

A Male-Produced Aggregation Pheromone of *Monochamus alternatus* (Coleoptera: Cerambycidae), a Major Vector of Pine Wood Nematode

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ABSTRACT The beetle *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) is an efficient vector of pine wood nematode, the causal pathogen of pine wilt disease, that has resulted in devastating losses of pines in much of Asia. We assessed the response of adult *M. alternatus* to 2-(undecyloxy)-ethanol, the male-produced pheromone of the congeneric *M. galloprovincialis* Dejean, in field experiments in Fujian Province, People's Republic of China. Both sexes of *M. alternatus* were attracted to lures consisting of 2-(undecyloxy)-ethanol combined with the host plant volatiles α -pinene and ethanol. A follow-up experiment showed that 2-(undecyloxy)-ethanol was synergized by both ethanol and α -pinene. Coupled gas-chromatography mass-spectrometry analyses of volatiles sampled from field-collected beetles of both sexes revealed that 2-(undecyloxy)-ethanol was a sex-specific pheromone component produced only by males. The combination of 2-(undecyloxy)-ethanol with ethanol and/or α -pinene will provide a valuable and badly needed tool for quarantine detection, monitoring, and management of *M. alternatus*.

KEY WORDS *Monochamus alternatus*, aggregation pheromone, 2-(undecyloxy)-ethanol, ethanol, α -pinene

In Asia, the beetle *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) is the principal vector of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle, the causal agent of the lethal pine wilt disease (Mamiya and Enda 1972). The pathogen was introduced into Japan from North America in the early 20th century, resulting in devastating losses of pines (e.g., an estimated 2.4 million m³ lost in 1979; Mamiya 1988). *M. alternatus* attacks primarily Japanese red and black pines, *Pinus densiflora* Siebold & Zucc. and *Pinus thunbergii* Parl., respectively, but it has been recorded in Japan from 18 species of *Pinus*, three species of *Picea*, and one species each of *Abies*, *Cedrus*, and *Larix* (Kobayashi et al. 1984). Pine wood nematode has spread from Japan to China, Korea, Taiwan, Laos and most recently, to Portugal (Kobayashi et al. 1984; Zhang et al. 2008). In China alone, an estimated 50 million trees were killed in an area of \approx 80,000 ha between 1982 and 2000 (Zhao

2008). Substantial effort has been directed toward minimizing losses through diverse means of vector control, and numerous studies have sought to identify semiochemicals for managing *M. alternatus*.

Observations of the attraction of *M. alternatus* to nematode-infested, and herbicide-treated pines (Kobayashi et al. 1970; Yamasaki et al. 1980) led to the identification of volatile terpenoids emitted by stressed hosts that were attractive to *M. alternatus*. Ikeda et al. (1980) identified α - and β -pinene as the most abundant among 11 attractive monoterpenes collected from the ether fraction of cold-trapped volatiles emitted by logs of *P. densiflora*. The water fraction contained ethanol, which enhanced attraction of beetles to the monoterpenes. These findings were confirmed by Fan et al. (2007a) who showed that four monoterpenes, (+)- α -pinene, (+)-(1S,6R)-3-carene, (-)-(1S,5S)- β -pinene, and terpinolene, elicited responses from antennae of both males and females in electroantennogram assays, and individually attracted beetles in the field, especially males. Among the four monoterpenes, (+)- α -pinene was most attractive, and attraction to all four was synergized by ethanol (Fan et al. 2007b).

Sakai and Yamasaki (1990) reported that the sesquiterpene (+)-juniperol and the diterpene (+)-pimeral, isolated from healthy *P. densiflora*, elicited flight responses from female *M. alternatus* in laboratory bioassays. Similarly, (+)-*cis*-3-pinen-2-ol isolated from paraquat-treated pines stimulated males but not females to fly in laboratory bioassays (Sakai and Ya-

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masaki 1991). However, the biological activities of these compounds were not assessed in field tests.

Several North American and European species of *Monochamus* have been shown to be attracted to combinations of conifer monoterpenes and pheromones of *Ips* bark beetles (Billings and Cameron 1984; Billings 1985; Raffa 1991; Allison et al. 2001; Miller and Asaro 2005; Pajares et al. 2004). In contrast, Fan et al. (2010) found that the bark beetle pheromone components ipsenol and ipsdienol inhibited attraction of *M. alternatus* to conifer volatiles.

The first evidence for pheromones of *M. alternatus* was reported by Fauziah et al. (1987), who observed that females were attracted to males in laboratory cage bioassays. These results were corroborated by Kim et al. (1992), who found that females were attracted to the odor of males in a Y-tube olfactometer but not to the odor of females or to clean air, and that males showed no preference for the odor of females over clean air. To date, there has been no further progress in the identification of a possible male-produced pheromone for this species. However, Pajares et al. (2010) recently reported the identification of a male-produced aggregation pheromone, 2-(undecyloxy)-ethanol, of the congeneric European species *Monochamus galloprovincialis* (Olivier).

Because several recent studies have shown that pheromone structures often are highly conserved among closely related species of cerambycids (Hanks et al. 2007, Silk et al. 2007, Lacey et al. 2009, Millar et al. 2009, Graham et al. 2010, Barbour et al. 2011), we hypothesized that 2-(undecyloxy)-ethanol, or a structurally related compound, might also be a pheromone component for *M. alternatus*. Here, we report the results of successful field trials that tested the attraction of *M. alternatus* to 2-(undecyloxy)-ethanol as a single component and in combination with host volatiles. We also identified 2-(undecyloxy)-ethanol as a sex-specific component of volatiles collected from male *M. alternatus*, confirming that this compound is indeed a pheromone component for this species. These results provide the crucial basic information required for the development of more effective attractant-baited traps for this notorious pest.

Materials and Methods

Synthesis of Pheromone Compounds. 2-(undecyloxy)-ethanol was synthesized as described by Pajares et al. (2010). Fuscumol was prepared as a mixture of stereoisomers by reduction of geranylacetone (Sigma-Aldrich Flavor and Fragrance Division, Milwaukee, WI) with lithium aluminum hydride as described by Silk et al. (2007). A portion of the resulting fuscumol was then acetylated with acetic anhydride and pyridine as described by Vidal et al. (2010).

Field Experiments. Field experiments were conducted at Fuzhou National Forest Park, Fujian, People's Republic of China, in two stands of 30-yr-old *Pinus massoniana* Lamb and mixed hardwoods where *M. alternatus* was causing significant mortality of pines. The first experiment (located at 119° 17'08" E, 26° 09'39" N) was

conducted as part of a screening study to test the responses of Asian cerambycids to known pheromones of North American and European species. Five compounds were tested in four lure combinations, as follows: 1) 2-(undecyloxy)-ethanol + α -pinene + ethanol, 2) 2-(undecyloxy)-ethanol alone, 3) α -pinene + ethanol, 4) α -pinene + racemic fuscumol (6,10-dimethyl-5,9-undecadien-2-ol), 5) α -pinene + fuscumol acetate (6,10-dimethyl-5,9-undecadien-2-yl acetate), and 6) controls consisting of traps without lures. Pheromone release devices consisted of polyethylene sachets (press-seal bags, 5 by 7.6 cm, part 01-816-1A, Thermo Fisher Scientific, Waltham, MA) that were loaded with 1 ml of isopropanol solutions of the pheromones (25 mg/ml). (\pm)- α -Pinene (20 ml, 98%; Aldrich, Shanghai, People's Republic of China) was released from dispensers prepared by absorbing the neat compound on a piece of plastic sponge which was then enclosed in a clear polyethylene pouch, with a release rate of \approx 2 g/d at 20°C (Alpha Scents, Portland, OR). Ethanol (2 ml) was formulated similarly and released at a rate of \approx 300 mg/d at 20°C (Alpha Scents). Lures were placed on panel traps (Alpha Scents) that had been coated with Fluon PTFE (AGC Chemicals Americas, Inc., Exton, PA) to increase trap efficiency (Graham et al. 2010). Trap cups contained a small amount of sand treated with 80% dichlorvos (*O,O*-dimethyl-*O*-2,2-dichlorovinyl phosphate, miscible oil; Shandong Dacheng Pesticide Co. Ltd., Zibo, People's Republic of China) to prevent the escape of captured beetles. Traps were set in the field on 18 June 2010 and trapped beetles were counted on 28 June and 4 July 2010. Traps and trap rows were spaced a minimum of 10 m apart, and the trap cups were placed \approx 1 m above the ground or liana layer, where present. Each treatment was replicated five times, and beetles were counted twice, for a total of 10 replicates (five spatial and two temporal). Each spatial replicate included one of each treatment in randomized order. When the traps were checked on 28 June, the treatment positions were rerandomized.

The second experiment (located at 119° 16'55" E, 26° 09'01" N) was designed to assess the individual effects of α -pinene and ethanol on the response of *M. alternatus* to 2-(undecyloxy)-ethanol. Treatments included 1) 2-(undecyloxy)-ethanol + α -pinene + ethanol, 2) 2-(undecyloxy)-ethanol + α -pinene, 3) 2-(undecyloxy)-ethanol + ethanol, 4) α -pinene + ethanol, and 5) controls consisting of unbaited traps. Lures, doses, and traps were as described above. Traps were deployed near the site of the first experiment on 25 July 2010, and beetles were counted on 2 and 15 August and 2 September 2010. There were five spatial and three temporal replicates (total 15). Each spatial replicate included one of each treatment in random positions. When the traps were checked on two and 15 August, the treatment positions were rerandomized. Voucher specimens of *M. alternatus* have been deposited in the Zoological Museum of the Institute of Zoology, Beijing, People's Republic of China.

Statistical Analysis. Raw and log-transformed trap catch data from the field experiments initially did not meet the assumption of homogeneity of variances due to the presence of nearly all zeros in control treat-

ments. Thus, trap catch data were analyzed to detect significant differences among the control and treatment trap catches with the nonparametric Kruskal–Wallis test. The main effects of lure, date, and spatial replicate were analyzed by three-way analysis of variance (ANOVA). To assess the effects of the various treatments, controls were removed from the analysis, and an ANOVA was performed on log-transformed trap catches (Finney 1989). In the second field experiment, one spatial replicate in one sampling period was removed to eliminate a significant replicate (position) effect (Dixon's Q test, $Q = 0.47$, $\alpha < 0.02$). The Newman–Keuls test was used to compare treatment and replicate means. Statistical analyses were performed using STATISTICA 6.1 (StatSoft 2003).

Collection and Analysis of Insect-Produced Volatiles. We collected adult *M. alternatus* for aeration and identification by setting up five traps in Fuzhou National Forest Park from 18 August 2010 to 14 October 2010. Traps were baited with 2-(undecyloxy)-ethanol + α -pinene + ethanol, and trap cups did not contain insecticide, so that adults could be captured alive. We captured 12 female and two male *M. alternatus*, held them in the laboratory for 2–3 d to confirm that they were healthy and active and then shipped them to the Institute of Chemistry, Chinese Academy of Sciences (ICCAS) in Beijing for pheromone collection. Along with the beetles, we separately shipped freshly cut branchlets of *P. massoniana* to be used as food for the adults. Aerations were performed from 2 September 2010 to 8 November 2010. Each aeration began in the morning, lasted ≈ 24 h, and host material (≈ 20 – 25 g) was changed daily. Individual beetles with cuttings of pine branchlets were held in a 500-ml glass Erlenmeyer flask fitted with inlet and outlet ports. Flasks were placed on a laboratory bench near a south-facing windowsill and were exposed to afternoon sunlight from ≈ 1200 to 1400 hours. Charcoal filtered air was drawn through flasks at a rate of 500 ml/min, and volatiles were trapped on two sequential adsorbent filters consisting of 200 mg of Porapak-Q (80–100 mesh; Supelco, Bellefonte, PA) packed between silanized glass wool plugs in a Pasteur pipette. The Porapak Q was first cleaned by rinsing with 5 ml of dichloromethane followed by 5 ml of pentane. Before experiments, the last 1 ml of the pentane wash was collected and analyzed to confirm that all volatile impurities had been stripped off the adsorbent by the cleaning treatment. Trapped volatiles were eluted from loaded adsorbent tubes with three 1-ml aliquots of pentane. Preliminary experiments determined that 2 ml eluted all detectable volatiles.

Upon arrival at ICCAS, beetles were separated and promptly aerated (on 2 September for the first male and 24 September for the second male). Aerations were performed on individual beetles for 24-h periods ($n = 9$ for the first male, $n = 43$ for the second male; $n = 55$ using 12 females separately). Separate controls ($n = 12$) consisted of empty aeration chambers, and aeration chambers loaded only with cuttings of pine branchlets.

Extracts of volatiles were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) on a Shimadzu GC-2010 interfaced to a Shimadzu GCMS-QP2010 Plus mass spectrometer using electron impact ionization (70 eV) (Shimadzu Corporation, Kyoto, Japan). The GC was fitted with a DB-5 fused silica capillary column (30 m by 0.25 mm i.d. by 0.25- μ m film thickness; J&W Scientific, Folsom, CA). Carrier gas was helium (1.0 ml/min), source and injector temperatures were 200 and 220°C respectively, with injections made in split mode (50:1 split). The oven temperature was programmed from 50°C for 1 min and then 10°C/min to 250°C, hold for 5 min. Pheromone identification was confirmed by matching the retention time and mass spectrum to those of an authentic standard of 2-(undecyloxy)-ethanol, and the amounts produced were calculated per beetle per day. Depending on the size of the pheromone peak, either 500 ng or 5 μ g of 2-(dodecyloxy)-ethanol (ethylene glycol monododecyl ether, $\geq 99.0\%$ pure, Aldrich, Shanghai, People's Republic of China) was added to a 500- μ l aliquot of an extract as an internal standard, and the aliquot was reanalyzed. The total volume of each extract was determined by measuring the volume with a calibrated syringe. External standard calibration curves were calculated at concentrations of 1, 2, 5, 8, and 10 ng/ μ l for 2-(undecyloxy)-ethanol ($r^2 = 0.96$) and 2-(dodecyloxy)-ethanol ($r^2 = 0.96$). Extracts from females, pine branchlet controls, and blank controls were analyzed using splitless injection in selected ion monitoring mode to maximize the sensitivity for detection of any trace of 2-(undecyloxy)-ethanol, scanning for ions of m/z 140, 154, and 185, by using the same temperature program.

Results

Field Experiments. In the first field experiment (Fig. 1), significantly more *M. alternatus* were captured in traps baited with 2-(undecyloxy)-ethanol + α -pinene + ethanol than in control traps (Kruskal–Wallis ANOVA: $H = 27.11$, $df = 5$, $P < 0.0001$; $n = 54$) or traps baited with any of the other treatments (ANOVA: $F = 9.7$; $df = 4, 36$; $P < 0.0001$). The sex ratio of *M. alternatus* (2.16:1, female:male) captured in traps baited with 2-(undecyloxy)-ethanol + α -pinene + ethanol was significantly different from 1:1 ($\chi^2 = 15.8$, $df = 1$, $P < 0.001$), whereas the sex ratio of *M. alternatus* captured in the remaining treatments combined was not significantly different from 1:1 ($\chi^2 = 0.60$; $df = 1$; $P > 0.1$; too few *M. alternatus* were captured in any one treatment to test separately). When the sexes were analyzed separately, the treatment with 2-(undecyloxy)-ethanol + α -pinene + ethanol trapped significantly more males and females than either the α -pinene + ethanol treatment (ANOVA, males: $F = 4.1$; $df = 4, 46$; $P < 0.01$, followed by Newman–Keuls range test; ANOVA, females: $F = 12.7$; $df = 4, 46$; $P < 0.0001$, followed by Newman–Keuls range test) or the control (Kruskal–Wallis ANOVA, males: $H = 19.90$, $df = 5$, $P < 0.01$; $n = 60$; Kruskal–Wallis ANOVA, females:

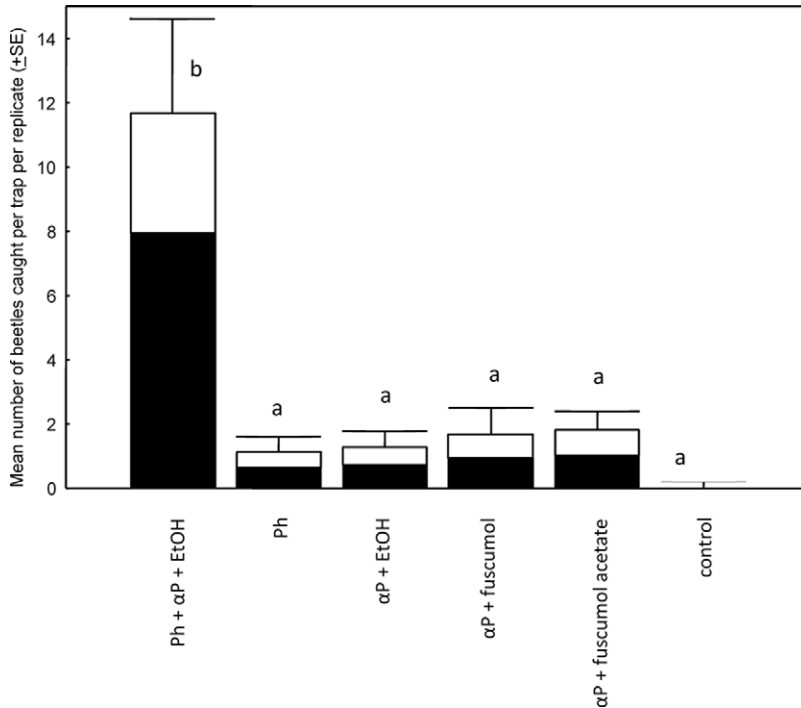


Fig. 1. Numbers of *M. alternatus* captured in panel traps baited with 2-(undecyloxy)-ethanol, α -pinene, ethanol, fuscumol, fuscumol acetate, and combinations thereof. White bars represent males and black bars represent females. Bars with the same letter are not significantly different ($P > 0.05$). Controls were compared with the treatments using the Kruskal-Wallis test; all other comparisons were made with ANOVA followed by Newman-Keuls range test. Ph, 2-(undecyloxy)-ethanol; α p, α -pinene; EtOH, ethanol.

$H = 24.66$, $df = 5$, $P < 0.001$; $n = 60$). No other insects were caught in sufficient numbers to suggest that they were being attracted by any of the lures.

In the second field experiment (Fig. 2), traps baited with 2-(undecyloxy)-ethanol and either α -pinene or ethanol caught as many *M. alternatus* as traps baited with all three components, but traps without 2-(undecyloxy)-ethanol caught significantly fewer females (ANOVA: $F = 3.10$; $df = 4, 53$; $P < 0.05$). Only traps baited with 2-(undecyloxy)-ethanol + α -pinene + ethanol, 2-(undecyloxy)-ethanol + α -pinene, or 2-(undecyloxy)-ethanol + ethanol were significantly more attractive than controls (Kruskal-Wallis ANOVA: $H = 20.97$, $df = 4$, $P < 0.001$; $n = 70$). An identical analysis with the outlier replicate included yielded the same grouping of treatments based on significance, i.e., deleting the outlier did not make any treatment significantly different from any other that was not significantly different when the outlier was included. However, inclusion of the outlier substantially increased the mean and standard error of the 2-(undecyloxy)-ethanol + ethanol treatment. The treatments containing 2-(undecyloxy)-ethanol attracted *M. alternatus* in a ratio of 1.98:1 (female:male), significantly different than 1:1 ($\chi^2 = 14.78$, $df = 1$, $P < 0.001$). When the sexes were analyzed separately with ANOVA, no significant treatment effect was detected, probably due to the second experiment being conducted late in the flight period and trap captures were low.

Collection and Analysis of Insect-Produced Volatiles. 2-(Undecyloxy)-ethanol was detected in 26 of the 52 24-h samples of volatiles from males on branchlets. No 2-(undecyloxy)-ethanol was detected in any sample from females on pine branchlets, branchlets only, or blank controls (Fig. 3; detection limit, ≈ 10 pg). The first male produced 2-(undecyloxy)-ethanol on days 3–6 with a peak of $6.3 \mu\text{g/d}$ on day 4 but died on the 10th day. The second male produced 2-(undecyloxy)-ethanol in 4–6 d cycles over 43 d with production lasting for 3–4 d and peaks of 1.9 – $14.6 \mu\text{g/d}$ ($\bar{x} \pm \text{SE} = 5.1 \pm 2.0$). Between each period of production, there was at least one day with undetectable quantities of 2-(undecyloxy)-ethanol.

Discussion

The results reported here support our hypothesis that pheromone structures might be highly conserved within the genus *Monochamus*. *M. alternatus* of both sexes were significantly attracted to 2-(undecyloxy)-ethanol when it was released in combination with the host volatiles α -pinene and ethanol. Furthermore, we identified this compound in aeration extracts prepared from male beetles on pine branchlets, confirming that it is indeed a pheromone component for this species. In parallel studies conducted in the United States, we also have shown that this compound is produced and

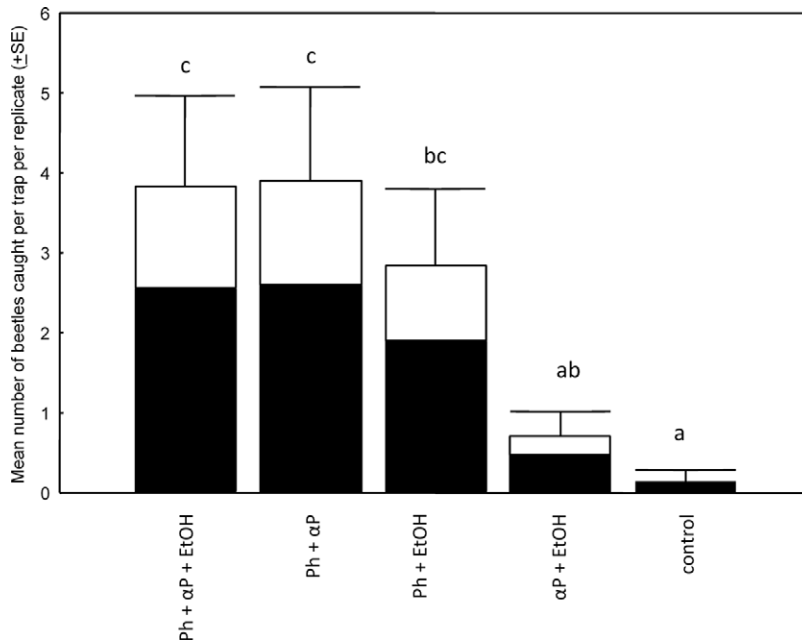


Fig. 2. Numbers of *M. alternatus* captured in panel traps baited with combinations of 2-(undecyloxy)-ethanol, α -pinene, and ethanol to assess the separate synergistic effects of α -pinene and ethanol. White bars represent males and black bars represent females. Bars with the same letter are not significantly different ($P > 0.05$). Controls were compared with the treatments using the Kruskal-Wallis test; all other comparisons were made with ANOVA followed by Newman-Keuls range test.

used as an aggregation pheromone by at least three other *Monochamus* spp. (unpublished data). Furthermore, the production of related hydroxyethers by males of the congeneric *Monochamus leuconotus* (Pascoe) (Hall et al. 2006, cited by Pajares et al. 2010), and males of *Anoplophora glabripennis* (Motschulsky) (Zhang et al. 2002), within the same tribe, suggests that the hydroxyether structural theme may be common in the tribe Monochamini, and perhaps other tribes of the subfamily Lamiinae.

Our results showed that 2-(undecyloxy)-ethanol plays an important role in the mating biology of *M. alternatus*. However, the sequence of behaviors leading to mating and the precise role of the male pheromone in that sequence warrant further investigation. In a time-budget analysis of the behavior of caged *M. alternatus*, Fauziah et al. (1987) reported that females were attracted to males from ≈ 1700 hours (< 1 h before sunset) until ≈ 2200 hours. Copulation typically lasted for about four hours, after which both sexes rested until the end of the scotophase. During the day both sexes engaged exclusively in feeding. Laboratory bioassays by Kim et al. (1992) supported the scenario of walking females being attracted to pheromone-producing males that remained motionless until contacted by the female. Our field results contradict previous laboratory bioassays in which only female *M. alternatus* responded to male-produced volatiles, and only by walking (Kim et al. 1992). That is, both sexes were trapped in our field bioassays, suggesting that the phero-

none is an aggregation pheromone rather than a sex pheromone. Second, our traps were suspended from wire hangers, so beetles that were captured must have flown into the traps. In contrast, the studies of Fauziah et al. (1987) and Kim et al. (1992) were based on observations of captive insects which were unable to fly, and these artificial constraints may have affected their bioassay results.

We suggest the following scenario for the reproductive biology of *M. alternatus*. First, host volatiles likely play a role in bringing the sexes together on the same tree (Hanks 1999, Fan et al. 2007a) followed by females walking and flying toward males. This scenario is supported by a reported strong male bias in terpene-baited traps even though the background population of emerging beetles was strongly female-biased (Fan et al. 2007a). Under this scenario, males are attracted to hosts, and then once on hosts, they produce pheromone to attract females over shorter distances (Ginzler and Hanks 2005). The production of pheromones by individual males may be affected by direct female contacts or other chemical cues, visual cues, or both and further experiments should be done with laboratory-reared beetles of known age and mating status to determine which factors, including host presence, influence pheromone production.

It remains to be determined whether other host volatiles such as 3-carene, β -pinene, and terpinolene (Fan et al. 2007a,b) may further enhance attraction to 2-(undecyloxy)-ethanol. Nevertheless,

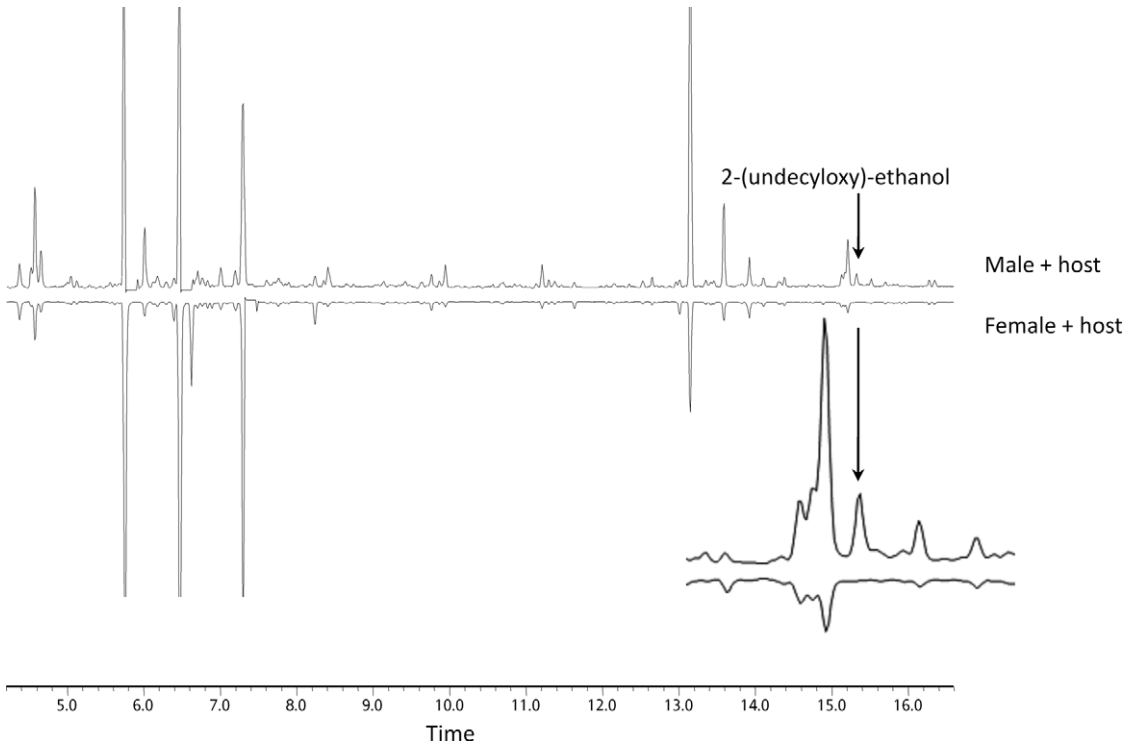


Fig. 3. GC-MS analyses showing representative total ion chromatograms of volatiles from 24-h aerations of male *M. alternatus* + host (*P. massoniana*) material (top) and females + host material (bottom). Inset shows magnified section of chromatogram that contains the 2-(undecyloxy)-ethanol peak. 2-(Undecyloxy)-ethanol was not found in females (lower limit of detection of the GC-MS in selective ion monitoring mode was 10 pg).

our studies clearly demonstrate that combining the pheromone component 2-(undecyloxy)-ethanol with the host volatiles α -pinene and ethanol resulted in a substantial improvement in the efficiency of trapping for *M. alternatus*. Our study strongly supports the immediate adoption of lures containing 2-(undecyloxy)-ethanol and host volatiles for quarantine monitoring of this notorious pest insect.

Acknowledgments

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