Behavioral Responses of Aphis citricola (Hemiptera: Aphididae) and Its Natural Enemy Harmonia axyridis (Coleoptera: Coccinellidae) to Non-Host Plant Volatiles

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Behavioral responses of *Aphis citricola* (Hemiptera: Aphididae) and its natural enemy *Harmonia axyridis* (Coleoptera: Coccinellidae) to non-host plant volatiles

Beizhou Song[1,2,#], Yinping Liang[1,#], Sizhou Liu[1], Linfeng Zhang[1], Guangbo Tang[1], Teng Ma[1], and Yuncong Yao[1,2,]*

Abstract

Plant volatiles can act as chemical signals that influence the behavior and distribution of insects. Although considerable information has been acquired on the effects of plant volatiles emitted from plants on herbivorous insects and their natural enemies, practical implementation of this knowledge is still lacking. We investigated 3 aromatic plant species, French marigold, *Tagetes patula* L. (Asteraceae), ageratum, *Ageratum houstonianum* Mill. (Asteraceae), and catnip, *Nepeta cataria* L. (Lamiaceae), to test their effectiveness in repelling or attracting spirea aphid, *Aphis citricola* van der Goot (Hemiptera: Aphididae), and its natural enemy, the multicolored Asian lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), in the field and the laboratory. We found that intercropping apple trees *Malus* spp. (Rosaceae) with aromatic plants in an orchard significantly reduced the number of *A. citricola* aphids present, but had the opposite effect on *H. axyridis*. In addition, the association between *H. axyridis* and *A. citricola* numbers was strengthened when the intercropping included French marigold. Using an H-tube olfactometer, we found that *A. citricola* was repelled by French marigold and catnip, whereas *H. axyridis* was attracted most by French marigold. Volatile analysis revealed that the sesquiterpenes D-limonene and terpinolene and the alcohol 2-ethyl-1-hexanol were the most abundant volatile compounds released by French marigold and catnip. *Harmonia axyridis* was significantly attracted by 12.5 µL/L D-limonene, 50 µL/L terpinolene, and 25 µL/L of a 1:1 mixture of the 2 compounds, but was repelled by higher concentrations of D-limonene. The results suggest that aromatic plants increase the resistance of apple trees to *A. citricola* both directly, by reducing the population of *A. citricola* through chemical repulsion, and indirectly, by increasing the *H. axyridis* population through chemical attraction.

Key Words: aphid; aromatic plant; repellency; attractancy

Resumen

Los volátiles de las plantas pueden actuar como señales químicas que influyen en el comportamiento y distribución de los insectos. Aunque se ha adquirido bastante información sobre los efectos de los volátiles vegetales emitidos por las plantas sobre los insectos herbívoros y sus enemigos naturales, todavía falta la aplicación práctica de estos conocimientos. Se investigaron 3 especies de plantas aromáticas, clavel de moro, *Tagetes patula* L. (Asteraceae), ageratum, *Ageratum houstonianum* Mill. (Asteraceae) y menta de gato, *Nepeta cataria* L. (Lamiaceae), para probar su efectividad en repeler o atraer al pulgón spirea, *Aphis citricola* van der Goot (Hemiptera: Aphididae), y su enemigo natural, la mariquita asiática de multicolores, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), en el campo y en el laboratorio. Se encontró que al intercalar manzanos *Malus* spp. (Rosaceae) con plantas aromáticas en un huerto redujeron significativamente el número de áfidos de *A. citricola* presentes, pero tuvieron el efecto opuesto en *H. axyridis*. Además, la asociación entre *H. axyridis* y el número de *A. citricola* se fortaleció cuando el clavel de moro fue intercalado. Usando un olfatómetro tubo-H, encontramos que *A. citricola* fue repelido por el clavel de moro y la menta de gato, mientras que *H. axyridis* fue atraído más por el clavel de moro. El análisis volátil reveló que los sesquiterpenos D-limoneno y terpinoleno y el alcohol 2-etil-1-hexanol eran los compuestos volátiles más abundantes liberados por el clavel de moro y la menta de gato. *Harmonia axyridis* fue atraído significativamente por 12,5 µL / L de D-limoneno, 50 µL / L de terpinoleno y 25 µL / L de una mezcla 1:1 de los 2 compuestos, pero fue repelido por mayores concentraciones de D-limoneno. Los resultados sugieren que las plantas aromáticas aumentan la resistencia de los manzanos a *A. citricola* tanto directamente, reduciendo la población de *A. citricola* mediante la repulsión química, como indirectamente, aumentando la población de *H. axyridis* a través de la atracción química.

Palabras Clave: áfido; planta aromática; repelencia; atraccion

Plant volatiles can function as important sources of information that mediate many interactions within a plant–arthropod community and can influence the behavior and distribution of insects (Dicke 2015). When plants are attacked by herbivores, they release complex mixtures of volatile compounds called herbivore-induced plant volatiles, which can trigger enhanced plant defenses that directly or indirectly protect the plant against herbivores (Lucas-Barbosa et al. 2011). Two types of plant defenses can be distinguished, the first of which involves

volatiles that affect the performance or behavior of herbivores and that may have a direct repellent effect on herbivores (Lucas-Barbosa et al. 2011). Examples include induced volatiles from tobacco, *Nicotiana tabacum* L. (Solanaceae), that are highly repellent to female moths of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) (Moraes et al. 2001) and induced volatiles from aromatic plants that are highly repellent to the beetles *Serica orientalis* Motschulsky, *Maladera verticalis* (Fairmaire), and *Anomala corpulenta* Motschulsky (all Coleoptera: Scarabaeidae) (Tang et al. 2013).

The other type of plant defense based on plant volatiles involves indirect defenses wherein induced volatiles attract the predators of herbivores, thereby exerting a degree of biological control (McCormick et al. 2012; Schausberger et al. 2012). For example, egg parasitoids (*Trichogramma bournieri* Pintureau & Babault; Hymenoptera: Trichogrammatidae) and larval parasitoids (*Cotesia sesamiae* [Cameron]; Hymenoptera: Braconidae) are attracted by volatiles induced by the stalk borer *Chilo partellus* Swinhoe (Lepidoptera: Crambidae) on teosinte, *Zea* sp. (Poaceae), which thereby provides an indirect defense mechanisms against the stalk borers (Mutyambai et al. 2015). As another example, treatment of seeds with the phytohormone jasmonic acid has also been shown to make tomato, *Solanum lycopersicum* L. (Solanaceae), plants more attractive to predatory mites (Smart et al. 2013).

The use of herbivore-induced plant volatiles to attract the natural enemies of crop herbivores, thereby reestablishing predator–prey relationships and reducing the need for additional pest suppression in agricultural habitats, has risen substantially over the past 10 yr (Kaplan 2012). These volatiles attract both parasitic and predatory insects that are natural enemies of the herbivores, and they may induce defense responses in neighboring plants (Paré & Tumlinson 1999; Pichersky & Gershenzon 2002). For example, plant volatiles from damaged cotton, *Gossypium hirsutum* L. (Malvaceae), were reported to increase the resistance of undamaged cotton and alfalfa, *Medicago sativa* L. (Fabaceae), plants to oviposition by *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) (Zakir et al. 2013). Sagebrush plants that were clipped in the field were found to release an epimer of methyl jasmonate that has been shown to be a volatile signal capable of inducing resistance in wild tobacco, and tobacco plants near the clipped sagebrush experienced greatly reduced levels of leaf damage by grasshoppers and cutworms compared with control plants (Karban et al. 2000).

However, the nature of the plant volatile compounds varies among plant and herbivorous insect species (Dudareva et al. 2006; Kappers et al. 2011), and plant volatiles can also attract herbivores. The redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae), for example, is attracted by the leaf volatiles of its host plants in North America and may use them as a cue for host location (Martini et al. 2015). Females of the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) are attracted to the place of oviposition by the volatiles emitted from host plants infested by the imported cabbageworm, *Pieris rapae* (L.) (Lepidoptera: Pieridae) (Shiojiri & Takabayashi 2003).

In addition, the intermingling of volatiles released by host plants and non-host plants may hamper searching behavior and reduce the host-finding success of parasitoids (Perfecto & Vet 2003; Gols et al. 2005). For instance, experienced parasitoid, *Oomyzus galerucivorus* (Hedqvits) (Hymenoptera: Eulophidae), females were shown to be attracted by the volatiles of yarrow, *Achillea millefolium* L. (Asteraceae), but showed no response when yarrow volatiles were offered simultaneously with those of a non-host plant (Randlkofer et al. 2007). However, insects can also identify volatile blends released by different plants through olfactory learning (Guillette et al. 2009; Shikano & Isman 2009), which may inform foraging insects of the identity and qual-

ity of the host plant. Taken together, these different factors and effects limit the use of plant volatiles to reduce herbivorous pest pressure and enhance biocontrol in agricultural production.

The spirea aphid, *Aphis citricola* van der Goot (Hemiptera: Aphididae), is a major apple tree pest in Chinese apple production areas and often causes serious economic losses; however, a generalist predator, the multicolored Asian lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), is successfully used to control this pest (Qiu et al. 2010; Wang et al. 2011). Conservation biological control of fruit tree aphids has been evaluated in many studies of orchard ecosystems (Fréchette et al. 2008; Song et al. 2010). Beginning in 2007, our group has studied the mechanism of intercropping aromatic plants with pear trees or apple trees in the ecological control of pests. We selected 10 aromatic plant species from 100 available species (Song et al. 2011, 2013; Wan et al. 2015), including French marigold, *Tagetes patula* L. (Asteraceae); ageratum, *Ageratum houstonianum* Mill. (Asteraceae); savory, *Satureja hortensis* L. (Lamiaceae); basil, *Ocimum basilicum* L. (Lamiaceae); catnip, *Nepeta cataria* L. (Lamiaceae); and others for further study.

Previous research by our group demonstrated that intercropping pear trees with aromatic plants can regulate the trophic structure of the arthropod community and significantly reduce the abundance of pests such as *Psylla chinensis* Yang & Li (Hemiptera: Psyllidae), *A. citricola*, and *Pseudococcus comstocki* Kuwana (Hemiptera: Pseudococcidae) (Wan et al. 2015). However, little is known about how the volatiles from the aromatic plants regulate the behavior of the spirea aphid and its lady beetle natural enemy; this is information that would be useful for biocontrol in apple production.

To test the behavioral responses of *A. citricola* and its natural enemy *H. axyridis* to aromatic plants and their volatiles, the volatile compounds from the aromatic plants were identified and their effectiveness in repelling or attracting *A. citricola* and *H. axyridis* were tested. We specifically addressed the following questions in this study: (1) Do aromatic plants increase the resistance of apple trees to *A. citricola*? (2) Do the volatile compounds from aromatic plants repel or attract *H. axyridis*? (3) What is the mechanism by which aromatic plants regulate *A. citricola* and *H. axyridis* populations?

Materials and Methods

INSECTS

Aphis citricola and *H. axyridis* adults were originally collected from apple trees in the experimental orchard in the Changping District, north of Beijing, China. The aphids were reared on fresh apple leaves for at least 2 generations and maintained in a growth chamber under controlled conditions: 28 ± 5 °C, 70 ± 5% RH, and a 16:8 h L:D photoperiod. *Harmonia axyridis* colonies were reared on *A. citricola* for at least 2 generations and maintained in a growth chamber under controlled conditions: 26 ± 2 °C, 70 ± 5% RH, and a 14:10 h L:D photoperiod (Li et al. 2010; Wang et al. 2011).

AROMATIC PLANTS

Seeds of French marigold, ageratum, and catnip were sown in a greenhouse in early Mar of 2014 and 2015, and seedlings (about 10 cm high) were transplanted individually to plastic pots (10 cm in diameter, 12 cm high). Each pot, containing 2 or 3 plants, was maintained in a climate chamber under controlled conditions: 25 ± 3 °C, 65 ± 5% RH, and a 16:8 h L:D photoperiod. The height of the aromatic plants used in the experiments was approximately 30 to 35 cm.

APPLE TREES

One-yr-old apple trees, *Malus domestica* Borkh. 'Fuji' (Rosaceae), were planted individually in plastic pots (40 cm in diameter, 50 cm high). The plants were kept in a greenhouse under controlled conditions (25 ± 3 °C, 65 ± 5% RH) and were watered weekly to full soil capacity. After 1 yr, the 2-yr-old trees were used for the experiments. The height and canopy width of the trees were approximately 110 and 35 cm, respectively.

H-TUBE OLFACTOMETER

The H-tube olfactometer used in this study, redesigned according to Liu (2001) as shown in Supplementary Fig. S1 (available online in Florida Entomologist 100[2] [Jun 2017] at http://purl.fcla.edu/fcla/entomologist/browse), consisted of 2 plastic boxes (80 cm L, 80 cm W, 150 cm H) with a 8.2 × 8.2 cm hole on one side 80 cm above the bottom. These were connected by a cross arm (20 cm L, 8 cm W, 8 cm H) with a 5 × 5 cm hole in the middle of the upper surface.

EXPERIMENTAL DESIGN IN THE ORCHARD

The experiment was performed in a 3.5 ha organic apple (*M. domestica* 'Fuji') orchard in the Changping District, north of Beijing, China. Beginning Mar 2012 and continuing to 2015, seeds from 2 of the 3 test plants (ageratum, French marigold, and catnip), were sown at a ratio of 1:1 in the area between 2 rows of apple trees and covered with plastic film to keep them warm. The experiment involved a randomized block design with 3 replicates, and 12 plots in total were created. The composition of each plot was as follows: catnip + French marigold, ageratum + French marigold, catnip + ageratum, and native vegetation (control). Each plot size was 18 × 40 m, and plots were spaced 18 m apart, separated by 3 clean tillage lines (only apple trees were present). Each plot contained 4 rows of fruit trees, and each row had 8 fruit trees. The distance between rows and distance between trees were 5 and 6 m, respectively.

The coverage rate of the intercrops in all the intercropped plots was >80% (the distance from intercrop margins to fruit trees was 0.6 m). The native vegetation had been removed entirely by rototilling before the intercropped pants were seeded, and emerging weeds were completely removed by weeding. In the control treatment plots, the native vegetation consisted mainly of 7 species: slender amaranth, *Amaranthus viridis* L. (Amaranthaceae); viola, *Viola philippica* Cav. var. *philippica* (Violaceae); plantain, *Plantago asiatica* L. (Plantaginaceae); dandelion, *Taraxacum officinale* (L.) [Weber] ex Wigg. (Asteraceae); green bristlegrass, *Setaria viridis* (L.) P. Beauv. (Poaceae); petunia, *Petunia hybrida* (Hook f.) Vilm. (Solanaceae); and shepherd's purse, *Capsella bursa-pastoris* (L.) Medik. (Brassicaceae). These were also kept at an 80% coverage rate. Plots were mowed 2 to 3 times per year and the clippings left in place to naturally degrade.

The numbers of *A. citricola* nymphs and adults and *H. axyridis* larvae and adults were recorded annually from May to Aug from 2012 to 2015. In each year, insects in apple trees and aromatic plants were collected from a series of sampling points. The specific methods used were as follows: Apple tree canopy surveys were performed by collecting samples on 1 d in each month from May to Aug each year. Within each plot, 4 apple trees were selected at random as sampling points, and each tree was sampled from 4 directions (east, south, west, and north). On each side, three 30 cm twigs from a high, middle, and low point of the tree were chosen to check for the presence of *A. citricola* and *H. axyridis*. The sampled twigs were covered with nylon sweep nets (30 cm in diameter, 50 cm deep) to prevent the escape of *H. axyridis* adults. Also, sweeping was used to sample *H. axyridis* present in the ground cover. The sweep net also was used to collect arthropods on intercrop vegetation in each plot. In the center of each plot, a 10-m-long area was selected as a sampling point, and each plot received 5 sweeps. A sweep was defined as a rapid 2-m-long movement of the net through the vegetation.

ANALYSIS OF AROMATIC PLANT VOLATILES

French marigold and catnip volatile compounds were collected in oven bags (355 × 508 mm; Reynolds, USA) using a dynamic headspace sampling method (Raguso & Pellmyr 1998; Oruna-Concha et al. 2002; Cozzolino et al. 2015). Both plants and plastic pots were placed in the oven bag when volatile compounds were collected. The volatiles were collected for 20 min and pulled out of the bag at a rate of 300 mL/min over a stainless steel column filled with 20 mg Tenax® Porous Polymer Adsorbent matrix Tenax GR (60–80 mesh; Chrompack, Varian, Palo Alto, California). A pot with soil but no plant was placed in the oven bag to collect ambient air at the same time and was used as a control. An automated thermal desorption–gas chromatograph/mass spectrometer (ATD-GC/MS) was used to analyze the volatiles. The volatiles were desorbed from the steel column by heating in an automated thermal desorber (TurboMatrix 650 ATD, PerkinElmer, Inc, Shelton, Connecticut) at 260 °C for 10 min and then cryo-focused for 3 min in a cold trap with a temperature maintained at −25 °C. The cold trap was then rapidly heated to 300 °C and maintained at that temperature for 5 min, allowing transfer of the volatiles to the GC (Clarus 600 GC, PerkinElmer, Inc, Shelton, Connecticut). The GC was equipped with a capillary DB-5MS column (30 m × 0.25 mm LD, with a 0.25 μm film thickness), and helium was used as the carrier gas. The GC oven was kept at 40 °C for 2 min, and then the temperature was increased at 4 °C/min to 160 °C, followed by an increase of 20 °C/min to 270 °C, where it was held for 3 min. The MS (Clarus 60 T, PerkinElmer, Inc, Shelton, Connecticut) was operated in the EI ionization mode at 70 eV and with a mass scan range of 29 to 600 amu. The temperatures of the interface and ion source were 250 and 220 °C, respectively. Compounds were identified by searching the NIST08 and Wiley online library in the TurboMass Ver5.4.2 software package (PerkinElmer, Inc, Shelton, Connecticut) and comparing their retention indices. The amounts of the compounds were calculated using the peak area normalization method (Raguso & Pellmyr 1998).

RESPONSES OF *A. CITRICOLA* AND *H. AXYRIDIS* TO CATNIP AND FRENCH MARIGOLD VOLATILES

The behavioral responses of *A. citricola* and *H. axyridis* were investigated using an H-tube olfactometer. Each treatment was performed as follows and replicated 3 times:

(a) Responses of *A. citricola* to French marigold and catnip: Two small apples trees grown in pots (40 cm diameter) and measuring about 110 cm in height were individually placed in the 2 plastic boxes of the H-tube olfactometer. Four pots of French marigold were simultaneously placed on one side, while the other side contained only the apple tree. The plants were left in this configuration for 1 d before testing to allow them to adapt to the new environmental conditions. The 2 boxes were not covered on the top and were not joined by the arm of the H-tube olfactometer before the formal test. Sixty *A. citricola* winged aphids (starved for 3 h) were introduced into the hole of the cross arm of the olfactometer and left for 2 h. Then, the number of aphids that chose the each box was recorded after 30, 60, 90, and 120 min. The aphids were considered to have made a choice when they walked at least 5 cm from the release point and remained there for at least 1 min. We also tested the response of *A. citricola* to catnip. The

experimental conditions were the same as above, but 4 pots of catnip were introduced instead of French marigold.

(b) Responses of *H. axyridis* to French marigold and catnip with no aphid: The experimental conditions were the same as in (a), but 30 *H. axyridis* adults (starved for 12 h) were introduced instead of *A. citricola*, and the number of individuals that made a choice was recorded at 5, 10, 30, 60, 90, and 120 min.

(c) Responses of *H. axyridis* to French marigold and catnip with aphids: The experimental conditions were the same as in (a), but 60 aphids (wingless spirea aphids, starved for 3 h) were applied to an apple tree on both sides at 2 h before testing. Subsequently, 30 *H. axyridis* adults, starved for 12 h, were introduced into the cross arm, and the number of *H. axyridis* adults that made a choice was recorded after 5, 10, 30, 60, 90, and 120 min.

(d) Responses of *H. axyridis* to French marigold and catnip with aphids that were removed after 2 h: The experimental conditions were the same as in (c), but all aphids were removed when the experiment started. Thirty *H. axyridis* adults (starved for 12 h) were introduced into the cross arm for testing, and the number of adults that made a choice was recorded at 5, 10, 30, 60, 90, and 120 min.

(e) Responses of *H. axyridis* to D-limonene and terpinolene with no aphids: Analytical reagent-grade pure D-limonene and terpinolene were used to test the behavioral responses of *H. axyridis*. The D-limonene and terpinolene samples were separately diluted to 12.5 µL/L, 25 µL/L, and 50 µL/L solutions, and a 1:1 D-limonene: terpinolene combination was also tested. The experimental conditions were the same as in (b), but cotton balls were dipped in 2 mL of each of the above solutions and used instead of aromatic plants on one side, and a cotton ball dipped in an equal volume of distilled water was used on the other side as a control. So one side was apple tree + plant volatiles, and the other side was apple tree + distilled water. The number of beetles that made a choice was recorded at 5, 10, 20, 30, and 60 min.

(f) Responses of *H. axyridis* to D-limonene and terpinolene with aphids: The experimental conditions were the same as in (e), but 60 aphids (wingless spirea aphids, starved for 3 h) were applied to an apple tree on both sides at 2 h before testing. Subsequently, 30 *H. axyridis* adults (starved for 12 h) were introduced into the cross arm, and the number of beetles that made a choice was recorded at 5, 10, 20, 30, and 60 min.

STATISTICAL ANALYSES

All statistical analyses were performed using the Origin® Pro 8.0 (OriginLab® Corporation, Northampton, Massachusetts) and SPSS 17.0 (IBM Corporation, Armonk, New York) software packages. To test the temporal dynamics of spirea aphids and multicolored Asian lady beetles in response to the different treatments in the orchard, the total individual number of *A. citricola* aphids and *H. axyridis* beetles were calculated for each year from 2012 to 2015, and the differences in the total numbers of *A. citricola* and *H. axyridis* individuals in the different treatment plots during the sampling years were tested by a 2-way analysis of variance with a Duncan multiple range test. We applied a linear regression model analysis to the relationship of the abundance of spirea aphids and multicolored Asian lady beetles, the ratio of multicolored Asian lady beetle abundance to spirea aphid abundance, and the sample years. Differences in the preferences of *A. citricola* and *H. axyridis* for pairs of plants and the *H. axyridis* responses to the relative amount of volatiles from aromatic plants were analyzed for significance using a paired *t*-test.

Results

NUMBERS OF *A. CITRICOLA* AND *H. AXYRIDIS* INDIVIDUALS

The numbers of *A. citricola* nymphs and adults present on the apple trees showed a trend of increased abundance from 2012 to 2015 in all treatment plots (Fig. 1A). However, the numbers of *A. citricola* nymphs and adults in catnip + French marigold, ageratum + French marigold, and catnip + ageratum treatments were significantly lower than in the native vegetation treatment in all years ($F_{3,9}$ = 12.84; $P < 0.001$), particularly in the catnip + French marigold treatment plot in 2013 (Fig. 1A; $F_{3,6}$ = 71.45; $P < 0.001$). The numbers of *H. axyridis* larvae and adults showed a trend of increasing abundance from 2012 to 2015 in the catnip + French marigold and the ageratum + French marigold plots, but the opposite trend from 2012 to 2015 in the catnip + ageratum and native vegetation treatments (Fig. 1B). The numbers of *H. axyridis* larvae and adults in
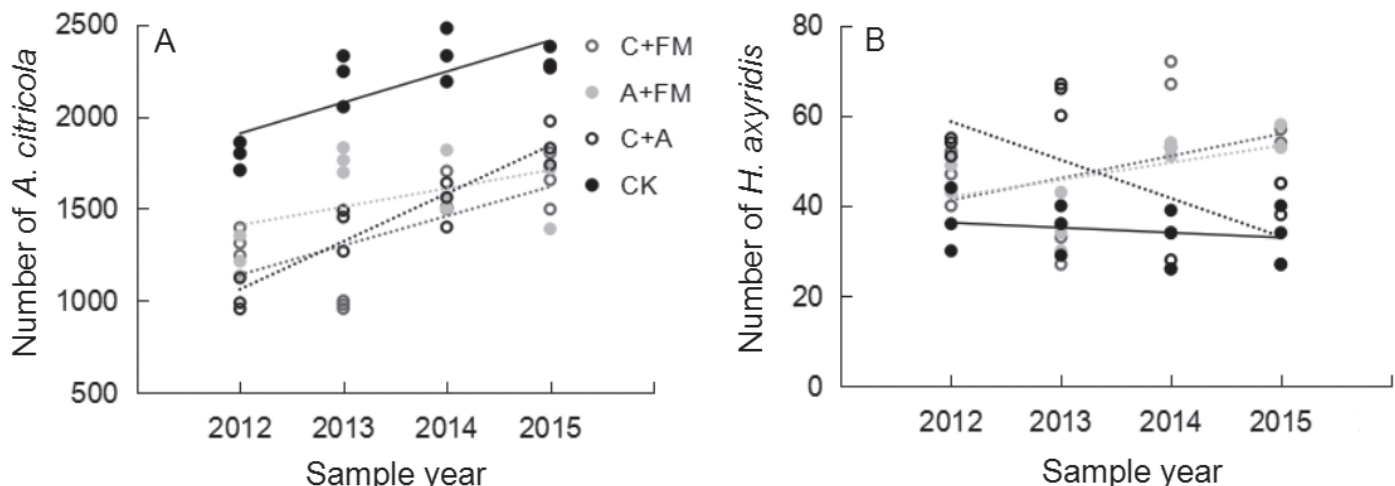

Fig. 1. Total numbers of *Aphis citricola* (A) and *Harmonia axyridis* (B) individuals from 2012 to 2015 in relation to ground cover vegetation. C + FM: catnip (*Nepeta cataria*) + French marigold (*Tagetes patula*), A + FM: ageratum (*Ageratum houstonianum*) + French marigold, C + A: catnip + ageratum; CK: native vegetation.

the catnip + French marigold and ageratum + French marigold plots were significantly higher than in the native vegetation plots in 2014 and 2015 (Fig. 1B; 2014: $F_{3,6}$ = 52.22; P < 0.0001; 2015: $F_{3,6}$ = 12.52; P = 0.005). In addition, the number in the catnip + ageratum plots was significantly higher than in the native vegetation plots in 2012 and 2013, whereas there were no differences between the two in 2014 and 2015 (Fig. 1B).

RELATIONSHIP BETWEEN NUMBERS OF *A. CITRICOLA* AND *H. AXYRIDIS* INDIVIDUALS

A linear regression model analysis showed that the numbers of *H. axyridis* larvae and adults were significantly and positively correlated with the numbers of *A. citricola* nymphs and adults in all sample years (Fig. 2, A: $F_{1,46}$ = 73.35; P < 0.0001; B: $F_{1,46}$ = 24.20; P < 0.0001; C: $F_{1,46}$ = 32.75; P < 0.0001; D: $F_{1,46}$ = 37.09; P < 0.0001), especially in the catnip + French marigold treatment plots (Fig. 2A). The ratio of *H. axyridis* to *A. citricola* abundance showed a negative correlation with the sample years, and the trend of a decreasing ratio was relatively less in the treatments containing French marigold (Fig. 3, A: $F_{1,46}$ = 2.58; P = 0.115; B: $F_{1,46}$ = 1.57; P = 0.694; C: $F_{1,46}$ = 15.69; P < 0.0001; D: $F_{1,46}$ = 4.81, P = 0.033).

RESPONSE OF *A. CITRICOLA* AND *H. AXYRIDIS* TO FRENCH MARIGOLD AND CATNIP

Fewer *A. citricola* aphids chose the apple tree + French marigold, or French marigold + catnip treatments, than the apple tree alone treatment (Fig. 4). The numbers of aphids that chose the apple tree alone treatment in the 120 min tests were 9-fold and 3.25-fold higher than those selecting the apple tree + French marigold or catnip treatments, respectively. We observed no differences in the numbers of *H. axyridis* beetles that chose the French marigold + apple tree treatment and the apple tree alone treatment from 5 min to 60 min, regardless of whether or not aphids had been present anywhere (Fig. S2). However, more beetles chose the French marigold + apple tree treatment than the apple tree alone treatment at 120 min when aphids were not present (Fig. 5A), and the choice was more significant when aphids were present (Fig. S2B). Fewer beetles choose the catnip + apple tree treatment than chose the apple tree alone treatment, whether aphids were present or not (Fig. 5B), but no significant differences were observed for the other treatments (Fig. S2).

FRENCH MARIGOLD AND CATNIP VOLATILES

Twenty-one and 24 compounds were separately identified from the French marigold and catnip volatiles, respectively, consisting
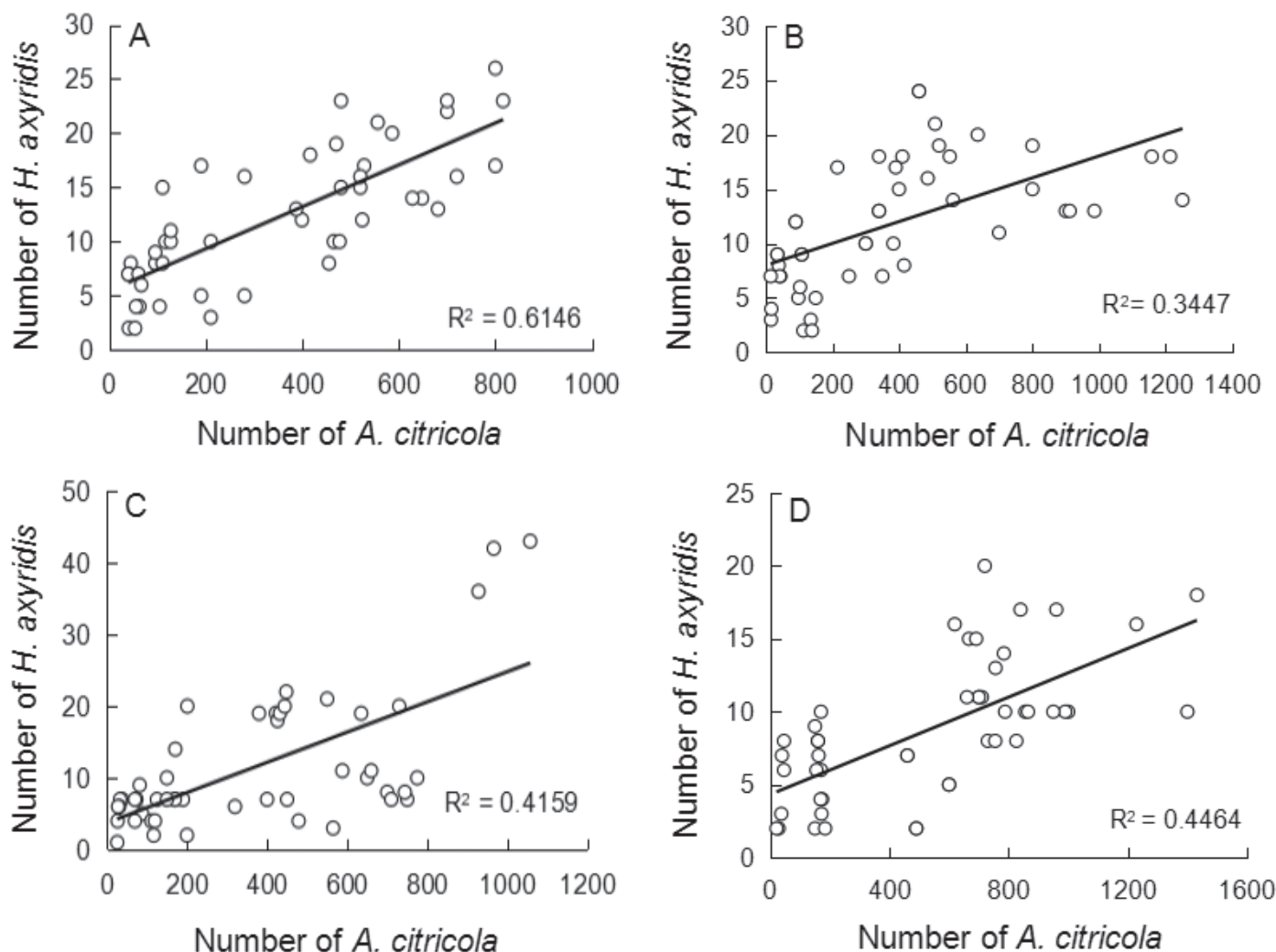

Fig. 2. Linear regression models showing the relationship between *Aphis citricola* and *Harmonia axyridis* abundance. A: Catnip (*Nepeta cataria*) + French marigold (*Tagetes patula*), B: ageratum (*Ageratum houstonianum*) + French marigold, C: catnip + ageratum, and D: native vegetation.
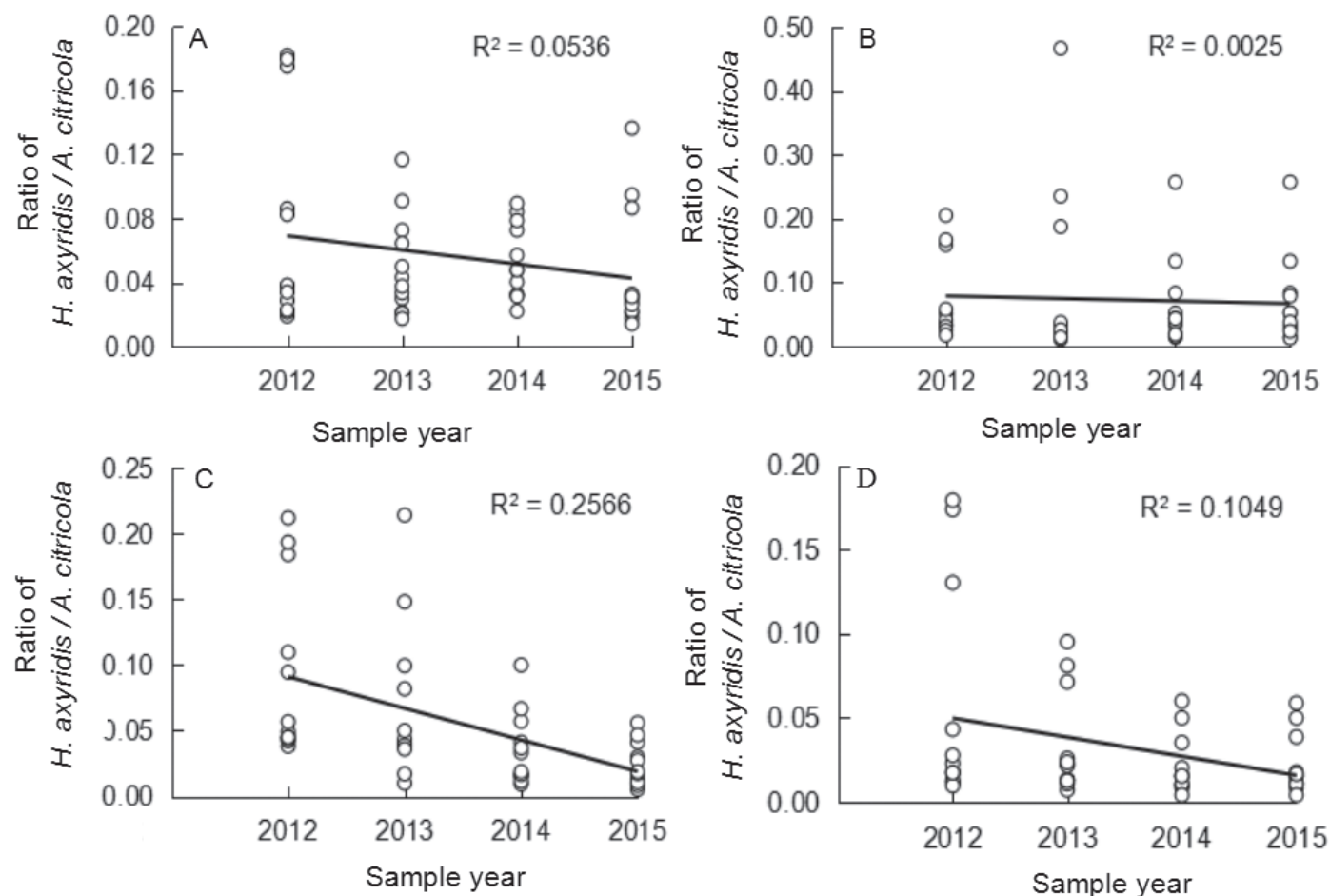
Fig. 3. Linear regression models showing the relationship in the ratio of *Harmonia axyridis* abundance to *Aphis citricola* abundance with sampling years. A: Catnip (*Nepeta cataria*) + French marigold (*Tagetes patula*), B: ageratum (*Ageratum houstonianum*) + French marigold, C: catnip + ageratum, and D: native vegetation.

mainly of alcohols, terpenoid, ketone, and ester compounds (Tables S1 and S2). The principal volatile compounds from French marigold were 2-ethyl-1-hexanol (25.90%), D-limonene (16.73%), terpinolene (14.82%), 1-methy-l-4-(1-methylethenyl)-benzene (7.76%), hexanal (4.76%), p-isopropyltoluene (3.59%), ocimene (3.40%), and a-pinene (2.18%) (Table S1; Fig. 6B). The main volatile compounds from catnip were 2-ethyl-1-hexanol (23.29%), o-xylene (9.05%), ethylbenzene (8.04%), p-xylene (7.6%), octamethyl cyclotetrasiloxane (6.41%), terpinolene (7.75%), and D-limonene (3.85%) (Table S2; Fig. 6C). The compound 2-ethyl-1-hexanol was also identified

from the control (air, Table S3), so there were only 2 major volatile compounds, terpinolene and D-limonene, that were identified from both French marigold and catnip and which had relatively high abundance. The ratio of D-limonene to terpinolene was 1.23:1 in French marigold plants, and the ratio of D-limonene to terpinolene was 0.50:1 in catnip plants. In addition, *A. citricola* showed the same response to French marigold and catnip, but the *H. axyridis* responses differed between the 2 plant species. We postulated that the different responses of *H. axyridis* to French marigold and catnip may be due to the different content of D-limonene and terpinolene or their ratio. Thus, D-limonene and terpinolene were used to test the behavioral responses of *H. axyridis*.
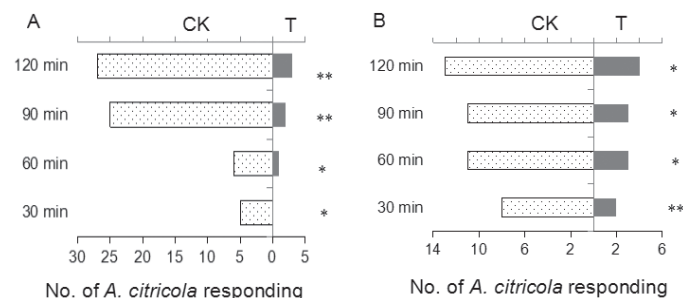
RESPONSE OF *H. AXYRIDIS* TO D-LIMONENE AND TERPINOLENE

The number of *H. axyridis* beetles that chose the 12.5 µL/L D-limonene + apple tree treatment was significantly greater than the number of beetles that chose the apple tree + distilled water treatment at 60 min, regardless of whether or not aphids had been present anywhere (Fig. S3A, D). However, fewer beetles chose the 50 µL/L D-limonene + apple tree treatment than the apple tree + distilled water treatment from 5 to 60 min (Fig. S3C, F). The numbers of beetles that chose the 12.5 µL/L D-limonene + apple tree treatment were significantly greater than the numbers of beetles that chose the 50 µL/L D-limonene + apple tree treatment at 60 min, whether or not aphids were present (Fig. 7A, B).

Fig. 4. Response of *Aphis citricola* adults to French marigold (*Tagetes patula*) (A) and catnip (*Nepeta cataria*) (B). T: Apple trees + aromatic plants; CK: apple trees only. The numbers of asterisks represent the level of significance: ** highly significant (*P* < 0.01); * significant difference (*P* < 0.05).
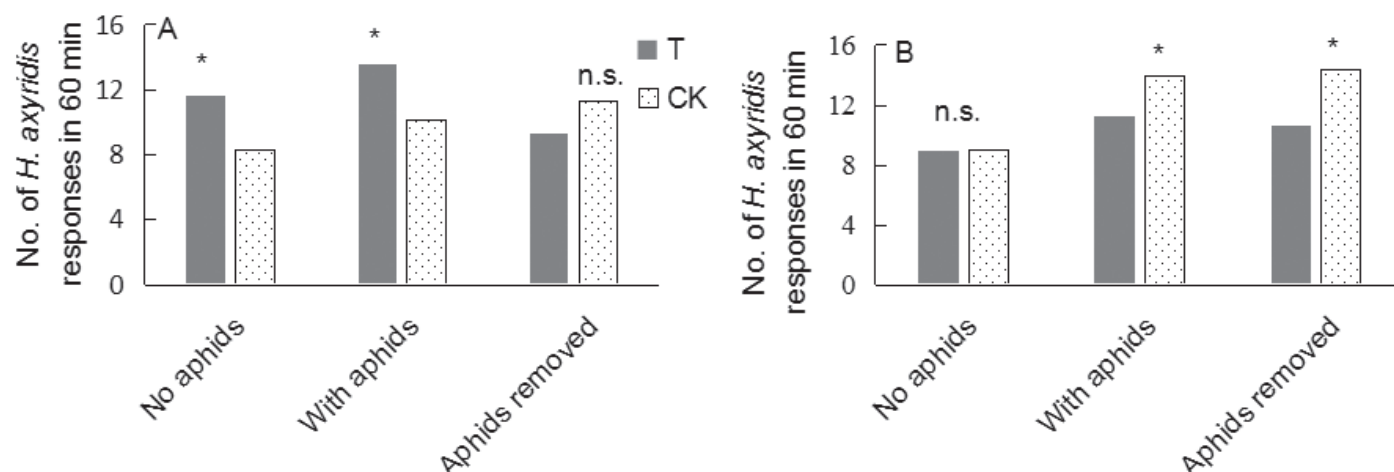
Fig. 5. Differences in the number of *Harmonia axyridis* adults responding to French marigold (*Tagetes patula*) (A) and catbip (*Nepeta cataria*) (B) after 60 min. T: Apple trees + aromatic plants; CK: apple trees. Aphids removed: aphids introduced for 2 h and then removed. The numbers of asterisks represent the level of significance: * significant ($P < 0.05$); n.s. no significant difference.

More beetles chose the 50 µL/L terpinolene + apple tree treatment than the apple tree + distilled water treatment from 30 to 60 min when aphids were present (Fig. S4F). Also, more beetles chose the 50 µL/L terpinolene + apple tree treatment than the 12.5 µL/L terpinolene + apple tree treatment at 60 min, whether or not aphids were present (Fig. 7C, D).

The numbers of beetles that chose the 25 µL/L 1:1 D-limonene to terpinolene + apple tree treatment were significantly greater than the numbers of beetles that chose the apple tree + distilled water treatment at 60 min, especially when aphids were present (Fig. S5B, E). The same results were observed in the 50 µl/L 1:1 D-limonene to terpinolene + apple tree treatment when aphids were introduced and then removed after infesting the apple tree (Fig. S5F). More beetles chose the 25 µl/L 1:1 D-limonene to terpinolene + apple tree treatment than the 12.5 µL/L and 50 µL/L treatments at 60 min, whether or not aphids were present (Fig. 8).

Discussion

Plant volatiles can act as important chemical signals that form communication links between insects and their host plants (Raguso & Pellmyr 1998; Tasin et al. 2010; McCallum et al. 2011; Sun et al. 2014; Thöming et al. 2014) and can function either as attractants or repellants. The interactions between insects and plants are usually affected by the environment, plant type, insect species, and others factors and are often disturbed when host plants and non-host plants are co-located (Bernasconi et al. 1998; Nottingham et al. 1991). In this study, aromatic non-host plants were introduced to an apple orchard environment and shown to have significant effects on populations of *A. citricola*. The volatiles from catnip and French marigold influenced the host location activities of *A. citricola*, and the olfactometer bioassays and field experiments demonstrated that they have a direct repellent effect on *A. citricola* (Figs. 1 and 4), thereby playing an indirect role in protecting the apple trees. These data provide supporting evidence for the chemical repellent hypothesis, as has been reported in other plant–herbivore systems (Ukeh et al. 2010; Kappers et al. 2011; Signoretti et al. 2012).

We expected to see a significant increase in the number of *H. axyridis* beetles in treatments that included aromatic plants. Indeed, this was the case in the treatment plots of catnip + French marigold and ag-

eratum + French marigold in 2014 and 2015, and significant increases were observed in the treatment plots of catnip + ageratum in 2012 and 2013 (Fig. 1). We believed that *H. axyridis* was attracted by the volatiles from the aromatic plants, and we noted a consistent increase in the number of the beetles in the plots containing French marigold. To verify this hypothesis, we chose French marigold and catnip to test the behavioral response of *H. axyridis*. The results showed that the beetles were indeed attracted by French marigold, regardless of whether aphids were present for up to 120 min (Fig. S2A, B), and were repelled by catnip after 120 min, again whether or not aphids were present or had been removed after 2 h (Fig. S2E, F). The number of beetles that chose French marigold increased with increasing duration of exposure, but there were no significant differences between the treatment and control until the 120 min time point (Fig. S2).

We propose two explanations: one is that the volatiles from French marigold may not have reached a sufficient concentration to act as attractants until that time point; the other is that the beetles may have required a learning, identification, and adaptation process to the French marigold volatiles that did not result in attraction until that time point. Insects are known to be able to identify volatile blends released by different plants through olfactory learning (Guillette et al. 2009; Shikano & Isman 2009), which may inform them about the identity and quality of the host plant. Our results also indicated that French marigold provided an indirect defense for the apple trees. In contrast, catnip had no effect on *H. axyridis*, because more beetles chose the control treatment than the catnip treatment after 120 min when aphids were present or had been present for 2 h (Fig. S2). We propose that aphid-induced volatiles released from the apple tree may be an important factor in *H. axyridis* attraction.

We next wanted to determine what plants and volatile compounds attracted *H. axyridis*. French marigold was selected for analysis and identification of volatile components, and because D-limonene and terpinolene were the most abundant, these were chosen to test the behavioral responses of *H. axyridis*. Subsequent experiments showed that the beetles were significantly attracted by low concentrations of D-limonene (Fig. S3A, D) but repelled by higher concentrations (Fig. S3C, F). We also observed that the beetles were significantly attracted by 50 µL/L terpinolene (Fig. S4, S5), but only in the presence of the aphids, suggesting that aphid-induced volatiles released from the apple trees may be important for *H. axyridis* attraction, but that terpinolene alone is not an attractant. In addition, 25 µL/L of a 1:1
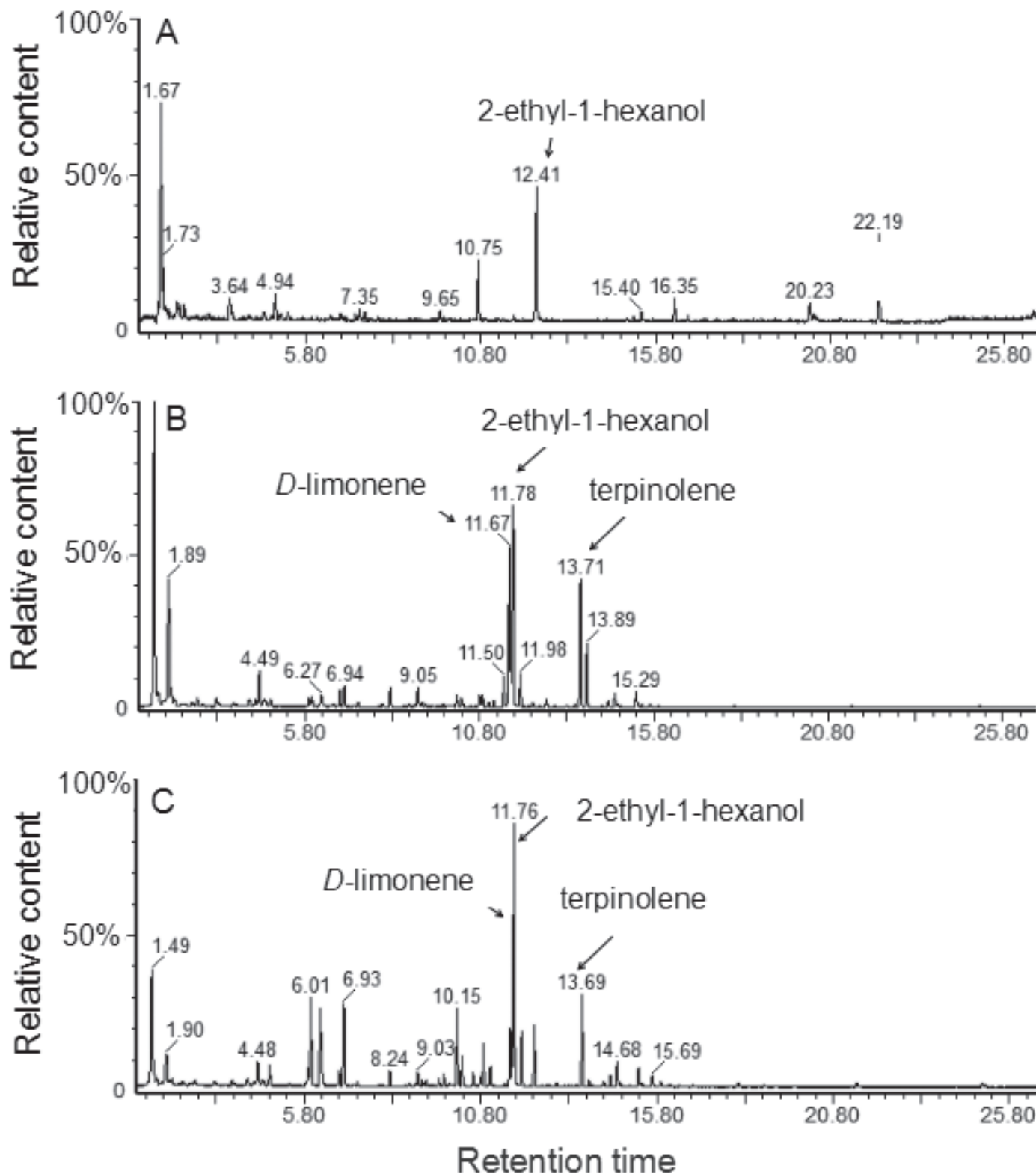
Fig. 6. Typical chromatograms obtained from headspace collections of volatiles from French marigold (*Tagetes patula*) (B) and catnip (*Nepeta cataria*) (C). A, air control.

mixture of D-limonene and terpinolene resulted in a significant attraction of the beetles (Fig. S5B, E). We suggest that there are two explanations for these results. One explanation is that D-limonene was diluted to a level that resulted in attraction, because it was mixed with terpinolene, which is consistent with our results reported above that

low concentrations of D-limonene attracted the beetles. The second explanation is that the aphid-induced volatiles released by the apple tree played a dominant role in *H. axyridis* attraction because a higher concentration of 1:1 mixed D-limonene and terpinolene also attracted the beetles.
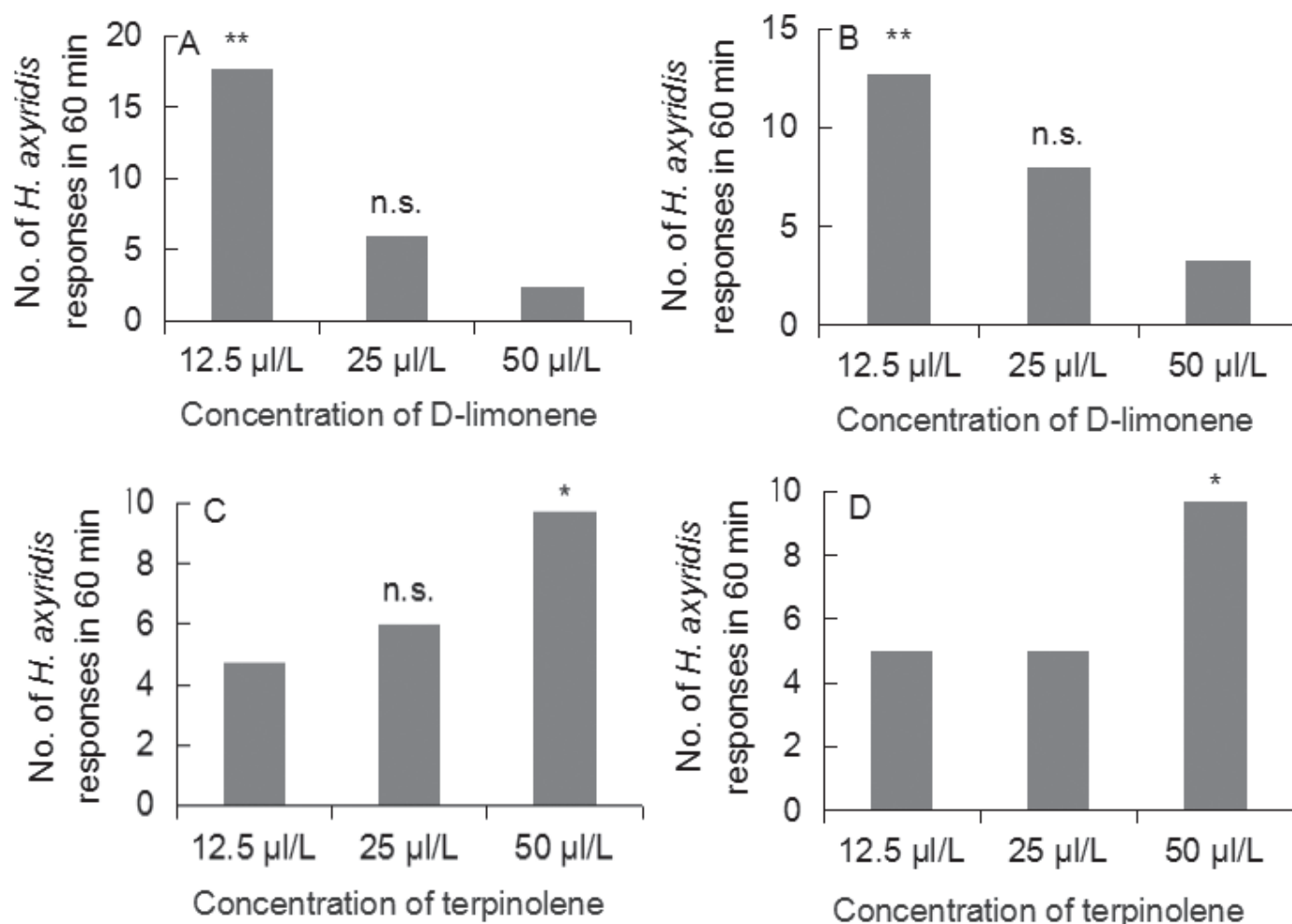
Fig. 7. Differences in the number *Harmonia axyridis* adults in response to 12.5 µL/L, 25 µL/L, and 50 µL/L D-limonene (A, B) and terpinolene (C, D) after 60 min. A, C: No aphids; B, D: aphids present. The numbers of asterisks represent the level of significance: ** highly significant (*P* < 0.01); * significant (*P* < 0.05); n.s. no significant difference.

These data provide evidence for the important role of plant volatiles in the behavior of the natural enemies of herbivorous insects. They also confirm previous reports that volatile plant compounds released in response to insect feeding serve as attractants and affect the behavior of the natural enemies of the herbivorous insects. Numerous studies have demonstrated the dynamic role of herbivore damaged plants in the attraction of natural enemies (Schaller & Nentwig 2000; Zhu & Park 2005; Tan & Liu 2014).
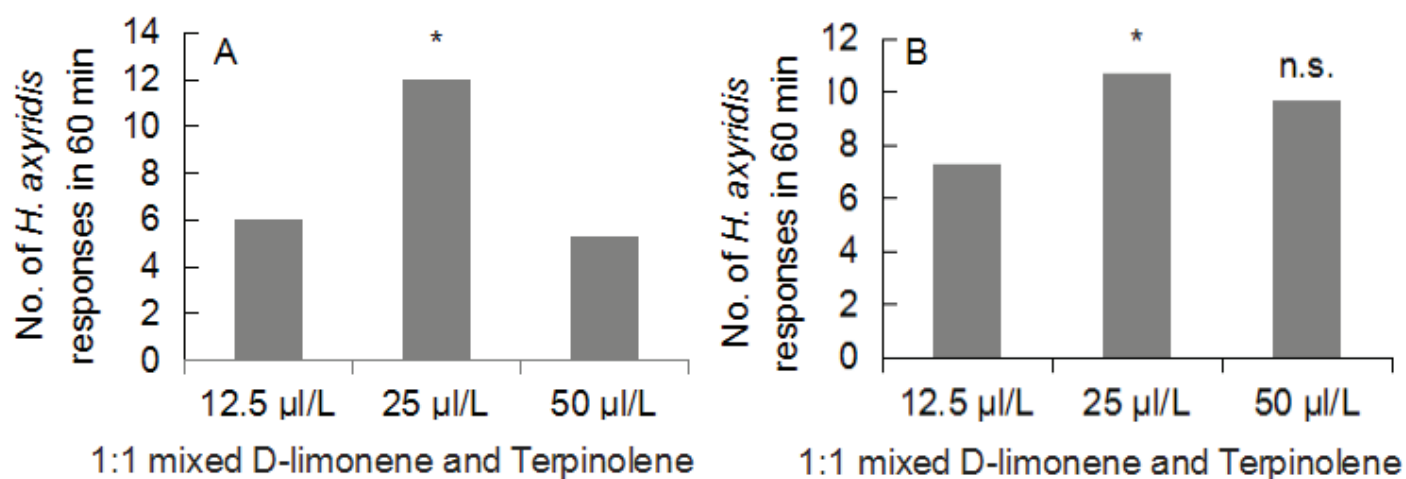

Fig. 8. Response of *Harmonia axyridis* adults to 12.5 µL/L, 25 µL/L, and 50 µL/L 1:1 mixed D-limonene and terpinolene after 60 min. A: No aphids; B: aphids present. The numbers of asterisks represent the level of significance: ** highly significant (*P* < 0.01); * significant (*P* < 0.05); n.s. no significant difference.
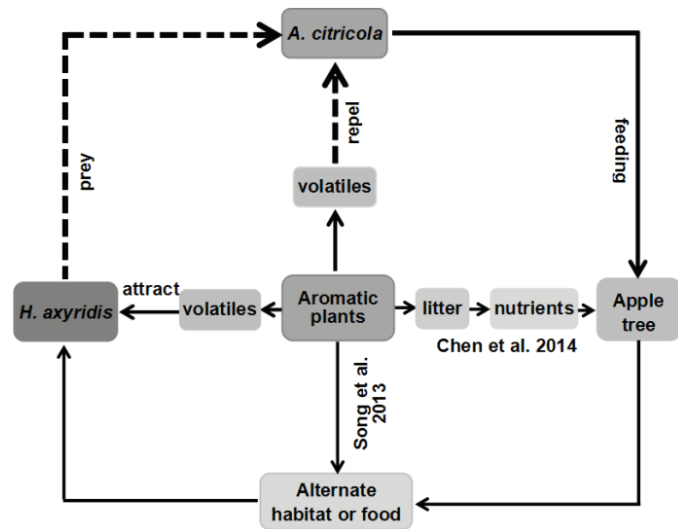
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Fig. 9. An *Aphis citricola* infestation model showing the effects of aromatic plant volatiles. Solid arrows refer to positive effects. Dotted lines refer to negative effect. The thickness of the arrows indicates the magnitude of the effects. The model includes data from this study and the studies by Song et al. (2013) and Chen et al (2014).

The interaction between *A. citricola* and *H. axyridis* and the ratio of their relative abundance were also influenced by the chemical repellency and attraction effects of the aromatic plants. Because the catnip + French marigold treatment plots were most effective in repelling *A. citricola* and attracting *H. axyridis*, the trend of a decreasing the ratio of *H. axyridis* to *A. citricola* abundance was relatively less in the treatments (Fig. 3B). The strongest positive association between *A. citricola* and *H. axyridis* was shown in the catnip + French marigold treatment (Fig. 2). The changes in these associations may affect biological control of *A. citricola* in the presence or absence of aromatic plants, because the effect of the aromatic plants on *A. citricola* was strong in plots containing aromatic plants, but the role of the natural enemies of *A. citricola* may be predominant in naturally vegetated plots.

Taken together, our results indicate that intercropping with aromatic plants in apple orchards can increase the resistance of apple plants to *A. citricola* directly or indirectly, either by significantly reducing the populations of *A. citricola* by chemical repulsion or by significantly increasing the population of *H. axyridis* by chemical attraction (Fig. 9). Of the volatiles involved, D-limonene and terpinolene play an important role in influencing the behavioral responses of *H. axyridis* adults. Future research will investigate whether these chemicals can be effectively applied for use in agricultural biocontrol programs. The development of an effective *A. citricola* repellent would greatly improve the ability to manage this herbivore.

Acknowledgments

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