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1-octadecene, A Female Produced Aggregation Pheromone of the Coffee White Stem Borer (*Xylotrechus quadripes*)

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Abstract: Coffee white stem borer, *Xylotrechus quadripes* is a serious insect pest of coffee across the world. Pheromone traps baited with male produced 2-hydroxy- 3-decanone (2H3D) attracted females, and also males. Interestingly, a pair of males and females in a cage attracted more beetles compared to 2H3D traps. We hypothesized volatiles emitted from females are perceived by males. Volatiles from females were collected through the air entrainment method and identified 1-octadecene by using gas chromatography coupled with mass spectrometry. Release of 1-octadecene was female specific, however, both male and female antennae elicited responses in chromatography-electroantennography. Electroantennogram responses were dose-dependent in both sexes and were higher in the antennae of females. Combination of male pheromone component 2H3D, and female produced 1-octadecene at 1:2 ratio increased both male (0.69 ± 0.13; mean ± SEM in -mV) and female (0.98 ± 0.20) antennal responses. Responses to constant doses of 2H3D and increasing doses of 1-octadecene (1:4, 1:6, 1:8, 1:10) were not different from male pheromone alone. Beetle captures to male pheromone alone at 3 different doses (75, 150 and 300 mg) were not significantly different from a pair of live beetle traps. A blend of 2H3D and 1-octadecene at 1:2 ratio trapped more beetles (3.50 ± 0.65; mean ± SEM) over 2H3D alone (2.00 ± 0.82), overall, our experiments suggest 1-octadecene is an aggregation pheromone. Our results support the hypothesis that males use 1-octadecene in mate finding, and potential to use it for monitoring and mass trapping.

Keywords: coffee; entrainment method; *Xylotrechus quadripes*; volatile; pheromone blend; live-beetle trap

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1. Introduction

Coffee is a famed preeminent plant in the world, Arabica coffee (*Coffea arabica* L.) and Robusta coffee (*Coffea canephora* Pierre) (Rubiaceae) are the two important species cultivated on a commercial scale and produce about 131.25 million bags in India [1]. Quality coffee is hindered mainly by insect pests and diseases. Among the insect-pests, coffee white stem borer (CWSB), *Xylotrechus quadripes* (Chevrolat) (Coleoptera: Cerambycidae), coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), shot hole borer, *Xylosandrus compactus* Eichhoff (Coleoptera: Curculionidae) and green scale, *Coccus viridis* Green (Hemiptera: Coccidae) cause serious threat to coffee production. However, CWSB alone causes an economic yield loss of 2 to 20% [2–5]. Females lay eggs singly or in batches

under bark crevices of the coffee plant, larvae bore and feed inside the stem, and the effect of conventional and chemical control strategies is relatively minimum [6,7].

Host plant identification is crucial for herbivorous insects to find relevant food sources and oviposition sites by the detection of secondary metabolites released by the plants [8]. Use of host plant volatiles and species-specific chemicals that alter insect behavior for the management of insect pests of agricultural crops is exploited after the identification of the first insect pheromone [9]. Pheromone based strategies do not affect natural enemies, efficient at low population densities, and bring down insect populations below the economic threshold level [9,10]. Thus, pheromone-based monitoring and/or mass trapping of CWSB populations can be a potential management tactic as it directly affects the egg laying.

Previous studies report a male CWSB produced aggregation pheromone compound, (*S*)-2-hydroxy- 3-decanone trapped both males and their conspecifics [11–15]. Similarly, cuticular hydrocarbons of female *Monochamus galloprovincialis* stimulate male copulatory behavior [16], and male produced 2-undecyloxy-1-ethanol attracts both males and females [17]. A blend of sesquiterpenes, α-longipinene a major compound and minor ones α-cubebene, and β-caryophyllene emitted by female Asian longhorn beetle, *Anoplophora glabripennis* elicited antennal responses in males and significant male captures in the field [18]. On the contrary, 8–10 days old live-female baited traps captured male white grubs, *Leucopholis lepidophora* (Coleoptera: Scarabaeidae) in the field conditions, and male-traps didn't attract any females [19].

Aggregation pheromones are known to cause clustering behavior, bring individuals into proximity to defend against predators, find shelter, overcome host resistance by the mass attack and find conspecifics [20]. In our pilot study, a trap with a pair of male and female trapped a greater number of beetles over a dispenser containing male produced aggregation pheromone 2-hydroxy- 3-decanone alone. We hypothesized the volatiles released from trapped females attract and/or synergize male CWSB. To determine the role of female produced volatiles, volatiles were collected, analyzed using gas chromatography coupled mass spectrometry. Further, volatiles were tested for their physiological and biological relevance using electroantennography (EAG) against both males and females. Field trapping studies were taken up to confirm the behavioral relevance of female produced volatiles.

2. Materials and Methods

2.1. Insects

Infested coffee stems were collected from the field (13.1365° N, 75.6403° E 970 m AMSL) and stored in a 3 × 3 × 3 m nylon net in an open area under shade trees. Freshly emerged adults were sexed and placed separately in a 100 mL plastic container with a cotton wad soaked in 10% sugar water at 23 °C, 70% relative humidity in the laboratory. Newly emerged 2–4 days old beetles were used for the volatile collection and bioassays.

2.2. Volatile Collection

Volatiles were collected from 50 sexually mature 2–4-day old males, and females separately for 2 h during their active period (10.00 h to 12.00 h, authors field observation, unpublished data) consecutively for 5 days using an air entrainment method. Where, air passing through an acrylic flask (5 × 5 × 12 cm) containing beetles was absorbed by a glass column filled with a polymer-conditioned 100 mg Porapak-Q 50–80 (Supelco, Bellefonte, PA, USA), and extracted with 1.5 mL of HPLC grade dichloromethane for 15 min. An airflow of 1.8 L/min (Gilmont #l flowmeter, PCI Analytics Pvt Ltd., Mumbai, India) was achieved using a vacuum system, and the air was pulled through activated charcoal (SD Fine Chem Ltd., Mumbai, India) to remove large hydrocarbon molecules and any oils present in the system and through a molecular sieve 5A, (Himedia, Mumbai, India) to remove smaller organic molecules. Collected volatiles were pooled, concentrated using nitrogen (99.99% purity) stream and used for downstream analysis. The concentrated extracts of the air-entrainment and synthetic compounds as standards were injected in the Gas Chromatography/Mass Spectrometry (GC/MS) analysis.

2.3. Chemicals and Dilutions

Male pheromone, (*S*)-2-hydroxy-3-decanone (97.5%), 2-octadecene (94%), 3-octadecene (95%) and 4-octadecene (94.5%) were synthesized at BCRL, India. 1-octadecene (97.34%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). For GC-MS injections, all compounds were diluted in dimethyl disulfide (DMDS) [21], and in hexane for GC-EAD, EAG and field experiments. 2% stock dilutions (20 mg/mL) were made in hexane for both (*S*)-2-hydroxy-3-decanone and 2-octadecene, and further serial dilutions up to 2 µg/µL were made. For example, 20 µL of 2 µg/µL was loaded onto the filter paper to get 0.04 mg. To test the combination of 2-hydroxy-3-decanone and 1-octadecene at ratios 1:0, 1:2, 1:4, 1:6, 1:8 and 1:10, in which 2-hydroxy-3-decanone was constant (8 mg) and 1-octadecene was loaded according to the ratio.

2.4. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The GC-MS (7820A, Agilent, Santa Clara, CA, USA) was fitted with a capillary HP-5 MS UI column (30 m × 0.25 mm i.d. × 0.25 µm film) and mass selective detector (5977E, Agilent) uses Triple Axis Detector). Helium was used as a carrier and makeup gas (1.2 mL/min) and the injections were in the split mode with a split ratio (10:1) GC oven temperature was programmed at 150 °C (hold for 2 min), raise to 10 °C min^{-1} to 220 °C (hold for 20 min) for the analysis of natural and synthetic compounds. The Detector temperature was set at 230 °C. For DMDS adducts, GC oven was programmed at 60 °C (held for 2 min) and raised at 10 °C per min up to 220 °C (held for 20 min). Sex-specific peaks were tentatively identified by matching their mass spectra to the GC-MS database (National Institute of Standards and Technology, Gaithersburg, MD, USA) and confirmed by comparing spectra and retention times with Kovat's Retention Index (KI) authentic standards. KI was calculated for the peak of interest and standards relative to blends of straight-chain hydrocarbons. Identification of peak was confirmed by co-injections of extracts with an authentic standard.

2.5. Gas Chromatography-Electroantennogram Detection (GC-EAD)

The GC (Agilent 7890A) was equipped with a HP-5 column (30 m × 0.32 mm i.d. × 0.25 µm film; make: J&W Scientific, Folsom, CA, USA). Nitrogen was used as a carrier and makeup gas (1.5 mL min^{-1}) and the injections were in the split mode with a split ratio (10:1). The GC oven was programmed at 150 °C for 2 min, raise 10 °C min^{-1} to 220 °C, hold for 20 min. The effluent from the columns was split using Agilent purged two-way splitter with half of the sample going to the flame ionization detector (FID) and the other half to the EAD. The portion directed to the EAD was dispersed in a humidified air stream (0.5 L/m). Flame ionization detection and EAD responses matching to the peaks along with their retention times were measured.

2.6. Electroantennography Assay

Antennae were excised from 2-day old male and female and placed between the electrodes using electroconductivity gel (Signa-gel, Parker Laboratory Fories. Inc., Fairfield, NJ, USA). A 0.5 L/min flow of charcoal-filtered, and humidified air blew continuously over the mounted antenna. Each stimulation generated by the stimulus controller was delivered for 0.5 s at 0.2 L/min and with an inter-stimulus interval of at least 90 s. Thirty seconds after loading the stimuli over a filter paper piece (60 × 5 mm, Whatman #1) and filter paper was inserted into a 14.6 cm Pasteur pipette, a given cartridge was not stimulated more than once. Order of stimulation was solvent followed by the lowest to the highest doses. The signal was pre-amplified using a custom-built amplifier (10X gain, Hanson B-102), and high pass filtered at 0.1 Hz, digitized, and analyzed using Autospike, Syntech, Germany. Only one antenna per beetle was used, antenna was first stimulated with hexane, followed by volatiles in the ascending order of dose. Total 5 antennae were tested for each sex in the male-female pheromone synergism experiment.

2.7. Field Trapping Studies

We conducted an experiment in one ha arabica coffee field at Mallenahalli, Chikmagalur (13°42′ N; 75°80′ E) to test different doses of 2-hydroxy- 3-decanone in 2012. Three different doses (75 mg, 150 mg, 300 mg) of 2-hydroxy- 3-decanone diluted in hexane were loaded onto the PVC vials with a lid and sticky cross-vane traps (Figure 1A). A pair of live beetles in a mosquito net (Galvanized iron wire cage, Figure 1B) served as a positive control, a cotton wad with 10% sugar solution was placed in the middle of the cage which created a 5 mm distance between male and female beetle. Hexane solvent was a negative control. A total of 45 traps (n = 9) were randomized, suspended at 1.83 m canopy height of coffee plants, traps were spaced 200 m apart between the traps. The experiment coincided with the beetle's flight activity (31 September 2012 to 10 January 2013). Traps were revised every 10 days, and captures were counted and sexed. Dead males and/or females in galvanized wire cages were replaced with healthy ones.

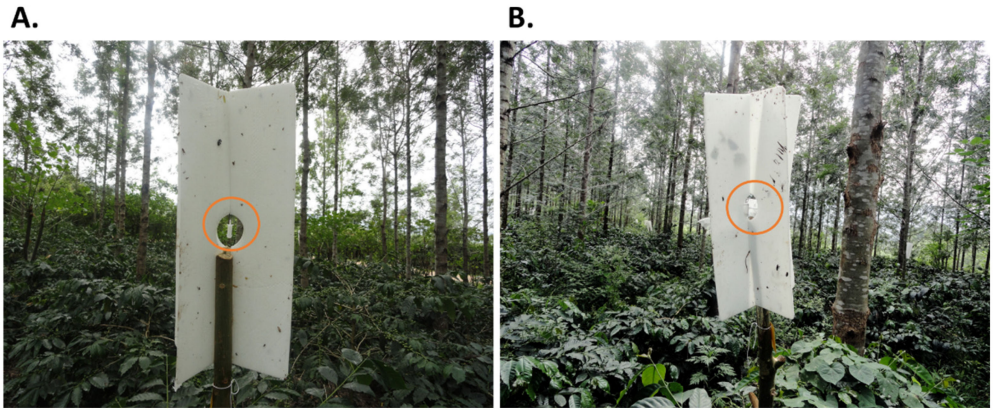

Figure 1. White sticky cross vane trap with a (**A**) plastic vial lure and (**B**) pair of live beetles in galvanized wire cage installed in the field (Orange color circles indicates the position of lure/live beetles).

EAG results indicated physiological synergism to a blend of 2-hydroxy-3-decanone and 1-octadecene. Thus, a field experiment was conducted at Mallenahalli, Chikmagalur (13°42′ N; 75°80′ E) to confirm the behavioral synergism. Sticky cross-vane traps were installed 200 m apart (a total of 20 traps/acre), and PVC vials were loaded with pheromone blends. Different ratios of 2H3D and 1-octadecene were tested. The treatments were randomized and replicated 4 times. The number of beetles captured was recorded at 10 days intervals for 50 days (from October 2013 to December 2013).

2.8. Statistical Analysis

Data on the number of males, females, and total beetles captured were square-root transformed to stabilize variances and subjected to analysis of variance (ANOVA). For EAG experiment, the maximum negative potential (-mV) generated by each stimulation was subjected to arcsine transformation followed by one-way ANOVA. Significant differences between the treatments were determined by Duncan's Multiple Range Test.

3. Results

3.1. Physiological Identification of Active Compounds by GC-EAD

Physiologically relevant compounds retention times were recorded from the headspace of female and male beetles using gas-chromatography coupled electroantennogram detection (GC-EAD). Antennae of both male and female beetles elicited a peak/response to headspace elute at retention time 7.91 min, and observed no difference in the antennal response of both males and females (Figure 2). There were multiple peaks in the female produced volatile headspace. Volatile headspace from males alone had no chromatogram peak at 7.91 min, and no EAD response suggested 1-octadecene is a female-produced compound. Volatile headspace from males had no chromatogram peak at 7.91 min, and

absence of antennal response suggested 1-octadecene is a female-produced compound. Gas chromatography quantification revealed the abundance of 1-octadecene samples collected from females alone was 33 µg compared to 13 g from males + females combined.

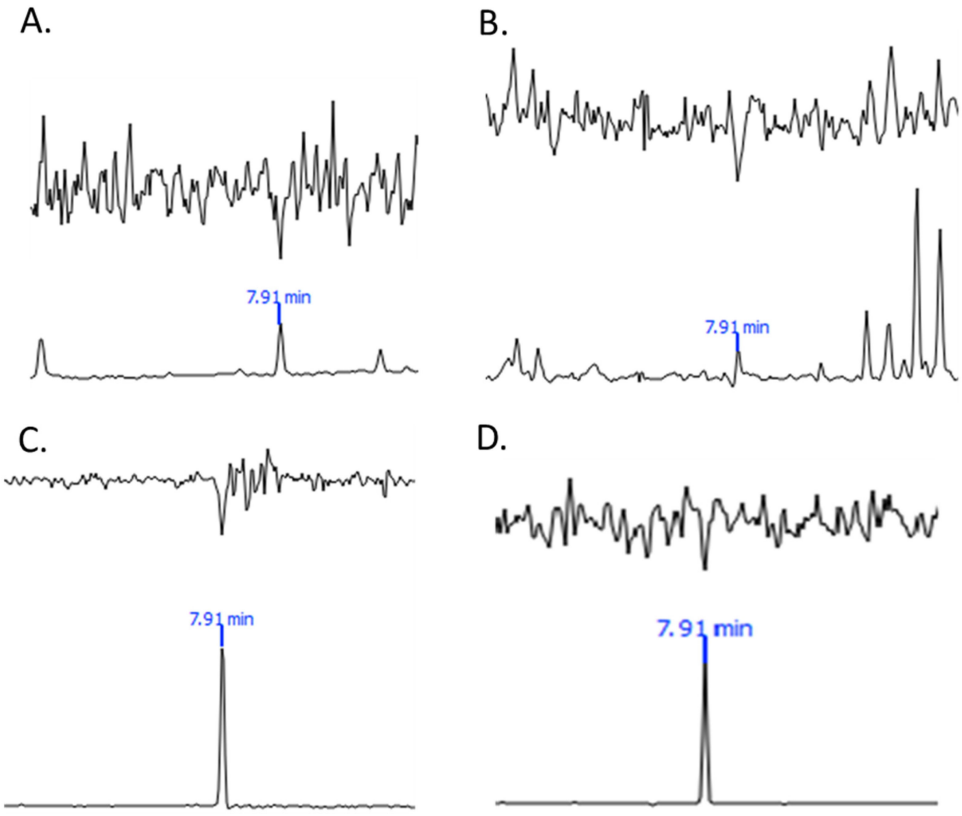

Figure 2. Representative traces of GC-EAD responses for volatiles collected from females and synthetic 1-octadecene. Antennal response (upper line) against gas chromatography-flame ionization detector (GC-FID) elute of female produced/synthetic compound (lower line), 7.91 min is the retention time of 1-octadecene. (**A**). Male antennal response to female produced volatiles; (**B**). Female antennal response to female produced volatiles; (**C**). Male antennal response to synthetic 1-octadecene; (**D**). Female antennal response to synthetic 1-octadecene.

3.2. Analysis of Air-Entrainment Samples by GC/MS

Analysis of the headspace of female and male beetles indicated quantitative differences in volatile profiles. Volatile headspace from females and synthetic compounds elicited a peak at 7.456 min (Figure 3A,B), and mass spectrometry confirmed the structure as 1-octadecene with M+ ion at m/z 252 and 224 [M+ -28] fragment after losing the C_2H_2 double bond fragment (Figure 4A,B). MS spectra, and KI's for synthetic 1-octadecene and female headspace compounds (physiologically active compounds) were identical as 1794 on the HP-5 column. GC-MS analysis of DMDS derivatives of the natural and synthetic 1-octadecene on HP-5 MS UI column revealed an adduct at 43.05 min, showed similar characteristic fragment ions at m/z 61 and 285 and M+ at m/z 346. Double bond at the first position in both natural and synthetic molecules confirmed the structure as 1-octadecene (Figure 5A–D). MS spectra of DMDS adduct of its position isomers 2-octadecene, 3-octadecene, and 4-octadecene (39.888 min, 38.115 min, and 36.835 min respectively in the FID profile) do not contain the same major fragment as in 1-octadecene. However, M+ 346 was the same for all isomers, having base peaks at m/z 271, 257 and 243 for 2-octadecene, 3-octadecene and 4-octadecene, respectively, confirmed the position of their double bonds (Figure 6).

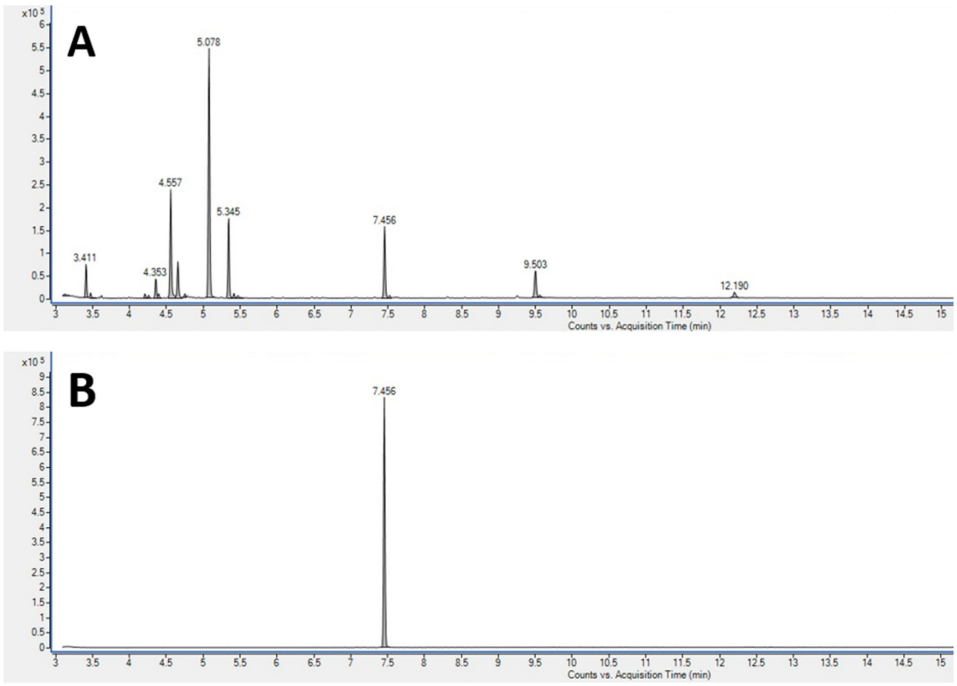

Figure 3. Gas chromatography analysis of Porapak extracts of air entrainment odors from female *X. quadripes*. (**A**) Natural female volatile, (**B**) Synthetic 1-octadecene, retention time at 7.45 min.

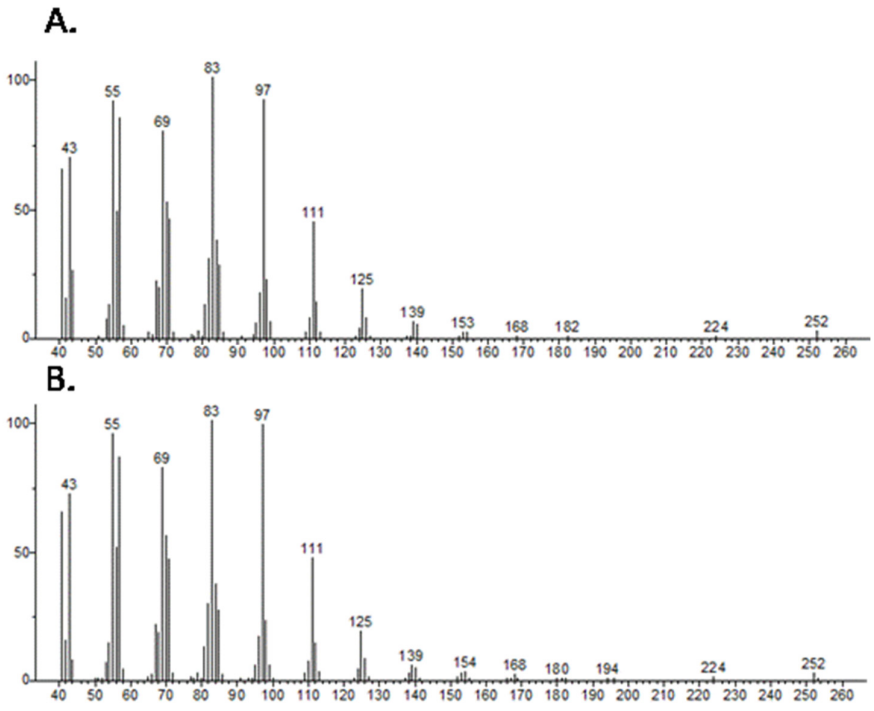

Figure 4. Electron impact ionization (70-eV) mass spectra of air entrainment extract from (**A**) female *Xylotrechus quadripes* and (**B**) synthetic 1-octadecene.

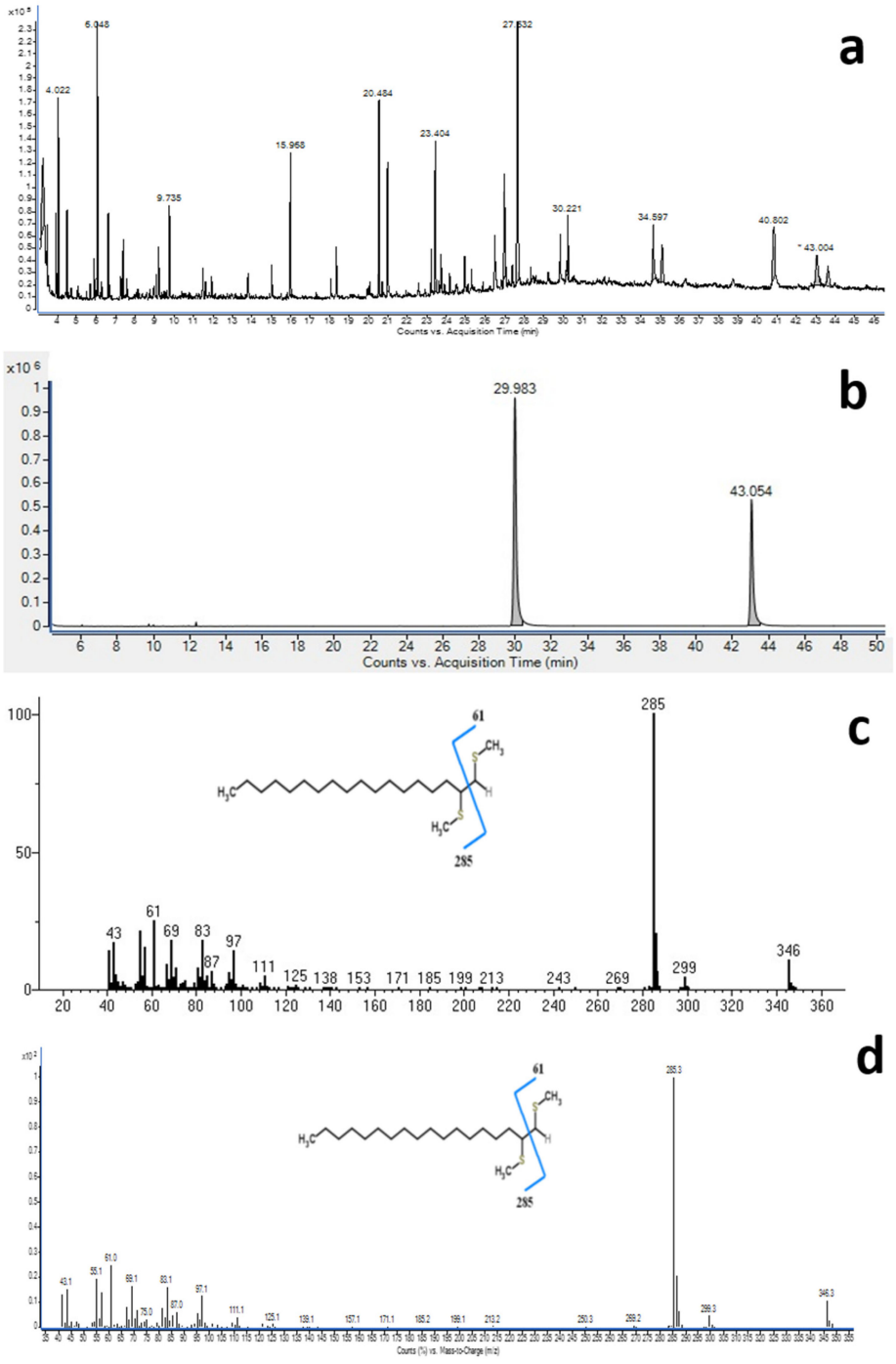

Figure 5. Analysis of DMDS adducts of the natural & synthetic female aggregation pheromone of *Xylotrechus quadripes* by GC (**a**) Natural volatile compounds; (**b**) Synthetic 1-octadecene; and by GC-MS (**c**) Natural volatile compounds; (**d**) Synthetic 1-octadecene.

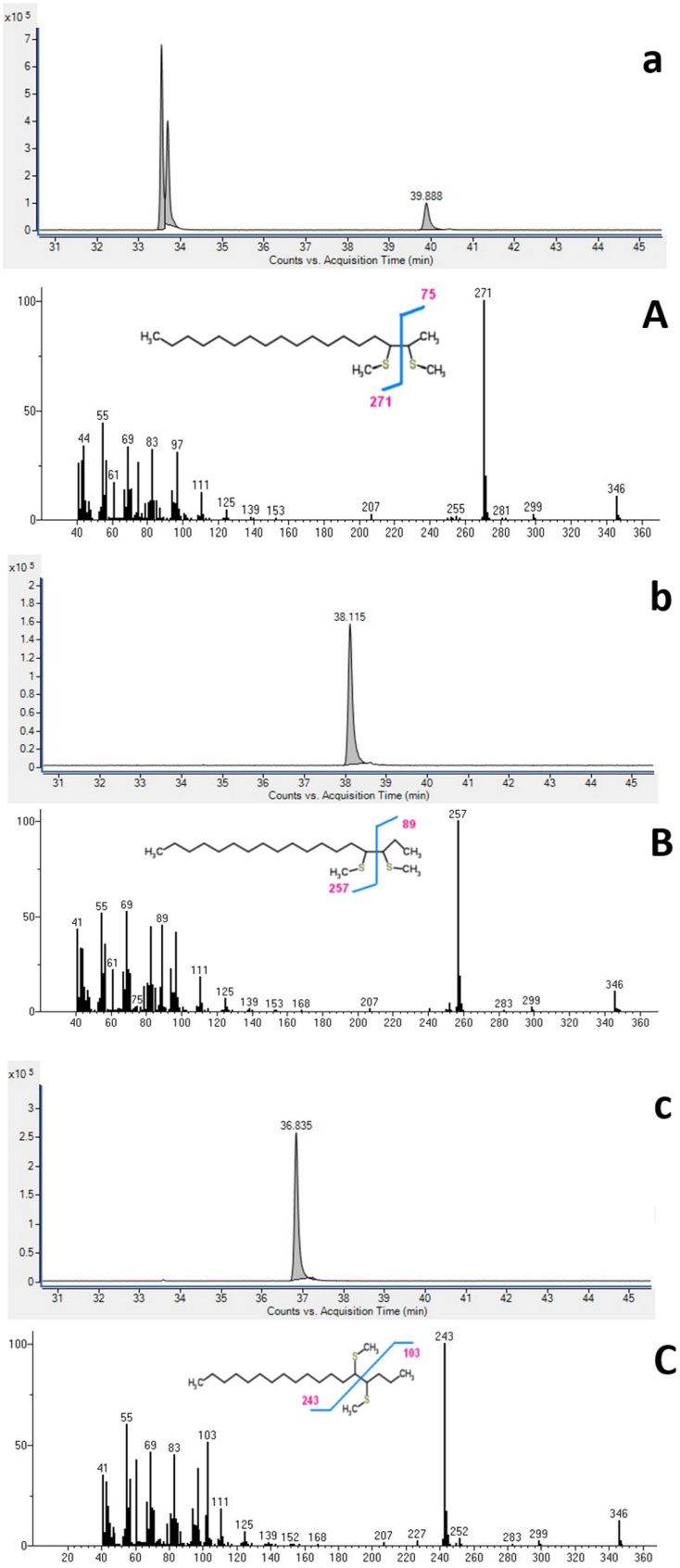

Figure 6. Analysis of DMDS adduct of closely related compounds of 1-octadecene by gas chromatography (**a**) 2-Octadecene; (**b**) 3-Octadecene; (**c**) 4-Octadecene, and by mass spectra (**A**) 2-Octadecene; (**B**) 3-Octadecene; (**C**) 4-Octadecene.

3.3. Physiological Response of Antennae

Electroantennography (EAG) revealed antennal responses of female and male CWSB to synthetic 1-octadecene were dose dependent (Figure 7). The antennal response of both female and male CWSB was significantly higher at 8 mg ($F_{4,20}$ = 5.742, $p < 0.001$, n = 5) and ($F_{4,20}$ = 7.131, $p < 0.001$, n = 5), respectively.

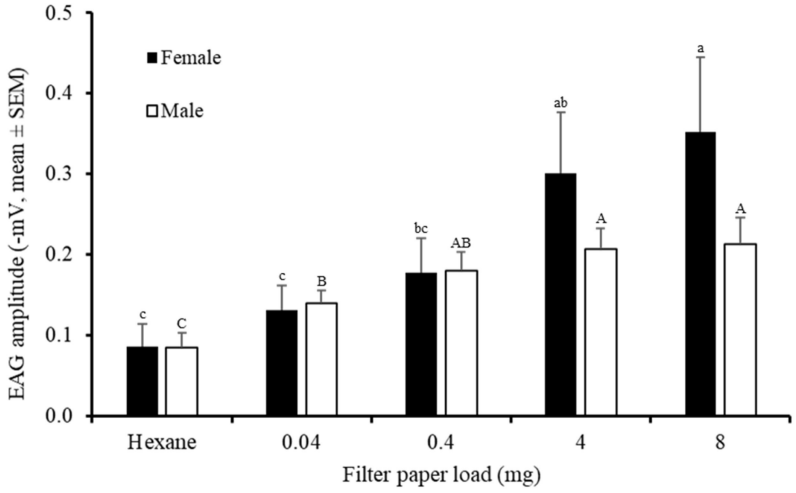

Figure 7. Dose-dependent response curve in mV of males and female antennae of CWSB to 1-octadecene. Same alphabetical letters indicate no significant difference between the doses tested, n = 5.

2-hydroxy-3-decanone:1-octadecene combined at different ratios (1:0, 1:2, 1:4, 1:6, 1:8 and 1:10) were evaluated separately against males and females, respectively. EAG response (mV) of female antennae was higher when stimulated with the 1:2 ratio (0.98 ± 0.44, mean ± SEM) ($F_{6,24}$ = 13.54, $p < 0.001$, n = 5), followed by 2-hydroxy-3-decanone alone (0.68 ± 0.21). Antennal responses to other ratios stimulated were at par with each other. A similar trend in antennal response was observed in males. EAG response of male antennae was higher with the 1:2 ratio (0.65 ± 0.31) ($F_{6,24}$ = 4.13, $p < 0.001$, n = 5) followed by 2-hydroxy-3-decanone alone (0.54 ± 0.24) and 1:6 (0.44 ± 0.16), 1:4 (0.41 ± 0.11), 1:8 (0.35 ± 0.15), 1:10 (0.30 ± 0.02) (Figure 8).

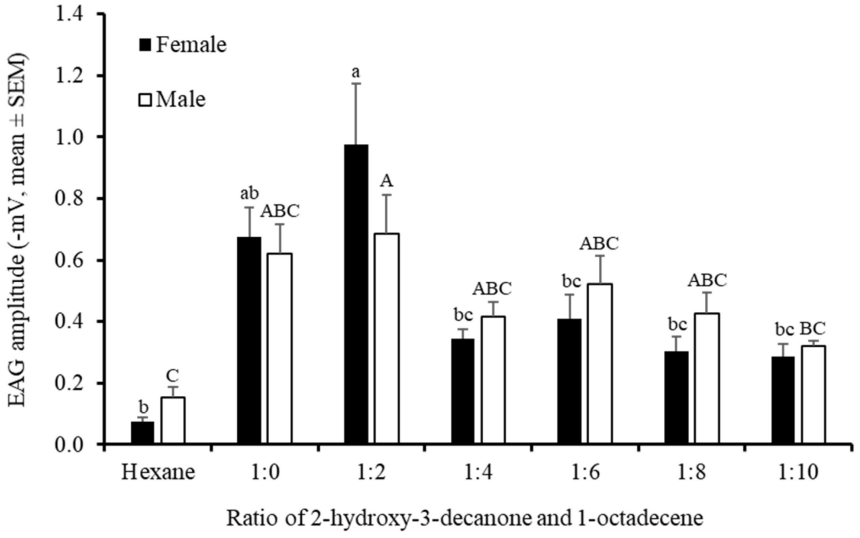

Figure 8. EAG response (Mean ± SEM) of CWSB female and male antennae stimulated with different ratios of 2-hydroxy-3-decanone and 1-Octadecene (n = 5). Same alphabetical letters indicate no significant difference among the different ratios tested.

3.4. Field Studies

More female catches (4.11 ± 1.03, mean ± SEM) were recorded in traps with a pair of male and female live beetles followed by traps baited with 150 mg of 2-hydroxy-3-decanone (2.78 ± 0.46), and control treatment trapped least number of beetles (1.33 ± 0.17). A similar pattern was observed in male CWSB beetles trapping. Only a few catches were found in the control traps (0.67 ± 0.17) (Figure 9). Field study results suggest 2-hydroxy-3-decanone, known to be an aggregation pheromone, did not catch a maximum number of beetles compared to the live beetles' trap.

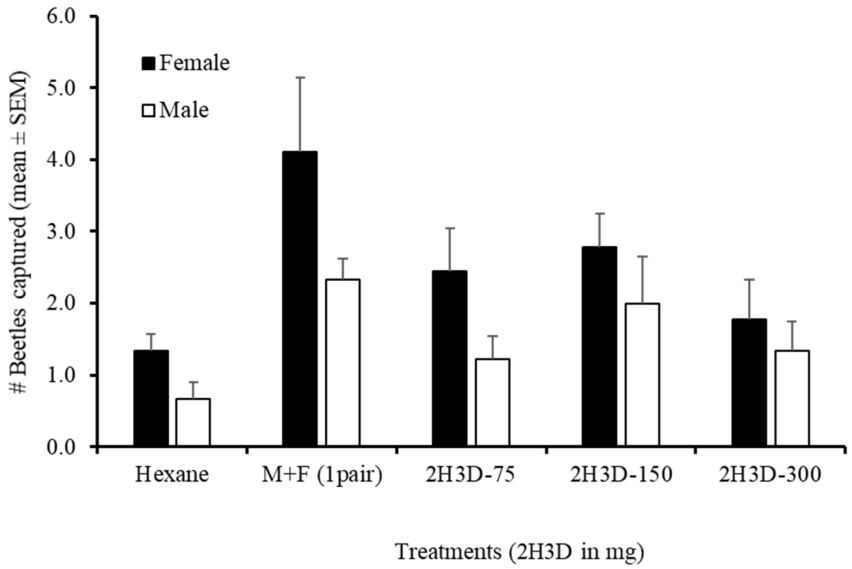

Figure 9. Number of male and female coffee white stem borer beetles in traps baited with different dose of male produced synthetic pheromone 2-Hydroxy-3-decanone (2H3D) and live insects (September–January 2012).

Field study to determine the synergistic effect of 2H3D combined with 1-octadecene revealed higher CWSB beetles (males and females). 2H3D and 1-octadecen at 1:2 ratio trapped significantly more number of beetles (1.98 ± 0.16) ($F_{4,12}$ = 3.47, p = 0.04, n = 4) followed by 1-octadecene alone (1.83 ± 0.28) and were significantly different from control. Although, other lures containing 2-hydroxy-3-decanone (1.50 ± 0.29) and 2-hydroxy-3-decanone:1-octadecene at 1:0.5 (1.26 ± 0.24) trapped more beetles but not significantly different from the control (0.93 ± 0.22) (Table 1).

Table 1. Number of coffee white stem borer male and female beetles trapped in traps baited with different ratios of 2-hydroxy-3-decanone and 1-octadecene (September–December 2013). Note: different letters in the column "CWSB beetle caught/trap" indicate significant differences between treatments.

Treatment No.	Treatments	CWSB Beetle Caught/Trap (Mean ± SEM)
T1	2-hydroxy-3-decanone:1-octadecene (1:0)	1.50 ± 0.29 ^{ab}
T2	2-hydroxy-3-decanone:1-octadecene (0:1)	1.83 ± 0.28 ^{a}
T3	2-hydroxy-3-decanone:1-octadecene (1:2)	1.98 ± 0.16 ^{a}
T4	2-hydroxy-3-decanone:1-octadecene (1:0.5)	1.26 ± 0.24 ^{ab}
T5	Hexane	0.93 ± 0.22 ^{b}
	F value	3.47

4. Discussion

Many studies have suggested the use of pheromones in insect communication [22], and here we report the 1-octadecene which acts as a female produced aggregation pheromone within Cerambycinae. A field study comparing different doses of male pheromone 2-hydroxy-3-decanone and trap with a pair of live male and female beetles trapped higher number of beetles in the latter. Similar observations were recorded where caged females attracted males in the field conditions [12]. Headspace collections in male and females indicated difference in the chromatogram. GC-MS volatile profile indicated that 1-octadecene was released by female CWSB and elicited GC-EAD and EAG responses from both antennae of male and female. 1-octadecene is found in chemical communication systems but not as a pheromone of Cerambycidae [22]. GC-MS analysis of natural volatile and synthetic 1-octadecene confirms the same molecular structure and the DMDS adduct of natural and synthetic. The terminal double bond was confirmed by the typical characteristic fragment ions in its MS. No match of retention time and MS fragmentation of DMDS adduct of other isomers *viz.*, 2-octadecene, 3-octadecene and 4-octadecene with that of the natural volatile and adducts, confirmed the structure as 1-octadecene. This was further confirmed by comparison of Kovat's retention Index of natural and synthetic compounds.

Electroantennography and GC-EAD results indicate 1-octadecene was found to elicit responses at both physiological and behavioral levels, and synergistic effect when combined with male produced pheromone 2-hydroxy-3-decanone. In both EAG and GC-EAD, female CWSB were quite sensitive to 1-octadecene compared to males. Responses were dose-dependent, which is a vital information for the standardization of pheromone loading in the dispenser. Similar observations were recorded in many species in the subfamilies Aseminae and Cerambycidae [23].

Male pheromone of CWSB identified as 2-hydroxy-3-decanone [11,12], used as a potential aggregation pheromone for the management of CWSB beetles. The present laboratory and field studies showed a stronger attraction to a blend of 2-hydroxy-3-decanone and 1-octadecene in 1:2 proportions than the single male pheromone 2-hydroxy-3-decanone corroborates our hypothesis. Previous behavioral study indicates that traps baited with live females and males capture large numbers of beetles than the traps baited with single-sex or male sex pheromone [15]. Traps with female pheromone 1-octadecene obtained a higher number of trapped beetles than the male pheromone 2-hydroxy-3-decanone indicating the potential of this new female pheromone. A study in the past proved 2H3D elicited antennal response but failed to trap any beetles with both multi-funnel and delta traps despite higher beetle density in the field condition [14]. However, cross-vane traps lured with 2H3D:1-octadecene trapped CWSB beetles despite the lower infestation level. This suggests 1-octadecene may synergize the CWSB attraction towards 2H3D. Cross-vane traps were more effective than multi-funnel traps for trapping cerambycid beetles [24], in our study we have tested the single and mixture of compounds using cross-vane traps may also have improved the CWSB captures.

1-octadecene must be considered as aggregation pheromone as a similar ratio of males and females in lured in traps with 2H3D and 1-octadecene. However, a study [12] reported traps with 2H3D trapped a smaller number of male CWSB, and were equal to traps without lures (control traps). Similar results were reported in other *Xylotrechus* species [25,26]. In *Neoclytus acuminatus acuminatus* male produced pheromone trapped both males and females, however, synthetic compounds were not tested [27]. We speculate that live *N. acuminatus* females emitted volatiles to elevate the aggregation behavior, which is similar to our study findings.

Addition of food attractants to synergize the trapping efficiency should be evaluated. However, *X. quadripes* adult's do not feed on the host-plant [5,28]. Addition of oviposition odorants in large quantities to mask the secondary metabolites released by coffee plants might reduce the egg load. The field studies reported here were limited two seasons and the beetle's density was comparatively lower. Further studies should be carried out in

coffee fields to determine the dosage, and the number of traps to be installed in the field for standardized monitoring, mass trapping and commercialization.

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