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Original article (Orijinal araştırma)

Attraction responses of ladybird beetle *Hippodamia variegata* (Goeze, 1777) (Coleoptera: Coccinellidae) to single and binary mixture of synthetic herbivore-induced plant volatiles in laboratory tests[1]

Laboratuvar koşullarında zararlılar tarafından teşvik edilen sentetik bitki kokularının tekli ve ikili karışımlarına uğurböceği *Hippodamia variegata* (Goeze, 1777) (Coleoptera: Coccinellidae)'nın yönelim cevabı

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Summary

The chemoreception response of an aphidophagous coccinellid predators [*Hippodamia variegate* (Goeze, 1777) (Coleoptera: Coccinellidae)] to the odors from four different synthetic HIPVs [methyl salicylate (MeSA), (E)-2-hexenal (E(2)H), farnesene (F) and benzaldehyde (Be)] was tested using two different doses (0.001 and 1 g/L) of the HIPVs, both alone and in five binary combinations [MeSA+F, MeSA+E(2)H, MeSA+Be, E(2)H+F and Be+F]. Insect responses were evaluated using two-choice experiments with a Y-tube olfactometer in laboratory conditions. The low single dose of MeSA attracted significantly more adults of *H. variegata* (71%) towards tubes containing the volatile source compared with the control volatile containing pure n-hexane. Adults of *H. variegata* did not significantly prefer single forms of either Be, E(2)H or F compared with MeSA alone. Additionally, this study showed that binary blends of MeSA with Be or F had significantly more attractiveness for *H. variegata* adults than controls. Thus, the compounds, Be and F, used together with MeSA were observed to increase adult attraction. In the future, additional studies that monitor the preferences of field populations of these predators treated with the attractive HIPV combinations should be conducted to confirm these findings.

Keywords: Attraction, biological control, *Hippodamia variegata*, predator, synthetic HIPV, Y-tube olfactometer

Özet

Yaprakbiti avcısı coccinellid türü *Hippodamia variegata* (Goeze, 1777) (Coleoptera: Coccinellidae)'nın zararlılar tarafından teşvik edilen (HIPV) kokularının dört farklı sentetik formunun [methyl salicylate (MeSA), (E)-2-hexenal (E(2)H), farnesene (F) ve benzaldehyde (Be)] tek başına ve ikili kombinasyonlarının [MeSA+F, MeSA+E(2)H, MeSA+Be, E(2)H+F ve Be+F] iki farklı dozuna (0.001 ve 1 g/L) olan kimyasal algılama cevapları test edilmiştir. Böceğin cevapları laboratuvar koşullarında iki seçenekli Y-tüp olfaktometre ile değerlendirilmiştir. Kontrol olarak kullanılan saf hekzana göre, MeSA'nın düşük dozu önemli bir şekilde daha fazla *H. variegata* erginini (%71) cezbetmiştir. Ayrıca, *H. variegata* erginleri Be, E(2)H ve F'nin tekli kombinasyonları ile önemli düzeyde cezbedilememiştir. Diğer taraftan, MeSA'nın Be ve F ile ikili kombinasyonlarının kontrole göre önemli bir şekilde daha fazla *H. variegata* dişisini çektiği belirlenmiştir. Sonuç olarak, bu bileşikler (Be ve F) MeSA ile birlikte kullanıldığında erginlerin cezbedilmesini arttırmışlardır. Gelecekte, bu bulguların desteklenmesi için bu avcı böceğin arazi koşullarında cezbedildiği kokulara yöneliminin izleneceği ek çalışmaların yapılması gerekmektedir.

Anahtar sözcükler: Çekicilik, biyolojik mücadele*, Hippodamia variegata*, avcı, sentetik HIPVler, Y-tüp olfactometre

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Introduction

Ladybird beetles are of great economic importance in agricultural production and have been used successfully for biological control of spider mites, aphids, coccids and other soft bodied insects (Hippa et al., 1978; Kring et al., 1985; Agarwala & Dixon, 1992; William, 2002). The coccinellid species, *Hippodamia variegata* (Goeze, 1777) (Coleoptera: Coccinellidae), is commonly found in Turkey (Bastug & Kasap, 2015; Bugday et al., 2015). This species feeds mainly on aphids such as the green apple aphid, *Aphis pomi* de Geer, 1773 (Homoptera: Aphididae) as well as psyllids, whiteflies, various lepidopterans and mealy bugs (Franzman, 2002; Khan & Mir, 2008).

Different blends of herbivore-induced plant volatiles (HIPVs) are released whenever a plant is fed upon by any herbivorous arthropod (Gaquerel et al., 2009; Kaplan, 2012). These volatiles are signals of anti-herbivore defense mechanisms in plants (Dicke, 1999; Pare & Tumlinson, 1999; Mumm & Dicke, 2010). Synthetic formulations of these HIPVs could have an important role as signals in tritrophic interactions among plants, pests and predators and might serve to aggregate members of the predatory species to locations with plants infested with mites and/or aphids (Rodriguez-Saona et al., 2011).

To date, although the attractant effects of some synthetic HIPVs that might be attractive for predatory and parasitoid species have been studied, often in both laboratory and field conditions, the attraction responses of *H. variegata* to these volatiles and their binary blends has not been studied. In recent years, methyl salicylate (MeSA) has been shown to attract some coccinellid species in vineyards, hop fields and sweet corn fields (James & Price, 2004; James, 2003b, 2005; James & Castle, 2005; Zhu & Park, 2005; Woods et al., 2011; Gadino et al., 2012; Maeda et al., 2015). MeSA has also been launched commercially as Predalure™, aimed at attracting ladybird beetles as well as lacewings and syrphids, *Orius* spp. (Anonymous, 2016). The attractiveness of benzaldehyde to some coccinellid species (i.e., *Coccinella septempunctata* (L., 1758), *Stethorus punctum picipes* (Casey, 1899)*, Stethorus gilvifrons* (Muls.,1850) has been demonstrated via electroantennogram and olfactometer laboratory and field tests (Han & Chen, 2002; James, 2005). It has also been revealed that farnesene, the aphid alarm pheromone, showed a kairomonic effect on *C. septempunctata, Hippodamia convergens* Guérin-Méneville, 1842, *Harmonia axyridis* (Pallas, 1773)*,* and *Adalia bipunctata* (L., 1758) (Coleoptera: Coccinellidae) (Abassi et al., 2000; Acar et al., 2001; Francis et al., 2004; Verheggen et al., 2007). Positive responses of *C. septempunctata* to (E)-2-hexenal, which is one of the nine components of tea aphid/tea shoot complex volatiles were shown using electroantennograms and wind tunnel bioassays by Han & Chen (2002). Furthermore, synthetic HIPVs have been verified to attract coccinellids under some laboratory and field conditions; thus, their potential for use in biological control strategies has been recognized (Yu et al., 2008; Lee, 2010). Although knowledge is limited, Y-tube olfactometer tests showed that *H. variegata* was attracted by olfactory stimuli from herbivore-infested plants in the laboratory (Tapia et al., 2010; Li et al., 2013). Still, information about the effectiveness of the attraction of synthetic HIPVs to this aphidophagous predatory species is lacking.

In a number of previous studies, each HIPV was considered individually to evaluate which exerts the strongest attraction to predators and parasitoids (James, 2003a, b; Yu et al., 2008; Simpson et al., 2011). However, plants damaged by herbivores release a complex of highly specific volatile blends consisting of multiple HIPVs. We hypothesized that instead of using only standard single synthetic HIPVs, blends consisting of two or more chemicals might be more attractive to insects (Szendrei & Rodriguez-Saona, 2010; Kaplan, 2012).

HIPVs attractive effects are usually evaluated by simple laboratory tests such as olfactometers and wind tunnels. Such tests can obtain prior knowledge about the potential role of HIPV regarding their attractiveness to any natural enemy species (Gols et al., 2003). Therefore, a Y-tube olfactometer setup was used in the laboratory tests in this study to better understand the response of *H. variegata* to some synthetic HIPVs that may have a role in attracting predators. Specifically, we compared the differences between two different doses (low and high) of single and binary combinations of four synthetic HIPVs, namely, MeSA, (E)-2-hexenal, farnesene and benzaldehyde.

Materials and Methods

Insects

Hippodamia variegata adults were obtained in early August 2012 from apple orchards of Uludag University, Bursa, Turkey.

Chemicals

Synthetic formulations of four HIPVs, benzaldehyde (Be) (99.5% purity; Merck, Kenilworth, NJ, USA), methyl salicylate (MeSA) (99% purity) (Acros Organics, Geel, Belgium), farnesene (F) (95% purity; Sigma-Aldrich, St. Louis, MO, USA), (E)-2-hexenal (E(2)H) (98% purity; Sigma-Aldrich) and a solvent that was mainly n-hexane (95% purity; Carlo Erba, Peypin, France) were used in this study.

Experimental design

The single and binary combinations of above mentioned synthetic agents used for olfactory tests were Be, E(2)H, F, MeSA, MeSA+F, MeSA+E(2)H, MeSA+Be, E(2)H+F and Be+F. Each synthetic HIPV was prepared at both low and high doses (0.001 and 1 g/L, respectively) in n-hexane. A total of 10 µl of each volatile source was dropped on two-layer filter paper (4-inch rectangles) using a micropipette. The n-hexane-diluted synthetic HIPVs were mixed in binary combinations in a microcentrifuge tube. A separate filter paper on which 10 µl of pure n-hexane was dropped was used as a control treatment. Before beginning the test, for provide odor flow stability, the olfactometer arms were prepared by forced airflow through the filter paper volatile sources for 5 min. The volatile sources (filter paper) were renewed for every test replicate or after every 30 min. The positions of the tube containing the HIPVs and the n-hexane were exchanged in each test. After each test, the Y-tube was cleaned with ethanol (≤100% purity) (Merck) and left to dry for 5 min in an incubator at 90°C.

Olfactometer set-up

The responses of *H. variegata* to the HIPV sources were tested using two-choice tests with a closed system Y-tube olfactometer as described in Gencer et al. (2009), which was a slight modification of Takabayashi & Dicke (1992). Distinctively, in the olfactometer, two Pyrex tubes (2.5 cm in diameter) connected to the Y-tube were used as volatile containers. These containers had an air inlet and outlet (0.8 cm in diameter) at opposite walls. An air pump was used to create an airflow from each container through the olfactometer arms; the airflow was adjusted with a flowmeter to 1.5 L/min and humidified with deionized water. The air passed through activated charcoal before reaching the cylinders. Airflow was measured in the entry arm. At the base of the Y-tube olfactometer, air was removed by a vacuum system.

Olfactometer bioassays

Adults of *H. variegata* that had been fed on green aphids (*A. pomi*) as prey were used in this study. The adults were kept in a small box (14 x 14 x 18 cm) without prey but with a small amount of water for 2-4 h prior to the test. The experiments were carried out in a climate controlled room (25°C, 60±5% RH). A single beetle was introduced into the tube and monitored until it had walked at least 7 cm up one of the arms. The behavior of each individual was observed for a maximum of 5 min. Adults that did not choose one of the side arms within 5 min were recorded as having made no choice and excluded from the statistical analysis (Takabayashi & Dicke, 1992; Cakmak et al., 2006). All olfactometer experiments for each HIPV alone and their blends were carried out on three different days. For each replicate, a total of 15 adult ladybirds were tested. Each individual insect was used only once.

Data analysis

Differences in the proportions of *H. variegata* adults' attraction by moving toward one of the HIPV sources or toward the n-hexane control were analyzed using the replicated goodness of fit test at a 5% critical level (Sokal & Rohlf, 1995). The null hypothesis was that the predator would exhibit a 1:1 distribution across the two odor sources for each replicate. Insects that did not make a choice were excluded from the statistical analysis.

Results

The response of *H. variegata* to different single synthetic HIPVs and their different doses is shown in Figure 1. *Hippodamia variegata* adults showed significant preference for MeSA at low dose compared to pure n-hexane (control) in one out of three replicates ($P < 0.01$, Figure 1). Pooled results (GP) also showed significant preference for MeSA at low dose ($P < 0.05$; Table 1). *Hippodamia variegata* did not show a preference between MeSA at high dose and control, and between other single synthetic HIPVs at both doses and control ($P > 0.05$, Figure 1, Table 1).

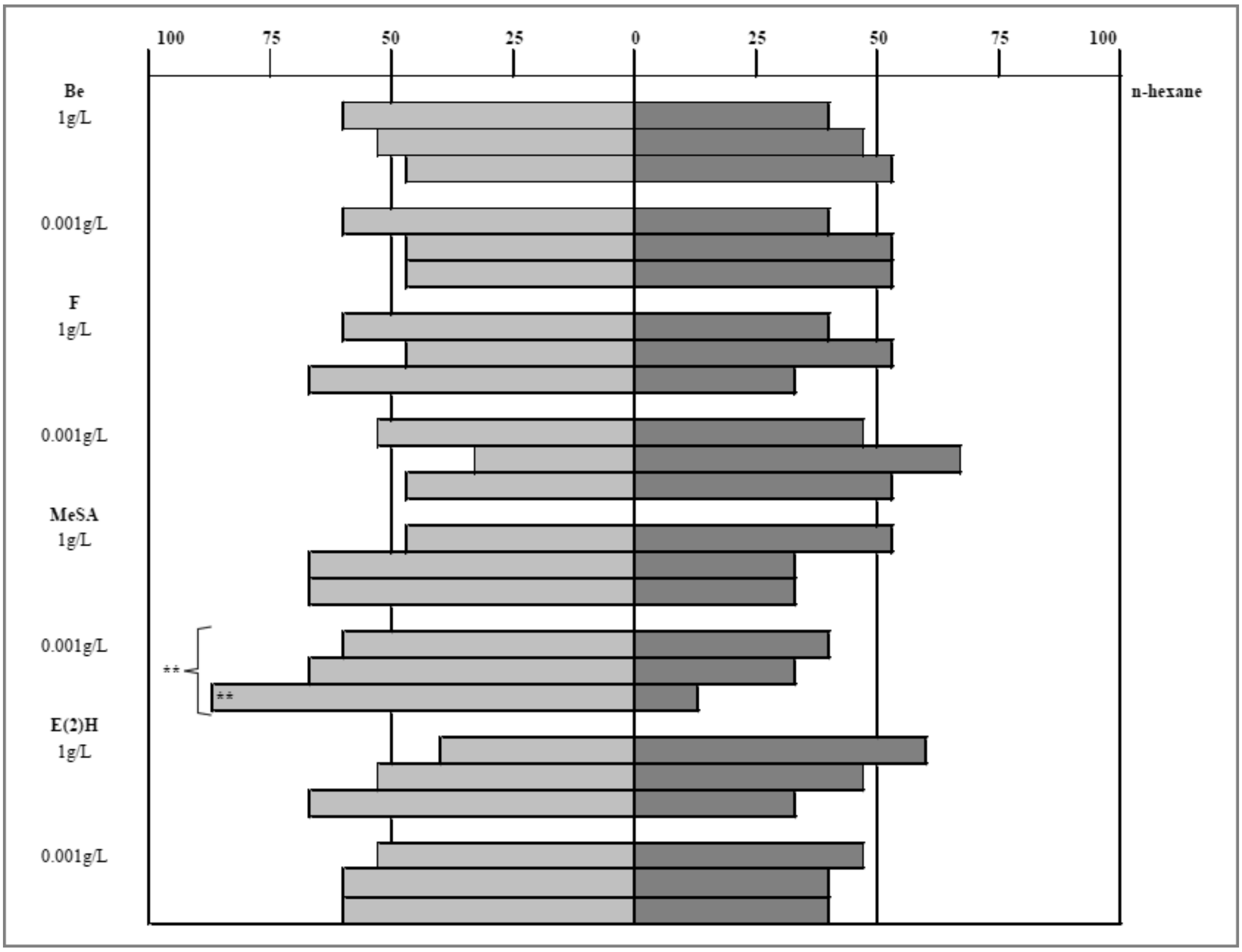

Figure 1. The response of *Hippodamia variegata* when offered high and low doses of four single synthetic HIPVs. Be, benzaldehyde; MeSA, methyl salicylate; F, farnesene; E(2)H, (E)-2-hexenal; *,**, significant P-values of 0.05 and 0.01, respectively, based on replicated goodness of fit test for G per replicate and G_P.

The response of *H. variegata* to the five binary combinations of the synthetic HIPVs and their different doses is shown in Figure 2. *Hippodamia variegata* showed a significant preference for the high dose of MeSA+F in one replicate and for the high dose of MeSA+Be in two replicates. Pooled results indicated that both doses of MeSA+F and the high dose of MeSA+Be significantly attracted *H. variegata* adults toward the volatile source ($P<0.05$, Table 1). However, pooled results indicated that *H. variegata* showed no preference for E(2)H+F, MeSA+E(2)H, Be+F at both doses and MeSA+Be at low dose compared to control, whereas it showed a significant preference in one out of three replicates for MeSA+E(2)H at high dose and Be+F at low dose (Table 1 and Figure 2). *Hippodamia variegata* showed the highest preference for the high dose of the MeSA+Be binary combination (overall, 87%), followed by MeSA alone at low dose (71%) and MeSA+F blend at high dose (69%) (Figures 1, 2).

Table 1. The results of replicated goodness of fit test on response of *Hippodamia variegata* to alone and five binary combination of four synthetic HIPVs

HIPVS	Doses (g/L)	n	G_H, df=2 P	G_P, df=1 P	G_T, df=3 P
Be	1	45	0.537 0.764	0.200 0.655	0.737 0.864
	0.001	45	0.715 0.699	0.022 0.881	0.737 0.864
E(2)H	1	45	2.170 0.338	0.200 0.655	2.370 0.499
	0.001	45	0.182 0.913	1.093 0.296	1.275 0.735
F	1	45	1.276 0.528	1.093 0.296	2.369 0.499
	0.001	45	1.276 0.528	0.557 0.456	1.833 0,608
MeSA	1	45	1.653 0.438	1.812 0.178	3.465 0.325
	0.001	45	3.038 0.219	8.279* 0.040	11.317 <0.01
MeSA+F	1	45	4.195 0.123	6.584** 0.010	10.779 0.013
	0.001	45	1.764 0.414	5.097* 0.024	6.861 0.076
E(2)H+F	1	45	0.537 0.764	0.200 0.655	0.737 0.864
	0.001	45	0.191 0.909	2.716 0.099	2.907 0.406
MeSA+E(2)H	1	45	2.644 0.267	3.810 0.05	6.554 0.088
	0.001	45	0.191 0.909	2.716 0.099	2.907 0.406
MeSA+Be	1	45	6.163* 0.046	27.043** <0.01	33.206 <0.01
	0.001	45	0.795 0.672	3.810 0.05	4.605 >0.05
Be+F	1	45	5.360 0.069	1.093 0.296	6.453 0.092
	0.001	45	1.353 0.508	3.810 0.050	5.163 0.160

Be, benzaldehyde; MeSA, methyl salicylate; F, farnesene; E(2)H, (E)-2-hexenal; G, replicated goodness of fit test; G_H, G for heterogeneity; G_P, pooled G; G_T, total G; P, probability; df, degree of freedom; n, 3 replicates x 15 individuals; *,**, significant *P*-values of 0.05 and 0.01, respectively.

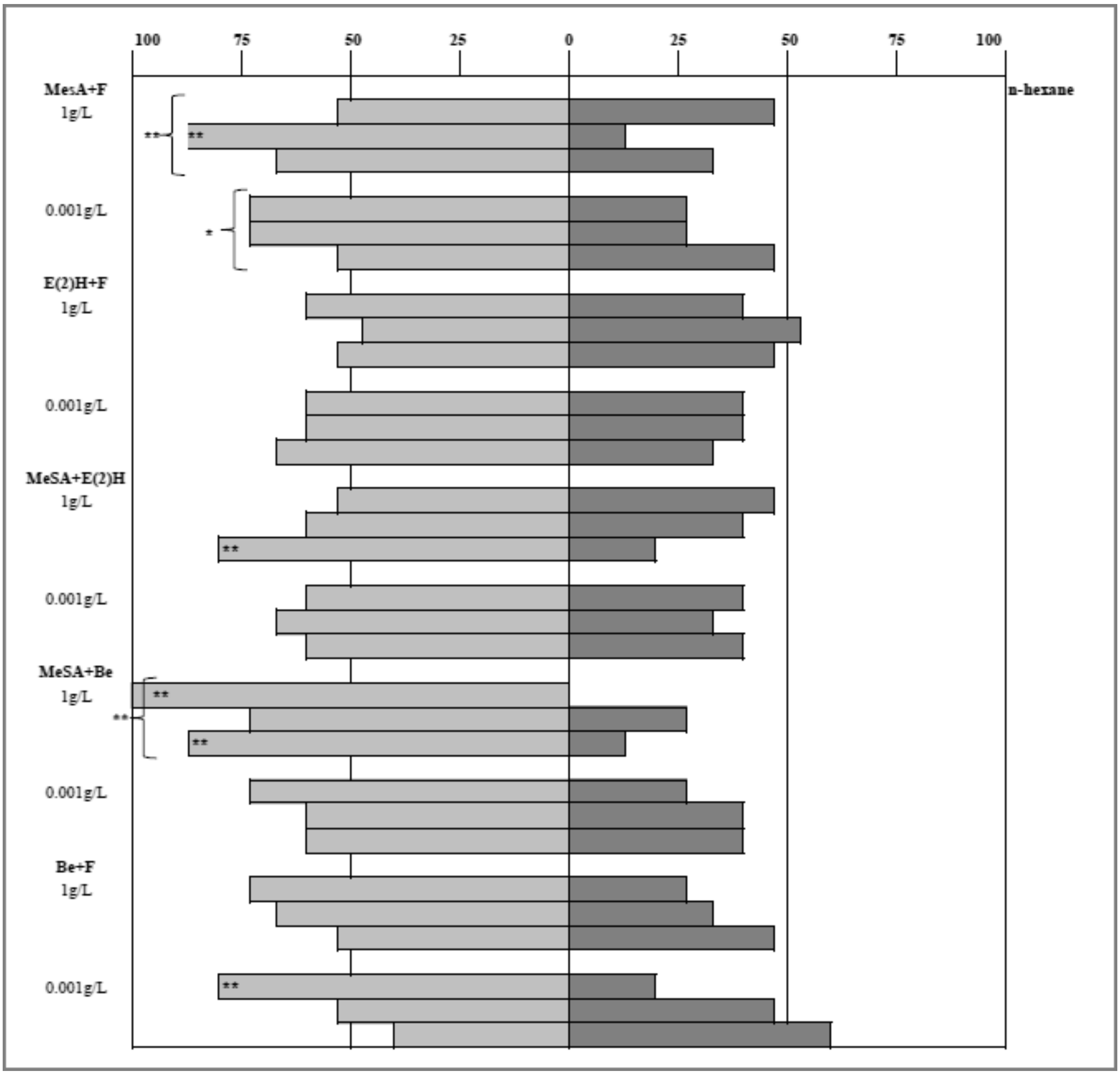

Figure 2. The response of *Hippodamia variegata* when offered high and low doses of five binary combination of four synthetic HIPVs. Be, benzaldehyde; MeSA, methyl salicylate; F, farnesene; E(2)H, (E)-2-hexenal; *,**, significant P-values of 0.05 and 0.01, respectively, based on replicated goodness of fit test for G per replicate and G_P.

Discussion

The current study demonstrated that MeSA among experimental synthetic HIPVs was significantly more attractive for *H. variegata* adults than the control. Similarly, some ladybird beetle species such as *Cycloneda polita* Casey, 1899, *C. septempunctata, H. axyridis, Propylea japonica* (Thunberg, 1780) and *Stethorus* spp., were attracted to MeSA-baited traps in hop fields (James, 2003b, 2005; Woods et al., 2011), vineyards (James & Price, 2004; Gadino et al., 2012), soybean fields (Zhu & Park, 2005; Mallinger et al., 2011), tea fields (Qi et al., 2008) and cranberry fields (Rodriguez-Sauna et al., 2011). In fact, MeSA lures have already been used as a commercial product to attract ladybird beetles (Lee, 2010; Anonymous, 2016). The findings obtained from this study are the first controlled study of HIPV attraction to *H. variegata*. Our results showed that *H. variegata* is one of the predators attracted by MeSA as a broad-spectrum lure.

Of the other tested HIPVs, farnesene is known as an important aphid alarm pheromone (Joachim et al., 2015). Furthermore, attractiveness of farnesene has been reported for two other ladybird species, *H. convergens* and *H. axyridis* (Colburn & Asquith, 1970). In addition, Li et al. (2013) showed that farnesene, one of the main components in volatiles released by *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae), elicited behavioral effects from *H. variegata.* Additionally, some researchers have shown that farnesene is attractive to some other ladybird species including *Coleomegilla maculata* (De Geer, 1877) (Coleoptera: Coccinellidae) and *P. japonica* in both laboratory and field tests (Zhu et al., 1999; Liu et al., 2014). In recent years, the attractant property of benzaldehyde has been demonstrated in some coccinellids (*C. septempunctata*, *S. gilvifrons* and *S. punctum picipes*) with olfactometer tests and in field studies (Han & Chen, 2002; James, 2003b; Sachin et al., 2008). In contrast, our test results were contradictory for pure farnesene and benzaldehyde. In the last pure synthetic HIPV (E(2)H) experiments, no evidence was obtained that ladybirds tended to be attracted to this compound due to no significant effect was found. This finding supports another study which found that the compound had no effect on females of *H. axyridis* even though it attracted males (Leroy et al., 2012).

Additionally, this study showed that binary blends of MeSA with Be or F had significantly more attractiveness for *H. variegata* adults than controls. Thus, compounds used together with MeSA were observed to have to increase adult attraction. The advantages of using mixtures instead of single compounds have been demonstrated in both field and laboratory studies in recent years (Maeda et al. 2015). Our mixture results are consistent with the findings of Szendrei & Rodriguez-Saona (2010), who revealed that individual volatiles were attractive overall, but that increased blend complexity corresponded with stronger attraction. Similarly, Jones et al. (2010) reported that the attractiveness of iridodial (a male-produced aggregation pheromone) increased when MeSA was added, doubling the number of lacewings captured in traps in apple orchards. Toth et al. (2009) achieved similar results for lacewings by adding MeSA to a blend of phenylacetaldehyde and acetic acid. Here, we demonstrated the attraction of MeSA+Be and MeSA+F combinations to coccinellids, especially *H. variegata.* This study constitutes a first report because there is no prior record in the literature.

Additionally, the binary combination (MeSA+E(2)H) were not attractive for the predator insect, though we obtained significant results concerning insect attraction to MeSA with Be or F. Our results are also in accordance with some results about the differences in olfactory responses in other predator and parasitoid species to various blends of HIPVs (Simpson et al., 2011; Van Wijk et al., 2011; Maeda et al., 2015). It seems likely that ecological differences may be the cause of such variations in the olfactory responses of different coccinellid species. The discrepancies in ladybird species attraction to different synthetic HIPVs or their combinations may depend on a number of different factors. Insects learn different volatile cues in their environments and have different experiences. In this study, we collected ladybirds from apple orchards, where their foraging host was *A. pomi*. Our data suggest that specific HIPV combinations should be tested for their effect on specially targeted organisms as well as particular agroecosystems. Our results are agreement with the results of Gencer et al. (2009), who showed that the olfactory responses of *S. gilvifrons* changed when its host plant or prey were altered. The volatile blends released by injured plants can vary between different combinations of plant and herbivore and between different herbivores on the same plant species as well as the same herbivore on different genotypes of the same plant species (Degen et al., 2004; Van Den Boom et al., 2004; Leitner et al., 2005). Our results lead us to two separate conclusions concerning the deployment of synthetic HIPVs for the purpose of conservation biological control. First, our data suggest that attraction of predator arthropods to synthetic volatiles is potentially dependent upon factors specific to the crop system (context dependency), to the local arthropod community, and to the specific field studied, which makes the implementation of HIPVs across systems challenging. Second, our results asserted that the effectiveness of HIPVs for attracting beneficial arthropods may be increased using different doses and combinations of synthetic HIPVs when a single compound is not sufficiently attractive. Our results are consistent with Qi et al. (2008) who noted that different dosages (10^{-6} to 10^{-2} g/ml) of HIPVs such as benzaldehyde, methyl salicylate, significantly affected the choices of *P. japonica* in Y-tube olfactometer tests. Furthermore, studies need to determine the most effective combination of HIPVs to more consistently and reliably attract predatory arthropods to these plant volatiles in the field.

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