Ambrosia Beetle (Coleoptera: Curculionidae) Responses to Volatile Emissions Associated With Ethanol-Injected Magnolia virginiana

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Environ. Entomol. 41(3): 636–647 (2012); DOI: http://dx.doi.org/10.1603/EN11299

ABSTRACT  Xylosandrus germanus (Blandford) and other species of ambrosia beetles are key pests of ornamental nursery trees. A variety of laboratory- and field-based experiments were conducted in pursuit of improved monitoring strategies and to develop a trap tree strategy for ambrosia beetles. Traps baited with bolts prepared from Magnolia virginiana L. injected with ethanol caught five times more X. germanus than ethanol-baited traps. Basal stem injections of ethanol into M. virginiana induced more ambrosia beetle attacks than irrigating or baiting with ethanol, and no attacks occurred on water-injected trees. A positive correlation was also detected between concentration of injected ethanol and cumulative attacks. Solid phase microextraction-gas chromatography-mass spectrometry characterized bark emissions from ethanol- and water-injected M. virginiana at 1, 2, 10, and 16 d after treatment. Ethanol emission from injected trees steadily declined from 1 to 16 d after treatment, but was not emitted from water-injected trees. A variety of monoterpenes were also emitted in trace amounts from the ethanol- and water-injected trees. Antennal responses of X. germanus via gas chromatography-electroantennographic detection to volatiles from ethanol-injected M. virginiana occurred for ethanol, but not the various monoterpenes. X. germanus and other ambrosia beetles were also equally attracted to traps baited with ethanol alone compared with a synthetic mixture of ethanol plus various monoterpenes formulated to mimic ethanol-injected M. virginiana. Injecting concentrated solutions of ethanol into trees may be useful for establishing odor-based trap trees, which could aid with monitoring programs and/or potentially deflect ambrosia beetles away from valuable nursery stock.

KEY WORDS  ambrosia beetles, Xylosandrus germanus, ethanol, solid phase microextraction
into bolts of elm (*Ulmus* sp.). Basal stem injections of ethanol into living trees also induced ambrosia beetle attacks under field conditions, and *X. germanus* was the most predominant species emerging from ethanol-injected trees in Ohio (Ranger et al. 2010). Injecting ethanol into *M. virginiana* also induced more attacks than other low molecular weight volatiles known to be emitted in response to physiological stress, namely, acetaldehyde, acetone, ethyl acetate, and methanol (Kimmerer and MacDonald 1987, Kimmerer and Kozlowski 1982, Ranger et al. 2010). Using the ethanol-injection technique to ensure ambrosia beetle pressure on experimental trees subsequently made it possible to assess the efficacy of botanical insecticides under field conditions in Ohio (Ranger et al. 2011b). The technique also allowed for determining the effectiveness of conventional insecticides against *Xylosandrus crassiusculus* (Motschulsky) and other ambrosia beetles in North Carolina, along with providing insight into secondary pest outbreaks resulting from the preventative insecticide applications (Frank and Sadof 2011). Field-based efficacy data for ambrosia beetles was previously difficult to obtain because of the inability to ensure pressure on treated and control trees (Mizell and Riddle 2004).

Inducing ambrosia beetle attacks on specific trees could aid with monitoring flight and colonization activity for properly timing insecticide applications within ornamental nurseries. Attacks by wood-boring beetles other than the Scolytinae have not been observed to occur as a result of ethanol-injection into living trees (Ranger et al. 2010). In addition, un.injected trees adjacent to ethanol-injected trees are not attacked by *X. germanus* or other ambrosia beetles (Ranger et al. 2010). Thus, strategic placement of ethanol-injected trees may also be useful for intercepting ambrosia beetles and deflecting them away from valuable nursery stock, particularly as part of a 'push-pull' management strategy (Pyke et al. 1987, Miller and Cowles 1990).

As part of our current study, a variety of laboratory- and field-based studies were conducted to aid in monitoring and developing a trap tree strategy for ambrosia beetles. Our objectives were to: 1) assess the attractiveness of traps baited with bolts from ethanol-injected *M. virginiana*; 2) compare injecting, irrigating, or baiting *M. virginiana* with ethanol for inducing attacks on specific trees; 3) examine the relationship between concentration of injected ethanol and attacks; 4) characterize volatile emissions from ethanol-injected trees and the corresponding antennal response by *X. germanus*; and 5) determine the attractiveness of a complex mixture of ethanol and monoterpenes based on emissions from ethanol-injected trees.

**Materials and Methods**

**Bolt Attractiveness.** To aid with developing improved detection and monitoring tactics, the attractiveness of bolts from ethanol-injected *M. virginiana* were compared with an ethanol lure. Based on Ranger et al. (2010), an Arborjet Tree I.V. system (Woburn, MA) was used to inject *M. virginiana* with 75 ml of 90% ethanol or water. Trees were ≈4 yr old, 1 m tall, and growing in 13 liter containers with a mixture of aged pine bark, peat, and coarse sand (60:30:10 volvol). Injection sites were initiated by drilling a single 9.5 mm hole ≈16 mm deep into the base of the trees, which had a base caliper of 40–50 mm. The hole was immediately plugged with an Arborjet injection port (9.5 mm diameter) and 75 ml of 90% aqueous ethanol solutions were injected at a delivery pressure of 4.14 bar (60 psi).

Stem sections of 10 cm in length were subsequently taken 24 h after injection at 5 cm above the injection port from the ethanol- or water-injected trees. A single bolt from either ethanol- or water-injected trees was then positioned and secured vertically within bottle traps prepared according to Ranger et al. (2010) and pictured in Reding et al. (2011). In short, a Tornado Tube (Steve Spangler Science, CO) was used to connect the mouths of a 1 liter plastic bottle and a 0.5 liter plastic bottle. Two rectangular openings ≈12.5 cm long × 6 cm wide were cut into the sides of the 1 liter bottle to allow the entrance of ambrosia beetles. Individual bolts were hung vertically within the 1 liter bottle. Additional traps were also baited with an individual heat-sealed, permeable membrane pouch filled with 10 ml of 95% ethanol (65 mg/d at 30°C; AgBio, Westminster, CO). Empty traps served as a blank control. The 0.5 liter collecting bottle was filled with propylene glycol as a killing and preserving agent. Traps were positioned 0.6 m above ground level by attaching the inverted end of the 1 liter bottle to a metal rod. Traps were arranged in a randomized complete block design along the edge of a woodlot at the Ohio Agricultural Research and Development Center (OARDC), Wooster, OH (Wayne Co.), with 6 m between traps and 15 m between blocks. In total, eight complete blocks were used, thus eight replicates for each treatment. Traps were held under field conditions from 4 May 2009 through 18 May 2009.

**Inducing Attacks on Specific Trees.** To aid in developing an odor-based trap tree for detecting, monitoring, and potentially mass trapping purposes, the effectiveness of injecting, irrigating, or baiting with ethanol for inducing ambrosia beetle attacks on specific trees was compared. The aforementioned techniques were used to inject potted *M. virginiana* with 75 ml of 90% ethanol or 75 ml of deionized/distilled water. Trees were also irrigated with 75 ml of 90% ethanol, or baited with one of the previously described ethanol lures at 40–50 cm above the tree base.

Immediately after injection, experimental trees were arranged in randomized complete blocks along the edge of a service road passing through a woodlot at the OARDC. Trees within each block were 3 m apart and replicated blocks were separated by 6 m. In total, five complete blocks were used, thus five replicates for each treatment. Trees were injected, irrigated, or baited on 21 July 2009 and maintained under field conditions until 6 August 2009 during which time they were thoroughly examined for new galleries at 1, 4, 7, 10, 13, and 16 d after treatment (DAT). Gallery
entrances were circled with a waterproof marker (Sharpie, Oak Brook, IL) to quantify attacks at each time point and subsequently totaled to calculate cumulative attacks.

**Concentration Response.** To optimize the attractiveness of ethanol-injected trees, the association between concentration of injected ethanol and ambrosia beetle attacks was examined. Potted *M. virginiana* were injected with 75 ml of 90, 45, 22.5, 11.25, or 5.6% solutions of ethanol based on the aforementioned techniques. Control trees were injected with 75 ml of water. Immediately after injection, trees were arranged in randomized complete blocks along the edge of a service road passing through a woodlot at the OARDC. Trees within each block were 3 m apart and replicated blocks were separated by 6 m. In total, five complete blocks were used, thus five replicates for each treatment. Trees were injected on 7 May 2009 and maintained under field conditions until 28 May 2009 during which time they were thoroughly examined for new galleries at 1, 3, 7, 14, and 21 DAT.

**Volatile Emissions.** The time-course emission of volatiles from *M. virginiana* at 1, 2, 10, and 16 DAT were assessed using trees injected with 75 ml of 90% ethanol or water. Four ethanol-injected and water-injected trees were examined while being maintained in a greenhouse under natural light conditions. Solid phase microextraction (SPME) was used to sample volatiles emanating from lower woody stem regions, which typically sustains the largest number of ambrosia beetle attacks (C. M. Ranger, personal observation). Volatile sampling chambers (6 × 1 × 7 cm; 1 × w × h) with an internal volume of 3 cm³ (Fig. 1A, B) were constructed by first using a razor blade to longitudinally slice a section of polytetrafluoroethylene (PTFE) tubing in half (10 mm internal diameter; 2 mm tubing thickness; Cole Parmer, Vernon Hills, IL). An 11 mm diameter circular membrane (Molded Thermogreen LB-2 Septa; Supelco, Bellefonte, PA), cut in half to form a semicircle, was then nestled within each end of the PTFE tubing. A 6 cm piece of PTFE microbore tubing (0.568 mm inner diameter × 1.07 mm outer diameter, Cole Parmer) was also fitted to pass through one of the septa, which would later allow for a SPME syringe to be inserted into the chamber (Fig. 1A). The free end of the microbore tubing that extended outside of the chamber was temporarily sealed by folding multiple times and compressing with a micro cable tie to prevent the loss of volatiles during equilibration. Cables ties were used to snugly secure the PTFE chamber against the stem in parallel, thereby confining 6 cm² of bark tissue. The particularly smooth bark associated with *M. virginiana* allowed for close contact between the tissue and sampling chamber, which effectively sealed the chamber from passive air flow. The lower end of the chambers were positioned =12–15 cm above and in-line with the injection port.

Chambers were held in place for 30 min to allow for volatile equilibration. After sufficient equilibration, the sealed microbore tubing extending outside of the chamber was cut within 0.5 cm of the septum and a syringe containing a retracted SPME fiber was immediately inserted through the microbore tubing and into the sealed chamber. Cable ties were used to secure the syringe holder against the stem in parallel, and then the SPME fiber was extruded from the syringe tip and exposed for 10 min within the chamber (Fig. 1B). The fiber was retracted into the syringe after sampling and immediately capped with a sealed section of PTFE microbore tubing to prevent contamination. Fibers were then wrapped in a Telfon bag and briefly held at −40°C until analysis. A fiber coating of divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS; 50/30 μm coating; Sigma-Aldrich, St. Louis, MO) was used, which is ideal for a range of volatile and semivolatile compounds (MW 40–275) (Sigma-Aldrich 2011).

Volatiles were thermally desorbed by exposing the fiber within the injection port of a gas chromatograph (Varian CP-3800) equipped with a Merlin Microseal septum system (Sigma-Aldrich). The injection port consisted of a deactivated glass liner with a 0.75 mm ID and a single tapered end specific for SPME applications. Fibers were held in the injection port for 2 min at 250°C, which was operated in splitless mode from 0 to 2.5 min and then split at a ratio of 1:20 for the remainder of the run. A capillary nonpolar DB-5 column (0.25 mm × 30 m × 0.25 μm; cross-linked/surface bonded 5% phenyl, 95% methylpolysiloxane; Agilent J&W, Santa Clara, CA) was used for analysis according to the following program: 40–150°C at 3°C/min and 150–246°C at 15°C/min. A ramp of 15°C/min was used after reaching 150°C because of the consistent absence of host-derived volatiles detected above this temperature. Helium was used as a carrier gas at 1 ml/min. A Varian 2200 mass spectral detector was operated in electron impact mode with a scan range of 14–415 m/z. System control was accomplished with Star Chromatography Workstation software (Star Toolbar, version 6.8; Varian, Palo Alto, CA). Fibers were conditioned before each analysis by exposure within the injection port for 20 min at 250°C.
All identifications were confirmed by comparing mass spectral fragmentation patterns and retention times with authentic standards, along with referencing Adams (2007) and the National Institute of Standards and Technology (NIST) MS database. For determining relative quantities, serial dilutions of ethanol (99.5% purity, Acros Organics, Geel, Belgium) ranging from 100 to 0.0001 g/L were made in water. Serial dilutions (100 to 0.0001 g/L) of the following monoterpenes were also prepared: (-)\text{-}\alpha\text{-}pinene (\geq 99.0\% ; Sigma-Aldrich), (+)\text{-}\alpha\text{-}pinene (\geq 99.0\% ; Sigma-Aldrich), (+)\text{-}\beta\text{-}pinene (\geq 99.0\% ; Sigma-Aldrich), (-)\text{-}\beta\text{-}pinene (\geq 99.0\% ; Sigma-Aldrich), (-)\text{-}\beta\text{-}pinene (\geq 98.5\% ; Sigma-Aldrich), myrcene (\geq 95.0\% ; Sigma-Aldrich), \rho\text{-}cymene (\geq 99.5\% ; Sigma-Aldrich), R- (+)\text{-}limonene (\geq 99.0\% ; Sigma-Aldrich), and eucalyptol (\geq 99.0\% ; Sigma-Aldrich). Monoterpenes standards were first diluted in ethanol because of their insolubility in water, but subsequent serial dilutions were made in water. Standards of various concentrations were analyzed by first using cable ties to loosely secure the previously described volatile sampling chamber in parallel with a glass test tube (2.5 cm \text{ diameter}). A 20 \mu L aliquot containing a known concentration of an individual compound was then applied to a piece of filter paper (2 \times 1 cm; 1 \times w) positioned within the volatile sampling chamber. The cable ties were then immediately pulled tight to effectively seal the chamber. SPME fibers were exposed for 10 min within the sampling chamber according to the previously described methods and analyzed immediately afterwards.

Peak areas associated with standard compounds were measured using the Star Chromatography Workstation software. Standard concentration curves were then calculated and used to determine the relative quantities of individual compounds associated with volatile emissions from \textit{M. virginiana}. Under the proper conditions, SPME can be used for comparing the relative quantities of an individual compound in different samples (Bartelt 1997, Romeo 2009). However, because of differences in affinities of SPME fibers for different compounds, relative quantitation by SPME does not allow one to compare the quantities of different compounds within the same sample (Bartelt 1997, Romeo 2009). Absolute quantitation of different compounds within the same sample can be achieved using SPME (Bartelt 1997, Romeo 2009), but was not conducted as part of our current study because of the lack of an internal standard during initial sampling. Thus, the relative quantities of individual compounds emitted from \textit{M. virginiana} over several points in time were only compared within (i.e., ethanol-injected or water-injected) and between (e.g., ethanol-injected versus water-injected) samples.

Electrophysiological Response. Gas chromatography-electroantennographic detection (GC-EAD) was used to assess the antennal response of 10 adult female \textit{X. germanus} to volatile emissions from ethanol-injected \textit{M. virginiana} at 2 DAT. Adult females were reared from maple bolts previously infested with \textit{X. germanus} that were confined to plastic containers with moist paper towels. Volatiles were collected from the bark of \textit{M. virginiana} using SPME according to the aforementioned procedures. The GC-EAD system was comprised of a Varian CP-3800 gas chromatograph equipped with a flame ionization detector (FID) and an electroantennographic detector (EAD) system (Syntech, Hilversum, The Netherlands). The GC was operated in splitless mode with the aforementioned column and program. Effluent from the column was split 1:1 between the FID and EAD. Nitrogen was used as a makeup gas (30 ml/min) at the junction of the splitter (SGE Analytical Science, Austin, TX). Deactivated, fused silica tubing (0.32 mm diameter) delivered half of the effluent to the FID while the other half passed through a heated transfer line maintained at 246°C. The terminal column end for the EAD extended 0.5 mm into a stainless steel odor delivery tube that was positioned 11 cm upwind from the antennal preparation. Humidified, charcoal filtered air passed through the odor delivery tube at 150 ml/min and over the antennal preparation at \approx 2.5 cm/s. Antennal preparations consisted of a glass pipette with an Ag/AgCl indifferent electrode containing Beadle-Ephrussi saline that was inserted into the foramen of \textit{X. germanus}’ head. The saline-filled Ag/AgCl recording electrode was micromanipulated to superficially puncture the center of the left antennal club as it was braced against the head.

Recording electrode signals were amplified by a high impedance probe and further amplified, filtered, and optimized using a two-channel data acquisition controller (IDAC-2; Syntech). Data were processed using GC-EAD 2010 software (Syntech). Amplitudes were calculated for all EAD peaks that occurred within a 15 min window that encompassed all of the compounds identified from ethanol-injected \textit{M. virginiana}. Spikes were only characterized as antennal responses if they occurred in at least 4 of the 10 runs.

Lure Formulation. Amounts of the following standards at the aforementioned purities were used to prepare a synthetic mixture of ethanol and selected monoterpenes approximating ratios associated with ethanol-injected \textit{M. virginiana} at 2 DAT: ethanol (118.35 g), (-)\text{-}\alpha\text{-}pinene (22.31 mg), (+)\text{-}\alpha\text{-}pinene (22.31 mg), (+)\text{-}\beta\text{-}pinene (43.78 mg), (-)\text{-}\beta\text{-}pinene (22.67 mg), (+)\text{-}\beta\text{-}pinene (22.67 mg), myrcene (15.88 mg), \rho\text{-}cymene (44.56 mg), limonene (43.74 mg), and eucalyptol (239.85 mg). A volume of paraffin oil comparable to the total volume of monoterpenes was added to ethanol as a control formulation. Test mixtures were then loaded into separate Pher-Emit dispensers (15 ml volume; Med-E-Cell, San Diego, CA) manufactured with a proprietary release mechanism of 394 mg/d at 23°C for ethanol.

Attractiveness of the test mixtures in the Pher-Emit dispensers was assessed using the aforementioned traps. Traps were arranged in completely randomized blocks along the edge of a woodlot in Wayne Co., OH, with 6 m between traps and 15 m between blocks. In total, eight blocks were used, thus eight replicates for each treatment. Field trapping was conducted from 12 May 2011 through 2 June 2011.
Statistics. One-way analysis of variance (ANOVA) and Duncan multiple range test (MRT) at $\alpha = 0.05$ were used to compare total ambrosia beetle counts in field trapping studies (SAS Institute 2001). Data sets involving cumulative ambrosia beetle attacks over time were first analyzed by repeated measures ANOVA to test for within-subject and between-subject treatment $\times$ time effects (SAS Institute 2001). One-way ANOVA and Duncan’s MRT at $\alpha = 0.05$ were subsequently used to compare means at specific time points when significant ($P < 0.05$) within-subject or between-subject treatment $\times$ time effects were detected. Pearson’s correlation coefficient analysis was also used to determine the degree of correlation between cumulative ambrosia beetle attacks and DAT (SAS Institute 2001). Pearson’s correlation coefficients characterize the relationship between two independent variables and range from an absolute negative correlation of $-1$ to an absolute positive correlation of $1$ (SAS Institute 2001). The general linear models procedure (SAS Institute 2001) was also used to first compare the overall equality of slopes associated with cumulative attacks and DAT. The general linear models procedure and predetermined contrast statements were then used to make specific comparisons among and between slopes (SAS Institute 2001).

The time-course emission of an individual compound within and between treatments was also examined using repeated measures ANOVA. One-way ANOVA and Duncan’s MRT were subsequently used to compare the relative concentrations of an individual volatile within a particular treatment when a significant within-subject treatment $\times$ time effect was detected. Pearson’s correlation coefficient analysis was also used to examine the correlation between emission of an individual volatile compound and DAT. When significant between-subject treatment $\times$ time effects were detected for an individual compound, relative concentrations associated with ethanol-injected and water-injected trees were compared at each time point using an unpaired $t$-test ($\alpha = 0.05$; SAS Institute 2001). In all cases, data were square root transformed before analysis, but untransformed data are presented.

Results

Bolt Attractiveness. A mean ($\pm SE$) of 23.88 ± 2.38 $X. \textit{germanus}$ were collected in traps baited with an individual bolt from ethanol-injected $M. \textit{virginiana}$, which was significantly higher compared with 4.75 ± 1.16 specimens collected from traps baited with an ethanol lure, 0.13 ± 0.13 from traps baited a bolt from a water-injected tree, and 0.0 ± 0.0 for the blank trap ($F = 50.98$; df = 3, 28; $P < 0.0001$). The ethanol lure was more attractive to $X. \textit{germanus}$ than the water-injected bolt and blank control, but significant differences did not occur between these latter two treatments.

Inducing Attacks on Specific Trees. The ability to induce ambrosia beetle attacks by injecting, irrigating, or baiting $M. \textit{virginiana}$ with ethanol, or baiting with an ethanol lure. Control trees were injected with water. Different letters indicate significant differences 16 DAT (Duncan’s MRT; $\alpha = 0.05$; see Results section for statistical analyses).

![Fig. 2. Cumulative mean ($\pm SE$) number of ambrosia beetle attacks associated with injecting or irrigating $M. \textit{virginiana}$ with ethanol, or baiting with an ethanol lure. Control trees were injected with water. Different letters indicate significant differences 16 DAT (Duncan’s MRT; $\alpha = 0.05$; see Results section for statistical analyses).](image-url)
between 1 DAT (0.0 ± 0.0) and 4 DAT (1.8 ± 1.0), which were both significantly lower than the remaining dates, but differences were not detected among 7 DAT (4.3 ± 1.4), 10 DAT (5.3 ± 1.3), 13 DAT (5.5 ± 1.4), and 16 DAT (6.3 ± 1.9) (F = 11.76; df = 5, 24; P < 0.0001) (Fig. 2). A positive correlation was detected between cumulative attacks and DAT for trees baited with ethanol (r = 0.76; P < 0.0001). Water-injected trees were not attacked throughout the duration of the experiment (Fig. 2).

Concentration Response. An association between concentration of injected ethanol ranging from 0 to 90% and cumulative ambrosia beetle attacks was assessed under field conditions (Fig. 3). A mean ± SE of 82.8 ± 11.0 cumulative attacks were associated with trees injected with 90% ethanol at 21 DAT, which was significantly higher compared with 11.2 ± 5.5 attacks on trees injected with 5.6% ethanol, 19.4 ± 8.2 attacks on trees injected with 11.25%, 46.8 ± 15.52 attacks on trees injected with 22.5%, and 50.8 ± 14.6 attacks on trees injected with 45% (F = 8.40; df = 5, 24; P < 0.0001) (Fig. 3). Significant differences were also detected in the overall equality of slopes associated with cumulative attacks and DAT for trees injected with a range of 0 to 90% ethanol (F = 11.44; df = 5; P < 0.0001). Subsequent specific contrasts determined the slope associated with cumulative attacks and DAT for trees injected with 90% ethanol (0.34) was significantly larger compared with trees injected with 45% (0.28), 22.5% (0.22), 11.25% (0.12), 5.6% (0.07), and 0% (0.0) (F = 23.02; df = 1; P < 0.0001). Pearson’s correlation coefficient analysis also detected a positive correlation between concentration of injected ethanol and corresponding cumulative attacks at 21 DAT (r = 0.76; P < 0.0001).

Repeated measures ANOVA detected a significant within-subject treatment × time effect (F = 7.44; df = 20; P < 0.0001). In particular, significant differences in mean (±SE) cumulative attacks were detected among trees injected with 90% ethanol at 1 DAT (4.8 ± 1.2), 3 DAT (15.2 ± 2.8), 7 DAT (46.4 ± 2.8), and 14 DAT (73.6 ± 8.9), but differences were not detected between 14 DAT and 21 DAT (82.8 ± 11.0) (F = 43.12; df = 4, 20; P < 0.0001) (Fig. 3). Pearson’s correlation coefficient analysis detected a positive correlation between cumulative ambrosia beetle attacks and DAT for trees injected with 90% ethanol (r = 0.87; P < 0.0001).

The mean (±SE) cumulative number of attacks occurring on trees injected with 45% ethanol were not significantly different between 1 DAT (2.0 ± 0.7) and 3 DAT (6.8 ± 2.4), but they were significantly lower compared with 7 DAT (25.8 ± 5.3), 14 DAT (44.0 ± 11.4), and 21 DAT (50.8 ± 11.6) (F = 12.27; df = 4, 20; P < 0.0001). However, no significant difference in cumulative attacks were detected among 7, 14, and 21 DAT. Pearson’s correlation coefficient analysis detected a positive correlation between cumulative ambrosia beetle attacks and DAT for trees injected with 45% ethanol (r = 0.78; P < 0.0001).

No significant difference in cumulative attacks occurred for trees injected with 22.5% ethanol between 1 DAT (3.4 ± 0.8) and 3 DAT (8.2 ± 0.9) (F = 6.65; df = 4, 20; P = 0.0014) (Fig. 3). A significant difference was also not detected between 3 DAT and 7 DAT (26.0 ± 5.9), or among 7 DAT, 14 DAT (39.6 ± 12.5), and 21 DAT (46.8 ± 15.5) Pearson’s correlation coefficient analysis detected a positive correlation between cumulative ambrosia beetle attacks and DAT for trees injected with 22.5% ethanol (r = 0.70; P = 0.0001).

No significant difference in cumulative attacks occurred for trees injected with 11.25% between 1 DAT (2.6 ± 0.9) and 3 DAT (6.4 ± 0.9), or among 3 DAT, 7 DAT (15.0 ± 4.9), 14 DAT (18.6 ± 7.6), and 21 DAT (19.4 ± 8.2) (F = 3.23; df = 4, 20; P = 0.034) (Fig. 3). A positive correlation between cumulative ambrosia beetle attacks and DAT was also detected for trees injected with 11.25% ethanol (r = 0.53; P = 0.007).

No significant difference in cumulative attacks occurred for trees injected with 5.6% ethanol among 1 DAT (1.6 ± 0.7), 3 DAT (4.2 ± 1.2), 7 DAT (8.6 ± 3.6), 14 DAT (10.0 ± 4.5), and 21 DAT (11.2 ± 5.5) (F = 1.52; df = 4, 20; P = 0.234) (Fig. 3). A positive correlation between cumulative ambrosia beetle attacks and DAT was detected for trees injected with 5.6% ethanol (r = 0.40; P = 0.045). No attacks occurred on trees injected with water throughout the duration of the experiment (Fig. 3).

Volatile Emissions. SPME-GC-MS was used to characterize the time-course emission of volatiles from ethanol-injected and water-injected M. virginiana at 1, 2, 10, and 16 DAT (Fig. 4; Table 1). Ethanol was detected in emissions from ethanol-injected trees at each time point, but was not associated with water-injected trees (Table 1; Fig. 4). A variety of monoterpenes were also associated with the ethanol-injected and water-injected trees, namely, α-pinene, (+)-β-pinene, (-)β-pinene, myrcene, p-cymene, limonene, and eucalyptol (Fig. 4; Table 1). Repeated measures ANOVA detected significant between-subject time × volatile effects between ethanol-injected and water-injected trees for ethanol (F = 56.94; df = 1, 6; P = 0.0003), α-pinene (F = 23.64; df =
Fig. 4. Representative chromatograms of volatile emissions from ethanol-injected (EI) and water-injected (WI) *M. virginiana* at 1, 2, 10, and 16 DAT. Peak numbers correspond to: (1) ethanol, (2) /H9251-pinene, (3) /H11001-camphene, (4) (-)-/H9252-pinene, (5) (+)-/H11001-pinene, (6) myrcene, (7) /H9267-cymene, (8) limonene, and (9) eucalyptol (see Tables 1 and 2).

Table 1. Time-course emission of volatile compounds from EI and WI *M. virginiana*

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>EI</th>
<th>WI</th>
<th>EI</th>
<th>WI</th>
<th>EI</th>
<th>WI</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>10</td>
<td>16</td>
<td>Mean (±SE) relative quantities (ng/chamber) <em>a</em></td>
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</tr>
<tr>
<td>1</td>
<td>Ethanol</td>
<td>2.96 × 10³ ± 7.2 × 10²</td>
<td>6.71 × 10³ ± 2.37 × 10³</td>
<td>9.26 × 10⁴ ± 3.64 × 10⁵</td>
<td>1.02 × 10⁶ ± 7.02 × 10⁵</td>
<td>17.10, 0.0001</td>
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<tr>
<td>2</td>
<td>α-pinene</td>
<td>276.48 ± 109.66a</td>
<td>481.33 ± 260.96a</td>
<td>681.90 ± 10.34b</td>
<td>22.90 ± 10.15b</td>
<td>7.46, 0.0042</td>
<td></td>
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<tr>
<td>3</td>
<td>(+)-camphene</td>
<td>25.73 ± 8.89a</td>
<td>64.06 ± 36.51a</td>
<td>21.08 ± 4.44a</td>
<td>2.36 ± 0.66a</td>
<td>2.85, 0.09</td>
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<tr>
<td>4</td>
<td>(−)-β-pinene</td>
<td>42.35 ± 9.02a</td>
<td>67.71 ± 10.99a</td>
<td>89.91 ± 56.10a</td>
<td>59.64 ± 39.29a</td>
<td>6.52, 0.007</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(+)-β-pinene</td>
<td>89.87 ± 17.65a</td>
<td>266.39 ± 116.82a</td>
<td>114.22 ± 54.41a</td>
<td>53.48 ± 27.27a</td>
<td>2.77, 0.12</td>
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<td>6</td>
<td>Myrcene</td>
<td>143.62 ± 50.04a</td>
<td>160.73 ± 87.31a</td>
<td>9.98 ± 3.18b</td>
<td>0.54 ± 0.53a</td>
<td>25.61, &lt;0.0001</td>
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</tr>
<tr>
<td>7</td>
<td>ρ-cymene</td>
<td>175.65 ± 80.21a</td>
<td>180.51 ± 81.87a</td>
<td>151.99 ± 20.58a</td>
<td>42.25 ± 8.52a</td>
<td>1.40, 0.29</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Limonene</td>
<td>175.65 ± 80.21a</td>
<td>180.51 ± 81.87a</td>
<td>151.99 ± 20.58a</td>
<td>42.25 ± 8.52a</td>
<td>1.40, 0.29</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Eucalyptol</td>
<td>175.65 ± 80.21a</td>
<td>180.51 ± 81.87a</td>
<td>151.99 ± 20.58a</td>
<td>42.25 ± 8.52a</td>
<td>1.40, 0.29</td>
<td></td>
</tr>
</tbody>
</table>

Means within a row and treatment (EI or WI) followed by different letters are significantly different (*Duncan's MRT; df* = 3, 12).

*a* Not detected (Ñ).

*b* Peak numbers correspond to Fig. 4.
Table 2. t-test statistics comparing time-course emission of individual volatile compounds from EI vs WI *M. virginianna* (see Table 1)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>1</th>
<th>2</th>
<th>10</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ethanol</td>
<td>t</td>
<td>11.26</td>
<td>6.64</td>
<td>5.15</td>
</tr>
<tr>
<td>2</td>
<td>α-pinene</td>
<td>t</td>
<td>3.77</td>
<td>2.50</td>
<td>8.43</td>
</tr>
<tr>
<td>3</td>
<td>(+)-camphene</td>
<td>t</td>
<td>3.64</td>
<td>3.42</td>
<td>7.41</td>
</tr>
<tr>
<td>4</td>
<td>(-)-β-pinene</td>
<td>t</td>
<td>3.53</td>
<td>4.03</td>
<td>0.96</td>
</tr>
<tr>
<td>5</td>
<td>(+)-β-pinene</td>
<td>t</td>
<td>7.23</td>
<td>3.47</td>
<td>2.63</td>
</tr>
<tr>
<td>6</td>
<td>Myrcene</td>
<td>t</td>
<td>3.60</td>
<td>2.55</td>
<td>3.16</td>
</tr>
<tr>
<td>7</td>
<td>ρ-cymene</td>
<td>t</td>
<td>3.04</td>
<td>10.36</td>
<td>7.33</td>
</tr>
<tr>
<td>8</td>
<td>Limonene</td>
<td>t</td>
<td>2.62</td>
<td>2.76</td>
<td>5.83</td>
</tr>
<tr>
<td>9</td>
<td>Eucalyptol</td>
<td>t</td>
<td>2.81</td>
<td>2.63</td>
<td>3.66</td>
</tr>
</tbody>
</table>

Unpaired t-test compared relative quantities of individual compounds between EI and WI trees at each time point (df = 6).

Peak numbers correspond to Fig. 4.

1. 6; P = 0.003), (+)-camphene (F = 19.97; df = 1, 6; P = 0.004), (-)-β-pinene (F = 7.15; df = 1, 6; P = 0.037), (+)-β-pinene (F = 98.28; df = 1, 6; P < 0.0001), myrcene (F = 22.54; df = 1, 6; P = 0.0032), ρ-cymene (F = 26.01; df = 1, 6; P = 0.0022), limonene (F = 28.03; df = 1, 6; P = 0.002), and eucalyptol (F = 10.30; df = 1, 6; P = 0.018). Emissions of all the monoterpenes were significantly higher from ethanol-injected trees compared with the water-injected trees at each time point, except for (-)-β-pinene at 10 and 16 DAT (Tables 1 and 2).

For ethanol-injected trees, repeated measures ANOVA revealed a significant within-subject time × volatile effect associated with ethanol (F = 18.77; df = 3; P = 0.0003) (Fig. 4; Table 1). A mean ± SE of 2.96 × 10^5 ± 7.2 × 10^4 and 6.71 × 10^5 ± 2.37 × 10^5 ng of ethanol per sampling chamber were associated with ethanol-injected trees at 1 and 2 DAT, which were significantly higher compared with 9.26 × 10^5 ± 3.64 × 10^5 and 1.02 × 10^6 ± 7.02 × 10^5 ng at 10 and 16 DAT, respectively (F = 17.10; df = 3, 12; P = 0.0001) (Table 1). Pearson’s correlation coefficient analysis detected a negative correlation between ethanol emission and DAT (r = −0.62; P = 0.011).

A significant within-subject time × volatile effect for ethanol-injected trees was also associated with α-pinene (F = 4.45; df = 3; P = 0.035) and myrcene (F = 5.57; df = 3; P = 0.019). A mean ± SE of 276.48 ± 109.66 and 451.33 ± 260.93 ng of α-pinene per sampling chamber were associated with ethanol-injected trees at 1 and 2 DAT, which were significantly higher compared with 68.19 ± 10.34 and 22.90 ± 10.15 ng at 10 and 16 DAT, respectively (F = 3.74; df = 3, 12; P = 0.042) (Table 1). Pearson’s correlation coefficient analysis detected a negative correlation between α-pinene emission and DAT (r = −0.66; P = 0.006). Similarly, a mean ± SE of 143.62 ± 50.04 and 160.73 ± 87.31 ng of myrcene per sampling chamber were associated with ethanol-injected trees at 1 and 2 DAT, which were significantly higher compared with 9.98 ± 2.18 and 1.07 ± 0.46 ng at 10 and 16 DAT, respectively (F = 5.03; df = 3, 12; P = 0.001) (Table 1). A negative correlation was detected between myrcene emission and DAT (r = −0.75; P = 0.001). Significant correlations between the emission of individual monoterpenes and DAT were not detected for the remaining compounds (P > 0.05).

For water-injected trees, repeated measures ANOVA revealed a significant within-subject time × volatile effect for α-pinene (F = 69.26; df = 3; P < 0.0001), camphene (F = 5.56; df = 3; P = 0.02), (-)-β-pinene (F = 6.50; df = 3; P = 0.013), (+)-β-pinene (F = 5.15; df = 3; P = 0.024), myrcene (F = 4.87; df = 3; P = 0.03), ρ-cymene (F = 8.21; df = 3; P = 0.006), limonene (F = 4.4; df = 3; P = 0.04), and eucalyptol (F = 5.51; df = 3; P = 0.02), which resulted in significant differences detected among various time points from 1 to 16 DAT (Table 1). However, significant correlations between the emission of individual monoterpenes and DAT for water-injected trees were not detected (P > 0.05).

**Electrophysiological Responses.** The antennal response of *X. germanus* to emissions from ethanol-injected *M. virginianna* at 2 DAT was successfully characterized using SPME-GC-EAD (Fig. 5). A consistent antennal response occurred to ethanol, with a mean (±SE) depolarization of 0.95 ± 0.18 mV. Antennal responses were not observed to the aforementioned monoterpenes.

**Lure Formulation.** An ethanol plus monoterpene mixture was prepared to mimic ratios associated with...
ethanol-injected *M. virginiana* at 2 DAT (Fig. 4). A significant difference in total trap captures was not detected between the ethanol plus monoterpene mixture and ethanol alone for *X. germanus*, *X. crassiusculus*, *Monarthrum mali* (Fitch), *Anisandrus sayi* (Hopkins), *Euwallacea validus* (Eichhoff), and *Hypothenemus eruditus* Westwood (Table 3). However, a mean (±SE) of 1.5 ± 0.5 Xyleborinus saxesenii (Ratzburg) were collected from traps baited with the ethanol plus monoterpene mixture, which differed significantly from the 0.6 ± 0.4 specimens collected from traps baited with ethanol alone (*F* = 5.93; df = 2, 21; *P* = 0.009) (Table 3).

*X. germanus* represented 91.2% of total captures for traps baited with ethanol plus the monoterpene mixture, followed by *E. validus* (4.9%), *A. sayi* (1.9%), *X. saxesenii* (1.4%), *M. mali* (0.3%), *H. eruditus* (0.3%), and *X. crassiusculus* (0.1%). Similarly, *X. germanus* represented 90.6% of total captures for traps baited with ethanol alone, followed by *E. validus* (3.8%), *A. sayi* (2.9%), *H. eruditus* (1.7%), *X. saxesenii* (0.6%), *M. mali* (0.1%), and *X. crassiusculus* (0.3%).

**Discussion**

Collectively, these results support the role of ethanol as a primary volatile cue that attracts and induces attacks by *X. germanus*. Traps baited with bolts from *M. virginiana* trees injected with ethanol were more attractive to *X. germanus* than an ethanol lure under the tested conditions. In addition, basal injections of ethanol into the woody stem tissue of *M. virginiana* induced more attacks than irrigating with a comparable volume of ethanol or baiting with an ethanol lure. Conceivably, more ethanol would have been delivered into the vascular system through pressurized irrigation. Our current study and previous studies have demonstrated that ethanol can be emitted from stem and leaf tissue (Ranger et al. 2010). However, the amount of ethanol emitted from an ethanol-injected or -irrigated *M. virginiana* tree was not calculated as part of our current study for comparison to the ethanol lure. Perhaps ethanol being emitted over a larger surface area also contributed to the ethanol-injected trees inducing more attacks than those baited with an ethanol lure. It should be noted that attacks on ethanol-injected trees were vertically distributed along the woody stem tissue of *M. virginiana*, but attacks on ethanol-baited trees almost exclusively occurred in the region where the ethanol lure rested against the bark tissue (C. M. Ranger, personal observation).

The positive concentration response by *X. germanus* to ethanol should be considered to optimize and make odor-based trap trees as attractive as possible. However, too high of an ethanol release rate could have a repellent effect on ambrosia beetle orientation (Salom and McLean 1990). For instance, Montgomery and Wargo (1983) found a 2 g/d ethanol release rate was more attractive to Scolytinae than higher release rates. Additional concentrations higher than those tested as part our current study are required to determine the upper concentration threshold of ethanol for attracting and inducing attacks by *X. germanus* and other selected ambrosia beetles. Understanding the duration such trap trees will remain attractive is also of

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**Table 3. Ambrosia beetle captures associated with traps baited with ethanol alone compared with synthetic mixture of ethanol plus various monoterpenes formulated to mimic ethanol-injected *M. virginiana***

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean (±SE) total trap counts</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol + monoterpene*</td>
<td>Ethanol</td>
<td>Blank</td>
</tr>
<tr>
<td><em>X. germanus</em></td>
<td>95.8 ± 19.9a</td>
<td>94.4 ± 15.9a</td>
<td>0.0 ± 0.0b</td>
</tr>
<tr>
<td><em>E. validus</em></td>
<td>5.1 ± 2.8a</td>
<td>4.0 ± 1.4a</td>
<td>0.0 ± 0.0b</td>
</tr>
<tr>
<td><em>A. sayi</em></td>
<td>2.0 ± 0.5a</td>
<td>3.0 ± 1.1a</td>
<td>0.0 ± 0.0b</td>
</tr>
<tr>
<td><em>X. saxesenii</em></td>
<td>1.5 ± 0.5a</td>
<td>0.6 ± 0.4b</td>
<td>0.0 ± 0.0b</td>
</tr>
<tr>
<td><em>M. mali</em></td>
<td>0.3 ± 0.2a</td>
<td>0.1 ± 0.1a</td>
<td>0.0 ± 0.0a</td>
</tr>
<tr>
<td><em>H. eruditus</em></td>
<td>0.3 ± 0.3a</td>
<td>1.8 ± 1.2a</td>
<td>0.0 ± 0.0a</td>
</tr>
<tr>
<td><em>X. crassiusculus</em></td>
<td>0.1 ± 0.1a</td>
<td>0.3 ± 0.2a</td>
<td>0.0 ± 0.0a</td>
</tr>
</tbody>
</table>

* See Materials and Methods for concentrations of individual components. Different letters within a row indicate significant differences (ANOVA, Duncan’s MRT; *α* = 0.05; df = 2, 21).

---
importance. Time-course analysis by SPME-GC-MS of emissions from ethanol-injected *M. virginiana* determined ethanol emission was negatively correlated with time, such that ethanol steadily declined over the course of 16 DAT. Temperature, light levels, and additional abiotic factors can affect the rate of volatile emissions (Nielsen et al. 1995, Staudt and Bertin 1998). Notably, 90% of cumulative ambrosia beetle attacks occurred on ethanol-injected *M. virginiana* by 10 DAT. Similarly, as part of the concentration response experiment, 90% of cumulative ambrosia beetle attacks occurred on trees injected with 90% ethanol by 14 DAT. In each case, attacks presumably began to subside as ethanol emission decreased. Ethanol was not emitted from water-injected trees, which were not attacked by ambrosia beetles during these experiments and others (Ranger et al. 2010).

In addition to ethanol, a total of eight acyclic (i.e., myrcene) and cyclic (i.e., α-pinene, β-pinene, camphene, ρ-cymene, limonene, and eucalyptol) monoterpenes were emitted in relatively trace amounts from ethanol-injected *M. virginiana*. Monoterpenes belong to the C_{10} class of isoprenoids and provide the characteristic aromas of essential oils, floral scents, and certain defensive resins (Mahmoud and Croteau 2002). These relatively common terpenoids are associated with the bark of *M. virginiana* (Sha et al. 2004), particularly tissue that has been mechanically damaged (Azuma et al. 1997). Considering that the aforementioned monoterpenes were generally emitted in higher amounts from the bark of ethanol-injected versus water-injected trees, ethanol-injection may have influenced monoterpene biosynthesis or increased their volatilization from the bark tissue.

The various monoterpenes emitted from ethanol-injected *M. virginiana* did not appear to act additively or synergistically with ethanol in attracting or inducing attacks by *X. germanus*. For instance, antennal responses by *X. germanus* via SPME-GC-EAD to volatile emissions from ethanol-injected *M. virginiana* only occurred to ethanol and not the various monoterpenes. To our knowledge, these results represent the first known SPME-GC-EAD responses measured for *X. germanus*. Dose–response electroantennogram (EAG) studies are warranted to determine if *X. germanus* antennae do in fact recognize the various monoterpenes identified in emissions from ethanol-injected *M. virginiana*. Such information could then lead to additional trapping studies aimed at developing an improved lure by testing mixtures containing antennally active monoterpenes in higher amounts than those naturally associated with ethanol-injected trees.

Because results from electroantennographic experiments should be compared with behavioral and/or field responses, the attractiveness of a synthetic mixture of ethanol and monoterpenes that mimicked ethanol-injected *M. virginiana* was also examined. However, the ethanol and monoterpene mixture was not more attractive to *X. germanus* than ethanol alone. Previous studies found (-)-α-pinene generally had a negligible effect on increasing the attraction of *X. germanus* to ethanol-baited traps (Miller and Rabaglia 2009, Gandhi et al. 2010, Ranger et al. 2011a). It should also be considered that the various monoterpenes were emitted from water-injected *M. virginiana*, but attacks did not occur on these trees. Because more *X. saxeseni* were attracted to traps baited with the ethanol plus monoterpene mixture compared with ethanol alone, additional field-trapping studies over a larger geographic distribution are warranted. *X. saxeseni* has been recovered from naturally infested ornamental field stock (Reding et al. 2010) and ethanol-injected trees (Ranger et al. 2010).

*X. germanus* is the most abundant species of ambrosia beetle emerging from infested nursery stock (Reding et al. 2010) and ethanol-injected trees (Ranger et al. 2010) in Ohio. It has traditionally been considered a pest of physiologically stressed trees (Hoffman 1941, Solomon 1985), presumably because of the association of ethanol with such hosts (Byers 1995, Kelsey 2001, Ranger et al. 2010). Notably, attacks have not occurred on neighboring water-injected or uninjected trees as part of our current study and previous ones (Ranger et al. 2010). Attacks by *X. germanus* have been suspected to occur on ‘apparently healthy’ trees (Weber and McPherson 1984, Grégoire 2001), but the possibility of such trees being ‘inapparently stressed’ at the time of attack cannot be ruled out. In some cases, physiologically stressed trees can appear visually healthy, but still emit stress-related volatiles that signify their suitability to colonization by *X. germanus* (C. M. Ranger, personal observation). For instance, black walnut (*Juglans nigra* L.) trees that were colonized by *X. germanus* exhibited slower growth rates in the season before attack than uncolonized trees, yet they were referred to as apparently healthy (Weber and McPherson 1984). Rather than randomly attacking apparently healthy trees, results from these experiments and others (Ranger et al. 2010) indicate that *X. germanus* specifically targets hosts based on the emission of ethanol. Indeed, Weber and McPherson (1984) concluded that *X. germanus* could differentiate between even slight differences in tree vigor.

Because of the implications of ethanol being associated with physiologically stressed hosts (Kelsey 2001), the importance of maintaining host vigor and minimizing stressors should be considered the primary foundation of a management plan for *X. germanus* and other selected ambrosia beetles in ornamental nurseries. For instance, water-logging and flooding, which are known to induce the production and emission of ethanol (Kimmerer and Kozlowski 1982, Liao and Lin 2001), have coincided with targeted ambrosia beetle attacks on such trees in ornamental nurseries (C. M. Ranger, personal observation). Additional stressors that can occur within ornamental nurseries, including pathogens, water deficits, heat, freezing, girdling, and pollutants can also lead to the production and emission of ethanol (Kimmerer and Kozlowski 1982, Kimmerer and MacDonald 1987, Sjödin et al. 1989, Joseph and Kelsey 1997, Kelsey and Joseph 1998). Maintaining host vigor is particularly important because preventative applications of conventional in-
secticides will not necessarily prevent ambrosia beetles from attacking trees that are emitting ethanol.

Acknowledgments

We thank James Moyseenko, Jennifer Barnett, Gerald Hammel, Leslie Morris, and Betsy Anderson for technical assistance. The lead author also thanks Brian Sullivan (USDA-FS) for training in ambrosia beetle antennal preparations for electrophysiological purposes. This research was supported by funding from the USDA-ARS Floriculture and Nursery Research Initiative, USDA-APHIS’ Request for Technology Development to Improve Detection and Survey for Exotic Wood Borers, and base funds associated with ARS Research Project 3607-22000-012-00D (National Program 304-Crop Protection and Quarantine).

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Received 22 November 2011; accepted 23 February 2012.