HERBIVORE-INDUCED RICE GRAIN VOLATILES AFFECT ATTRACTION BEHAVIOR OF HERBIVORE ENEMIES

QingFeng Tang, TianSheng Yang and JunQi Jiang

SUMMARY

The responses of female Lariophagus distinguendus (Hymenoptera: Pteromalidae) to volatile signals derived from herbivore-induced rice grain was investigated in the static four-chamber olfactometer. Results showed that the L. distinguendus females, irrespective of experience, were apparently attracted by the odors released from rice grain. The active chemicals in the saliva of the maize weevil, Sitophilus zeamais, that elicit the production of rice volatiles was investigated. Results showed that artificially damaged grains do not emit large amounts of volatiles that attract the experienced parasitoid females emitted by the S. zeamais larvae damaged grains. Further experiments revealed the experienced L. distinguendus females were apparently attracted by the odors released from rice induced by the saliva of the weevil larvae. Moreover, we compared the responses of female parasitoids to odors released from rice induced by protein substances of regurgitant and non-protein substances. The experiments revealed that volatiles attracting parasitoid L. distinguendus are possibly induced by the some protein substances from weevil larvae saliva. The effect of experience on the response of the parasitoids to several stimuli was discussed.

Introduction

In many parts of the world rice is the major component of the diet. Unfortunately, a large proportion of the annual yield is lost during storage, and these losses have been partly attributed to insect pests resulting in the deterioration of the quality and the quantity of stored rice grains (Machado et al., 2008). To protect grains from the insect damages, tons of insecticides have been applied to control the pests, which have resulted in environmental damages, pest resurgence, pest resistance to insecticides, and lethal effects on non-target organisms. Furthermore, because of cost, these pesticides are becoming increasingly inaccessible to farmers, particularly in developing countries. This fact, combined with the consumer’s demand for residue-free food, prompted researchers to evaluate other alternative reduced-risk control methods for stored-grain protection (Tang et al., 2009). To resolve the above problems, new environmental friendly methods to control the insect damages have become of interest to researchers; biological control of pests using parasitoids or predators is an example (Scholler et al., 1997). The use of natural enemies such as parasitoids for the control of stored-product pests has been suggested for many years (Hase, 1924; Ryabov, 1926). However, little is known about tritrophic interactions among rice grain, insect herbivores, and natural enemies of the herbivores.

The use of specific attractants might represent an option to increase the presence of the parasitoid wasp in granary or could be coupled with biocontrol programmes to improve biocontrol services. The option is effective in controlling insect pests of stored products and has a positive impact on the quality of stored cereal products. These chemicals that are beneficial for both the carnivores and the plant have been termed ‘herbivore induced synomones’ (HIS). Since the first description of this interaction between plants, herbivores and carnivores mediated by HIS there have been numerous studies on this phenomenon. There is evidence to suggest that HIS can regulate tritrophic interactions among plants, insect herbivores, and natural enemies of the herbivores (Steinberg et al., 1993; Ockroy et al., 2001; Tinzara et al., 2005; Dalessandro et al., 2009; Deshpande and Kainoh, 2012; Peñaflor and Bento, 2013; Desurmont et al., 2015; Yoneya and Miki, 2015). To date, HIS have been demonstrated mostly in the somatic tissue of plants, particularly for foliage. It is well documented that carnivorous arthropods often use volatile chemical cues that are released from the food plants of their victims when searching for prey or hosts. By way of comparison, little is known about the release of HIS from reproductive tissue such as rice grain (Tang et al., 2009).

The present paper is devoted to a tritrophic system consisting of the rice grains (Oryza sativa L.), larvae of the maize weevil Sitophilus zeamais (Coleoptera: Curculionidae) that are endophytic in rice grains, and the pteromalid wasp Lariophagus distinguendus (Hymenoptera: Pteromalidae), an idiobiont ectoparasitoid of beetle larvae, infesting seeds and grains. The maize weevil S. zeamais is one of the most common and destructive storage pests of stored cereal grains in the world. The adult beetles can attack whole grains, and eggs are laid...
SUSTANCIAS VOLÁTILES DE GRANOS DE ARROZ INDUCIDAS POR HERVÍBOROS
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RESUMEN

En un olfatómetro de cuatro cámaras se estudiaron las respuestas de hembras de Lariophagus distinguendus (Hymenoptera: Pteromalidae) a señales volátiles provenientes de granos de arroz e inducidas por herbívoros. Los resultados mostraron que las hembras de L. distinguendus, independientemente de su experiencia, son atraídas por los olores desprendidos por los granos de arroz. Se investigaron las sustancias activas presentes en la saliva del gorgojo del maíz, Sitophilus zeamais, que inducen la producción de sustancias volátiles en el arroz. Los resultados mostraron que los granos de arroz artificialmente dañados no emiten cantidades de sustancias volátiles capaces de atraer a parasitoides hembras experimentadas emitidas por las larvas de S. zeamais de granos dañados. Experimentos adicionales revelaron que las hembras experimentadas de L. distinguendus eran aparentemente atraídas por los olores desprendidos de los granos de arroz e inducidos por la saliva de las larvas de gorgojos. Adicionalmente se compararon las respuestas de los parasitoides hembras a olores emanados del arroz inducidos por proteínas del vómito y por sustancias no proteicas. Los experimentos mostraron que los productos volátiles que atraen los parasitoides de L. distinguendus son inducidos probablemente por algunas sustancias proteicas de la saliva del gorgojo. Se discute el efecto de la experiencia previa en la respuesta de los parasitoides a diversos estímulos.

SUBSTÂNCIAS VOLÁTEIS DE GRAÓS DE ARROZ INDUZIDAS POR HERVÍBOROS
AFETAM A ATRAÇÃO DE INIMIGOS HERBÍVOROS
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RESUMO

Em um olfatómetro de quatro câmaras se estudaram as respostas de fêmeas de Lariophagus distinguendus (Hymenoptera: Pteromalidae) a sinais volátiles provenientes de grãos de arroz e induzidas por herbívoros. Os resultados mostraram que as fêmeas de L. distinguendus, independentemente de sua experiência, são atraídas pelos odores desprendidos pelos grãos de arroz. Investigaram-se as substâncias ativas, presentes na saliva do gorgojo do milho, Sitophilus zeamais, que induzem à produção de substâncias voláteis no arroz. Os resultados mostraram que os grãos de arroz artificialmente danificados não emitem quantidades de substâncias voláteis capazes de atrair parasitoides fêmeas experimentadas emitidas pelas larvas de S. zeamais de grãos danificados. Experimentos adicionais revelaram que as fêmeas experimentadas de L. distinguendus eram aparentemente atraídas pelos odores desprendidos dos grãos de arroz e induzidos pela saliva das larvas de gorgulhos. Adicionalmente se compararam as respostas dos parasitoides fêmeas a odores emanados do arroz induzidos por proteínas do vómito e por substâncias não proteicas. Os experimentos mostraram que os produtos voláteis que atraem os parasitoides de L. distinguendus são induzidos provavelmente por algumas substâncias proteicas da saliva do gorgojo. Discute-se o efeito da experiência prévia na resposta dos parasitoides a diversos estímulos.

Inside kernels in which the larvae feed and develop entirely and pupate. The oviposition hole is closed with an egg plug (Hill, 1983; Storey, 1987; Kouninki et al., 2007). L. distinguendus has been suggested as a biological control agent against the maize weevil in grain stores. The parasitoid egg is laid into the infested grains beside the beetle larva. Females of L. distinguendus have been reported to locate its host by volatiles derived from infested grain (Steidle and Scholler, 1997). However, the attractiveness mechanisms of herbivore-induced rice grain volatiles to parasitoid are still unknown.

The present study was initiated to elucidate the effects of herbivore-induced rice grains volatiles on the taxis behavior of L. distinguendus and its mechanisms. Such research was of importance to reveal the mechanism of indirect defense of rice grains against larvae of S. zeamais and, it was also very important in the understanding of the ecological functions of herbivore-induced rice grains volatiles. The results would provide new information to the biological control efficiency of parasitic wasp on stored-grain pests, which represents a technical support of great demand in green grain storage.

Materials and methods

Insect rearing

All insect rearing was carried out at 26 ±1°C, 70 ±5% relative humidity (r.h.) and a photoperiod of L14: D10. To rear L. distinguendus, 50 newly emerged adult wasps were placed into Petri dishes (9cm diameter, 1cm high) with ~50g of rice grains infested by 3rd-4th instar larvae of S. zeamais and kept there until their death. After a developing time of 19-21 days, parasitoids emerged from the next generation were collected daily from each Petri dish. To rear S. zeamais, 30 adults were allowed to oviposit into 300ml of rice grains with about 14% moisture content in glass jars (8cm diameter, 10cm high). To obtain unmated males or females of S. zeamais; adults were separated by dimorphic rostral characteristics within 12h of emergence (Halstead, 1963).

Insects for bioassays

Parasitoids used in experiments were about 3 days old. To obtain naïve parasitoids for bioassays, freshly emerged male and female parasitoids were collected from the infested grains within 1h of emergence and kept in Petri dishes on moistened filter paper in a climatic chamber without host odors under the same conditions as described above.

To obtain experienced parasitoid females, recently emerged (<24h) wasps were placed into Petri dishes containing rice grains infested by weevil larvae and imagines of S. zeamais. Females were allowed to mate and oviposit for 2 days. Subsequently, they were removed and kept in Petri dishes.
with moistened filter paper until they were used in the experiments on the following day.

**Static four-chamber olfactometer**

The response of female parasitoids to different odor samples was examined using a static four-chamber olfactometer, as described by Ruther and Steidle (2000). The olfactometer (Figure 1) was made of acrylic and consisted of a cylinder (4cm high, 19cm diameter) divided by vertical plates into four chambers. On the top of the cylinder, a walking arena (1cm high, 19cm diameter) was placed consisting of plastic gauze (0.5mm mesh) with a rim of acrylic (0.9cm high) and covered with a glass plate to prevent parasitoids from escaping. No airflow was generated. An odor sample was placed in a Petri dish (5.5cm diameter) with brown filter paper (4cm diameter) in one chamber or in two opposite chambers. Volatiles were allowed to diffuse through the gauze, resulting in an odor field in the walking arena above. The remaining chambers contained Petri dishes with brown filter paper as controls.

**General methods for bioassays**

Evaluations were performed under constant temperature and humidity at 26 ±1ºC and 70±5% r.h., in the darkness under red light to avoid distraction of parasitoids by light but to enable observations. Behavioral data were visually recorded using a stopwatch. To avoid biased results due to possible side preferences of the parasitoids, the position of the olfactometer was rotated 90º clockwise after every test. Contamination of the walking arena with sample odors or by possible pheromones of the parasitoids was avoided by cleaning the walking arenas and glass plates with ethanol and demineralized water before each insect. For all experiments, odor samples were renewed after five parasitoids had been tested.

Fifty parasitoids were tested for each type of sample. Each individual parasitoid was used only once. At the start of each bioassay, the parasitoids were released individually in the center of the walking arena and their standing times in the four sectors above the arena were registered for 600s. The time the parasitoids spent walking in the areas directly above the Petri dishes with odor samples was compared to the areas with control Petri dishes and used to assess the stopping effect of an odor sample. Parasitoids that walked for less than 50% of the total observation time were not included in the statistical analysis.

**Collection of weevil regurgitant**

Instars of the maize weevil, *S. zeamais*, were obtained from rearing facilities. They were placed on rice for 12-16h before their regurgitant was collected. The collection procedure was described by Turlings et al. (1993). Briefly, well-fed instars were held with a pair of lightweight forceps and gently squeezed in the head region with another pair. This caused the instars to empty their foregut content (regurgitant), which was collected by drawing it via a 100ml capillary tube into a vial under low vacuum. The samples were preserved with 5ml of 0.05M sodium phosphate (pH 8.0) and used for bioassays within 12h.

**Responses of *L. distinguendus* females to *S. zeamais* larvae induced rice grains volatiles**

A series of experiments were conducted to test the attraction of *L. distinguendus* females to herbivore-induced odors emitted from rice grains using the static four-chamber olfactometer described above. Fifty weevil larvae infested grains from which larvae, faeces, and egg plugs had been removed (infested grain only; LIGO) vs 50 healthy grains, which had been artificially damaged (artificially damaged grain; AG). Two empty petri dishes (C) were used as control.

The infested grain tested by weevil larvae was obtained by dissecting grains infested by 3rd-4th instar weevil larvae. The infested grain material was cleaned from faeces using a fine brush. Artificially damaged grains were cut with scissors, knives or needles in order to better mimic damages caused by the gnawing larvae.

**Responses of *L. distinguendus* females to *S. zeamais* larvae regurgitant induced rice grains volatiles**

A series of experiments was conducted to test the attraction of *L. distinguendus* females to regurgitant-induced odors emitted from rice grains, using the original regurgitant (OR) or boiled regurgitant (BR). The original regurgitant was centrifuged at 1000rpm for 10min; the pH of the supernatant adjusted with citric acid and centrifuged again at 15000rpm for 30min. The resulting supernatant (protein substances; RP) was used for bioassays, while the resulting precipitate (non-protein substances; RNP) was washed with 0.05M sodium phosphate (pH 8.0) and re-dissolved in the same volume of the original solution (RNP). Finally, part of the protein-containing supernatant (RP) was boiled in order to denature the proteins (BP).

A 1mm diameter hole, ~2mm deep, was drilled on healthy rice grains. Three different experiments were conducted using the static four-chamber olfactometer to test the attraction of *L. distinguendus* females to regurgitant induced rice grains volatiles: 1) Rice grains (50g) were infused 2µl of the OR extract vs 50g of grains infused 2µl of the BR extract, and two empty petri dishes. 2) Grains (50g) infused 2µl of the RP extract vs 50g of grains infused 2µl of RNP extract, and two empty petri dishes. 3) Rice grains (50g) infused 2µl of RP extract vs 50g infused 2µl BP extract, and two empty petri dishes. The infused rice was placed separately in the Petri dishes and kept in humidifiers containing a saturated sodium chloride solution at 65-70% r.h. for seven days before being used.

**Statistical analysis**

The Friedman ANOVA was used to test for differences between the four areas. In case that significant differences were found, the Wilcoxon-Wilcoxon-test for multiple comparisons (Sachs, 1992) was used to determine which sectors are different from each other.

**Results**

**Responses of female parasitoids to *S. zeamais* induced rice grain volatiles**

Naive and experienced *L. distinguendus* females spent significantly (P<0.05) more time in treatment odor fields compared to control odor fields, in experiments involving either rice grains infested by *S. zeamais* from which weevil, faeces, and egg plugs had been removed or artificially damaged rice grains (Figures 2 and 3). Naive *L. distinguendus* females showed no statistically significant difference (P>0.05) in their choice between rice grains infested by *S. zeamais* and artificially damaged rice grains (Figure 2). However, in contrast to inexperienced parasitoids, experienced *L. distinguendus* females were

![Figure 1. Static four-chamber-olfactometer used for bioassays.](image-url)
Females were in a four chamber olfactometer. OR, BR and C as in Figure 2. Bars with different letters are significantly different at P<0.05.

strongly (P<0.05) attracted to the rice grains infested by weevil larvae from which weevil, faeces, and egg plugs had been removed over the artificially damaged rice grains (Figure 3). The results strongly suggested that the experienced L. distinguendus females were attracted by the specific defensive chemicals released from infested grains.

Responses of female parasitoids to original regurgitant induced rice grain volatiles

Naive and experienced L. distinguendus females spent significantly (P<0.05) more time in treatment odor fields compared to control odor fields (Figures 4 and 5). Naive L. distinguendus females showed no statistically significant difference (P>0.05) in choice between rice grains which had been infused 2μl OR and rice grains which had been infused 2μl BR (Figure 4). However, in contrast to inexperienced parasitoids, experienced L. distinguendus females were strongly (P<0.05) attracted to the rice grains which had been infused 2μl OR over the rice grains which had been infused 2μl BR (Figure 5).

Responses of female parasitoids to protein substances from regurgitant induced rice grain volatiles

Naive and experienced L. distinguendus females spent significantly more (P<0.05) time in treatment odor fields compared to control fields of the olfactometer in all experiments (Figures 6 and 7 and, 8 and 9). Naive L. distinguendus females showed no statistically significant difference (P>0.05) in choice between grains which had been infused 2μl of original regurgitant, BR: areas above Petri dishes with grains which had been infused 2μl of boiled regurgitant, C: areas above control Petri dishes. Bars with different letters are significantly different at P<0.05.

Discussion and Conclusion

Many parasitoids are able to learn to locate their hosts using cues associated with the hosts or host’s environment (Tumlinson et al., 1992; Godfray, 1994; Canale et al., 2014). They appear to associate them with the presence of a host (associate learning) or generally increase their response to stimuli by a certain experience, as shown for parasitoids of many families (Vet and Dicke, 1992). They direct host location by using innate cues from different, taxonomically non-related hosts and host plants (seeds). Like numerous other insects, L.
distinguentes has been shown to learn from experience (Steidle, 2000). The results obtained are consistent with previous studies, in which L. distinguentes showed learning ability in an olfactometer. It will be able to locate further hosts more easily by using these additional stimuli.

Herbivore-induced plant odors have been reported to be used as chemical cues in the foraging behavior of natural enemies. When plants are invaded by larvae of insects or other arthropods, they usually produce and release defensive chemicals that can be recognized by predators and parasitoids to locate their victims. These plant chemicals may also serve as ‘alarm signals’ that are exploited by predators and parasitoids to locate their victims. There is considerable evidence that the volatile ‘alarm signals’ are induced by interactions of substances from the herbivore with the damaged plant tissue (Steowe et al., 1995). However, so far few experimental evidences have been reported to support this, and the release of herbivore-induced synomones (HIS) has almost exclusively been demonstrated in somatic plant tissues. In 2005, Steidle et al. concluded that the plants have the ability to ‘whisper for help’ of the parasitoid L. distinguentes. However, it was not demonstrated whether potential HIS were actively produced by infested grains or a by-product of grain metabolism.

Experienced L. distinguentes females were strongly attracted to the grains to which protein substances from original regurgitant had been applied. As demonstrated by Turlings et al. (1991) for the parasitoid Cotesia marginiventris (Cresson) and its host Spodoptera exigua (Höfner) and, by Mattiacci et al. (1995) for Cotesia glomerata (L.) and its host Pieris brassicae (L.), plant volatiles can be induced by the regurgitate of caterpillars. Thus, it seems likely that parasitoid attracting volatiles are also induced by the saliva of the weevil larvae or females. Although theoretically less likely, some generalist predators appear to make some use of plant volatiles to locate prey. Yellow jacket wasps in the Vespula vulgaris species group are attracted to mixtures of (E)-2-hexenal with either a-terpineol or linalool-all ubiquitous components of volatiles from plants under attack by herbivores (Steowe et al., 1995).

Herbivore-induced plant odors play major roles in the foraging behavior of natural enemies. When the larval stages of insects or other arthropods are the targets, a foraging strategy is employed. Under the condition that some specific chemical cues are used in the communication between insect pests and their natural enemies (Cox, 2004), the strategy of applying semiochemicals into the biological controls has been considered to attract parasitoids or predators into a crop or increase the amount of time they spend in a field. Therefore, increased understanding of the chemically mediated interactions between arthropod hunters and their victims will be useful for the biological controls of pests on a crop. Our ultimate goal is to be able to develop an environmentally friendly method to control the pest resurgence on a crop and reduce the currently heavy dependence on pesticides.
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