

## Priming of indirect defences

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### Abstract

Leaf damage induces in many plant species the secretion of extrafloral nectar (EFN) and/or the release of specific odours (volatile organic compounds, VOCs). Both traits attract carnivorous arthropods and function as indirect plant defences by increasing the predation pressure on attacking herbivores. We have conducted field experiments in Mexico and found that plants that had been exposed to VOCs thereafter responded to subsequent leaf damage with an increased EFN secretion. VOCs ‘primed’ the plants to respond more efficiently once they were attacked themselves. Such priming effects are well known for induced resistance to pathogens, yet this is the first description of VOCs priming EFN secretion, a taxonomically widespread anti-herbivore defence. Plants can use chemical signals in their environment to assess the risk of herbivory and make use of this information to adjust their own defensive strategy accordingly.

### Keywords

Ant–plant interaction, extrafloral nectar, herbivore-induced volatiles, indirect defence, induced defence, Lima bean, Mexico, *Phaseolus lunatus*, plant–plant communication, talking trees.

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## INTRODUCTION

Many plant species have evolved induced resistance against pathogens or herbivores and thus correlate the expression of a defensive trait with the current need for resistance (Karban & Baldwin 1997; Gatehouse 2002). A major drawback of induced defences is the time lag between the first attack and the activation of the defence, during which plants remain vulnerable (Heil & Baldwin 2002). A possibility to circumvent this disadvantage is to use information from the environment or from earlier attacks to assess the future risk of being attacked and adjust the defensive phenotype accordingly. In the case of induced pathogen resistance, plants have repeatedly been shown to use a first infection as a cue to respond faster and stronger to subsequent attack (Zimmerli *et al.* 2000; Conrath *et al.* 2002). For induced defences to herbivores such within-plant priming processes have so far not been described.

In response to herbivore damage, many plants release volatile organic compounds (VOCs). These VOCs can attract predators such as carnivorous mites or parasitic wasps (Turlings *et al.* 1995; Takabayashi & Dicke 1996; Dicke *et al.* 1998), or they can repel herbivores (DeMoares *et al.* 1998; Heil 2004a). Caterpillars placed underneath the canopies of tomato plants induced with exogenously applied jasmonic acid (JA, a plant hormone frequently involved in

defences induced in response to mechanical wounding or herbivore attack) suffered from significantly higher rates of parasitism (Thaler 1999). A field study on native tobacco demonstrated it was indeed the presence of volatiles that increased predation rates of herbivore eggs (Kessler & Baldwin 2001).

Such VOCs provide information about the status of attack of the emitting plant, which can be used not only by higher trophic levels, but also by neighbouring plants of the same or another species (Baldwin & Schultz 1983; Arimura *et al.* 2000; Karban *et al.* 2000; Farmer 2001; Karban 2001). VOCs thus are putatively involved in ‘plant–plant communication’ processes too. Whether plants growing near to a VOCs-releasing plant can respond to this information has been discussed controversially, as many authors criticized the effects observed under experimental conditions with respect to their relevance under natural conditions (Baldwin *et al.* 2002; Dicke & Hilker 2003; Paschold *et al.* 2006), but evidence of plant–plant communication is accumulating. Plants respond to VOCs by changing transcription patterns of defence-related genes (Bate & Rothstein 1998; Arimura *et al.* 2000; Farag *et al.* 2005; Paschold *et al.* 2006), and they may increase the production of defence-related plant hormones such as JA and of other VOCs (Engelberth *et al.* 2004; Ruther & Kleier 2005), of proteinase inhibitors (Tschardt *et al.* 2001) or of phenolic compounds (Baldwin

& Schultz 1983). VOCs can even induce another type of indirect defence, i.e. the secretion of extrafloral nectar (EFN) (Choh *et al.* 2006; Kost & Heil 2006). VOCs identified to cause such changes in undamaged plants include (*Z*)-3-hexenyl acetate (Kost & Heil 2006) and several structurally related  $C_6$ -volatiles (Bate & Rothstein 1998; Engelberth *et al.* 2004; Farag *et al.* 2005; Ruther & Kleier 2005), whose stereochemistry apparently is of great significance (Farmer 2001; Ruther & Fürstenau 2005).

Most interestingly, Engelberth *et al.* (2004) reported that corn plants are primed by volatiles that are released from damaged plants: exposure to such volatiles caused yet undamaged corn plants to produce JA and terpenes more intensively and/or rapidly in response to caterpillar-caused damage than plants that were damaged without this pre-treatment (Engelberth *et al.* 2004). In contrast, no such priming effects could be observed in tobacco plants (Paschold *et al.* 2006), which also did not respond to volatile methyljasmonate at levels consistent with concentrations that occur under natural conditions (Preston *et al.* 2004).

Further studies are therefore needed to elucidate whether or not VOC-elicited priming plays a role in induced plant defences to herbivores. In the present study, we made use of the existence of two types of inducible, indirect defensive mechanisms in Lima bean (Heil 2004b). In response to damage, Lima bean not only releases VOCs but also secretes EFN, a nectar that is functionally not involved in pollination but rather in the attraction of carnivorous arthropods such as ants (Heil & McKey 2003). In the present study, we investigated whether VOCs can also prime EFN secretion. EFN alone or the induction of both types of indirect defence (EFN and VOCs) benefit Lima bean under natural conditions (Heil 2004b; Kost & Heil 2005); a presumed priming effect thus was believed to have a high ecological relevance in this study system.

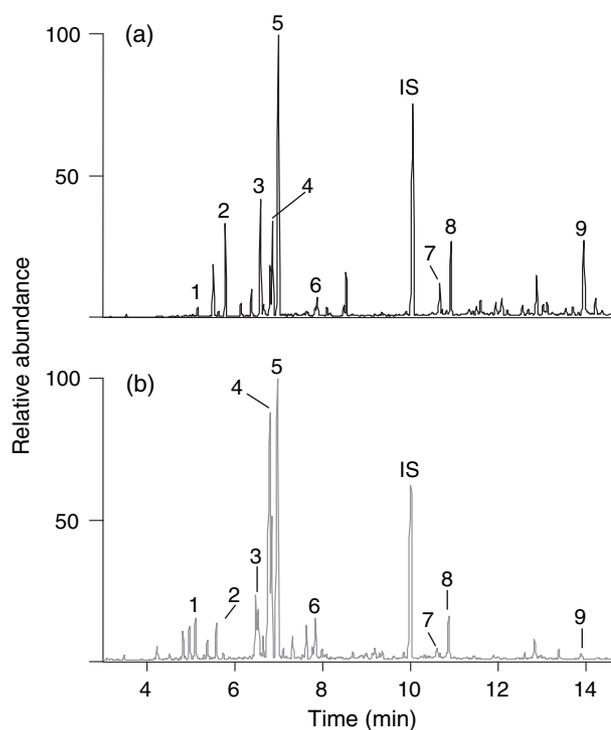
## MATERIAL AND METHODS

Lima bean plants (*Phaseolus lunatus* L., Fabaceae) growing naturally at a site *c.* 15 km west of Puerto Escondido (state of Oaxaca, Mexico; 15°55.596' N and 097°09.118' W, elevation 15 m a.s.l.) were used for this experiment. Lima bean grows as a climber. To exclude genetic and site effects, eight groups consisting of four tendrils each were selected as experimental unit with all tendrils of one group belonging to the same plant individual.

### Artificial volatile blend

Release of VOCs from naturally induced plants depends qualitatively and quantitatively on factors such as the type and intensity of damage and the spatiotemporal distribution of damage (Mithöfer *et al.* 2005; CK and MH unpublished

data). In order to ensure a reproducible experimental setup, we thus prepared an artificial volatile blend and dissolved it in lanolin (Sigma-Aldrich: <http://www.sigmaaldrich.com>) as a matrix, from which the dissolved volatiles could evaporate. Such VOCs-releasing lanolin pastes have already been successfully used in this and in other study systems (Kessler & Baldwin 2001; Kost & Heil 2006). The mixture used in the present study mimics the herbivore-induced bouquet of Lima bean both quantitatively and qualitatively as to emission within 24 h under the experimental conditions chosen (Fig. 1 and Fig. 1 in Kost & Heil 2006). It consisted of 0.12 µg linalool, 0.13 µg β-caryophyllene, 0.19 µg methyl salicylate, 0.26 µg (*Z*)-jasmone (all purchased of Sigma-Aldrich), 0.02 µg (*Z*)-3-hexenyl acetate (Avocado Research Chemicals Ltd., Leysham, Lancaster, UK), 0.85 µg (*E/Z*)-β-ocimene [mixture of *E* (70%) and *Z* (30%) isomers kindly provided by Roger Snowden, Firmenich, Geneva, Switzerland], 0.63 µg (*3E*)-4,8-dimethylnona-1,3,7-triene (DMNT) and 0.9 µg (*3E,7E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene



**Figure 1** Representative gas chromatographic profiles of volatiles collected from an herbivore-induced bean tendril (a) and a bean tendril treated with the synthetic volatile blend dissolved in lanolin paste (b). VOC release of 24 h was quantified under the same conditions as applied in the present study as described in Heil (2004b) and Kost & Heil (2006). Identified compounds are: 1, (*Z*)-3-hexenyl acetate; 2, (*E,Z*)-β-ocimene; 3, (*R*)-(-)-linalool; 4, DMNT; 5, C<sub>10</sub>H<sub>14</sub>; 6, methyl salicylate; 7, (*Z*)-jasmone; 8, β-caryophyllene; 9, TMTT; IS, internal standard (1-bromodecane).

(TMTT) (synthesized by standard methods, see Pattenden & Weedon 1968) per  $\mu\text{L}$  lanolin. Purity of all compounds was  $> 98\%$ .

### Priming experiment

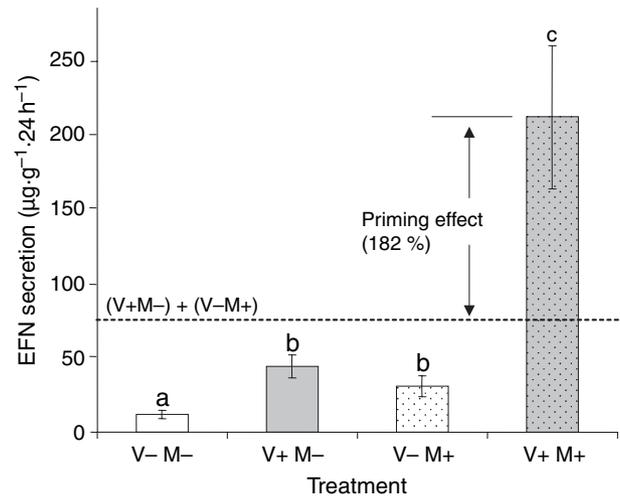
On day 1, two of the four bean tendrils were treated with the artificial volatile paste by applying 0.5 mL of the VOC paste in five portions of 0.1 mL each on green plastic strips attached to these tendrils. Thereby, we avoided any direct contact of the paste with the plant surface to prevent diffusion of compounds into the plant. To the remaining two tendrils we attached plastic strips with 0.5 mL pure lanolin paste as control. All tendrils were then packed into a perforated PET plastic bag ('Bratenschlauch'; Toppits, Minden, Germany). Bags were perforated with 1 hole  $\text{cm}^{-2}$  (diameter of holes 0.3 cm). On the second day, plastic bags and plastic strips with lanolin paste were removed. Leaves of each one tendril that had been exposed to VOCs and one control tendril per group were damaged mechanically by punching holes into the leaf blade with a metal brush ( $\approx 15$  holes  $\text{cm}^{-2}$ , diameter of individual holes  $\approx 0.2$  mm) and tendrils were packed in nets to prevent consumers to having access to the nectaries (Heil 2004b). The production rate of EFN was quantified after 24 h as amounts of soluble solids as described previously (Heil *et al.* 2000) by using microcapillaries for determination of volume and a portable refractometer for determination of concentration. Leaves then were collected and dried to calculate EFN secretion rates as  $\mu\text{g}$  soluble solids per 24 h and per g leaf dry mass.

### Statistical analyses

We analysed our data by applying a mixed-effect model with 'treatment' as a fixed factor and 'tendril group' as a random factor. The amount of EFN in  $\mu\text{g}$  produced per 24 h and per g dry weight was square-root-transformed to meet the assumption of homogeneity of variances. The Student–Newman–Keuls (SNK) test was used as a *post hoc* analysis. All statistical evaluations were done using SPSS 13.0 (SPSS for Windows; SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSION

Plants damaged mechanically that had been exposed to VOCs on the day before produced higher amounts of EFN than plants that received the same level of damage without having been exposed to VOCs (Fig. 2). Mechanical damage alone and exposure to VOCs alone also significantly induced EFN secretion, thus confirming earlier results (Heil 2004b; Choh *et al.* 2006; Kost & Heil 2006). The secretion rates of the plants first exposed to VOCs and then mechanically damaged were even significantly higher (182%) than the



**Figure 2** Priming of extrafloral nectar (EFN) secretion by VOCs. Mean EFN ( $\pm$  SEM) secretion in  $\mu\text{g}$  soluble solids per 24 h and per g leaf dry mass is displayed for the 24 h upon mechanical damage of plants. Treatments are: V – M – control, no volatiles or mechanical damage; V + M – volatiles on day 1 with no mechanical damage on day 2; V – M + mechanical damage inflicted on day 2, without a pre-treatment of volatiles on day 1; V + M + damage inflicted on day 2 after tendrils have been pre-exposed to VOCs on day 1. The dashed line indicates the added EFN productions of the two treatment groups V – M + and V + M –. The difference between the additive effect of these two treatments (dashed line) and the effect of the combined application of volatiles and mechanical damage (V + M +) represents the magnitude of the priming effect. Different letters indicate significant differences (SNK *post hoc* test,  $P < 0.05$ ,  $n = 8$ ).

sum of the amounts produced in response to either VOCs only or mechanical damage only (univariate ANOVA:  $P < 0.0001$ ,  $n = 8$ ). The phenomenon thus cannot be explained by a simple additive effect of VOCs plus damage. It can neither be explained by a more effective uptake of the VOCs by damaged leaves, as the plastic strips containing the VOCs-releasing lanolin paste were removed before leaves were damaged. During and after damaging leaves, these were thus only exposed to the ambient atmosphere. Apparently, the exposure to VOCs primed plants, which then responded more strongly to the damage inflicted on the next day.

Interestingly, corn plants showed priming effects on JA production only in response to herbivore damage, while there was no priming in response to mechanical wounding alone (Engelberth *et al.* 2004). In the present study, plants were damaged mechanically. Endogenous JA production and EFN secretion in Lima bean or in plants such as *Macaranga tanarius* (Euphorbiaceae) and several *Acacia* species and other Fabaceae are induced by mere mechanical damage, and JA is both required and sufficient to induce

EFN secretion in these species (Heil *et al.* 2001, 2004; Heil 2004b). Although both synthesis of sesquiterpenes and JA in corn and EFN secretion by Lima bean show priming effects, the details of the underlying signalling mechanisms seem not to be identical.

While priming effects are involved in volatile-mediated indirect resistance of plants to herbivores (Engelberth *et al.* 2004), this is the first report that VOCs can prime EFN secretion, a trait whose protective effect on Lima bean has been demonstrated in nature (Heil 2004b; Kost & Heil 2005). Airborne volatiles that usually are emitted upon herbivore damage can be recognized by yet undamaged Lima bean plants as a signal for an impending herbivore attack and prime their own indirect defence. As already pointed out (Kost & Heil 2006), the tangled growth of Lima bean make a plant-internal signal less efficient as long as it is transported through the shoots. VOCs thus might serve as a cue to elicit EFN secretion in exactly those parts of another – or even the same – plant individual, where resistance actually is required: in the spatially (yet not necessarily anatomically) neighbouring parts. Both EFN secretion and the release of VOCs are taxonomically widespread (Koptur 1992; Karban & Baldwin 1997). Volatile-elicited priming may thus represent a ubiquitous mechanism that adds a new facet to our understanding of the complex interactions among different trophic levels.

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