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Electrophysiological and Behavioral Responses of the Multicolored Asian Lady Beetle, *Harmonia axyridis* Pallas, to Sesquiterpene Semiochemicals

François J. Verheggen · Quentin Fagel · Stéphanie Heuskin · Georges Lognay · Frédéric Francis · Eric Haubruge

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Abstract The role of two volatile sesquiterpenes, (*E*)-β-farnesene and (−)-β-caryophyllene, in the chemical ecology of the multicolored Asian lady beetle, *Harmonia axyridis* Pallas, was investigated by using both electrophysiological and behavioral techniques. (*E*)-β-Farnesene is the major component of the alarm pheromone of most aphid species, which are preyed on by *H. axyridis*. (−)-β-Caryophyllene was previously isolated from the headspace volatiles above overwintering and aggregated *H. axyridis* females. These sesquiterpenes elicited significant electroantennogram (EAG) activity from both *H. axyridis* male and female antennae. In a four-arm olfactometer, male and female *H. axyridis* were highly attracted toward (*E*)-β-farnesene, whereas only males were attracted to (−)-β-caryophyllene. In a bioassay technique that used a passively ventilated plastic box, both male and female *H. axyridis* aggregated in the (−)-β-caryophyllene-treated side of the box. These results support the potential usefulness of (*E*)-β-farnesene and (−)-β-caryophyllene in push–pull strategies that use *H. axyridis* as a biological control agent in aphid-infested sites or to control this new urban pest in residential structures.

Keywords Aggregation pheromone · Behavioral assays · (−)-β-Caryophyllene · Electroantennography · (*E*)-β-Farnesene · Four-arm olfactometer · *Harmonia axyridis*

Introduction

Several insect species release aggregation pheromones that attract conspecifics for mating, thus optimizing resource use, or that lead to aggregation in an environmentally favorable

F. J. Verheggen (✉) · Q. Fagel · F. Francis · E. Haubruge
Department of Functional and Evolutionary Entomology, Gembloux Agricultural University,
Passage des Déportés 2, 5030 Gembloux, Belgium
e-mail: entomologie@fsagx.ac.be

S. Heuskin · G. Lognay
Department of Analytical Chemistry, Gembloux Agricultural University, Gembloux, Belgium

spot to overwinter. Aggregation pheromones, as well as sex pheromones, are essential components of integrated pest management methods that are used in push–pull strategies to repel the target pests from a protected host, while luring them toward an attractive trap (Cook et al. 2007). Although the multicolored Asian lady beetle, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), was introduced in Europe and North America as an effective natural enemy of various pests that include aphids and mites, adverse effects of *H. axyridis* on nontarget insects, humans, and crops have been identified. Furthermore, *H. axyridis* negatively impacts native coccinellids, as *H. axyridis* is the top predator in the aphidophagous guild. It occupies the ecological niches of endemic lady beetles and other aphid-specific predators such as the two-spotted lady beetle, *Adalia bipunctata* L., or the seven-spotted lady beetle, *Coccinella septempunctata* L. (Koch 2003; Sato et al. 2005). *Harmonia axyridis* is also considered a nuisance pest because it migrates from the field into houses and other structures when temperatures decline, and forms mass overwintering aggregations in elevated, dark, and concealed portions of structures (Huelsman et al. 2002). In addition to its invasive behavior, *H. axyridis*, when disturbed, secretes hemolymph that imparts an unpleasant odor to any surfaces (walls, furnishings, etc.) where beetles aggregate (Huelsman et al. 2002; Magnan et al. 2002). The secretions and the unpleasant odor can lead to allergic reactions in human occupants.

To develop efficient push–pull strategies, better knowledge is required of the potential semiochemicals involved in the establishment and persistence of overwintering aggregations of *H. axyridis*. While observing *H. axyridis* aggregation sites, Nalepa et al. (2000) concluded that there was little evidence for volatile aggregation pheromones and that contact chemoreception with conspecifics probably mediates the aggregation behavior. However, Brown et al. (2006) reported the identification of (−)-β-caryophyllene from the headspace of *H. axyridis* females under conditions that simulated natural overwintering conditions. (−)-β-Caryophyllene is also commonly released from various plant families, including the Brassicaceae (Rohloff and Bones 2005), Solanaceae (Farag and Paré 2002), and Poaceae (Dean and De Moraes 2006).

To demonstrate the potential role of (−)-β-caryophyllene in the aggregation behavior of *H. axyridis*, and the impact of (*E*)-β-farnesene (major component of the aphid alarm pheromone) on the foraging behavior of this aphid predator, we conducted both electrophysiological and behavioral experiments. These two methodological approaches have been used widely in the identification of lady beetle-related semiochemicals (Zhu et al. 1999; Al Abassi et al. 2000; Acar et al. 2001; Francis et al. 2001, 2004; Ninkovic et al. 2001).

Methods and Materials

Insects Larvae of *H. axyridis* were collected in May 2007 on the edges of a bean field and placed in aerated plastic boxes (up to 25 individuals per container). The larvae and the resulting adults were provisioned daily *ad libitum* with aphids, *Acyrthosiphon pisum* Harris, reared on beans. Sugar, water-impregnated cotton, and multiflower pollen were also provided. Boxes were placed in controlled environment incubators (16-hr-light photoperiod; 25±2°C; 70% RH). Males and females were separated at least 1 wk prior to electrophysiological and behavioral bioassays.

Electrophysiology (EAG) The *H. axyridis* antenna was carefully excised from the head so that all segments and the basal nerve were still attached. The antenna was mounted between two glass Ag–AgCl electrodes (Harvard Apparatus, Holliston, MA, USA; 1.5 mm o.d.×

1.17 mm i.d.) filled with saline solution (NaCl, 7.5 g/l; $CaCl_2$, 0.21 g/l; KCl, 0.35 g/l; $NaHCO_3$, 0.2 g/l) and in contact with a silver wire. The base of the antenna was first inserted into the ground glass electrode. The tip of the recording electrode was bowl-shaped, and half of the last distal antennal segment was immersed into the saline solution. This setup was previously shown to produce elegant results in the study of the olfactory responses of the confused flour beetle, *Tribolium confusum* Du Val (Coleoptera: Tenebrionidae), to its aggregation pheromone (Verheggen et al. 2007). The DC potential was recorded on a computer (Auto Spike v. 3.0) by using an amplifier (IDAC-4, Syntech®, Hilversum, The Netherlands) with 100-fold amplification. A 0.5-cm^2 piece of filter paper that was impregnated with 10 μl of the chemical under examination was placed in a Pasteur pipette and used to puff an air sample in a constant 1.5 l/min airstream. As a negative control, the antennae were first stimulated with semiochemical-free filter paper (=mechanical stimulus). Later, the antennae were stimulated with (−)-β-caryophyllene or (*E*)-β-farnesene (EβF). (−)-β-Caryophyllene was purchased from Sigma-Aldrich (Chemie Gmbh, Steinheim, Germany) and had a chemical purity of >97% (determined by GC). EβF was synthesized from farnesol (Tanaka et al. 1975) and had a chemical purity of 98% (also determined by GC). Ten antennae were tested for each sex.

Four-arm Olfactometer Assays The four-arm olfactometer was similar to that previously described by Vet et al. (1983). It was constructed entirely of Teflon® and was closed with a removable glass roof, both previously cleaned with *n*-hexane. The walking arena was 40 cm wide (from center to odor source) and 1.5 cm high (from Teflon® walking arena to glass ceiling). Charcoal-filtered air was pushed in each of the four olfactometer arms through Teflon® tubing and adjusted to 100 ml/min with a digital flowmeter. A pump ventilated the walking arena by removing air from the center at 400 ml/min. A 0.5-l glass chamber was connected to one of the four olfactometer arms and was used to dispose of the odor source. Three stimuli were tested on both *H. axyridis* males and females: (1) the four arms of the olfactometer were connected to pure air sources, the glass chamber remained empty; (2) 20 unwinged adult aphids, *Myzus persicae* Sultzer, were rapidly crushed inside of the glass chamber by using a small glass pestle left inside the chamber [as a natural source of EβF: according to previous studies, the volatiles released by crushed *M. persicae* consist exclusively of EβF (Edwards et al. 1973; Francis et al. 2005)]; (3) a 0.5-cm^2 piece of filter paper was impregnated with 10 μl of (−)-β-caryophyllene and placed inside the glass chamber. The glass chamber was randomly connected to one of the four arms of the olfactometer. Both the walking arena and the glass ceiling were washed with *n*-hexane after each lady beetle was tested. The olfactometer was divided into one central 10-cm squared area and four other areas related to the four odor sources. The choice of the lady beetle was determined by (a) the first area it entered, (b) the first area where it stood for 30 consecutive seconds, and (c) the area where it stood at the end of the 3 min of observation (=last entered area). The behavioral observations were conducted in a laboratory at 22±1°C and under uniform lighting to avoid interference with behavior of the test insects.

Aggregation Assays Three 5-cm-diameter holes were cut into a 30×15×15-cm plastic box, and the holes were covered with metal screening. One hole was located on each lateral side (30 cm apart) and a third hole was located on the top. A 4-cm^2 piece of filter paper impregnated with 10 μl of (−)-β-caryophyllene was attached to the screen cover of one of the lateral holes by using a rubber band. Ten males and 10 females *H. axyridis* were introduced into the box for 1 hr, and the side where they stood was recorded after 30 and 60 min. Behavioral observations were conducted under the same conditions as mentioned above.

Statistical Analyses A Student's *t* test was performed to compare the mean EAG responses to the semiochemical stimuli with the EAG responses to the mechanical stimulant, as well as to compare EAG responses between the sexes. Observed frequencies related to the choice of *H. axyridis* in olfactometer assays (four-arm and aggregation bioassays) were compared to corresponding theoretical frequencies by using a χ^2 goodness-of-fit test. All statistical tests were conducted using Minitab® release 14.2.

Results

Electroantennography To the best of our knowledge, this is the first report of successful EAG recordings from *H. axyridis* antennae. Both pure EβF and (−)-β-caryophyllene elicited good activity from antennae of both sexes. Female and male antennal responses to the latter semiochemical were higher than those produced mechanically (Student's *t* test, t_{obs}=4.89, P<0.001, and t_{obs}=5.49, P<0.001, for females and males, respectively). Male antennae were more sensitive to (−)-β-caryophyllene (338±31 μV, N=10) than female antennae (178±19 μV, N=10) (t_{obs}=4.41, P=0.001).

The mean EAG responses to EβF were significantly higher than those to mechanical stimuli (t_{obs}=2.43, P=0.027, and t_{obs}=2.53, P=0.022, for females and males, respectively). The mean EAG responses to EβF was higher in males (202±17 μV) than in females (120± 18 μV) (t_{obs}=3.30, P=0.004). (−)-β-Caryophyllene induced higher EAG responses than EβF (t_{obs}=2.22, P=0.040, and t_{obs}=3.84, P=0.002, for females and males, respectively).

Four-arm Olfactometer Assays In the four-arm olfactometer, EβF (crushed *M. persicae* as a natural source) and (−)-β-caryophyllene elicited significant behavioral activity from both *H. axyridis* males and females (Table 1). According to the three behavioral criteria that we observed and recorded, males were attracted to (−)-β-caryophyllene. Females were less attracted than males to (−)-β-caryophyllene, as significant results were obtained only while observing the last area entered (χ^2=7.92, P=0.048). EβF attracted both male and female *H. axyridis* (Table 1).

Aggregation Assays After 30 min, both sexes of *H. axyridis* aggregated on the (−)-β-caryophyllene side of the box (χ^2=20.48, P<0.001, and χ^2=18.00, P<0.001, for males and females, respectively) (Fig. 1). Similar results were observed after 1 hr, as both males and females were still aggregated in the side of the box where (−)-β-caryophyllene was released (χ^2=11.52, P=0.001, and χ^2=13.52, P<0.001, respectively).

Discussion

Comprehension of the chemical ecology of *H. axyridis* is essential before developing push–pull strategies for the efficient use of this lady beetle in aphid control or for the control of this new urban pest in structures. (−)-β-Caryophyllene and EβF are ubiquitous plant volatiles, and previous studies have demonstrated the role of plant semiochemicals in the searching behavior of aphid natural enemies (Tumlinson et al. 1992). In our experiments, (−)-β-caryophyllene and EβF elicited antennal activity in both sexes and significant attraction in a four-arm olfactometer. It has been suggested that lady beetles orient

Table 1 Behavioral responses [observed frequencies (N=50) were compared to expected frequencies assuming random distribution (one odor source and three controls) by using a χ^2 test] of *H. axyridis* to two sesquiterpenes

Odor Source	*H. axyridis* Gender	Behavioral Observations	Observed Frequencies	χ^2	P
(−)-β-Caryophyllene	Female	First entered area	0.38	4.56	0.207
		First area entered for 30 sec	0.38	4.56	0.207
		Last entered area	0.42	7.92	0.048
	Male	First entered area	0.44	9.68	0.021
		First area entered for 30 sec	0.46	11.92	0.008
		Last entered area	0.44	9.84	0.020
(*E*)-β-Farnesene[a]	Female	First entered area	0.50	16.72	0.001
		First area entered for 30 sec	0.52	16.60	<0.001
		Last entered area	0.54	22.80	<0.001
	Male	First entered area	0.52	19.60	<0.001
		First area entered for 30 sec	0.54	22.48	<0.001
		Last entered area	0.58	29.20	<0.001

[a] Natural source of EβF was 20 unwinged adult aphids, *M. persicae* Sultzer (crushed in the olfactometer release chamber)

themselves toward aphid prey by discriminating aphid punctual and EβF emission from the continuous plant release of EβF, usually joined with (−)-β-caryophyllene emission (Dawson et al. 1984). The latter has also been shown previously to counteract the attraction of lady beetles to EβF, acting thus as a natural alarm pheromone inhibitor, allowing the beetles to differentiate between EβF of plant and aphid origin. As *H. axyridis* releases its aggregation pheromone at the end of autumn, when the temperatures get lower and aphids are present in lower densities, no inhibition happens.

Our results demonstrated that (−)-β-caryophyllene and EβF act as semiochemicals for *H. axyridis*. Indeed, (1) there are some specific neuronal receptors that allow their perception and (2) a behavioral attraction/aggregation was noted in the two behavioral assays. Al Abassi et al. (2000) obtained similar electrophysiological results while studying *C. septempunctata* olfactory cell responses toward the same two sesquiterpenes: Cells having high specificity for (−)-β-caryophyllene and EβF were identified. In the 12-spotted lady beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae), the highest EAG responses were obtained while testing EβF, (−)-β-caryophyllene, and two other terpenoid alcohols (Zhu et al. 1999). Therefore, it is not surprising that *H. axyridis*, which also belongs to the Coccinellidae, possesses similar specific receptors.

(−)-β-Caryophyllene is specifically released by *H. axyridis* females in overwintering aggregation conditions (Brown et al. 2006). The production of (−)-β-caryophyllene by females of *H. axyridis* was confirmed in the present study by volatile collection and subsequent GC-MS analysis (data not shown). Whereas cases of aggregation pheromone release by one sex only are common in the Coleoptera (e.g., Verheggen et al. 2007), the use of a chemical commonly found in the headspace of various plants as an aggregation pheromone might be inappropriate. One might put forth the assumption that lady beetles have different antennal sensitivity to (−)-β-caryophyllene according to the season, being more sensitive during winter conditions. (−)-β-Caryophyllene has an attractive effect on *H.*

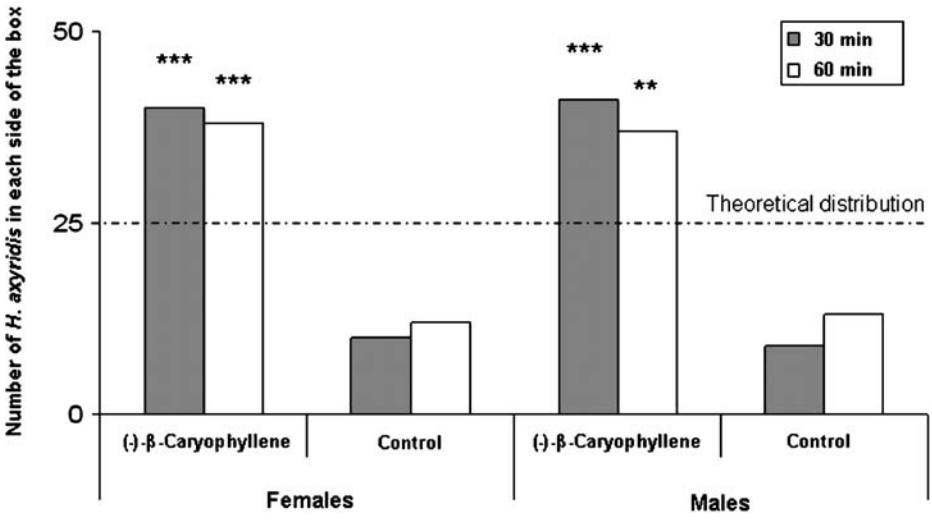

Fig. 1 Observed distribution of female and male *H. axyridis* (N=50 for each sex) relative to the release site of (−)-β-caryophyllene in an aggregation bioassay. *Double* and *triple asterisks* indicate significant differences between observed and expected distributions at $P<0.01$ and $P<0.001$, respectively (χ^2 goodness-of-fit test)

axyridis, although the responses of the females in the four-arm olfactometer were significant in only one of the three behavioral observations. However, in the second set of behavioral assays, the distribution of both males and females in the (−)-β-caryophyllene-treated side of the box was significant. These behavioral data, the antennal perception of (−)-β-caryophyllene, and its specific period of release (i.e., in the early winter) support the hypothesis that it acts as a component of an aggregation pheromone rather than a sex pheromone.

Like other lady beetle species, including *A. bipunctata* (Hemptinne et al. 2000; Francis et al. 2004), *H. convergens* (Acar et al. 2001), and *C. septempunctata* (Nakamuta 1991; Al Abassi et al. 2000), *H. axyridis* is able to perceive and orient itself toward EβF, the main component of the aphid alarm pheromone. Our electrophysiological and behavioral results support previous research that demonstrated that *H. axyridis* has a high ability to track aphid populations in space and time (Osawa 2000). Indeed, this aphid predator showed fast and pronounced orientation behavior toward the EβF source in the four-arm olfactometer. Having the ability to localize aphids under predation represents an undeniable advantage for a mobile and voracious predator like *H. axyridis*. Although previous studies demonstrated that *H. axyridis* responded to volatiles from aphids and aphid-damaged plants (Han and Chen 2002), our results do not support previous work that demonstrated that *H. axyridis* was attracted toward a colony of *A. pisum*, but not toward cornicle secretions (Mondor and Roitberg 2000), which are known to contain EβF (Francis et al. 2005).

In summary, push–pull strategies that target the use of *H. axyridis* as a biological control agent in aphid-infested sites, or that are designed to control this new urban pest in human dwellings, should take into account the potential attractive effect that (−)-β-caryophyllene could have in outdoor conditions. Our data also suggest that, in aphid biological control strategies, one should incorporate EβF as an attractant for various aphid natural enemies, including *H. axyridis*.

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