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Herbivore-induced plant volatiles induce an indirect defence in neighbouring plants

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Summary

1 Many plant species respond to herbivory with increased emission of volatile organic compounds (VOCs): these attract carnivorous arthropods and thereby function as an indirect defence mechanism. Whether neighbouring plants can 'eavesdrop' on such airborne cues and tailor their defences accordingly, remains controversial.
2 We used Lima bean plants (*Phaseolus lunatus*) to investigate whether herbivore-induced VOCs induce another indirect defence strategy, i.e. the secretion of extrafloral nectar (EFN) in conspecific plant neighbours, and whether this enhances the defence status of the receiving plant under natural conditions.
3 EFN secretion was induced by VOCs released from herbivore-damaged bean tendrils as well as by a synthetic VOC mixture resembling the natural one. One constituent of the herbivore-induced blend – the green leaf volatile (3*Z*)-hex-3-enyl acetate – was sufficient to elicit the defence reaction.
4 A long-term experiment comparing the defensive effect of EFN alone with the VOC-mediated effect (EFN induction plus attraction of plant defenders) suggested that Lima bean benefits from both indirect defences. Repeated treatment of tendrils with either an artificial blend of VOCs or with EFN led to the attraction of a higher cumulative number of predatory and parasitoid insects (i.e. ants and wasps) as well as to less herbivore damage and an increased production of inflorescences and leaves.
5 Our results demonstrate that one indirect defence mechanism can induce another one in conspecific plants, and that Lima bean plants can benefit from this VOC-induced EFN secretion under natural conditions. Both extrafloral nectaries and the capability to release VOCs upon herbivory are present in many plant taxa and airborne signalling may thus represent a common mechanism for regulating the secretion of EFN in plant parts which face an increased risk of herbivory.

Key-words: airborne signalling, ants, indirect defence, extrafloral nectar, herbivore-induced plant volatiles, herbivory (3*Z*)-hex-3-enyl acetate, Lima bean, Mexico, plant-plant communication

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Introduction

A large number of plants respond to leaf damage with the induction of a variety of defences. Many of these defences are regulated via the octadecanoid pathway, with jasmonic acid acting as a central signalling molecule (Creelman & Mullet 1997; Wasternack & Parthier 1997). In addition to direct defences, such as the production of leaf toxins, plants may also respond to herbivory with the emission of volatile chemicals that serve as host-location cues for carnivores (for review see Pichersky & Gershenzon 2002; Arimura *et al.* 2005). Plant-mediated attraction of carnivorous arthropods to actively feeding herbivores is generally believed to function as an indirect defence that enhances the fitness of the volatile-emitting plant by increasing predation

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pressure on the herbivores (Takabayashi & Dicke 1996; Paré & Tumlinson 1999).

Moreover, such plant volatiles provide chemical information about the status of attack of the emitting plant, which might be used not only by higher trophic levels (Turlings *et al*. 1995; Kessler & Baldwin 2001; van Poecke & Dicke 2004), but also by neighbouring plants of the same or another species (Baldwin & Schultz 1983; Arimura *et al*. 2000a; Karban & Maron 2002).

Plants that can activate and tailor their defences according to information derived from their neighbour may gain a selective advantage over plants that are unable to make use of this information. Whether plants positioned downwind are indeed capable of such 'eavesdropping' on airborne cues has been debated intensively (Fowler & Lawton 1985; Shonle & Bergelson 1995), but evidence for such plant–plant communication is accumulating (e.g. Bruin *et al*. 1992; Karban *et al*. 2000; Tscharntke *et al*. 2001).

An increasing number of laboratory studies on different plant species suggest that plants can perceive volatile signals, as evidenced by changes in transcription of defence-related genes (Arimura *et al*. 2000b; Gomi *et al*. 2003; Paschold *et al*. 2006). Furthermore, both laboratory and field studies have revealed that exposure to herbivore-induced (HI) volatile organic compounds (VOCs) results in changes in the abundance of phytohormones (Arimura *et al*. 2002; Engelberth *et al*. 2004), as well as in increased production of defence-related metabolites such as terpenoids (Engelberth *et al*. 2004; Ruther & Kleier 2005), proteinase inhibitors (Farmer & Ryan 1990; Tscharntke *et al*. 2001) or phenolic compounds (Baldwin & Schultz 1983; Tscharntke *et al*. 2001). Relatively few studies, however, report such effects under field conditions (e.g. Fowler & Lawton 1985; Preston *et al*. 2001) and, in particular, evidence for the expression of defence-related plant metabolites after exposure to VOCs under field conditions is largely lacking (but see Karban *et al*. 2000).

Here we attempt to bridge this gap by identifying a new mechanism of communication between herbivore-damaged and undamaged plants growing under natural conditions. Lima bean (*Phaseolus lunatus* L., Fabaceae), a model plant commonly employed in studies of induced defences, is known to emit HI-VOCs, and also has extrafloral nectaries in which increased secretion (Heil 2004) acts as an inducible indirect defence mechanism under natural conditions (Kost & Heil 2005). These traits make Lima bean an ideal system to investigate whether VOCs can induce another indirect defence trait (i.e. secretion of extrafloral nectar) in undamaged plants and whether this has fitness consequences for the receiver of the signal.

We used experiments performed on plants growing under natural field conditions to address four questions. (i) Does the emission of HI-VOCs induce the secretion of extrafloral nectar (EFN) in undamaged, neighbouring tendrils? (ii) Which compounds within the complex blend of HI-VOCs are responsible for the induction of EFN secretion? (iii) Does the Lima bean benefit from a volatile-induced secretion of EFN under natural growing conditions? (iv) Are putative plant defenders (ants and wasps) attracted to VOCs and EFN?

Materials and methods

STUDY SITE AND SPECIES

Field work was done in 2003 and 2004 during the transition from wet to dry season (October–December). All field experiments were performed on a native population of Lima bean growing in the coastal area near Puerto Escondido in the state of Oaxaca, Mexico. The plants investigated were growing under natural field conditions along dirt roads leading to extensively used pastures or plantations at sites where previous experiments had been performed (Heil 2004; Kost & Heil 2005). Due to the tangled growth of Lima bean, it was not always possible to ensure that the bean tendrils used in the experiment belonged to one single plant individual.

All laboratory experiments were performed with potted plants which were grown from seeds derived from plants of our study sites. According to preliminary analyses with AFLP markers (M. Heil, unpublished data), these plants belong to the 'Mesoamerican genotype' (*sensu* Gutiérrez Salgado *et al*. 1995). Plants were grown in plastic pots with a diameter of 14 cm. Growing conditions were 23 °C, 60% humidity, and 270 $\mu E\ m^{-2}\ s^{-1}$ during a 14 h photoperiod. Experiments in the laboratory were performed with 6-week-old plants (i.e. 7–8 leaf stage).

EMISSION OF HI-VOCS

Experiment 1

We enclosed pairs of naturally growing, basifixed tendrils with five leaves in separate gauze bags (mesh size 0.5 mm) and supplied one of the two bags with herbivores that had been previously observed feeding on Lima bean. Herbivores involved were mainly the beetles *Cerotoma ruficornis* and *Gynandrobrotica guerreroensis* (both Chrysomelidae), *Epilachna varivestis* Mulsant (Mexican bean beetle, Coccinellidae), and a curculionid species, as well as one species of Ensifera and one of Caelifera. Abundance and species composition of the caged herbivores mirrored the frequency of occurrence during insect sampling and thus represented a cross-section through the local herbivore community typically attacking Lima bean plants. Each gauze bag was supplied with six Ensifera or Caelifera and 10 beetles of various species.

After 48 h, the naturally induced tendrils were detached, immediately supplied with a water reservoir and the herbivores carefully removed. The tendrils

were bagged in a PET foil ('Bratenschlauch', Toppits, Minden, Germany) that does not itself emit detectable amounts of volatiles. The emitted VOCs were collected continuously over 24 h on charcoal traps (1.5 mg charcoal, CLSA-Filters, Le Ruissaeu de Montbrun, France) using air circulation as described previously (Donath & Boland 1995). After 24 h, leaves were dried for dry mass determination and volatiles were eluted from the carbon trap with dichloromethane (40 μL) containing 1-bromodecane (200 ng μL^{-1}) as an internal standard. Samples were then transferred to glass capillaries, sealed by melting the open end and stored at < 5 °C for transport to Germany. Samples were analysed on a GC-Trace mass spectrometer (Thermo Finnigan, www.thermofinnigan.com) according to Koch *et al.* (1999). Individual compounds (peak areas) were quantified with respect to the peak area of the internal standard and related to the dry weight of the measured tendril.

Experiment 2

We used laboratory grown plants to test whether the detachment of Lima bean tendrils alters the qualitative and quantitative composition of the blend of emitted HI-VOCs. The five youngest leaves of two potted Lima bean plants were exposed to five adult *E. varivestis* for 2 days at room temperature, while two control plants were left undamaged. After 24 h, the herbivory rate of the two herbivore-damaged tendrils was estimated according to Kost & Heil (2005) to ensure that the mean leaf area consumed was similar in the two plants (paired *t*-test: $P > 0.05$, $n = 9$). One damaged and one undamaged tendril were then cut and supplied with a water reservoir and the two other plants remained potted. Volatiles were collected from the five youngest leaves of all four plants for the next 24 h as described for Experiment 1. This experiment was replicated nine times.

EFN COLLECTION: GENERAL PROCEDURE

The production rate of EFN was determined as amounts of secreted soluble solids (i.e. sugars, amino acids; see, e.g. Heil *et al.* 2000), by quantifying the nectar volume with micro capillaries and the nectar concentration with a portable, temperature-compensated refractometer (Heil *et al.* 2000, 2001).

We had previously verified that the amount of secreted EFN depends on the dry weight of the secreting leaf. The accumulated EFN of 30 potted Lima bean plants at the 7–8 leaf stage was washed off with pure water. Fourteen of these plants were sprayed with an aqueous solution of 1 mmol jasmonic acid (JA) until the surfaces of all leaves were covered, while the remaining 16 control plants remained untreated. After 24 h, the amount of newly secreted EFN of the five youngest leaves was quantified as described above and the leaves were dried for dry weight (DW) determination. Significant relationships were found between the amount of secreted EFN and dry weight of the secreting leaves for both control (linear correlation $r = 0.811$, $n = 16$, $P < 0.001$) and JA-treated tendrils (linear correlation after reciprocal transformation $r = 0.57$, $n = 14$, $P < 0.05$).

EFN-INDUCTION EXPERIMENTS

The amount of secreted EFN strongly depends on parameters such as light intensity (C. Kost, personal observation; Helder 1958; Michaud 1990; Pacini *et al.* 2003) or leaf age (C. Kost, unpublished data; Heil *et al.* 2000). We therefore used a paired experimental design with the maximum distance between two tendrils of each pair being < 1 m (to reduce environmental variation) and matched tendril pairs of similar leaf age. Due to this experimental approach, the relative difference between treated and control tendrils, rather than the absolute amount of EFN secreted, is the appropriate measure to assess any given effect.

Tendrils used for all EFN-induction experiments were undamaged basifixed tendrils with five leaves, which were placed in mesh bags (mesh size 0.5 mm). A ring of sticky resin (Tangletrap®, Tanglefoot Company, Grand Rapids, Michigan, USA) was applied at their base to protect the tendrils from flying and crawling nectar consumers.

Experiment 3

Wild growing tendrils were exposed to herbivores naturally feeding on Lima bean as described above (Experiment 1). After 48 h, the naturally induced tendrils were detached, immediately supplied with a water reservoir and the herbivores carefully removed. These 'emitter' tendrils were then packed together with a basifixed bean tendril ('receiver tendril') into a perforated PET-plastic bag ('Bratenschlauch', Toppits, Minden, Germany). Receiver tendrils packed with detached but otherwise undamaged tendrils served as controls. The two tendrils were arranged so that the emitter tendril entered the plastic bag from above and the receiver tendril from below. Both ends of the bag were tied up with a cord, with the water reservoir projecting out of the lower end of the assembly. EFN production rates of receiver tendrils were quantified after 24 h. The plastic bags used for this experiment were perforated with a conventional office hole-punch (hole area 0.78 cm^2) at regular intervals (previous trials showed that, even after direct exposure to sunlight, the packed tendril did not wilt nor did moisture accumulate on the inside of the foil within 24 h when the perforation ratio was 11%).

Experiment 4

A blend of synthetic VOCs was prepared and dissolved in lanolin paste (Sigma-Aldrich, www.sigmaaldrich.com) as a

matrix from which the volatiles could evaporate (Kessler & Baldwin 2001). The results from previous measurements were used to adjust the mixture so that it mimicked, both quantitatively and qualitatively, the emission induced by herbivores within 24 h. The artificial blend consisted of 0.12 μg (*R*)-(–)-linalool, 0.13 μg β-caryophyllene, 0.19 μg methyl salicylate, 0.26 μg (*Z*)-jasmone (all purchased from Sigma-Aldrich), 0.02 μg (3*Z*)-hex-3-enyl acetate (Avocado Research Chemicals Ltd, Leysham, Lancaster, UK), 0.85 μg (*E*,*Z*)-β-ocimene (mixture of (*E*)-isomer (70%) and (*Z*)-isomer (30%)) (kindly provided by Roger Snowden, Firmenich, Geneva, Switzerland), 0.63 μg (3*E*)-4,8-dimethylnona-1,3,7-triene (DMNT) and 0.9 μg (3*E*,7*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) (synthesized by standard methods (Pattenden & Weedon 1968)) per μL lanolin. Purity of all compounds was > 98%.

To verify whether the VOCs released from the lanolin paste also mimicked the bouquet induced by herbivores under field conditions, green plastic strips, on which 40 μL of either the artificial VOC mixture or lanolin only were spotted, were applied to seven pairs of detached Lima bean leaves. The plastic strips were used to prevent any diffusion of compounds into the plant. The headspace of these leaves was sampled for 24 h as described for Experiment 1.

Experiment 5

Lanolin paste containing the artificial volatile blend (40 μL), or lanolin alone, was spotted on green plastic strips attached close to each of five leaves of a bean tendril. The production rate of EFN was quantified after 24 h. This experiment was conducted in 2003 (n = 10) as well as in 2004 (n = 12).

Experiment 6

Pairwise comparisons between tendrils, which have been treated with lanolin alone and untreated tendrils (n = 12), tested for an effect of lanolin on the EFN production rate.

Experiment 7

With the same experimental design as used in Experiment 5, we performed single-compound comparisons with all eight constituents of the complete blend. For this purpose we mixed blends that contained 1 μg of every compound per μL lanolin and 40 μL of this mixture were applied per leaf as described above. Sample sizes for this experiment are given in Table 1.

LONG-TERM EXPERIMENT

Experiment 8

This experiment was conducted to verify whether VOC-induced EFN secretion indeed enhances the defence status of wild growing Lima bean plants. We therefore compared the defensive effect of EFN alone to the volatile-mediated effect, i.e. attraction of plant defenders to both VOCs and VOC-induced EFN. For this purpose, we used a paste containing volatiles (as in Experiment 4) and an artificial EFN (an aqueous solution of 4.01 g sucrose L^{-1}, 24.24 g L^{-1} of each fructose and glucose). Both mixtures were adjusted to mimic natural production within three days post-induction (C. Kost, unpublished data; Kost & Heil 2005).

Seventeen groups of four neighbouring tendrils each growing < 3 m apart from each other were selected as experimental units. All tendrils selected were trained along supporting ropes, and the tendrils of each group were randomly assigned to one of four treatments. Tendrils were either left untreated (control group) or treated every 3 d with lanolin paste only (treatment control group), artificial volatile blend (volatile group) or artificial nectar mixture (nectar group). The lanolin paste was applied as described above, whereas the EFN mimic (40 μL per trifoliate leaf) was applied directly to the extrafloral nectaries of every leaf of each tendril of the nectar group. To determine the effect of the four treatments on the fitness of the treated tendrils, we quantified three fitness-relevant plant parameters, herbivory rate (as percentage leaf loss, Kost & Heil 2005)

Table 1 Extrafloral nectar (EFN) secretion from natural growing Lima bean tendrils after treatment with single constituents of the herbivore-induced volatile blend (numbered as in Fig. 1). Pairwise comparisons between wild growing, neighbouring tendrils of five leaves, each treated with either 40 μL of lanolin paste (Control) or lanolin paste containing 1 μg μL^{-1} of a single compound (Compound), are displayed. Mean (± SEM) EFN secretion rates in mg soluble solids per g leaf dry mass per 24 h and P-values of paired t-tests between both treatment groups are given

		EFN secretion rate (mg g^{-1} 24 h^{-1})		
Compound (no.)	n	Control	Compound	P
(3*Z*)-Hex-3-enyl acetate (1)	10	2.6 (± 0.6)	4.6 (± 0.9)	0.040
(*E*,*Z*)-β-Ocimene (2)	7	2.2 (± 0.8)	2.5 (± 0.6)	0.542
(*R*)-(–)-Linalool (3)	10	3.5 (± 0.9)	4.9 (± 0.9)	0.300
DMNT (4)	8	2.8 (± 0.9)	4.3 (± 0.8)	0.246
Methyl salicylate (6)	7	4.4 (± 0.7)	3.5 (± 0.4)	0.104
(*Z*)-Jasmone (8)	8	4.8 (± 0.7)	4.5 (± 0.7)	0.746
β-Caryophyllene (9)	6	3.9 (± 0.6)	3.1 (± 0.8)	0.296
TMTT (10)	8	4.7 (± 1.0)	6.3 (± 1.5)	0.405

and the numbers of newly developed leaves and inflorescences at the beginning of the experiment and after 25 days. The differences between these two values were calculated to determine the development of the respective parameter.

Experiment 9

The influence of our treatments on the presence of putative plant defenders was investigated by assessing the number of ants and wasps that visited the study tendrils. Fourteen censuses were performed within 3 days at two sites (at site 1 on day 7 and at site 2 on day 18 after the beginning of the experiment) and insect numbers were pooled for each tendril.

STATISTICAL ANALYSES

The high variability of the EFN secretion rates measured in Experiments 3, 5, 6 and 7, which is caused by environmental factors (see above), allowed comparisons between two groups of paired tendrils, but multiple statistical testing between different tendril pairs was not possible. These experiments were therefore considered independent and analysed by using paired *t*-tests without a multiple-comparison correction (such as Bonferroni). Differences in the emission rate of VOCs (Experiments 1, 2 and 4) were estimated using exact Mann–Whitney *U*-tests and Wilcoxon signed rank tests for matched pairs. Despite deploying multiple comparisons to one data set, we did not use a multiple-comparison correction. By adjusting the Type I error (i.e. error of incorrectly declaring a difference) downwards, such a procedure would have increased the chance of making a Type II error (i.e. no difference is declared, while in fact there is a difference). Experiments 1, 2 and 4 aimed at comparing our treatments to the natural situation. Thus, accepting Type I rather than Type II errors was the more conservative approach.

Data from the long-term experiment (Experiments 8 and 9) were analysed by applying a mixed-effect model with 'treatment' as a fixed and 'tendril group' as a random factor. The number of leaves were ln-transformed and the number of inflorescences log-transformed to meet the assumption of homogeneity of variances. *Post hoc* comparisons (LSD) were performed to test for statistically significant differences between treatments. Data were analysed using SPSS 13.0 (SPSS for Windows, SPSS Inc., Chicago, USA).

Results

EMISSION OF HI-VOCS

In response to herbivore attack, Lima bean plants released a blend of VOCs comprised of eight main constituents (Experiments 1 and 2; Figs 1 and 2). Detachment of bean tendrils had little effect on either the total amount of volatiles emitted (Wilcoxon signed rank test: $P > 0.05$, $n = 9$), or the emission rate of the eight main constituents of the blend in comparison to potted plants (Experiment 2; Fig. 2). The only significant effects were seen in otherwise undamaged plants, where detachment caused a decrease in the emission rate of β-caryophyllene and the homoterpene TMTT (Wilcoxon signed rank tests: $P < 0.01$ and $P < 0.05$, $n = 9$).

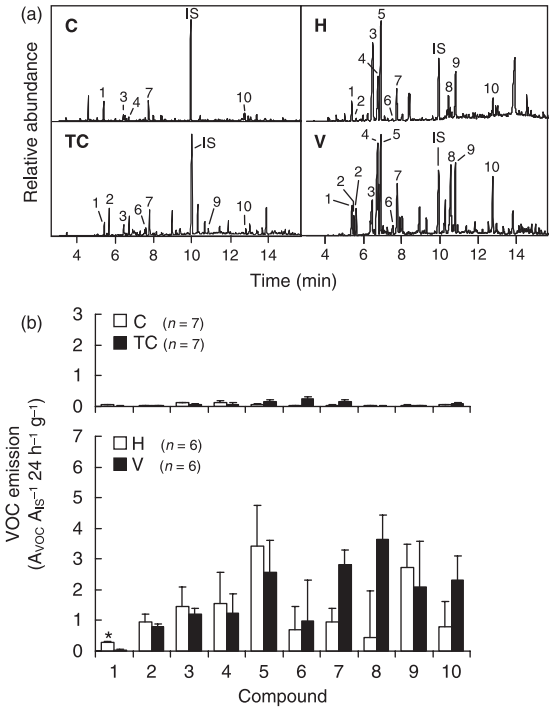

Fig. 1 Volatile blends resulting from different treatments (Experiments 1 and 4). (a) Representative gas chromatographic profiles of an undamaged bean tendril (C), an herbivore-induced bean tendril (H), a single leaf treated with lanolin paste (TC) or a single leaf treated with artificial volatile blend dissolved in lanolin paste (V). (b) Pairwise comparisons between mean (+ SEM) VOC emission of undamaged tendrils (C) and tendrils treated with lanolin paste (TC) as well as between herbivore-induced bean tendrils (H) and tendrils treated with the artificial volatile blend dissolved in lanolin paste (V). The amount of emitted VOCs is given as peak area relative to the peak area of an internal standard per 24 h and per g dry weight. Asterisks denote significant differences between C and CT as well as between H and V for every analysed VOC (exact Mann–Whitney *U*-test, $*P < 0.05$). Identified compounds are: 1 (3*Z*)-hex-3-enyl acetate; 2 (*E*,*Z*)-β-ocimene; 3 (*R*)-(–)-linalool; 4, DMNT; 5, $C_{10}H_{14}$; 6, methyl salicylate; 7, $C_{10}H_{16}O$; 8 (*Z*)-jasmone; 9, β-caryophyllene; 10, TMTT; IS, internal standard (1-bromodecane).

EFN-INDUCTION EXPERIMENTS

Quantification of EFN after 24 h indicated that receiver tendrils exposed to naturally induced VOCs (i.e. from detached, herbivore-damaged emitter tendrils) showed a two-fold increase in EFN secretion rate as compared to controls (Experiment 3; Fig. 3, C′ vs. H comparison; paired *t*-test: $P < 0.001$, $n = 9$).

If airborne VOCs were responsible for the observed effect, then synthetic mixtures which mimic the blend

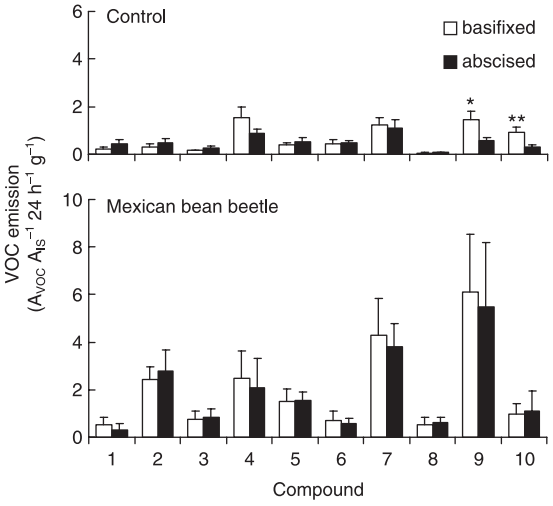

Fig. 2 Comparison of the mean (+ SEM) VOC emission between basifixed and abscised tendrils from undamaged plants (Control) and plants damaged by five adult individuals of *Epilachna varivestis* (Mexican bean beetle) (Experiment 2). VOCs as in Figure 1. Asterisks denote significant differences between basifixed and abscised tendrils for every analysed VOC (Wilcoxon signed rank test, $*P < 0.05$; $**P < 0.01$). Sample size was nine plants per treatment.

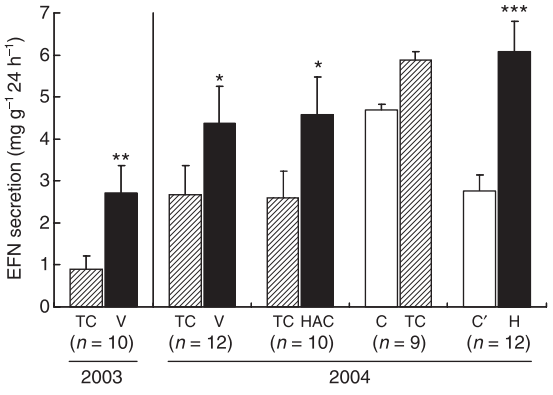

Fig. 3 Extrafloral nectar (EFN) secretion in response to different treatments. Mean (+ SEM) EFN secretion rates are given in mg soluble solids per g leaf dry mass per 24 h. Pairwise comparisons between tendrils treated with lanolin paste (TC) and the artificial volatile blend (V) (Experiment 5) or (3*Z*)-hex-3-enyl acetate (HAC) dissolved in lanolin (Experiment 7) are depicted. Furthermore, the EFN secretion rate of untreated tendrils (C) and tendrils treated with lanolin paste (TC) (Experiment 6) as well as between tendrils exposed to detached, undamaged tendrils (C′) and tendrils exposed to detached, herbivore-damaged tendrils (H) (Experiment 3) are displayed. Asterisks denote significant differences between pairs of tendrils (paired *t*-test, $*P < 0.05$, $P < 0.01$, $***P < 0.001$).

emitted from naturally induced tendrils should also be capable of inducing EFN secretion in undamaged plants. Our artificial mixture was both qualitatively and – to a large degree – quantitatively similar to the natural blend, although significantly lower amounts of (3*Z*)-hex-3-enyl acetate were released (Experiment 4; Fig. 1; exact Mann–Whitney *U*-test: $P < 0.05$).

Application of this artificial volatile blend, and the resulting increased amount of VOCs in the headspace of Lima bean tendrils, significantly increased the EFN secretion rate as compared to control tendrils that had been treated with lanolin only (Experiment 5; Fig. 3, TC vs. V; paired *t*-test: $P < 0.01$ in 2003, $P < 0.05$ in 2004). A comparison of the EFN production rate between untreated tendrils and tendrils, to which lanolin only had been applied, revealed no statistically significant difference (Experiment 6; Fig. 3, C vs. TC; paired *t*-test: $P > 0.05$, $n = 12$).

When the EFN secretion rate of control tendrils (lanolin only) were pairwise compared with tendrils that had been treated with a lanolin mixture containing only one of the eight main constituents of the complete herbivore-induced blend (Experiment 7; Table 1), only (3*Z*)-hex-3-enyl acetate significantly increased EFN secretion rates (Fig. 3, TC vs. HAC), although some other constituents ((*R*)-(–)-linalool, DMNT and TMTT) did increase EFN secretion (Table 1). EFN-induction by (3*Z*)-hex-3-enyl acetate was similar in strength to the complete mixture of synthetic VOCs (Fig. 3).

LONG-TERM EXPERIMENT

After 25 days of repeatedly applying a synthetic mixture of EFN and VOCs (i.e. every 3 days), fitness-relevant plant parameters such as the number of inflorescences (Fig. 4; univariate ANOVA: $P < 0.01$, $n = 17$) and leaves (Fig. 4; univariate ANOVA: $P < 0.001$, $n = 17$) were significantly increased in the two treatment groups (nectar and volatiles) as compared to both control groups (control and treatment control) (Experiment 8). Moreover, tendrils treated with the artificial blends of VOCs and EFN had received significantly less damage by leaf-chewing herbivores (Fig. 4; univariate ANOVA: $P < 0.01$, $n = 17$) than did control tendrils. Remarkably, the intensity of the defensive effects observed in the nectar and the volatile group were quantitatively indistinguishable (Fig. 4).

The numbers of ants and wasps that visited the studied tendrils at the end of the experiment were significantly increased on treated tendrils (univariate ANOVA for both ants and wasps: $P < 0.001$, $n = 17$) as compared to controls (Experiment 9; Fig. 5). Again, no statistically significant difference could be detected between nectar and volatile group. However, wasps were generally 25-times less frequently observed than ants (Fig. 5).

Discussion

Our results demonstrate that HI-VOCs were recognized by undamaged, neighbouring Lima bean plants as indicating the likelihood of an impending herbivore attack, prompting them to activate their own defence mechanisms (secretion of EFN). Both naturally emitted VOCs (Experiment 3) and a mixture of synthetic VOCs (Experiment 5) were capable of inducing EFN secretion in receiver tendrils. (3*Z*)-hex-3-enyl acetate was identified as a biologically active substance

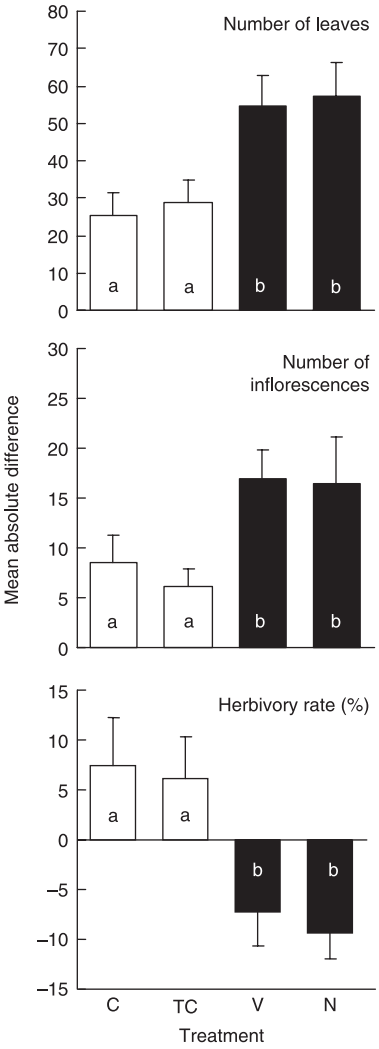

Fig. 4 Effects of volatile and nectar treatment on fitness-relevant plant parameters (Experiment 8). Differences between measurements at $t = 0$ days and $t = 25$ days are displayed (mean + SEM). Groups of tendrils were left untreated (control, C), or were treated at regular intervals (3 days) with lanolin paste (treatment control, TC), artificial volatile blend dissolved in lanolin paste (volatiles, V) or an artificial extrafloral nectar (nectar, N). Different letters indicate significant differences among treatments (univariate ANOVA, $P < 0.05$ according to LSD *post hoc* test). Sample size was 17 groups of tendrils.

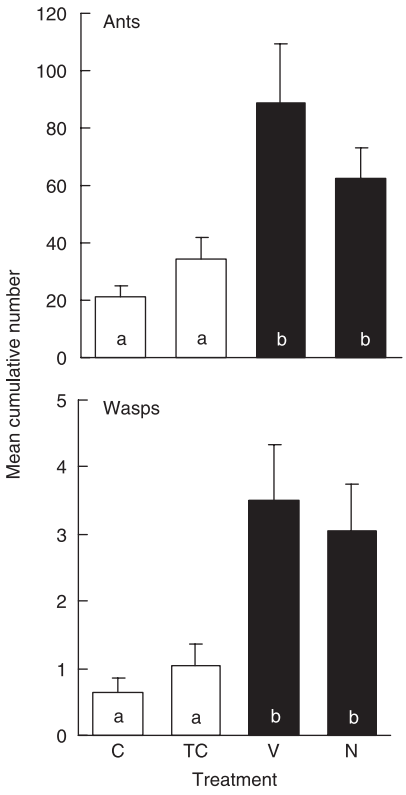

Fig. 5 Effects of volatile and nectar treatment on insect numbers (Experiment 9). Insects, residing on bean tendrils that were left untreated (control, C), or were treated at regular intervals (3 days) with lanolin paste (treatment control, TC), artificial volatile blend dissolved in lanolin paste (volatiles, V) or an artificial mixture of extrafloral nectar (nectar, N), were counted. Fourteen censuses were performed within 3 days at two sites (site 1 on day 7 and site 2 on day 18 after the beginning of the experiment). Cumulated insect numbers (mean + SEM) are presented. Different letters indicate significant differences among treatments (univariate ANOVA, $P < 0.05$ according to LSD *post hoc* test). Sample size was 17 groups of tendrils.

(Experiment 7) and the application of a mixture of synthetic VOCs benefited Lima bean under natural growing conditions (Experiments 8 and 9).

Contrasting the VOC profiles emitted from Lima bean plants, which had been attacked by either a mixture of natural folivores (Experiment 1; Fig. 1) or Mexican bean beetles *E. varivestis* (Experiment 2; Fig. 2) revealed quantitative rather than qualitative differences. The VOCs released from *P. lunatus* upon herbivore damage largely matched those of previous reports (Dicke *et al*. 1999; Ozawa *et al*. 2000; Mithöfer *et al*. 2005). However, it appears that the particular type of damage inflicted has a major influence on the qualitative composition of the emitted VOC blend. Chewing herbivores, whether caterpillar, beetle or even snail, provoke a similar blend of VOCs (this study; Mithöfer *et al*. 2005), whereas cell-content feeders such as mites frequently induce compounds which are absent in the VOC profile induced by chewing herbivores (Dicke *et al*. 1999; Arimura *et al*. 2000a). As a first step in investigating whether VOCs can induce EFN secretion, we focused our analysis on the eight most dominant compounds, which are induced independently of the feeding mode of the attacking herbivore.

Although it is known that ablation of leaves or branches may alter the amount and blend composition of VOCs emitted from herbivore-infested plants (Arimura *et al*. 2001; Schmelz *et al*. 2001), the HI-VOC profiles emitted from abscised tendrils and basifixed plants were very similar (Experiment 2; Fig. 2), and we were therefore confident in using detached tendrils as VOC-emitters for our EFN induction experiment (Experiment 3).

Studying airborne communication between plants under field conditions is a daunting task: plants in their natural environment are exposed to a multitude of

additional signals, and factors such as wind currents may hamper the detectability of a given effect. Because this study was intended to test whether airborne VOCs can induce EFN secretion in Lima bean plants at all, our approach was designed to increase the probability of detecting an effect under field conditions.

We enclosed the receiver and the naturally induced emitter tendrils in bags of PET foil (Experiment 3). Because previous studies (e.g. Bate & Rothstein 1998; Arimura *et al*. 2000a; Tscharntke *et al*. 2001) have often been criticised for using airtight chambers, which may alter the physiological status of the enclosed plants by causing a shortage of CO_2 (Baldwin *et al*. 2002; Dicke *et al*. 2003), we perforated the foil (perforation with relatively large holes). The enclosed tendrils did not show any symptoms of wilting and there was no moisture on the inside of the foil, even after exposure to direct sunlight. These conditions may thus have corresponded to a windless day. The observation that the artificial VOC mixture, which closely mimicked the naturally released blend, was also capable of inducing EFN secretion in tendrils which were not enclosed at all (Experiment 5), suggests that enclosure *per se* had little influence. However, future investigations in which Lima bean plants are exposed to herbivore-damaged neighbouring plants without any enclosure are necessary to clarify whether this phenomenon also occurs under unconstrained environmental conditions.

Information exchange between emitter- and receiver-tendrils via a systemic signal within the same plant (Dicke & Dijkman 2001; Wäckers *et al*. 2001) or via the rhizosphere between two different individuals (Chamberlain *et al*. 2001; Pickett *et al*. 2003) was excluded in Experiment 3, because we used detached emitter tendrils to expose basifixed receiver tendrils to the headspace of either undamaged or herbivore-damaged tendrils. Although within-plant or rhizosphere signalling may have occurred. In the EFN-induction experiments with synthetic VOCs (Experiments 5 and 7), both mechanisms should have resulted in an induction of the control tendrils and thus to underestimation of any effect. The presence of an effect therefore suggests that the signal, which elicited an increased EFN secretion, has been transmitted aerially, rather than through the plant or the soil.

The use of synthetic VOCs allowed identification of (3*Z*)-hex-3-enyl acetate as the compound eliciting the observed defence induction (Experiment 7; Table 1). (3*Z*)-hex-3-enyl acetate belongs to the group of so-called green leaf volatiles (GLVs), which are known to be emitted rapidly after leaf-damage (Loughrin *et al*. 1994; Turlings *et al*. 1995; Arimura *et al*. 2000a), but are also produced *de novo* in response to herbivore damage by many plant species (Kalberer *et al*. 2001; Kessler & Baldwin 2001; Röse & Tumlinson 2004).

(3*Z*)-hex-3-enyl acetate has been previously shown to induce defence genes in uninfested leaves of Lima bean (Arimura *et al*. 2001) and *Arabidopsis* (Bate & Rothstein 1998) and to prime corn plants against subsequent herbivore damage (Engelberth *et al*. 2004). An increased emission rate of (3*Z*)-hex-3-enyl acetate directly after mechanical wounding (i.e. within the first hour) of Lima bean plants (Arimura *et al*. 2000a) may facilitate a fast induction of the EFN secretion in neighbouring tendrils and thus allow a rapid response to a current threat.

Since (3*Z*)-hex-3-enyl acetate has been identified as the biologically active substance, the decreased rate at which this GLV is emitted from the mixture of synthetic VOCs (Fig. 1) should have led to an underestimation of the inductive effect and could explain the weaker effect of the synthetic VOC blend compared to herbivore-damaged bean tendrils (Fig. 3). Other constituents of the synthetic VOC mixture may also have contributed to EFN induction. Compounds that have been identified in other studies as eliciting VOC-induced plant responses, but which did not show an effect on the secretion rate of EFN in this study, include (*Z*)-jasmone (Birkett *et al*. 2000; Bruce *et al*. 2003) (*E*,*Z*)-β-ocimene, DMNT, TMTT (Arimura *et al*. 2000a) and methyl salicylate (Shulaev *et al*. 1997). Our methodology for headspace sampling and VOC analysis does not allow distinction between an inductive effect of a single compound and the combined action of several.

Few studies conducted under field conditions have clearly demonstrated fitness consequences for the receiver of the volatile signals (Dolch & Tscharntke 2000; Karban *et al*. 2000; Karban & Maron 2002). To verify whether VOC-induced EFN secretion enhances the defence status of Lima bean plants, and thus plant fitness, we treated tendrils repeatedly with the artificial VOC mixture (Experiment 8): such tendrils lost less leaf area to herbivores and produced more leaves and inflorescences than controls (Fig. 4). In a previous study, conducted at the same sites, Lima bean plants that had been repeatedly induced with the chemical elicitor jasmonic acid suffered from less herbivore damage and showed a significant increase in the number of leaves, inflorescences and fruits (Heil 2004). The increased number of inflorescences observed in this study will likely translate into increased seed set and thus enhance reproductive success.

Because VOCs induced EFN secretion, the tendrils of the volatile treatment experienced the combined defensive effects of EFN and VOCs, but none of the fitness-related parameters were different from those in the nectar treatment (Fig. 4). EFN therefore played a more important role as indirect defence in our study system than did volatile chemicals, perhaps with VOCs functioning more as EFN-inducing signals among bean tendrils located within a patch of increased herbivore pressure than as long-distant cues for flying plant defenders. However, our experimental approach does not exclude a protective effect exerted by volatile-attracted defenders. This issue should be addressed in future studies.

The architecture of the Lima bean is characterized by (i) short distances between individual bean tendrils

and (ii) a tangled growth that creates microenvironments with reduced wind current. A systemic defence-inducing signal which is transported within the plant would therefore be very ineffective (Jones *et al*. 1993). In contrast, an airborne signal would create a gradient of the infochemical around the site of attack, to which both parts of the emitting plant and neighbouring plants can respond. Moreover, the intricate growth structure may minimize dilution of airborne signals (Thistle *et al*. 2004) and thus favour plant–plant communication.

Regarding the widespread taxonomic distribution of both extrafloral nectaries (Koptur 1992) and the capability of plants to respond to herbivore damage with the emission of VOCs (van Poecke & Dicke 2004), such a plant–plant communication response may represent a common mechanism which adds a new facet to our understanding of the complex interactions among different trophic levels. Thus, the elucidation of this mechanism opens up new avenues for further studies that range from the underlying signalling cascades to the ecological relevance of this mechanism in various study systems.

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