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Identification of *Mythmna separata*-induced maize volatile synomones that attract the parasitoid *Campoletis chlorideae*

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Abstract: *Campoletis chlorideae* Uchida (Hym., Ichneumonidae) is an important solitary koinobiont larval endoparasitoid of many noctuid species including *Mythmna separata* (Lep., Noctuidae). In the present study, maize volatiles induced by feeding damage of *M. separata* were found to be attractive to *C. chlorideae* in a Y-tube olfactometer. Eleven compounds were released in significant amounts from *M. separata*-infested maize seedlings, of which five compounds, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, linalool and phenylethyl acetate were chosen for electrophysiological and behavioural tests. All five compounds elicited electroantennogram responses in *C. chlorideae*. However, only pure (*Z*)-3-hexenyl acetate and linalool were attractive to the parasitoid in the Y-tube. Interestingly, linalool was also attractive to starved parasitoids over a range of doses tested. These results suggested that (*Z*)-3-hexenyl acetate and linalool may be key infochemicals in the host-foraging behaviour of *C. chlorideae*, and linalool may act as a food-source signal for the parasitoid.

Key words: *Campoletis chlorideae*, *Zea mays*, behavioural response, electrophysiological response, herbivore-induced plant volatiles, synomones

1 Introduction

Adult parasitoids must not only find hosts for reproduction purposes but must also locate food sources to meet their short-term nutritional needs. Ample evidence demonstrates that plant volatiles play an important role as food- or host-location cues in the foraging behaviour of parasitoids (Vet and Dicke 1992; Wäckers 1994), and that the physiological states of parasitoids influence their foraging behaviour (Lewis et al. 1998). Starved parasitoids actively responded to food-related odours, whereas satiated parasitoids preferred host-associated odours (e.g. Wäckers 1994). However, there is an overlap between the spectrum of volatiles induced by herbivore feeding on vegetative plant tissues and that of floral volatiles (Turlings et al. 1993; Röse and Tumlinson 2004). Some of these shared compounds may serve as cues for host and food location of parasitoids. It is essential to understand the function of the plant volatiles in mediating foraging behaviour of a parasitoid species considered as a potential biological control agent.

Recording of electroantennograms (EAGs) is useful for screening volatile compounds with potential attractiveness to parasitoids. An EAG response profile is thought to reflect the sensitivity and/or relative abundance of olfactory antennal receptor neurones that respond to the compounds tested. This profile is helpful in the identification of potentially active compounds, but EAG activity does not necessarily indicate behavioural activity of the pure compound (Li et al. 1992; Park et al. 2001). Behavioural bio-assays are therefore indispensable to demonstrate that single volatile compounds or blends are used as infochemicals by the parasitoid.

Campoletis chlorideae Uchida is an important larval endoparasitoid of the early instars of many noctuid species, including *Helicoverpa armigera*, *Helicoverpa assulta*, *Spodoptera litura*, *Spodoptera exigua*, *Agrotis ipsylon*, *Anomis flava*, *Mythmna separata* and *Acantholeucania loreyi* (Sato 1988; Li et al. 1997; Yan et al. 2001; He et al. 2002a,b; Hou et al. 2002; Guo et al. 2003). The parasitoid has been extensively studied as a potential biological control agent of *H. armigera* in China, Korea and India (e.g. Zheng and Lu 1981; Dai 1990; Nandihalli and Lee 1995a,b; Kumar et al. 2000; Wang 2001; You et al. 2002; Liu et al. 2004; Pandey et al. 2004). However, little is known about its host-foraging behaviour. In the present study, we investigated the behavioural responses of *C. chlorideae* to several single compounds that were emitted by *M. separata*-infested maize plants to establish the relative importance of these plant volatiles in host location of *C. chlorideae*.

2 Materials and Methods

2.1 Plants

Zea mays L. (Maize: Gramineae) cultivar 'Zhongdan-306' obtained from the Institute of Crop Breeding and Cultivation, Chinese Academy of Agricultural Sciences (CAAS), Beijing was used in all the experiments. Four maize seeds were planted in a flowerpot [16 cm (diameter) × 15 cm (deep)] using fertilized soil obtained from the Institute of Vegetables and Flowers, CAAS, Beijing. The pots were kept outdoors under natural conditions at temperatures of 24–33°C, day length 14–15 h from June to September 2004, and were watered every day. A gauze cage (3 m length, 3 m width, 2 m height) was later installed on the growing potted seedlings to prevent infestation by naturally occurring herbivores.

Two-week-old maize seedlings with three to four leaves were used for volatile extraction and behavioural bioassays. Maize seedlings were uprooted from the pots and the soil was gently washed off the roots with tap water. The seedlings were then transferred to a vial (100 ml) filled with water. A batch of three maize seedlings were either left undamaged (control) or subjected to infestation by nine fourth-instar *M. separata* larvae, which have been previously starved for about 10 h. All these were performed between 08:00 and 09:00 hours (local time).

2.2 Insects

Mythmna separata were reared on artificial diets at 26 ± 1°C, 75% relative humidity (RH), 16 : 8 h (light : dark) as described in Zhang and Wang (2003). A colony of the parasitoid *C. chlorideae* was reared with cocoons collected from Zhengzhou, Henan province, China and was maintained on *M. separata* larvae fed with artificial diet. Mated female wasps were allowed to sting host larvae in their late second or early third instar once or twice, and these parasitized host larvae were kept in an incubator under the rearing conditions earlier described until cocoon formation. Fifteen cocoons were collected and kept in a glass tube (2 cm diameter, 10 cm length) plugged with cotton wool until adult emergence. Twenty adults were kept in a cage (10 cm diameter, 20 cm length) in a sex ratio of 1 : 1. Two- to 3-day-old mated female parasitoids were used for behavioural and EAG tests. Satiated parasitoids were allowed to feed on honey solution (20% v:v) every day, while starved parasitoids were deprived of food supply after 2 days of feeding on honey solution and were given water only during the 24 h preceding the behavioural test.

2.3 Test compounds

Chemicals, (*Z*)-3-hexen-1-ol (98%) and (*Z*)-3-hexenyl acetate (97%) from Roth KG Company (Karlsruhe, Germany), (*E*)-2-hexenal (97%), linalool (97%) and phenylethyl acetate (99%) from Fluka Chemie Company (Buchs, Switzerland), were used for EAG and behavioural tests. Each compound was dissolved into mineral oil (Sigma Chemical Company, St Louis, MO, USA) to obtain 10 μg/μl solutions. For dose–response tests of linalool, 100 μg/μl solution was prepared and diluted to give 0.01, 0.1, 1, 10 μg/μl solutions. The solutions were kept in a refrigerator at −20°C until used.

2.4 Volatile collection

After receiving nine fourth-instar caterpillars, plants with insects were immediately put into a glass jar (12 cm diameter, 21 cm high) for volatile collection. Volatiles were collected using a push–pull technique (compressed air and vacuum). Clean air was led through a flowmeter for measuring and regulating the air flow, a charcoal filter for purification, and a water bubbler for humidification. The pure and moist air then entered the jar at 300 ml/min from the lower part of the jar, passed over the infested plants, and then passed through an outlet in the top of the jar. Volatiles were trapped in a glass tube (10 cm long, 6 mm diam) containing 25 mg of 80/100 mesh Super Q adsorbent (Altech Assoc., Deerfield, IL). The trap was connected through Teflon tubing to the outlet of the jar at one end, and via another flowmeter at the other end to a vacuum pump. During the collection, the jar containing the plants was kept on ice to keep the inner temperature 25 ± 2°C, and two fluorescent lamps (each 40 W) were suspended over the jar to illuminate the plants, producing a light intensity of about 2000 lx. Two collection systems, one containing uninfested control plants and the other infested plants, were used in parallel every time and the collection was run for 12 h. Collection was repeated five times with fresh batches of plants on different days.

After collection, the trap was rinsed with 200 μl of redistilled hexane. Two internal standards (800 ng of n-decane and benzyl acetate in 10 μl hexane) were added based on the knowledge obtained from preliminary experiments showing that the two compounds were not released from both undamaged and herbivore-infested plants. Identifications and quantifications of volatiles were carried out by coupled gas chromatography-mass spectrometry (GC-MS) on a Hewlett-Packard 6890 GC–5973 MSD (Agilent Technologies Inc., Wilmington, DE, USA). The GC was equipped with a DB-WAX column (Agilent Technologies, Palo Alto, CA) (polyethylene glycol 20000, 60 m × 0.25 mm ID; film thickness 0.15 μm). Helium was used as the carrier gas with a flow velocity of 26 cm/s. A 2-μl aliquot of volatile samples was injected, and then immediately split with a purge flow of 30 ml/min. The injector temperature was 250°C and the GC-MS transfer line temperature was 280°C, source 230°C, quadrupole 150°C, ionization potential 70 eV, and scan range 30–300 m/z. Following injection, the column temperature was increased from 55 to 200°C at 8°C /min. Compounds were identified by comparing mass spectra with NIST library spectra (Agilent Technologies), and (*E*)-2-hexenal, hexyl acetate, (3*E*)-4,8-dimethyl-1,3–7-nonatriene, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, linalool, (*E*)-β-farnesene and phenylethyl acetate were confirmed with authentic reference compounds. Compounds were quantified by their total ion abundances relative to that of the internal standards.

2.5 Antennal preparation and EAG recording

Two to 3-day-old adult female parasitoids were used for EAG tests. The head was detached, and one or two distal segments of the antenna used for signal recording were cut off. A glass capillary tube filled with Ringer's solution was inserted into a compound eye and served as a reference electrode. Another glass capillary was connected to the cut antennal tip and served as the recording electrode. Silver-silver chloride junctions were used in the capillary electrodes and connected to the entrance of the amplifier. The EAG signal recorded with the electrode was first amplified with a DC/AC preamplifier (Syntech UN-06), and further processed with an Autospike software (Syntech®, Hilversum, The Netherlands). Each compound was tested on 15 individuals. Each antenna was used to test 20–30 compounds.

An aliquot of 10 μl of test solution was pipetted to a piece of filter paper (5 × 50 mm), which was immediately inserted into a glass Pasteur pipette (5 mm in diameter, 15 mm in

length). After loading the filter paper, the pipette was sealed with Parafilm at the two ends until it was used for odour presentation (within 1 h). The narrow end of the Pasteur pipette was inserted through a small hole in the wall of a steel tube (3 cm from the hole to the outlet of the tube). Antennae were positioned approximately 2 cm from the outlet of the steel tube. For stimulation, 2 ml of purified, charcoal-filtered air was led through the Pasteur pipette cartridge for 0.1 s into the main air stream running through the tube (30 ml/min). An electrically controlled airflow controller (Syntech CS-05) triggered by manual operation was used for the stimulation. At least 60 s was allowed between successive stimulations, and the stimulations with different odorants were made in random order. For dose–response experiments, exposure proceeded from lowest to highest concentration to minimize the effect of olfactory adaptation possibly resulting from strong stimulation. Ten microlitres of mineral oil and 100 μg of (*Z*)-3-hexen-1-ol in 10 μl mineral oil were used as control and standard stimulus, respectively. In a test series of odorants or dosages, control and standard stimuli were applied subsequently after three successive stimulations. Normalization was made by dividing the peak EAG amplitude of the test puff with the average EAG amplitude of the two nearest standard stimulations after the subtraction of the amplitude recorded in response to the control.

2.6 Choice experiments

A Y-tube olfactometer was used to test the attractiveness of plant volatiles and single compounds to *C. chlorideae* females while Erlenmeyer flasks (1000 ml) were used as odour containers. The system consisted of a central tube (10 cm long, 4 cm diameter) and two lateral arms (20 cm long, 4 cm diameter). The arms were divided into basal and terminal segments (10 cm each) connected to each other with tight glass–glass joints, and the terminal segments were connected to the flask with Teflon tubing. A white paper was placed under the Y-tube, and two fluorescent lamps (each 40 W) were suspended over the Y-tube to produce a light intensity of 2000 lx. Charcoal-purified and water-humidified air was passed into each flask and then into each arm of the olfactometer at 500 ml/min and was drawn out of the base of the olfactometer by a vacuum pump.

After receiving nine fourth-instar caterpillars, a batch of three plants used for bioassay was caged in a fine gauze bag. Undamaged plants were also caged in a similar bag. The bag and caterpillars were removed after 6 h of treatment, and the plants were subsequently transferred to the flask. For the tests of single compounds, a 10-μl of solution was applied onto a filter paper (1×2 cm), and the filter paper was immediately placed into the flask.

Each parasitoid was used only once. They were offered the choice between odours from herbivore-infested and intact maize plants, or between single compounds and mineral oil (control). Using a small glass vial (2 ml), naïve (no experience with hosts, plants and tested compounds) female parasitoids were individually released at the base of the central arm of the Y-tube and observed for 5 min. If a parasitoid did not make a choice after this period, it was removed and recorded as 'no choice'. Parasitoids that walked to the end of the basal arms or into the terminal arms and stayed there at least 5 s were recorded as having made a choice for the odour offered through that arm. After five individuals were tested, the olfactometer was turned such that the position of the arms was reversed, and the flasks were switched. Then another five parasitoids were tested. During the test, the room temperature was kept at 25 ± 1°C. The olfactometer and the glass jar were cleaned with water, ethanol and acetone at the end of each day.

Only one choice situation was tested each day. The choice between herbivore-infested plants and undamaged plants was repeated three times each with new plants on different days. For choices between single compounds and mineral oil, tested compounds and mineral oil were renewed every 5–10 min (with two to three individuals tested), and each combination was repeated three times on different days. In total, at least 70 parasitoids were tested for each choice situation.

2.7 Statistical analysis

Tukey multiple comparison test after ANOVA was used to determine statistical differences of the EAG responses of female parasitoids to compounds. Chi-squared analysis was performed to test the significance of differences between numbers of wasps that made a choice between the two odour sources offered (a 50:50 probability was set between the numbers of wasps walking to the control side and the test side in the Y-tube). Wasps that were scored as *no choice* were excluded from statistical analysis. Analyses were carried out with SPSS 10.0 for Windows (SPSS Inc., Chicago, IL) (Lu 2000).

3 Results

3.1 Volatile compounds emitted from herbivore-infested maize seedlings and their attractiveness to *C. chlorideae*

Gas chromatography-MS analysis identified 11 compounds in *M. separata*-infested maize plants, whereas only two compounds, (*E*)-2-hexen-1-ol and (*E*)-β-farnesene, were released in significant amounts from undamaged maize plants (fig. 1). In a Y-tube olfactometer, parasitoids showed a significant preference ($\chi^2 = 9.80$, $P < 0.01$) for herbivore-induced maize volatiles to that emitted from undamaged maize plants (fig. 2).

3.2 EAG and behavioural responses of *C. chlorideae* to single compounds

We chose five compounds that were commonly emitted from both our maize variety and other maize varieties (Fritzsche Hoballah et al. 2002) for EAG and behavioural tests. Mineral oil causes little EAG response (0.47 ± 0.04 mV, $n = 15$) in *C. chlorideae*. For the first stimulations, the EAG amplitude in response to the standard stimulus (100 μg of (*Z*)-3-hexen-1-ol) was 14.82 ± 0.65 mV (mean ± SE, $n = 15$). All other tested compounds triggered significant EAG responses (fig. 3). When the attractiveness of all compounds was tested in a Y-tube olfactometer with starved and satiated wasps, only (*Z*)-3-hexenyl acetate ($\chi^2 = 4.19$, $P = 0.041$) and linalool ($\chi^2 = 7.19$, $P < 0.01$) were attractive to the food-satiated parasitoids (fig. 4). Interestingly, linalool was also attractive to the parasitoids that were starved for 24 h ($\chi^2 = 32.34$, $P < 0.01$; fig. 4).

We further investigated EAG and behavioural dose–response relationships of *C. chlorideae* to linalool. With doses >10 μg in the odour cartridge, linalool elicited significant EAG responses (fig. 5). Doses of

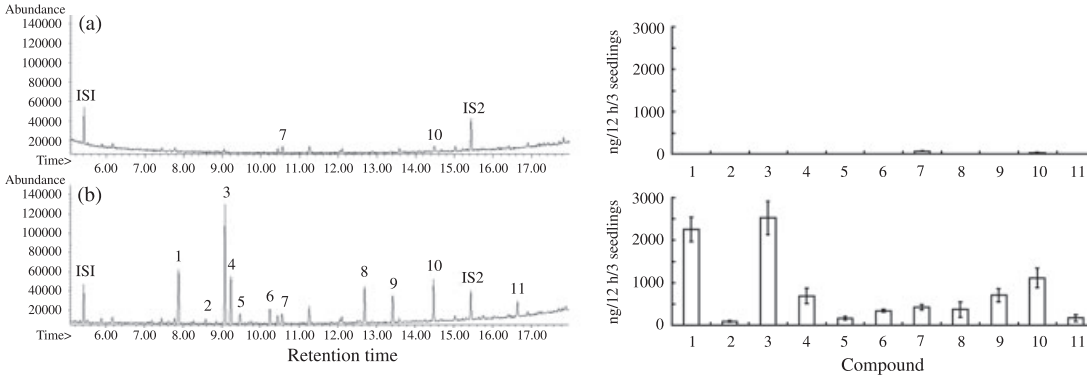

Fig. 1. *Representative total ion current chromatograms of the headspace volatiles from undamaged (a) and* M. separata*-infested (b) maize seedlings. Volatiles were collected for 12 h (daytime). Peak identities are 1, (E)-2-hexenal; 2, hexyl acetate; 3, (3E)-4,8-dimethyl-1,3–7-nonatriene; 4, (Z)-3-hexenyl acetate; 5, '(E)-2-hexenyl acetate'; 6, (Z)-3-hexen-1-ol; 7, (E)-2-hexen-1-ol; 8, linalool; 9, '(E)-α-bergamotene'; 10, (E)-β-farnesene; 11, phenylethyl acetate (compounds within quotation marks were tentatively identified based on more than 90% identity of mass spectrum with that in the NIST-library). IS1 and IS2 are the internal standards n-decane and benzyl acetate. The bar graph to the right of each chromatogram represents an average amount of each volatile from five replications (mean ± SE, n = 5)*

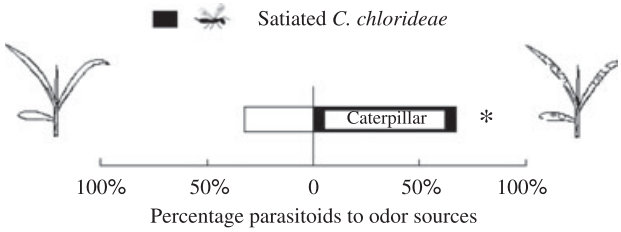

Fig. 2. *Choice distribution (%) of satiated* C. chlorideae *in dual choice tests between undamaged and* M. separata*-infested maize seedlings in a Y tube. Asterisk indicates a significant difference (chi-squared test, P < 0.05)*

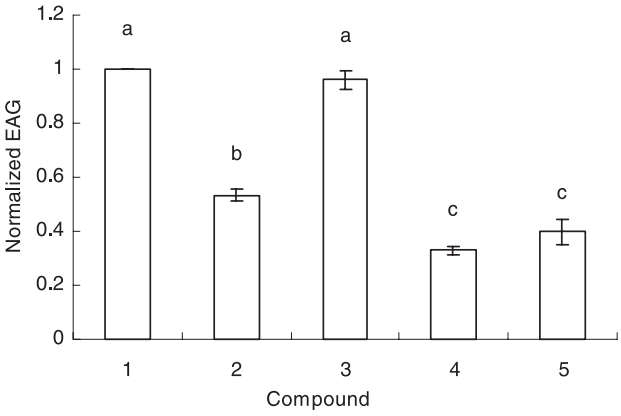

Fig. 3. *EAG response profiles of female* C. chlorideae *to 100 μg of five compounds. EAG responses were normalized against mean EAG response to 100 μg of standard stimulus, 1, (Z)-3-hexen-1-ol (mean ± SE, n = 15); 2, (Z)-3-hexenyl acetate; 3, (E)-2-hexenal; 4, linalool; 5, phenylethyl acetate. Different letters indicate significant differences (Tukey's multiple comparison test after ANOVA, P < 0.05)*

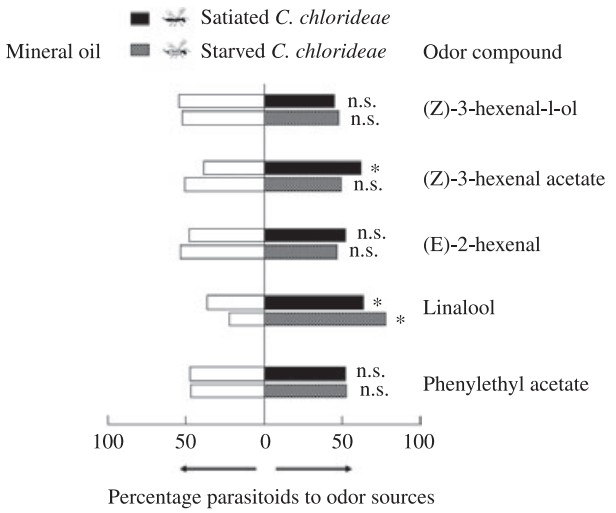

Fig. 4. *Choice distribution of satiated (■) and starved (▩)* C. chlorideae *in dual choice tests between mineral oil (control) and five pure compounds (dissolved in mineral oil) in a Y tube. Asterisks indicate a significant difference (chi-squared test, P < 0.05). n.s., not significant*

1–100 μg produced attraction to the parasitoids in the Y-tube (fig. 6). In each choice situation, individuals that made no choice made up <15% of the total number of parasitoids tested.

4 Discussion

In the present study, *M. separata*-induced maize volatiles produced attraction of the generalist parasitoid *C. chlorideae* (fig. 2). Linking plant volatiles analysis to behavioural observations, we demonstrated that two herbivore-induced components, (*Z*)-3-hexenyl acetate and linalool, were effective

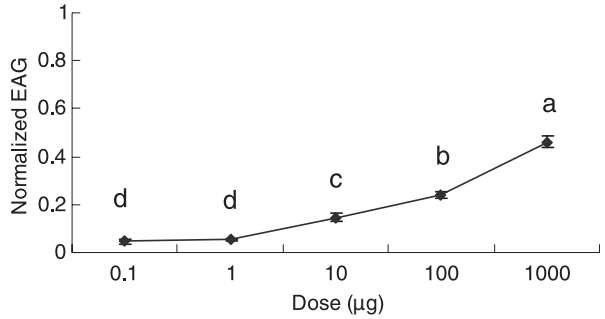

Fig. 5. *EAG dose–response curves of female* C. chlorideae *to linalool. Dose indicates the amount of compound (µg) loaded onto a piece of filter paper in the odour cartridge. EAG responses were normalized against the mean EAG response to 100 µg of standard stimulus, (Z)-3-hexen-1-ol (mean ± SE, n = 15). Different letters indicate significant differences (Tukey's multiple comparison test after* ANOVA, *P < 0.05)*

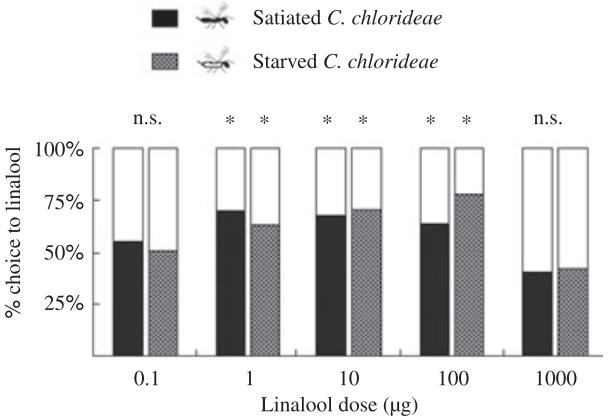

Fig. 6. *Choice distribution of satiated (■) and starved (▩)* C. chlorideae *in dual choice tests between mineral oil (control) and a range doses of linalool (dissolved in mineral oil) in a Y tube. Asterisks indicate a significant difference within a choice test (chi-squared test, P < 0.05). n.s., not significant*

attractants to satiated female parasitoids. Interestingly, linalool also resulted in attraction of starved parasitoids.

Herbivore-induced plant volatiles are blends of 20–200 compounds. These compounds are used to a different extent in different species of natural enemies (Steidle and van Loon 2003). In some cases, the presence of certain single key compounds is sufficient to attract natural enemies (Kessler and Baldwin 2001; De Boer and Dicke 2004), while in other cases natural enemies seem to require a mixture for a full behavioural response (De Bruyne et al. 1991; Smid et al. 2002). It is a difficult task to identify which herbivore-induced plant volatiles in a blend are responsible for the attraction of parasitoids. Only in a few plant–herbivore–natural enemy systems are some behaviourally active components out of the total blend of such volatiles are known (Dicke et al. 1990; Turlings et al. 1991; Scutareanu et al. 1997; Du et al. 1998; Turlings and Fritzsche 1999). The approach that combines GC with electrophysiology can reveal those compounds that are perceived by peripheral chemoreceptors of the parasitoids (Smid et al. 2002). Based on such knowledge, blend components that show electrophysiological activities are selected for behavioural testing. Using EAG recordings, we demonstrated that all five compounds, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, linalool and phenylethyl acetate could be perceived by *C. chlorideae* (fig. 3). However, in the Y-tube only linalool and (*Z*)-3-hexenyl acetate showed attractiveness to the satiated parasitoids (fig. 4). Thus, these two compounds might be key components in herbivore-induced maize volatiles for the attraction of *C. chlorideae*. Considering the quite often occurrence of (*Z*)-3-hexenyl acetate and linalool as herbivore induced synomones, the results of the current study agree with the notion that generalist parasitoids tend to innately use general plant compounds for host location (Vet and Dicke 1992; Steidle and van Loon 2003).

Parasitoids are also known to use plant volatiles for food location (Takasu and Lewis 1993a; Wäckers 1994; Sirot and Bernstein 1996; Lewis et al. 1998; Jacob and Evans 2001). It is expected that compounds commonly occurring in floral blends will be the main triggers of innate flower responses in generalist flower foragers, such as insect parasitoids (Wäckers 1994). Linalool is one of such commonly occurring floral compounds (Knudsen et al. 1993). This compound, accounted for 95% of total floral fragrance of *Daphne mezereum* (Borg Karlson et al. 1995). Linalool is known to be attractive to a broad spectrum of pollinators, herbivores and parasitoids (Henning et al. 1992; Borg Karlson et al. 1995; Du et al. 1998; Raguso and Pichersky 1999). Here we present evidence that linalool is a food foraging infochemical for the parasitoid *C. chlorideae* (fig. 6).

Parasitoids were also stimulated and attracted by nectar odours (Patt et al. 1997). It is usually assumed that parasitoids have innate responses to food-associated scents, but learning processes can modify these responses (Lewis and Takasu 1990; Takasu and Lewis 1993b, 1996). The rearing history of *C. chlorideae* on honey solution may have exposed the parasitoid to linalool, a common scent compound of honey. It is possible therefore that the attractiveness of linalool to starved *C. chlorideae* might be the result of learning or experience.

In summary, the current study demonstrated that two herbivore-induced maize components, (*Z*)-3-hexenyl acetate and linalool, were attractive to the generalist parasitoid *C. chlorideae*. In addition, linalool also evoked attraction of starved parasitoids. These results indicate that (*Z*)-3-hexenyl acetate and linalool may be infochemicals used by *C. chlorideae* in its host foraging behaviour, and linalool may also be an indicator of food for the parasitoid. In our future work, we will evaluate the infochemical function of the two compounds under field conditions.

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