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Sex Attractant Pheromone from the Rice Stalk Stink Bug, *Tibraca limbativentris* Stal

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Abstract The male-produced sex pheromone from the Brazilian rice stalk stink bug *Tibraca limbativentris* is reported. Olfactometer bioassays with sexually mature males and females showed that males attracted females, which suggests that males release a sex pheromone. Males were not attracted to either sex, nor were females attractive to conspecific females. Attraction of the females to males was highest at night. The headspace volatiles collected from male and female bugs were analyzed by gas chromatography (GC) and GC–mass spectrometry. Two male-specific compounds were identified as isomers of 1′*S*-zingiberenol, whereas a series of defensive compounds were identified in extracts from both sexes. Zingiberenol has three chiral centers, and the nonselective syntheses used produced two groups of isomers, zingiberenol I containing four isomers, namely (1*RS*,4*RS*,1′*R*)-4-(1′,5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol, and zingiberenol II containing the other four isomers, namely (1*RS*,4*RS*,1′*S*)-4-(1′,5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol. Both groups of stereoisomers were more attractive than hexane

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controls. The absolute configuration of the insect-produced pheromonal components remains to be elucidated, but the 1′*S* stereochemistry was established for at least one of the isomers.

Key words Semiochemical · zingiberenol · (1*RS*, 4*RS*, 1′*S*)-4-(1′, 5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol · allomone

Introduction

The rice stalk stink bug, *Tibraca limbativentris* Stal, 1860 (Hemiptera: Pentatomidade), is a neotropical pest of paddy rice in Brazil, Argentina, and Uruguay (Panizzi et al., 2000). Recently, it was reported in the Dominican Republic (Jorge, 1999) and also in Colombia, Ecuador, Panama, Costa Rica, and Suriname, and is a potential threat to the United States. Introduction into North America may result in serious losses to U.S. and Mexican rice production. Other economically important crops may serve as host plants as well (North American Plant Protection Organization-Phytosanitary Alert System; http://nappo.org/).

Tibraca limbativentris is a damaging insect to rice in Brazil. Feeding of the insects at the base of the plants in the vegetative stage causes the phenomenon known as "dead heart", and feeding during the reproductive stage causes the plants to exhibit damage known as "white panicle" (Ferreira et al., 1997). Bug populations have been high for several consecutive years (up to 200 individuals/m^2), causing production losses varying from 5% to 80% (Ferreira et al., 1997 and references therein). This species occurs in almost all states of Brazil and is sometimes found on alternative hosts such as soybean, tomato, wheat, and native Gramineae (Panizzi et al., 2000 and references therein).

The control of *T. limbativentris* is heavily based on chemical pesticides (Usta et al., 1994; Ferreira et al., 1997; Costa and Link, 1998/99; Panizzi et al., 2000). For example, in Colombia, stink bugs in rice require up to three insecticide applications per season (Pantoja et al., 1995).

Because of the habit of *T. limbativentris* feeding in the upside-down position at the base of the plants, when the damage is perceived by growers, the economic threshold has already been reached. Thus, pheromone-baited traps might be a useful tool for monitoring these pests. The objectives of the research described herein were to determine whether *T. limbativentris* produces a male-specific sex pheromone, as reported for other phytophagous stink bug species, and, if so, to identify the chemical structure(s) of the biologically active compound(s).

Methods and Materials

Insects

Tibraca limbativentris adults were from a colony started with adults collected near Embrapa Rice and Beans, Goiania-Goiás (16°4′S and 49°14′W). Insects were reared on rice plants, *Oryza sativa*, potted in a large (5 kg) pot in a green house and kept as a stock colony. In the laboratory, where the knowledge of sexual maturity (age) was necessary for bioassays and collection of volatiles (aeration experiments), bugs were reared in 5-l plastic containers with the food supply being renewed 3 times per wk (Cavalcante et al., 2004), at 26 ± 1°C and 65 ± 10% relative humidity under 14:10 light/dark photoperiod (from 06:00 to 20:00 hr).

Most of the egg masses were laid on the rice plants, and 3 times a week these plants were transferred to new plastic containers where the nymphs were reared as above.

To prevent interactions between the sexes, males were separated from females after their imaginal molt and cuticular hardening (ca. 24 hr). For all experiments, sexually mature adults (>15-d-old) were used (Cavalcante et al., 2004).

Collection of Volatiles

Volatiles were collected from individual groups of 15 male or female *T. limbativentris* ($N = 50$). The insects were gently introduced into 1-l glass containers (Zhang et al., 2003) to minimize emission of defensive secretions. Air was drawn into the container through 4–12 mesh-activated charcoal (Fisher Scientific, Pittsburgh, PA, USA), and out of the container through two traps (15 × 1.5 cm OD) containing Super Q (200 mg each; Alltech Associates, Inc., Deerfield, IL, USA) by vacuum pump (~1 l/min). The adsorbent traps were eluted with hexane (4 × 0.5 ml per sample) and each sample was concentrated to approximately 100 µl under a gentle flow of nitrogen for further analysis. Insects were fed with fresh green beans (*Phaseolus vulgaris*; replaced three times a week) and aerated continuously for 15 d, changing the adsorbent traps daily, to get enough material for bioassays and chemical analysis. To check the emanation of any volatiles from the green beans that would contaminate the airborne collection from the above experiment, the same procedure was carried out with three green beans inside the glass chambers and the volatiles were collected for 24 hr for 4 d ($N = 12$).

Olfactometer Bioassays

A two-choice olfactometer modified from Borges and Aldrich (1994) was used to test the biological activity of live insects, insect aeration extracts of *T. limbativentris*, and synthetic compounds. The olfactometer release chamber was a 500-ml, three-neck, round-bottomed flask (all 24/40 joints, Kontes, Vineland, NJ, USA). Two 250-ml rotary evaporator trap adapters (24/40 joints) were attached to each side arm of the release flask (the treatment and control arms). A charcoal (20/40 mesh) filter (130 × 10 mm ID) was attached to the side arms using two 40-cm-long pieces of a Silastic tubing (3/16 inch ID × 5/16 inch OD; VWR Scientific Corporation, Darmstadt, Germany), inserted in a "Y" connector of the same diameter and connected to adapters (24/40 joint) on each side arm of the olfactometer. The air was humidified by passage through a container of distilled water between the charcoal filter and the arms of the olfactometer. The middle neck of the flask was connected to the vacuum pump with an adapter and the air flow was adjusted with a "Clear Flow Rotameter" (Accura Flow Products, Warminster, PA, USA) to a flow of 0.8 l/min. The apparatus was positioned horizontally on a countertop in a room with bright fluorescent lights (4 × 40 W) during daytime conditions, and lighted with red lamps (4 × 60 W) during scotophase. The temperature in the bioassay room was maintained at 26.0 ± 1.0°C. The positions of the olfactometer arms were inverted between control and treatments after each three repetitions to avoid any positional bias. The apparatus was cleaned with fragrance-free liquid soap, rinsed thoroughly with water, and dried at 80°C, after each five replicates. The insects were placed in the round-bottom flask (release chamber), and the treatments were placed at the end of the reducing adapter chamber (treatment arms).

A single *T. limbativentris* adult was gently introduced into the release chamber of the Y-tube olfactometer with the aid of an artist's paint brush (Camel Hair, number 1), and its pattern of behavior (response) was recorded for 15 min/replicate. The duration of each

bioassay replicate was monitored using a stopwatch. Before testing, the insects were allowed to acclimatize for a short period (ca. 3 min) in the release chamber while assembling the treatment chambers. The first choice of the insect was recorded, i.e., the first arm of the olfactometer that the insect chose and remained in for at least 100 sec. The test insects were used only once during the bioassays.

Determination of Period of Maximum Activity

To test if *T. limbativentris* had a daily cycle of sexual activity, bioassays were carried out to determine the cycle of maximum response by testing insect responses to volatiles from live bugs used as the pheromone source. For this experiment, the following periods were designated: morning (M = 06:00–12:00), afternoon (A = 12:00–18:00), and night (N = 18:00–24:00). Fifty replicates were carried out for each time period and the number of insects attracted to the pheromone source was recorded.

Bioassays with Living Insects

Experiments were carried out to determine whether males were the pheromone-producing sex, analogous to other phytophagous stink bug species (Borges et al., 1987; Aldrich et al., 1987, 1994; Borges and Aldrich, 1994; Borges, 1995; McBrien and Millar, 1999; Millar, 2005; Moraes et al., 2005). The attraction of insects of both sexes to odors from males or females was tested, respectively, by offering test individuals a choice between odors from live insects and a clean air control ($N = 50$ for each combination). Because observations during rearing indicated peak mating activity occurred at night, bioassays were performed in the evening and scotophase.

Bioassays with Insect Extracts and Synthetic Compounds

The same bioassay procedures described above were used to compare the biological activity of aeration extracts and authentic synthetic standards. The solution of test stimulus was one individual equivalent (IE) of aeration extract spotted on a strip of filter paper (1.5 cm long and 0.5 cm wide). Controls consisted of filter papers treated with hexane. Because females were attracted to male odor, and all other combinations with live insects did not show a positive response, only females were used as responders in all subsequent bioassays. Bioassays ($N = 15$–31) with an aeration extract from males or females were carried out using an extract obtained from a 24-hr aeration of 15 bugs.

Based on experimental findings with 0.01, 0.1, and 1 mg/ml, bioassays were conducted with 10 μl of 0.1 mg/ml, (i.e., 1000 ng of the synthetic compound) loaded on a strip of filter paper (1.5 cm long and 0.5 cm wide). The responses of *T. limbativentris* females to two sets of zingiberenol isomers (zingiberenol I comprising the four stereoisomers of the group (1*RS*, 4*RS*, 1′*R*)-4-(1′,5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol ($N = 56$) and zingiberenol II comprising the four stereoisomers of the group (1*RS*, 4*RS*, 1′*S*)-4-(1′,5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol ($N = 71$) were compared to a hexane control. In addition, zingiberenol I was compared against zingiberenol II ($N = 41$).

Chemical Analysis

Extracts were analyzed by gas chromatography (GC; HP-6890, HP-1 column, 50 m × 0.25 mm ID, 0.25 μm film, J&W Scientific, Folsom, CA, USA), by using a

temperature program of 30°C for 1 min, 5°C/min to 150°C for 0.1 min, 10°C/min to 250°C for 20 min. Samples (1 μl) were injected in cool on-column mode with hydrogen carrier gas, and a flame ionization detector (FID) at 270°C.

Extracts were analyzed by coupled GC–mass spectrometry (GC–MS) by using a Thermo-Finnigan MAT95XP magnetic sector mass spectrometer, which was directly coupled to a TRACE 2000 GC. The GC was equipped with a DB-1 column (50 m × 0.25 mm ID, 0.25 μm film, J&W Scientific) and a cool on-column injector. The carrier gas was helium. Ionization was by electron impact (70 eV, source temperature 200°C). Data were collected and analyzed with Xcalibur software.

The components of aeration extracts from both sexes of *T. limbativentris* were tentatively identified by comparison of GC retention times with those of synthetic standards using polar and nonpolar columns (HP-Wax column, 30 m × 0.3 mm ID, 0.45 μm film or HP-1 column, 50 m × 0.25 mm ID, 0.25 μm film, both from J&W Scientific) with the temperature programs described above. Chemical identifications were confirmed by comparison of retention time and MS data with authentic standards and with MS library data (NIST, Saturn library 2000).

To confirm the presence of an alcohol group in the putative pheromone components, microsilylation was carried out using *N*-methyl-(trimethylsilyl)-trifluoroacetamide (MSTFA) in a 1-ml glass vial. An aliquot of 50 μl was taken from an extract of volatiles of 20 males of *T. limbativentris*, concentrated under a stream of nitrogen flow almost to dryness, and redissolved in 100 μl of MSTFA. The sample was heated in a water bath at 60°C for 1 hr, and analyzed directly by GC–MS using the conditions described above. The same reaction was conducted with a standard solution of zingiberenol (2 μl of 100 mg/ml), prepared as described below.

Synthesis of 4-[(1′*R*)-1,5-Dimethylhex-4-enyl]-1-Methylcyclohex-2-en-1-ol

Zingiberenol mixtures were prepared by Fuji Flavor Co., Ltd. (Tokyo, Japan), with the help of Dr. Tatsuji Chuman. The eight stereoisomers of zingiberenol were prepared in two samples, with each of the samples containing four diastereoisomers. The zingiberenol I mixture contained the (1*R*,4*R*,1′*R*), (1*S*,4*R*,1′*R*) (1*R*,4*S*,1′*R*) and (1*S*,4*S*,1′*R*) stereoisomers, whereas Zingiberenol II contained the (1*R*,4*R*,1′*S*), (1*S*,4*R*,1′S), (1*R*,4*S*,1′*S*), and (1*S*,4*S*,1′*S*) stereoisomers (Figure 1). The syntheses of zingiberenols I and II were conducted following method II from Hagiwara et al. (2002). In brief, the 4-[(1′*R*)-1,5-dimethylhex-4-enyl]-1-methylcyclohex-2-en-1-ols were synthesized from (*R*)-citronellal. GC–MS *m/z* 222, 207, 204, 189, 179, 161, 151, 137, 123, 119, 109, 95, 77, 69, 55. IR (KBr): 3352, 2927, 2858, 1450, 1377, 1122, 980 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$), δ: 0.81 (d, 1.2 H, J = 6.9 Hz), 0.84 (d, 0.3 H, J = 7.5 Hz), 0.86 (d, 1.2 H, J = 6.9 Hz), 0.88 (d, 0.3 H, J = 7.5 Hz), 1.10–2.14 (m, 11 H), 1.27 (s, 3 H), 1.60 (s, 3 H), 1.69 (broad s, 3 H), 5.06–5.12 (m, 1 H), 5.50–5.65 (m, 2 H). The compounds 4-[(1′*S*)-1,5-dimethylhex-4-enyl]-1-methylcyclohex-2-en-1-ol were synthesized using the same procedure starting from *S*-citronellal, and gave analogous spectra.

Chemicals

Decanal, nonanal, limonene, 6-methyl-5-hepten-2-one, decane, (*E*)-2-octenal, tridecane, tetradecane, and pentadecane were purchased from Sigma Aldrich (Gillingham, Dorset, UK). α-Copaene, α-pinene, β-pinene, and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were purchased from Fluka (Steinheim, Germany).

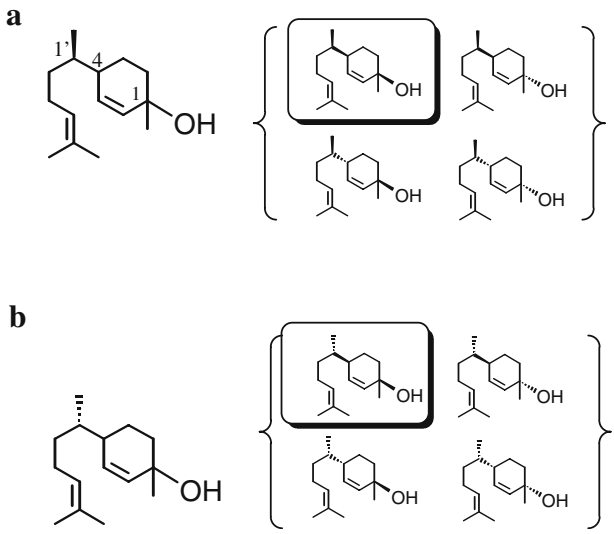

Fig. 1 (a) Zingiberenol I, containing the four isomers, (1*RS*,4*RS*, 1R)-4-(1′,5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol. (b) Zingiberenol II, containing the four isomers (1*RS*, 4*RS*, 1′*S*)-4-(1′,5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol

Statistical Analysis

Data on the responses of males and females to different treatments (live insects, aeration extracts, and synthetic compounds vs. control) were analyzed using χ^2 tests, for first choice. Only the insects that left the release chambers of the olfactometer were considered for the analyses. Treatments in which the "nonresponders" (insects that fail to respond in each of the treatments) were >70% were not statistically analyzed.

Results

Determination of Period of Maximum Activity

Bioassays with virgin sexually mature females showed that the attraction to males was strongest during the night period (18:00–24:00 hr). During the morning and afternoon period, >90% of the insects tested did not leave the release chamber of the olfactometer,

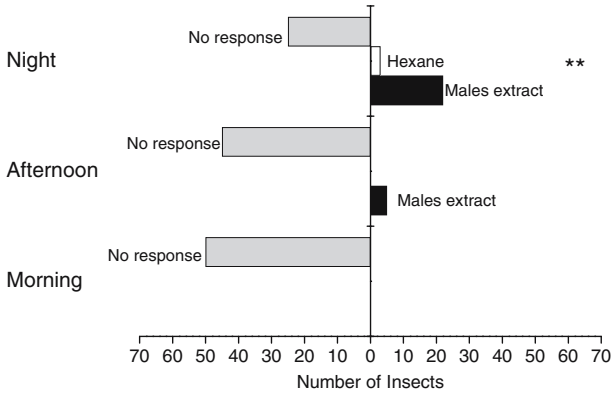

Fig. 2 Number of female *T. limbativentris* responding to live males during different periods of the day and night in laboratory bioassays. Morning; 8:00–12:00 hr; afternoon, 12:00–18:00 hr; and night, 18:00–24:00 hr ($\chi^2 = 14.44$, $P < 0.001$, $N = 50$). Left-hand side: Number of insects that failed to respond in each of the treatments. $^{**}P < 0.01$

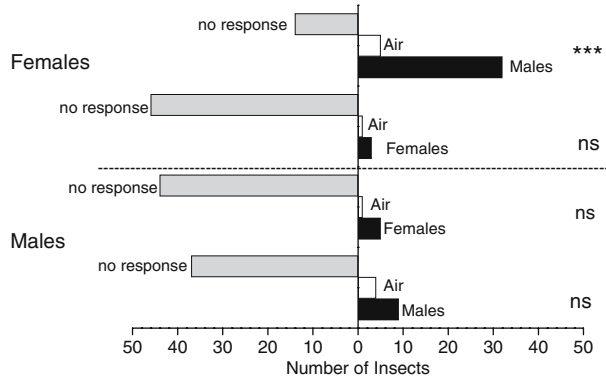

Fig. 3 Response of *T. limbativentris* (first choice) males and females in olfactometer arms responding to different treatments: females attracted by males ($\chi^2 = 19.70$, $P < 0.001$, $N = 50$), females attracted by females, males attracted by females, and males attracted by males, recorded during evening and scotophase under laboratory conditions ***$P < 0.001$

indicating that this species has a crepuscular/nocturnal sexual activity cycle (Figure 2). Therefore, all subsequent bioassays were performed during this period.

Bioassays with Live Insects

In the Y-tube olfactometer bioassays, female bugs were attracted to odors of live males, but not to odors of live females (Figure 3). Males were not attracted to odors of either sex (Figure 3). These results, in concordance with the analyses of insect extracts, show that males produce a sex pheromone.

Bioassays with Insect Extracts and Synthesized Compounds

Tibraca limbativentris females were more attracted to male extract (treatment arm) than to the hexane control (Figure 4). Male extract did not attract males, and extracts of females were not attractive to either sex (Figure 4). Following results with live insects and extracts, and chemical analyses, the responses of *T. limbativentris* females to zingiberenol I comprising the four stereoisomers, (1*RS*, 4*RS*, 1′*R*)-4-(1′,5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol, and to zingiberenol II comprising the four stereoisomers, (1*RS*, 4*RS*, 1′*S*)-4-(1′,5′-dimethylhex-4′-enyl)-1-methylcyclohex-2-en-1-ol, were tested in olfactometer bioassays. First, zingiberenols I and II were tested against male extract and in both bioassays the synthetic compounds were as attractive as extracts from males (Figure 5). When zingiberenols I and II were compared against each other, the two were equally

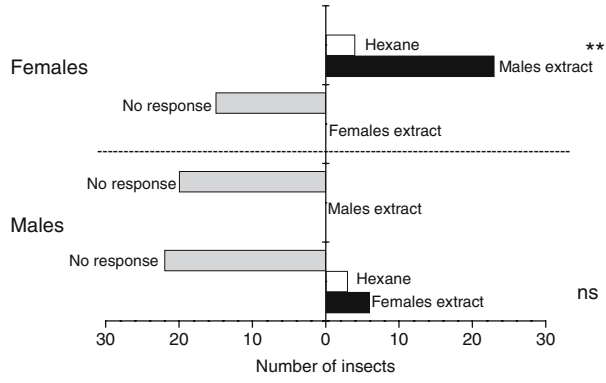

Fig. 4 Number of *T. limbativentris* (first choice) males and females in olfactometer arms responding to different treatments: females attracted by extracts from males ($\chi^2 = 13.37$, $P < 0.001$, $N = 27$), females attracted by extracts from females, males attracted extracts from males, and males attracted by extracts from females ***$P < 0.001$

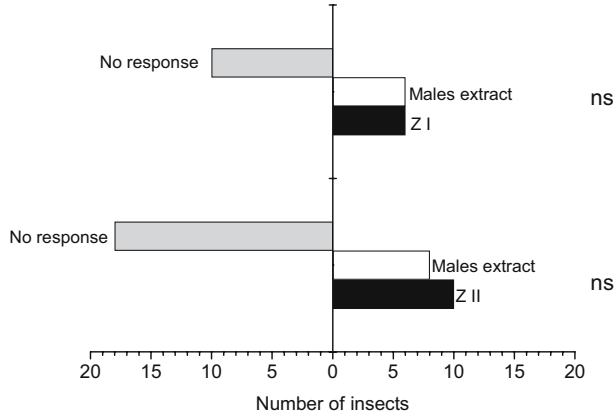

Fig. 5 Number of *T. limbativentris* (first choice) females in olfactometer arms responding to different treatments: females tested to extracts from males (1 IE) and zingiberenol I (Z I; 1000 ng), and females tested to extracts from males (1 IE) and zingiberenol II (Z II; 1000 ng). ns: not significant

attractive (Figure 6). However, when zingiberenols I and II were compared against neutral controls, only zingiberenol II was attractive (Figure 6). These results suggest that at least one of the stereoisomers in the zingiberenol II blend is a sex pheromone component for this species.

Chemical Analysis

Comparison of aeration extracts collected from both sexes of *T. limbativentris* by GC and coupled GC–MS showed that aeration extracts from males contained at least two male-specific compounds, in addition to several compounds that were common to both sexes (Figure 7). Compounds found in both sexes were identified by comparison with authentic standards as α-pinene (1), β-pinene (2), 6-methyl-5-hepten-2-one (3), decane (4), limonene (5), (*E*)-2-octenal (6), nonanal (7), dodecane (8), decanal (9), tridecane (10), α-copaene (11), tetradecane (12), pentadecane (13), and hexadecane (14), by using both polar and apolar columns. The GC analysis of control extracts obtained from aerations of green beans for 4 d did not show any compound detectable by FID.

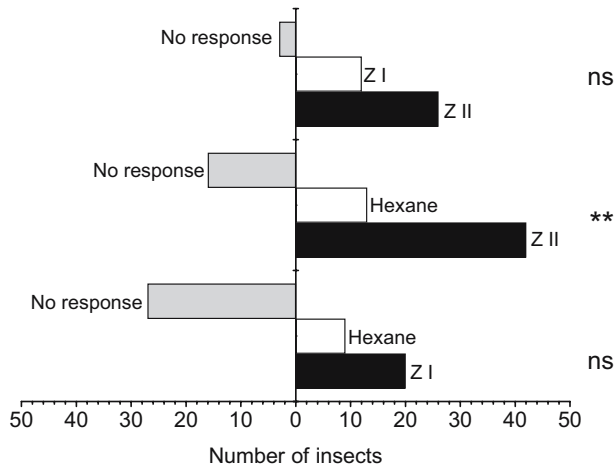

Fig. 6 Number of *T. limbativentris* (first choice) females in olfactometer arms responding to two isomer groups of zingiberenol: 1000 ng of the zingiberenol I blend ($\chi^2 = 4.17$, $P = 0.041$, $N = 29$) versus hexane as a control and to 1000 ng of zingiberenol II versus hexane as a control ($\chi^2 = 7.64$, $P < 0.01$, $N = 55$), and zingiberenol I versus zingiberenol II. $^{**}P < 0.01$

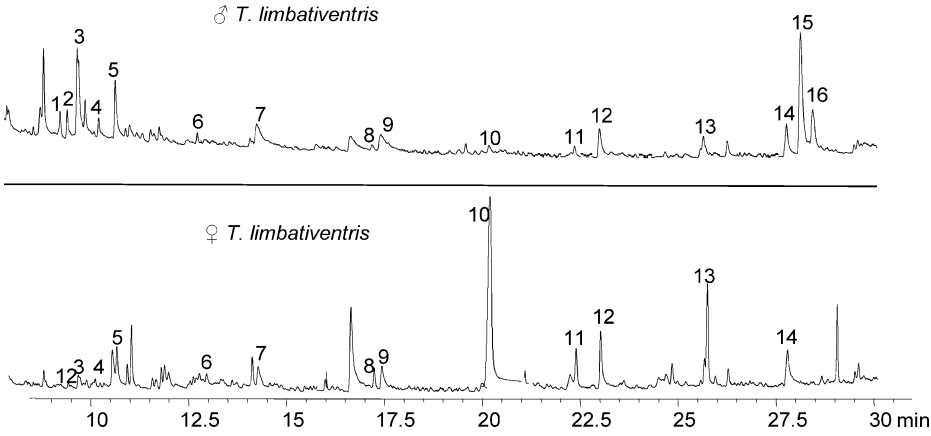

Fig. 7 Gas chromatograms of the volatile chemicals collected from live male (top) and female (bottom) *T. limbativentris*. 1, α-pinene; 2, β-pinene; 3, 6-methyl-5-hepten-2-one; 4, decane; 5, limonene; 6, (*E*)-2-octenal; 7, nonanal; 8, dodecane; 9, decanal; 10, tridecane; 11, α-copaene; 12, tetradecane; 13, pentadecane; 14, hexadecane; 15 and 16, isomers of (1*RS*,4*RS*,1′*S*)-zingiberenol II

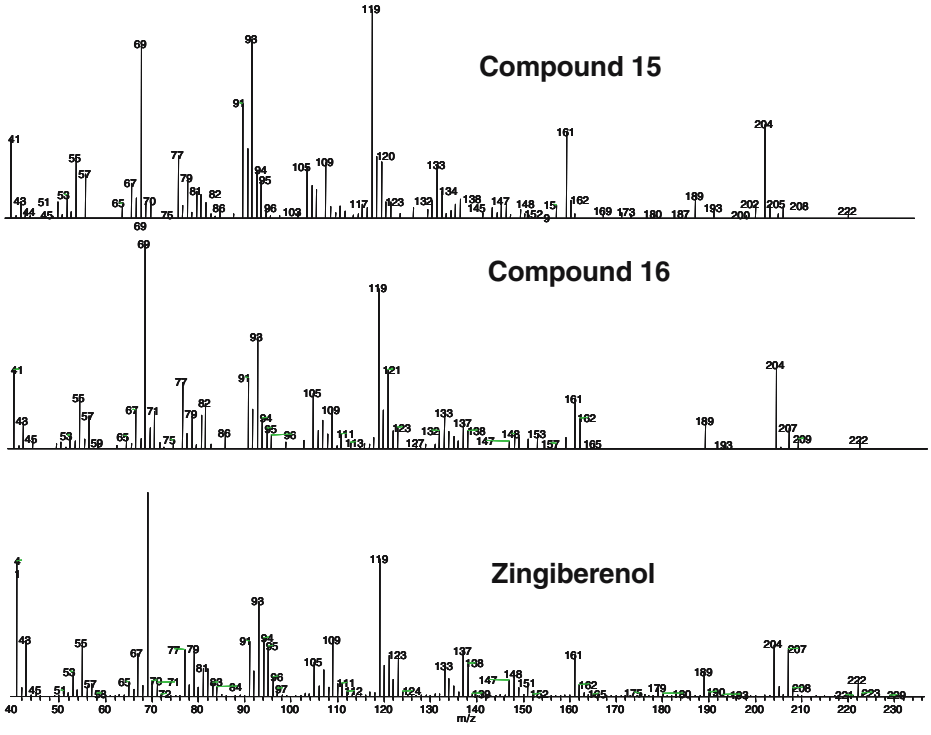

Fig. 8 Mass spectral data of compounds 15 (top) and 16 (center) obtained from volatiles collected from live males, and mass spectra from synthetic zingiberenol (bottom)

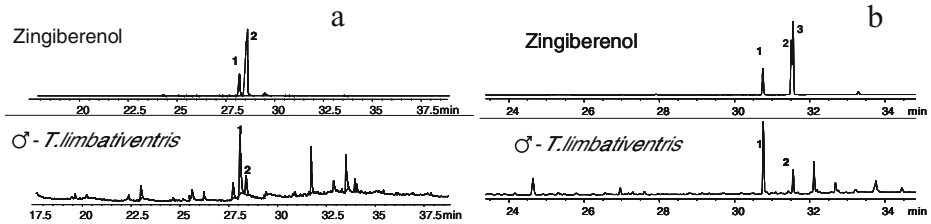

Fig. 9 Gas chromatogram of volatile chemicals collected from live male *T. limbativentris* and of a standard solution of synthetic zingiberenol containing the four diastereoisomers. (a) Polar column, DB-WAX. (b) Nonpolar column, HP-1. Numbers 1 and 2 indicate isomers of zingiberenol. Two diastereoisomers were resolved on the HP-1 column and three diastereoisomers were resolved on the DB-WAX column

The fragmentation patterns for compounds 15 and 16 suggested a sesquiterpene-type structure with an alcohol group, with a possible molecular ion at *m*/*z* 222, and an ion at *m*/*z* 204 (M-18), arising from loss of water (Figure 8). The NIST library matched the fragmentation pattern of compounds 15 and 16 with the fragmentation pattern of sesquisabinene hydrate or zingiberenol (Figure 8). Comparison of the GC retention times of compounds 15 and 16, by using polar and nonpolar columns, with synthetic zingiberenol isomers (I and II) containing the four diastereisomers, was carried out (Figure 9a and b). Compounds 15 and 16 coeluted with the two isomers of zingiberenol solution II that were resolved on the HP-1 column. Because zingiberenol has three chiral centers (positions 1′, 1, and 4), there are eight isomers (four enantiomeric pairs of diastereomers). Zingiberenols I and II contained four diastereoisomers each and are "enantiomers." Although diastereoisomers frequently can be separated on an achiral column, the zingiberenol I and II mixtures separated into only two peaks on the HP-1 column. Therefore, zingiberenols I and II were silylated to obtain a better separation of the four diastereoisomers on an achiral column. The derivatization was carried out using *N*-methyl-(trimethylsilyl) trifluoroacetamide (MSFTA) with the male aeration extract and the zingiberenol (I and II) mixtures. The four derivatized diastereoisomers in zingiberenols I and II were indeed separated on the HP-1 column (Figure 10a). For the derivatized aeration extract of male *T. limbativentris*, compounds 15 and 16 disappeared and three new compounds were generated, which

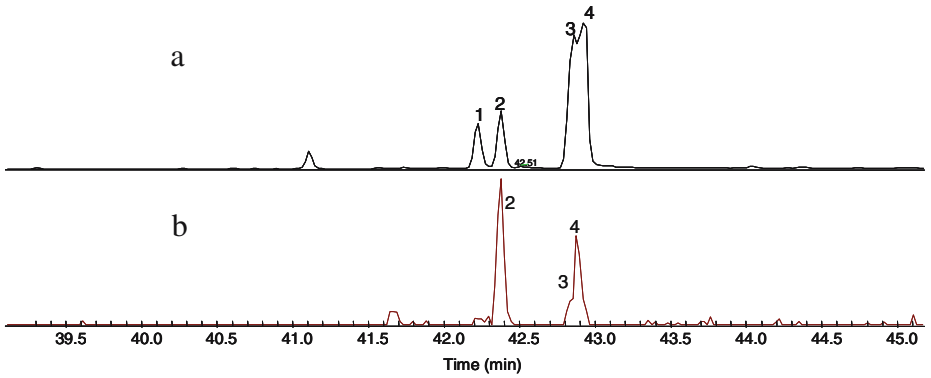

Fig. 10 Gas chromatogram showing four isomers of silanized zingiberenol ($C_{15}H_{28}O$-TMS) (top) and the three peaks obtained from derivatization of the sample obtained from airborne collection of volatiles of male *T. limbativentris* (bottom)

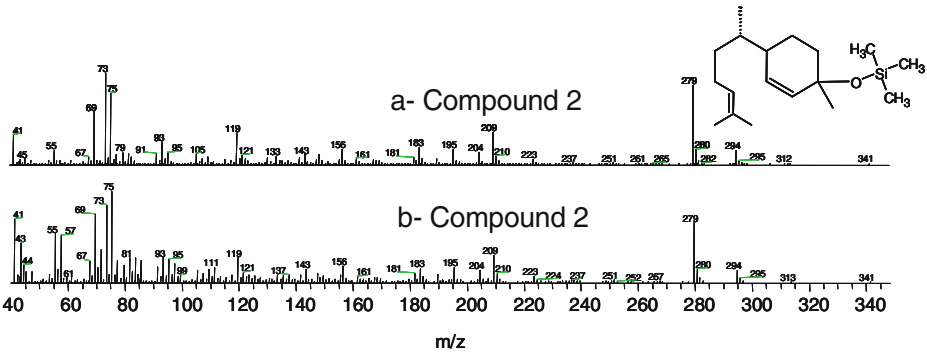

Fig. 11 Mass spectral data showing the pattern of fragmentation. (a) Compound 2: silanized zingiberenol (from Fig. 5a) ($C_{15}H_{28}O$-TMS). (b) Compound 2 (from Fig. 5b), obtained from derivatization of samples obtained from collection of volatiles of male *T. limbativentris*

matched the retention times of three of the four derivatized isomers in zingiberenols I and II (Figure 10b). Coinjection of MSFTA-derivatized zingiberenols I and II confirmed that each solution contained only the four diastereoisomers and all peaks matched each other. GC–MS analysis of the derivatized zingiberenol I and II mixtures and the male *T. limbativentris* aeration extract showed that the four derivatized compounds in zingiberenols I and II closely matched fragmentation patterns to the three derivatized compounds in the male extract. Figure 11 shows the fragmentation pattern of compound 2 of zingiberenol II mixture, identical to compound 2 of the male extract.

Discussion

Some of the compounds identified in the aeration extracts from *T. limbativentris* are common in stink bugs, such as (*E*)-2-octenal, (*E*)-2-decenal, nonanal, tridecane, and dodecane (Borges and Aldrich, 1992; Aldrich et al., 1993, 1995; Ho and Millar, 2001; McBrien et al., 2002; Moraes et al., 2005). In addition, males produced at least three isomers of zingiberenol, one or more of which appear to be sex pheromone components for this species.

In a direct comparison, female *T. limbativentris* were equally attracted by the zingiberenol I and II blends. However, fewer insects responded to zingiberenol I than to zingiberenol II when each blend was tested versus a control. This suggests that one or more of the isomers in zingiberenol II constitute the natural pheromone. Although the stereoisomeric mixture of zingiberenol II was attractive to females in this study, further electrophysiological and behavioral bioassays with individual isomers and specific blends need to be carried out to determine the precise configurations of the male-produced *T. limbativentris* sex pheromone component(s). There are several examples known of stink bug species that use different proportions of stereoisomers to produce a unique signal, such as *Acrosternum hilare* (McBrien et al., 2001) and *Nezara viridula* (Brézot et al., 1994).

When tested during different times of the day, females responded best during the period between 18:00 and 24:00 hr. This is similar to the behavior patterns found for other pentatomid species, such as *Euschistus heros* and *Thyanta perditor*, where the strongest responses were recorded during the afternoon to evening (Borges et al., 1998a,b; Moraes et al., 2005). However, this pattern differs from that found in other pentatomids such as

Nezara viridula (Borges et al., 1987) and *E. obscurus* (Borges and Aldrich, 1994), which show no variation in the level of responsiveness of females to males throughout the day.

In this study, both genders released some compounds that are seldom found or reported from stink bug airborne collections, such as α-pinene, β-pinene, and 6-methyl-5-hepten-2-one. The specific functions of these compounds, as well as the impact of factors such as different rearing conditions and diets upon their production, remain to be studied. The compound 6-methyl-5-hepten-2-one is clearly insect-produced and not from diet, because extracts of the metathoracic glands of both sexes contain this compound. However, some of the other compounds, such as α-pinene, β-pinene, limonene, and α-copaene, were not found in metathoracic gland extracts. Additionally, the green bean control aerations did not show the presence of any of these compounds. Thus, all these compounds appear to be released by *T. limbativentris*.

In summary, this work demonstrated that female *T. limbativentris* were attracted to a pheromone blend released by conspecific males and that females were attracted to a blend of synthetic zingiberenol isomers. The biological role of zingiberenol in the *T. limbativentris* male-specific emissions still needs to be determined under natural conditions. Furthermore, the role and absolute configuration of each of the zingiberenol isomers produced by males remain to be determined.

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References

ALDRICH, J. R., OLIVER, J. E., LUSBY, W. R., KOCHANSKY, J. P., and LOCKWOOD, J. A. 1987. Pheromone strains of the cosmopolitan pest, *Nezara viridula* (Heteroptera: Pentatomidae). *J. Exp. Zool.* 244: 171–175.

ALDRICH, J. R., WAITE, G. F., MOORE, C., PAYNE, J. A., LUSBY, W. R., and KOCHANSKY, J. R. 1993. Male-specific volatiles from Nearctic and Australasian true bugs (Heteroptera: Coreidae and Alydidae). *J. Chem. Ecol.* 19:2767–2781.

ALDRICH, J. R., OLIVER, J. E., LUSBY, W. R., KOCHANSKY, J. P., and BORGES, M. 1994. Identification of male-specific volatiles from Nearctic and Neotropical stink bugs (Heteroptera: Pentatomidae). *J. Chem. Ecol.* 20:1103–1111.

ALDRICH, J. R., ROSSI, M. C., and BIN, F. 1995. Behavioral correlates for minor volatile compounds from stink bugs (Heteroptera: Pentatomidae). *J. Chem. Ecol.* 21:1907–1920.

BORGES, M. 1995. Attractant compounds of the southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae). *Ann. Soc. Entomol. Brasil* 24:215–225.

BORGES, M. and ALDRICH, J. R. 1992. Instar-specific defensive secretions of stink bugs (Heteroptera: Pentatomidae). *Experientia* 48:893–896.

BORGES, M. and ALDRICH, J. R. 1994. Attractant pheromone for nearctic stink bug, *Euschistus obscurus* (Heteroptera: Pentatomidae): insight into a neotropical relative. *J. Chem. Ecol.* 20:1095–1102.

BORGES, M., JEPSON, P. C., and HOWSE, P. E. 1987. Long-range mate location and close-range courtship behaviour of the green stink bug, *Nezara viridula* and its mediation by sex pheromones. *Entomol. Exp. Appl.* 44:205–212.

BORGES, M., MORI, K., COSTA, M. L. M., and SUJII, E. R. 1998. Behavioural evidence of methyl-2,6,10-trimethyltridecanoate as a sex pheromone of *Euschistus heros* (Heteroptera: Pentatomidae). *J. Appl. Entomol.* 122:335–338.

BORGES, M., SCHMIDT, F. G. V., SUJII, E. R., MEDEIROS, M. A., MORI, K., ZARBIN, P. H. G., and FERREIRA, T. B. 1998b. Field responses of stink bugs to the natural and synthetic pheromone of the Neotropical brown stink bug, *Euschistus heros* (Heteroptera: Pentatomidae). *Physiol. Entomol.* 23:202–207.

BRÉZOT, P., MALOSSE, C., MORI, K., and RENOU, M. 1994. Bisabolene epoxides in sex pheromone of Nezara viridula (L.) (Heteroptera: Pentatomidae). Role of *cis* isomer and relation to specificity of pheromone. *J. Chem. Ecol.* 20:3133–3147.

CAVALCANTE, A. C. S., CORDEIRO, D. M., LAUMANN, R. A., MORAES, M. C. B., BARRIGOSSI, J. A., and BORGES, M. 2004. Ciclo de vida e metodologia de criação de *Tibraca limbatriventris* Stal. 1860 (Heteroptera: Pentatomidae) para estudos de ecologia química. Brasília: Embrapa, Centro Nacional de Recursos Genéticos e Biotecnologia, 2004, p. 18 (Embrapa-Cenargen, Boletim de Pesquisa, 78).

COSTA, E. C. and LINK, D. 1998/99. Efeito de inseticidas sobre predadores em arroz irrigado. *Ver. Fac. Zootec. Vet. Agro. Uruguaiana.* 5/6:29–33.

FERREIRA, E., ZIMMERMANN, F. J. P., SANTOS, A. B., and NEVES, B. P. 1997. O percevejo-do-colmo na cultura do arroz. Goiânia: Embrapa, Centro Nacional de Pesquisa de Arroz e Feijão, 1997, p. 43 (*Embrapa-CNPF, Documentos, 75*).

HAGIWARA, H., OKABE, T., ONO, H., KAMAT, V. P., HOSHI, T., SUZUKI, T., and ANDO, M. 2002. Total synthesis of bisabolane sesquiterpenoids, α-bisabol-1-one, curcumene, curcuphenol, and elvirol: utility of catalytic enamine reaction in cyclohexenone synthesis. *J. Chem. Soc., Perkin Trans.* 1:895–900.

HO, H.-Y. and MILLAR, J. G. 2001. Compounds in metathoracic glands of adults and dorsal abdominal glands of nymphs of the stink bugs, *Chlorochroa uhleri*, *C. sayi*, and *C. ligata* (Hemiptera: Pentatomidae). *Zool. Stud.* 40:193–198.

JORGE, P. 1999. Management of the brown pentatomid of rice in the Dominican Republic. *Craphin News* 18:8–9.

MCBRIEN, H. L. and MILLAR, J. G. 1999. Phytophagous bugs, pp. 1797–1818, *in* J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CABI Publishing, London, UK.

MCBRIEN, H. L., MILLAR, J. G., GOTTLIEB, L., CHEN, X., and RICE, R. E. 2001. Male produced sex attractant pheromone of the green stink bug, *Acrosternum hilare* (Say). *J. Chem. Ecol.* 27:1821–1839.

MCBRIEN, H. L., MILLAR, J. G., RICE, R. E., MCELFRESH, J. S., CULLEN, E., and ZALOM, F. G. 2002. Sex attractant pheromone of the red-shouldered stink bug *Thyanta pallidovirens*: a pheromone blend with multiple redundant components. *J. Chem. Ecol.* 28:1797–1818.

MILLAR, J. G. 2005. Pheromones of true bugs, pp. 37–84, *in* S. Schulz (ed.). The Chemistry of Pheromones and Other Semiochemicals II. Topics in Current Chemistry vol. 240. Springer Verlag, Berlin, Heildel, Germany.

MORAES, M. C. B., MILLAR, J. G., LAUMANN, R. A., SUJII, E. R., PIRES, C. P. S., and BORGES, M. 2005. Sex attractant pheromone from the neotropical red-shouldered stink bug, *Thyanta perditor* (F.). *J. Chem. Ecol.* 31:1405–1417.

North American Plant Protection Organization-Phytosanitary Alert System, (http://nappo.org/). *Archived Pest Alert*. Jul-11/01. Rice stalk stinkbug; Punaise (Fr.); Chinche grande del arroz (Span.); Percevejo da haste (Port.) *Tibraca limbativentris* (Stal) Stinkbug pest of rice introduced into the Dominican Republic.

PANIZZI, A. R., MCPHERSON, J. E., JAMES, D. G., JAVAHERY, M., and MCPHERSON, R. M. 2000. Stink bugs (Pentatomidae), pp. 421–474, *in* C. W., Schaefer and A. R., Panizzi (eds.). Heteroptera of Economic Importance. CRC Press, Boca Raton, FL. 828 pp.

PANTOJA, A., DAZA, E., GARCIA, C., MÉJÍA, O. I., and RIDER, A. D. 1995. Relative abundance of stink bugs (Hemiptera: Pentatomidae) in southwestern Colombia rice fields. *J. Entomol. Sci.* 30:463–467.

USTA, G. A., ORTEGA, M. E., PEREZ, C. R., and LOBOTON, V. 1994. Aspectos biologicos y fluctuacion poblacional de *Tibraca limbatriventris* (Stal) 1860 (Hemiptera: Pentatomidae) en arroz. *Arroz* 391: 20–27.

ZHANG, A., BORGES, M., ALDRICH, J. R., and CAMP, M. J. 2003. Stimulatory bug volatiles for the neotropical brown stink bug, *Euschistus heros* (F.) (Heteroptera: Pentatomidae). *Neotropical Entomol.* 32:713–717.