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Effect of *Solenopsis invicta* (Hymenoptera: Formicidae) on flower-visiting behavior of insects on *Brassica napus* (Brassicales: Brassicaceae)

Duan Wu^{1,2}, Ling Zeng¹, Yongyue Lu¹, and Yijuan Xu^{1,*}

Abstract

This study used field trials to investigate how the fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae) affects the flower-visiting behavior of insects on rapeseed *Brassica napus* L. (Brassicales: Brassicaceae). Chemicals from fire ants were identified, and their electrophysiological effects on the cabbage white butterfly *Pieris rapae* (L.) (Lepidoptera: Pieridae) were analyzed by gas chromatography–electroantennogram detection (GC-EAD) bioassays. The results showed that *S. invicta* significantly reduced the abundance of pollinators. The number of striped flea beetles (*Phyllotreta striolata* [F.]; Coleoptera: Chrysomelidae) and hover flies (*Eristalinus quinquestriatus* [F.] and *E. quinquelineatus* [F.]; Diptera: Syrphidae) in fire ant–included and fire-ant-and-aphid-included plots was 43.5 and 39.4% lower, respectively, than in control plots. The visiting duration at a single flower for *P. rapae* in the plots that had fire ants and aphids excluded was significantly longer than in the fire ant–included (1.42 fold) and fire-ant-and-aphid-included plots (1.29 fold). However, the visiting frequency of *P. rapae* and of the Chinese honey bee *Apis cerana* F. (Hymenoptera: Apidae) and the duration of each visit of *A. cerana* were not significantly changed. Antennae of *P. rapae* showed obvious electrophysiological reactions to the volatiles of *S. invicta*, and n-heptacosane elicited the largest EAD response. Our results suggest a negative effect and its chemical mechanism of *S. invicta* on the behavior of several flower-visiting insects.

Key Words: biological invasion; species abundance; GC-EAD

Resumen

Este estudio utilizó pruebas de campo para investigar cómo la hormiga de fuego *Solenopsis invicta* Buren (Hymenoptera: Formicidae) afecta el comportamiento de insectos que visitan flores de colza, *Brassica napus* L. (Brassicales: Brassicaceae). Se identificaron los productos químicos de las hormigas de fuego, y sus efectos electrofisiológicos sobre la mariposa blanca de repollo *Pieris rapae* (L.) (Lepidoptera: Pieridae) que fueron analizados por medio de bioensayos con cromatografía de gases-detección electroantennograma (CG-DEA). Los resultados mostraron que *S. invicta* redujo significativamente la abundancia de polinizadores. El número de escarabajos pulga rayado (*Phyllotreta striolata* [F.]; Coleoptera: Chrysomelidae) y moscas cernícolas (*Eristalinus quinquestriatus* [F.] y *E. quinquelineatus* [F.]; Diptera: Syrphidae) en parcelas con la hormiga de fuego incluida y las de hormigas y áfidos incluidos fue del 43,5 y el 39,4% menor, respectivamente, que en las parcelas de control. La duración de cada visita a una sola flor para *P. rapae* en las parcelas que tenían los áfidos y hormigas de fuego excluidos fue significativamente más largo que a las flores con la hormiga de fuego incluida (1,42 veces) y en parcelas que tenían los áfidos y hormigas de fuego (1,29 veces). Sin embargo, la frecuencia de visita por *P. rapae* y de la abeja de la miel china *Apis cerana* F. (Hymenoptera: Apidae) y la duración de cada visita de *A. cerana* no cambiaron significativamente. Las antenas de *P. rapae* mostró reacciones electrofisiológicas obvias a los volátiles de *S. invicta*, y el n-heptacosano provocó la mayor respuesta DEA. Nuestros resultados sugieren un efecto negativo y su mecanismo químico de *S. invicta* sobre el comportamiento de varios insectos que visitan las flores.

Palabras Clave: invasión biológica; abundancia de las especies; CG-DEA

Invasive ants occur commonly from temperate to tropical ecosystems in both artificial (such as agricultural) and natural systems (Buckley 1987; Way & Khoo 1992; Bluthgen et al. 2000). Due to high colony density, extremely aggressive and omnivorous fire ants can have a significant impact on native arthropod communities, highlighting why the fire ant is known as one of the most ecologically destructive species (Moller 1996; Holway et al. 2002). Meanwhile, honeydew-producing hemipterans have the potential to generate strong indirect effects on arthropod communities by manipulating the behavior and abundance of ants foraging on plants (Eubanks 2001; Holway et al. 2002; Kaplan & Eubanks 2002, 2005). Although the red imported fire ant (*Solenopsis invicta* Buren; Hymenoptera: Formicidae) has been well recognized for

its impact on arthropod diversity, the effect of this invasive ant on plant pollinators is not yet understood.

Rapeseed *Brassica napus* L. (Brassicales: Brassicaceae) is a common cross-pollinated plant and an insect-pollinated (entomophilous) crop, and its nectar attracts flower-visiting insect pollinators. Our previous study found that fire ants frequently foraged *B. napus* flowers, possibly producing negative effects on flower-visiting insects, including the Chinese honey bee *Apis cerana* F. (Hymenoptera: Apidae) and the cabbage white butterfly *Pieris rapae* (L.) (Lepidoptera: Pieridae). These flower-visiting insects usually play an important role in the reproduction of plants (Hickman 1974; Gómez et al. 1996; Raju & Ezradanam 2002; de Vega et al. 2009; Luo et al. 2011, 2012). Furthermore, fire ant

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workers foraged more frequently on plants when aphids were present, potentially leading to an increased problem for the pollinators. Chemicals, including hydrocarbons, esters, and fatty acids, mediate the recognition and communication between insects (Howard 1993; Kaib et al. 2004; Dani et al. 2005). We hypothesized that fire ants may cause interference on the flower-visiting behavior of insects via their volatiles, which have been well researched in other studies (Oliveira 1997; Blancafort & Gómez 2005; Ballantyne 2011).

To test this hypothesis, we conducted a series of field experiments to examine how *S. invicta* and its interaction with aphids affected the flower-visiting behavior of insects on *B. napus*. We also identified chemicals from fire ants and analyzed their electrophysiological effect on *P. rapae* by gas chromatography–electroantennogram detection (GC-EAD) bioassays. The results of these experiments may provide insights to evaluate the impact that *S. invicta* has on crops and the mechanisms involved.

Materials and Methods

ORGANISMS

Brassica napus seeds (Nanwu kemao Co., Ltd., Beijing, China) were purchased from a farm market. For field bioassays, cotton aphids *Aphis gossypii* Glover and corn leaf aphids *Rhopalosiphum maidis* (Fitch) (Aphidomorpha: Aphididae) were collected from Chinese hibiscus *Hibiscus rosa-sinensis* L. (Malvales: Malvaceae) and maize *Zea mays* L. (Poales: Poaceae), respectively. Green peach aphids *Myzus persicae* (Sulzer) and mustard aphids *Lipaphis erysimi* (Kaltenbach) (Aphidomorpha: Aphididae) were collected from *B. napus* in the experimental fields of South China Agricultural University (23.235814°N, 113.655616°E). These aphids were reared on *B. napus* in the laboratory, where the temperature was maintained at 27 ± 2 °C with a relative humidity of 60 to 70%, and used in the field experiments 24 h later.

For laboratory bioassays, cabbage white butterfly *P. rapae* and Chinese honey bee *A. cerana* adults were collected from *Bidens bipinnata* L. (Asterales: Asteraceae) on the campus of South China Agricultural University. These butterflies and honeybees were maintained in cages for 1 to 2 h until bioassays could be performed.

EXPERIMENTAL DESIGN

Experiment I: Effects of *S. invicta* on the Flower-Visiting Behavior of Insects on *B. napus*

This investigation was conducted in the experimental fields of South China Agricultural University from Sep to Nov 2013. Fifteen plots were prepared for growing *B. napus*. The area of each plot was approximately 6 m² (2 × 3 m). The plots were separated by 1 m wide ditches that contained water to prevent ant migration.

Our experiments were conducted as follows: (1) 5 plots were supplied with colonies of *S. invicta*; (2) 5 plots were supplied with colonies of *S. invicta* and aphids; (3) 5 plots without fire ant (confirmed by baiting) and aphid infestations were chosen to exclude colonies of *S. invicta* and aphids. When necessary, imidacloprid (Daguangming Agrochemical Co., Ltd., Jiangmeng, China) was used to eliminate aphids on *B. napus*. In each treatment, we cultivated 70 to 100 *B. napus* plants in each plot. Sizable colonies of *S. invicta* were inoculated on the appointed plots when *B. napus* began to bloom. Specifically, 50 g (approx. 50,000 individuals) of colony, which included workers, brood, and 5 queens, was introduced. We then verified whether the transferred colonies had survived by placing sausage baits, and a 2nd colony of *S.*

invicta was provided if the 1st colony had not successfully established. Seven days after fire ant colonization, 600 to 800 aphids for each of 5 plots were transferred onto the plants via small plastic tubes with cotton plugs prior to their introduction to the study site.

Before the field investigation, we determined the flower-visiting insects by net sweeping and microscopic observation. The main insects that visited *B. napus* were as follows: *P. rapae*; *A. cerana*; striped flea beetle *Phyllotreta striolata* (F.) (Coleoptera: Chrysomelidae); flower thrips *Frankliniella intonsa* (Trybom) (Thysanoptera: Thripidae); and the hover flies *Eristalinus quinquestratus* and *E. quinquelineatus* (Diptera: Syrphidae). *Pieris rapae* and *A. cerana* were considered as effective pollinators of *B. napus* flowers by pollen inspection (Free 1993).

From 22 Oct to 8 Nov, we measured the following variables during the day. (1) Ten flowers were collected from each plot to count the number of *P. striolata*, and a 10 min observation of each plot was conducted to determine the number of other insects. (2) Visiting frequency, that is, the number of flowers visited by a pollinating insect, was observed for 1 min. To obtain this measurement, the time when an insect pollinator started to visit the first flower until it left the plot was recorded, and the number of flowers it visited and the duration for each visit were then calculated (Arroyo et al. 1985; Martén-Rodríguez & Fenster 2008). Observations of *P. rapae* and *A. cerana* were replicated 30–40 and 65 times, respectively, for each plot. (3) We measured visiting duration for each flower to establish the length of time a pollinating insect visited each flower (Arroyo et al. 1985; Martén-Rodríguez & Fenster 2008). We recorded the time when an insect pollinator first visited a flower until it left the flower. Observations of *P. rapae* and *A. cerana* were replicated 30–40 and 90 times, respectively, for each plot.

Experiment II: Electroantennogram Reaction of *P. rapae* to the Volatiles of *S. invicta*

Extraction of Volatiles of Fire Ant Workers by Solid Phase Microextraction (SPME). All of the analyses were performed following the SPME procedure described below. A fiber was exposed to the headspace vapor of a 20 mL vial containing 2 g of living fire ant workers for 30 min. SPME experiments were carried out using a 100 µm polydimethylsiloxane fiber (Supelco, Bellefonte, Pennsylvania, USA). The fiber was conditioned in the injection port of the gas chromatograph at 300 °C under helium flow for 1 h before use. Desorption was carried out in a splitless mode at a temperature of 310 °C for 3 min. A fiber blank was run between each sample to reduce memory effects.

Gas Chromatography–Mass Spectrometry (GC-MS) Analyses. The volatiles of *S. invicta* workers were analyzed by GC-MS (Shimadzu QP2010 Plus, Kyoto, Japan). Volatiles were separated in an Agilent DB-5 capillary column (5% phenyl-methylpolysilicone; 30 m × 0.25 mm ID × 0.25 µm; Agilent Technologies, Santa Clara, California) using helium (99.99%) as carrier gas at a 1.0 mL/min flow rate. The oven temperature was held at 150 °C for 1 min and increased by 10 °C/min until 280 °C, where it was held for 10 min.

GC-EAD Analyses. The antennae of *P. rapae* adults were exposed to volatiles and subsequently subjected to GC-EAD analyses to detect biologically active peaks (components). The GC-EAD techniques used were similar to those described in previous studies (Ngumbi et al. 2009). The system consisted of an Agilent 7890 gas chromatograph (Agilent Technologies) equipped with a flame ionization detector (FID) and coupled to an EAG detector. The dimension of the GC capillary column was the same as described above. The column effluent was mixed with 30 mL/min helium and split at a ratio of 1:2 (v/v), with 1 part going to the FID and the other through a heated (280 °C) transfer line (Syntech®, Hilversum, the Netherlands) into a charcoal-filtered and humidified air-

stream (500 mL/min) directed at the antenna preparation (EAG detector). The GC oven was programmed as above. The antenna preparation and EAG techniques were the same as previously described (Andersson & Dobson 2003). The antenna for the GC-EAD was removed from the male butterfly, and the base was connected to the grounding electrode while the tip, which was cut (approx. 2 mm removed) to obtain better contact, was connected to the recording electrode. The identification of each volatile compound was made by GC-MS (see above), and the approximate amount of each compound was calculated from the GC-MS analyses. The analog signal was detected through a probe (INR-II, Syntech®), captured and processed with a data acquisition controller (IDAC-4, Syntech®), and later analyzed with computer software (GcEad 32, Syntech®). Five successful GC-EAD recordings were obtained for each treatment. GC-EAD traces were overlaid on the computer monitor and inspected for significant and consistent qualitative and quantitative differences among treatments (Ngumbi et al. 2009).

Electroantennographic Recording. The olfactory sensitivity of male butterflies to 3 chemical standards, namely, tricosane, pentacosane, and heptacosane from Dr. Ehrenstorfe (Augsburg, Germany), was tested. EAG (Syntech®) recordings were conducted with the recording electrode. The antenna preparation and EAG techniques were the same as above. The recordings were taken by pulsing air (1,000 ms) containing the identified cue diluted in hexane to produce a concentration of 0.01 mg/mL and 1 mg/mL over the antenna.

EAG Response of *P. rapae* to Volatile Compounds. Ten microliters per concentration of each compound was deposited onto a filter paper strip (5 × 20 mm), which was then inserted into a glass tube (6 × 95 mm). Each sample was examined 3 times for each antenna. Five males were used for the EAG recording of each sample, and the average response was calculated.

STATISTICAL ANALYSES

Repeated measures analysis of variance (ANOVA) was performed to compare the species richness of flower-visiting insects on the *B. napus* plants among treatments and investigation dates. One-way ANOVA was performed to compare the number of flower-visiting insects on the *B. napus* plants, the visiting frequency and duration, and the EAG relative values (EAG response value to chemical standards/EAG response value to hexane) of butterflies and honeybees. ANOVA were followed by Tukey's post hoc test for multiple comparisons. All statistical analyses were conducted with SPSS version 14.0 (SPSS Inc., Chicago, Illinois).

Results

EFFECT OF *S. INVICTA* ON THE SPECIES ABUNDANCE AND THE NUMBER OF FLOWER-VISITING INSECTS ON *B. NAPUS*

Field trials showed that the survey date ($F = 7.116$, $df = 6$, $P < 0.001$) had a significant effect on the species richness of the flower-visiting insects, whereas no significant difference was found among the treatments ($F = 1.67$, $df = 2$, $P = 0.229$) (Fig. 1A). Survey date and treatment (survey date: $F = 37.718$, $df = 6$, $P < 0.001$; treatment: $F = 9.054$, $df = 2$, $P = 0.004$) had significant effects on the total number of observed flower-visiting insects. The number of flower-visiting insects that appeared in the control plots was significantly higher than that in the plots of the 2 treatments, which contained fire ants or fire ants and aphids together (Fig. 1B).

The number of flea beetles in the plots with fire ants present was 43.5% lower than the number of flea beetles present in the control, but no difference was apparent between the 2 treatments ($F = 7.539$,

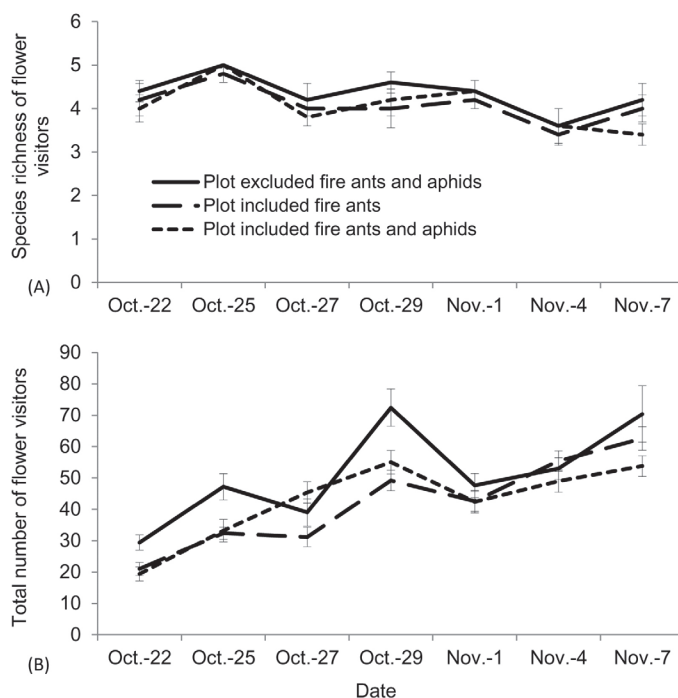


Fig. 1. Means (\pm SE) of species richness (A) and total number (B) of flower visitors on *Brassica napus* in fire ant-excluded, fire ant-included, and fire-ant-and-aphid-included plots.

$df = 2$, $P = 0.008$) (Fig. 2). The interaction between fire ants and aphids significantly decreased the number of the 2 hover fly species *E. quinquestratus* and *E. quinquelineatus* (39.4% lower than in control; $F = 4.202$, $df = 2$, $P = 0.041$), whereas no significant differences were found for *P. rapae* ($F = 2.716$, $df = 2$, $P = 0.106$), *A. cerana* ($F = 2.306$, $df = 2$, $P = 0.142$), and *F. intonsa* ($F = 0.216$, $df = 2$, $P = 0.809$).

EFFECT OF *S. INVICTA* ON THE VISITING FREQUENCY AND DURATION OF *P. RAPAE* AND *A. CERANA*

Solenopsis invicta had no significant effect on the visiting frequency of *P. rapae* ($F = 0.163$, $df = 2$, $P = 0.852$) and *A. cerana* ($F = 3.305$, $df = 2$, $P = 0.072$) (Fig. 3). Cumulative probability through the investigation course varied similarly among the treatments for both *P. rapae* and *A. cerana* (Fig. 3). The visiting duration at a single flower for *P. rapae* in the plots that had fire ants and aphids excluded was significantly longer (1.42 and 1.29 fold) than in the plots of the 2 treatment ($F = 10.83$, $df = 2$, $P = 0.002$), whereas no significant difference was found for *A. cerana* ($F = 1.684$, $df = 2$, $P = 0.227$) (Table 1).

COMPOSITION OF THE VOLATILES OF *S. INVICTA* WORKERS AND THEIR ELECTROANTENNOGRAPHIC ACTIVITIES IN *P. RAPAE*

Five chemicals elicited an electroantennographic response in *P. rapae* adult males (Fig. 4). The 1st and 4th chemicals indicate n-alkanes, which had no electrophysiological effects on *P. rapae* males. One unknown chemical (No. 3) could not be identified because of low concentration.

EAG RESPONSE OF *P. RAPAE* TO VOLATILE COMPOUNDS

Three chemical standards were available for the electroantennographic detection. At 0.01 mg/mL, the EAD relative value of *P. rapae* to heptacosane was significantly higher than to tricosane and penta-

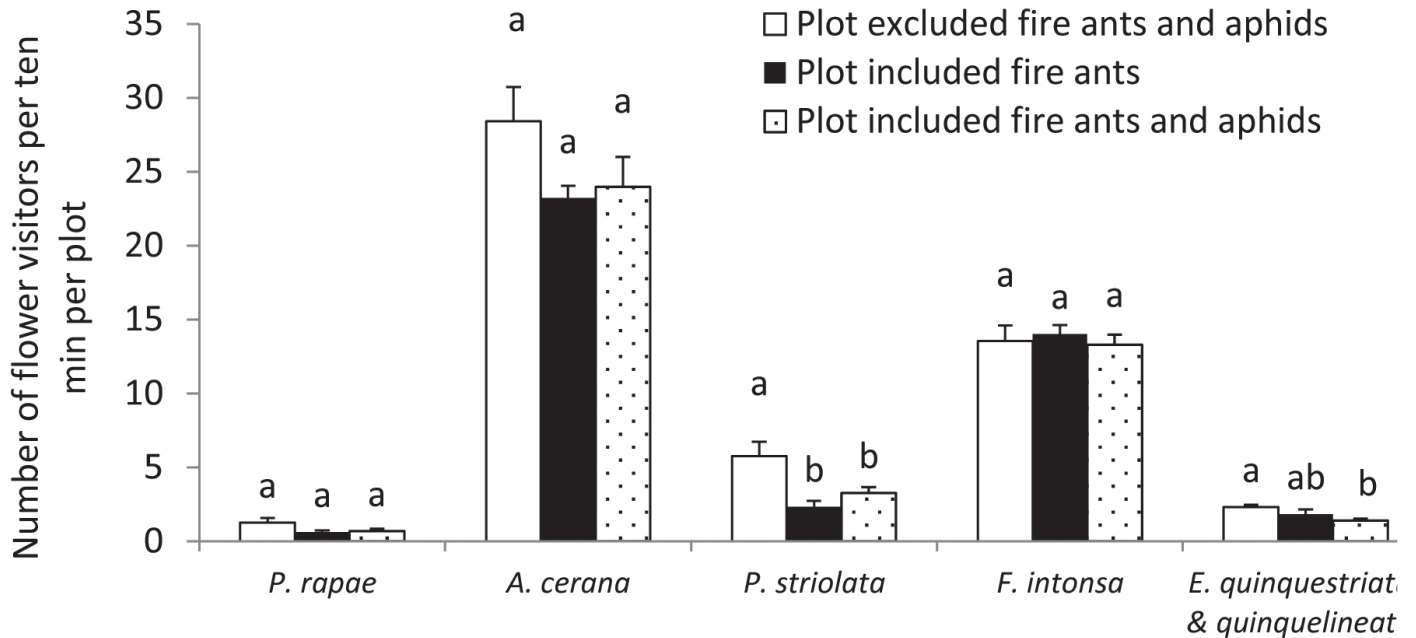


Fig. 2. Daily number (mean \pm SE, the number of *Frankliniella intonsa* was calculated per 10 min per 10 flowers) of flower visitors on *Brassica napus* in fire ant-excluded, fire ant-included, and fire-ant-and-aphid-included plots.

cosane ($F = 4.362$, $df = 2$, $P = 0.023$), whereas no significant difference between the compounds was found at 1 mg/mL ($F = 3.084$, $df = 2$, $P = 0.062$) (Table 2).

Discussion

Insects are known to use a variety of sensory modalities in foraging, and visual, olfactory, and gustatory cues are usually involved in their orientation to and finding of food sources (Barth et al. 1985; Willmer & Stone 1997; Kats & Dill 1998; Dicke & Grostal 2001; Tsuji et al. 2004; Gonzalez & Rodriguez-Girones 2013; Gonzalez et al. 2013). In butterfly species, attraction to flowers is modulated by olfaction of floral scents (Honda et al. 1998; Andersson 2003; Tasin et al. 2010), and ant visitation to plants has been shown to hinder pollinators (Blancafort & Gómez 2005).

In the field investigation, we found that fire ant workers usually foraged on the stems near the ground but climbed higher and foraged more frequently on upper stems and flowers when aphids were present. Fire ants foraging on plants did not significantly re-

duce the number of *A. cerana* and *P. rapae*, but they did reduce the number of 2 hover fly species, *E. quinquestratus* and *E. quinquelineatus*, in addition to *P. striolata*, which is an important pest of *B. napus* (Yan et al. 2013). This result indicates that fire ants may reduce the infestation by *P. striolata* as well as the pollination activity of *P. striolata*. Visiting frequency and duration of visits of *A. cerana* showed no difference among the control and treatment plots, suggesting that fire ants did not affect their pollination efficiency on the *B. napus* plants. The fire ants did not affect the visiting frequency of *P. rapae*, one of the insect visitors on *B. napus*; however, the visiting duration at a single flower for *P. rapae* in the plots that had fire ants and aphids excluded was significantly longer than in the fire ant-included (1.42 fold) and fire-ant-and-aphid-included plots (1.29 fold). A decrease in the visiting duration of this butterfly may result in reduced pollination and further reduced fruit set (Thomson & Goodell 2001).

It was reported that a reduction in the diversity and abundance of insects in *Protea nitida* Mill. (Proteales: Proteaceae) inflorescenc-

Table 1. Effect of *Solenopsis invicta* and its interaction with aphids on the flower-visiting efficiency of *Pieris rapae* and *Apis cerana* on *Brassica napus*.

Visitor	Parameter	Plot without fire ants and aphids	Plot with fire ants	Plot with fire ants and aphids	F	P
<i>P. rapae</i>	Visiting frequency	7.32 \pm 0.77a	7.24 \pm 0.50a	7.42 \pm 0.58a	0.163	0.852
	Duration of each visit	8.85 \pm 1.30a	6.24 \pm 0.59b	6.87 \pm 0.73b	10.83	0.002
<i>A. cerana</i>	Visiting frequency	12.42 \pm 0.49a	13.31 \pm 0.79a	13.05 \pm 0.30a	3.305	0.072
	Duration of each visit	2.81 \pm 0.35a	2.72 \pm 0.26a	2.51 \pm 0.14a	1.684	0.227

Data (mean \pm SE) in the same row followed by the same letter are not significantly different from each other ($P > 0.05$, Tukey's post hoc comparison).

Table 2. Quantification of GC-EAD responses of *Pieris rapae* males to the volatile compounds of *Solenopsis invicta*.

Concentration	n-Tricosane	n-Pentacosane	n-Heptacosane	F	P
0.01 mg/mL	0.836 \pm 0.082ab	0.744 \pm 0.052a	1.032 \pm 0.073b	4.362	0.023
1 mg/mL	0.786 \pm 0.101a	0.842 \pm 0.063a	1.034 \pm 0.039a	3.084	0.062

Data (mean \pm SE) in the same row followed by the same letter are not significantly different from each other ($P > 0.05$, Tukey's post hoc comparison).

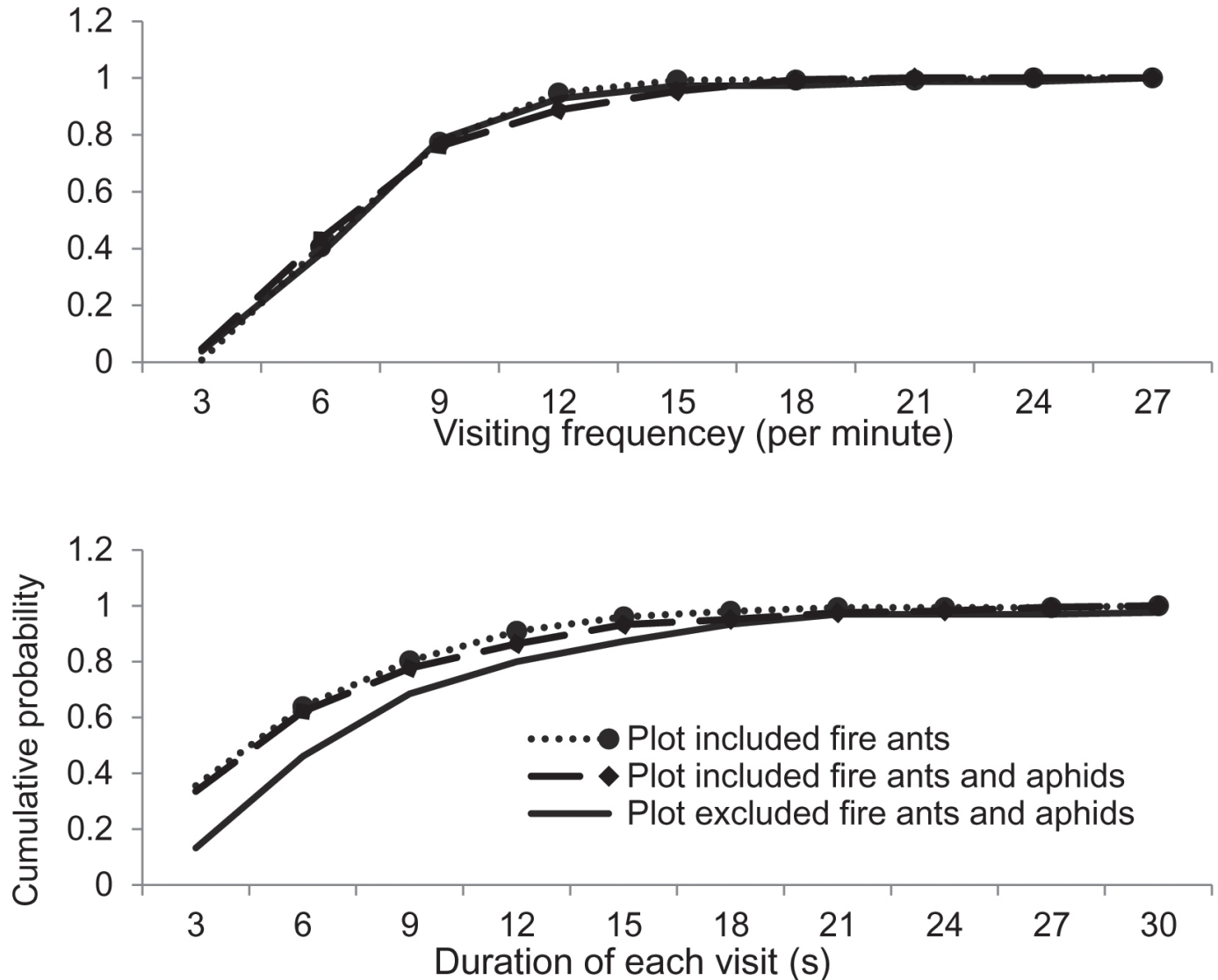


Fig. 3. The cumulative probability of visiting frequency and duration of each visit of *Pieris rapae* on *Brassica napus* in fire ant-excluded, fire ant-included, and fire-ant-and-aphid-included plots.

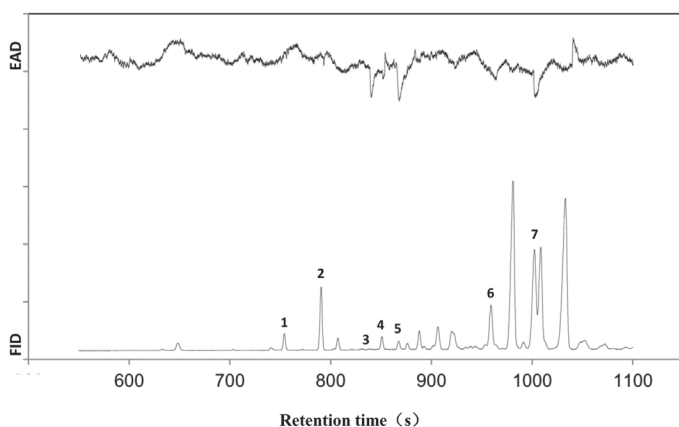


Fig. 4. GC-EAD responses of *Pieris rapae* males to volatiles of *Solenopsis invicta*. GC-EAD active compounds: (1) n-tricosane; (2) 3-methyl tricosane; (3) unknown; (4) n-pentacosane; (5) 13-methyl pentacosane; (6) n-heptacosane; (7) 13,15-dimethyl heptacosane.

es infested by *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) could retard pollination and thus the reproductive capacity of the plants (Visser et al. 1996). In our experiments, *P. rapae* adults reduced their stay on the plants, possibly owing to the aggressive behavior of fire ants, which prey on other arthropods. Although our laboratory bioassays showed fire ant odors could induce the EAG response in *P. rapae* males, this finding only indicates that the test material was sensed by the olfactory system. It is to be stressed that EAG data should be interpreted carefully and more bioassays are needed to elucidate the behavioral significance of bioactive compounds.

Our results indicate that fire ants may have affected the behavior of several of the potential pollinators, but probably had little effect on successful pollination of the crop because of the low impact on the behavior and abundance of *A. cerana*. More data are needed to evaluate the threat posed by fire ants to plant and pollinator conservation in the invaded crop fields. Moreover, the extent of the impact that fire ants have on crop production in agricultural systems in southern China needs further study.

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