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The Role of Indole and Other Shikimic Acid Derived Maize Volatiles in the Attraction of Two Parasitic Wasps

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Abstract After herbivore attack, plants release a plethora of different volatile organic compounds (VOCs), which results in odor blends that are attractive to predators and parasitoids of these herbivores. VOCs in the odor blends emitted by maize plants (*Zea mays*) infested by lepidopteran larvae are well characterized. They are derived from at least three different biochemical pathways, but the relative importance of each pathway for the production of VOCs that attract parasitic wasps is unknown. Here, we studied the importance of shikimic acid derived VOCs for the attraction of females of the parasitoids *Cotesia marginiventris* and *Microplitis rufiventris*. By incubating caterpillar-infested maize plants in glyphosate, an inhibitor of the 5-enolpyruvylshikimate-3-phospate (EPSP) synthase, we obtained induced odor blends with only minute amounts of shikimic acid derived VOCs. In olfactometer bioassays, the inhibited plants were as attractive to naive *C. marginiventris* females as control plants that released normal amounts of shikimic acid derived VOCs, whereas naive *M. rufiventris* females preferred inhibited plants to control plants. By adding back synthetic indole, the quantitatively most important shikimic acid derived VOC in induced maize odors, to inhibited plants, we showed that indole had no effect on the attraction of *C. marginiventris* and that *M. rufiventris* preferred blends without synthetic indole. Exposing *C. marginiventris* females either to odor blends of inhibited or control plants during oviposition experiences shifted their preference in subsequent olfactometer tests in favor of the experienced odor. Further learning experiments with synthetic indole showed that *C. marginiventris* can learn to respond to this compound, but that this does not affect its choices between natural induced blends with or without indole. We hypothesize that for naïve wasps the attractiveness of an herbivore-induced odor blend is reduced due to masking by nonattractive compounds, and that during oviposition experiences in the presence of complex odor blends, parasitoids strongly associate some compounds, whereas others are largely ignored.

Keywords *Cotesia marginiventris* · *Microplitis rufiventris* · *Spodoptera littoralis* ·

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Zea mays · Parasitoids · Volatile Organic Compounds (VOCs) · Herbivore-Induced Plant Volatiles (HIPVs) · Host location · Associative learning · Tritrophic interactions · Indole · Shikimic acid · Glyphosate · Induced defenses

Introduction

Plants that are attacked by herbivorous arthropods are known to release a complex blend of volatile organic compounds (VOCs). These herbivore-induced plant volatiles (HIPVs) are exploited by predators and parasitoids as foraging signals that help them locate their herbivorous prey or hosts (Turlings and Wäckers, 2004; Arimura et al., 2005). At present, more than 1000 low-molecular-weight organic compounds have been reported to be emitted from plants, including alkanes, alkenes, alcohols, ketones, aldehydes, ethers, esters, and carboxylic acids (Dudareva et al., 2004; Niinemets et al., 2004). Although some of these compounds are constitutively emitted by undamaged, healthy plants, considerably higher amounts are emitted after herbivore damage and various HIPVs may even be synthesized *de novo* in response to damage (Turlings et al., 1990, 1998; Paré and Tumlinson, 1997). Some HIPVs are specific to certain plant taxa, for example, sulfur containing compounds in *Allium* plants (Dugravot et al., 2004) or glucosinolate breakdown products in Brassicaceae species (Scascighini et al., 2005), but others are common to many species (Fritzsche Hoballah et al., 2002; van den Boom et al., 2004). Common compounds include “green-leaf volatiles” (C_6 aldehydes, alcohols, and derivatives), cyclic and acyclic terpenoids, phenolic compounds, and nitrogenous compounds (Dicke, 1999; Paré and Tumlinson, 1997). These derive from at least three pathways. Green leaf volatiles are products of the enzymatic activity of hydroperoxide lyase (HPL), a component of the lipoxygenase (LOX) pathway, which results in multiple rearrangement of (*Z*)-3-hexenal (Bate and Rothstein, 1998; Blee, 1998). Terpenoids are synthesized via the isopentenyl pyrophosphate (IPP) intermediate following the classical mevalonate pathway or via an alternative IPP pathway with glyceraldehyde-3-phosopate and pyruvate identified as the direct precursors of IPP (Lichtenthaler et al., 1997). Finally, aromatic compounds, such as methyl salicylate (MeSA) and indole, are formed via the shikimic acid pathway (Bennett and Wallsgrove, 1994; Paré and Tumlinson, 1997).

In maize plants, the mechanisms of biosynthesis, induction, and release of HIPVs are well characterized (Turlings et al., 1998; Frey et al., 2000; Shen et al., 2000; Schnee et al., 2002; Gouinguené et al., 2003; Schmelz et al., 2003a,b,c; Köllner et al., 2004; Lawrence and Novak, 2004; Ruther and Kleier, 2005), and the ecological significance of these compounds in tritrophic signaling has been demonstrated in laboratory and field experiments (Turlings et al., 1990; Bernasconi et al., 1998; Hoballah and Turlings, 2001; Fritzsche Hoballah et al., 2002; Rasmann et al., 2005). In particular, the role of green leaf volatiles and terpenoids in attracting natural enemies of the herbivores has been investigated in various experiments (D’Alessandro and Turlings, 2005; Hoballah and Turlings, 2005). Yet, it remains unclear which compounds are essential for attraction (D’Alessandro and Turlings, 2006). One group of compounds that has hardly been studied in the context of parasitoid attraction are the shikimic acid derived VOCs.

The main shikimic acid derived VOC released by maize seedlings after infestation with larvae of *Spodoptera* moths is indole (Turlings et al., 1998; D’Alessandro and Turlings, 2005). This compound is induced after treatment of maize seedlings with volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine], a fatty acid-amino acid conjugate found in the regurgitate of *Spodoptera* larvae (Alborn et al., 1997; Turlings et al., 2000). Indeed, jasmonic acid, which is involved in the induction of HIPVs in maize (Schmelz et al., 2003a), also appears to be an integral part of volicitin-mediated induction of indole (Frey et al., 2000). Frey et al. (2004) identified an enzyme in maize, indole-3-glycerol phosphate lyase (Igl), which converts indole-3-glycerol phosphate to free indole. This differs from the enzyme BX1, which catalyzes the conversion of indole-3-glycerol phosphate to indole to form the direct defense compounds 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-2*H*-,1,4-benzoxazin-3(4*H*)-one (DIMBOA), or tryptophane synthase, which produces the amino acid tryptophane (Frey et al., 1997). The selective activation of the evolutionarily similar genes *igl* and *bx1* suggests that the plants are capable of selecting direct or indirect defense mechanisms depending on the type of stress they are exposed to. Therefore, volatile indole was expected to be a key compound in the attraction of natural enemies of the herbivores.

Here we studied the importance of shikimic acid derived HIPVs, in particular indole, in attracting females of two parasitoid species, *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) and *Microplitis rufiventris* (Kokujev) (Hymenoptera: Braconidae). Both attack early instars of numerous lepidopteran moths, including many pests, and are known to use plant-provided VOCs in host location (Gouinguené et al., 2003; Hoballah and Turlings, 2005). We manipulated the volatile blend emitted by maize seedlings (*Zea mays* var. Delprim) that had been fed upon by larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) by incubating the plants in glyphosate [*N*-(phosphonomethyl)-glycine]. This compound inhibits the enzyme 5-enolpyruvylshikimate-3-phospate (EPSP) synthase (Haslam, 1993; Schönbrunn et al., 2001) and strongly reduces the amounts of shikimic acid derived VOCs. Attraction of the odor from these inhibited plants was compared to that of the natural blends emitted by control plants. Subsequently, we tested an inhibited blend against an inhibited blend to which we added back a natural amount of synthetic indole, the major shikimic acid derived HIPVs.

C. marginiventris females, like many other female parasitoids, are able to learn and associate plant VOCs with the presence of suitable hosts during oviposition experiences (Turlings et al., 1993; D'Alessandro and Turlings, 2005). We therefore compared the responses of naïve and experienced female parasitoids, and in a series of learning experiments with *C. marginiventris* females, we estimated how well the wasps can learn to associate indole with host presence.

Methods and Materials

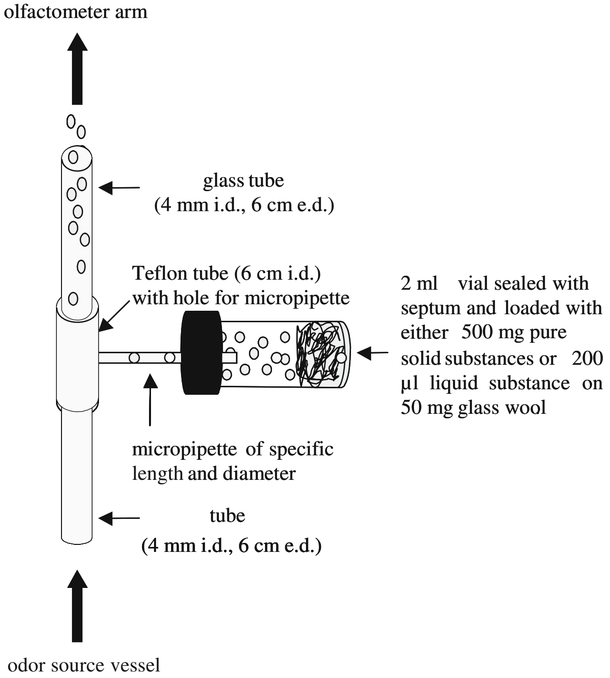
Insects and Insect Treatments The caterpillar *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the solitary endoparasitoids, *C. marginiventris* (Cresson) (Hymenoptera: Braconidae) and *M. rufiventris* (Kokujev) (Hymenoptera: Braconidae) were reared as previously described (Turlings et al., 2004). Adult parasitoids were kept in plastic cages at a male/female ratio of approximately 1:2 and were provided with moist cotton wool and honey as food source. Cages were kept in incubators (*C. marginiventris*, 25±1°C; *M. rufiventris*, 23±1°C; 16:8 hr L/D) and transferred to the laboratory 30 min before the experiments. We tested mated 2- to 4-d-old naive and experienced females. The latter were given experiences by allowing them to oviposit 3–5 times into second instar *S. littoralis*. A metal screen was attached to the top opening (2 cm diam) of an odor source vessel of the same type as used in the olfactometer (see below). Approximately 20 larvae were placed on the screen, and individual wasps were allowed to oviposit in one or two larvae, while they were exposed to the odor of the source that was placed inside the vessel. This source was either an infested control plant, an infested inhibited plant, or synthetic indole only. Airflow and concentrations of volatiles were the same as during the olfactometer bioassays (see below). Naïve females were neither exposed to the volatiles nor given any oviposition experience before testing. The different groups of wasps were kept separately in small plastic boxes with moist cotton wool and honey and released into the olfactometer 1–3 hr after the oviposition experiences.

Plants and Odor Sources Maize (*Z. mays*, var. Delprim) was sown in plastic pots (10 cm high, 4 cm diam) with commercial potting soil (Ricoter Aussaaterde, Aarberg, Switzerland) and placed in a climate chamber (23±2°C, 60% r.h., 16:8 hr L/D, and 50,000 lm/m^2). Plants used for the experiments were 10–12 d old and had 3 fully developed leaves. The evening before the experiments, plants were cut with a razor blade at soil level, while the stem was held under water to prevent air entering the vascular system. Subsequently, they were placed in a vial (8 ml) filled with either deionized water (control plant) or in a 1 mM glyphosate [*N*-(phosphonomethyl)-glycine; Fluka, Buchs, Switzerland] solution (inhibited plant). Vials were wrapped in aluminum foil, and one vial containing a single plant was placed in an open odor source vessel of the olfactometer (described by Turlings et al., 2004). Two hr after incubation, plants were infested with 20 second instar *Spodoptera*, which were released in the whorl of the youngest leaf. After infestation, plants were kept under laboratory conditions (25±2°C, 40±10% r.h., 16:8 hr L/D, and 8000 lm/m^2) and were used for the experiments the following day, between 10 A.M. and 4 P.M.

To check whether the treatment of the plants with the inhibitor affected the larval feeding behavior, plants were collected after the bioassays, and the leaves were scanned into Adobe Photoshop 6.0. The total leaf area removed during the 24-hr feeding period was compared for the different treatments based on differences in pixels that indicated tissue removal.

Indole (purity ≥ 99%; Fluka) was released from a device consisting of a 2-ml glass vial that contained 500 mg synthetic indole (Fig. 1), which was connected, via a glass capillary,

Fig. 1 Schematic representation of the odor delivery system for synthetic VOCs



to a Teflon tube placed between two glass tubes connecting the top of an odor source vessel to an olfactometer arm. Preliminary experiments with various synthetic volatile compounds (e.g., MeSA, linalool, indole) showed that at room temperature this device allowed the constant release of pure compounds, and that their release rates could be controlled by adjusting the length and the diameter of the capillary tube (Duran, Hirschmann EM). The release rate was calibrated to the lower range of amounts of indole that was found for infested maize plants. Vials were prepared freshly the evening before the experiments and connected the following morning to the odor source vessels used for training or testing.

Olfactometer Bioassays All odor sources were tested for attractiveness to parasitoids in a four-arm olfactometer (described by D'Alessandro and Turlings, 2005) as indicated in Table 1. Cleaned and humidified air entered the odor source vessel at 1.2 l/min (adjusted by a manifold with four flowmeters; Analytical Research System, Gainesville, FL, USA) via Teflon tubing and carried the VOCs through to the olfactometer compartment. Half of the air (0.6 l/min/olfactometer arm) was pulled out via a volatile collection trap that was attached to the system above the odor source vessels (see "Collection and analyses of VOCs"). Incoming and outgoing air were balanced by a Tygon tube connected to a vacuum pump via another flow meter and a pressure gauge. Empty arms were connected to empty vessels and carried clean, humidified air only.

Wasps were released in groups of six into the central part of the olfactometer, and after 30 min the wasps that had entered an arm of the olfactometer were counted and removed. Wasps that did not enter an arm after this time were removed from the central part of the olfactometer and considered as "no choice." Experiments were replicated on eight different days, and for each replicate a total of six groups of six wasps were tested during a 3-hr sampling period, alternating between groups of naive and experienced wasps in experiments where three different groups were tested (total of 96 wasps/group), or only naïve wasps in the other experiments (total 288 wasps). Ten neon tubes attached to a metal frame above the olfactometer provided approximately 7000 lm/m^2 at the height of the odor source vessels. All bioassays were carried out between 10 A.M. and 4 P.M.

Collection and Analyses of VOCs VOCs of each odor source were collected during the olfacotemeter bioassay on a Super-Q trap (25 mg, 80–100 mesh; Alltech Associates, Deerfield, IL, USA, described by Heath and Manukian, 1992). Each trap was attached horizontally to the elbow of the olfactometer and connected via Tygon tubing to a flowmeter (Analytical Research System) and a vacuum pump. Air carrying the volatiles was pulled through each trap for 3 hr at a rate of 0.6 l/min during each behavioral bioassay. Afterwards, the traps were extracted with 150 μl dichloromethane (Suprasolv; Merck, Dietikon, Switzerland), and 200 ng of *n*-octane and *n*-nonyl acetate (Sigma, Buchs, Switzerland) in 10 μl dichloromethane were added to the samples as internal standards. All extracts were stored at −76°C until analyses. Traps were washed with 3 ml dichloromethane before they were reused for a next collection.

VOCs of the experiments with control and inhibited plants were analyzed with a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectrometer that 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). A 2-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). Helium at constant flow (0.9 ml/min) was used as carrier gas. After injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and

Table 1 Odor sources and experimental design

Figure	Odor sources				Wasp	Wasp treatment	Replications of experiment
	Arm 1	Arm 2	Arm 3	Arm 4			
3A	Control plant (infested)	Empty	Inhibited plant (glyphosate, infested)	Empty	*C. marginiventris*	Naive, control, inhibited	8
3B	Control plant (infested)	Empty	Inhibited plant (glyphosate, infested)	Empty	*M. rufiventris*	Naive, control, inhibited	8
4A	Inhibited plant and indole (gylphosate, infested)	Empty	Inhibited (glyphosate, infested)	Empty	*C. marginiventris*	Naive	8
4B	Inhibited plant and indole (gylphosate, infested)	Empty	Inhibited (glyphosate, infested)	Empty	*M. rufiventris*	Naive	8
5A	Indole	Empty	Empty	Empty	*C. marginiventris*	Naive, indole, control	8
5B	Control plant (infested)	Empty	Inhibited plant (glyphosate, infested)	Empty	*C. marginiventris*	Naive, indole, control	8

Further details on odor sources, number, and treatment of wasps are described in the text and in the figures. Treatments of the plants are given in parentheses.

subsequently to 200°C at 5°C/min followed by a postrun of 5 min at 250°C. The detected volatiles were identified by comparison of their mass spectra with those of the NIST 02 library, by comparison of their spectra and retention times with those of authentic standards, and by comparison of retention times with those in previous analyses (D'Alessandro and Turlings, 2005). Compounds that were not identified by comparing retention times and spectra with those of pure standards are indicated in Fig. 2 with superscript N, and their identity should be considered tentative. Twelve samples per treatment were analyzed in the SIM mode (ion 117, qualifier 95), and indole was quantified based on a calibration curve with known amounts of synthetic indole. All other compounds were only quantified in the full scan range based on comparison of their peak area with those of the internal standards (*n*-octane for compounds 1–14, *n*-nonyl acetate for compounds 14–27). A total of 18 samples were injected per treatment.

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 (R: A language and Environment for Statistical Computing, Version 1.9.1, Vienna, Austria, 2006, ISBN 3-900051-07-0 http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals. We

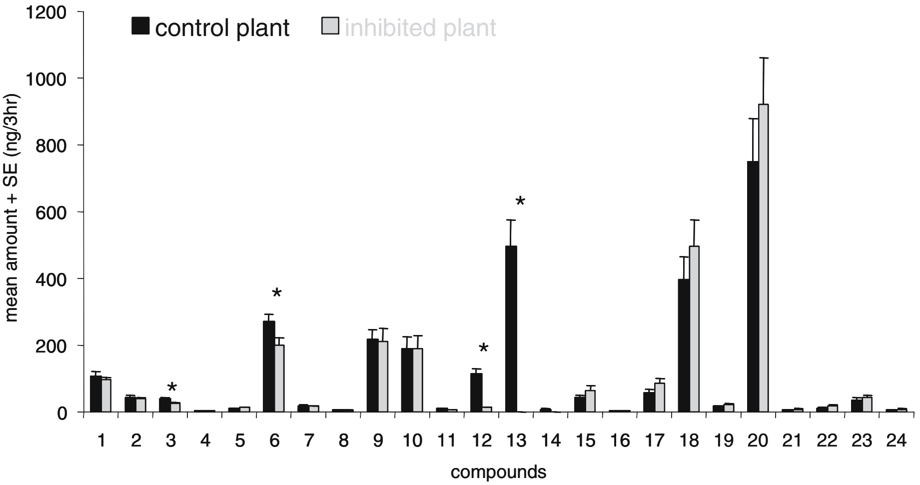


Fig. 2 Mean amount (±SE) (ng) of major VOCs recollected from cut *Spodoptera*-induced maize seedlings during 3 hr. Control plants were incubated in water, inhibited plants were incubated in a 1 mM glyphosate solution. Asterisks above bars indicate significant differences (*t*-test on log(x+1) transformed data, $P<0.05$) in the amount of a specific compound. $N=18$ per treatment. The compounds are as follows: **1**, (*Z*)-3-hexenal; **2**, (*E*)-2-hexenal; **3**, (*Z*)-3-hexen-1-ol; **4**, (*Z*)-2-penten-1-ol acetate; **5**, β-myrcene; **6**, (*Z*)-3-hexenyl acetate; **7**, (*E*)-2-hexenyl acetate; **8**, (*Z*)-β-ocimene^{N}; **9**, linalool; **10**, (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT); **11**, benzyl acetate; **12**, phenethyl acetate; **13**, indole; **14**, methyl anthranilate; **15**, geranyl acetate; **16**, unknown sesquiterpenoid; **17**, (*E*)-β-caryophyllene; **18**, (*E*)-α-bergamotene; **19**, unknown sesquiterpenoid; **20**, (*E*)-β-farnesene; **21**, unknown sesquiterpenoid; **22**, unknown sesquiterpenoid; **23**, β-sesquiphellandrene^{N}; **24**, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). The compounds are ordered in accordance with their retention on a nonpolar capillary column

tested "treatment" effects (=odor sources) for naive and experienced wasps separately, and we included "release" as an explanatory variable to avoid "pseudo-replications." In addition, we tested if there was a significant effect of "experience" and an interaction between "treatment × experience."

The amounts of VOCs were analyzed by using *t*-tests. Amounts of VOCs that were not normally distributed were $\log(x+1)$ transformed prior to analysis. The amounts of indole quantified in the single ion mode were analyzed with a Kruskal–Wallis test. Differences between the treatments were analyzed with the Tukey's test. Differences between the removed leaf areas after caterpillar feeding were analyzed with a *t*-test. All analyses were run on SigmaStat (Version 2.03).

Results

VOCs of Control and Inhibited Plants Herbivore-infested maize seedlings that were incubated with their cut stem in water (control plants) released a volatile blend consisting of 24 detectable VOCs, including green leaf volatiles, terpenoids, and shikimic acid derivatives (Fig. 2). Seedlings that were incubated in a 1 mM glyphosate solution released only trace amounts of indole and methyl anthranilate and had strongly reduced amounts of other shikimic acid derived VOCs (*t*-test: benzyl acetate, $t_{34}=1.741$, $P=0.092$; phenethyl acetate, $t_{34}=11.218$, $P<0.001$). The amounts of VOCs derived from other biochemical pathways were similar to those of the control plants, except for the somewhat reduced amounts of (*Z*)-3-hexen-1-ol ($t_{34}=2.306$, $P=0.027$) and 3-*Z*-hexen-1-ol acetate ($t_{34}=2.577$, $P=0.014$). In addition to the compounds quantified in Fig. 2, we also detected trace amounts of (*E*)-nerolidol, (*Z*)-jasmone, and some minor, unidentified compounds. These VOCs were not included in quantification analyses.

The single ion mode analyses of indole revealed that during the 3-hr bioassay periods we collected 835.44±135.91 ng from control plants and only 0.55±0.17 ng from inhibited plants. In bioassays where synthetic indole was added to the air stream with the odor of an inhibited plant, we detected 192.77±11.46 ng. There was a significant difference between the amounts released by these three treatments (Kruskal–Wallis followed by Tukey test, $H_2=30.294$, $P<0.001$).

Inhibitor treatment did not affect the larvae's feeding rate; the leaf areas removed by the larvae during a 24-hr feeding period were similar for control plants (4.73±0.40 cm^2) and for inhibited plants (4.81±0.38 cm^2) (*t*-test: $t_{20}=-0.15$, $P=0.882$).

Attractiveness of Inhibited vs. Control Plants Neither naïve nor experienced *C. marginiventris* females significantly distinguished between VOC blends emitted by *Spodoptera*-induced maize seedlings with strongly reduced amounts of shikimic acid derived VOCs (see above) and VOCs emitted by control plants (Fig. 3a, GLM, naive: $F_{1,15}=0.78$, $P=0.39$; experienced on inhibited blend: $F_{1,15}=3.91$, $P=0.067$; experienced on control blend: $F_{1,15}=0.43$, $P=0.52$). Yet, the type of experience had a significant effect on the choice of the wasps ($F_{1,60}=4.566$, $P=0.037$), implying that *C. marginiventris* females were able to detect the difference between the two odor sources. The responsiveness (the proportion of wasps entering an arm with one of the two treatments) was high and similar for all treatments, and only few wasps entered an empty arm (Fig. 3a).

Naive and experienced females of *M. rufiventris* significantly preferred the inhibited blend (Fig. 3b, GLM, naive: $F_{1,15}=11.04$, $P=0.005$; experienced on inhibited blend: $F_{1,15}=$

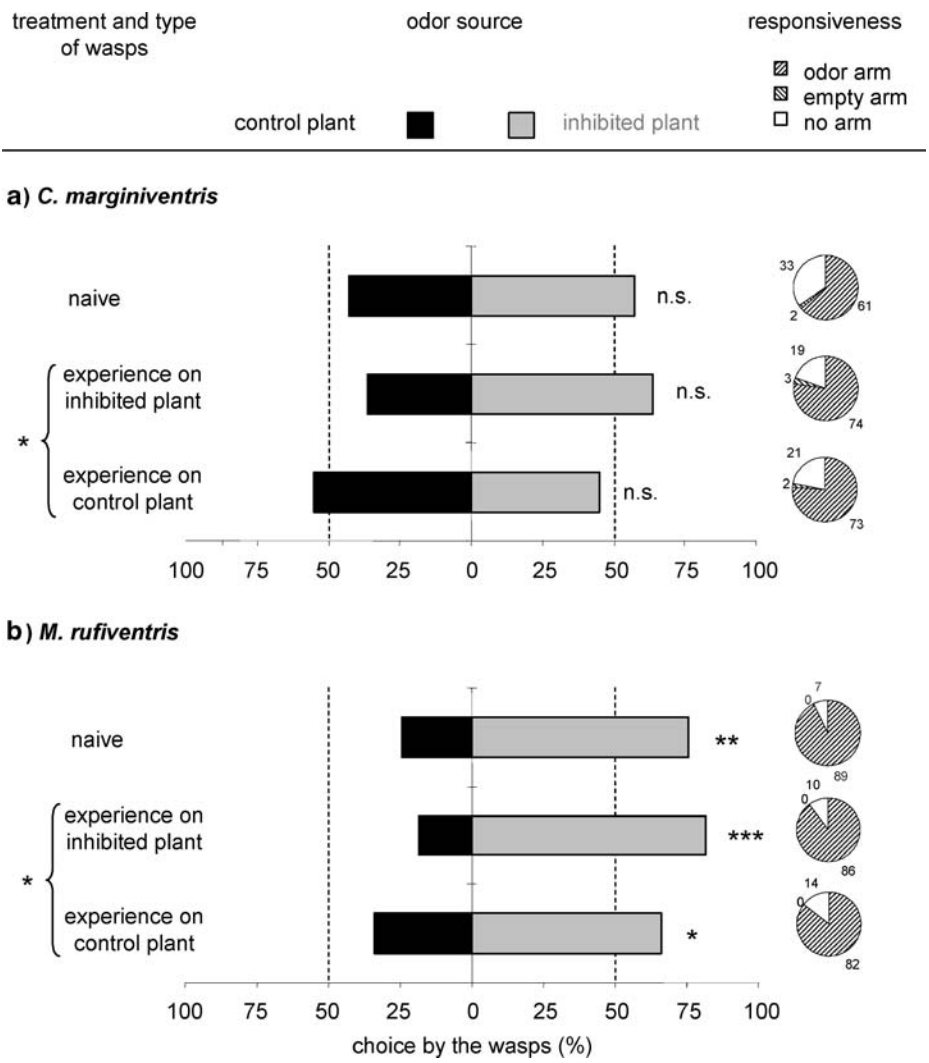


Fig. 3 Importance of shikimic acid derived VOC for the attraction of two parasitoid species. (a) Choice of *C. marginiventris* females and (b) *M. rufiventris* females between arms carrying VOCs of *Spodoptera*-induced maize seedlings that were either incubated in water (control plants) or in a 1 mM glyphosate solution (inhibited plants). Pretreatment of the wasps (=type of experience) is indicated on the left. Pie charts indicate overall responsiveness (=number of wasps entering the different types of arms). GLMs were performed in order to test for differences between the two odor arms within one group of wasps as well as to compare the types of experiences. ***$P<0.001$, **$P<0.01$, *$P<0.05$, n.s. = no significant difference, $P>0.05$

26.19, $P<0.001$; experienced on control blend: $F_{1,15}=6.19$, $P=0.025$). As with *C. marginiventris*, the type of experience had an effect on the choice of the wasps ($F_{1,60}=7.073$, $P=0.010$). The responsiveness was high, and none of the *M. rufiventris* wasps entered an empty arm.

Role of Indole Indole was the major shikimic acid derived VOC released by *Spodoptera*-induced maize seedlings (Fig. 2). We tested its role in the attraction of the two parasitoid species by comparing the attractiveness of HIPV blends released by two inhibited plants, whereby we added synthetic indole back to one of the blends. The amounts of indole added (192.77±11.46 ng/3 hr) fell within the lower ranges of indole detected in a natural induced maize blend (see above). *C. marginiventris* did not distinguish between the two blends (Fig. 4a, GLM: $F_{1,47}$=0.87, P=0.36). In contrast, *M. rufiventris* significantly preferred the inhibited blend without synthetic indole (Fig. 4b, $F_{1,47}$=43.16, P<0.001). Results for both wasps are consistent with those from the previous experiment, showing an insignificant role of shikimate-derived compounds for *C. marginiventris* attraction, whereas they have a repellent effect on *M. rufiventris*.

Learning of Indole When *C. marginiventris* females were given a choice between one arm with synthetic indole (192.77±11.46 ng/3 hr) and three arms with clean air only, they neither showed an innate (naive wasps) attraction towards indole (Fig. 5a, GLM: $F_{1,47}$= 0.60, P=0.44), nor were they attracted to indole after having experienced a natural blend that contained similar amounts of indole ($F_{1,47}$=0.29, P=0.60). However, if they were exposed to pure indole during oviposition experiences they preferred an arm carrying indole over arms with clean air ($F_{1,47}$=11.62, P=0.001), although the type of experience had only a marginal effect on their choices ($F_{1,124}$=2.919, P=0.090) and the overall responsiveness was rather low (Fig. 5a).

In an additional experiment, we tested whether exposing the wasps to indole during ovipositions increases the attraction towards an induced maize blend containing indole (control plant) compared to a blend with only trace amounts of indole (inhibited plant). As

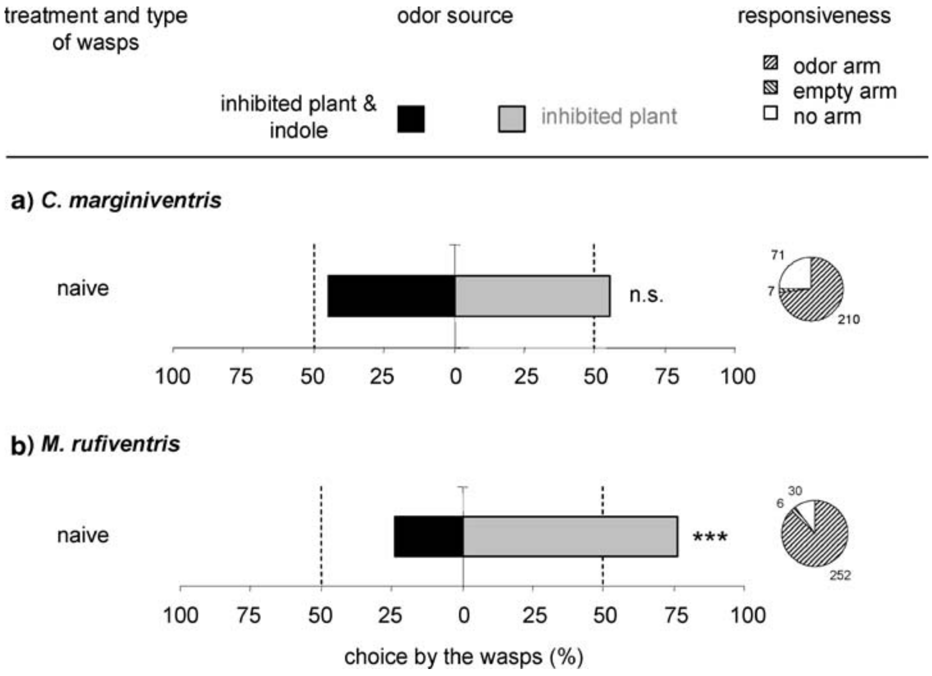


Fig. 4 Importance of indole for the attraction of two parasitoid species. (a) Choice of naïve *C. marginiventris* females and (b) naïve *M. rufiventris* females between arms carrying *Spodoptera*-induced maize VOCs of inhibited plants and of inhibited plants to which synthetic indole was added. See Fig. 3 for further explanations

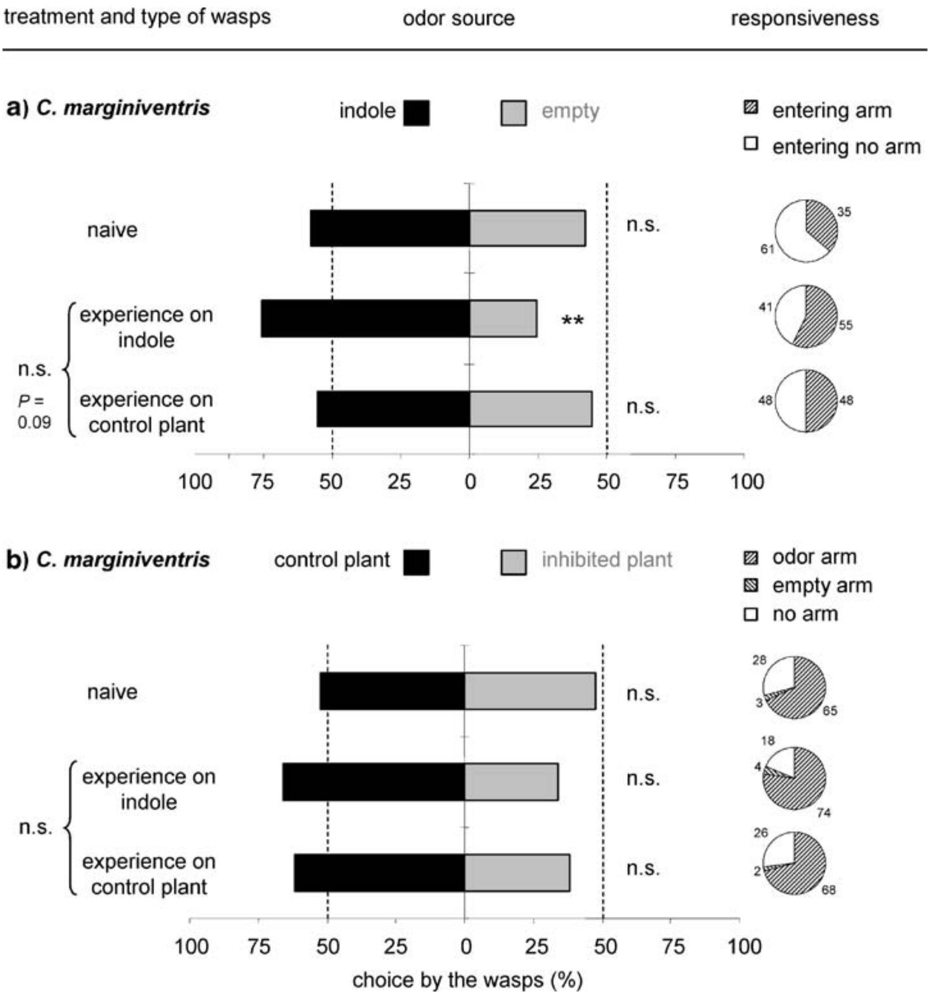


Fig. 5 Importance of indole in learning experiments by *C. margininventris*. (a) Choice of females with different learning experiences between arms carrying synthetic indole and empty arms with clean air only. (b) Choice of females with different learning experiences between arms carrying *Spodoptera*-induced maize VOCs of control and inhibited plants. See Fig. 3 for further explanations

in the experiments above, naive wasps and wasps that had experienced the control blend did not distinguish between the two blends (Fig. 5b, GLM: $F_{1,15}$=0.062, P=0.81; $F_{1,15}$=1.92, P=0.19, respectively). Interestingly, even an experience with pure synthetic indole did not result in a change in preference ($F_{1,15}$=3.70, P=0.074), and the type of experience did not have a significant effect on the wasps' choice ($F_{1,60}$=0.214, P=0.646).

Discussion

One way of studying the importance of individual VOCs for the attraction of natural enemies is to compare the attractiveness of an incomplete with a normal blend of HIPVs (D'Alessandro and Turlings, 2005). By restoring the incomplete blend with synthetic compounds that are missing, the importance of the added compounds can be confirmed (de Boer and Dicke, 2004). Here, we used glyphosate, an inhibitor of the enzyme EPSP synthase (Haslam, 1993; Schönbrunn et al., 2001), to inhibit the production of shikimic acid derived HIPVs. As expected, this inhibitor treatment resulted in a strong reduction of the emission of indole, methyl anthranilate, phenethyl acetate, and benzyl acetate, whereas the amounts of most other compounds, except two green-leaf volatiles, were not affected by the inhibitor treatment (Fig. 2). Treating plants with glyphosate normally results in lower amounts of chorismate, a precursor for the production of salicylic acid (SA) (Shah, 2003). SA has been shown to synergistically or antagonistically interact with jasmonic acid (Thaler et al., 2002; Bostock, 2005), an important hormone involved in the induction of maize VOCs (Schmelz et al., 2003c). We speculate that SA does not play a major role in induced volatile emissions in this maize variety. Indeed, MeSA, the volatile form of SA, was only occasionally detected in trace amounts in our control plants.

Glyphosate-treated plants are also expected to contain lower levels of phenolic compounds, which should have a positive effect on herbivores. However, we did not observe any difference in the amount of leaf damage inflicted by *Spodoptera* larvae between glyphosate-treated and glyphosate-untreated plants. A possible negative effect of glyphosate through direct toxicity has been ruled out in ecotoxicological risk assessment studies with arthropods (Giesy et al., 2000). Furthermore, glyphosate is not readily volatilized and is primarily degraded by microbial metabolism in the soil (Tu et al., 2001). Indeed, we did not detect additional VOCs from glyphosate-treated seedlings, making glyphosate a suitable inhibitor to study the attractiveness of shikimic acid derived VOCs.

Attractiveness of Shikimic Acid Derived VOCs It is generally assumed that HIPVs are exploited by natural enemies of the herbivores in order to locate their host or prey (Dicke, 1999; Turlings and Wäckers, 2004), but which compounds of a volatile blend are actually important in the foraging behavior of natural enemies is not yet known for most tritrophic systems (Dicke and van Loon, 2000). Previous studies on caterpillar-induced maize volatiles showed that qualitative differences in the odor blend may be more important for the attraction of parasitic wasps than quantitative differences (Fritzsche Hoballah et al., 2002). Indeed, a blend of induced maize volatiles with reduced amounts of the three major sesquiterpenoids, (*E*)-β-caryophyllene, (*E*)-α-bergamotene, and (*E*)-β-farnesene, was equally attractive to naive *C. marginiventris* as control blends with high amounts of these compounds, whereas removing some minor, polar compounds from the blend rendered it completely unattractive to *C. marginiventris* (D'Alessandro and Turlings, 2005). Here, we provide another example showing that some common HIPVs, i.e., shikimic acid derived volatiles, are not involved in the initial attraction of two parasitoids to host infested plants (Figs. 3 and 4). Indole, which is one of the dominating compounds in *Spodoptera*-induced maize volatiles (Turlings et al., 1998; D'Alessandro and Turlings, 2005), might even be repellent or might mask the attractiveness of compounds used for host location. Interestingly, several studies on maize volatiles indicate specific induction of volatile indole by herbivore-derived elicitors and not by excision stress or mechanical damage (Frey et al., 2000; Schmelz et al., 2003b). However, in other studies, many of the common herbivore-induced VOCs, including indole and several terpenoids, have been detected in analyses of VOCs released by plants exposed to other forms of stresses, as for example mechanical wounding (van den Boom et al., 2004), exposure to other VOCs (Ruther and Fürstenau, 2005), or infection by microorganism (Huang et al., 2003). Hence, the emission of various volatiles can be induced by a number of enemies and stresses, yet natural enemies are able to discriminate between different forms of stresses (Takabayashi et al., 1995; De Moraes et al., 1998; de Boer et al., 2004; Vuorinen et al., 2004). Selection must have favored parasitoids with an ability to distinguish between host- and nonhost-related compounds and an innate response to compounds that are specifically correlated with host presence is the most likely mechanism that allows them to make such distinctions (Vet and Dicke, 1992). We hypothesize that naïve females of generalist parasitic wasps are attracted only to a few key compounds within a complex blend of volatiles, and that most other compounds within such a blend contribute little to its initial attractiveness. They may mask the attractive compounds, or may even be repellent. Still, these compounds may become attractants when the wasps have associated them with host presence.

Importance of Learning One way for parasitoids to deal with highly complex and variable blends is their ability to learn by association (Turlings et al., 1993; Vet et al., 1995). It is assumed that such learning processes are specifically important for generalist wasps such as *C. marginiventris* and *M. rufiventris*, parasitizing various host, feeding on different plant species (Vet and Dicke, 1992; Steidle and van Loon, 2003). Indeed, *C. marginiventris* shows a keen ability for associative learning, whereas this form of learning is less clear for *M. rufiventris* (D'Alessandro and Turlings, 2005; Hoballah and Turlings, 2005; Tamò et al., 2006). Here again, *C. marginiventris* showed a significant shift in its preference in favor of the blend that it had experienced during multiple ovipositions (Fig. 3a). The response of *M. rufiventris* also changed significantly after experience, but it maintained a significant preference for the odor of inhibited plants even after having experienced the odor of control plants (Fig. 3b).

The predatory mites (*Phytoseiulus persimilis*) have been found to strongly associate MeSA with the presence of their prey if they are reared in the presence of a complex blend of herbivore-induced VOCs that includes MeSA (de Boer et al., 2004). Similarly, Vet et al. (1998) found that the *Drosophila* parasitoid *Leptopilina heterotoma* learned to discriminate between odors from substrates that were qualitatively different, but failed to discriminate when differences were small, unless unrewarding experiences provided evidence of the absence of hosts in one of the substrates. That some compounds are more important than others for associative learning was found for the parasitoid *M. croceipes*. After conditioning to a complex mixture, females of this species established a hierarchy among various components, with some of them accounting for a major part of the behavioral activity evoked by the mixture (Meiners et al., 2003). In our study, *C. marginiventris* was able to learn and subsequently respond to pure synthetic indole; however, this learning of indole had no effect on the females' responses to natural, complex blends with or without indole (Fig. 5). Apparently, indole is not a compound that is strongly associated during learning processes, especially if it is not offered in a complex volatile environment. Although indole is strongly induced after *Spodoptera* infestation on maize plants, it is also found in a variety of other stress-induced plant volatile blends (see above), and may not provide foraging parasitic wasps with specific information on the presence or absence of hosts.

In summary, we studied the role of shikimic acid derived VOCs, in particular indole, in the host searching behavior of two parasitoid species, *C. marginiventris* and *M. rufiventris*. This group of VOCs forms a substantial part of the volatile blend released by maize seedlings in response to feeding by lepidopteran larvae, but the results show that they are not important for the attraction of the two wasp species tested here. Attraction of *C. marginiventris* was not affected by the presence or absence of indole, the major shikimic acid derived VOC, whereas this compound was repellent rather than attractive to *M. rufiventris*. Learning of indole during oviposition experiences did not greatly alter these responses. Hence, this study suggests that parasitoids do not use all herbivore-induced VOCs for habitat and host location to a similar degree, but rather pay selective attention to a few compounds. Identifying these key compounds seems crucial for a good understanding of the host searching process in parasitoids and for the development of strategies to increase the efficiency of natural enemies for the control of pest insects (Turlings and Ton, 2006).

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