***Electronic Supplementary Materials for Ge, Li, Yang, Wei & Kang:***

**Female adult puncture-induced plant volatiles promote mating success of the pea leafminer via enhancing vibrational signals**

METHODS

*(b) Experiment 1: Olfactory responses to adult leafminer volatiles*

*Behavioural bioassays*

The behavioural setup was designed as previously described [1] with some modifications. The olfactometer has a central glass chamber (6 cm internal diameter [ID] and 5 cm length) connected to four arms (15 mm ID and 5 cm length) that each had an upward insect-trapping bulb (50 mL). We used four glass jars (10 cm ID and 22 cm length) to provide different volatile sources. A manifold of four flowmeters modulated the flow rate of purified and humidified air to each glass jar at 300 mL/min. Each glass jar was connected to one arm with Tygon tubing (8 mm ID). During the experiment, 2-day-old females or males were introduced into one glass jar as an odour source, and the three other empty jars were used as the controls. A group of four flies was released into the central glass chamber. After 15 min, we counted the number of flies in the trapping bulb of each arm, and flies that localised at other positions was regarded as ‘no-choice’. The flies were then removed. Each odour treatment was tested with nine groups comprising four flies each. The experiments were performed between 10:00 AM and 4:00 PM. After each experiment were carried out, all of the glass and tube components were cleaned with water or hexane and then oven baked at 180 °C and 80 °C, respectively, for 3 h.

*Gas chromatography–electroantennographic detection recording*

A solid-phase microextraction (SPME) fibre (PDMS/DVB at 65 μm) was used to adsorb chemicals from a group of 30 two-day-old adult females for 30 min in a screw-top Supelco vial (2 mL). Thus, SPME extracts included volatiles and nonvolatiles. SPME fibres used for adsorption in empty vials served as the control. Antennae were prepared as previously reported [2]. In brief, one antenna was removed from the head of a male leafminer. The tip of the antenna was cut off. Then, the clipped antenna was connected to the recording electrode (glass capillary Ag–AgCl electrode filled with Ringer’s solution). The reference electrode was connected to the base of the antenna. Signals were passed through a high-impedance DC ampliﬁer (UN-06, Syntech, Hilversum, Netherlands) into a signal connection interface box (Auto Spike, IDAC 2/3, Syntech). A PC-based interface and software package (EAD version 2.3, Syntech) was used for data storage and processing. Electroantennographic detection (EAD) was performed in accordance with a previously described procedure with some modifications [3]. An SPME fibre with fly body chemicals was injected splitless into a modiﬁed Agilent 7890 A GC equipped with an HP-5 column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies, Inc.). This column was equipped with a column splitter (OSS-2, SGE, Ringwood, Victoria, Australia) and an extra outlet (temperature controller, TC-02, Syntech), which allowed for simultaneous ﬂame ionisation detection (FID) and EAD. The efﬂuent from the column was split into two streams: one (20%) was transferred to the FID, and the other (80%) was transferred to the EAD. The injector, detector and EAD transfer line temperatures were 250 °C, 280 °C and 200 °C, respectively. The GC oven temperature was maintained at 60 °C for 1 min and then increased to 200 °C at 30 °C/min, increased to 280 °C at the programmed rate of 5 °C/min and finally increased to 320 °C at 30 °C/min. The oven temperature was held at 320 °C for 2 min.

*Collections and analyses of adult body extracts*

Cuticular hydrocarbons (CHCs) were extracted by immersing 30–40 virgin two-day-old adult flies of each sex in hexane (5 µL per fly) for 10 min. CHC extracts were transferred to 2 mL screw cap vials (Waters Co., USA) with Teflon/rubber septa and then stored at −20 °C until further analysis. The Collected chemical samples were analysed and identified through gas chromatography–mass spectrometry (GC–MS) (Hewlett Packard 6890N GC model coupled with 5973 MSD) as previously described [4]. An HP-5 column (as described above) was used to separate CHCs. Approximately 2 µL of each sample was injected splitless into the GC–MS injector. The injector temperature was maintained at 280 °C at a constant helium flow rate of 1.0 mL/min. The oven temperature was maintained at 40 °C for 1 min, increased to 300 °C at a rate of 8 °C/min and increased to 320 °C at a programmed rate of 20 °C/min. The oven temperature was held at 320 °C for 2 min. An *n*-alkane (C6-C40, AccuStandard, New Haven, USA) standard was also run for the calculation of retention indices (RI) (table S1). CHCs were identified by comparing their retention times, spectra and RI with those of other insect CHCs [4-7]. Reference mass spectra from the NIST02 library (Scientific Instrument Services, Inc., Ringoes, NJ, USA) were also used. In addition, some synthetic compounds (e.g., 2-methyl-octacosane and 7-methyl-nonacosane) were run through GC–MS to confirm the identity of the compounds derived from adult leafminers (table S1). Given that the precision of FID is better than that of MS in chemical quantification, GC-FID was performed. In brief, an Agilent GC (7890A) coupled with an auto-injector (7683 autoinjector module, CAT No. G2913A, Agilent Technologies, Inc. USA) was equipped with an HP-5 column, and the same thermal program was adopted. Mixed samples consisting of pentacosane and heptacosane at different concentrations (1, 5, 20, 50, or 100 ng/μL) were used as external standards to develop standard curves for quantifying volatiles.

*(c) Experiment 2: Olfactory responses to HIPVs*

*Behavioural bioassays*

Female adult-punctured leaves were prepared by placing one healthy bean plant in a mesh cage containing 20 female flies and then transferred to the environmental chamber for 16 h (from 5:00 PM to 9:00 AM). This treatment typically yielded leaves with approximately 1,000 punctures [8]. Male and female responses to punctured plants were quantified as described above. Seven groups of four flies were tested for each odour treatment. To test the possible stimulatory effects of plants on the emission of sex pheromones, we introduced 20 sexually mature females to a glass jar containing punctured plants as an odour source for male selection. Furthermore, 15 groups of four flies were tested for each odour treatment.

The preferences of females and males for individual compounds were assessed in a Y tube (stem, 10 cm; arms, 23 cm at 60°; ID, 2.3 cm) as previously reported [3]. A flowmeter was used to maintain the flow of purified air at 300 mL/min through each arm of the olfactometer. Each synthetic compounds at a dosage of 10 µL of hexane (table S2) were added to a glass capillary tube (10 µL; Sigma-Aldrich, Steinheim, Germany) and placed in one arm of the Y tube. The hexane control (equal volume of HPLC-grade hexane) was placed in the other arm. The dosage of synthetic compounds in 10 µL of hexane was equivalent to 1 h of the entrainment of volatiles in the blend of female–plant complex (table S2). Each adult fly was placed in the Y tube for 5 min. Adults that remained inactive for 5 min were recorded as having made ‘no choice’. Glass capillary tubes with odour and hexane were used for 1 h during which 5–8 flies finished the dual-choice tests. Then, new odour and control were supplied, and the position of arms was reversed to avoid position bias. The Y tube was replaced with a clean one after 10–16 individuals were tested. A total of 140–160 flies of each sex were used for each synthetic compound.

*GC–EAD recording*

To identify the bioactive volatiles that are present in female puncture-induced HIPVs, we measured the antennal responses of both sexes to common GLVs, terpenoids and oximes by using the same GC–EAD recording setup and method described above with some modifications. To avoid the dosage effect on the variation in antennal responses to natural volatiles of female puncture-induced blends and to directly compare them without bias, we used a synthetic blend containing equal amounts of each volatile to compare the variation in the EAD responses of adult leafminers to different compounds. The volatile mixture was prepared by mixing and diluting synthetic standards in approximately 20 ng/μL of each compound present in the headspaces of vials containing female puncture-induced volatiles (table S2). We used a HP-INNOWAX column (30 m × 0.25 mm × 0.25 µm) to separate the volatile blend. The GC oven temperature was maintained at 50 °C for 2 min, increased to 180 °C at a rate of 8 °C/min, increased again to 230 °C at a rate of 15 °C/min and finally held at 230 °C for 10 min. Five successful GC–EAD recordings with different fly antennae were obtained for each synthetic blend.

*Collections and analyses of HIPVs*

To test if the females emited specific volatiles in the presence of host plants, we compared the profiles of volatiles from punctured leaves and females plus punctured leaves (table S2). Female-punctured leaves were prepared as described above. A headspace plant volatile collection setup was established as previously described with minor changes [8]. Plastic bags were replaced by glass jars (40 cm high × 17 cm ID) for volatile accumulation, and a potting medium was wrapped with aluminium foil (Xindan Co., Shanghai, China). In the two treatments, collections were replicated four times. Extracts in the absorbent (Porapak Q, Supelco, Bellefonte, PA) were rinsed with 700 µL of HPLC-grade dichloromethane. Aeration extracts were stored at −20 °C until they were used in chemical analyses. A DB-WAX polydimethylsiloxane column (60 m × 0.25 × mm × 0.15 µm, Agilent Technologies) was used for plant volatile analysis [8]. The initial oven temperature was maintained at 40 °C for 2 min, increased to 180 °C at a rate of 5 °C/min and increased to 230 °C at a rate of 10 °C/min. Plant volatiles were identified through the comparison of retention times with the retention times of synthetic standards obtained through analyses on the same column and spectra obtained from the NIST02 library [8].

REFERENCES

[1] D'Alessandro, M. & Turlings, T.C. 2005 In situ modification of herbivore-induced plant odors: a novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chem Senses* **30**, 739-753. (doi:10.1093/chemse/bji066).

[2] Zhao, Y.X. & Kang, L. 2002 The role of plant odours in the leafminer *Liriomyza sativae* (Diptera: Agromyzidae) and its parasitoid *Diglyphus isaea* (Hymenoptera: Eulophidae): orientation towards the host habitat. *Eur J Entomol* **99**, 445-450. (doi:DOI: 10.14411/eje.2002.056).

[3] Wei, J., Wang, L., Zhu, J., Zhang, S., Nandi, O.I. & Kang, L. 2007 Plants attract parasitic wasps to defend themselves against insect pests by releasing hexenol. *PLoS One* 2, e852. (doi:10.1371/journal.pone.0000852).

[4] Xue, H.-J., Wei, J.-N., Magalhães, S., Zhang, B., Song, K.-Q., Liu, J., Li, W.-Z. & Yang, X.-K. 2016 Contact pheromones of 2 sympatric beetles are modified by the host plant and affect mate choice. *Behav Ecol* **27**, 895-902. (doi:10.1093/beheco/arv238)

[5] Katritzky, A.R., Chen, K., Maran, U. & Carlson, D.A. 2000 QSPR correlation and predictions of GC retention indexes for methyl-branched hydrocarbons produced by insects. *Anal Chem* **72**, 101-109. (doi:Doi 10.1021/Ac990800w).

[6] Chernenko, A., Holman, L., Helantera, H. & Sundstrom, L. 2012 Cuticular chemistry of males and females in the ant *Formica fusca*. *J Chem Ecol* **38**, 1474-1482. (doi:10.1007/s10886-012-0217-4).

[7] Carlson, D.A., Bernier, U.R. & Sutton, B.D. 1998 Elution patterns from capillary GC for methyl-branched alkanes. *J Chem Ecol* **24**, 1845-1865. (doi:Doi 10.1023/A:1022311701355).

[8] Wei, J.N., Zhu, J. & Kang, L. 2006 Volatiles released from bean plants in response to agromyzid flies. Planta 224, 279-287. (doi:10.1007/s00425-005-0212-x).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **table S1|Comparison of cuticular hydrocarbon compounds in whole-body extracts between 2 day old virgin male and female leafminers** | | | | | |  |
|  | Compound Name | RI\* | Absolute amount of cuticular hydrocargbons per fly (ng)† | | Diagnostic ions (MI‡) | |
| # | Male | Female |
| 1 | 7-Methyltricosane | 2344 | 49.78±9.46a | 56.81±6.32a | 112, 252, (338) | |
| 2 | 5-Methyltricosane | 2351 | 8.00±1.30a | 8.61±0.74a | 85, 281 (338) | |
| 3 | 2-Methyltricosane | 2362 | 55.58±7.40a | 68.18±7.86a | 295, 323, (338) | |
| 4 | 3-Methyltricosane | 2375 | 8.88±0.77a | 10.46±1.24a | 309 (338) | |
| 5 | Tetracosane | 2400 | 2.87±1.44a | 5.18±0.37a | (338) | |
| 6 | 2-Methyltetracosane | 2462 | 32.26±4.97a | 36.37±4.64a | 309, 337, (352) | |
| 7 | Unknown | 2466 | 45.41±5.11a | 63.79±7.93a | (352) | |
| 8 | Unknown | 2473 | 3.05±0.30a | 4.26±0.44a | (352) | |
| 9 | Unknown | 2481 | 22.74±1.30a | 34.37±3.44b | (352) | |
| 10 | Pentacosane | 2500 | 228.45±6.89a | 298.31±29.05a | (352) | |
| 11 | 7-methyl-Pentacosane | 2541 | 850.04±75.79a | 1291.45±147.64a | 280, 351, (366) | |
| 12 | Hexacosane | 2600 | 56.25±3.74a | 84.47±11.04a | (366) | |
| 13 | 3,11-Dimethyltetracosane | 2609 | 248.00±10.79a | 378.89±42.72b | 323, (366) | |
| 14 | 13-Methylhexacosane | 2634 | 50.71±1.05a | 79.94±10.11b | 239, (380) | |
| 15 | 2-Methylheptacosane | 2661 | 247.35±3.83a | 379.74±47.23b | 337, 365, (380) | |
| 16 | 6,14-Dimethylhexacosane | 2675 | 47.78±1.47a | 78.79±9.96b | 99, 225, 323, (380) | |
| 17 | Heptacosane | 2700 | 339.78±31.06a | 471.58±65.03a | (380) | |
| 18 | 7-Methylheptacosane | 2742 | 105.11±7.06a | 173.20±18.70b | 112, 309, (394) | |
| 19 | 3-Methylheptacosane | 2774 | 310.20±15.74a | 453.01±53.83a | 365, (394) | |
| 20 | Octacosane | 2800 | 44.39±7.23a | 91.94±12.33b | (408) | |
| 21 | Nonacosane | 2900 | 122.32±16.35a | 159.87±32.58a | (422) | |
| 22 | 7-Methylnonacosane | 2941 | 138.56±13.50a | 192.60±28.57a | 112, 337, (436) | |

\* RI：Retention indices were determined by run an alkane-mixture under the same GC condition as used for adult body extracts.

† Absolute amount of CHCs was quantified by GC-FID.

‡ MI: Molecular ion of CHCs was shown in the parenthesis.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **table S2|Comparison of percentages and amount of the headspace volatiles of female-punctured leaves and female-leaf complex** | | | | | | |
| # |  | Female-puncture leaves | | Female-leaf Complex | | Dosages in behavioural assays (ng in 10ul hexane) |
| Compound name | Percentage (%) | Amount (ng/h/1000 punctures) | Percentage (%) | Amount (ng/h/1000 punctures) |
| 1 | (3*E*)-4,8-dimethyl-1,3,7-nonatriene | 3.28 ± 0.34a | 21.91 ± 6.36 | 2.25±0.20a | 28.19 ±5.90 | 20 |
| 2 | z-3-hexenyl-acetate | 25.66 ± 5.59a | 214.46 ± 90.23 | 49.61±5.46b | 610.78±123.57 | 500 |
| 3 | z-3-hexenol | 1.46 ± 0.75a | 14.02 ± 7.17 | 7.51 ± 1.02b | 86.63±9.90 | 80 |
| 4 | (syn)-2-Methylpropanal oxime | 22.44 ± 3.38a | 133.68 ± 20.97 | 13.13 ± 3.34a | 155.28±37.38 | 200 |
| 5 | (anti)-2-Methylpropanal oxime | 5.34 ± 0.50a | 33.33 ± 6.46 | 3.62 ± 0.75a | 43.85±10.84 |
| 6 | (syn)-2-Methylbutanal oxime | 28.35 ± 3.93a | 167.35 ± 23.69 | 16. 33 ± 2.30a | 193.75±39.01 | 200 |
| 7 | (anti)-2-Methylbutanal oxime | 6.43 ± 0.61a | 39.94 ± 7.38 | 3.90 ± 0.62a | 47.6±12.02 |
| 8 | Caryophyllene | 7.02 ± 1.99a | 62.79 ± 34.06 | 3.65 ± 0.68a | 42.23±9.83 | 50 |