



Sitophilus zeamais - Induced Rice Grain Volatiles: Attractiveness Towards the Generalist Parasitoid Wasp, *Theocolax elegans*

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ABSTRACT

One of the most damaging insect pests to stored grain is the maize weevil, *Sitophilus zeamais*. Current control methods are the use of fumigants and residual chemical insecticides and not ecologically friendly, leading to the development of new strategies based on its natural enemies. In this study, we evaluated the ability of *Theocolax elegans* to discriminate *S. zeamais* - infested rice grains versus non-infested rice grains. We subsequently identified the active chemicals in the saliva of the maize weevil that elicit the production of rice grain volatiles. Behavioral assays were conducted in a multiple-choice olfactometer. Our results demonstrated that the experienced *T. elegans* females were apparently attracted by the odors released from rice grains induced by the saliva of the *S. zeamais* larvae. Moreover, the active chemicals in the saliva of *S. zeamais* larvae that elicit the production of rice volatiles were 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 *T. elegans* females were apparently attracted by the odors released from rice induced by the saliva of the *S. zeamais* larvae. Moreover, we compared the responses of female parasitoids to odors released from rice induced by protein substances of saliva and non-protein substances. Further experiments revealed that volatiles attracting parasitoid *T. elegans* are possibly induced by some protein substances from *S. zeamais* larvae saliva.

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Authors' Contribution

QT designed and performed the experimental work, analyzed the data and wrote the article.

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Volatile organic compounds,
Saliva, Biological control

INTRODUCTION

The maize weevil (*Sitophilus zeamais* Motschulsky; Coleoptera: Curculionidae) is one of the most common and destructive storage pests in the world (Araujo *et al.*, 2008; Wang *et al.*, 2015). Damage caused by *S. zeamais* on stored rice grains may be extremely high in tropical and sub-tropical regions (Ribeiro *et al.*, 2014). Infestation may start in the mature crop standing in the field and continue in the granary where considerable losses can occur (Giles, 1969). Adult female *S. zeamais* cause damage by boring into the kernel and laying eggs (ovipositing). Then, larvae and pupae eat the inner parts of the kernel, resulting in a damaged kernel and reduced grain weight (Tang *et al.*, 2008). Apart from weight losses, the damaged kernels have low nutritional value, low rate of germination, low commercial value and decreased weight, and increased susceptibility to fungal infestation (Nwosu *et al.*, 2015; Guedes *et al.*, 2006). *S. zeamais* cause extensive losses in quality and quantity of the grain in the field as well as in storage (Carvalho *et al.*, 2014). Currently, the primary means of maize weevil

control in warm climates is the use of fumigants and residual chemical insecticides (Faruki *et al.*, 2005; Negahban and Moharramipour, 2007). Synthetic chemical pesticides are usually applied by the farmers to reduce losses during storage (Adane *et al.*, 1996). In recent years, however, the overreliance and the use of chemical insecticides in crop pest control programs (around the world) has resulted in environmental damage, pest resurgence, pest resistance to insecticides, and lethal effects on non-target organisms (Memon and Gilbert, 2013). Furthermore, because of cost, these pesticides are becoming increasingly inaccessible to farmers, particularly in developing countries (Tang *et al.*, 2008; Slater *et al.*, 2012). 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.

Theocolax elegans is a solitary ectoparasitoid that parasitizes larvae and pupae of *Rhyzopertha dominica* (F.) (Coleoptera : Bostrichidae), *Sitophilus* spp. (Coleoptera : Curculionidae), *Stegobium paniceum* (L.) (Coleoptera : Anobiidae), *Callosobruchus* spp. (Coleoptera : Bruchidae), and *Sitotroga cerealella* (Olivier) (Lepidoptera : Gelechiidae) that develop inside cereal grains or legume seeds (Flinn *et al.*, 1996). *T. elegans* has been shown to have potential as a biocontrol agent. This parasitoid wasp spontaneously colonizes

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granary, but its population density is rarely sufficient to control the pests (Toews *et al.*, 2001; Flinn *et al.*, 2006). Thus, the use of specific attractants might represent an option to increase the presence of this parasitoid wasp in granary or could be coupled with augmentative biocontrol programmes and improve its biocontrol services. It is effective in controlling insect pests of stored products and has a positive impact on the quality of stored cereal products.

Several studies have demonstrated that many species of natural enemies are attracted by volatile compounds emitted by infested plants, with these compounds called 'Herbivore Induced Synomones' (HIS). These chemicals are beneficial for both the carnivores and the plant (Machado *et al.*, 2014). 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 much evidence to suggest that HIS can regulate tritrophic interactions among plants, insect herbivores, and natural enemies of the herbivores (Yoneya and Miki, 2015; Desurmont *et al.*, 2015; Deshpande and Kainoh, 2012; Dalessandro *et al.*, 2009; Tinzaara *et al.*, 2005; Ockroy *et al.*, 2001; Steinberg *et al.*, 1993). To date, HIS have mostly been demonstrated 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 the victims of potential hosts when searching for prey. By way of comparison, little is known about the release of HIS from reproductive tissue such as rice grain seeds (Tang *et al.*, 2009).

This study aimed to evaluate the ability of *T. elegans* to discriminate *S. zeamais* - infested versus non-infested rice grains, and to identify the active chemicals in the saliva of the maize weevil that elicit the production of rice grain volatiles. The results are expected to provide baseline information for the development of pest control measures based on the enemies of *T. elegans*. This research was of great importance to reveal the mechanism of indirect defense of grain seeds against stored-grain insect. And it was also very important in the understanding of the ecological functions of herbivore-induced grains volatiles.

MATERIALS AND METHODS

Insects cultures

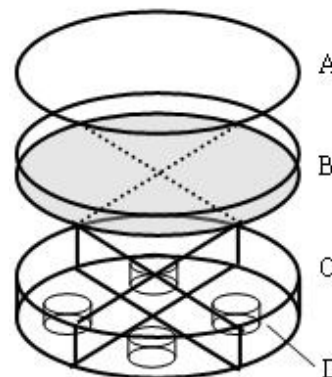
All insect cultures were kept at $26 \pm 2^\circ\text{C}$, $70 \pm 5\%$ relative humidity (r.h.) and a photoperiod of L14: D10. To rear *T. elegans*, 50 newly emerged adult wasps were placed into Petri dishes (9 cm diameter, 1 cm high) with about 50g of rice grains infested by 3rd-4th instar larvae

of *S. zeamais* and kept there until their death. After a developing time of 19-25 days, emerged parasitoids 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 (8 cm diameter, 10 cm high). To obtain unmated males or females of *S. zeamais*, adults were separated by dimorphic rostral characteristics within 12 h of emergence (Halstead, 1963).

Insects for bioassays

Parasitoids used in experiments were about 2 d old. To obtain experienced parasitoid females, recently emerged (< 24 h old) 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 3 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.

In accordance with Vet and Groenewold (1990), we define inexperienced parasitoids as insects that had no experience with the host beyond that that occurred during development within and eclosion from the host. To obtain naive parasitoids for bioassays, freshly emerged male and female parasitoids were collected from the infested grains within 1 h 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.



A. Glass plate. B. Arena. C. Cylinder. D. Petri dishes.

Fig. 1. Static four-chamber olfactometer used for bioassays. For details see text.

Static four-chamber olfactometer

The response of female parasitoids towards different odor samples was examined using a static four-chamber olfactometer as described by Ruther and Steidle (2000). The olfactometer (Fig. 1) was made of acrylic glass and

consisted of a cylinder (4 cm high, 19 cm diameter) divided by vertical plates into four chambers. On the top of the cylinder, a walking arena (1 cm high, 19 cm diameter) was placed consisting of plastic gauze (mesh 0.5 mm) with a rim of acrylic glass (0.9 cm 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 (5.5 cm diameter) with brown filter paper (4 cm 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 only as controls.

General methods for bioassays

Evaluations were performed in a constant temperature and humidity room at $26\pm 2^\circ\text{C}$ and $70\pm 5\%$ 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 clockwise by 90° after every insect. 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 each.

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 arrestment times in the four sectors above the arena were registered for 600 sec. 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 arrestment 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 saliva

Instars of the maize weevil, *S. zeamais*, were obtained from rearing facilities. They were placed on rice grains for 12-16 h before their saliva was collected. The collection procedure has been 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 (saliva), that was collected by drawing it via a 100-ml capillary tube into a vial under low vacuum. The samples were preserved with 5 ml of

0.05 M sodium phosphate (pH 8.0) and used for bioassays within 12 h.

Responses of T. elegans females to S. zeamais induced rice grains volatiles

We conducted a series of experiments to test the attraction of *T. elegans* females to herbivore-induced odors emitted from rice grains. (1) 50 grains infested with weevil larvae from which larvae, faeces, and egg plugs had been removed [infested grain only (LIGO)] versus 50 healthy grains, that had been artificially damaged [artificially damaged grain (AG)] and two empty petri dishes (C). (2) 50 grains infested with adult *S. zeamais* males from which weevils and faeces had been removed [infested grain only (MIGO)] versus 50 healthy grains, that had been artificially damaged (AG) and two empty petri dishes (C). (3) 50 grains infested with unmated adult *S. zeamais* females from which the weevils and faeces had been removed [infested grain only (FIGO)] versus 50 healthy grains, that had been artificially damaged (AG) and two empty petri dishes (C).

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 or adults.

Responses of T. elegans females to S. zeamais larvae saliva induced rice grains volatiles

We conducted a series of experiments to test the attraction of *T. elegans* females to saliva-induced odors emitted from rice grains. 1, Original saliva (OS); 2, boiled saliva (BS); 3, The original saliva were centrifuged at 1000 r/min for 10 min; 4, The supernatant was adjusted the pH to acidic with citric acid. Then the supernatant were centrifuged at 15000 r/min for 30 min. The supernatant was used for bioassays (RP). 6, The resulting precipitate (non-protein substances) was washed with 0.05M sodium phosphate (pH 8.0) and re-dissolved in the same volume of the original solution (RNP); 7, Boiled protein substances from 5 (BP).

A hole 2 mm deep with/min depth was drilled in the base of healthy rice grains with the help of a needle. Three different experiments were conducted using the static four-chamber olfactometer to test the attraction of *T. elegans* females to saliva induced rice grains volatiles. (1) 50 g rice infused with 2 μ l OS extract versus 50 g rice infused with 2 μ l BS extract, and two empty petri dishes. (2) 50 g rice infused with 2 μ l RP extract versus 50 g rice infused 2 μ l extract RNP, and two empty petri dishes. (3) 50 g rice infused 2 μ l RP extract versus 50 g rice infused with 2 μ l BP extract and two empty petri dishes. The rice

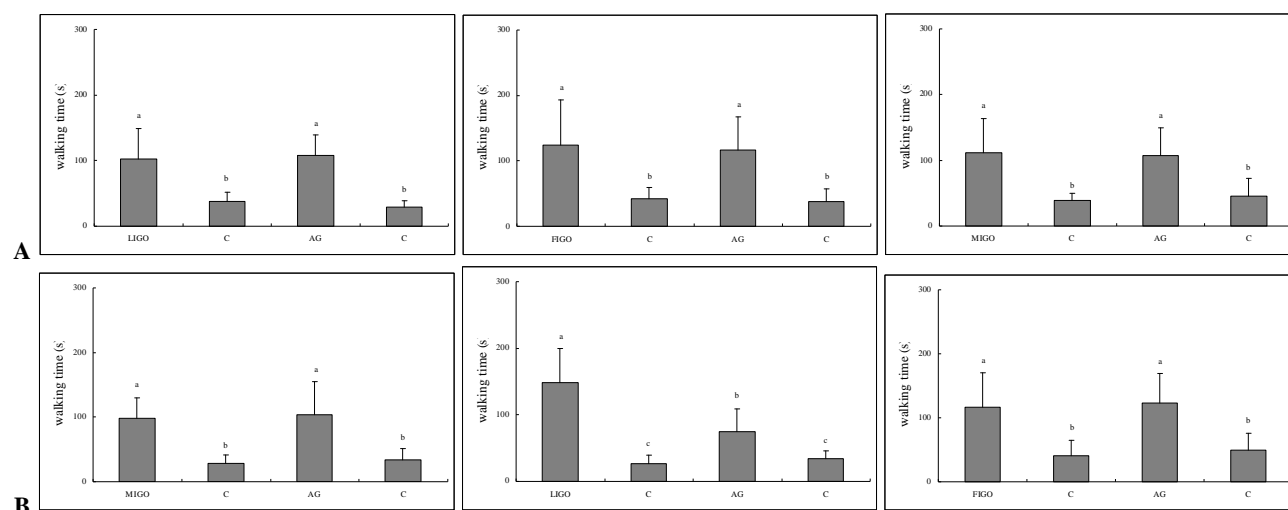


Fig. 2. Mean walking time (\pm SD; $n = 50$) of naïve females (A) and experienced females (B) of *Theocolax elegans* in a four chamber olfactometer. LIGO, areas above Petri dishes with infested grains by weevil larvae from that larvae, feces, and egg plugs had been removed, MIGO, areas above Petri dishes with infested grains by adult *S. zeamais* males from that weevils and feces had been removed, FIGO, areas above Petri dishes with infested grains by unmated adult *S. zeamais* females from that weevils and feces had been removed, AG: artificially damaged grains, C: areas above control Petri dishes. Bars with different letters are significantly different at $P < 0.05$ (Friedman ANOVA followed by Wilcoxon-Wilcox-test for multiple comparisons).

was allowed separately to place in the Petri dishes 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 of significant differences the Wilcoxon-Wilcox-test for multiple comparisons was used to determine which sectors are different from each other.

RESULTS

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

The results of the multiple choice bioassay are presented in Figures 2 and 3. Naive and experienced *T. elegans* females spent significantly ($P < 0.05$) more time in treatment odor fields compared to control odor fields in experiments involving either infested rice grains by *S. zeamais* from which weevil, faeces, and egg plugs had been removed (Naive, LIGO, 102 ± 47 s; Naive, MIGO, 112 ± 51 s; Naive, FIGO, 124 ± 69 s; Experienced, LIGO, 148 ± 52 s; Experienced, MIGO, 98 ± 32 s; Experienced, FIGO, 117 ± 53 s) or artificially damaged rice grains (Naive, LIGO, 108 ± 31 s; Naive, MIGO, 107 ± 42 s; Naive, FIGO, 117 ± 50 s; Experienced, LIGO, 75 ± 34 s; Experienced, MIGO, 104 ± 51 s; Experienced, FIGO, 123 ± 46 s). The results suggested that the *T. elegans*

directs the host location by using innate cues from host rice grains.

Naive *T. elegans* females showed no statistically significant difference ($P > 0.05$) in choice between infested rice grains by *S. zeamais* (LIGO, 102 ± 47 s; MIGO, 112 ± 51 s; FIGO, 124 ± 69 s) and artificially damaged rice grains (LIGO, 108 ± 31 s; MIGO, 107 ± 42 s; FIGO, 117 ± 50 s) (Fig. 2A). However, in contrast to inexperienced parasitoids, a significantly ($P < 0.05$) higher proportion of experienced *T. elegans* females orientated towards the area associated with the infested rice grains by weevil larvae from that weevil, faeces, and egg plugs had been removed (LIGO, 148 ± 52 s), rather than towards the artificially damaged rice grains (LIGO, 75 ± 34 s) (Fig. 2B). When evaluating experienced *T. elegans* females' choice between the infested rice grains by adult *S. zeamais* from which faeces had been removed (MIGO, 98 ± 32 s; FIGO, 117 ± 53 s) and artificially damaged rice grains (MIGO, 104 ± 51 s; FIGO, 123 ± 46 s), we observed no preference. The results strongly suggested that the experienced *T. elegans* had the ability to discriminate *S. zeamais* larvae - infested versus non-infested rice grains and the specific defensive chemicals were released from infested grains.

Responses of female parasitoids to original saliva induced rice grain volatiles

The results of the multiple choice bioassay are presented in Figure 3A. Experienced *T. elegans* females

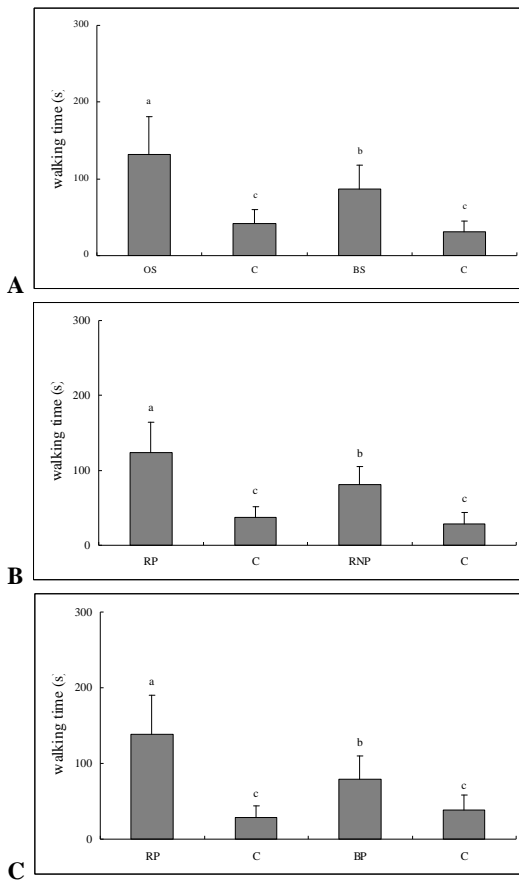


Fig. 3. Mean walking time (\pm SD; n = 50) of experienced females of *T. elegans* in a four chamber olfactometer. (A) OS: areas above Petri dishes with grains that had been infused 2 μ l original saliva, BS: areas above Petri dishes with grains that had been infused 2 μ l boiled saliva. (B) RP, areas above Petri dishes with grains that had been infused 2 μ l protein substances; RNP, areas above Petri dishes with grains that had been infused 2 μ l non-protein substances. (C) RP, areas above Petri dishes with grains that had been infused 2 μ l protein substances; BP, areas above Petri dishes with grains that had been infused 2 μ l boiled protein substances. C, areas above control Petri dishes. Bars with different letters are significantly different at $P < 0.05$ (Friedman ANOVA followed by Wilcoxon-Wilcoxon-test for multiple comparisons).

spent significantly ($P < 0.05$) more time in treatment odor fields (OS: 132 \pm 49s; BS: 87 \pm 31s) compared to control odor fields (C: 42 \pm 18s, 31 \pm 14s). Also, experienced *T. elegans* females were strongly ($P < 0.05$) attracted to the rice grains that had been infused 2 μ l original saliva (OS: 132 \pm 49s) over the rice grains that had been infused 2 μ l boiled saliva (BS: 87 \pm 31s). The results strongly

suggested that the *S. zeamais* larvae saliva had the active chemicals that induced the indirect defense of rice grains.

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

The results of the multiple choice bioassay are presented in Figure 3B, 3C. Experienced *T. elegans* females spent significantly more ($P < 0.05$) time in treatment odor fields compared to control fields of the olfactometer in all experiments.

Experienced *T. elegans* females were strongly ($P < 0.05$) attracted to rice grains that had been infused 2 μ l protein substances (RP, 124 \pm 40s) over 10 rice grains that had been infused 2 μ l non-protein substances (RNP, 81 \pm 24s) (Fig. 3B). Experienced *T. elegans* females showed statistically significant difference ($P < 0.05$) in choice between grains that had been infused 2 μ l protein substances (RP, 138 \pm 52s) and grains that had been infused 2 μ l boiled protein substances (BP, 79 \pm 31s) (Fig. 3C). The results strongly suggested that the experienced *T. elegans* females were attracted by the grains that had been infused protein substances from *S. zeamais* larvae saliva.

DISCUSSION

In this paper, we investigated the ability of a generalist parasitoid wasp to respond to grains chemical cues associated with damage induced by a phytophagous pest. We found that *T. elegans* was able to discriminate rice grains infested by *S. zeamais* larvae from a non-infested rice grains using olfactory cues. Subsequently, we confirmed protein substances in the saliva of *S. zeamais* larvae induced the indirect defense of rice grains.

Parasitoids use cues associated directly with the hosts or indirectly with host's environment to locate their hosts. Many parasitoids are able to learn to host-related cues (Canale *et al.*, 2014; Godfray, 1994; Tumlinson *et al.*, 1992). They associate them with the presence of a host (associate learning) or generally increase the response to stimuli by a certain experience, as shown for parasitoids of many families (Vet and Dicke, 1992; Fernandez-Grandon and Poppy, 2015). These results are consistent with this study, that *T. elegans* in an olfactometer showed learning ability. Under these conditions it is not surprising that *T. elegans* females rely heavily on learning, and their response to plant volatile mixtures is reinforced by hunting success. The generalist parasitoid *T. elegans* is able to use innate cues from different, taxonomically non-related hosts and host seeds for directed host location, then learning to respond to stimuli emanating from the host habitats as well. It will

be able to locate further hosts more easily by using these additional stimuli.

Plant odors convey ecologically relevant information to other organisms and thereby play an important role in mediating diverse interactions within terrestrial communities (Heil, 2008). For example, changes in plant volatile emissions elicited by insect feeding damage can influence the behavior of foraging arthropods, with implications for tri-trophic plant–herbivore–natural enemy interactions (Vet and Dicke, 1992; Mauck *et al.*, 2010; Gossner *et al.*, 2014). Indeed, herbivore-induced plant volatiles are thought to function as an indirect form of plant defense by recruiting natural enemies of feeding herbivores (Kaplan and Lewis, 2015). This study provides the experiment data demonstrating that the experienced *T. elegans* females respond to volatiles released by rice grains following *S. zeamais* larvae feeding.

HIS blends elicited by arthropod feeding (or oviposition) are typically chemically distinct from blends induced by mechanical damage alone, and their production is influenced by the presence of chemical elicitors in the oral secretions of feeding herbivores (Reymond, 2013; De Moraes *et al.*, 2001; Alborn *et al.*, 1997). Consequently, HIS provide information-rich cues regarding the presence and identity of feeding herbivores that have been implicated in a wide range of above-ground and below-ground interactions among plants and other organisms (Rasmann *et al.*, 2005). As noted above, many predatory and parasitic arthropods utilize HIS cues to locate otherwise cryptic herbivorous prey in complex olfactory environments (Takabayashi and Dicke, 1996). As demonstrated by Turlings *et al.* (1991) for the parasitoid *Cotesia marginiventris* (Cresson) and its host *Spodoptera exigua* (Hübner) and by Mattiacci *et al.* (1995) for *Cotesia glomerata* (L.) and its host *Pieris brassicae* (L.), plant volatiles can be induced by the saliva of caterpillars. Thus it seems likely that parasitoid *T. elegans* attracting volatiles are also induced by the saliva of the weevil larvae. 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 α -terpineol or linalool—all ubiquitous components of volatiles from plants under attack by herbivores (Steowe *et al.*, 1995). Herbivores themselves also frequently utilize HIS as foraging cues, often exhibiting aversive responses to the odors of damaged plants, but in some cases utilizing such odors as aggregation cues (Loughrin *et al.*, 1996). The characteristics and functions of herbivory insect induced plant volatiles are results of the co evolution among plant, herbivore and natural enemy.

CONCLUSIONS

This study confirmed that the generalist parasitoid wasp *T. elegans* uses common herbivore-induced plant volatiles to locate prey. Moreover behavioral assays have demonstrated that protein substances from *S. zeamais* larvae saliva contribute towards attracting *T. elegans* to infested rice grains. This information may be a baseline to further studies aiming to develop semiochemical strategies that could be incorporated into an integrated management approach to improve existing pest control techniques of the stored-grain insect.

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Conflict of interest declaration

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “*Sitophilus zeamais* - Induced Rice Grain Volatiles: Attractiveness Towards the Generalist Parasitoid Wasp *Theocolax elegans*”.

Statement of conflict of interest

Authors have declared no conflict of interest.

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