In Situ Modification of Herbivore-Induced Plant Odors: A Novel Approach to Study the Attractiveness of Volatile Organic Compounds to Parasitic Wasps

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Abstract

Many parasitic wasps (parasitoids) exploit volatile organic compounds (VOCs) emitted by herbivore-infested plants in order to locate their hosts, but it remains largely unknown which specific compounds within the volatile blends elicit the attractiveness to parasitoids. One way of studying the importance of specific VOCs is to test the attractiveness of odor blends from which certain compounds have been emitted. We used this approach by testing the attraction of naive and experienced females of the two parasitoids Cotesia marginiventris and Microplitis rufiventris to partially altered volatile blends of maize seedlings (Zea mays var. Delprim) infested with Spodoptera littoralis larvae. Adsorbing filter tubes containing carbotrap-C or silica were installed in a four-arm olfactometer between the odor source vessels and the arms of the olfactometer. The blends breaking through were tested for chemical composition and attractiveness to the wasps. Carbotrap-C adsorbed most of the sesquiterpenes, but the break-through blend remained attractive to naive C. marginiventris females. Silica adsorbed only some of the more polar VOCs, but this essentially eliminated all attractiveness to naive C. marginiventris, implying that among the adsorbed compounds there are some that play key roles in the attraction. Unlike C. marginiventris, M. rufiventris was still attracted to the latter blend, showing that parasitoids with a comparable biology may employ different strategies in their use of plant-provided cues to locate hosts. Results from similar experiments with modified odor blends of caterpillar-infested cowpea (Vigna unguiculata) indicate that key VOCs in different plant species vary greatly in quality and/or quantity. Finally, experienced wasps were more strongly attracted to a specific blend after they perceived the blend while ovipositing in a host. Considering the high number of distinct adsorbing materials available today, this in situ modification of complex volatile blends provides a new and promising approach pinpointing on key attractants within these blends. Advantages and disadvantages compared to other approaches are discussed.

Key words: host location, indirect plant defense, induced plant odors, olfactometer, parasitoids, tritrophic interactions

Introduction

Herbivore-induced plant volatiles are known to play an important role in the interactions between plants and arthropods (Dicke et al., 2003b; Turlings and Wackers, 2004; van Poecke and Dicke, 2004; Arimura et al., 2005). These highly detectable volatile organic compounds (VOCs) may act either directly, for example, by deterring oviposition by lepidopteran herbivores (Landolt, 1993; De Moraes et al., 2001; Kessler and Baldwin, 2001), or indirectly, by attracting natural enemies of herbivores (Dicke and Sabelis, 1988; Turlings et al., 1990). In addition, there is growing evidence that herbivore-induced VOCs are involved in chemical information transfer between plants (Arimura et al., 2000; Baldwin et al., 2002; Engelberth et al., 2004).

Plants are known to emit more than 1000 different VOCs, including alkanes, alkenes, alcohols, ketones, aldehydes, ethers, esters, and carboxylic acids (Dudareva et al., 2004; Niinemets et al., 2004). Some VOCs are taxon specific, such

as the glucosinolate breakdown products in Brassica species (Mattiacci et al., 1995), whereas others appear to be common to many different plant families (Van Den Boom et al., 2004). These common VOCs include the "green-leaf volatiles" (C6 aldehydes, alcohols, and derivatives), cyclic and acyclic terpenes, phenolic compounds, and nitrogenous compounds (Dicke, 1999b; Paré and Tumlinson, 1999). The induction and release of such compounds is dependent on the interaction of biotic factors, such as plant hormones (de Bruxelles and Roberts, 2001; Thaler et al., 2002; Farmer et al., 2003; Rojo et al., 2003; Schmelz et al., 2003; Ament et al., 2004; van Poecke and Dicke, 2004), herbivore-derived elicitors (Mattiacci et al., 1995; Alborn et al., 1997; Halitschke et al., 2001; Spiteller and Boland, 2003; Merkx-Jacques and Bede, 2004), and associated microorganisms (Cardoza et al., 2002), and abiotic factors, such as wounding (Schmelz et al., 2001; Howe, 2004; Mithöfer et al., 2005), O_3 and CO_2

concentration (Vuorinen *et al.*, 2004a,b), UV radiation (Johnson *et al.*, 1999), heavy metals (Mithöfer *et al.*, 2004), temperature, and light (Takabayashi *et al.*, 1994; Gouinguené and Turlings, 2002). In addition, there is great variability in the composition of volatile blends among different plant genotypes within a plant species (Gouinguené *et al.*, 2001; Degen *et al.*, 2004). It is unlikely that every VOC emitted by plants serves as an ecological or physiological signaling compound (Penuelas and Llusia, 2004), but in only a few systems behavioral active compounds of the total blend have been identified (Du *et al.*, 1998; Powell *et al.*, 1998; de Boer and Dicke, 2004; de Boer *et al.*, 2004).

In this study, we address the question whether in complex odor blends emitted by *Spodoptera littoralis*–infested maize and cowpea seedlings there are key VOCs that mediate the attraction of two parasitoid species, *Cotesia marginiventris* and *Microplitis rufiventris*. Both species have previously been shown to be highly attracted by herbivore-induced VOCs, which are the main cues used by these parasitoids to locate their host habitat (Turlings *et al.*, 1991a,b, 2004; Fritzsche Hoballah *et al.*, 2002; Gouinguené *et al.*, 2003). Still, the use of induced volatiles differs between the two species. While naive *C. marginiventris* preferred blends with high amounts of green-leaf volatiles over blends with high amounts of sesquiterpenes, *M. rufiventris* did not show such a preference (Hoballah and Turlings, 2005). Here, we study the role of herbivore-induced VOCs for the attraction of these parasitoids in more detail. One way of studying the importance of individual VOCs is to compare the attractiveness of volatile blends differing in only few known compounds. These blends can be obtained by using different chemical elicitors (Dicke *et al.*, 1999; Turlings *et al.*, 2000) or by silencing genes involved in indirect defenses (Degenhardt *et al.*, 2003; van Poecke and Dicke, 2003; Kessler *et al.*, 2004). Confirmation of the importance of the missing VOCs can then be obtained by adding back synthetic compounds to the incomplete blends (de Boer and Dicke, 2004).

Here, we introduce a novel approach to obtain volatile blends of only partially different composition. Volatile blends were passed over adsorbing filters, which resulted in the adsorption of some VOCs, while others broke through and were measured and tested for attraction to naive and experienced parasitoid females. The results show that *C. marginiventris* and *M. rufiventris* use different cues and that some commonly induced VOCs have little or no impact on attraction, whereas other, minor, compounds are essential and highly attractive.

Materials and methods

Plants and plant treatments

Maize (*Zea mays* var. Delprim) and cowpea (*Vigna unguiculata*, Haeflinger, Herzogenbuchsee, Switzerland) were sown in plastic pots (10 cm high, 4-cm diameter) with fertilized commercial soil (Balkonerde, Coop, Switzerland) and grown at $27 \pm 2°C$, 60% relative humidity, 16:8 h light:dark (16L:8D), and 50,000 lm/m^2. Maize plants used for the experiments were 10–12 days old and had three fully developed leaves. Cowpea plants were 14–16 days old and had the cotyledons and six small leaves.

The evening before the experiments, plants and pots were introduced into the odor source vessels of an olfactometer (described by Turlings *et al.*, 2004) and infested with 20 second-instar *S. littoralis* larvae by releasing them in the whorl of the youngest leaf. After infestation, plants were kept under laboratory conditions with supplemented light ($26 \pm 3°C$, $40 \pm 10\%$ relative humidity, 16L:8D, and 10,000 lm/m^2) and were used for the experiments the day after, between 11:00 AM and 4:00 PM.

Insects and insect treatments

The caterpillar *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the solitary endoparasitoids *C. marginiventris* (Cresson) (Hymenoptera: Braconidae) and *M. rufiventris* (Kokujev) (Hymenoptera: Braconidae) were reared as described before (Turlings *et al.*, 2004). Adult parasitoids were kept in plastic cages at a sex ratio of approximately 1:2 (male:female) and were provided with moist cotton wool and honey as food source. The cages were kept in incubators (*C. maginiventris*: $25 \pm 1°C$; *M. rufiventris*: $23 \pm 1°C$; 16L:8D) and transferred to the laboratory 30 min before the experiments. We tested mated 2- to 4-day-old naive and experienced females. The latter were given experiences by allowing them to oviposit three to five times into second-instar *S. littoralis* larvae while simultaneously being exposed to the complete blend ("no filter", see subsequently). Experienced wasps were kept separately in small plastic boxes with moist cotton wool and honey and released in the olfactometer 1–3 h after their oviposition experience.

Olfactometer bioassays

To test the attractiveness of various herbivore-induced volatile blends to *C. maginiventris* and *M. rufiventris*, we used a four-arm olfactometer (Figure 1), which was modified after the six-arm olfactometer used in earlier studies (Turlings *et al.*, 2004). The olfactometer consisted of a central glass chamber [6-cm internal diameter (ID), 5-cm length] with four arms (15-mm ID, 5-cm length), each with a glass elbow (5-cm length) and an upward connection for an insect-trapping bulb (50 ml). Each glass elbow had a horizontal opening for a volatile collection trap (see subsequently) and was connected via a glass tube (4-mm ID, 8-cm length) to a glass vessel that contained the odor source. This connecting tube was either empty (controls) or contained an adsorbing material to filter out specific compounds from the blend emitted by the odor source. All parts were connected either via male/female ground glass connectors or via Teflon-coated GL-screw cap fittings.
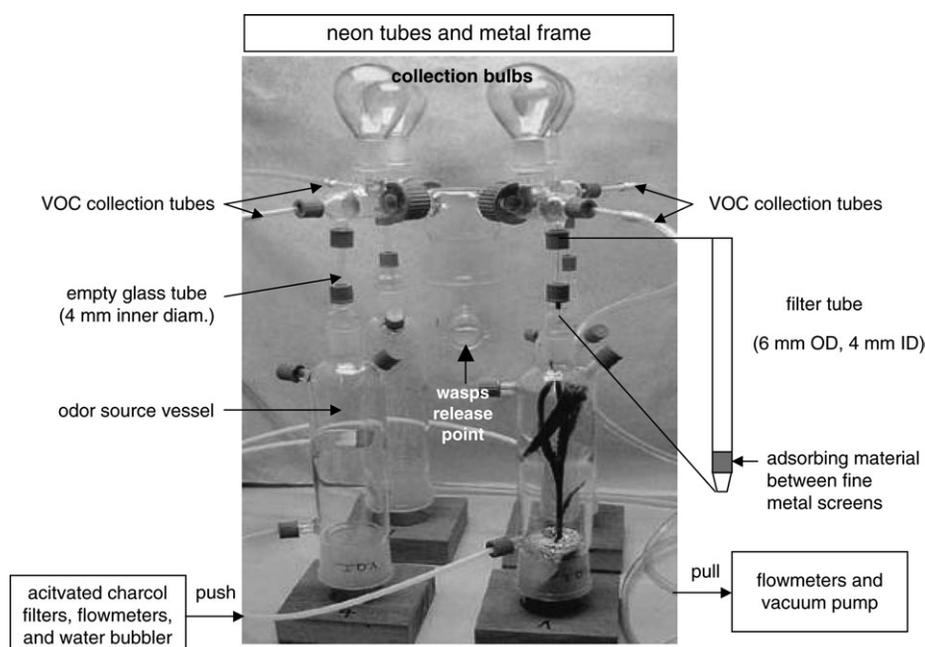
Figure 1 Picture and schematic representation of the four-arm olfactometer and the adsorbing filter tube.

Purified and humidified air entered each odor source vessel at 1.2 l/min (adjusted by a manifold with four flow meters; Analytical Research System, Gainesville, FL) via Teflon tubing and carried the VOCs through the connector tube to the elbows of the olfactometer. In these elbows, half of the air (0.6 l/min) was pulled out via the volatile collection traps (see subsequently) and the other half entered the central glass chamber.

Wasps were released in groups of six into the central glass chamber via a horizontally attached glass tube (6-cm ID, 10-cm length) with a 2.5-cm-ID opening. Wasps that entered an arm reached the elbow, where a stainless steel screen blocked their path. Eventually, they walked up in the direction of the light source above the olfactometer and into a trapping bulb, where they could easily be counted and removed. Ten neon tubes attached on a metal frame above the olfactometer provided approximately 7000 lm/m^2 at the height of the odor source vessels. To eliminate any visual distractions, a white cardboard cylinder was placed around the central chamber between the four odor source vessels (not shown in Figure 1). Wasps that did not enter a bulb or an elbow after 30 min were removed and considered having made "no choice." A total of four groups of six wasps were tested during a 2-h period, with naive and experienced groups released alternately. All experiments were run between 11:00 AM and 4:00 PM and repeated several times as indicated in Table 1.

Adsorbing filters and odor sources

Odour blends differing quantitatively and qualitatively in specific VOCs were obtained by passing air at 1.2 l/min containing the natural herbivore-induced blend (see Plants and Plant Treatments) over adsorbing filters of carbotrap-C (20–40 mesh, Supelco, Bellefonte, PA) or silica (63–200 mesh, 60 Å, Brunschwig, Basel, Switzerland). The adsorbing filters were positioned as indicated in Figure 1, and the resulting blends were tested in various experiments against positive controls, which consisted of the full odor blend of herbivore-infested seedling (no filter), and against negative controls, which consisted either of an empty vessel only (empty) or of an empty vessel with an adsorbing filter (e.g., empty and carbotrap filter) or with solvent on a filter paper (solvent) (Table 1). To obtain well-defined modified VOC blends, we selected different amounts of adsorbing materials, and we passed the whole blend for a certain prerun time (Table 1) over the filter before testing and sampling the blend for 2 h. Filters were prepared by filling various amounts (Table 1) of the adsorbents into the connection tube (8-cm length, 4-mm ID) sealed on both sides with a stainless steel screen mesh. Prior to each experiment, filters were rinsed with 3 ml of dichloromethane (Suprasolv, GC-grade, Merck, Dietikon, Switzerland) and baked for 4 h at 200°C. To standardize the adsorption of water, silica filters were rinsed with 100 μl Milli-Q water and dried in the humidified air stream of the olfactometer for 15 min before installing them into the olfactometer. Carbotrap-C filters were not rinsed with water because of the hydrophobic properties of this material. The silica extract consisted of VOCs that were extracted with 300 μl of dichloromethane from a 25-mg silica filter of a previous experiment. An aliquot of 100 μl of this extract was placed on a filter paper (1/2 disk, 50-mm diameter, Nr. LS 14, Schleicher and Schuell, Bottmingen, Switzerland) and

Table 1   Odour sources and experimental design

Experiment	Plant	Odor sources				Wasp	Replications of experiment
		Arm 1	Arm 2	Arm 3	Arm 4		
1	Maize	No filter (whole blend)	Carbotrap low (30 mg, prerun 3.5 h)	Carbotrap high (150 mg, prerun 0.5 h)	Empty	*C. marginiventris*	6
2	Maize	Carbotrap high (150 mg, prerun 0.5 h)	Empty and carbotrap filter (150 mg, prerun 15 min)	Empty	Empty	*C. marginiventris*	6
3	Maize	No filter (whole blend)	Silica low (12.5 mg, prerun 3.5 h)	Silica high (25 mg, prerun 0.5 h)	Empty	*C. marginiventris*	6
4	Maize	Silica high (25 mg, prerun 0.5 h)	Empty and silica filter (25 mg, prerun 15 min)	Empty	Empty	*C. marginiventris*	6
5	Maize	Silica high (25 mg, prerun 0.5 h)	Empty and silica filter (25 mg, prerun 15 min)	Empty	Empty	*M. rufiventris*	6
6	Maize	Silica extract (100 µl, prerun 2 h)	Solvent (100 µl, prerun 2 h)	Empty	Empty	*C. marginiventris*	4
7	Maize	Restored blend (silica high and extract)	Silica high and solvent	Empty	Empty	*C. marginiventris*	6
8	Cowpea	Silica high (25 mg, prerun 0.5 h)	Empty and silica filter (25 mg, prerun 15 min)	Empty	Empty	*C. marginiventris*	6

Further details on odor sources, number, and treatment of wasps are described in the text. Amounts of adsorbing materials or extracts and prerun time (time that the VOCs were passed over the filter before sampling and testing the blends) are indicated in parentheses.

introduced into an empty glass tube (4-mm ID, 8-cm length) that connected the odor vessel with the central chamber (Figure 1). The "restored blend" was obtained with a combination of the "silica high" filter and a silica extract, which was placed on filter paper after the silica filter. The positions of the odor sources were randomly chosen for different replications of the experiments.

Collection and analyses of VOCs

VOCs of each odor source were collected on a Super-Q trap (25 mg, 80–100 mesh, Alltech Associates, Inc., Deerfield, IL, described by Heath and Manukian, 1992) that was attached horizontally to the elbow of an olfactometer arm (Figure 1) and connected via Tygon tubing to a flow meter (Analytical Research System) and a vacuum pump. Air carrying the VOCs was pulled through each trap during the 2-h bioassay period at a rate of 0.6 l/min. Afterward, the traps were extracted with 150 µl dichloromethane, and 200 ng each of *n*-octane and *n*-nonyl acetate (Sigma, Buchs, Switzerland) in 10 µl dichloromethane was added to the samples as internal standards. Traps were washed with 3 ml of dichloromethane before reusing them for a next collection. VOCs adsorbed on the silica filters were extracted with 150 µl dichloromethane and VOCs on the carbotrap filters with 300 µl dichloromethane for subsequent analyses. Internal standards were added as described earlier. All solutions were stored at −76°C until analyses or bioassays.

VOCs were analyzed with a Hewlett Packard HP 6890 series gas chromatograph equipped with an automated col-

umn injection system (HP G1513 A) and a flame ionization detector. A 3-µl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25-mm ID, 0.25-µm film thickness, Alltech Associates, Inc.). Helium at constant pressure (18.55 psi) was used as carrier gas flow. Following injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C/min followed by a postrun of 5 min at 250°C. The detected VOCs were quantified based on a comparison of their peak areas with those of the internal standards (*n*-octane for compounds 1–14, *n*-nonyl acetate for compounds 15–27) and identified by comparison of retention times with those from previous analyses (Turlings *et al.*, 1998; Gouinguené *et al.*, 2001; Fritzsche Hoballah *et al.*, 2002). To confirm these identities, at least one sample per odor source was analyzed using a gas chromatograph (Agilent 6890 Series GC system G1530A), with the same kind of apolar column (HP-1) and an identical temperature program, coupled to a mass spectrometer operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33–280 amu). Mass spectra were compared with those of the NIST 02 library, and where necessary, spectra and retention times were compared with those of authentic standards. Compounds that were not identified by comparing retention times and spectra with those of pure standards are indicated in Table 2 and are labeled with a superscript N in the text, and their identification should be considered tentatively.

Statistical analyses

The functional relationship between parasitoids' behavioral responses and the different odor sources offered in the four-arm olfactometer was examined with a log linear model (a generalized linear model, GLM). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the overdispersion of wasps within the olfactometer (Turlings *et al.*, 2004). The model was fitted by maximum quasi-likelihood estimation in the software package R (version 1.9.1), and its adequacy was assessed through likelihood ratio statistics and examination of residuals. We tested treatment effects (=odor sources) for naive and experienced wasps individually. In addition, we tested if there was a significant effect of the experience and an interaction between treatment and experience.

The amounts of VOCs were analyzed using analyses of variance (ANOVAs) and *t*-tests. Amounts of VOCs that were not normally distributed were log(x + 1) transformed prior to analysis. Differences between the treatments were analyzed using the Tukey's test. All analyses were run on SigmaStat (version 2.0).

Results

Modification of induced maize blends over carbotrap-C filters

We detected 27 VOCs in quantifiable amounts in the unfiltered induced maize blend (no filter) (Figure 2A, Table 2). Terpenes (compounds 5, 8–10, 16–27) were the most abundant VOCs and made up more than 80% of the whole blend. Within the terpenes, the sesquiterpenes (*E*)-α-bergamotene and (*E*)-β-farnesene were the most dominant ones and made up more than 65% of all quantified terpenes. Furthermore, green-leaf VOCs (compounds 1–4, 6, 7), shikimic acid–derived compounds (11–13, 15), and an unknown compound (14) were detected in quantifiable amounts. (*Z*)-Jasmone, two oximes, and some other compounds were detected by gas chromatography–mass spectrometry in low quantities after concentrating the extract over nitrogen (data not shown). The blend "carbotrap low" (Figure 2B, Table 2) was lacking two unknown minor sesquiterpenes (21, 23) and (*E*)-nerolidol (26) and contained only trace amounts of the minor terpenes (compounds 17, 27) and of the unknown compound (14). Additionally, there was a significant reduction in the amounts of the two major sesquiterpenes, (*E*)-α-bergamotene (*t*-test, t_{16} = 2.64, P = 0.018) and (*E*)-β-farnesene (t_{16} = 4.06, P < 0.001), of the sesquiterpenes 18 (t_{16} = 2.82, P = 0.012), 24 (t_{16} = 5.31, P < 0.001), and 25 (t_{16} = 4.69, P < 0.001), and of geranyl acetate (t_{16} = 4.66, P < 0.001). The blend "carbotrap high" (Figure 2C, Table 2) was lacking all sesquiterpenes (compounds 16–26) and the homoterpene (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) and contained only trace amounts of (*Z*)-β-ocimene, benzyl acetate, and phenethyl acetate. One-way ANOVA indicated significant differences in the amounts of β-myrcene ($F_{2,27}$ = 9.19, P < 0.001), linalool ($F_{2,27}$ = 15.32, P < 0.001), (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) ($F_{2,27}$ = 58.71, P < 0.001), and indole ($F_{2,27}$ = 7.13, P = 0.003). No VOCs in quantifiable amounts were detected in the clean air or in clean air passed over filter tubes. Therefore, these blends are not shown in Figure 2.

In a first experiment, wasps had the choice between the whole blend, the modified blends, and clean air (experiment 1, Table 1). GLM indicates a significant difference between the four treatments for naive wasps ($F_{3,33}$ = 13.20, P < 0.001) as well as for experienced wasps ($F_{3,33}$ = 25.67, P < 0.001). Both groups of wasps were strongest attracted to the unfiltered blend no filter (Figure 3A). Naive wasps were also strongly attracted toward carbotrap low, and this attraction was not significantly different from attraction to the unfiltered blend (P = 0.35). In a following experiment, carbotrap high was tested alone against three arms with clean air (experiment 2, Table 1), and both naive and experienced wasps were still clearly attracted to this modified maize blend (naive: $F_{2,34}$= 16.59, P < 0.001; experienced: $F_{2,34}$= 21.43, P < 0.001) (Figure 3B). However, the overall responsiveness (=wasps that entered an arm) was relatively low (naive: 54%, experienced: 54%).

Modification of induced maize blends over silica filters

The VOCs detected in the blend no filter (Figure 2A, Table 2) were similar to those from the experiment with carbotrap filters described previously. The blend "silica low" (Figure 2D, Table 2) did not contain geranyl acetate, (*E*)-nerolidol, and a minor unknown compound (14). The blend silica high (Figure 2E, Table 2) was lacking the same compounds as well as methyl anthranilate and contained only trace amounts of linalool and phenethyl acetate. There was also a significant difference in the amounts of β-myrcene (one-way ANOVA, $F_{2,47}$ = 4.00, P = 0.025) and TMTT ($F_{2,47}$ = 25.87, P < 0.001). No VOCs were detected in quantifiable amounts in the clean air.

As in the first experiment with carbotrap filters, wasps had the choice between the whole blend, the modified blends, and clean air only (experiment 3, Table 1). The GLM revealed a significant difference between the four treatments for the choice of naive wasps ($F_{3,33}$ = 13.20, P < 0.001) as well as for those of experienced wasps ($F_{3,33}$ = 25.67, P < 0.001), and both groups of wasps were strongest attracted to the whole blend (Figure 4A). In a following experiment (experiment 4, Table 1), with only the silica high blend versus three arms with clean air, neither naive nor experienced wasps were attracted to the modified maize blend, and there was no significant difference between the treatments (Figure 4B). Less than 35% of all tested wasps entered an arm, confirming the absence of attraction toward this blend.

Unlike *C. marginiventris*, naive and experienced females of *M. rufiventris* were attracted to the blend silica high

Table 2 Overview of VOCs and quantities (ng ± SE) collected during the bioassays

Nr.	Compounds	Experiments 1 and 2			Experiments 3, 4, and 5			Experiments 6 and 7			Experiment 8
		No filter (12)	Carbotrap low (6)	Carbotrap high (12)	No filter (12)	Silica low (12)	Silica high (24)	Silica extract (12)	Restored blend	Silica high solvent (6)	Silica high (6)
1	(Z)-3-Hexenal	134.9 ± 18.9	160.9 ± 41.8	110.4 ± 11.3	134.9 ± 18.9	91.8 ± 8.6	123.8 ± 14.2	8.2 ± 2.3	141.4 ± 24.6	131.0 ± 20.0	39.0 ± 8.2*
2	(E)-2-Hexenal	22.7 ± 4.5	18.8 ± 3.4	25.3 ± 3.5	22.7 ± 4.5	21.8 ± 1.9	27.6 ± 3.6	tr	32.4 ± 9.0	21.2 ± 3.4	10.8 ± 3.2
3	(Z)-3-Hexenol	25.6 ± 3.6	29.4 ± 5.9	19.7 ± 1.6	25.6 ± 3.6	19.7 ± 1.9	21.1 ± 2.5	4.4 ± 0.6	23.8 ± 6.6	20.4 ± 4.0	18.8 ± 5.8
4	(Z)-2-Penten-1-ol acetate[N]	3.8 ± 0.5	5.5 ± 0.9	4.8 ± 0.5	3.8 ± 0.5	3.5 ± 0.2	4.4 ± 0.4	tr	3.8 ± 0.4	4.6 ± 0.6	nd
5	β-Myrcene	12.1 ± 1.2 (a)	19.4 ± 2.6 (b)	9.2 ± 1.2 (a)	12.1 ± 1.2 (a)	16.5 ± 0.7 (ab)	18.5 ± 1.6 (b)	tr	20.8 ± 1.4	21.8 ± 3.0	tr
6	(Z)-3-Hexenyl acetate	96.9 ± 13.1	144.5 ± 27.8	102.1 ± 16.6	96.9 ± 13.1	86.5 ± 6.7	84.0 ± 11.4	51.0 ± 7.5	77.2 ± 11.8	85.6 ± 17.0	29.0 ± 6.0*
7	(E)-2-Hexenyl acetate	5.5 ± 1.1	7.1 ± 1.5	11.6 ± 2.7	5.5 ± 1.1	5.5 ± 0.6	7.7 ± 1.3	4.1 ± 1.4	6.8 ± 2.6	6.8 ± 2.4	tr
8	(Z)-β-Ocimene	3.9 ± 0.9	5.3 ± 1.0	tr	3.9 ± 0.9	5.3 ± 0.6	5.2 ± 0.6	nd	5.4 ± 1.2	5.4 ± 1.2	tr
9	Linalool	207.7 ± 31.4 (a)	379.7 ± 67.4 (b)	86.0 ± 17.5 (c)	207.7 ± 31.4	132.9 ± 10.1	tr	517.5 ± 65.7	tr	tr	nd
10	DMNT	112.7 ± 22.3 (a)	194.7 ± 51.8 (a)	3.2 ± 1.8 (b)	112.7 ± 22.3	163.9 ± 14.3	196.8 ± 25.9	2.9 ± 0.5	166.8 ± 26.4	187.2 ± 36.6	71.6 ± 20.2*
11	Benzyl acetate	3.3 ± 0.9	5.3 ± 1.8	tr	3.3 ± 0.9	5.1 ± 0.8	4.2 ± 1.0	8.7 ± 1.1	5.6 ± 1.4	4.0 ± 1.8	nd
12	Phenethyl acetate	26.8 ± 4.9	41.7 ± 8.4	tr	26.8 ± 4.9	15.4 ± 1.6	tr	74.9 ± 11.0	tr	tr	nd
13	Indole	125.9 ± 37.0 (a)	269.2 ± 66.7 (a)	41.1 ± 24.7 (b)	125.9 ± 37.0	119.4 ± 11.4	278.7 ± 41.7	104.1 ± 23.3	296.0 ± 78.8	290.4 ± 92.2	37.2 ± 16*
14	Unknown	3.2 ± 1.8	tr	nd	3.2 ± 1.8	nd	nd	5.9 ± 2.0	nd	nd	nd
15	Methyl anthranilate	12.2 ± 5.0	13.8 ± 4.4	nd	12.2 ± 5.0	7.5 ± 1.0	nd	37.6 ± 11.5	nd	nd	nd
16	Geranyl acetate	76.4 ± 10.5 (a)	21.5 ± 3.1 (b)	nd	76.4 ± 10.5	nd	nd	262.9 ± 42.9	nd	nd	nd
17	Unknown sesquiterpenoid	6.1 ± 0.9	tr	nd	6.1 ± 0.9	7.3 ± 0.4	7.1 ± 0.9	nd	6.2 ± 0.4	7.0 ± 1.4	nd
18	Unknown sesquiterpenoid	14.3 ± 2.2 (a)	5.2 ± 0.6 (b)	nd	14.3 ± 2.2	16.2 ± 1.0	15.8 ± 2.0	2.2 ± 0.4	17.0 ± 3.0	15.4 ± 4.0	nd
19	(E)-β-Caryophyllene	143.1 ± 18.8	163.7 ± 16.9	nd	143.1 ± 18.8	174.0 ± 9.9	189.4 ± 27.2	19.0 ± 3.8	142.2 ± 22.4	144.0 ± 38.0	6.9 ± 3.0*
20	(E)-α-Bergamotene	501.9 ± 70.0 (a)	234.4 ± 19.1 (b)	nd	501.9 ± 70.0	548.5 ± 33.5	571.1 ± 78.1	59.3 ± 11.9	453.6 ± 44.8	537.8 ± 101.0	tr
21	Unknown sesquiterpenoid	13.6 ± 2.2	nd	nd	13.6 ± 2.2	17.5 ± 1.1	12.0 ± 2.2	3.6 ± 0.8	13.0 ± 1.4	13.0 ± 3.2	nd
22	(E)-β-Farnesene	998.4 ± 141.2 (a)	172.7 ± 26.9 (b)	nd	998.4 ± 141.2	1099.5 ± 69.1	943.1 ± 139.7	505.3 ± 110.7	778.0 ± 77.0	874.0 ± 184.8	15.8 ± 5.6*
23	Unknown sesquiterpenoid	10.0 ± 1.4	nd	nd	10.0 ± 1.4	12.8 ± 0.9	10.2 ± 1.7	3.6 ± 1.4	7.8 ± 0.8	8.8 ± 2.0	nd
24	Unknown sesquiterpenoid	21.7 ± 2.5 (a)	2.4 ± 1.1 (b)	nd	21.7 ± 2.5	26.2 ± 1.5	22.1 ± 3.0	9.0 ± 1.8	19.4 ± 2.2	20.8 ± 4.4	nd
25	β-Sesquiphellandrene[N]	59.8 ± 7.4 (a)	9.9 ± 1.3 (b)	nd	59.8 ± 7.4	66.4 ± 4.2	56.1 ± 8.4	25.8 ± 5.6	46.4 ± 4.8	50.6 ± 11.4	nd
26	(E)-Nerolidol	6.0 ± 1.5	nd	nd	6.0 ± 1.5	nd	nd	9.2 ± 2.6	nd	nd	nd
27	TMTT	20.6 ± 2.1	tr	nd	20.6 ± 2.1 (a)	16.8 ± 1.1 (a)	5.1 ± 1.2 (b)	33.0 ± 5.2	6.2 ± 1.2	4.6 ± 1.0	nd

Superscript N = compound identified by comparison with the NIST 02 library only. tr = compound found in trace amounts only (average peak area below 1% of internal standard) or in less than half of the samples. nd = compound not detected during the 2-h sampling period. Same treatments were pooled from different experiments, and the number of replicates is given in parentheses. Letters in parentheses indicate significant differences between the treatments within one experiment (see Materials and Methods for statistical procedures). The amounts of the silica extract were not taken into statistical consideration. The amounts of the cowpea experiment were compared to the amounts of the same filter treatment in the silica experiments (maize), and significant differences are indicated by asterisks. Only cowpea VOCs identified also in maize blends are indicated in the table; additional VOCs and VOCs found in trace amounts are reported in the text.
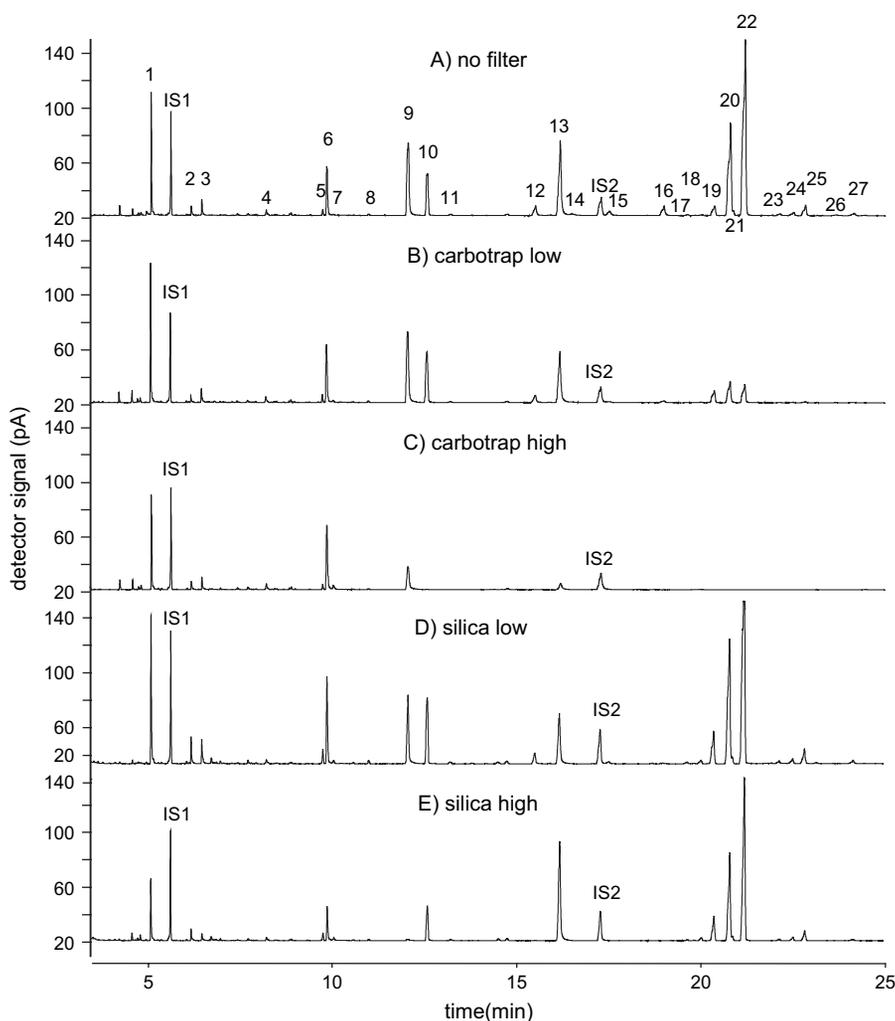
Figure 2 Chromatograms of *Spodoptera*-induced maize VOCs: **(A)** whole blend without a filter, **(B)** after filtration over a 30-mg carbotrap-C filter, **(C)** after filtration over a 150-mg carbotrap-C filter, **(D)** after filtration over a 12.5-mg silica, and **(E)** after filtration over a 25-mg silica filter. Main peaks are labeled in graph A. A complete list and mean quantities of individual compounds are given in Table 2. IS1 and IS2 correspond to internal standards.

(Figure 5; experiment 5, Table 1), resulting in a significant difference between the treatments (naive: $F_{2,45} = 71.21$, $P < 0.001$; experienced: $F_{2,45} = 57.76$, $P < 0.001$). In addition, there was a significant treatment × experience effect ($F_{2,90} = 4.20$, $P = 0.018$).

Attractiveness of VOCs adsorbed by silica

Earlier, we found that *C. marginiventris* females are not attracted to the modified induced maize blend silica high (Figure 4B), which was a surprising result considering that many volatiles readily break through the filter. We extracted the VOCs that were adsorbed on the silica filters during these experiments and found that the extracts indeed contained the compounds that were missing in the breakthrough as well as the compounds that were found in reduced amounts in the breakthrough (Table 2, silica extract). In experiment 6 (Table 1), we tested this extract on filter paper in

the olfactometer and found that it was extremely attractive to naive *C. marginiventris* females (Figure 6A, GLM; $F_{2,46} = 65.30$, $P < 0.001$). We also tested if the missing attraction of the blend silica high could be restored by adding silica extract to this blend (experiment 7, Table 1). Wasps were highly attracted to this restored blend (Figure 6B), and the difference between the treatments was significant ($F_{2,70} = 33.36$, $P < 0.001$). Analyses of the VOCs collected during this experiment indicated that the restored blend was similar to silica high (Table 2). Indeed, no significant differences were found in the amounts of individual VOCs between the two blends, indicating that most VOCs of the silica extract evaporated fast from the filter paper.

Modification of induced cowpea blends over silica filters

The modified cowpea blend was qualitatively and quantitatively very different from the modified maize blend (Table 2).
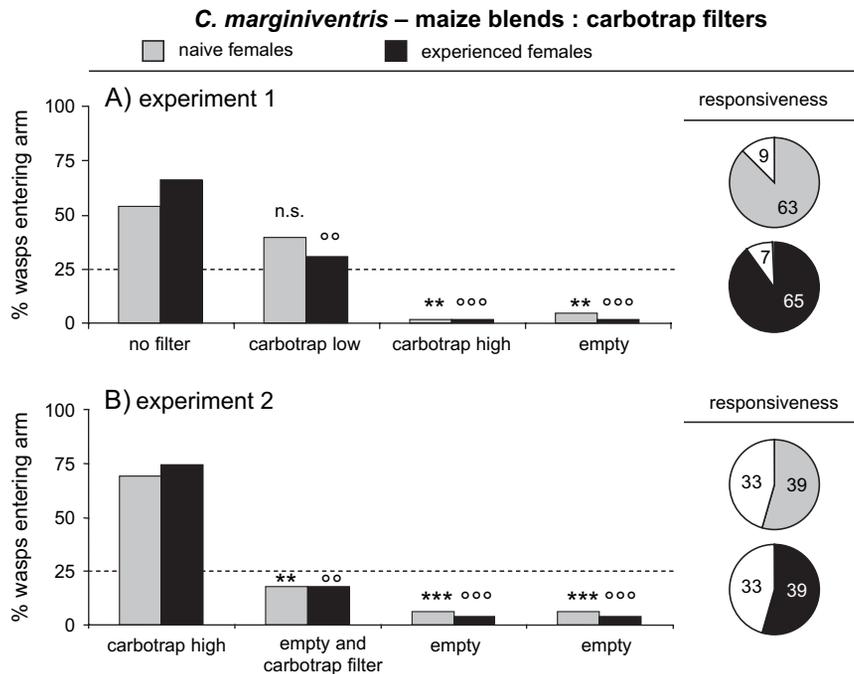
Figure 3 Response of naive and experienced *Cotesia marginiventris* females to whole and modified *Spodoptera*-induced maize blends. Carbotrap-C filters were used to modify the blends. **(A)** Wasps had the choice between three plant-derived blends and clean air only (=empty). **(B)** Wasps had the choice among one plant-derived blend, clean air passed over an empty filter, and clean air only. Composition of the plant-derived blends is given in Figure 2 and Table 2. The responsiveness (proportion of wasps choosing an arm) is indicated by the pie charts with the white part showing the total number of wasps that did not enter any olfactometer arm. Data were analyzed using a GLM, and symbols indicate significant differences between the odor sources within one treatment of wasps (* for naive and ° for experienced wasps: 1 symbol $P < 0.05$, 2 symbols $P < 0.01$, 3 symbols $P < 0.001$) based on comparisons to a reference odor source (=odor source with highest attraction).

It contained significantly less (*Z*)-3-hexenal (*t*-test: $t_{10} = 2.63$, $P = 0.026$), (*Z*)-3-hexen-1-ol acetate ($t_{10} = 2.33$, $P = 0.042$), DMNT ($t_{10} = 3.18$, $P = 0.010$), indole ($t_{10} = 4.14$, $P = 0.002$), (*E*)-β-caryophyllene ($t_7 = 6.43$, $P < 0.001$), and (*E*)-β-farnesene ($t_7 = 12.37$, $P = 0.001$) and only trace amounts of β-myrcene, (*E*)-2-hexenyl acetate, (*Z*)-β-ocimene, and (*E*)-α-bergamotene. In addition to the VOCs listed in Table 2, we also detected the sesquiterpene α-cubebene[N] and trace amounts of (*E*)-2-hexen-1-ol, methyl salicylate, eucalyptol[N], and some unknown compounds. Analyses of the VOCs adsorbed on the filter (not shown in Table 2) showed that the silica filter adsorbed mainly (*Z*)-3-hexen-1-ol acetate, indole, and (*E*)-nerolidol. In addition, we detected trace amounts of (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol benzaoate[N], methyl anthranilate, methyl salicylate, (*Z*)-jasmone, eucalyptol[N], and (*E*)-β-farnesene in the filter extract.

Unlike the maize blend, *C. marginiventris* females were readily attracted to the modified cowpea blend by passing it over the silica filter (Figure 7; experiment 8, Table 1) (GLM: $F_{2,70} = 41.64$, $P < 0.001$).

Discussion

The complexity and variability of VOC blends emitted by herbivore-infested plants have proven to greatly complicate the identification of the principal compounds mediating inter-

actions between the emitting plants and associated organisms. Here, we introduce a novel approach to study the attractiveness of herbivore-induced plant VOCs to parasitoids. Typical blends of VOCs released by herbivore-infested plants were altered by filtration over adsorbing filters that were installed in-line between the odor source vessels and the arms of a four-arm olfactometer. This resulted in the adsorption of several VOCs, while others broke through and were tested simultaneously for chemical identity and for attractiveness to the wasps.

Attractiveness of herbivore-induced VOCs to parasitoids

Our results show that a partial reduction of the sesquiterpenes of *Spodoptera*-induced maize blends did not have a significant effect on the attraction of naive *C. marginiventris* females, while experienced females preferred the unfiltered blend with higher amounts of sesquiterpenes (Figures 2 and 3, Table 2). These findings are consistent with earlier studies (Turlings and Fritzsche, 1999; Hoballah and Turlings, 2005) in which, after oviposition experiences in the presence of *Spodoptera*-induced maize VOCs, the wasps were highly attracted to blends that contained sesquiterpenes. During contact with hosts, many parasitoids are known to associate the perceived odor with the presence of hosts and subsequently exhibit an attraction to the experienced
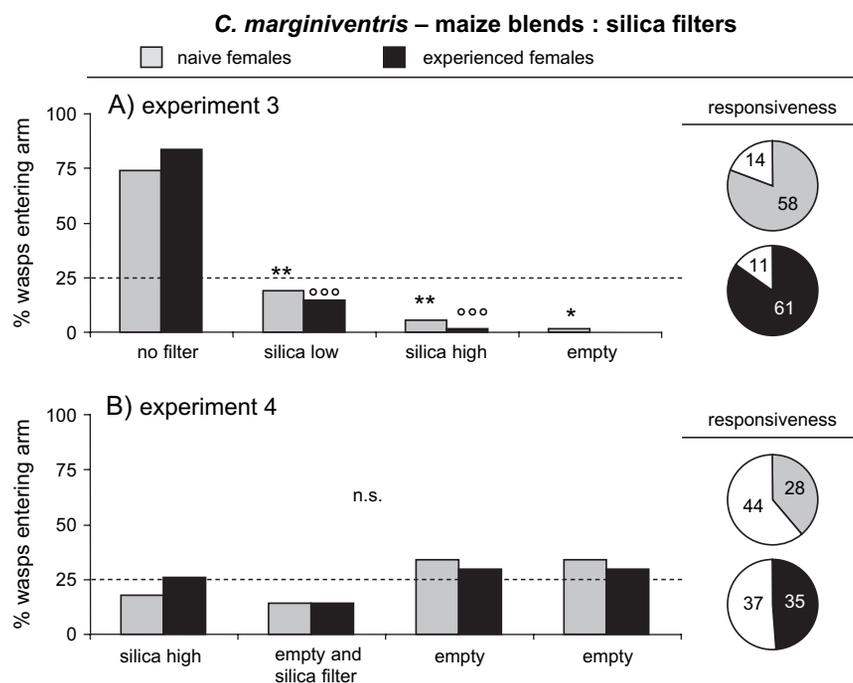
Figure 4   Response of naive and experienced *Cotesia marginiventris* females to whole and *Spodoptera*-induced maize blends. Silica filters were used to modify the blends. **(A)** Wasps had the choice between three plant-derived blends and clean air only (=empty). **(B)** Wasps had the choice among one plant-derived blend, clean air passed over an empty filter, and clean air only. Composition of the plant-derived blends is given in Figure 2 and Table 2. See the caption of Figure 3 for further explanations.

odor (Turlings *et al.*, 1993b; Vet *et al.*, 1995). Studies on associative learning by the parasitoid *M. croceipes* (Meiners *et al.*, 2003) and by honeybees (Laloi *et al.*, 2000) have shown that, after conditioning to a complex mixture, these insects established a hierarchy among various components, with some of them accounting for a major part of the behavioral activity evoked by the mixture. In our experiments, it remains to be determined whether the stronger attraction toward the blends with high amounts of sesquiterpenes is due to an association of these compounds during oviposition or due to increased attraction to compounds correlated with the sesquiterpenes. Interestingly, in the current study, both naive and experienced females were still attracted to a blend that did not contain any detectable amounts of sesquiterpenes and only 20% of the total quantified VOCs compared to the no filter blend. These results imply that the sesquiterpenes are not essential for the attraction of *C. marginiventris* females.

In contrast, a reduction of a few rather polar compounds strongly affected the attraction of this parasitoid species (Figures 2 and 4, Table 2). The blend silica high that still contained more than 80% of all VOCs and about 70% of the total quantity detected in the unfiltered blend had completely lost its attractiveness to naive and experienced wasps. This suggests that some compounds that are essential for the attraction of the wasps were filtered out. Support for this notion comes from the experiment that tested the attractiveness of the VOCs that were filtered out by the silica filter. Dichloro-

methane extracts of these compounds on filter paper were highly attractive to the wasps, and adding the extract to the unattractive silica high blend completely restored its attractiveness to naive females (Figure 6). Barely detectable amounts of VOCs were collected from the headspace of the filter paper (Table 2), suggesting that the implicated compounds are behaviorally active at very low doses. Indeed, arthropod chemoreceptors are much more sensitive than the detectors of analytical instruments (Dicke, 1999a; Rains *et al.*, 2004), and responses can be triggered by fewer than six molecules of a specific VOC (Angioy *et al.*, 2003). Further studies will attempt to identify which compounds in the silica extract attract the wasps at such low doses.

Unlike *C. marginiventris*, *M. rufiventris* females were readily attracted to the blend silica high (Figure 5). Hence, different parasitoid species exploit different VOCs to locate their hosts. Although *M. rufiventris* is less of a generalist than *C. marginiventris*, the biology and host range of these wasps imply that there is an overlap in the potential plant cues that they could use (Hegazi and El-Minshawy, 1979; Maes, 1989). The difference between the two species is consistent with earlier studies (Hoballah and Turlings, 2005), showing that *M. rufiventris* responds differently to induced maize VOCs than *C. marginiventris*. Differences in the use of plant cues to locate the hosts have also been found for other generalist and specialist parasitoid species (Röse *et al.*, 1998; De Moraes and Lewis, 1999) and even for closely related species (Geervliet *et al.*, 1998; Smid *et al.*, 2002).
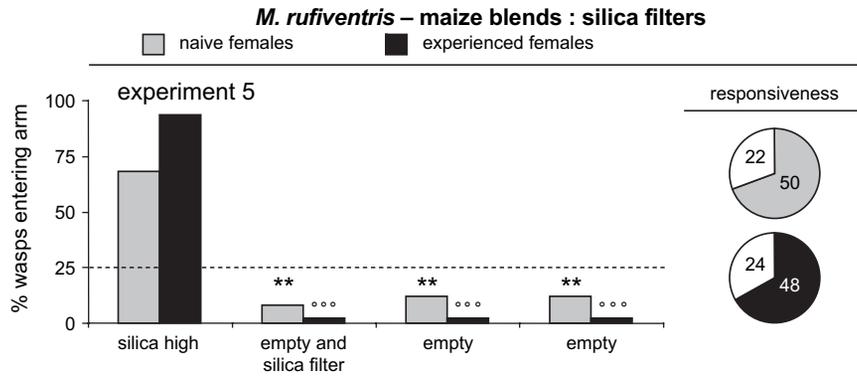
Figure 5   Response of naive and experienced *Microplitis rufiventris* females to a modified *Spodoptera*-induced maize blend. See the caption of Figure 3 for further explanations.

Interestingly, filtration of an induced cowpea blend over silica resulted in a blend that contained only 15% of the total amount of VOCs compared to the similarly modified maize blend, but this blend was still very attractive to naive *C. marginiventris* females (Figure 7). This indicates that cowpea contains different or larger amounts of highly attractive compounds, which supports the conclusion of an earlier study comparing the attractiveness of nonmodified cowpea and maize blends (Fritzsche Hoballah *et al.*, 2002). Indeed, the modified cowpea blend contained some VOCs that were not detected in the maize blend. Specifi-

cally, the behavioral importance of the trace amounts of methyl salicylate found in the cowpea blend should be further investigated. Gas chromatography electroantennogram detector analyses using *C. marginiventris* females showed that this compound was electrophysiologically active at very low dosages (Gouinguené and Turlings, 2005). Furthermore, methyl salicylate has been shown to be attractive to several carnivorous arthropods in the laboratory (Dicke *et al.*, 1990; Pickett *et al.*, 1999; de Boer and Dicke, 2004), as well as to parasitic wasps in the field (James and Price, 2004).
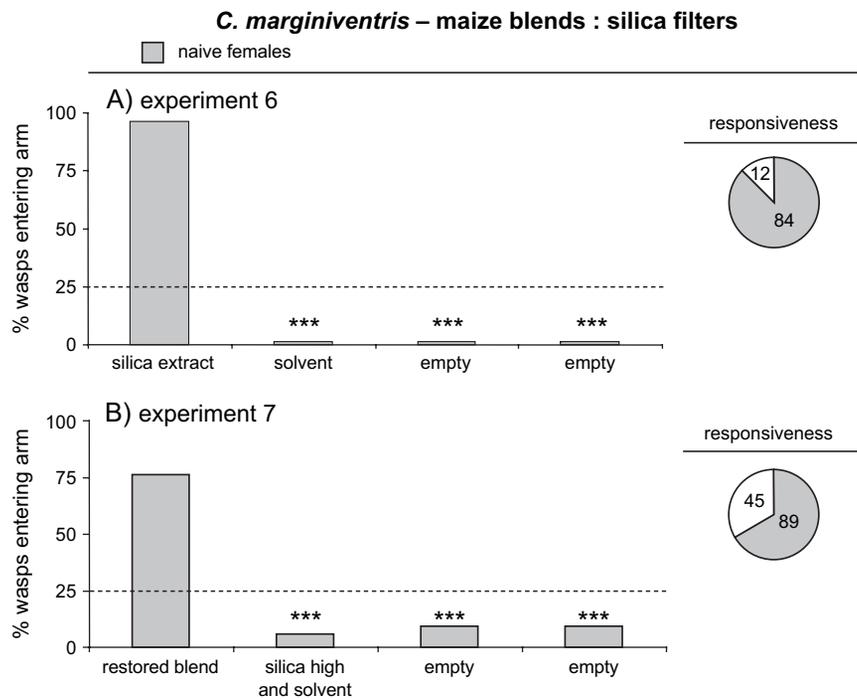
Figure 6   **(A)** Response of naive *Cotesia marginiventris* females to an extract of *Spodoptera*-induced maize VOCs adsorbed by a silica filter in a prior experiment. **(B)** Response of naive *C. marginiventris* females to modified *Spodoptera*-induced maize blends. Silica filters were used to modify two maize blends, and to one such blend an extract of silica-adsorbed volatiles was added on filter paper. Compositions of the blends are given in Table 2. See the caption of Figure 3 for further explanations.
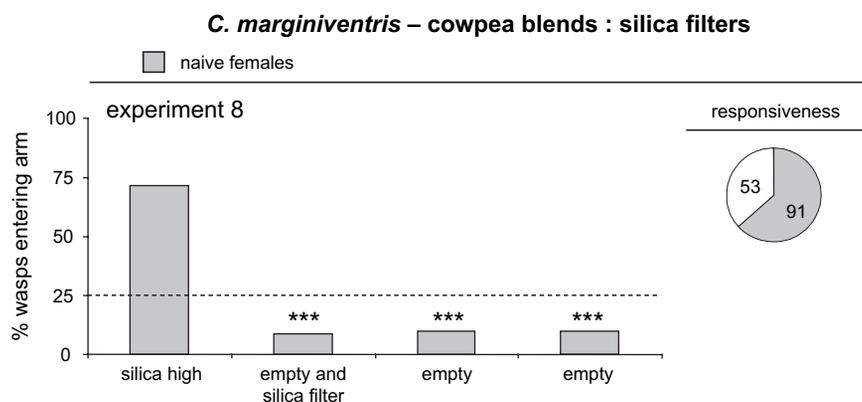
Figure 7 Response of naive *Cotesia marginiventris* females to a modified *Spodoptera*-induced cowpea blend. The composition of the cowpea blend is given in Table 2. See the caption of Figure 3 for further explanations.

Advantages and disadvantages of *in situ* modification of VOC blends

The differential attraction of the two parasitoid species tested in our study as well as the differential response of naive and experienced wasps and the differential attractiveness of VOCs from two plant species illustrate the complexity of the exploitation of plant-derived VOCs by parasitoids for host location. The approach we used here takes this complexity into account. It is a top-down approach starting with the whole herbivore-induced VOC blend and reducing its complexity by selectively adsorbing some compounds. Simultaneous testing and collecting of VOCs allow a direct linking of the VOC profile to the wasp behavior. We used two different adsorbing materials, carbotrap-C and silica. Carbotrap-C is a graphitized carbon that is usually used for adsorptive enrichment and thermal desorption of VOCs in the sampling range of C12 to C20 (Dettmer and Engewald, 2002), whereas silica is mainly used to adsorb very polar compounds (Harper, 2000). The breakthrough of VOCs from an adsorbing bed depends on many factors, including vapor concentration, air flow and volume, bed geometry, flow rates, and temperature (Harper, 2000; Dettmer and Engewald, 2002). We ran our experiments at room temperature for a relatively short bioassay period (2 h), and we adjusted the flow rates, amount of adsorbent, and prerun times to obtain VOC blends with well-defined quantitative and qualitative differences from a natural blend. In only two of 12 experiments, we found a significant "release time × treatment" effect (statistical test not shown), suggesting that the blends tested in this study remained more or less equally attractive over the 2-h bioassay period. Furthermore, this *in situ* modification of plant-emitted VOC blends has little impact on the interaction between the plant and the herbivore, and it avoids pleiotropic effects, which might occur in studies using genetically modified organisms (van Poecke and Dicke, 2003).

Other studies have used bottom-up approaches by, for example, identifying VOC profiles and testing individual or blended synthetic compounds (Dicke *et al.*, 1990; Whitman and Eller, 1990; Turlings *et al.*, 1991b). Such studies face the problem that plants emit numerous different compounds (Dudareva *et al.*, 2004; Niinemets *et al.*, 2004) with various isomeric forms. Each of these compounds could be of key importance, but it is unfeasible to study them all. Many of the minor compounds will not have been identified, and not all are readily available for individual testing. In addition, insect responses to different VOCs in a blend are often of a nonadditive nature (Visser and de Jong, 1988). For example, neither nonanal nor gernaylacetone alone attracts females of *Apanteles carpatus*, a parasitoid of the cloth moth *Tinea pennionella*, but a one-to-one blend of both compounds is as attractive as an extract of all volatiles from moth-infested beaver pelt (Takacs *et al.*, 1997). Synergistic effects have also been found in field experiments (Hammack, 2001), and the attraction of insects to VOCs can be influenced by background odors as well (Reddy *et al.*, 2002; Dicke *et al.*, 2003a; Mumm and Hilker, 2005). Moreover, compounds that normally attract insects can be repellent or even toxic at elevated concentrations (Read *et al.*, 1970). Releasing different fractions of VOCs from filter papers (Udayagiri and Jones, 1992; Turlings and Fritzsche, 1999) allows virtually no control of release rates and might lead to ratios of VOCs that are far different from natural. Relative ratios are important in the attraction of many insects, which is particularly evident from studies on pheromones, but may also be important for specific recognition of herbivore-induced VOCs (Turlings *et al.*, 1993a; De Moraes *et al.*, 1998; Bruce *et al.*, 2005). The approach we suggest in this study significantly altered the odors, but the compounds that broke through the filter had similar concentrations and ratios as in the natural blend (Table 2).

On the other hand, this approach is faced with the problem that VOCs on certain types of adsorbent material may create artifacts by causing reactions with reactive atmospheric species (Hoffmann, 1995; Kleno *et al.*, 2002), and compounds might be rearranged or decomposed (Rothweiler *et al.*, 1991). Although we did not detect additional peaks in the modified blends, we cannot exclude the possibility that

some minor artifacts were produced while passing VOCs over the adsorbents. We specifically tested for this possibility by passing clean air over adsorbing filters (negative control) and by adding back the adsorbed fraction to a nonattractive blend, which restored the attraction (positive control). Furthermore, the specificity and efficacy of the technique could be improved by using adsorbent materials coated with a specific reagent. Such microchemical reactions have played crucial roles in the determination of the structure of insect pheromones (Attygalle and Morgan, 1988; Jones and Oldham, 1999) and could easily be adapted to study the importance of plant-derived VOCs *in situ*. We are currently testing silica filters coated with 2, 4-dinitrophenylhydrazine (Supelco), which selectively adsorb compounds with carbonyl groups.

Conclusions

The *in situ* modification of herbivore-induced VOC blends appears to be an effective new approach to study the importance of specific VOCs involved in tritrophic interactions. Considering the large number of different adsorbing materials that are commercially available, this approach could easily be adapted to study the role of VOC blends in other biological systems, including VOCs involved in attracting pollinators or herbivores. To our knowledge, only one study has used a similar approach to assess the attraction of insects toward different fractions of plant-derived VOCs (Natale *et al.*, 2003), but the breakthrough VOCs were not recollected and identified in that study. Information on the relative attractiveness of individual VOCs within complex blends is highly desired, not only as it may aid in the development of crop varieties with odor emissions that facilitate biological control of pests and diseases (Degenhardt *et al.*, 2003; Wei *et al.*, 2004) but also for a comprehensive understanding of insect olfaction.

Acknowledgements

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