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Electrophysiological and Behavioral Responses of a Parasitic Wasp to Plant Volatiles Induced by Two Leaf Miner Species

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Abstract

In the present study, Y-tube olfactometric assays demonstrated that headspace volatile extracts collected from leaf miner–damaged, or artificially damaged, bean plants were more attractive to naive females of the parasitoid insect *Opius dissitus* than those collected from healthy plants. Headspace extracts from both *Liriomyza huidobrensis* and *Liriomyza sativae* second-instar larvae–damaged beans were analyzed by coupled gas chromatography–electroantennographic detection (GC–EAD). Of nine EAD-active volatiles identified, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (3*Z*)-hexenyl acetate, (*syn*)-2-methylpropanal oxime, and (*syn*)-2-methylbutanal oxime were the most abundant compounds that evoked significant electroantennogram (EAG) responses. Compounds (3*Z*)-hexen-1-ol, (*anti*)-2-methylbutanal oxime, linalool, β-caryophyllene, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene also elicited clear EAG responses but were present in smaller amounts. Choice experiments in a Y-tube olfactometer indicated that synthetic versions of (3*Z*)-hexen-1-ol, 2-methylpropanal oxime, 2-methylbutanal oxime, 3-methylbutanal oxime, linalool, (*E*,*E*)-α-farnesene, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were attractive individually, while (3*Z*)-hexenyl acetate and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were unattractive at concentrations similar to those obtained from the headspace collection. Moreover, a blend of nine EAD-active volatiles was significantly more attractive relative to hexane (solvent). A mixture of oximes, tereponids, and green leaf volatiles may facilitate host location by female *O. dissitus*.

Key words: behavioral experiments, gas chromatography–electroantennographic detection, *Liriomyza huidobrensis*, *Liriomyza sativae*, *Opius dissitus*, plant volatiles

Introduction

It has been demonstrated that parasitoid insects can use volatile compounds released by insect herbivore–damaged plants to locate their hosts (e.g., Vet and Dicke, 1992; Turlings *et al.*, 1995; De Moraes *et al.*, 1998; Paré and Tumlinson, 1999; Mumm and Hilker, 2005). It has been suggested that it might be possible to exploit such volatile substances to attract parasitoids and thereby improve biological control in the field (Vet and Dicke, 1992; Cortesero *et al.*, 2000; Aldrich *et al.*, 2003). In this regard, it was anticipated that so-called "minimally effective blends" of volatile compounds attractive to parasitoids would be developed. However, unlike the relatively simple composition of insect pheromones, volatile blends collected from herbivore-damaged plants may comprise hundreds of compounds (e.g., Dicke *et al.*, 1999; Zhu *et al.*, 1999; Birkett *et al.*, 2003; de Boer and Dicke, 2004; Turlings and Wäckers, 2004; Bichão *et al.*, 2005; Pophof *et al.*, 2005). This complexity makes it difficult to establish which specific compound, or compounds, actively attracts parasitoid insects (Dicke and van Loon, 2000).

Recent research (Du *et al.*, 1998; Ngi-Song *et al.*, 2000; Park *et al.*, 2001, 2002; Smid *et al.*, 2002; Zhao and Kang, 2002) has revealed that the olfactory sensilla of parasitoids only responds to a limited number of the compounds released by insect-damaged plants thereby substantially reducing the number of compounds that require testing. Plant volatile compounds that have elicited antennal responses were also attractive to parasitoids in behavioral experiments (Du *et al.*, 1998; Ngi-Song *et al.*, 2000). The summed neural activity of antennal olfactory receptors can be measured using the GC–EAD technique (Wadhams, 1990). Although electrophysiological techniques have the advantage of online identification of the electrophysiologically active components of volatile blends, these compounds are not always behaviorally active to insects (Bjostad, 1998). The behavioral significance of these compounds therefore needs to be evaluated in behavioral experiments. Using a combination of electrophysiological and behavioral experiments, a few studies have identified plant volatile substances that enhance

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the ability of parasitoid insects to locate host habitat (e.g., Du *et al.*, 1998; Ngi-Song *et al.*, 2000). Furthermore, recent research has demonstrated that predatory insects and parasitoids are attracted to insect-damaged plants in nature (e.g., Bernasconi Ockroy *et al.*, 2001; Kessler and Baldwin, 2001).

Opius dissitus Muesebeck (Hymenoptera: Braconidae) is an important biological control agent with a worldwide distribution (Murphy and Lasalle, 1999) that parasitizes the larvae of *Liriomyza sativae* and *Liriomyza huidobrensis* in both outdoor crops and greenhouses from the north to south of China (Xu *et al.*, 1999; Song *et al.*, 2003). Previous research on the chemicals used by leaf miner parasitoids to locate their hosts has shown that odors from plant–leaf miner complexes play an important role in host location (Dicke and Minkenberg, 1991; Petitt *et al.*, 1992; Finidori-Logli *et al.*, 1996; Zhao and Kang, 2002). However, very little is known about the volatile chemicals released from plant–*L. sativae* and plant–*L. huidobrensis* complexes and which of these facilitate host location.

Recently, we identified a large number of volatiles in headspace samples from healthy, mechanically damaged, and leaf miner–damaged bean, *Phaseolus vulgaris* L., plants (Wei *et al.*, 2006). These volatiles can be divided into three major categories: green leaf volatiles (GLVs), terpenoids, and oximes. Among 25 emitted volatiles identified, 11 compounds were proven to be induced only by leaf miner damage. They were β-caryophyllene, α-humulene, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (*anti*)-2-methylbutanal oxime, (*syn*)- and (*anti*)-2-methylbutanal oxime, (3*Z*)-hexenyl butyrate, (3*Z*)-hexenyl iso-valerate, 2-ethyl-cyclopentanone, 1-pentanol, and methyl salicylate. Furthermore, our previous study showed that there are no qualitative differences between volatile emissions from bean plants induced by adults of two *Liriomyza* species, but significant quantitative differences are found in the blends of volatiles released; *L. huidobrensis*–damaged plants produce larger amounts of volatiles than those of *L. sativae*–damaged plants (Wei *et al.*, 2006). The current paper addresses the question: which of these volatiles are perceived by the antennae of female *O. dissitus* and are used by this parasitoid to locate its host? Specifically, we report 1) the results of choice experiments in a Y-tube olfactometer designed to test responses of naive female *O. dissitus* to different bean plant headspace extracts, 2) the identity of electrophysiologically active compounds in headspace extracts of both *L. huidobrensis*– and *L. sativae*–damaged beans, and 3) the results of choice experiments in a Y-tube olfactometer designed to test the preferences of naive female *O. dissitus* for single synthetic standards of EAD-active compounds, or a mixture of these compounds.

Materials and methods

Plants and insects

Bean plants (*P. vulgaris* L. cv Naibai) were planted individually in 12-cm-diameter plastic pots containing a 4:1 peat:vermiculite mixture at 25 ± 5°C, 14 h:10 h light: dark (L:D) photoperiod, and 60% relative humidity (RH). Approximately 2-week-old bean plants with two fully developed true leaves were used in all experiments and for insect rearing. The parasitoid *O. dissitus* was reared on colonies of two leaf miners, the pea leaf miner, *L. huidobrensis*, and the vegetable leaf miner, *L. sativae*, both of which were fed on bean plants. Parasitoid and leaf miner cultures were maintained in separate environmental chambers operating at 25 ± 1°C, 70 ± 10% RH, and a 14 h:10 h L:D photoperiod. Female parasitoids from pupae that had been isolated in glass tubes (70 × 8 mm) were mated within 24 h of emergence and then kept in glass vials (80 × 23 mm) with access to honey solution (10%) under the same environmental conditions described above. All parasitoids used in choice experiments were 2- to 4-day-old naive female adults, which had no previous exposure to either leaf miners or plants. Each individual was used only once in the experiments. Parasitoid cultures were propagated for 2 years under these conditions.

Plant treatments

The plant treatments used for behavioral experiments were done in the same way as described by Wei *et al.* (2006). Briefly, 1) clean bag (CTRL); 2) healthy, undamaged plants (UP); 3) needle-punctured plants (NPP); 4) plants with cut leaves (CLP); 5) plants on which *L. huidobrensis* females had fed and oviposited (Lh-FOP): three intact plants exposed to 150–200 adult *L. huidobrensis* (sex ratio 1:1) in an environmental chamber for 2 h, usually between 8:00 AM and 10:00 AM; 6) plants on which *L. sativae* females had fed and oviposited (Ls-FOP); (7) plants on which *L. huidobrensis* second-instar larvae had fed (Lh-LP): three plants were exposed to 150–200 adult flies for 2 h; (8) plants on which *L. sativae* second-instar larvae had fed (Ls-LP); (9) plants with empty *L. huidobrensis* mines (Lh-EMP): three plants were exposed to about 100 adult *L. huidobrensis* for 2 h so that they had empty leaf mines after about 10 days; and (10) plants with empty *L. sativae* mines (Ls-EMP).

Headspace volatile collection

The headspace volatile collection system was the same as described by Wei *et al.* (2006). Briefly, three potted bean plants were enclosed in a 40.6 × 44.4–cm plastic oven bag (Reynolds, about 7500 ml in volume during collection) sealed with self-sealing strips around each stem approximately 4–5 cm above soil level, and purified and humidified air was pumped into the bag through a molecular sieve (0.5 nm), freshly activated charcoal trap, and a water jar by a compressor. Effluent volatiles were extracted at a rate of 400 ml/min with a membrane pump (Beijing Institute of Labour Instruments, China) and absorbed on 100 mg of Porapak Q (mesh size = 80–100, Supelco, Bellefonte, PA) in a glass tube for 10 h. Three bags, each enclosing three potted plants, were prepared in parallel. Odors collected from bag that did not contain plants, but had otherwise been treated in the same way as those that did, served as control (CTRL). Volatile compounds absorbed on the Porapak Q filter were extracted with 600 μl HPLC-grade dichloromethane (Tedia Company, Fairfield, OH) in a glass vial. The extracts of each treatment from the three bags were pooled and concentrated to 400 μl in a nitrogen (purity 99.999%) atmosphere. All aeration extracts were stored at −20°C until used in behavioral experiments. The extracts from plants infested with second-instar larvae were concentrated to about 100 μl for GC–EAD studies (see below). After headspace sampling, plants were severed where the bag had been sealed and the part enclosed by the bag weighed immediately. Numbers of punctures and larvae were recorded by examining leaves with a stereomicroscope (Table 1).

GC–EAD recording

The antennae of parasitoids were prepared as described by Zhao and Kang (2002) with a few modifications. The head of the insect was removed, and the tips of the antennae were cut off. The reference electrode (Ringer's filled glass capillary Ag–AgCl electrode) was inserted in the hemocoel of the cranial cavity, while the recording electrode was connected to the two antennae in one individual. Signals from the antennae were passed through a high-impedance DC amplifier (UN-06, Syntech, Hilversum, The Netherlands) in a signal connection interface box (Auto Spike, IDAC 2/3, Syntech), and data storage and processing were carried out with a PC-based interface and software package (EAD version 2.3, Syntech). About 3 μl of samples were injected splitless into a modified HP 5890 series II GC equipped with an HP-INNOWAX column (30 m × 0.25 mm × 0.25 μm), with a column splitter (OSS-2, SGE, Ringwood, Victoria, Australia) and an extra outlet (temperature controller, TC-02, Syntech), which allowed simultaneous flame ionization [flame ionization detector (FID)] and EAD. The injector, detector, and EAD transfer line temperatures were 250°C, 280°C, and 200°C, respectively. The oven temperature was set at 50°C for 2 min, increased to 180°C at a rate of 8°C/min, then increased again to 230°C at 15°C/min, and finally held at this temperature for 10 min. Nitrogen was used as the carrier gas (1.5 ml/min) and the makeup gas (40 ml/min). The effluent from the column was split into two parts, one (12.5%) transferred to FID and the other (87.5%) to EAD in a ratio of approximately 1:7. The column of the EAD outlet was introduced into an 8-mm-diameter glass tube with a constant airstream of 250 ml/min. The supplied air was filtered through activated charcoal and humidified by passage over a water jar. The mounted antennae were placed 0.5 cm from the end of the glass tube (12 cm from the EAD outlet on the GC). Heptanoic acid, ethyl ester and dodecanoic acid, ethyl ester (40 ng/μl, >98% pure) were used as external standards for quantification of active chemical compounds in the extracts and a standard blend (Table 2). The nine-component mixture of compounds was obtained by mixing and diluting different synthetic compounds in amounts that resembled the composition of headspace volatiles (Table 2). Our previous study (Wei *et al.*, 2006) showed that the larvae-damaged plants can emit the most abundant volatiles in both quantity and quality, and the compounds collected in other eight treatments were all present in larvae-damaged volatile blends. Therefore, it is enough just to use two typical extracts for GC–EAD studies and confirm with a standard blend. Five successful GC–EAD recordings with different female antennae were performed for each extract. Compounds that elicited consistent responses in at least four recordings are marked with numbers in Figure 2A,B. Electrophysiological activity of compounds was confirmed by a further five successful recordings using a nine-component mixture containing standards of each EAD-active compound (Figure 2C).

Table 1 Source of different headspace extracts and dosages of extracts used in behavioral experiments

Extract sources[a]	Plant fresh weight (means ± SEs, g)	Degree of damage (means ± SEs)	Dosage in behavioral test (equivalent to collection time)[b]
CTRL	—[c]	—	0.6
UP	17.6 ± 1.6	—	0.6
NPP	17.4 ± 2.7	3000 ± 648	0.6
CLP	15.4 ± 1.3	626 ± 10.6	0.6
Lh-FOP	16.2 ± 1.5	2113 ± 241.5	0.6
Ls-FOP	14.3 ± 1.5	2864 ± 787.5	0.6
Lh-2ILP	15.2 ± 1.2	74.1 ± 11.8	0.5
Ls-2ILP	14.5 ± 0.6	138.4 ± 16.8	0.5
Lh-EMP	14.7 ± 1.8	77 ± 9.8	0.6
Ls-EMP	15.4 ± 0.9	48.9 ± 8.7	0.6

[a] For abbreviations of extract sources, please see Materials and Methods for explanation.

[b] Mean dosage of each extract used in behavioral experiments was equivalent to 0.5–0.6 h entrainment of volatiles; 0.6 h entrainment = 8 μl extracts; 0.5 h entrainment = 6 μl extracts.

[c] "—" denotes absence.

Y-tube olfactometer behavioral experiments

A Y-tube olfactometer was used to investigate the responses of *O. dissitus* females to different volatile extracts or eletrophysiologically active compounds in the absence of any visual cues. The Y-tube olfactometer (stem, 10 cm; arms, 23 cm at 60° angle; internal diameter (ID), 2.3 cm) and the observation chamber were similar to those described by Kang and Hopkins (2004). The Y-tube olfactometer was put into an observation chamber (95 × 60 × 45 cm). Four 22-W cool, white fluorescent tubes located above the device provided uniform lighting. The temperature in the chamber was maintained at 26 ± 1°C at all times by an air conditioner. A pump

Table 2 Quantification of GC-EAD–active compounds in headspace extracts and a blend of standards and mean EAG response of antennae to them[a]

Compounds[b]	Extract from Lh-LP and EAG response (means ± SEs)[c]		Extract from Ls-LP and EAG response (means ± SEs)[d]		Standard blend and EAG response (means ± SEs)	
	ng	−mV	ng	−mV	ng	−mV
1 (3*E*)-4,8-Dimethyl-1,3,7-nonatriene	289.3 ± 17.7	0.36 ± 0.02	170.0 ± 13.6	0.29 ± 0.01	117.2 ± 9.4	0.34 ± 0.02
2 (3*Z*)-Hexenyl acetate	539.1 ± 29.4	0.43 ± 0.02	252.0 ± 12.4	0.38 ± 0.03	221.2 ± 19.8	0.44 ± 0.03
3 (3*Z*)-Hexen-1-ol		0.33 ± 0.03		0.26 ± 0.02		0.36 ± 0.03
4 (*syn*)-2-Methylpropanal oxime	292.2 ± 11.6	0.39 ± 0.02	91.6 ± 7.3	0.31 ± 0.03	336.4 ± 10.9	0.39 ± 0.04
5 (*syn*)-2-Methylbutanal oxime	280.4 ± 12.8	0.32 ± 0.01	172.0 ± 7.7	0.3 ± 0.01	190.5 ± 20.2	0.37 ± 0.04
6 (*anti*)-2-Methylbutanal oxime	73.8 ± 2.2	0.27 ± 0.01	58 ± 4.1	0.21 ± 0.02	64 ± 6.5	0.32 ± 0.03
7 Linalool	64.5 ± 4.0	0.35 ± 0.02	14.4 ± 2.1	0.28 ± 0.02	85.9 ± 3.9	0.42 ± 0.03
8 β-Caryophyllene	78.6 ± 5.2	0.23 ± 0.01	42.4 ± 6.5	0.19 ± 0.01	105.9 ± 16.9	0.29 ± 0.01
9 (3*E*,7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	74.0 ± 6.1	0.28 ± 0.01	42.6 ± 4.5	0.23 ± 0.01	72.9 ± 9.1	0.33 ± 0.02

[a] Amount of each compound in 3 μl headspace extracts or a mixture of standards. Five replicates for each sample.

[b] The compounds are listed based on their GC retention time on an HP-INNOWAX column (30 m × 0.25 mm × 0.25 μm). The retention time of compounds (3*Z*)-hexen-1-ol and (*syn*)-2-methylpropanal oxime overlapped on this column. The value represents the total amount of two compounds in each column in the table.

[c] Lh-LP = bean plants damaged by *L. hudobransis* second-instar larvae.

[d] Ls-LP = bean plants damaged by *Liriomyza sativae* second-instar larvae.

(Beijing Institute of Labour Instruments, China) was used to draw air through activated charcoal and a water jar so that it was purified of any contaminating odors and humidified before passing over the target and entering the Y-tube. Airflow through each of the olfactometer arms was maintained at 250 ml/min by a flowmeter. Headspace extracts (Table 1) were applied to 1 × 2–cm pieces of filter paper (Fuyuan Papermill Company, Hangzhou, Zhejiang, China) and placed in the airstream entering one of the olfactometer arms and with a control (equal volume of HPLC-grade dichloromethane) in the other. Each individual parasitoid was introduced into the Y-tube at the entrance of the stem and thus had a choice between the test odor and the control. New filter papers with the extracts and dichloromethane were used for every parasitoid. Female parasitoids were tested individually between 0900 and 1800. The position of the arms containing treatment and control odors was reversed to avoid position bias after every five individuals had been tested. The Y-tube was replaced with a clean one after 10 individuals had been tested.

Each parasitoid spent 5 min in the olfactometer. A "no choice" was recorded when a parasitoid remained inactive for the duration of this period. The experimental protocol used was that described by Du *et al.* (1996) and Colazza *et al.* (2004) with some modifications. A parasitoid was considered to have made a first choice when it moved >5 cm into either arm (visually assessed by a line marked on both arms). Parasitoids' final choice was the arm they were in at the end of the 5-min experimental period. The amount of time parasitoids spent in each arm of the device was also recorded. Preliminary tests had shown that *O. dissitus* had no preference for either solvent control (dichloromethane or hexane) (Figures 1 and 3). This confirmed that Y-tube olfactometers could be used for further behavioral experiments.

Chemicals

Headspace volatiles were identified by gas chromatography–mass spectrometry (Wei *et al.*, 2006). Standard compounds, such as (3*Z*)-hexen-1-ol (≥95% pure), linalool (97% pure), (3*Z*)-hexenyl acetate (99% pure), (*E*,*E*)-α-farnesene (>80% pure), and β-caryophyllene (95% pure), were purchased from Sigma-Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland), or Tokyo Kasei Kogyo Co. (Tokyo, Japan). Homoterpens, (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, were kindly provided by Dr W. Boland of the Max Planck Institute for Chemical Ecology, Jena, Germany. Oximes, (*syn*)- and (*anti*)-2-methylbutanal oxime, (*syn*)- and (*anti*)-3-methylbutanal oxime, and (*syn*)- and (*anti*)-2-methylpropanal oxime, were generously donated by Dr R. Kaiser of Givaudan Schweiz AG, Dubendorf, Switzerland. The purity of these compounds was measured with GC and was at least 95%. For behavioral experiments, single compounds, or a mixture of them, were diluted in HPLC-grade hexane in amounts equivalent to those in volatile emissions collected from bean plants infested with approximately 100 leaf miner larvae for ≈1 h (Figure 3). Individual compounds or the mixture of compounds were applied to 1 × 2–cm pieces of filter paper and placed inside one arm of the Y-tube olfactometer with

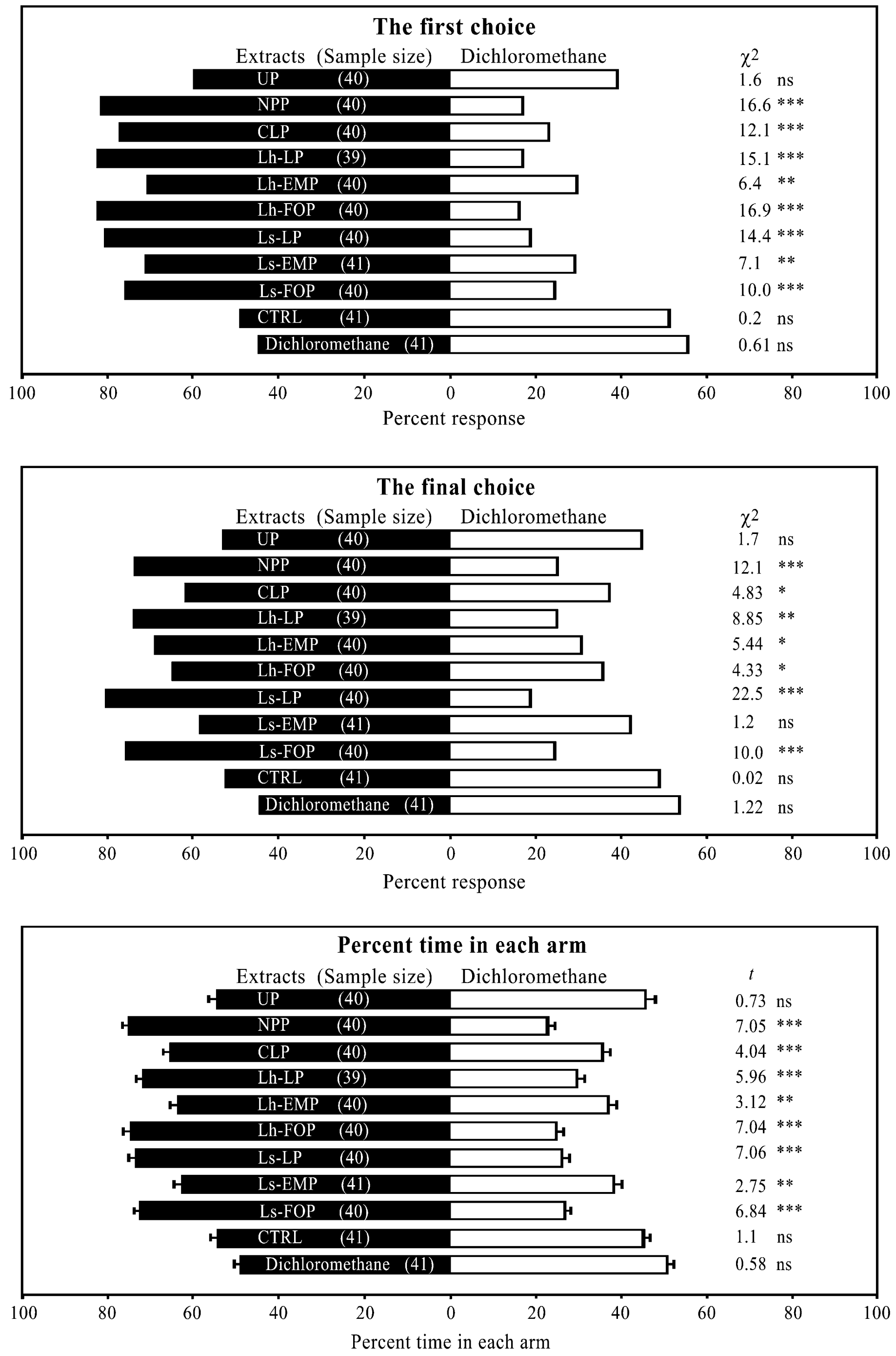

Figure 1 Responses of naive female *Opius dissitus* in a Y-olfactometer to headspace volatile extracts of bean plants versus a solvent control (dichloromethane). First choice, final choice, and time spent in each arm were measured during each experiment. For abbreviations of different treatments, please see Materials and Methods for explanation. χ^2 test for differences between numbers of parasitoids in each arm and a paired-sample *t*-test for differences in percent time spent in each arm. Numbers in parentheses represent sample size; $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant. Bars indicate mean values (±SE) of the percent time spent in each arm by about 40 females over an observation period of 5 min.

a control (equal volume of HPLC-grade hexane) in the other (Figure 3). New filter papers with the synthetic compounds and hexane were used for every parasitoid.

Quantification of each reference sample (Figure 3) was confirmed by injecting 1 μl of each sample in HP 6890 GC equipped with a DB-WAX column (60 m × 0.25–mm ID, 0.15-μm film thickness). Heptanoic acid, ethyl ester and dodecanoic acid, ethyl ester (1 ng/μl) were used as external standards. The GC oven temperature was first kept at 30°C for 2 min, then increased at a rate of 5°C/min to

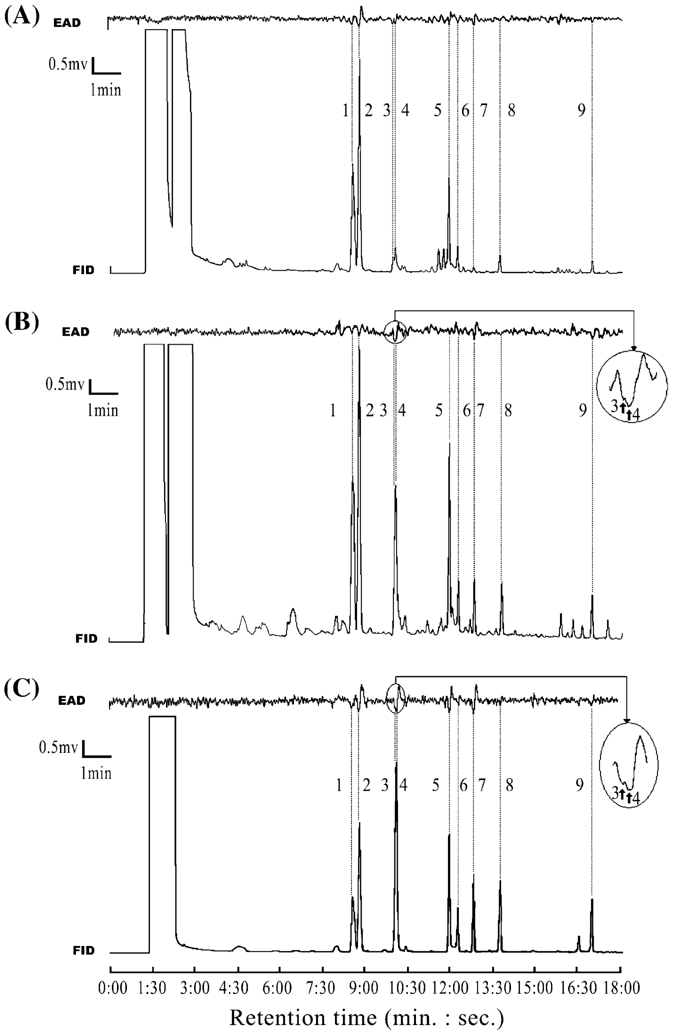

Figure 2 Simultaneously recorded antennal responses of naive female *Opius dissitus* to volatiles and gas chromatogram of headspace volatiles from *Liriomyza sativae* second-instar larvae–damaged bean plants **(A)**, *Liriomyza huidobrensis* second-instar larvae–damaged bean plants **(B)**, and a blend of standards **(C)**. Peaks as described in Table 2. Five successful replicates for each chemical sample. Compounds that elicited consistent responses (in at least four recordings) are marked with numbers.

180°C, and then finally increased to 230°C at 10°C/min. Although they were not EAD-active compounds, (*E*,*E*)-α-farnesene and 3-methylbutanal oxime were used in behavioral experiments because the former was collected from plants that had been subject to a variety of damage while the latter was only collected from second-instar larvae–infested bean plants (Wei *et al.*, 2006).

Data analysis

A χ^2 test was used to determine the significance of differences between the number of parasitoids choosing the treatment or control arm of the olfactometer. A paired-sample *t*-test (two tailed) was employed to analyze the differences between the percentage of time spent in each arm of the olfactometer. Data was arcsine ($x^{-1/2}$) transformed to correct for heterogeneity of variances before analysis (statistical analysis software, SPSS 11.0). Parasitoids that did not make a choice were excluded from statistical analyses. Unsuccessful parasitoid response rates in choice experiments ranged from 8% to 27%.

Results

Responses to volatile extracts in the olfactometer

As their first choice, naive female *O. dissitus* always significantly preferred volatile extracts collected from injured plants to the solvent control (percent responses > 70% in all cases, $P < 0.01$; Figure 1). The strongest upwind responses were to odors collected from NPP, plants on which adult *L. huidobrensis* had oviposited and fed (Lh-FOP) for 2 h, and plants that had been infested with second-instar larvae of *L. huidobrensis* (Lh-LP) and *L. sativae* (Ls-LP) (from 79.5% to 82.5% response to these treatments, respectively). Odors collected from healthy plants (UP) and clean bags (CTRL) were unattractive to parasitoids tested (48.8% and 43.9%, respectively). The olfactometer arm containing volatile extracts from damaged plants was the final choice of significantly more insects (percent responses > 60%, $P < 0.05$; except for treatment of Ls-EMP, $P > 0.05$), but, given a choice between odors collected from healthy plants and the solvent control, there was no significant difference in the number of insects that eventually settled in each arm of the olfactometer ($P > 0.05$). Female *O. dissitus* spent a greater amount of time exploring the arm containing extracts from injured plants relative to the solvent control (percent time > 62% in all cases, $P < 0.01$); however, when presented with a choice between extracts from healthy plants or the control, there was no difference in the amount of time spent exploring each arm of the device (percent time around 50%, $P > 0.05$).

GC–EAD responses to volatiles from bean plant–leaf miner complexes

Headspace collections from *L. sativae* and *L. huidobrensis* larvae–infested plants were analyzed by GC–EAD (Figure 2A,B). Nine volatile compounds in the headspace collections consistently elicited significant EAD responses from the antennae of female *O. dissitus*. Among them, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (3*Z*)-hexenyl acetate, (*syn*)-2-methylpropanal oxime, and (*syn*)-2-methylbutanal oxime were the most abundant volatiles in the blends collected and also elicited high electroantennogram (EAG) responses (Table 2). However, antennae also responded to compounds that were relatively small components of these extracts, such as (3*Z*)-hexen-1-ol + (*syn*)-2-methylbutanal oxime, linalool, β-caryophyllene, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Table 2). The EAG responses of antennae of this parasitoid to small components were also in high values such as to linalool, (3*Z*)-hexen-1-ol, and

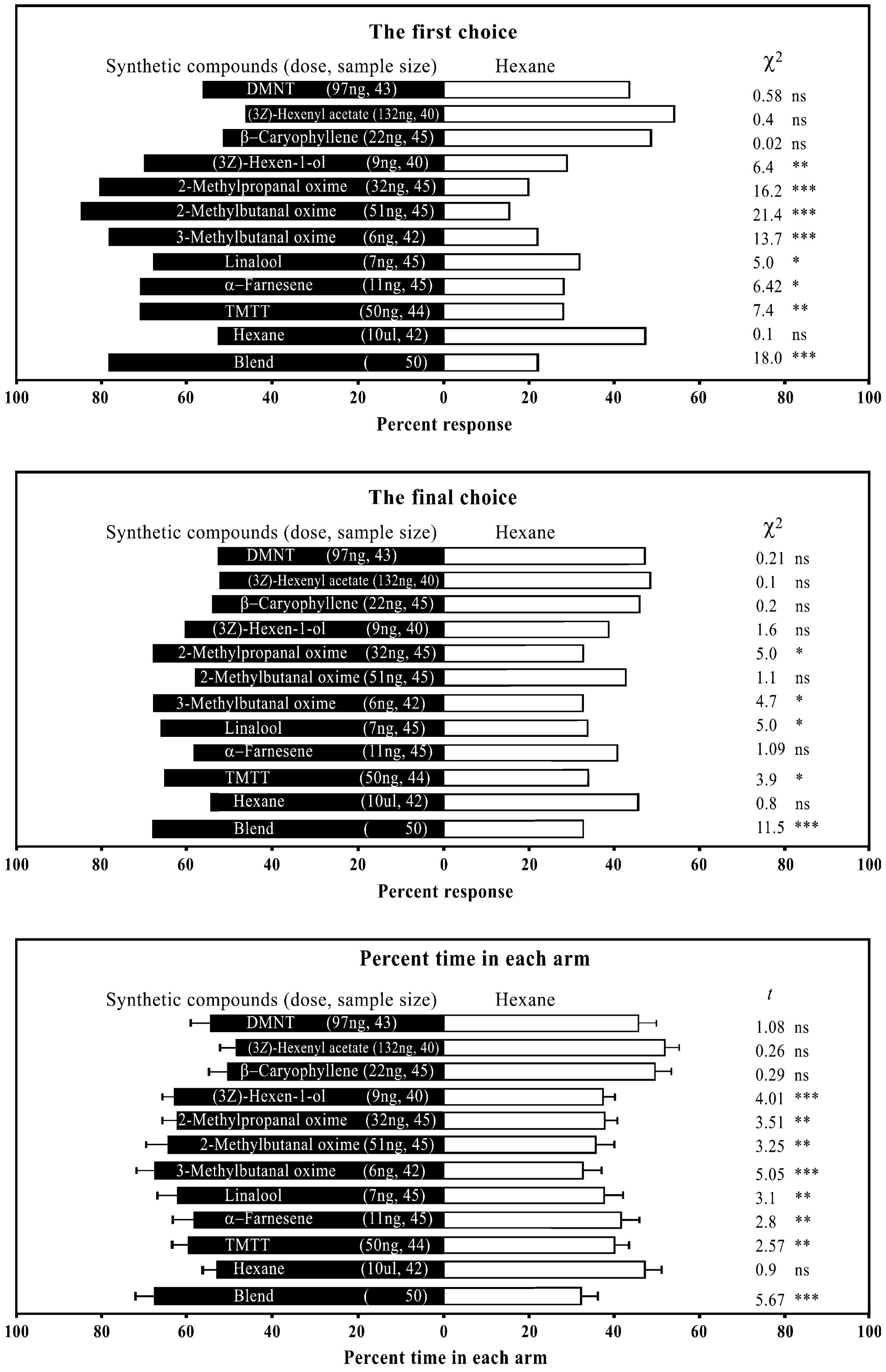

Figure 3 Responses of naive female *Opius dissitus* in Y-olfactometer to individual or a mixture of standard compounds versus a solvent control (hexane). First choice, final choice, and percent time spent in each arm were measured during each experiment. DMNT: (3*E*)-4,8-dimethyl-1,3,7-nonatriene; TMTT: (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Blend includes DMNT (140 ng), (3*Z*)-hexenyl acetate (145 ng), (3*Z*)-hexen-1-ol (21 ng), (*syn*)-2-methylpropanal oxime (78 ng), (*syn*)- and (*anti*)-2-methylbutanal oxime (58 and 23 ng), linalool (17 ng), β-caryophyllene (28 ng), and TMTT (31 ng). χ^2 test for significant differences between numbers of parasitoids in each arm and paired-sample *t*-test for significant differences in percent time spent in each arms. Numbers in parentheses represent dose and sample size. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant. Bars indicate mean values (±SE) of the percent times spent in each arm by about 45 females over an observation period of 5 min.

(*syn*)-2-methylbutanal oxime. The activity of these compounds was confirmed by GC–EAD using a nine-component mixture of standards formulated to approximate the composition of headspace volatiles (Figure 2C).

Parasitoid responses to single or mixed synthetic compounds in the olfactometer

EAD-active compounds together with *E*,*E*-α-farnesene and 3-methylbutanal oxime were introduced individually to one arm of an olfactometer in amounts approximating their natural abundance in headspace collections with a hexane control in the other arm (Figure 3). (3*E*)-4,8-Dimethyl-1,3,7-nonatriene, (3*Z*)-hexenyl acetate, and β-caryophyllene were unattractive to naive female *O. dissitus*, either as their first (55.8%, 46.7%, and 51.1% responses to these three compounds, respectively, $P > 0.05$) or final choice (53.5%, 50.8%, and 53.3% responses, respectively, $P > 0.05$). Therefore, insects spent similar amounts of time in both arms (54.2%, 51.2%, and 51% time spent in treatment arm, $P > 0.05$). As a first choice, female *O. dissitus* always significantly preferred the other seven volatiles to the solvent control (percent response > 66%, $P < 0.05$). Three oximes (2-methylpropanal oxime, 2-methylbutanal oxime, and 3-methylbutanal oxime) were the compounds initially most attractive to naive female *O. dissitus* (80.0%, 84.4%, and 78.6% responses, respectively, $P < 0.01$). (3*Z*)-hexen-1-ol, linalool, and (*E*,*E*)-α-farnesene also showed varying degrees of attractiveness. However, in terms of the final choice, only 2-methylbutanal oxime, 3-methylbutanal oxime, linalool, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were significantly attractive (66.7% response to them in each case, $P < 0.05$). Despite the observed differences in parasitoids responding to these seven compounds between the first choice and the final choice, the time they spend in the arm containing each of the seven compounds is significantly longer than that in the control arm (percent time > 61% in each case, $P < 0.01$). Furthermore, a blend of synthetic standards, all EAD-active volatiles in amounts approximating those in headspace collections, was significantly attractive (80% response as first choice, $P < 0.01$; 74% response as final choice, $P < 0.01$; and 69.1% residence time in treatment arm, $P < 0.001$; Figure 3).

Discussion

Herbivore-inflicted injury induced plants to release volatile terpenoids (e.g., linalool, (*E*)-β-ocimene, (*E*)-β-caryophyllene, *cis*-α-bergamotene, and (3*E*)-4,8-dimethyl-1,3,7-nonatriene), and the parasitoids and predators used the terpenoids as major cues to locate hosts (Dicke *et al.*,1990; Turlings *et al.*, 1990; Turlings and Tumlinson, 1992; De Moraes *et al.*,1998; Ngi-Song *et al.*, 2000; Kessler and Baldwin, 2001; Birkett *et al.*, 2003; Colazza *et al.*, 2004; de Boer and Dicke, 2004; Mumm and Hilker, 2005). Our data verify that terpenoids (linalool, (*E*,*E*)-α-farnesene, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene) collected from bean plant–leaf miner complexes are attractive to female *O. dissitus.* Furthermore, we found that the oximes, 2-methylpropanal oxime and 2-methylbutanal oxime, were the predominant compounds emitted from leaf miner–infested bean plants and, together with 3-methylbutanal oxime, were also electrophysiologically and behaviorally active (Figures 2 and 3). To the best of our knowledge, this is the first demonstration that herbivore-induced volatile oximes function as synomones attracting parasitoid insects to their prey.

Typical volatiles released by plants in the early stages of insect damage are the so-called GLVs, such as six-carbon chain length alcohols, aldehydes, and esters (e.g., Turlings *et al.*, 1995; Paré and Tumlinson, 1999). Some GLVs such as (3*Z*)-hexenyl acetate and (3*Z*)-hexen-1-ol appear to predominate in volatile blends collected from almost all plants (Fritzsche Hoballah *et al.*, 2002). Whitman and Eller (1990) reported that two parasitoids were attracted to GLVs in a flight tunnel, especially to different hexenols and hexenals. Another parasitoid, *Aphidius rhopalosiphi*, was strongly attracted to the two GLVs (3Z)-hexenyl acetate and (2*E*)-hexenal in Y-tube experiments (Wickremasinghe and Van Emden, 1992). Du *et al.* (1998) isolated six electrophysiologically active compounds from *Vicia faba* plants infested with *Acyrthosiphon pisum*, including (3*Z*)-hexenyl acetate and (3*Z*)-hexen-1-ol, and these EAG-active compounds produced significantly more oriented flights in a wind tunnel than a solvent control. Similarly, Ngi-Song *et al.* (2000) used GC–EAD and behavioral experiments to demonstrate that (3*Z*)-hexenyl acetate was one of six electrophysiologically active compounds collected from maize seedlings infested with *Chilo partellus* larvae that was also attractive to *Cotesia flavipes*. Furthermore, Birkett *et al.* (2003) reported that in wind tunnel experiments, a single synthetic compound of (3*Z*)-hexen-1-ol elicited a significant increase in oriented flight and landing on the odor source by the whitefly parasitoid, *Encarsia formosa*, but the most effective attractant was a mixture of (3*Z*)-hexen-1-ol and 3-octanone. Fritzsche Hoballah *et al.* (2002) hypothesized that release of GLVs was a characteristic of freshly damaged plants and may play a role in the initial attraction of naive predatory insects and insect parasitoids to such plants and damaged sites on them. Our data demonstrate that low amounts of the compound (3*Z*)-hexen-1-ol can both be perceived and attract the naive female *O. dissitus*. In contrast, naturally occurring amounts of (3*Z*)-hexenyl acetate were unattractive relative to a solvent control (Figure 3). In addition to parasitoids, predators can also be attracted by GLVs (e.g., Kessler and Baldwin, 2001). In the above-mentioned examples, the roles of each GLV appear to differ in different tritrophic systems.

Comparing activities of compounds in GC–EAD and choice experiments, we found that (3*Z*)-hexenyl acetate, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and β-caryophyllene elicited significant responses in the antennae of naive female *O. dissitus* but that amounts of these compounds similar to those encountered in nature were unattractive in choice experiments (Figure 3). Recently, we found that a blend of (3*Z*)-hexenyl acetate and (3*E*)-4,8-dimethyl-1,3,7-nonatriene was also unattractive to female *O. dissitus*, although (3*Z*)-hexenyl acetate and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were both the most abundant volatiles in plant extracts (Table 2) and were EAD active (Figure 2). However, electrophysiologically active compounds in plant headspace collections are not always behaviorally active (Bjostad, 1998). The study of Mumm and Hilker (2005) suggested that egg parasitoids, *Chrysonotomyia ruforum*, responded specifically to (*E*)-β-farnesene, but only when this compound was contrasted with a background odor of non–oviposition-induced pine volatiles. Furthermore, inexperienced and experienced parasitoids responded differently to volatile compositions (D'Alessandro and Turlings, 2005). The Y-tube olfactometer detects short-range responses to volatile compounds by walking insects, whereas a wind tunnel detects long-range flight responses (Du *et al.*, 1996). Du *et al.* (1996, 1998) showed that the same plant semiochemicals were attractive to insects in both kinds of apparatus. Despite these findings in other systems, further work needs to be done in our tritrophic system to answer this interesting question.

In contrast with electrophysiological studies of plant–herbivore and flower–pollinator interactions (e.g., Bjostad, 1998; Zhu *et al.*, 1999; Park *et al.*, 2002; Aldrich *et al.*, 2003; Andersson, 2003), there have been few similar studies on parasitoid wasp species (Du *et al.*, 1998; Park *et al.*, 2001, 2002; Smid *et al.*, 2002). This is probably due to differences in antennal sensitivity to plant volatiles between herbivores or pollinators and carnivores (Bjostad, 1998). In most cases, a Y-splitter was used to provide equal amounts of effluent from the column to parasitoid antennae and to a FID (Du *et al.*, 1998; Ngi-Song *et al.*, 2000; Park *et al.*, 2001, 2002; Smid *et al.*, 2002). In order to enhance the detection of volatiles by parasitoid antennae, headspace extracts have always been condensed to relatively high concentrations (e.g., Ngi-Song *et al.*, 2000; Smid *et al.*, 2002). In this study, instead of using a Y-splitter, we used a flexible connector with makeup gas to transfer a greater proportion of the effluent from the column to the insect antennae and a lesser proportion to the FID and obtained the promising results (Figure 2).

In our GC–EAD experiments, at the beginning, the retention time of the compounds (3*Z*)-hexen-1-ol and (*syn*)-2-methylpropanal oxime and the EAD responses of the parasitoid to these were separated by an HP-INNOWAX column (Figure 2A). However, after about 4 months, with several changes of the column, the retention time of these two compounds overlapped on this column in some experiments (Figure 2B,C). Nonetheless, we still detected separate EAD responses to these two compounds when responses were amplified by the software (Figure 2B,C and Table 2). A tentative explanation for this phenomenon is that the efficiency of the column decreased during the course of experiments (Prof. C.H. Zhao, Institute of Zoology, Chinese Academy of Science, Beijing, personal communication). In our study, headspace extracts from *L. sativae* larvae–damaged bean plants were analyzed by coupled GC–EAD with this column before we measured the other two samples.

A legitimate criticism of many Y-tube experiments is the failure to take into account mistakes made by insects during initial exploration of the apparatus (Du *et al.*, 1996). Du *et al.* (1996) suggested that recording insects' final choices after a set time period would solve this problem. In addition, we assumed that parasitoids might spend longer time in the arm of the device containing the preferred odor. Therefore, we also measured the time they spent in each arm of the olfactometer. Our data show that the first choice responses of naive female *O. dissitus* are always consistent with the arm of the device in which they spent the greatest amount of time but not with the insects' final choice (Figures 1 and 3). For instance, irrespective of these differences in the insects' first and final choices, they tended to spend a greater amount of time exploring the arm of the olfactometer containing any of the seven volatile compounds than that containing the hexane control (Figure 3). This suggests that recording the time insects spend in each arm of the Y-tube, in addition to recording their first choice, is more reliable than just recording final choice. Just recording insects' first choice may be sufficient and would greatly reduce experimental time.

In the wind tunnel study of Petitt *et al.* (1992), *O. dissitus* preferentially landed on leaf miner–infested, rather than noninfested, potted lima bean plants, and this preference was confirmed by choice experiments in a four-armed olfactometer. The present study further confirms that volatile plant extracts can have the same effect on insect behavior as entire plants. Petitt *et al.* (1992) demonstrated that female *O. disstus* that had been exposed to lima bean plants preferred the odor of infested lima bean plants over that of infested aubergine or cotton plants but that an oviposition experience on these plant species caused a dramatic change in preference that was due, at least in part, to associative learning. This suggests that *O. dissitus* has a level of behavioral plasticity in host selection that involves learning and context-dependent analysis of chemical cues as other parasitic wasps (e.g., Turlings *et al.*, 1990, 1995; Vet and Dicke, 1992; Meiners *et al.*, 2003; Olson *et al.*, 2003). It is possible that learning may allow *O. dissitus* to distinguish between the odors of plants with different types of damage, thereby enabling them to distinguish those that have been damaged by potential hosts from those damaged by other factors.

Summary

This study provides an example of online detection (GC–EAD) of active compounds in headspace collections and subsequent behavioral responses of insects to these volatiles. Through this dual approach, we were able to identify active compounds in complex headspace blends and confirm that these compounds attracted female *O. dissitus* to their host. To the best of our knowledge, this is the first evidence that herbivore-induced volatile oximes function as synomones attracting parasitoids to hosts. We conclude that, in the *P. vulgaris–Liriomyza* spp.–*O. dissitus* tritrophic system, feeding by *L. huidobrensis* or *L. sativae* larvae on bean plants induces the emission of synomones, green leafy, tereponids, and oximes that individually or collectively attract the parasitoid, *O. dissitus*, to its host habitat. It is evident that, in this tritrophic system, *O. dissitus* can overcome the reliablility–detectablility problem (Vet and Dicke, 1992) by following cues released by plants on which the appropriate host leaf miners have fed. We hypothesize that the predominant compounds in herbivore-induced volatile blends play an important role in mediating parasitoid search behavior over relatively long distances, while secondary and minor compounds improve the precision of host location over short distances. Testing this hypothesis will require further research on the behavioral responses of parasitoids to different volatile compounds by comparative studies.

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