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## Behavioral response of *Coccinella septempunctata* (L.) (Coleoptera: Coccinellidae) to plant volatiles infested by a chewing herbivore, *Epilachna vigintioctopunctata* versus a sucking pest, *Aphis gossypii*

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### Abstract

Plants modify the volatile profile in response to herbivore damage, which can influence the foraging behavior of predators in tri-trophic interactions. This study aimed to investigate the olfactory response of the ladybird predator *Coccinella septempunctata* to cucumber, bean, potato, and brinjal leaves infested by a normal prey, *Aphis gossypii*, and an alternative prey, *Epilachna vigintioctopunctata*. An olfactometer bioassay was applied for this investigation using the four plants with or without infestation by the two herbivorous prey having different feeding guilds. The behavioral response was analyzed in terms of selectiveness and readiness in the olfactometer test. Our study found that *C. septempunctata* attracted selectively to leaves damaged by either aphids or *Epilachna* over undamaged leaves, but the magnitude of attraction depended on plant species. In terms of selectiveness, the ladybird did not distinguish between leaves infested by the two herbivores, however, it responded more quickly to aphid-infested plants than plants infested by the alternative prey. Plants infested simultaneously by both herbivores were more attractive in terms of readiness than those damaged by *A. gossypii* or *E. vigintioctopunctata* alone in three plants except bean plants. Thus, it is evident that the ladybird beetle uses volatiles emitted from damaged plants to locate its prey. The present study highlights that the behavioral response of *C. septempunctata* to herbivore-induced plant volatiles is complex and can depend on plant species and herbivore types. Our study also suggests the potential of *C. septempunctata* as a predator in the biological control of *E. vigintioctopunctata* for cucumber and potato fields.

**Keywords:** Different feeding guilds, biological control, herbivore-induced plant volatiles, olfactory response

### Introduction

Natural enemies, such as insect predators and parasitoids, commonly play a vital role in suppressing the populations of insect herbivores, which makes them useful in pest management in agriculture [6, 16]. They can effectively search their target prey or host using a variety of cues to detect them. An important search cue is plant odors [25]. Although intact plants themselves may serve as a cue, many previous studies have demonstrated that insect predators and parasitoids are strongly attracted to odors emitted from infested plants, *i.e.*, herbivore-induced plant volatiles (HIPVs) [2, 24]. Therefore, the foraging behavior of predators and parasitoids depends on the herbivore and the host plants with which they interact. Understanding how different plant volatile cues are involved in the function and effectiveness of natural enemies can provide significant insights and ideas into the biological control of agricultural pests [26].

Plants synthesize volatiles and nonvolatile chemicals; for example, undamaged plants emit green leaf volatiles in minor quantities [44], which can even attract natural enemies [43].

Plants also produce various other volatile and non-volatile chemicals in response to the damage or presence of herbivores and pathogens [29]. HIPVs are the volatile organic compounds released by plants in comparatively higher amounts when attacked by herbivores.

Although insect predators use volatiles from certain herbivores and the host plants to locate their prey [12, 22, 38, 43, 53], the volatile compounds released by plants can differ qualitatively and quantitatively depending on the combinations of plant and herbivore species [10, 12, 14, 47]. A given plant species may produce different volatiles in response to different herbivores [27]. Also, different genotypes of the same plant species may emit different volatiles even when the same species of herbivores attack them [9]. In any case, insect predators appear to rely greatly on plant volatiles since the plant volatiles are more detectable than the herbivore-derived volatiles due to their large biomass [49] though they may use the volatiles from the prey at a close distance [20, 35] or those from their by-products, such as frass, honeydew, exuviate, mandibular gland secretions, defense secretions, etc. [30, 49].

Olfactory cues from plants and prey enhance predatory efficiency, reducing the searching time, increasing the attack rates [3, 32, 49] and influencing the reproductive success of predators [33]. However, sensory and physical ability toward the odor source is species-dependent [1, 22]. Natural enemies selectively respond to plant volatile chemicals induced by suitable and unsuitable hosts or prey [7, 10]. The production of volatile chemicals depends on plant species, plant cultivars, development stages of plants, herbivore species, and their development stages [4, 43]. Activation of different biochemical pathways involved in volatile chemicals generates selection specificity [52].

Over the past two decades, plant-herbivore interactions have been studied extensively on predators and parasitoids [38, 46]. However, plant systems support multiple herbivores for the survival of natural enemies [7]. Different natural enemies foraging in the same tri-trophic environment respond to HIPVs or multiple herbivores affect the multi-trophic interactions when the herbivores belong to different feeding guilds [20, 52]. Numerous herbivores are involved in the same plant system and their influence on predators is studied. It may reduce the attraction of natural enemies [52], enhance attraction [8, 21, 28, 36] or no effect [28, 36]. Based on predator effects on their prey populations, predators can interact synergistically, additively, or antagonistically [45].

*Coccinella septempunctata* (L.) (Coleoptera: Coccinellidae) (After that, C7) is generally considered an oligophagous predator of several aphid species in diverse habitats [23, 34] and can play a prominent role in suppressing aphid populations. However, several studies have shown that C7 also feeds on other groups of insects as alternative prey, such as coleopterans [16] and this predatory beetle has the potential

as an important predator of some agricultural pests other than aphid pests.

*Epilachna vigintioctopunctata*, i.e., Epilachna beetle (Coleoptera: Coccinellidae) is an economically significant herbivorous pest [40] damaging mainly Solanaceae, Cucurbitaceae, Fabaceae, Convolvulaceae, and Malvaceae [40, 42, 48]. Although chemical control is the main measure to combat this pest, other measures, such as biological control with insect natural enemies, are on demand to reduce pesticide use or to apply IPM practices due to environmental and health issues [41].

We have previously shown that larvae and adults of C7 readily attack and prey on larvae of *E. vigintioctopunctata* though they are rather alternative prey items. Our previous study has suggested that C7 can play a vital role in suppressing Epilachna beetles [39]. However, it is not unclear whether C7 can sense the presence of Epilachna larvae infesting agricultural crops from a distance and whether C7 can discriminate between plants with and without Epilachna infestation. If C7 could recognize and be attracted to crops damaged by Epilachna larvae, C7 may be usable in the biological control of Epilachna beetles.

Our primary aim of the present study is thus to examine whether C7, like other insect predators, can recognize HIPVs from Epilachna-infested crops. In addition, the present study focuses on the behavioral response of C7 to HIPVs from crops damaged by multiple pests. Epilachna beetles are commonly found on crops with the cotton aphid *Aphis gossypii* (Hemiptera: Aphididae), another serious pest and a main prey for C7. These 2 pests are different in terms of feeding habits: chewing and sucking pests. Therefore, we investigate the behavioral response of C7 to the 4 major crops attacked by Epilachna and cotton aphids with the aid of the olfactometer.

## Materials and Methods

### Host plants

In our experiments, we used 4 crop species. Cucumber (*Cucumis sativus*, Cucurbitaceae) variety “Suyo Kyuri” and bean (*Phaseolus vulgaris* L., Fabaceae) variety “Dover ingen-mame” seeds were germinated on growing starter cubes (40 cm x 25 cm with 187 seedling holes) and kept in the laboratory at 25±2 °C, photoperiod L16:D8, 65 ± 5% RH conditions until they reached the 4-5 leaves stage. Brinjal (*Solanum melongena*, Solanaceae) variety “Kumamoto naganasu” seedlings transplanted into pots (13 cm diameter) containing the garden soil mixture were grown to 5-6 leaves. Potato (*Solanum tuberosum*, Solanaceae) variety “Dansyaku-imo” seeds were sprouted and grown until they reached the 4-5 leaves stage. These plants were used for the following experiments.

### Insects

All insects for stock culture and testing were reared for generations in the laboratory. Adults of *Coccinella septempunctata* were collected from natural habitats close to

Kyushu University, Fukuoka, Japan, and were reared in the incubator kept at  $25 \pm 2$  °C, photoperiod L16:D8,  $65 \pm 5\%$  RH condition. They were daily fed a mixture of cotton aphids, *Aphis gossypii* (Hemiptera: Aphididae) reared on cucumber seedlings.

Cotton aphids were collected from farmer fields where cucumbers were grown, close to Kyushu University, and were reared on cucumber seedlings in the incubator kept at  $25 \pm 2$  °C, photoperiod L16:D8,  $65 \pm 5\%$  RH conditions.

*Epilachna vigintioctopunctata* were collected from farmer fields where potatoes were grown and were reared on brinjal seedlings in the same conditions as above. For details on rearing *Epilachna*, refer to our previous study [39].

### Olfactometer bioassays

A Y-tube olfactometer (22 cm stem and 16 cm arms long, 3 cm radius, 120° Y angle) was placed horizontally on a white bench for the bioassay. Before and after the experiment, all the glassware was cleaned with ethanol. Newly eclosed adults of C7 were arbitrarily selected from the rearing stock and were left in the bioassay room for 24 h before testing for acclimatization and starvation. For each experimental treatment, 20 C7 were used, and each C7 beetle was tested only once in the bioassay.

Damaged (=infested) and undamaged leaves of cucumber, bean, potato, or brinjal were used in our experiments. Twenty insects of *A. gossypii* and/or first instars of *Epilachna* beetle were placed on one test leaf using a soft brush. The leaf with *A. gossypii* and/or *Epilachna* was enclosed in a tissue bag for ventilation. The insects were allowed to feed on all test leaves for 24 h and the infested leaves with the herbivores were used for the bioassay. We confirmed that *Epilachna* larvae fed on all 4 crop leaves prior to testing. Damaged and/or undamaged leaves were placed in odor-source tubes in two arms of the olfactometer, respectively. Test adult C7 were singly introduced into the base of the olfactometer to see their preference for either arm. The number of C7 adults that moved toward the odor source within 5 min was recorded. The time taken until the test beetle reached contact with an odor source in each arm was also recorded to assess the responsiveness of C7 to test odors. If a beetle did not reach the odor source of the Y-tube within 5 min, it was counted as a “not-responded”. The following pairwise tests were performed to test the attraction of C7.

1. Clean air versus clean air
2. Undamaged versus damaged by *A. gossypii*
3. Undamaged versus damaged by *E. vigintioctopunctata*
4. Undamaged versus damaged by *A. gossypii* + *E. vigintioctopunctata*
5. Damaged by *A. gossypii* versus damaged by *E. vigintioctopunctata*
6. Damaged by *A. gossypii* versus damaged by *A. gossypii* + *E. vigintioctopunctata* and
7. Damaged by *E. vigintioctopunctata* versus damaged by *A. gossypii* + *E. vigintioctopunctata*.

### Data analysis

The number of C7 beetles responding to either arm of the olfactometer was statistically analyzed using Pearson's chi-square tests with a 50% expected response (Goodness Fit Test). The mean time taken until the test beetle reached an odor source was calculated and was separated with the Wilcoxon Rank Sum test ( $\alpha = 0.05$ ). If test insects did not respond to any odor sources, they were excluded from the analysis. All data were analyzed using JMP version 13 software.

## Results

### Attraction of C7 to the olfactory cues

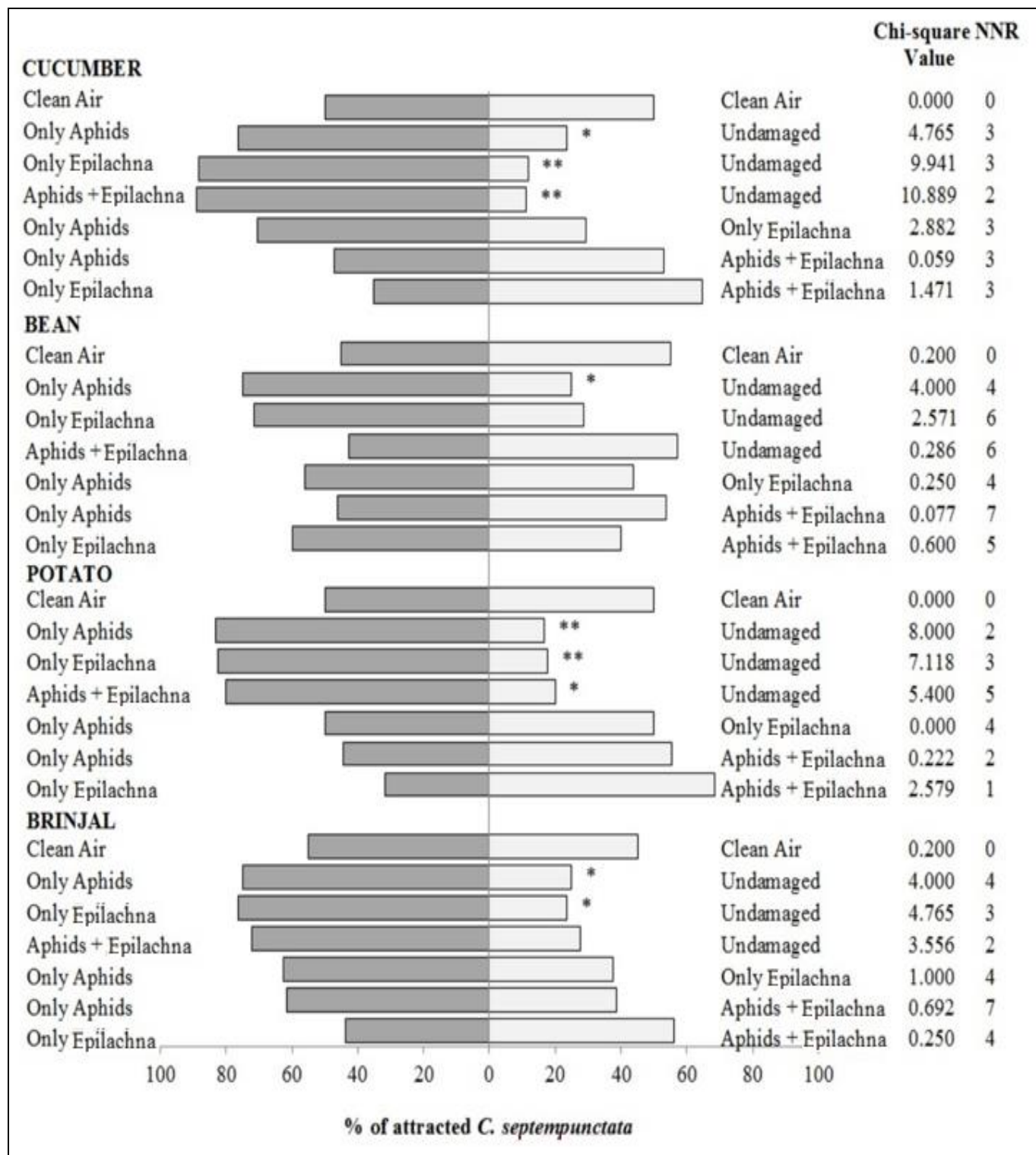
First, an introductory experiment was conducted to check for directional bias, *i.e.*, clean air versus clean air for all crops, and the results confirmed that the bioassay arena was unbiased as none of the C7 beetles attracted significantly for either side of the Y-tube olfactometer arms ( $p > 0.05$ ) (Fig. 1). C7 beetles significantly preferred odors from infested cucumber, bean, and brinjal over intact plants ( $p < 0.05$ ) (Fig. 1). The response of C7 appeared stronger when potato was tested, showing a highly significant attraction to aphid-infested leaves ( $\chi^2 = 8.0$ ,  $p = 0.005$ ) (Fig. 1). Bean leaves damaged by *Epilachna* were not attracted to C7 than undamaged leaves ( $\chi^2 = 2.571$ ,  $p = 0.109$ ) but significant attraction was detected on brinjal ( $p = 0.029$ ), or cucumber ( $p = 0.002$ ), or potato ( $p = 0.008$ ) (Fig. 1). C7 was also attracted to cucumber and potato leaves damaged by both herbivores significantly over undamaged leaves ( $p < 0.05$ ). There was no significant difference in the number of attractions between aphid-damaged and *Epilachna*-damaged leaves, regardless of crop species. Similarly, no significant differences were detected for all crop species when the response was tested between odors from leaves damaged by single versus multiple species (Fig. 1).

### Quickness of C7 response

Responsiveness of adult C7 to olfactory cues was also analyzed with the time taken until test C7 had reached an odor source in the Y-tube olfactometer and was summarized in Fig. 2. The analyses showed that C7 responded more quickly to odor cues when tested with aphid-infested versus undamaged leaves in cucumber ( $Z = 2.55$ ,  $p = 0.011$ ) and potato ( $Z = 2.61$ ,  $p = 0.009$ ) but this was not the case for bean ( $Z = 1.15$ ,  $p = 0.249$ ) and brinjal ( $Z = 1.70$ ,  $p = 0.089$ ). Time taken when examined between *Epilachna*-infested and intact leaves was longer in all crops compared with the responsiveness to aphid-infested leaves, whereas the time to infested leaves was shorter in cucumber ( $Z = 2.16$ ,  $p = 0.031$ ) and potato ( $Z = 2.33$ ,  $p = 0.020$ ) than intact control leaves. Time to be attracted to leaves damaged both by aphids and *Epilachna* was significantly shorter than undamaged in all crops (cucumber  $Z = 2.19$ ,  $p = 0.029$ ), (brinjal  $Z = 2.12$ ,  $p = 0.034$ ) and potato ( $Z = 2.53$ ,  $p = 0.011$ ) except beans for which the time to the

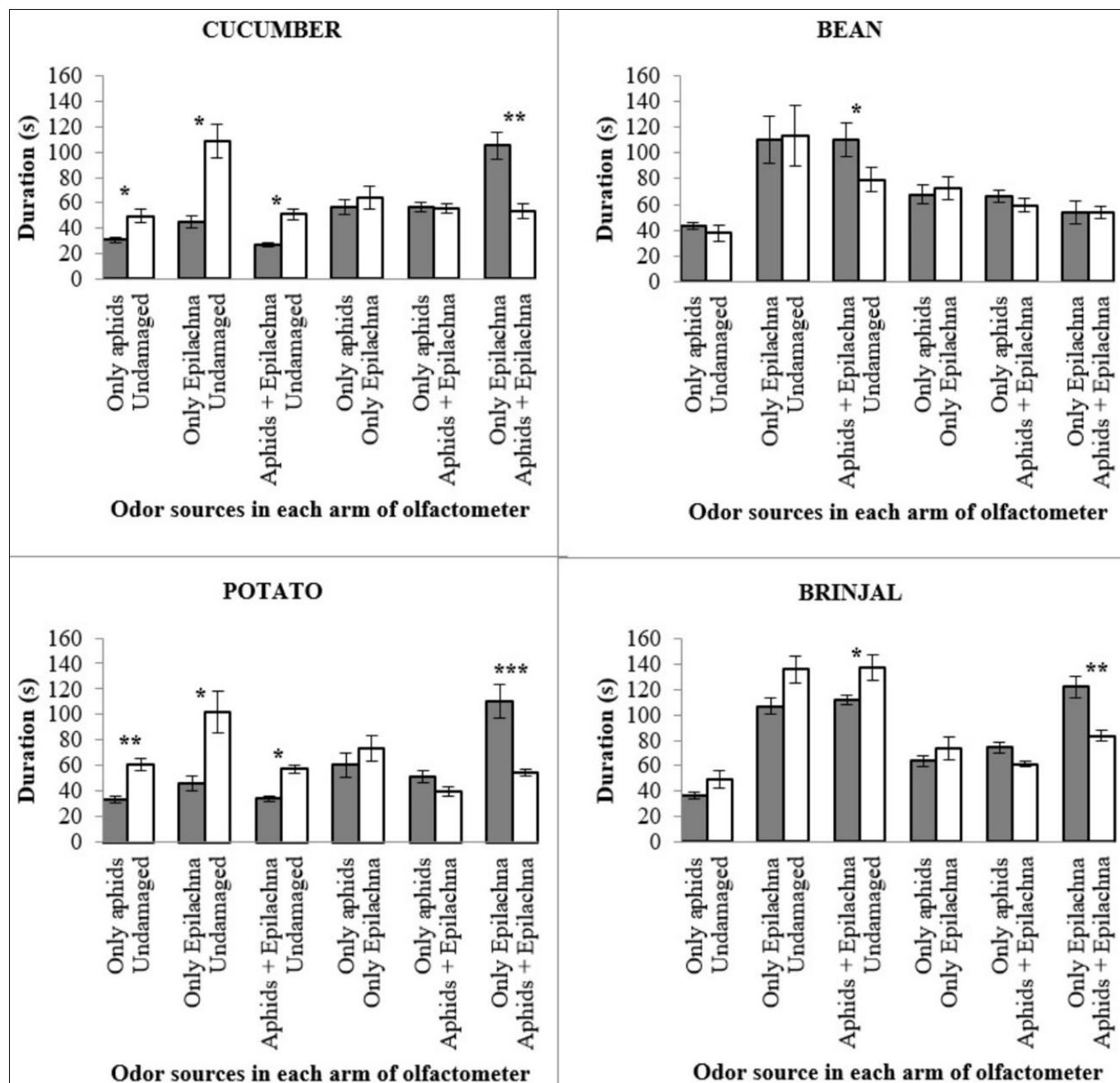
control source was longer ( $Z=2.00$ ,  $p = 0.045$ ).  $C7$  took a shorter time toward the mixed odor source than *Epilachna* infested in cucumber ( $Z=3.17$ ,  $p = 0.002$ ), potato ( $Z=3.38$ ,  $p =$

$<0.001$ ), and brinjal ( $Z=2.96$ ,  $p = 0.003$ ) but this was not the case when aphid-infested leaves were used (Fig. 2).



**Fig 1:** Olfactory response (%) of *C. septempunctata* (n =20) toward undamaged vs. aphid, *Epilachna*, or both damaged four crops (cucumber, brinjal, potato, and bean). \* and \*\* sign indicate the significance level  $0.01 < p < 0.05$  and  $0.001 < p < 0.01$  respectively. NNR represents the number of insects not responded to either of the odor sources.





**Fig 2:** Mean ( $\pm$  SE;  $n=20$ ) time (s) of C7 beetle to reach an odor source in the Y-tube experiment. \*, \*\*, and \*\*\* signs indicate  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$  significant difference between odor sources respectively (Wilcoxon rank sum test). Infested (= damaged) and intact leaves were tested as odor sources. Shorter time indicated a faster response to the source.

## Discussion

The present study provides experimental evidence that (1) adult C7 are attracted to the 4 crop plants damaged by pest herbivores, regardless of their feeding type, *i.e.*, sucking or chewing, though the responsiveness can differ depending on crop species, (2) adult C7 may innately recognize the infestation by *Epilachna* beetles via plant volatiles though they are rather an alternative prey, and (3) the presence of multiple herbivores on plants can enhance the orientation or foraging response of C7 via plant volatiles.

First, the results of the present study also proved that newly eclosed C7 uses plant volatile induced by *A. gossypii* and acts as a reliable cue to find its prey. Our experiment confirmed that adult C7 discriminated plant volatiles from aphid-infested versus intact plants and responded positively to the former,

and this is true for all tested crops, *i.e.*, cucumber, bean, potato, and brinjal (Fig. 1). The results suggest that adult C7 can use HIPVs to search their prey. Likewise, previous studies demonstrated that some ladybird beetle species preferentially choose odors from aphid-infested plants [21, 32]. Therefore, ladybird beetles can sense the presence of their main prey, aphids, with olfactory cues from a distance, and this ability will allow them to find their prey effectively. Plants, when intact, release minor quantities of volatile compounds. It is now well-documented that, when attacked by herbivores or pathogens, plants enhance the production of volatiles quantitatively [18] and emit specific odors that differ from intact plants, which are called herbivore-induced plant volatiles [37]. Several previous studies have given evidence that odors from infested or infected plants or synthesized

HIPV compounds can positively attract ladybeetles [5, 17, 21, 32, 50, 53], including *C7* [33]. HIPVs are important cues for predators to find their prey [20, 49] in a reliable and well-detectable way [13]. According to the “cry for help” theory [13], plants can defend themselves by attracting natural enemies with HIPVs [11, 19, 32]. Thus, HIPVs are a form of plants’ indirect defense, and both plants and predators mutually benefit via HIPVs in the context of tri-trophic interactions.

In our study, we reared *C7* using cotton aphids on cucumber, and, hence, adult *C7* for testing should have experienced a combination of cotton aphids and cucumber. Then, it is likely that test *C7* has learned the odor of these prey and plants. A relatively strong response of tested *C7* to such odors (Fig. 1) may be explained by a learning effect. However, tested *C7* also showed a strong positive response to the other 3 tested plants with cotton aphid damage (Fig. 1) suggesting that odor-learning may not be the sole explanation and that *C7* can innately recognize aphid-infested plants. Alternatively, the 4 crop plants may release the same volatile compound(s) when infested by cotton aphids. To conclude which is the likely reason, it is necessary to examine and compare the volatile compounds from the 4 crops, *i.e.*, cucumber, bean, potato, and brinjal. Relatively few studies examine the learning behavior of *C7* and other ladybird beetles to plants and the effect of prior experience [32]. Hence, learning the prey-searching of ladybirds in the context of HIPVs would be a significant subject in the future.

Second, adult *C7* exhibited a positive foraging response to cucumber, potato, and brinjal when the alternative prey had damaged plants, *E. vigintioctopunctata*. This result suggests that *C7* may widely respond to plants attacked by various agricultural pests. It will be advantageous for *C7* to recognize the presence of potential prey, other than main prey aphids because aphids may not be available or abundant enough. And the use of alternative prey can enhance survival and reproduction under such circumstances. Since *C7* in our study had no prior experience with *Epilachna*, the observed positive response to *Epilachna*-infested plants is not a learned response but can be rather an innate response. An explanation is that the 4 crop plants tested in the present study produce and release, at least in part, the same volatile compounds regardless of herbivore species. Attraction to the same or shared odors, regardless of inducer herbivores, may be advantageous for *C7* to exploit a wide variety of prey items. It is well-documented that *C7* preys on numerous insects, such as aphids, thrips, leaf beetles, etc. [16], which makes *C7* a very abundant and widespread Coccinellidae.

The present results also suggest that the responsiveness of *C7* is stronger on aphid-infested than *Epilachna*-infested leaves in terms of the time taken to reach an odor source when a choice was given with non-infested intact plants (Fig. 2). This makes sense because aphids are the main prey for *C7*. Curiously, *C7* did not distinguish the odors between aphid- and *Epilachna*-infested plants in all crops (Fig. 1 and Fig. 2). It is known that HIPVs produced from a plant can contain the same or shared compounds even when different species of herbivores are

involved [8]. It is therefore likely that the plant volatiles from aphid-infested and *Epilachna*-infested leaves smell similar to *C7*, which makes it difficult to distinguish between the volatiles. However, it is also true that the biosynthetic pathways induced by chewing versus sucking herbivores are different. For example, damage by phloem-feeding insects is related to the activation of salicylic acid-dependent responses whereas that by chewing insects is related to the activation of Jasmonic acid-dependent responses [31]. They interfere with each other negatively [31, 51] or enhance the behavior [7, 8] and no interference was observed in the behavior of aphids when *P. xylostella* simultaneously damaged the plant [7].

Although our study proved that *C7* could be attracted to plants damaged by both herbivores, the attractiveness of the plants appeared stronger in cucumber and potato than in bean and brinjal (Fig. 1). It is evidenced that HIPVs can shorten the time for predators to find their prey. The time taken by *C7* to reach the odors emitted by prey in sole or mixed over undamaged was shorter in cucumber and potato but *C7* spent more time attracting towards HIPVs emitted by beans damaged by both herbivores (Fig. 2). It is not known why the response of *C7* can differ depending on crop species. A possible explanation may be the magnitude of damage caused by the test herbivores different among the tested crop species. This possibility will be examined in future studies. Another explanation may be volatile compounds differ qualitatively and/or quantitatively among the crops, though they could produce, at least in part, the same compounds, and the odors from the infested bean and brinjal contain fewer compounds to attract *C7*. To test this idea, volatile compounds should be analyzed and compared. Such an analysis is also a future subject.

Third, the present study added evidence that simultaneous infestation by different feeding guilds could enhance the attraction of *C7* in some circumstances (Fig. 1 and Fig. 2). The enhanced response of *C7* to plant odors with multiple herbivores’ infestation could take place because the plant damage of tested leaves was greater due to greater number of herbivores, causing greater production of plant volatiles and/or because the plant produced different volatiles in response to different type of herbivores, changing a volatile profile having a greater number (rather than quantity) of attractive chemical compounds. The biosynthetic pathways induced by chewing and sucking herbivores are different. Damage by phloem-feeding insects is related to the activation of salicylic acid-dependent responses whereas damage by chewing insects is related to the activation of Jasmonic acid-dependent responses [31]. Consequences of simultaneous attack chewing and sucking herbivores on HIPVs can therefore be complicated; it may or may not cause infested plants more attractive to natural enemies, and, in some cases, attraction may even be hindered [7, 15].

Plant individuals are commonly attacked by multiple species of herbivores simultaneously or sequentially. In this case, the HIPV profile may differ from that with a single-species damage. Also, HIPVs should depend on plant species whereas

generalist herbivores can attack multiple plant species. Then, the difference in HIPVs can affect plant-herbivore-predator interactions by affecting the response of predators to the plant [36]. It is therefore important to investigate how predators may respond to various combinations of different plant and herbivore species and plants damaged by multiple herbivores under various volatile mixtures released from plants [7]. The present study demonstrated that the C7 beetle could enhance the attraction to the odors emitted by damaged leaves infested simultaneously by two herbivore pests, however, the enhancement depended on the combination with crop species (Fig. 1 and Fig. 2).

A positive effect of simultaneous infestation to natural enemies can occur because of enhanced production of volatile emissions [36]. In addition to an additive effect on volatile emission, the presence of multiple herbivore species on the same plant individual may have a synergistic effect by expressing biosynthetic pathways synergistically [8]. At the same time, however, simultaneous attack by multiple herbivores can result in suppression of some volatile compounds, i.e., antagonistic effect [8]. Thus, simultaneous attacks by multiple herbivores on a plant individual should cause a somewhat more complicated influence on tri-trophic interactions.

Finally, this study concluded that the behavioral response of *C. septempunctata* to herbivore-induced plant volatiles is complex and it depends on both plant species and the herbivore types. However, the potential of *C. septempunctata* as a predator in the biological control of *E. vigintioctopunctata* in cucumber and potato crops was elucidated for future studies.

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