana* after attacks were induced by trunk injections of stress-related volatiles.

	Total number of emerged adults[a]				
	Xylosandrus germanus	*Monarthrum fasciatum*	*Xyleborus sayi*	*Xylosandrus saxeseni*	*Monarthrum mali*
Ethanol	531	85	46	17	3
Acetaldehyde	57	6	3	1	0
Acetone	29	0	0	0	0
Methanol	8	0	0	0	0
Uninjected	0	0	0	0	0

[a]Cumulative emergence up to 50 days after branches were excised and confined to rearing containers.

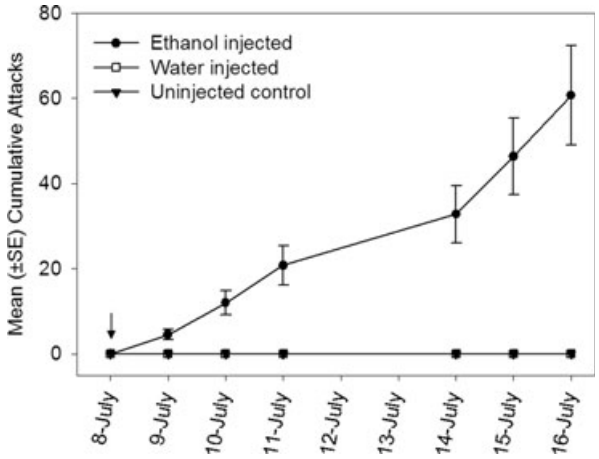

Figure 3 Mean ± SE cumulative attacks by ambrosia beetles in *Magnolia virginia* injected with 75 mL of 50% ethanol, 75 mL of deionized water, or an uninjected control. Arrow indicates injection date. Cumulative attacks on the ethanol-injected trees were significantly greater than the water-injected and uninjected-control trees on each sampling date, except 8 July 2008 ($n = 6$ trees per treatment; one-way analysis of variance; $\alpha = 0.05$; honestly significant difference; for statistical values, see Results).

detected as a major compound, minor or trace amounts of the remaining three volatiles were also detected. For example, ethanol was the most abundant of the four stress-related volatiles emitted 24 h after its injection into *M. virginiana* but minor/trace amounts of acetone, acetaldehyde and methanol were also detected. The same phenomenon was documented for acetaldehyde and acetone injected trees but acetone was not detected from methanol injected trees (Fig. 4). Trace amounts of methanol were detected from the uninjected control trees but acetaldehyde, acetone and ethanol were not detected.

Discussion

The results obtained in the present study provide the first demonstration that injecting trees with stress-related volatiles, particularly ethanol, reliably induces attacks by natural populations of *X. germanus* under field conditions. Baiting trees with ethanol lures has proved unreliable for inducing attacks by *X. germanus* (C. M. Ranger, personal observation), which subsequently led to the evaluation of injection treatments. Using trunk injections to induce attacks by ambrosia beetles on select trees may prove to be a useful strategy for detection and mass trapping, particularly for *X. germanus*. For example, apple (*Malus* spp.) trees baited with lures dispensing the host volatile benzaldehyde and the pheromone grandisoic acid act as sentinel trees for the plum curculio, *Conotrachelus nenuphar* (Herbst), which improves the proper timing and reduces the incidence of insecticide applications (Prokopy *et al*., 2004; Leskey *et al*., 2008). At the very least, reliably inducing attacks by *X. germanus* and other ambrosia beetles on select experimental trees will provide an opportunity to evaluate conventional, reduced-risk and botanical insecticides. However, the intensity of attacks is probably dependent in part on population density, which may vary within and between years and localities.

Injections of ethanol solutions into *M. virginiana* induced the heaviest attacks and *X. germanus* was the most abundant species emerging from this treatment, followed by *M. fasciatum*, *X. sayi*, *X. saxeseni* and *M. mali*, respectively. Each of these ambrosia beetles is considered to be a secondary pest that prefers stressed, injured or dying deciduous trees (Solomon, 1995). Acetaldehyde induced fewer ambrosia beetle attacks than ethanol, whereas the injection of methanol and acetone only induced a minimal number of attacks. Wounding *M. virginiana* trees by drilling and subsequently plugging with an injection port did not induce ambrosia beetle attacks, nor did injecting trees with water.

Xylosandrus germanus was the only ambrosia beetle that emerged from acetone and methanol injected trees, which may prove useful for selectively inducing attacks by this particular species. Because none of the uninjected or water-injected *M. virginiana* control trees were attacked during any of the tree-injection experiments, an olfactory-based trap tree strategy may ultimately be useful for reducing ambrosia beetle attacks on valuable nursery stock. Conceivably, trap trees could become a component of a push–pull strategy (Pyke *et al*., 1987; Miller & Cowles, 1990; Cook *et al*., 2007), whereby repellents/deterrents push host seeking ambrosia beetles away from vulnerable nursery stock and injections of stress-related volatiles pull beetles into neighbouring sacrificial trap trees. The effectiveness of repellents under field conditions may actually improve by offering insects a choice between attractive host-plant odours and host-masking repellents (Van Tol *et al*., 2006). Studies are currently underway aiming to identify promising

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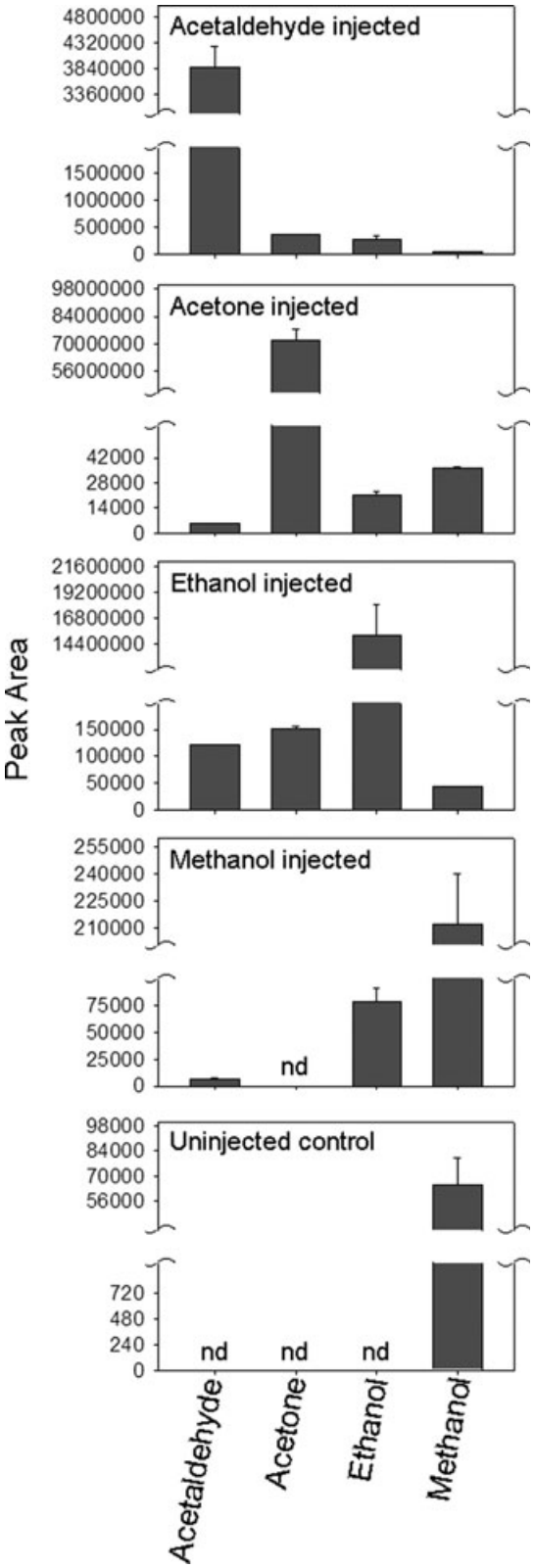

Figure 4 Mean ± SE emission of acetaldehyde, acetone, ethanol, and methanol 24 h after their injection into *Magnolia virginiana*, along with their emission from uninjected control trees ($n = 3$ trees per treatment); nd, not detected.

repellent formulations against *X. germanus* and other nursery-infesting ambrosia beetles to be used as part of a push–pull strategy (C. M. Ranger, unpublished data). Aggregation and antiaggregation pheromones have shown promise as part of a push–pull strategy for Scolytinae pests of forest crops, including *Dendroctonus pseudotsugae* (Ross & Daterman, 1994) and *Dendroctonus ponderosae* (Lindgren & Borden, 1993). Push–pull strategies have considerable potential in horticulture as a result of the associated production areas and high crop value but only a few systems are being pursued for development (Cook *et al*., 2007).

Similar to the tree injection experiments, ethanol was the most effective volatile for attracting *X. germanus* and *H. dissimilis* to baited bottle traps. Methanol was slightly attractive to *X. germanus*, whereas acetaldehyde and acetone were both inactive as lures in bottle traps for ambrosia beetles. During olfactometer bioassays, Moeck (1970) also found that ethanol and methanol were highly and slightly attractive to the ambrosia beetle *Trypodendron lineatum* (Olivier), respectively, whereas acetaldehyde was inactive. Similarly, Montgomery and Wargo (1983) found that traps baited with ethanol were attractive to the Scolytinae but that acetaldehyde and acetone exhibited little or no attractiveness. Mixtures of ethanol, methanol and acetaldehyde were not synergistic with ethanol (Montgomery & Wargo, 1983). In general, the proportion of ambrosia beetle species caught in baited traps was similar to that of the same species emerging from infested trees, which indicates that bottle traps provide insight into ambrosia beetle reproductive success.

MacDonald and Kimmerer (1993) reported that ethanol emission occurred after its uptake by excised leaves of poplar (*Populus* sp.). Similarly, SPME-GC-MS confirmed that individual compounds injected into *M. virginiana* were subsequently the most abundant of the four stress-related volatiles measured 24 h after their injection. Emission of minor or trace amounts of the remaining three volatiles may have resulted from metabolic and/or degradative processes. For example, the emission of ethanol by trees injected with acetaldehyde, as confirmed by SPME headspace sampling, may have resulted from metabolism of acetaldehyde via alcohol dehydrogenase. Because acetaldehyde was inactive as a lure in bottle traps, attacks induced by its injection into *M. virginiana* are probably attributed to some of the acetaldehyde being converted into ethanol. Minor to trace amounts of ethanol emitted from trees injected with acetone and methanol also probably contributed to attacks by *X. germanus* and other ambrosia beetles.

The difference in attacks induced by injecting each of the stress-related volatiles into *M. virginiana* indicates that *X. germanus* has an efficient mechanism for olfactory orientation and assessment. Attacks by *X. germanus* on *M. virginiana* trees injected with stress-related volatiles indicate positive flight orientation to a specific point source based on olfactory cues (i.e. primary attraction). Saint-Germain *et al*. (2007) concluded that primary attraction functioned at a relatively large scale by pulling Scolytinae into patches containing an attractant-baited tree but random landing on adjacent unbaited trees occurred at a finer spatial scale. However, it remains unclear whether *X. germanus* and other ambrosia beetles chose not to land on the control trees as a result of an absence of stress-related cues, or whether the trees were rejected for colonization after landing.

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Xylosandrus germanus has traditionally been viewed as a pest of physiologically-stressed hosts (Hoffman, 1941; Maksymov, 1987; Bruge, 1995), although a few studies have indicated that apparently-healthy trees are also colonized (Weber, 1982; Grégoire *et al*., 2001). However, referring to a tree as being 'apparently healthy' may be a misnomer. Trees experiencing a certain degree of physiological stress may appear to be healthy but still emit stress-related volatiles signalling their quality to host-seeking ambrosia beetles. Kühnholz *et al*. (2001) noted that a complex chemical ecology may allow for secondary ambrosia beetles to perceive stress-related volatiles produced by otherwise apparently-healthy trees. Thus, anthropologically speaking, trees viewed as being apparently healthy may actually be inapparently stressed. Ethanol is not a normal product of plants growing under healthy, aerobic conditions (Kimmerer & Kozlowski, 1982).

As an example, apparently-healthy black walnut (*Juglans nigra* L.) that were subsequently colonized by *X. germanus* exhibited slower growth rates in the season prior to attack (Weber & McPherson, 1984). Similarly, pre-existing physiological stress may have predisposed apparently-healthy beech (*Fagus sylvatica* L.) trees to attack by *X. germanus* and other ambrosia beetles (Nageleisen, 1994; Grégoire *et al*., 2001, Henin & Versteirt, 2004). The colonization of *M. virginiana* trees injected with stress-related volatiles but an absence of attacks on adjacent control trees provides a further indication that *X. germanus* has an efficient mechanism for assessing and differentiating host quality. Weber and McPherson (1984) also considered that *X. germanus* could differentiate between even slight differences in tree vigour.

In conclusion, the present study demonstrates that injection of *M. virginiana* with stress-related volatiles is a reliable procedure for inducing attacks by *X. germanus* and other ambrosia beetles. Because the procedure has potential to become a component of a push–pull strategy for *X. germanus*, additional studies are therefore warranted to assess the efficacy of such an approach for ornamental nurseries. For example, the ability of ethanol injected trees to remove ambrosia beetles from the nursery agroecosystem needs to be compared with ethanol-baited traps. Injecting trees with ethanol is certainly more laborious than assembling an ethanol-baited trap, although recent studies have determined that ethanol-injected *M. virginiana* bolts hung in bottle traps attracted more *X. germanus* than ethanol lures (C. M. Ranger, unpublished data). Additional studies are also warranted aiming to identify the optimum blend of volatile organic compounds for inducing attacks by *X. germanus.*

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