A supplemental component of aggregation attractant pheromone in the bean bug *Riptortus clavatus* (Thunberg) (Heteroptera: Alydidae)[†], related to food exploitation

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Abstract

Traps baited with dried soybeans and a synthetic aggregation attractant pheromone were more effective than traps baited with synthetic aggregation pheromone alone against the bean bug, *Riptortus clavatus*. Dried soybeans alone did not attract bean bugs, so this difference was not due to the attractiveness of the dried soybean itself. The known components of the aggregation pheromone were detectable only in extracts of a few individual attracted bugs; however, all the attracted male bugs had (*E*)-2-hexenyl hexanoate, which has been identified as an alarm pheromone in this species. This component was present in higher amounts in extracts of male or female *R. clavatus* that had been fed on soybeans than that of starved males or females. In the experiments, the attractiveness of tetradecyl isobutyrate, which is an essential component of aggregation pheromone, was increased by the addition of (*E*)-2-hexenyl hexanoate. These results suggested that (*E*)-2-hexenyl hexanoate is one of the components of the attractant aggregation pheromone of *R. clavatus* and that it may act as a synergistic composition but not a repellent. It is hypothesized that the pheromonal process related to food exploitation in *R. clavatus* includes positive feedback since attracted bugs stay to feed at sites and continuously release (*E*)-2-hexenyl hexanoate.

Key words: (*E*)-2-Hexenyl hexanoate; tetradecyl isobutyrate; aggregation attractant pheromone; alarm pheromone; supplemental component

INTRODUCTION

The adult males of the bean bug, *Riptortus clavatus* (Thunberg) (Heteroptera: Alydidae), attract both conspecific males and females in field experiments (Numata et al., 1990). A three-component mixture (3 mix) of (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-hexenyl (*Z*)-3-hexenoate and tetradecyl (=myristyl) isobutyrate has been identified as an aggregation attractant pheromone of male *R. clavatus* (Leal et al., 1995). It attracts not only adults of both sexes but also nymphs. Mizutani et al. (1997) revealed that male and female *R. clavatus* were captured in traps baited with tetradecyl isobutyrate alone as well as with the 3 mix.

The synergistic effect of (*E*)-2-hexenyl (*E*)-2-hexenoate and (*E*)-2-hexenyl (*Z*)-3-hexenoate was confirmed in Japan (Endo et al., 2005) and Korea (Huh et al., 2005) when these compounds were mixed together with tetradecyl isobutyrate. Recently, we found that octadecyl isobutyrate is released from male bugs and acts as a synergistic component of the *R. clavatus* aggregation pheromone (Yasuda et al., 2007).

In some heteropterans, contagious distribution on host plants was often observed (e.g. Schaefer, 1980; Ventura and Panizzi, 2003). Y. Honda, one of the authors of this manuscript, observed that many more *R. clavatus* were captured with traps baited with dried soybeans and the synthetic aggre-

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[†] Recently, Kikuhara (2005) proposed that the scientific name of this species is changed to *R. pedestris* (Thunberg).

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Fig. 1. Typical catches of *R. clavatus* in traps baited with soybeans and the synthetic aggregation pheromone.

gation pheromone than with synthetic pheromone alone (Fig. 1). If this preference could be explained, the synthetic agent for monitoring could be improved and the role of aggregation pheromone in food exploitation (Morishima et al., 2005) could be defined.

The present paper reports that (*E*)-2-hexenyl hexanoate, which has been identified as an alarm pheromone of *R. clavatus* (Leal and Kadosawa, 1992), is present in male and female *R. clavatus*, and that this component enhanced the attraction to traps already baited with tetradecyl isobutyrate. We discuss the role of (*E*)-2-hexenyl hexanoate in the food exploitation strategy of *R. clavatus*.

MATERIALS AND METHODS

Insects. *R. clavatus* adults were collected in soybean fields in Tsukuba and maintained on soybean seeds, red clover (*Trifolium pretense*) seeds, and water at 25°C with a 14L:10D photoperiod in the laboratory. The emerged adults were segregated by sex and the males were separately transferred to other cages.

Chemicals. Tetradecyl isobutyrate was obtained from 1-tetradecanol reacted with isobutyric anhydride and pyridine. A plastic pellet formulation (4–5 mm diameter, 5 mg) impregnated with a mixture (1:5:1) of synthetic tetradecyl isobutyrate, (*E*)-2-hexenyl (*E*)-2-hexenoate and (*E*)-2-hexenyl (*Z*)-3-hexenoate (3 mix) (Fuji Flavor Co., Ltd., Japan) was used in Field experiment 1 as described below. (*E*)-2-Hexenyl hexanoate was purchased

Fig. 2. Trap apparatus in Field experiment 1. Trap (A) consisted of a plastic plate (18 cm×25.5 cm), a pole (1 m) and a stainless-mesh cage (B: 10 cm outside diameter×8 cm height).

from the Tokyo Chemical Industry Co., Ltd., Japan.

Synthetic chemicals for Field experiment 2 were impregnated into gray septa made of halo-butyl isoprene blend elastomer (8 mm outside diameter, West Pharmaceutical Services Singapore Pte Ltd., Singapore) by applying 300 μ l of hexane solution into the depression. Each septum was placed in a draft chamber for ca. 1 h at room temperature to allow the solvents to evaporate. For the negative control, a septum containing 300 μ l of the hexane was used.

Field experiment 1. Field experiments to check the additional effect of dried soybeans on the attractiveness of the 3 mix were conducted in a field of the National Agricultural Research Center (NARC, 140°06'20"E, 36°01'20"N) in Tsukuba, Japan. The field was ca. 1,000 m² of a grassy field with rows of coniferous trees. Two plastic pellet formulations, containing 10 mg comprised of the 3 mix chemicals, and/or ten seeds of dried soybeans, were put in a stainless steel-mesh cage (10 cm outside diameter×8 cm height) (Fig. 2). An empty cage was used as a control. The dried soybeans in the cage were placed so that *R. clavatus* could feed from outside the cage. The cage was fixed in front of a plastic plate (18 cm×25.5 cm) at a height of ca. 1 m from ground. Replicates of each treatment (a total of eight traps) were set in the field at intervals of about 10 m on 12–14 October and 17–21

October 2005. *R. clavatus* on the cage and plastic plate were visually counted and collected four times a day on 12–14 October and twice a day on 17–21 October. All the males and selected females collected were separately extracted with hexane and analyzed as described below. The trap locations were rotated in turn after the last observation every day. The numbers of insects caught in the trap were pooled for 1 day per trap (x) and transformed to $\log(x+0.5)$ for ANOVA and the subsequent Tukey-Kramer test.

Feeding of males and females. To test the effect of the male and female diet on the levels of (*E*)-2-hexenyl hexanoate, emerged males and females were maintained with water only for zero or four days and then divided into two groups with the respective soybeans or water only. The groups of five males and females were fed for one day, and then extracted with hexane, as described below. The groups of five males and females with water only for two or five days were also extracted.

Preparation of whole-body extracts of *R. clavatus*. Intact male and female *R. clavatus* were separately dipped in 2 ml of hexane for 1 h at room temperature. The hexane solution of *n*-hexadecane (2 μ g) was added as an internal standard into each sample. The extract was decanted from the male or female body into a glass vial. The residual body was rinsed with 1 ml of hexane, and the rinse was added to the extract. The extracts were stored at -20°C until gas chromatography-mass spectrometry (GC-MS) analyses. The extracts of hexane solution were concentrated to ca. 100- μ l volumes just before GC-MS analyses.

GC-MS analyses. GC-MS analyses were done on an Agilent 6890N GC with an HP-INNOWax column (30 m $\times$ 0.25 mm inside diameter $\times$ 0.25 μ m film thickness) by on-column injection combined with an Agilent 5973 Network Mass Selective Detector. Injection was made directly onto the capillary column through a cool-column injector and the injector temperature was programmed at oven temperature plus 3°C . Helium was used as the carrier gas. The initial GC oven temperature was 50°C (1 min hold), increased to 180°C at $10^{\circ}\text{C min}^{-1}$, increased again to 240°C at $20^{\circ}\text{C min}^{-1}$, and was then held for 5 min.

Field experiment 2. Field experiments to check the additional effect of (*E*)-2-hexenyl hexanoate (10 mg) on the attractiveness of tetradecyl iso-

butyrate (10 mg) was also conducted in the same NARC. A septum containing 300 μ l of hexane was used as a control. Double-sided sticky plates (18 cm $\times$ 25.5 cm, Fieldcatch, Fuji Flavor Co., Ltd.) were set at a height of ca. 1 m from the ground. Each septum impregnated with test chemicals was fixed at the center position on one side of the plate. Replicates of each chemical (a total of eight traps) were set in the field at intervals of about 10 m from 28 to 31 October and from 1 to 4 November 2005. Trap catches were checked on 31 October and 4 November, respectively. The numbers of insects caught in a trap (x) were transformed to $\log(x+0.5)$ for ANOVA and the subsequent Tukey-Kramer test.

RESULTS

Traps baited with dried soybeans and the 3 mix captured significantly more *R. clavatus* than traps baited with the 3 mix alone (Field experiment 1, Fig. 3), and no *R. clavatus* were captured in traps baited with dried soybeans alone.

The hexane extracts of 43 individual *R. clavatus* males, which had been captured with soybean and 3 mix bait in Field experiment 1, were separately analyzed with GC-MS. Two dominant peaks were observed in each extract. Two peaks were identified as (*E*)-2-hexenal and (*E*)-2-hexenyl hexanoate, and the mean amount of the latter was ca. 0.7 μ g/male

Fig. 3. Mean trap captures of *R. clavatus* with the 3 mix (a 1:5:1 mixture of synthetic tetradecyl isobutyrate, (*E*)-2-hexenyl (*E*)-2-hexenoate and (*E*)-2-hexenyl (*Z*)-3-hexenoate) (10 mg) and/or soybeans (10 seeds). Values are the means and SE (bar) for 1 trap for 1 day ($n=16$). Means accompanied by the same letter are not significantly different by Tukey-Kramer test at $p=0.05$.

Fig. 4. Mean contents of (*E*)-2-hexenyl hexanoate in the whole-body extracts of 1- and 5-day-old male (A) and female (B) *R. clavatus* with soybeans (fed) or without soybeans (starved) for one day. Values are the mean contents and SE (bar) ($n=5$). The mean values of the difference in contents of (*E*)-2-hexenyl hexanoate between fed and starved *R. clavatus* were applied to paired *t*-test.

($n=43$). In 10 extracts of 43 males, (*E*)-2-hexenyl (*E*)-2-hexenoate and/or (*E*)-2-hexenyl (*Z*)-3-hexenoate were detected. The mean amounts were 0.2 and 0.06 µg/male, respectively. Only one extract of an individual male had tetradecyl isobutyrate, at ca. 8 ng/male. Female extracts also had (*E*)-2-hexenal and (*E*)-2-hexenyl hexanoate; the mean amount of the latter was ca. 0.5 µg/female ($n=4$).

The contents of (*E*)-2-hexenyl hexanoate in the whole body extracts of laboratory-reared males and females of *R. clavatus* were measured by GC-MS (Fig. 4). One-day-old males with or without soybeans had 1.18 (± 0.36 SE) and 0.41 (± 0.06 SE) µg/male of (*E*)-2-hexenyl hexanoate, respectively ($n=5$). This difference was marginally significant (*t*-test, $p=0.053$). Five-day-old males with soybeans for 1 day or without soybeans had 0.98 (± 0.07 SE) and 0.54 (± 0.12 SE) µg/male of (*E*)-2-hexenyl hexanoate, respectively ($n=5$). This difference was significant (*t*-test, $p<0.01$). Five-day-

Fig. 5. Mean trap captures of *R. clavatus* with tetradecyl isobutyrate (14:iBu, 10 mg) and/or (*E*)-2-hexenyl hexanoate (E2-6:Hx, 10 mg). Values are the means and SE (bar) for 1 trap for 3 days ($n=4$). Means with the same letter are not significantly different by Tukey-Kramer test at $p=0.05$.

old females with soybeans for 1 day or without soybeans had 1.24 (± 0.03 SE) and 0.42 (± 0.21 SE) µg/female of (*E*)-2-hexenyl hexanoate, respectively ($n=5$). This difference was significant (*t*-test, $p<0.01$); however, (*E*)-2-hexenyl hexanoate contents of the extracts of one-day-old females with or without soybeans were not significantly different (*t*-test, $p>0.05$).

Traps baited with tetradecyl isobutyrate+(*E*)-2-hexenyl hexanoate captured significantly more *R. clavatus* than traps baited with tetradecyl isobutyrate, (*E*)-2-hexenyl hexanoate or control ($p<0.01$) (Field experiment 2, Fig. 5).

DISCUSSION

Greater numbers of *Riptortus clavatus* were captured in traps baited with dried soybeans and the synthetic aggregation pheromone than those with the synthetic aggregation pheromone alone (Figs. 1 and 3). Furthermore, no *R. clavatus* were captured by traps baited with dried soybeans alone (Fig. 3). GC-MS analysis of the extracts of attracted bugs indicated few or no known components attractive to conspecific bugs. Only one extract of an individual male had a major attractive component, tetradecyl isobutyrate; its amount was ca. 8 ng/male. When the attraction of individual males was examined, males with less than ca. 30 ng of tetradecyl isobutyrate did not attract conspecific bugs (Mizutani et al., unpublished data).

All attracted bugs, both male and female, had (*E*)-2-hexenyl hexanoate. This compound has been identified as an alarm pheromone of *R. clavatus*

(Leal and Kadosawa, 1992), and unconditionally accepted as such in examinations of the attraction of the aggregation pheromone of *R. clavatus* (Leal et al., 1995). The attractiveness of tetradecyl isobutyrate, which is an essential component of attractant aggregation pheromone, to conspecific males and females was increased by the addition of (*E*)-2-hexenyl hexanoate (Fig. 5). These results suggested that (*E*)-2-hexenyl hexanoate is one of the components of the aggregation pheromone of *R. clavatus* and may act in the synergistic composition of the pheromone of this bug.

In the present study, field experiments were conducted from October to early November. The critical daylength for the induction of diapause was 13.5 h at 25°C (Numata and Hidaka, 1982), and the adults of *R. clavatus* caught in soybean fields at NARC in this period of 2006 were in the diapause state (Mizutani et al., unpublished data). Traps baited with synthetic aggregation pheromone caught diapausing adults in autumn as well as non-diapausing adults in summer in the field (Masuta et al., 2001; Mizutani et al., 2002; Tabuchi et al., 2006). Further detailed experiments will be necessary to reveal the effects of diapause of *R. clavatus* on chemical communication.

Feeding and attractiveness of conspecific individuals are closely related in *R. clavatus* (Mori-shima et al., 2005). The close relationship between the feeding conditions of *R. clavatus* males and their release of attractants suggests that the attractants might play a key role in food exploitation. A similar strategy was proposed in the pheromone system of the brown-winged green bug *Plautia stali* (= *Plautia crossota stali*) Scott (Heteroptera: Pentatomidae) (Shiga and Moriya, 1989) and predatory stink bugs (Aldrich et al., 1976; Sant'Ana et al., 1997). *P. stali* males attract conspecific males and females (Moriya and Shiga, 1984). The authors noticed that no individuals attracted to the males copulated on cages that included males (Moriya and Shiga, 1984), and that most of the attracted adults had far less food in their stomach and showed less developed fat bodies and sexual organs (Shiga and Moriya, 1989). It is believed that specific male individual(s) that have fed enough emit the aggregation pheromone, which then attracts conspecific adults and nymphs with an empty stomach.

However, the results of this paper suggest that

not only a specific male individual but also independently attracted individuals play important roles in the food exploitation strategy. In four selected observations in Field experiment 1 (Fig. 3), we observed the behavior of attracted *R. clavatus* on the cages. When there were 4, 6, 4 and 5 adults on the cage baited with soybeans and the 3 mix, we found that 4, 4, 2 and 3 were feeding on the soybeans, respectively (Mizutani, unpublished data). All the attracted bugs had considerable amounts of (*E*)-2-hexenyl hexanoate in GC-MS analyses. This component in extracts of males and female *R. clavatus* was greater in individuals fed on soybeans than those of starved males in the present study (Fig. 4). When attracted adults stay on or near a food source to feed, they can release (*E*)-2-hexenyl hexanoate. This suggests that *R. clavatus* population attractiveness could increase with the positive feedback process, and that a repetition of 'attraction and release' could cause the contagious distribution of *R. clavatus*. In another context, this component may be instrumental in securing feeding locations. If the concentration of (*E*)-2-hexenyl hexanoate increases in response to the essential component of the aggregation pheromone, tetradecyl isobutyrate, this may indicate that individuals gain feeding opportunities through the pheromone system. It has not been reported that developmentally heterogeneous populations are more attractive than bugs releasing the essential component of the aggregation pheromone alone. It is necessary to consider the details of the influence of the pheromone components and/or individuals attracted in the food exploitation strategy of *R. clavatus*.

In many heteropterans, the observation of repellent actions of tested insects to secretions or chemicals in small observation areas have been used for bioassays for alarm pheromones. A typical alarm pheromone component, (*E*)-2-hexenal, has shown both attractant (e.g. Ishiwatari, 1976; James et al., 1996) and repellent (e.g. Ishiwatari, 1974) functions in many heteropterans. (*E*)-2-Octenyl acetate, which is one of the attractant pheromone components of *Leptocorsa chinensis* (Heteroptera: Alydidae) (Leal et al., 1996), exhibits repellent action in adults in such bioassays (Yamashita and Isayama, 2005). (*E*)-2-Hexenyl hexanoate had been identified as the alarm pheromone of *R. clavatus* (Leal and Kadosawa, 1992). No repellent effect was observed in the present study. Furthermore, nymphs

of *R. clavatus* were also captured in traps baited with the 3 mix (50 mg) and (*E*)-2-hexenyl hexanoate (5 mg) as well as those with the 3 mix alone (50 mg) (Endo et al., unpublished data). These results suggested that bioassays in small observation areas and short intervals for alarm pheromone could be insufficient. Re-examination of the so-called alarm pheromone of heteropterans may therefore be necessary.

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