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Shin, Hyo Seob

Department of Applied Biology, College of Agriculture and Life Sciences, Chungnam National University

Ryu, Tae Hee

Department of Applied Biology, College of Agriculture and Life Sciences, Chungnam National University

Kwon, Hye Ri

Department of Applied Biology, College of Agriculture and Life Sciences, Chungnam National University

Seo, Mi Ja

Department of Applied Biology, College of Agriculture and Life Sciences, Chungnam National University

他

<https://doi.org/10.5109/1526395>

出版情報：九州大学大学院農学研究院紀要. 60 (2), pp.357-362, 2015-09-18. Faculty of Agriculture, Kyushu University

バージョン：

権利関係：



Aggregation Pheromone Secretion Change of *Riptortus pedestris* (Hemiptera: Alydidae) Depending on Diet

Hyo Seob SHIN¹, Tae Hee RYU¹, Hye Ri KWON¹, Mi Ja SEO¹, Yong Man YU¹,
Chisa YASUNAGA–AOKI* and Young Nam YOUN^{1*}

Laboratory of Insect Pathology and Microbial Control, Institute of Biological Control,
Faculty of Agriculture, Kyushu University, Fukuoka 812–8581, Japan
(Received May 7, 2015 and accepted May 19, 2015)

To confirm the difference in aggregation pheromone levels in *Riptortus pedestris* depending on host plants, *R. pedestris* was measured after feeding 6 legumes: mung bean [*Vigna radiata* var. *radiata* (L.) R. Wilczek], small black bean (*Rhynchosia nulubilis*), hairy vetch (*Vicia villosa* Roth), yellow soybean [*Glycine max* (L.) Merr.], cranberry bean (*Phaseolus vulgaris*), and black soybean [*Glycine max* (L.)]. For the *R. pedestris* collected at bean fields, hairy vetch field, and outdoor field, the pheromones were also analyzed using gas chromatography. We focused on tetradecyl isobutyrate (TI) which is a known insect attractant. The bugs provided with cranberry beans secreted the most TI and those provided with hairy vetch secreted the least TI; the amount of TI secreted was different for each of the beans. The bean bugs' TI secretion percentage was the highest for those that ingested black soybean and lowest for those that ingested hairy vetch. Aggregation pheromone secretions were affected by food sources; with cases of supporting plants displaying the lowest amount of secretion, whereas cases of supporting plants and seeds displayed the highest amount of secretion as well as the highest percentage of secreting bean bugs. Bean bugs captured in bean fields secreted the highest amount of TI component.

Key words: Aggregation pheromone, *Riptortus pedestris*, Secretion change

INTRODUCTION

Riptortus pedestris causes massive damage to soybeans as well as other crops. Plant damage attributed to *R. pedestris* is found in persimmons and citrus orchards. It has been reported that *R. pedestris* occurs in foxtail millet, proso millet, and sorghum as a dominant species (Chung *et al.*, 1995; Choi *et al.*, 2000; Lee *et al.*, 2009; Kim *et al.*, 2010). As *R. pedestris* has high mobility and female adults have an excellent ability to distinguish host plants from various crops (Ikeura and Kuroda, 2013), it moves to the neighboring woods or fields when chemical control is applied and returns later. Thus, controlling this pest is difficult. The aggregation pheromone trap has proven to be effective and has been recently used in order to attract and capture *R. pedestris* (Masuta *et al.*, 2001; Huh and Park, 2006; Park *et al.*, 2012; Shin *et al.*, 2012).

Aggregation pheromone of *R. pedestris* secreted by male adults can attract conspecific adults as well as nymphs (Numata *et al.*, 1990; Leal *et al.*, 1995). Aggregation pheromone is composed of (E)–2–hexenyl (Z)–3–hexenoate (E2HZ3H), (E)–2–hexenyl (E)–2–hexenoate (E2HE2H) and tetradecyl isobutyrate (TI) (Leal *et al.*, 1995). It has been reported that the composition of aggregation pheromone may vary depending on geographical locations (Leal *et al.*, 1995). Among its components, TI is the main component to which *R. pedestris* is attracted while E2HE3H is a component

that attracts egg parasitoids such as *Ooencyrtus nezarae* (Hymenoptera: Encyrtidae) (Leal *et al.*, 1995; Mizutani *et al.*, 1997; Masuta *et al.*, 2001; Huh and Park, 2005). According to a study on the secretion properties of the aggregation pheromone of *R. pedestris*, the secretion increases as days pass and as temperature increases (Huh *et al.*, 2009). Resting and sexually immature males do not secrete E2HZ3H and E2HE2H, and the amount of secretion varies depending on the feeding condition (Mizutani *et al.*, 2008).

In previous studies, it has been reported that the daily growth and survival rate of *R. pedestris* vary depending on soybean cultivars, and that the host preference and degree of damage caused by the bugs also vary depending on traits such as pubescence and pod and hilum color (Son *et al.*, 2000). Many other studies have also reported that secretion of the aggregation pheromone is dependent on the surrounding environment and on physiological conditions in other bean bugs. However, no studies on the differences in aggregation pheromone secretions depending on soybean cultivars have been conducted.

Therefore, in this study, we measured the amount of aggregation pheromone under different feeding conditions in each soybean cultivar to determine how they affected the variation in the amount of aggregation pheromone secreted by *R. pedestris*.

MATERIALS AND METHODS

Culture and collection of test insect

Culture of test insect

R. pedestris was cultured over successive generations in an acrylic cage (400×440×500 mm) in the insect

¹ Department of Applied Biology, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305–764, Korea

* Joint corresponding authors (E-mail: youngnam@cnu.ac.kr; yasunaga@grt.kyushu-u.ac.jp)

culture room ($25\pm 1^{\circ}\text{C}$, 14L:10D). Wild type insects were irregularly added in order to prevent inbreeding depression. A layer of paper towel was placed in the acrylic cage to easily remove secretions. The insects were fed soybean cultivars and plants. Seeds from the six legumes used in this experiment were mixed and placed on a Petri dish (150 mm). Cranberry beans (*Phaseolus vulgaris*) were supplied as foliated plants without pods. In addition, eggs were collected by placing cotton wool in the cage. A water-filled Falcon tube (50 ml) topped with cotton wool was placed in the cage as water source.

All adults used in the experiment were 7–8 days old. Nymphs in unknown life stages were randomly selected and used for the preference experiments.

Collection of test insects

Field populations were collected by using pheromone traps and a sweeping net in a soybean field ($1,207\text{ m}^2$, $36^{\circ}22'03.8''\text{N}$ $127^{\circ}21'10.8''\text{E}$) in Gung-dong and a hairy vetch field ($1,070\text{ m}^2$, $36^{\circ}21'40.5''\text{N}$ $127^{\circ}19'33.8''\text{E}$) in Noeun-dong, both in Yuseong-gu, Daejeon. Straight-line distance between the collection sites was 2.51 km. Test insects were also caught in various places of the hills near the Chungnam National University in Gung-dong. For the collection using pheromone traps, four pheromone traps were installed at 50 m intervals between the traps in each test field. Bean bugs were collected from the traps every two weeks. For collection using a sweeping net, it was swept for 10 cycles and repeated three times in each field.

Experimental plants and culture

Six legumes (Fabaceae) without pods including mung bean, small black bean, hairy vetch, yellow soybean, cranberry bean, and black soybean were used in the laboratory assay. Plants were cultured in a growth chamber ($25\pm 1^{\circ}\text{C}$, 14L:10D). The size and shape of leaves varied depending on the legumes, although legumes were all 2–3 weeks old from the seeding.

Aggregation pheromone extraction and GC analysis

Aggregation pheromone extraction and analysis

Aggregation pheromone of *R. pedestris* was extracted from one male adult as follows. First, it was placed and soaked in 1 mL Hexane (99%, Merck, Germany) in 1.5 ml micro tube for 5 minutes. Gas chromatography (7890A GC Chemstation, Agilent Technologies, USA) was used for GC analysis. Conditions described in the study of Huh *et al.* (2008) were modified for the analysis to create quantitative curves and measure the amount of secretion. DB-WAX column (ID 0.25 mm \times 30 m, Agilent Technologies, USA) was used in this experiment. GC conditions are as follows. We increased the oven temperature up to 210°C by $10^{\circ}\text{C}/\text{minute}$ after it was maintained at 50°C for 1 minute. Then, it was maintained for 15 minutes. Carrier gas was H_2 and the inlet temperature was 250°C . A detector was FID and temperature was 250°C .

Creation of quantitative curves of each component

In this study, standards were ordered from Practica

Chem Company and used to analyze the three main pheromone components, E2HZ3H, E2HE2H and TI, among other components of aggregation pheromone. Purity was 95%, 92% and 95%, respectively. GC conditions were used for the quantitative analysis on the same three components. On the basis of results, quantitative curves were created for each component. After the aggregation pheromone lure was diluted with Hexane (99%, Merck, Germany) solution to make 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 mg/ml, DB-WAX column was applied. Curves were created by using the area values and concentrations on the GC graph; these curves were then used to calculate aggregation pheromone secretions. Every curve's determination coefficient value was 0.99 and above.

Difference in amount of aggregation pheromone secretion in each legume

We determined the difference in the amounts of aggregation pheromone secretion of *R. pedestris* fed with six legumes. Six podless legume plants were placed in each acrylic cage (400 \times 400 \times 500 mm) and 10 male adults of *R. pedestris* were put in a cage and cultured for 10 days. Then, aggregation pheromone was extracted and quantified. Mating status and body weight were unknown for the tested bugs. Extraction methods and conditions for GC analysis are as mentioned above. Experiments were repeated a total of five times.

Difference in the amount of secretion based on feeding conditions

Cranberry bean seeds, foliated plants without pods, and plants with seeds were placed in each rearing cage to examine how the difference in the amount of pheromone secretion depended on feeding conditions. Each of the conditions used one plant and 100 seeds. The water-filled tube was placed in the acrylic cage as described above. Ten male adults of *R. pedestris* were placed in the cage and were cultured for seven days before the extraction of aggregation pheromone. Mating status and body weight are unknown for the tested bugs. The extraction method and conditions for GC analysis are described above. Experiments were repeated five times.

Comparison of difference in aggregation pheromone secretion aggregation pheromone secretion in each crop of bean bugs caught in the bean field, hairy vetch field and outdoor field

Test insects were obtained by sweeping an insect net in the bean field, hairy vetch field and outdoor field. As various soybean cultivars were mixed in the bean field, bean bugs were captured during the period when pods were made and harvested after planting. In the case of hairy vetch, it was planted in the middle of April and pods were made in the middle of June. Bean bugs were captured during the period when hairy vetch seeds fell and regenerated. In the outdoor bean field, bean bugs were captured in the area which was farthest from the bean field or hairy vetch field and where other kinds of crops (pepper, Proso Millet, etc.) grew.

Statistical analysis

Statistical analysis used in this experiment is as follows: When the distribution of aggregation pheromone secretion depending on soybean cultivars and variation in amount of aggregation pheromone secretion depending on the number of bean bugs were analyzed, graphs were created using a Sigma plot 12.0. When the aggregation pheromone secretion resulting from the feeding conditions and soybean cultivars and the secretions from each crop in the outdoor field were analyzed, a one-way ANOVA test was performed by using an SPSS (PASW statistics 20.0) within the range of significant probability of 0.05. A significance test was performed through Tukey's HSD test.

RESULTS

Difference in aggregation pheromone secretion based on legumes

The bug that ingested the black soybeans secreted highest amount of E2HZ3H while hairy vetch had the smallest amount of secretion of E2HZ3H. Mung bean had the largest amount of E2HE2H secretion and small black bean had the smallest amount of E2HE2H secretion. In addition, TI has been known to be the most associated

with the attractiveness of aggregation pheromone to *R. pedestris*. Cranberry bean had the largest amount of TI secretion, followed in decreasing order by small black bean, mung bean, black soybean, yellow soybean, and hairy vetch (Table 1).

According to the following results, the secretion of three main components of aggregation pheromone except E2HE2H was statistically significant in six legumes. For the ratio of bean bugs secreting each component, more than 90% of bean bugs used in the experiment secreted E2HZ3H and E2HE2H in all tests. However, bean bugs that secreted TI showed a difference of percentage for each legume. Bean bugs that secreted TI showed the highest percentage for black soybean and showed the lowest percentage for hairy vetch (Fig. 1).

When looking at the composition ratio (E2HZ3H:E2HE2H:TI) in aggregation pheromone secreted from bean bugs used in the experiment, cranberry bean showed the highest ratio for TI in 1:1.51:0.54 and hairy vetch showed the lowest ratio in 1:1.77:0.12 (Table 2).

According to the results of experiment, there were variations in the amount of aggregation pheromone produced by *R. pedestris* between the six legumes used in the experiment.

Table 1. Difference of aggregation pheromone components ($\mu\text{g}/\text{bug} \pm \text{SE}$) of *R. pedestris* according to host seeds and foliated plants

Plant	E2HZ3H*	E2HE2H	TI
Cranberry bean	12.01 \pm 5.45ab**	18.15 \pm 8.71a	6.55 \pm 3.04a
Small black bean	10.69 \pm 3.41ab	17.30 \pm 4.93a	3.16 \pm 0.92bc
Mung bean	13.23 \pm 3.45ab	22.69 \pm 6.37a	5.70 \pm 2.41abc
Yellow soybean	13.98 \pm 4.64a	22.12 \pm 7.46a	4.36 \pm 2.69ab
Black soybean	14.17 \pm 2.72a	22.56 \pm 4.18a	4.32 \pm 2.31ab
Hairy vetch	10.43 \pm 4.99b	18.48 \pm 7.56a	1.26 \pm 0.21c

*E2HZ3H: (E)-2-hexenyl (Z)-3-hexenoate, E2HE2H: (E)-2-hexenyl (E)-2-hexenoate, TI: tetradecyl isobutyrate. **Test by Tukey's HSD ($\alpha=0.05$) in SPSS (PASW statistics 20.0).

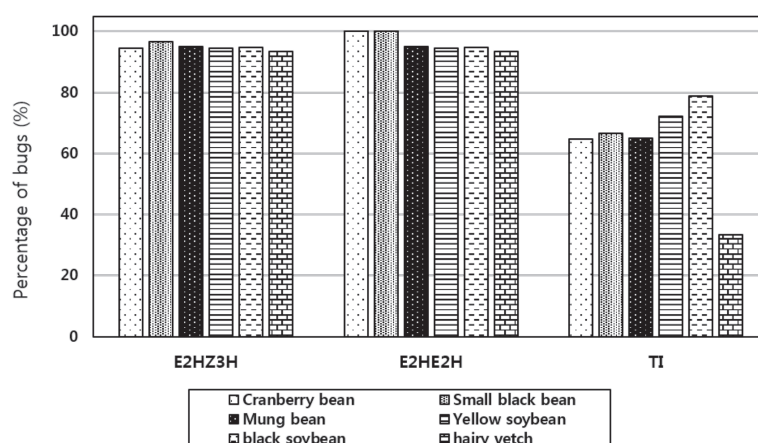


Fig. 1. Percentage of *R. pedestris* that secreted the three major components of aggregation pheromone according to the host plants.

Table 2. Aggregation pheromone components ratio of *R. pedestris* according to host plant and different field

Ratio of aggregation pheromone components (E2HZ3H* : E2HE2H : TI)		
Plant	Cranberry bean	1 : 1.51 : 0.54
	Small black bean	1 : 1.62 : 0.29
	Mung bean	1 : 1.72 : 0.43
	Yellow soybean	1 : 1.58 : 0.31
	Black soybean	1 : 1.59 : 0.30
	Hairy vetch	1 : 1.77 : 0.12
Field	Hairy vetch	1 : 1.53 : 0.12
	Bean	1 : 1.84 : 0.32
	Other	1 : 1.46 : —

*E2HZ3H: (E)-2-hexenyl (Z)-3-hexenoate, E2HE2H: (E)-2-hexenyl (E)-2-hexenoate, TI: tetradecyl isobutyrate.

Table 3. Amount of aggregation pheromone secreted ($\mu\text{g}/\text{bug} \pm \text{SE}$) by *R. pedestris* according to different feed sources

	E2HZ3H*	E2HE2H	TI
Plant	$7.19 \pm 3.43\text{b}^{**}$	$13.74 \pm 7.48\text{a}$	$0.49 \pm 0.05\text{a}$
Seed	$12.88 \pm 3.55\text{a}$	$18.99 \pm 5.99\text{a}$	$3.85 \pm 2.03\text{ab}$
Plant + Seed	$12.01 \pm 5.45\text{a}$	$18.14 \pm 8.70\text{a}$	$6.55 \pm 3.04\text{b}$

*E2HZ3H: (E)-2-hexenyl (Z)-3-hexenoate, E2HE2H: (E)-2-hexenyl (E)-2-hexenoate, TI: tetradecyl isobutyrate. **Test by Tukey's HSD ($\alpha=0.05$) in SPSS (PASW statistics 20.0).

Difference in aggregation pheromone secretion based on feeding conditions

When plants, seeds and plants mixed with seeds were supplied, the amount of aggregation pheromone secreted by test bean bugs was compared for each group (Table 3).

It was found that E2HZ3H, E2HE2H and TI showed the lowest amount of secretion when the bugs only ingested plants. The ratio of bean bugs that secreted the pheromone was also the lowest. When seeds were supplied in the diet, production of E2HZ3H and E2HE2H and the ratio of bugs that secreted the pheromone were similar. However, TI showed the most statistically significant difference for each feeding condition. Therefore, the above results suggest that there are some variations

in the amount of aggregation pheromone secreted from *R. pedestris* that are depending on diets.

Comparison of difference in aggregation pheromone secretion in each crop of bean bugs caught in the field

The amount of aggregation pheromone secretion from each bean bug captured in the field with various crops by using insect nets was compared. Both E2HZ3H and E2HE2H were detected in bean bugs captured in the outdoor field, but TI was not detected. Nevertheless, all three components were detected in bean bugs captured in the hairy vetch and bean fields. The secretions from bean bugs captured in the bean field were the same as those from bean bugs captured in the hairy vetch field

Table 4. Amount of aggregation pheromone secreted ($\mu\text{g}/\text{bug} \pm \text{SE}$) by *R. pedestris* caught in different collection fields

Field	E2HZ3H*	E2HE2H	TI
Hairy vetch	$8.91 \pm 3.63\text{a}^{**}$	$13.60 \pm 7.33\text{ab}$	$1.09 \pm 0.30\text{a}$
Bean	$9.88 \pm 4.23\text{a}$	$18.22 \pm 8.95\text{a}$	$3.18 \pm 1.95\text{a}$
Other	$7.44 \pm 3.86\text{a}$	$11.05 \pm 7.11\text{b}$	—

*E2HZ3H: (E)-2-hexenyl (Z)-3-hexenoate, E2HE2H: (E)-2-hexenyl (E)-2-hexenoate, TI: tetradecyl isobutyrate. **Test by Tukey's HSD ($\alpha=0.05$) in SPSS (PASW statistics 20.0)

(Table 4).

When looking at the ratio of the three components of aggregation pheromone, bean bugs captured in the hairy vetch field and bean field secreted the ratio of 1:1.53:0.12 and 1:1.84:0.32, respectively (Table 2). Taken all results together, it was found that bean bugs captured in the bean field secreted different amounts of aggregation pheromone than those captured in the outdoor bean field or hairy vetch field did.

DISCUSSION

In this study, we investigated the variation in the amount of aggregation pheromone secretion aggregation pheromone secretion from a bean bug, *R. pedestris* (Hemiptera: Alydidae), depending on diets by using GC-MS.

Aggregation pheromone of *R. pedestris* consists of E2HZ3H, E2HE2H and TI. Since then, there have been recent reports that Octadecyl isobutyrate (OI) is included. Research shows that OI, combined with the other three components, plays a role to increase the attractiveness (Yasuda *et al.*, 2007a). However, it was not detected for any of the six legumes in this experiment and there was no similarity between bean bugs secreting aggregation pheromone. In addition, it has been found that (E)-2-hexanyl hexanoate, known as the alarm pheromone, creates a synergistic effect along with TI and thus facilitates the secretion of aggregation pheromone (Yasuda *et al.*, 2007b).

When quantitative analysis was performed on aggregation pheromone, we focused on TI, a known insect attractant. E2HZ3H and E2HE2H were measured at a certain amount or higher and the ratio of bean bugs secreting them was high in all experiments. However, there were variations in the secretion of TI among the six legumes. TI secretion also varied depending on diets. The ratio of aggregation pheromone components varied depending on different geographical locations. When the ratio of the three components was normalized by the amount of E2HZ3H (=1), the ratio of Tsukuba and Kumamoto populations in Japan was 1:2.8:0.2 and 1:1.5:0.1, respectively. In addition, the ratio of Jinju and Iksan populations in Korea was 1:1.4:0.2 and 1:0.8:0.2, respectively (Leal *et al.*, 1995; Huh *et al.*, 2008). The results of analysis on the bean bugs captured in the outdoor bean field in Daejeon indicated that the composition ratio was 1:1.84:0.32, which was different from that in Japan but similar to that of the Jinju population (Table 2).

As in other bugs, the amount of aggregation pheromone secretion from *R. pedestris* varied depending on the surrounding environment, the bugs' physiological state, or their nutritional state (Mizutani *et al.*, 2008). Male adults do not secrete aggregation pheromone when there are no foods available, but they secrete it to attract other populations when foods are available (Leal *et al.*, 1995). Male adults attracted to live bait males did not have foods in their stomachs and TI was not detected (Mizutani *et al.*, 2008). For this reason, bean bugs captured by using

pheromone traps or sweeping showed small amounts of aggregation pheromone regardless of their field of origin. This suggested that bean bugs that were attracted to pheromone traps seemed to eat less food. According to the studies of Huh and Park (2006) and Shin *et al.* (2012), when additional foods were supplied in a pheromone trap, the trap's attractiveness increased. Persimmon and soybeans showed greater damage when they were supplied together rather than when only persimmon was supplied (Lee *et al.*, 2009). The previous study indicated that when *R. pedestris* was cultured under the single condition (10L:14D), male adults that entered reproductive dormancy secreted aggregation pheromone. However, the function of aggregation pheromone secretion was not directly associated with mating (Wada *et al.*, 1997). The amount of aggregation pheromone secretion was not different, regardless of mating, weight, or time zone. However, it varied depending on the number of elapsed days and the time of capture. (Huh *et al.*, 2009).

Both mung bean and hairy vetch were legumes which are known to receive damage from *R. pedestris*. However, when those two legumes were supplied as foods, nymph could not complete their metamorphosis into adults, resulting in an incomplete life cycle. Therefore, these legumes were not suitable for foods (Bae *et al.*, 2009; Seo *et al.*, 2011). Despite the small amount of aggregation pheromone in hairy vetch fields, *R. pedestris* could infest and inflict damage on hairy vetch. They may use the hairy vetch field for a temporary food source due to the lack of other available food items. However, mung bean increased the aggregation pheromone secretion even though it contained resistant materials affecting the growth of *R. pedestris* (Jung *et al.*, 2005). In the future, we would like to examine the components of this study's test legumes and investigate the correlation between these components and the synthesis of aggregation pheromone in *R. pedestris*.

ACKNOWLEDGEMENTS

This work was carried out with the support of "Cooperative Research Program for Chungnam National University (Project No. is 2014-1194-01)".

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