Oviposition Preferences of Oriental Fruit Moth *Grapholita Molesta* (Lepidoptera: Tortricidae) To Pear HIPVs

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Research Article

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Abstract

Herbivore-induced plant volatiles (HIPVs) are regarded as an indirect plant defense against herbivores, since they attract natural enemies to the infested plant. On the other hand, HIPVs also affect behavioral responses of herbivores. In a previous study, *Lytopylus rufipes*, a parasitoid of the oriental fruit moth (*Grapholita molesta*), showed positive responses to pear HIPVs, but the influence of pear HIPVs on *G. molesta* is still unclear. To better understand these responses, we first tested oviposition preferences of *G. molesta* to uninfested and infested pear shoots (US and IS, respectively) and uninfested mature leaves (ML) in dual-choice bioassays with a cylindrical tube. Then, preferences to volatile components were also assessed. Results show that *G. molesta* females preferred US-, IS-, and ML-treated areas compared to the control area (no leaves), respectively. Subsequently, *G. molesta* females preferred IS over US, and US more than ML when comparing pear tissues. Furthermore, *G. molesta* females didn't show any significant preference to individual volatile components, but more eggs were laid in the area treated with a synthetic pear HIPV blend, compared to the hexane-treated area (control). These results indicate that *G. molesta* females do not avoid infested pear shoots nor HIPVs. Moreover, HIPVs may recruit not only natural enemies, but herbivores to the release point of HIPVs. Thus, it is important to examine the responses of *G. molesta* females to HIPVs in the field before employing them for pest management.

Introduction

Insects have a sensitive olfactory system that enables them to detect volatile chemicals carrying various types of information, and appropriate responses to that information help them to survive in complex environments. For instance, herbivorous insects use host plant volatiles (HPVs) to locate suitable habitats for feeding or oviposition (Schoonhoven et al. 2005). Then, subsequent herbivory may alter the plant metabolic pathways, inducing modifications to foliar volatile composition (Dicke and Hilker 2003; Schoonhoven et al. 2005). Herbivore-induced plant volatiles (HIPVs) mediate multiple-trophic interactions, such as repelling herbivores or attracting their natural enemies, and are regarded as an indirect defense strategy of plants (Collatz and Dorn 2013; de Moraes et al. 2001).

For herbivores, release of HIPVs not only indicates the presence of conspecific competitors, which may reduce the quantity and/or quality of the host plant, but also implies a higher risk of encountering natural enemies. Thus, avoiding HIPVs can benefit herbivores with adequate food and/or safer habitats, increasing their fitness. de Moraes et al. (2001) found that the tobacco budworm, *Heliothis virescens* (Noctuidae: Lepidoptera), tends to oviposit on uninfested tobacco plants (*Nicotiana tabacum*) compared to tobacco plants infested by conspecific larvae. This is strong evidence indicating that female moths can recognize HIPVs, enabling them to oviposit in uninfested areas where their progeny may have better fitness. Nevertheless, some herbivores show positive responses to HIPVs that conflict with previous observations (El-Sayed et al. 2016, 2018; Giacomuzzi et al., 2016, 2017; Yoneya and Miki 2015). An herbivore attraction to HIPVs may lead to a higher density of herbivores that reduces parasitism pressure (Rusch et al. 2015). El-Sayed et al. (2016) found that the light brown apple moth, *Epiphyas postvittana* (Tortricidae: Lepidoptera), prefers apple seedlings infested by conspecific larvae. In a subsequent study,

both male and female *E. postvittana* were attracted by a synthetic blend (phenylacetonitrile: acetic acid = 100:3) suggesting the potential to apply HIPVs for trapping *E. postvittana* females in orchards (El-Sayed et al. 2019).

The oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae), is a widely distributed invasive pest that is assumed to be native to Asia (Kirk et al. 2013; Rothschild and Vickers 1991). The eggs of *G. molesta* are usually found on young tissues and newly hatched neonates excavate tunnels and feed inside the host plant. This feeding habit protects *G. molesta* larvae from natural enemies and pesticides. For practical treatments, farmers apply insecticides and sex pheromones to suppress field populations of *G. molesta* (Pastori et al. 2012; Trimble et al. 2001). Unfortunately, the efficacy of insecticides may be lower than expected because of the feeding habits of *G. molesta* larvae and the rapid development of insecticide resistance (de Lame et al. 2001; Kanga et al. 2003; Navarro-Roldán et al. 2017). On the other hand, a high density of *G. molesta* may lower the efficacy of mating disruption using sex pheromone against *G. molesta*, suggesting that other approaches are necessary (Allen 1962; Allen and Yetter 1949). For example, HIPVs are potential attractants to keep natural enemies in the field and to enhance efficacy of biological control (Mills and Heimpel 2018).

In a previous study, *G. molesta* larvae induced the release of HIPVs from pear trees, and positive responses of its parasitoid, *Lytopylus rufipes* Nees (Hymenoptera: Braconidae) to the HIPVs (as a volatile bouquet) were observed (Liu et al. 2019). However, responses of *G. molesta* females to pear HIPVs (attractive, neutral, or deterrent) are another important factor in this tri-trophic interaction system. In this study, the oviposition preferences of gravid *G. molesta* to different parts of pear trees and pear HIPVs were evaluated to discover possible effects of pear HIPVs on *G. molesta* in the field.

Materials And Methods

Insect colonies and rearing procedures

A *G. molesta* colony was obtained from the Nagano Fruit Tree Experiment Station in 2015, and the rearing procedure was described by Liu et al. (2019). A transparent plastic container (I.D. 15 cm, height 8 cm, a container hereafter) with a lid was used for rearing *G. molesta*. An egg sheet (ca. 200 eggs) was put in a container with a block of artificial diet (100g, Silkmate 2M, Nosan, Yokohama, Japan). In addition, a corrugated cardboard strip (ca. 3 cm × 47 cm) was attached to the wall in the same container as a shelter for pupation at the same time. Pupae were sexed and kept in separate containers, and newly emerged adults (ca. male: female = 60:30) were transferred to another container with a water dispenser. The inner wall of a container was lined with wax paper for egg collection. Rearing conditions were maintained at 25 ± 1°C and 60 ± 10% RH, with a photoperiod of 16L:8D.

Plant Materials

Potted pear trees (*Pyrus pyrifolia* variety Kosui, 8 years old) were obtained from the Laboratory of Pomology and Postharvest Physiology of Fruit (University of Tsukuba). To prevent accidental infestation,

pear shoots (including the tip and 2 immature leaves) and mature leaves were covered with fine mesh bags until the experiment. Newly hatched (within 24 h) neonates of *G. molesta* were placed near axils (5 neonates/shoot) for infestation 24 h before experiments. Uninfested shoots (US), infested shoots (IS) and mature leaves (ML) were kept on the pear tree and harvested 15 mins prior to experiments.

Daily Fecundity and Oviposition Time of *G. molesta*

To determine conditions for oviposition preference in *G. molesta* females, we observed oviposition time and daily fecundity. Newly emerged adults (male: female = 20:10) were placed in a container (the size and condition are described above) and allowed to mate. Successful mating was checked in the evening, and mated females were then placed in separate containers and provided with wet cotton. When we estimate daily fecundity, each moth was transferred to a new container and the number of eggs in the old container was recorded ($n = 17$). When we observed oviposition time, the number of eggs laid on the wall and cover of the container were recorded every hour from 17:00 until 06:00 the next day ($n = 53$). During the scotophase, red light bulbs (40 W, 10 - 20 lx) were used for observations. Individual differences in egg production (number and pre-oviposition period) were observed in preliminary experiments.

Oviposition Preference of *G. molesta* on Pear Tissues

To examine whether volatiles released from different host plant parts affect oviposition preferences of female oriental fruit moths, dual choice bioassays among control, US, IS, and ML were conducted. This bioassay was modified from the method described by Piyasaengthong et al. (2016). According to preliminary observations of oviposition behaviors, mated female moths showed stable egg production (in number) on the 3rd day after mating, if they laid 1-10 eggs on the first day after mating. Therefore, mated females were prepared as described above and moths that laid 1-10 eggs on the first day after mating were used in bioassays on the 3rd day. An acrylic cylinder (I.D. 9.6 cm, length 32 cm, a cylindrical container hereafter) was used as an area for oviposition bioassays (Fig. S1). Wax paper was attached to the inner surface of the cylindrical container before an experiment. Quarter lines and centerlines were labeled to identify locations of samples (on quarter lines) and the release point (on the centerline). Plant samples were harvested and weighed, and then soaked in water immediately for ca. 15 min prior to the experiments. Pear shoots were harvested from the tip of a branch and ML were harvested at least 30 cm from the tip. The cut surface was covered with wet cotton to prevent desiccation during the experiment and then wrapped with aluminum foil. Samples were covered with a white mesh (double layer) to prevent possible effects of visual or tactile stimulation and then hung at the 1st and 3rd quarter positions in the cylindrical container to give a 16-cm distance to each sample. Wet cotton covered with aluminum foil was used as a blank (control). Ten mated females were anesthetized with CO₂ and placed at the center of the cylindrical container. Wet cotton as a water source was placed in a plastic cup (I.D. 2.5 cm, height 1.2 cm) at the center of the cylindrical container. Each opening of the cylindrical container was covered with a piece of mesh to prevent the escape of moths during experiments. Experiments were set up at 18:00 and collected at 06:00 on the next day. After every experiment, wax paper was collected and the number of eggs on the wax paper was counted. Bioassays were conducted in an air-controlled room at $25 \pm 1.0^{\circ}\text{C}$ and $60 \pm 10\%$ RH.

Oviposition Response to Pear HIPVs

To investigate the effects of pear HIPVs on oviposition preferences of *G. molesta*, volatile components of pear HIPVs were applied as odor sources instead of plant tissues in bioassays. Pear HIPVs, including (*Z*)-3-hexenyl acetate (H), (*E*)- β -ocimene (O), linalool (L), (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT, D), and (*E,E*)- α -farnesene (F) were identified by Liu et al. (2019), and the authentic chemicals of these HIPVs components were adjusted to 6-h quantities and used in bioassays (Table S2). A Teflon disk was separated from a Teflon/silicon septum (diameter 1.2 cm, TS-12712, GL Sciences Inc., Tokyo, Japan) and used as a volatile carrier. Chemicals (as hexane solutions, 10 μ L) and solvent control (hexane, 10 μ L) were loaded onto the Teflon disks and allowed to evaporate prior to experiments.

Statistical Analysis

All data were analyzed using the software package R v.3.4.0 (R Core Team, 2017) for statistical analyses. The number of eggs laid in each area was compared using Wilcoxon matched-pairs signed-ranks test. The total number of eggs laid in each treatment in plant- or chemical-treated bioassays was compared using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with Bonferroni correction.

Results

Daily Fecundity and Oviposition Time of *G. molesta*

Female *G. molesta* were isolated after mating and the number of eggs was recorded every day. Most mated females started to oviposit 1 day after mating, and the number of eggs was 7.5 ± 2.0 eggs (Mean $\pm$ SE). The largest number of eggs was 11.4 ± 3.0 , 3 days after mating, and then steadily decreased thereafter (Fig. S2). A decline in oviposition behavior could have caused a bias in the bioassays, so female moths were used in bioassays within 3 days after mating. On the other hand, *G. molesta* females started laying eggs from 17:00, but the number was relatively low. Female moths tended to lay eggs from 1 h before scotophase, and the highest number was observed from 22:00 to 23:00 (Fig. S3). After 00:00, moths stopped moving until the end of observations.

Oviposition Preference of *G. Molesta* to Pear-Leaves under Different Conditions

Pear leaves in different conditions (US, IS, and ML) were prepared as odor sources for dual-choice bioassays to determine when the gravid *G. molesta* showed any preference among pear volatiles. Female moths preferred to lay eggs in any type of pear-leaf (treatment) area compared to control areas (blank). They laid 53.3 ± 3.7 (Mean $\pm$ SE) eggs in US areas vs. 24.8 ± 2.2 eggs in control areas ($Z = -2.80$, $P = 0.002$, Fig. 1, I), 51.8 ± 2.6 eggs in IS areas vs. 18.6 ± 2.8 eggs in control areas ($Z = -2.80$, $P = 0.002$, Fig. 1, II), and 16.2 ± 4.4 eggs in ML areas vs. 17.0 ± 3.1 eggs in control areas ($Z = -0.77$, $P = 0.484$, Fig. 1, III). Furthermore, when evaluating oviposition preference between areas containing different types of pear

leaves, female moths laid 78.2 ± 7.7 eggs in US areas vs. 39.4 ± 5.2 eggs in ML areas ($Z = -2.80$, $P = 0.002$, Fig. 1, IV), and 36.9 ± 4.4 eggs were laid in IS areas vs. 22.0 ± 3.0 eggs at US areas ($Z = -2.64$, $P = 0.006$, Fig. 1, V). On the other hand, female moths laid the most eggs in bioassay IV (mean $\pm$ SE = 117.6 ± 10.9), followed by bioassay I, II, and V (mean $\pm$ SE = 78.1 ± 4.0 , 70.4 ± 4.0 , and 58.9 ± 6.0 , respectively), and the least number of eggs was observed in bioassay III (mean $\pm$ SE = 27.3 ± 5.1) ($\chi^2 = 33.97$, $df = 4$, $P < 0.001$).

Oviposition Response to HIPVs Released from Pear Shoots

To investigate the effect of HIPVs released from larva-infested pear shoots on oviposition of female moths, comparisons were conducted between pear HIPVs (5 volatile components and a quinary blend) versus a solvent control. When comparing volatile components with the solvent control, mated females did not show a significant preference in bioassays ($P > 0.05$, Fig. 2, H, O, L, F, and D). In contrast, female moths preferred to lay eggs in the HIPV-treated area (mean $\pm$ SE = 99.6 ± 8.3) compared to the control area (41.8 ± 6.1) ($Z = -3.41$, $P < 0.001$, Fig. 2, VM). Considering the quantity effects on oviposition preference, 10x-diluted VM (VM 10×dil.) was also tested. Female moths laid more eggs in the HIPV-treated area (41.6 ± 4.0) compared to the control area (28.2 ± 3.7) ($Z = -2.56$, $P < 0.008$, Fig. 2, VM10d). Moreover, the total number of eggs laid in the bioassay between HIPVs and solvent (141.4 ± 12.1) was significantly more than in other bioassays ($\chi^2 = 33.91$, $df = 6$, $P < 0.001$, Fig. 2).

Discussion

We hypothesized that plant volatile profiles could affect the oviposition preference of *G. molesta* females when they find suitable habitat for their offspring. To investigate this hypothesis, we conducted dual-choice bioassays in cylindrical tubes with pear leaves, and the results show a clear preference for pear shoots, but not mature leaves (ML). Moreover, *G. molesta* females laid more eggs in the IS-treated area than in the US-treated area. Subsequently, the effect of each component and volatile mixtures on *G. molesta* oviposition preference was evaluated. Females showed a significant preference for the VM and the 10x-diluted VM, but not for individual components. These results indicate that the volatile bouquet may be important in host selection by *G. molesta*. Furthermore, *G. molesta* females laid more eggs when treated with the volatile mixture than with individual components and diluted VM, implying that the concentration of pear HIPVs may affect the performance of egg deposition.

As a deciduous perennial plant, the phenology of yearly growth from blooms to leaf abscission in plants of the family Rosaceae follows a scenario that is dominated by environmental factors such as temperature and photoperiod (Kurokura et al. 2013). Volatile emissions also fluctuate according to the seasonal stage, which may be correlated with different resources (flowers, leaves, or fruits) in each period (Schoonhoven et al. 2005). Therefore, recognizing and responding appropriately to plant volatiles is essential for herbivores, especially when exploring suitable habitats for oviposition (Najar-Rodriguez et al. 2013; Vallat and Dorn 2005). In addition to phenology, plant volatiles can also reflect the quality of host plants. For instance, HIPVs may indicate higher levels of secondary compounds (direct defense), and the fitness of offspring would decrease because of higher predation risk (indirect defense) (Yoneya and Miki

2015). Hence, herbivores tend to avoid host plants that release HIPVs (Bernasconi et al. 1998; de Moraes et al. 2001). Nevertheless, our results showed that female *G. molesta* laid more eggs in HIPV-treated areas, which indicates that *G. molesta* females do not avoid pear HIPVs, contrary to the general pattern of herbivore responses to HIPVs. Positive responses of herbivores to HIPVs suggest a complex relationship in various tri-trophic systems (El-Sayed et al. 2016, 2018; Giacomuzzi et al. 2016, 2017; Yoneya and Miki 2015).

(Z)-3-Hexenyl acetate is a constitutive volatile component of plants in the family Rosaceae, which may be recognized by herbivores as an indicator of suitable habitat (Il'ichev et al. 2009; Lu and Qiao 2020; Najjar-Rodriguez et al. 2013). Recently, Chen et al. (2019) found a general odorant receptor, *GmolOR9*, of *G. molesta*, which may be related to detection of host plant volatiles. Injection of double-stranded RNA for *GmolOR9* decreased electroantennogram responses to (Z)-3-hexenyl acetate of *G. molesta* females, but this phenomenon was not observed in males. These results imply that the perception of (Z)-3-hexenyl acetate is more important for females than males of *G. molesta*. Nevertheless, our results do not support direct causality between perception of (Z)-3-hexenyl acetate and oviposition preference. The entire volatile bouquet of host plant may be necessary for *G. molesta* females when searching for suitable habitat for their progeny.

Volatile emissions of plants may relate to their susceptibility to detection by herbivores, which was defined as “apparency” by Feeny (1976). Although higher apparency for host plants indicates a higher risk of parasitism, avoidance of natural enemies may not be the priority for herbivores if the parasitism pressure would be reduced by a higher density of host larvae (Jaenike 1990; Scutareanu et al. 1997; Rusch et al. 2015). Female moths laid the least number of eggs in the ML-Blank bioassay. Besides, the number of eggs laid in the IS-treated area is more than the US-treated area, which in turn is more than the MS-treated area. These behavioral responses to pear leaves may be related to the quantity of pear leaf volatiles, as the amount of volatiles released from IS was higher than US followed by ML (Table S1). In addition, results in subsequent bioassays with VM and diluted VM provide strong evidence for quantity effects and support the apparency hypothesis. Some herbivores use HIPVs as a “lighthouse” to discover the right habitat because uninfested plants usually have lower volatile emission rates (Liu et al. 2019), but they ultimately avoid laying eggs on infested plants (Landolt 1993). On the other hand, Yoneya and Miki (2015) suggested a model based on herbivory levels (uninfested, lightly infested, and highly infested). After herbivory, host plant quality starts to decrease followed by an increased risk of predation and the apparency of host plants (Halitschke et al. 2008). On the other hand, successful colonization of conspecifics on a plant implies a safe food for herbivores (Yoneya et al. 2010). Therefore, if the capacity (remaining food) is sufficient to support more herbivores, using HIPVs to improve search efficacy and/or to accept habitats may be a good strategy to produce more offspring during a limited lifespan (Jaenike 1990).

Furthermore, the capacity of *G. molesta* larvae on a pear tree may remain constant or even increase because of pear tree responses after infestation. When a larva feeds on a primary shoot of a pear branch, lateral shoots develop shortly after the infestation. After frequent infestations, a series of tolerance

responses of a pear tree may result in a bushy morphology, which increases the quantity of food. Consequently, a larger capacity for conspecific larvae would result. On the other hand, the bushy pear may also disrupt host location by natural enemies, since it provides shelter for *G. molesta* larvae, and the increased population would reduce the parasitism risk (Rusch et al. 2015). Thus, laying eggs on pear trees that are infested by conspecific larvae may benefit *G. molesta* with more abundant food and mitigated predatory/parasitism risk.

In summary, this study demonstrates that pear HIPVs not only attract *L. rufipes*, but also *G. molesta* females for oviposition. This phenomenon makes interactions more complex in apple, pear, peach, and apricot orchards, and raises more questions that are worth answering, such as (1) whether seasonal changes affect the perception of volatile components or mixtures by *G. molesta* females, (2) whether fitness of *G. molesta* differs when breeding in habitats with or without infested shoots, and (3) whether application of supplementary pear HIPVs affects *G. molesta* and *L. rufipes*. Answering these questions may help to develop novel pest management systems in orchards, to reduce insecticide use.

Declarations

The authors have no conflicts of interest to declare. All coauthors have seen and agreed with the contents of the manuscript and there was no financial interest to report.

Author Contributions

CML, SM, and YK conceived the study. SM carried out chemical synthesis. CML designed the bioassay and carried out analytical works. CML and SP carried out bioassays. CML drafted the manuscript with assistance from SM and YK, and WA provided technical suggestions. All authors edited the manuscript and approved the final version.

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Figures

Fig. 1. Oviposition preferences of *Grapholita molesta* to pear leaves in dual-choice bioassays (n = 10 in I, II, III, and IV; n = 16 in V). Bars represent the number of eggs laid in each treated area (□ Blank, □ US: uninfested shoots, IS: ▨ infested shoots, and ■ ML: mature leaves). Error bars represent $\pm$ SEM and asterisks indicate significance in Wilcoxon matched-pairs signed-ranks test ($P < 0.05$) and “ns” means not significantly different ($P \geq 0.05$).

Figure 1

See image above for figure legend

Fig. 2. Oviposition preferences of *Grapholita molesta* to different volatile components and the volatile mixture in dual-choice bioassays (n = 16). Bars represent numbers of eggs laid in each treated area (□ Control, ■ Volatiles,). Error bars represent $\pm$ SEM and asterisks indicate significance from Wilcoxon matched-pairs signed-ranks test ($P < 0.05$) and “ns” means not significantly different ($P \geq 0.05$). H = (Z)-3-hexenyl acetate, O = (E)- β -ocimene, L = linalool, D = (E)-4,8-dimethyl-1,3,7-nonatriene, F = (E,E)- α -farnesene, VM=volatile mixture (synthetic mixture of pear HIPVs, VM10d=10x diluted VM).

Figure 2

See image above for figure legend

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