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(*R*)-(+)-citronellal identified as a female-produced sex pheromone of *Aromia bungii* Faldermann (Coleoptera: Cerambycidae)

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Abstract

Aromia bungii Faldermann (Coleoptera: Cerambycidae) is an important wood-boring pest of peach, apricot, plum and cherry in China. It is difficult to control it because of the cryptic feeding behaviour of larvae within the bark. In order to facilitate monitoring and control strategies for adult *A. bungii*, a series of experiments to verify its sex pheromone was conducted. Firstly, Y-tube experiments showed that *A. bungii* males were significantly attracted to volatiles from living female *A. bungii*. Combined with our earlier laboratory results showing that *A. bungii* females could emit (*R*)-(+)-citronellal, we evaluated the antennal responses of male *A. bungii* to (*R*)-(+)-citronellal using coupled gas chromatography-electroantennograms (GC-EAD) and electroantennography (EAG). (*R*)-(+)-citronellal elicited male antennal responses. In Y-tube behavioural bioassays, (*R*)-(+)-citronellal was attractive to male *A. bungii*. Therefore, (*R*)-(+)-citronellal which is a sex pheromone component produced by female *A. bungii* was hypothesised. The efficiency of using (*R*)-(+)-citronellal, alone or in combination with other attractants, to monitor and control *A. bungii* now requires further field experimentation and optimisation.

Keywords: Long-horned beetle, Wood borer, Chemical ecology, Olfactory response, Bioassay, GC-EAD

Background

Aromia bungii Faldermann (Coleoptera: Cerambycinae) is an important wood-boring pest of peach, apricot, plum and cherry in China (Huang 1978; Men et al. 2017 and Fukaya et al. 2017). Although it is native to China, it has been also reported in South Korea, Vietnam, Russia and elsewhere (Gong et al. 2013) and has recently invaded and established in Japan and several European countries, including Germany and Italy (Burmeister et al. 2012 and Garonna et al. 2013). It has also been intercepted in the UK, USA and Australia causing widespread national concern (Xu et al. 2017 and Fukaya et al. 2017).

The larvae of *A. bungii* bore into the lower trunk of mature host trees, often reducing tree longevity. For example, in the Beijing region, more than 13,000 hm^2 of peach and apricot orchards (about 1/3 of the local acreage of these trees) were infested by *A. bungii* and more than 90% of infested trees were seriously damaged (Liu et al. 1999). In orchards at the centre of Hebei province, adults of *A. bungii* can be found from late May until the middle of August. In Sichuan, Anhui, Shandong, Henan and Shaanxi provinces, *A. bungii* requires 2 years to complete one generation, while in Shanxi and Hebei provinces, 3 years is necessary for completion of one generation (Bai et al. 2017). Larvae usually have five larval instars and the adults emerge in spring in southern China and in summer in Northern China. *A. bungii* generally infests peach trees that are older than 10 years (do not oviposit on trees younger than 10 years of age), with trees over 15 years in age being most seriously infested (Bai et al. 2017). All varieties of peach tree are susceptible.

Because of their specificity and safety, insect pheromones have long been used to monitor and/or control insect pests (Rodstein et al. 2011). The pheromones of cerambycid beetles are of two types: aggregation pheromones that are produced by the males and attract both sexes and sex pheromones that are produced by the females and attract only males (Carde 2014, 2016 and Millar and Hanks 2016). Pheromones identified for the subfamilies Cerambycinae, Spondylidinae and Lamiinae have all been male-produced

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aggregation pheromones, whereas that for the subfamilies Prioninae and Lepturinae, all pheromones identified have been female-produced sex pheromones (Hanks and Millar 2016). To date, research on pheromones of Cerambycinae has mainly focused on the male-produced aggregation pheromone (Reddy et al. 2005 and Lacey et al. 2004, 2009). Female-produced sex pheromones have not been reported in the Cerambycinae.

Xu et al. (2017) identified and synthesised the male-produced aggregation pheromone of *A. bungii*, which attracted both sexes in field experiments done in China and Japan. Wei et al. (2013) detected (*R*)-(+)-citronellal from the bodies of adult female *A. bungii* and speculated that it may have a role in sexual behaviour.

Here, laboratory behaviour experiments to determine whether *A. bungii* adults are attracted to the same-sex or opposite-sex individuals were carried out and followed by evaluation of electrophysiological responses of antennae from males to (*R*)-(+)-citronellal (90%, Aldrich) and then Y-tube olfactometer experiments with *A. bungii* males.

Material and methods

Source of insects

Heavily-infested peach logs were collected from orchards in Shunping County, Hebei province, China, and transferred to the laboratory. Virgin adults of *A. bungii* emerged from these logs were collected. Specifically, in early April 2017, infested trees were felled and transported back to the laboratory. Restricted by the tree shapes, the lengths of 57 logs varied between 30 and 120 cm and the diameter between 10 and 30 cm. The cut edges were sealed by paraffin wax and a plastic film to avoid desiccation. Logs were placed in steel gauze mesh cages (20 mesh sieve), and incubated at 25 ± 4 °C, RH 60 ± 10%, until adults emerged. Adults of *A. bungii* were collected twice daily, at 08:00 and 20:00, starting late May until the mid-July. Emerged adults were placed individually in plastic chambers (PE, 18 cm × 11 cm × 8 cm) and maintained at room temperature (25 ± 2 °C). They were fed daily with peach jelly (a child's snack) purchased from the supermarket. In general, *A. bungii* lived for approximately 40–50 days in the lab; adults used for experiments were all over 3 days of age.

Olfactory responses of *A. bungii* adults to same-sex or opposite-sex individuals

A glass Y-tube olfactometer was used to investigate the behavioural responses of *A. bungii* adults to other individuals (Fig. 1). Dimensions of the Y-tube olfactometer were stem length, 40 cm; arm lengths, 30 cm at a 75° to each other; and internal diameter, 8 cm. A ball-shaped trap (2000 ml in volume and 35 cm in length) formed from a flask was placed at the end of each arm. Any beetle entering the trap was unable to crawl out and back down the arm. The two traps were connected to the odour source vessels, the flow meter, the activated carbon filter and the air generator which included an activated carbon and silica gel filter. All devices were connected by Teflon tubing. The odour source for one arm (treatment arm) came from four living adult *A. bungii* (either male or female depending on treatment and held in separate vessels to avoid fighting) and the other was clean air (control arm). The Y-tube olfactometer was placed in a room with the two arms beneath a natural light source (approximately 4.80 × 10^6-lx). The flow rate of clean air through each arm was 400 ml/min. During the bioassay, a beetle was introduced into the main arm of the Y-tube olfactometer. The response time ranged from 0 to 10 min. If the beetle crawled beyond halfway along an arm, or fell into the trap, it was considered to have made a choice. In the absence of a choice within 10 min, the test beetle was removed and the data excluded from statistical analysis. Each beetle was used only once, and at least 30 replicate responsive adults were required for each choice test. In order to eliminate any positional effects, the treatment and control arms of the Y-tube olfactometer were exchanged after every five beetles tested. Before each experiment, the components of the Y-tube olfactometer were cleaned in absolute ethanol (Kermel, AR), rinsed thoroughly in distilled water and dried in hot air. The responses of either individual males or females to volatiles from four females vs clean air and the responses of individual males or females to volatiles from four males vs clean air were compared. The Y-tube was cleaned after every five individuals run. All experiments were carried out at 20 ± 2 °C and the RH was approximately 50%. Before the test, adults were placed

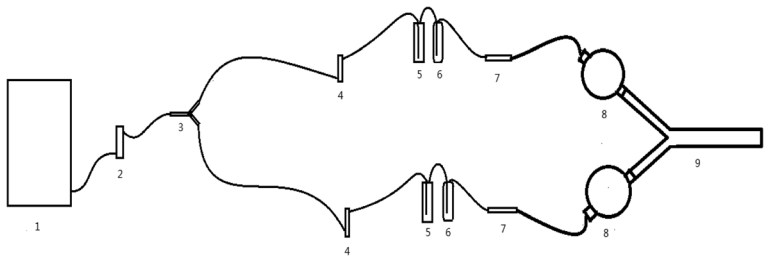

Fig. 1 Diagram of Y-tube olfactometer. 1: air generator; 2: main flow meter; 3: split-flow Y-tube; 4: flow meter; 5: activated carbon filter; 6: pure water; 7: the odour of treatment and the control; 8: ball-shaped trap; 9: Y-tube olfactometer

individually in the PE box (18 cm × 11 cm × 8 cm) at 26 ± 0.5 °C, RH 55 ± 5% for 24 h without a food supply.

Coupled gas chromatography-electroantennogram detection (GC-EAD)

(*R*)-(+)-citronellal was analysed by GC-EAD on an Agilent 7890A GC (Agilent, USA) with an injection port temperature of 250 °C, equipped with a HP-5 capillary column (30 m × 0.32 mm × 0.25 μm) in a splitless mode, and the GC oven programmed to run from 45 °C (held for 2 min) to 220 °C at 8 °C/min (held for 10 min). Column effluent was split 1:1 between the flame ionisation detector (FID) and the electroantennogram detector (EAD) with two deactivated capillary columns of equal length used as transfer lines with a deactivated Y splitter (Agilent, part number: 5181-3398). The column to the EAD preparation passed through the wall of the GC via a GC-EAD transfer (SYNTECH), heated to 220 °C. The two terminal flagella of individual antenna from male *A. bungii* were cut and approximately 1 mm from the tip removed. This section of the antenna was then mounted between two glass capillary electrodes (formed on a Narisshige electrode puller) and filled with physiological saline; the electrical contact to the amplifier was provided by a silver wire. The antennal preparation was centred in the effluent air stream from the GC, and antennal signals were amplified and filtered using a single-step high-input impedance DC amplifier and recorded in parallel with the FID signal.

Electroantennography (EAG)

In order to further verify the electrophysiological activity in response to (*R*)-(+)-citronellal, a series of EAG tests was conducted. The EAG recording operating system was composed of an intelligent data acquisition controller IDAC-4, a micro operation instrument Syntech MN-151, a stimulated air flow controller Syntech CS-55 and Syntech software processing system (Syntech, Germany). Recording electrodes and indifferent electrodes were both Ag-AgCl in physiological saline. The glass capillaries (1.5 mm OD) were filled with physiological saline. The airflow velocity and the continuous airflow were set at 400 ml/min. The stimulation time was 0.5 s, and the interval between two stimulations was 60 s. The qualitative filter paper was cut into 1.5 cm × 0.8 cm and folded into 1.5 cm × 0.4 cm and used as the carrier for the test volatile. A head-stage amplifier, a 'signal interface box' (model ID-04) and a PC-based acquisition system were used to amplify and record the EAG signal. At intervals of 1 min, stimulations were made beginning with the lowest concentration of the compounds and ending with the highest concentration. Ten antennae from male adults of over 3 days in age were stimulated with a dilution series of (*R*)-(+)-citronellal (90%, Aldrich): 5 ng/μl, 50 ng/μl, 500 ng/μl, 5 μg/μl, 50 μg/μl, 100 μg/μl and 300 μg/μl. Each time, 10 μl of solution was added to the filter paper for each concentration. Before and after stimulation by each concentration, the antennae were stimulated with hexane (Kermel, HPLC).

Olfactory response of *A. bungii* males to (*R*)-(+)-citronellal

Ten microlitres of (*R*)-(+)-citronellal (100 μg/μl) was placed onto filter paper (length, 4 cm; width, 0.2 cm) as the treatment odour source. Ten microlitres of hexane was placed on a similar filter paper to provide the control. After approximately 1 min, the two filter papers were connected to the two arms of the Y-tube as the odour sources. The other procedures were as described previously.

Statistical analysis

Bivariate non-parametric Wilcoxon tests were used to analyse and compare the results of the selection of volatile from *A. bungii* and (*R*)-(+)-citronellal. Variance analysis and HSD multiple comparisons of the experimental data were made. The mean values of EAG reactions were analysed by variance analysis ($P < 0.05$) using SPSS 21.0 statistical software.

Results and discussion

Olfactory responses of *A. bungii* adults to same-sex or opposite-sex individuals

The results of behavioural response showed that females were not significantly attracted to volatiles from either living males or females. Males were not attracted to the volatiles from other males, but males were significantly attracted to the volatiles from females ($Z = -2.90$, $P < 0.01$) (Fig. 2).

This result indicated that there was no aggregation pheromone emitted from the living male or female body to attract the same-sex adults. However, since the living females could attract the males, it was hypothesised that females produced pheromone in *A. bungii*.

Coupled GC-EAD

Results of GC-EAD showed that (*R*)-(+)-citronellal elicited responses in the antennae of *A. bungii* males (peak 1, Fig. 3), but there was no responses in the antennae of *A. bungii* females.

Since (*R*)-(+)-citronellal was detected from the female body of *A. bungii* (Wei et al. 2013), and it could elicit the response of antennae of *A. bungii* males in GC-EAD system, it might be an active compound emitted from the living female.

EAG responses of male *Aromia bungii*

All quantities of (*R*)-(+)-citronellal, except 0.5 μg, evoked an EAG response in adult male *A. bungii*. The relative value of the EAG response reached its peak when the quantity of (*R*)-(+)-citronellal was 1000 μg (Fig. 4). There

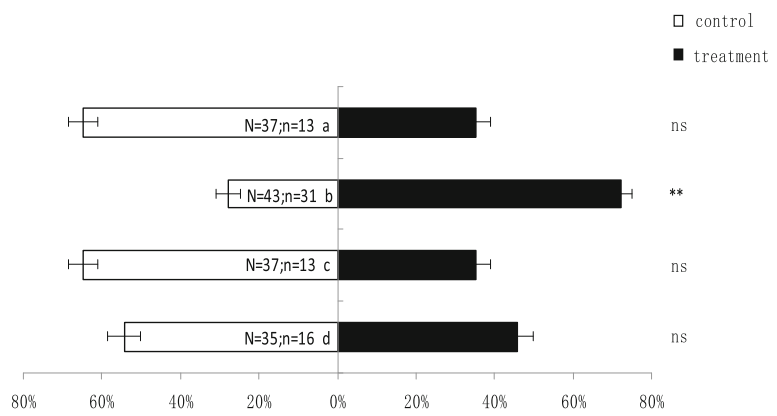

Fig. 2 Behavioural responses of *Aromia bungii* individuals to living *Aromia bungii* adults of the same or opposite sex. a Responses of *A. bungii* males to the odour of four males. b Responses of *A. bungii* males to the odour of four females. c Responses of *A. bungii* females to the odour of four females. d Responses of *A. bungii* females to the odour of four males. N, number of responsive *A. bungii*; n, number of *A. bungii* choosing the treatment group; significant difference at the 0.01 level (nonparametric test, bivariate correlation, Wilcoxon test)

was a significant difference in values amongst the different quantities of (*R*)-(+)-citronellal ($F = 10.23$, $df_1 = 6$, $df_2 = 63$, $P < 0.05$). EAG responses to 500 μg were significantly higher than response to those with lower quantities ($F = 12.009$, $df_1 = 4$, $df_2 = 45$, $P < 0.05$), and there was a statistically significant difference between responses to 1000 and 50 μg ($F = 9.209$, $df_1 = 1$, $df_2 = 18$, $P < 0.05$).

Since it was emitted in trace amount but needed a relative higher amount to elicit the antennal response of male *A. bungii*, which was different with the traditional sex pheromone of the moth, it might also be an intermediate metabolite for other chemical cues or defence substances. In addition, the compound emitted from the cerambycid might need a bit higher amount to elicit the antennal response (Xu et al. 2017). Therefore, it was not a surprise that 5 μg of (*R*)-(+)-citronellal only elicited a weak EAG responses in *A. bungii* males.

Y-tube olfactometer bioassays

Results showed that 23 males of *A. bungii* were attracted to (*R*)-(+)-citronellal and only 11 males chose the control arm ($Z = -2.06$, $P < 0.05$). It indicated that (*R*)-(+)-citronellal could significantly attract the *A. bungii* males and might play a role as female-produced pheromone.

Female-produced pheromone (*R*)-(+)-citronellal

Amongst cerambycids, species in three genera have been shown to use female-produced sex pheromones (Leal et al. 1994; Ray et al. 2012 and Wickham et al. 2016). Until this study, female-produced sex pheromones had not been

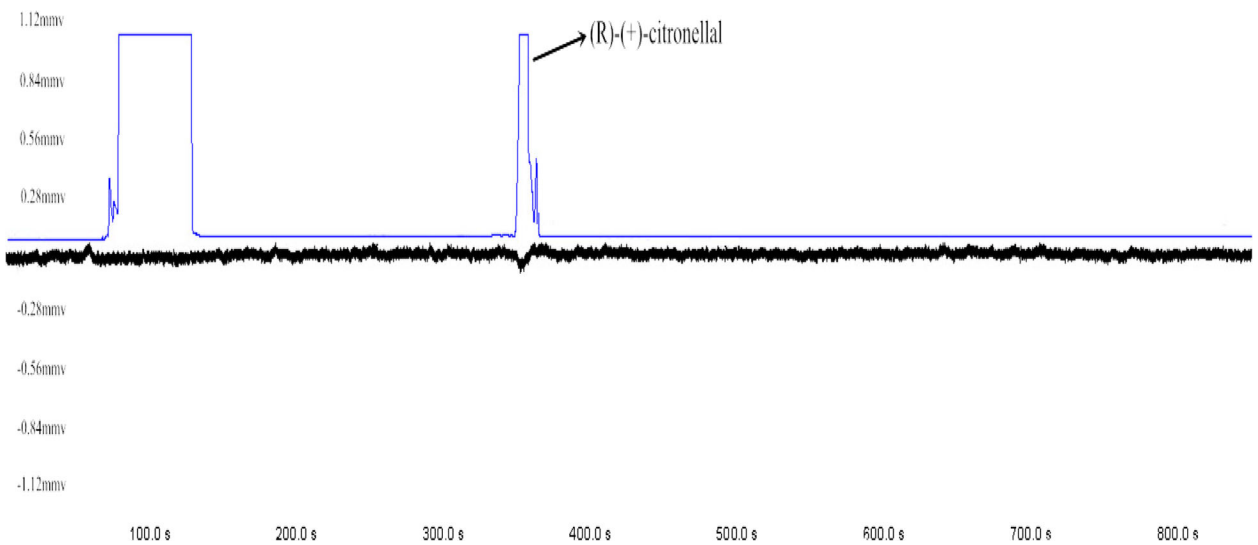

Fig. 3 Coupled gas chromatography-electroantennogram analysis of (*R*)-(+)-citronellal (7.5 μg). The upper trace is the chromatogram; the lower inverted trace is the electroantennogram signal from the antenna of *A. bungii* male

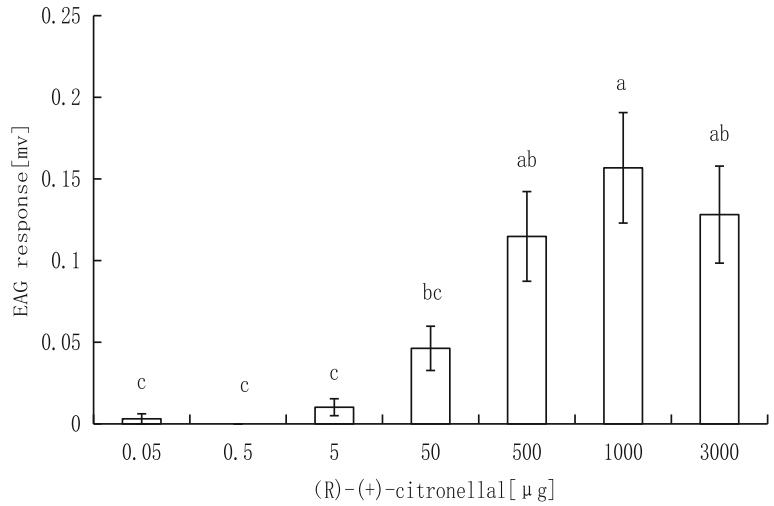

Fig. 4 Responses of *A. bungii* antennae to different quantities of (*R*)-(+)-citronellal. Data are means ± SE. Values followed by different lowercase letters in the columns are significantly different to each other at $P < 0.05$, Tukey's multiple comparison test

reported in the sub-family Cerambycinae. Our previous study showed that (*R*)-(+)-citronellal was produced by female *A. bungii* (Wei et al. 2013). Because (*R*)-(+)-citronellal could only be detected from the live female body by chance and it was in trace amount, so it was not been quantified. Due to its attraction to male *A. bungii*, (*R*)-(+)-citronellal, the sex pheromone produced by *A. bungii* females, was hypothesised. This is the first time that a female-produced sex pheromone has been reported in Cerambycine.

Citronellal is used by insects to identify their host plants and may also have other roles in insects' behaviour. For example, citronellal found in the host plants of *Demonax transilis* Bates (Coleoptera: Cerambycidae) was only attractive to females (Ikeda et al. 1993). As a volatile from non-host plants, citronellal also elicited strong EAG responses in the antennae of female *Monochamus alternatus* (Coleoptera: Cerambycidae) beetles and deterred females from ovipositing (Li et al. 2007). There are three important sources of pheromone precursor compounds in insects: de novo biosynthesis, further processing of host plant-derived compounds and direct integration of molecules from compounds (Yew and Chung 2015). (*R*)-(+)-citronellal elicited responses in the antennae of *A. bungii* males and was only attractive to males. Although this volatile in peach trees was not found, the possibility that (*R*)-(+)-citronellal is derived from the host plant of *A. bungii* could not be completely excluded.

Whether (*R*)-(+)-citronellal alone, or in combination with other chemicals, could attract male *A. bungii* in the field needs further research. Semiochemicals from host plants may also play important roles in attracting *A. bungii* adults to aggregate and mate.

Conclusions

In conclusion, the results of the present study hypothesised that (*R*)-(+)-citronellal is a sex pheromone component produced by female *A. bungii*. The identification of sex pheromones of *A. bungii* female will provide a theoretical and technical basis for the integrated management of *A. bungii*.

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Availability of data and materials

The data and material are included in the dissertation of the first author but has not yet been published formally.

Authors' contributions

W-CW is responsible for conducting the EAG and GC-EAD experiment and data analysis and writing the draft manuscript. D-DC contributed in the rearing of the beetle and conducting part of the olfactory test. JM conducted a part of the olfactory test. J-RW is responsible for designing and supervising the study, revising the paper scientifically and checking the analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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