

Induction of two indirect defences benefits Lima bean (*Phaseolus lunatus*, Fabaceae) in nature

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Summary

1 Herbivore damage often induces the release of herbivore-induced plant volatiles (HIPVs) or the secretion of extrafloral nectar (EFN). The purpose of this study was to induce such proposed indirect defence responses in plants growing in their natural environment and to monitor the effects on vegetative and generative traits such as herbivory, plant growth and fruit set.

2 I tested whether mature wild Lima beans (*Phaseolus lunatus*, Fabaceae) growing in Mexico could be reliably induced to produce HIPVs and EFN in response to application of the phytohormone, jasmonic acid (JA), and investigated whether this treatment leads to any benefit to the plants.

3 Eleven HIPVs were released from JA-treated leaves at significantly higher amounts than from control leaves, and only one (methylsalicylate) at significantly lower amounts than from controls. Treatment with JA increased EFN secretion on leaves on average by more than 30 times and on bracts by more than 20 times. EFN production, which has not previously been described in Lima bean, represents a second induced indirect resistance trait of this species.

4 JA treatment reduced both the number of dead shoot tips and leaf damage by herbivores. Treated tendrils grew faster and produced more leaves than controls. At the end of the experiment, treated tendrils bore *c.* two times more inflorescences and three times more fruits than controls.

5 Repeated treatment with JA resulted in a combined induction of HIPVs and EFN and strongly benefited wild Lima beans. The plants suffered less from the consequences of herbivore attack, supporting the use of the term ‘induced defences’ for these traits. JA-induced defences thus can significantly benefit Lima bean under natural conditions.

Key-words: ants, antiherbivore defence, extrafloral nectar, induced defence, Leguminosae, octadecanoid signalling, plant–herbivore interaction, Mexico, VOCs, volatiles

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Introduction

Many plants respond to herbivore damage by the up-regulation of traits that attract ‘enemies of their enemies’ (Price *et al.* 1980; Karban & Baldwin 1997), which then mediate a defensive function, for instance by preying on herbivores. Such so-called indirect defences include the release of herbivore-induced plant volatiles (HIPVs) and the secretion of extrafloral nectar (EFN, i.e. nectar that is functionally not involved in pollination, Elias 1983). Most of the responses occurring after herbivore damage appear to be regulated via the octadecanoid signalling cascade, in which the plant hormone jasmonic acid (JA) acts as a central signalling molecule

(Creelman & Mullet 1997; Wasternack & Parthier 1997). The same signalling pathway is involved in many direct defences such as the synthesis of toxic compounds (Karban & Baldwin 1997).

HIPVs released by damaged plants can attract predators such as carnivorous mites or parasitic wasps (Turlings *et al.* 1995; Takabayashi & Dicke 1996; DeMores *et al.* 1998; Dicke *et al.* 1998). Caterpillars placed on tomato plants induced with exogenously applied JA suffered from significantly higher rates of parasitism (Thaler 1999). A field study on native tobacco demonstrated that it is indeed the release of volatiles that increases predation rates of herbivore eggs (Kessler & Baldwin 2001). Volatile compounds are, however, an openly presented signal that can be used by all organisms, including the herbivores themselves. High levels of HIPVs,

which indicate a high density of potential competitors and enemies, can act as a direct defence mechanism by deterring herbivores (overview in Dicke & van Loon 2000). For example, exposure to spider mite-infested leaves of Lima bean usually induces the release of HIPVs from uninfested leaves (Dicke & Dijkman 1992) and their subsequent avoidance by spider mites. The ecological relevance of such effects has been demonstrated in a field study on *Nicotiana attenuata*: Lepidopteran females avoided oviposition on plants releasing HIPVs (Kessler & Baldwin 2001).

Although induced direct and indirect resistance traits are currently the object of intensive research, studies showing that such traits can benefit plants in nature are scarce (Agrawal 1998; Baldwin 1998; see Kessler & Baldwin 2001 for effects of HIPVs). Karban & Baldwin (1997) suggested that the term 'induced resistance' is used for all plant responses that reduce herbivore survival or reproduction, or preference for a plant, and that 'induced defence' is reserved for traits that decrease the negative fitness consequences of herbivore attacks on plants. The status of HIPVs thus remains unclear for most plant species.

Much knowledge has recently been accumulated on the processes that are involved in the elicitation and transduction of internal signals modulating the release of HIPVs, the synthesis of both signalling compounds and HIPVs, and on the responses of individual predators to the released HIPVs. *Phaseolus lunatus* L. (Fabaceae, Lima bean) is among the species most widely used in genetic, biochemical and ecological studies. Feeding by herbivores on Lima bean leaves leads to extensive changes in patterns of gene expression (Arimura *et al.* 2000). More than 60 HIPVs may be released only, or at much higher rates, from leaves damaged by herbivores (Dicke *et al.* 1999), and *de novo* synthesis in response to herbivore damage has been demonstrated experimentally (Donath & Boland 1994; Boland *et al.* 1995; Bouwmeester *et al.* 1999) for some. Mechanical damage of Lima bean leaves induces only a subset of those HIPVs that are released in response to chewing or sucking herbivores (Dicke 1994), suggesting that herbivore-derived elicitors are required for the induction of the whole blend (Dicke 1994; Spiteller *et al.* 2000). A transient increase in endogenous JA in leaves is regularly involved in the synthesis of HIPVs after herbivore-caused damage (Koch *et al.* 1999), and volatile blends similar (although not identical) to those released after such damage are also released by plants treated with exogenously applied JA (Hopke *et al.* 1994; Boland *et al.* 1995; Dicke *et al.* 1999; Ozawa *et al.* 2000). HIPVs can also be induced by chemical elicitors that activate octadecanoid signalling (Krumm *et al.* 1995; Piel *et al.* 1997; Engelberth *et al.* 2000). HIPVs released by Lima bean leaves treated with JA or damaged by herbivores attract carnivorous animals such as mites (Dicke 1994; Dicke *et al.* 1999, 2003a), thrips (Shimoda *et al.* 1997) and braconid wasps (Petitt *et al.* 1992).

Surprisingly little is known, however, about the functional aspects of the ecology and, ultimately, the

evolution of this response. We know only of studies on cultivated Lima bean seedlings under controlled laboratory or glasshouse conditions. The few animal species to which they were exposed represented a restricted number of feeding guilds, both of herbivores and of carnivores, and many of the species used have natural geographical ranges that do not overlap with that of wild Lima beans. The synthesis of HIPVs varies with both plant genotype (Loughrin *et al.* 1995; Halitschke *et al.* 2000; Fritzsche-Hoballah *et al.* 2002) and attacking herbivore (Dicke 1994, 1999; Takabayashi & Dicke 1996; Ozawa *et al.* 2000) and might also be affected by the developmental stage of the herbivores (Takabayashi *et al.* 1995). Carnivores are able to distinguish between different HIPV blends (DeMoaers *et al.* 1998; Dicke *et al.* 1999), and their previous experience may affect their behaviour towards the particular HIPVs emitted by a specific plant (Petitt *et al.* 1992; Krips *et al.* 2001). Moreover, the various carnivore species present, with different responses to HIPVs, may show complex interactions under natural conditions. Finally, plants naturally grow in mixed stands, and carnivores must be able to distinguish their blends from the general background, an aspect overlooked in almost all laboratory studies (Dicke *et al.* 2003a).

The purpose of the present study was to investigate whether induced responses have beneficial effects on wild Lima beans. Like many other species of the Fabaceae, Lima bean bears extrafloral nectaries on the stipules of its leaves (A. Delgado, personal communication, Fig. 1). Many studies have demonstrated the protective effect of facultative ant-plant interactions (Bentley 1977; Koptur 1992; Heil & McKey 2003). In several plant species, extrafloral nectar (EFN) secretion is induced by herbivory (Heil *et al.* 2001; Stephenson 1982; Wäckers *et al.* 2001; Ness 2003). In *Macaranga tanarius* (Euphorbiaceae) this response is regulated via the octadecanoid pathway, and repeated induction of EFN flow can significantly benefit plants due to a nearly 10-fold reduction of

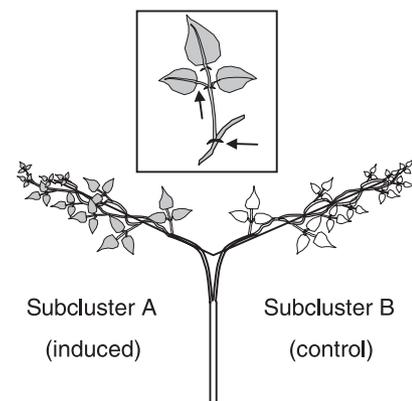


Fig. 1 Experimental set-up and location of extrafloral nectaries. An individual plant was trained along two separate ropes to create two 'subclusters', one of which was randomly chosen to receive JA treatment, while the other served as control. Insert: A trifoliate leaf with EFN-producing stipules marked in black.

herbivore damage (Heil *et al.* 2001). The applicability of these results to other plant species remains to be proven.

Exogenous JA was repeatedly applied to parts of plants, which were then compared with untreated parts of the same plants in order to answer the following questions: (i) Do mature wild Lima bean plants respond to JA treatment with induced release of HIPVs similarly to seedlings of cultivated forms? (ii) Is EFN secretion by Lima bean induced by JA application? (iii) Does repeated treatment with JA benefit Lima bean at its natural growing site?

Materials and methods

PLANT MATERIAL AND STUDY SITE

Phaseolus lunatus L. (Lima bean, Fabaceae) occurs naturally throughout Meso- and South America. Studies using isoenzyme and genetic markers found two different gene pools, the 'Mesoamerican' type being widely distributed in neotropical lowlands, while the 'Andean' appears to be restricted to the western Andes (Maquet *et al.* 1990; Gutiérrez Salgado *et al.* 1995; Salgado *et al.* 1995; Maquet & Baudoin 1997; Caicedo *et al.* 1999). Two gene pools, corresponding to the wild ones, also exist in cultivated Lima beans, suggesting independent domestication events (Gutiérrez Salgado *et al.* 1995). Preliminary analyses with AFLP markers revealed that the Lima bean cultivar that is used in many laboratory studies (Jackson Wonder Bush) belongs to the Mesoamerican genotype (M. Heil, unpublished data).

The present study was conducted in the coastal area of the state of Oaxaca, Mexico, close to the centre of genetic diversity of wild Lima beans and the putative site of domestication of the 'Mesoamerican' gene pool (Gutiérrez Salgado *et al.* 1995). Lima bean grows here as a liana covering trees and shrubs along roadsides and also in extensively used pastures. AFLP analyses showed that the plants form a distinct cluster within the 'Mesoamerican' gene pool and are genetically close to cv. Jackson Wonder Bush (M. Heil, unpublished data). Voucher specimens are deposited at the Herbario MEXU (UNAM, Mexico City, Mexico) and in the personal collection of MH. Plants were selected at two sites close to Puerto Escondido, 1–2 km from the shore and about 3 km apart.

The main rainy season lasts from June to August and most of the above-ground parts of the perennial Lima bean die back during the dry season. Plants start to germinate or bud in June or July and the first inflorescences usually appear in October or November. Depending on water supply, the production of flowers and fruits ends between February and April (A. Delgado, personal communication; M. Heil, personal observation).

GENERAL EXPERIMENTAL DESIGN

I selected 25 clusters (groups of tendrils apparently belonging to a single plant) spread across the two sites.

In order to reduce effects of site and genotype, the tendrils in each cluster were divided into two subclusters and trained along two separate ropes (Fig. 1). One randomly selected subcluster per pair was sprayed with a 1 mmol litre⁻¹ aqueous solution of JA until the surfaces of all leaves were covered, while the other (control) was sprayed with an equal amount of water. Control subclusters were covered with plastic foil during JA treatment. Starting on 4 November, plants were sprayed every 3 days till 10 November (days 0, 3, 6), then weekly (days 13, 20) and then every 3 or 4 days (days 23, 27, 30, 33, 36) till 10 December. During this time, human and cattle impacts had reduced the initial number of clusters to a final sample size of 14.

PRODUCTION OF VOLATILES

To confirm whether, and to what degree, treated and control subclusters differed in their rate of HIPV release, one or two clusters were chosen randomly after JA treatment, and five leaves were randomly selected from both the treated and the control subcluster. Leaves were removed *c.* 0.5 h after spraying, transported to the laboratory in sealed plastic bags and the five leaves belonging to one subcluster were then placed together with their petioles in water. This procedure took less than 20 min and leaves showed no symptoms of wilting. A closed air volume of about 1 L was created around the leaves using plastic foil ('Bratenschlauch', Toppits, Minden, Germany, a PET foil designed to protect meat during broiling), which does not emit detectable amounts of volatiles even after exposure to temperatures of up to 150 °C. Leaves were placed so that they received a natural photoperiod without being exposed to direct sunlight. Volatiles emitted were continuously collected over the following 24 h on small charcoal traps (1.5 mg charcoal, CLSA-Filters, Le Ruissaeu de Montbrun, Damauzan sur Aurize, France) using air circulation as described previously (Donath & Boland 1994, 1995; Koch *et al.* 1999). After 24 h, leaves were dried over silica gel for the quantification of leaf dry mass, and volatiles were desorbed from the carbon trap with dichloromethane (2 × 20 µL) to which 1-bromodecane at a concentration of 200 ng µL⁻¹ had been added as standard. Samples then were transferred to glass capillaries, sealed by melting the open end of the capillaries and then stored at < 5 °C for transport to Germany. Samples were analysed without further preparation with a GC-Trace mass spectrometer (2000 series, Thermo Quest, Finnigan, Rodano, MI, USA). Identification was conducted by comparison with mass spectra of reference compounds (see Koch *et al.* 1999 for details and Hopke *et al.* 1994; Piel *et al.* 1997 for original identification of compounds) with the program Xcalibur 1.2 (Finnigan Corp.).

PRODUCTION OF EFN

Lima bean secretes extrafloral nectar (EFN) on three pairs of stipules per leaf (at the base of the leaf stalk, at

the branch point of the two lateral leaflets and at the base of the stalk of the terminal leaflet) (Fig. 1). Nectaries on the bracts have yet to be classified as extrafloral, although they too may attract ants, which are not involved in pollination (M Heil, personal observation). To test whether EFN secretion responds to JA treatment, eight further clusters were selected and treated once with JA solution as described above. EFN secretion by five leaves and two inflorescences each on one treated and one control tendril per cluster was measured 24 h after JA application. Nectar secretion was quantified as amounts of secreted soluble solids by measuring both nectar volume with micro capillaries and nectar concentration with a refractometer as described previously (Heil *et al.* 2000a, 2001). The leaves and inflorescences were then collected and dried over silica gel.

EFFECTS OF JA TREATMENT ON VEGETATIVE TRAITS AND FITNESS PARAMETERS

About 10 days after the beginning of the experiment I observed that numbers of dead shoot tips appeared to differ between treated and untreated subclusters. Starting on 17 November, shoot tips on all subclusters were counted and categorized as alive or dead after each treatment. Only shoot tips that were dry, but still present, were regarded as dead: broken tips and those obviously removed by chewing herbivores were excluded from the analysis.

All shoot tips were individually marked with coloured tape on 28 November and the length of each tendril from the tape to its tip and the number of leaves present were measured. Measurements were repeated on 10 December in order to estimate shoot growth and numbers of newly produced leaves. Fitness parameters were estimated by counting the total number of inflorescences per subcluster and the number of beans (i.e. fruits) present at the end of the experiment. As no inflorescences were present on any of the clusters at the beginning of the experiment, these counts represented newly produced reproductive parts. To quantify rates of herbivory, 35 leaves were chosen randomly from each subcluster and dried between sheets of paper (leaves shrink by less than 5% during this procedure). Dried leaves were scanned, and their area was quantified by comparison with a structure of known area. Using a standard image processing software, intact leaves were reconstructed by filling all herbivore-caused holes in the leaf blades. Missing leaf area was estimated as difference between the actual and the reconstructed area of each individual leaf and expressed as percentage of the reconstructed total leaf area.

STATISTICAL DATA ANALYSIS AND PRESENTATION

The experiment was set up to compare treated and control subclusters from the same individual. The statistical analysis therefore used Wilcoxon tests for matched

pairs wherever possible, i.e. in all cases in which single (or average) values per subcluster had to be compared. Data on herbivory comprised values of 35 leaves per subcluster and were therefore evaluated with Mann–Whitney *U*-tests conducted separately for each cluster. All statistical evaluations were done with SPSS (10.0). Data are presented as box-whisker plots, which indicate the overall distribution of the data by showing the first and third quartile (lower and upper margin of the box), the median (line within the box) and the 5% as well as the 95% percentile.

Results

EFFECTS OF JA TREATMENT ON VOLATILES AND EFN

Release of HIPVs within 24 h after treating plants with JA solution varied considerably among clusters. Although most clusters showed a very strong response (see example in Fig. 2a,b), others showed no obvious effect, either because large amounts of HIPVs were released by the control subcluster or due to a lack of a response by the JA-treated subcluster. Overall, however, 11 of 14 HIPVs regularly occurring after JA treatment (*cis*- β -Ocimene, C_{11} Homoterpene, $C_{10}H_{14}$, $C_{10}H_{16}O$, unidentified compounds 8, 9 and 10, *cis*-Jasmone, β -Caryophyllene, TMTT [4,8,12-trimethyltrideca-1,3,7,11-tetraene], and MeJA) were released at significantly higher amounts from JA-treated than from control subclusters, while significantly more MeSA was released from controls than from treated subclusters (Fig. 2c). Putative identities for the three peaks that could not be identified by direct comparison with reference compounds are: 8 (3*Z*)-hexenylpentanoate; 9 (3*Z*)-hexenylhexanoate and 10, hexenylbenzoate, according to the mass spectra library NIST (1.7).

JA treatment significantly affected secretion of EFN by both vegetative and generative plant parts (Fig. 3). On average, EFN secretion was *c.* 0.02 mg soluble solids g^{-1} leaf dry mass $24 h^{-1}$ on control leaves and *c.* 1.5 mg g^{-1} $24 h^{-1}$ on induced leaves. EFN secretion by inflorescences on treated tendrils was about 30 times higher than on control tendrils (induced tendrils *c.* 3 mg g^{-1} $24 h^{-1}$, controls *c.* 0.1 mg g^{-1} $24 h^{-1}$). For both types of EFN secreting organs, this difference was significant ($P < 0.01$ according to Wilcoxon pairs tests, $n = 8$ clusters).

EFFECTS OF JA TREATMENT ON VEGETATIVE AND GENERATIVE TRAITS

Lima bean plants suffered from severe mortality of young shoot tips, with 50% of all tendril tips in control subclusters being classified as dead at the end of the experiment (Fig. 4). Both census date and induction significantly affected the percentage of dead shoot tips (Table 1). The percentage of dead shoot tips increased during the experiment on control subclusters, but decreased on induced subclusters (Fig. 4), leading to a significant census date–induction interaction (Table 1).

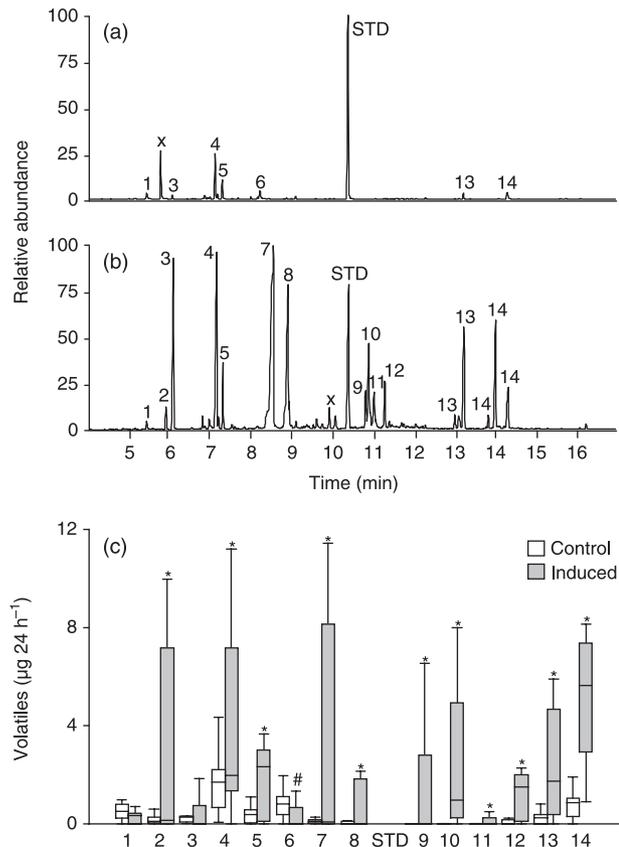


Fig. 2 Response of the production of volatiles to JA treatment. Fourteen regularly occurring HIPVs (1, *cis*-Hexenylacetate; 2, *cis*- β -Ocimene; 3, *trans*- β -Ocimene; 4, C₁₁ Homoterpene; 5, C₁₀H₁₄; 6, MeSA; 7, C₁₀H₁₆O; 8, unidentified; STD, standard [1-bromodecane]; 9, 10, unidentified; 11, *cis*-Jasmone; 12, Caryophyllene; 13, TMTT [4,8,12-trimethyltrideca,1,3,7,11-tetraene]; 14, MeJA) were used for evaluation. See 'Results' section for putative identities of compounds 8, 9, 10. Gas chromatographic spectra of HIPVs released from five leaves each from a control (a) and the corresponding JA-treated (b) subcluster over 24 h after treatment (x = contaminants). Panel c: average amounts of volatiles released on different days by 15 clusters (see insert in Fig. 3 for the explanation of box-whisker plots). *HIPVs released at significantly higher amounts from induced than from control subclusters. #HIPV released at significantly higher amounts from control than from induced subclusters (both cases, $P < 0.05$ according to Wilcoxon pair tests conducted for individual compounds with the amount released on the same day by the control and the induced subcluster of one cluster serving as a pair, $n = 15$ clusters in all cases).

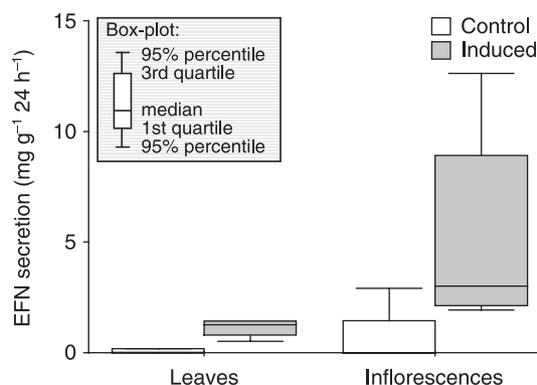


Fig. 3 Extrafloral nectar (EFN) secretion on leaves and inflorescences in response to JA treatment. EFN secretion is measured as mg of soluble solids secreted per g dry mass during 24 h after JA treatment.

Repeated JA treatment reduced the final proportion of dead shoot tips to less than 10%. Wilcoxon pair tests on the percentage of dead shoot tips observed on control and on induced subclusters, conducted as posthoc tests

Table 1 Results of repeated measures ANOVA on effects of JA treatment ('Induction') on numbers of dead shoot tips. These were counted repeatedly (see Fig. 4 for census dates) on each cluster and both date and induction therefore serve as within-subject variable. Sample size = 14 clusters

	SS	d.f.	<i>F</i>	<i>P</i>
Date	5261	6	9.03	< 0.001
Error	6993	72		
Induction	36149	1	190.63	< 0.001
Error	2275	12		
Date × induction	4093	6	7.50	< 0.001
Error	6547			

separately for each census date, revealed significant differences at six of seven census dates ($P < 0.05$, Fig. 4).

When tendrils die, a new shoot tip has to be established from a lateral bud, thus slowing down both the growth rate of the tendrils and the rate at which new leaves are produced (M. Heil, personal observation). Tendrils forming part of induced subclusters consequently grew

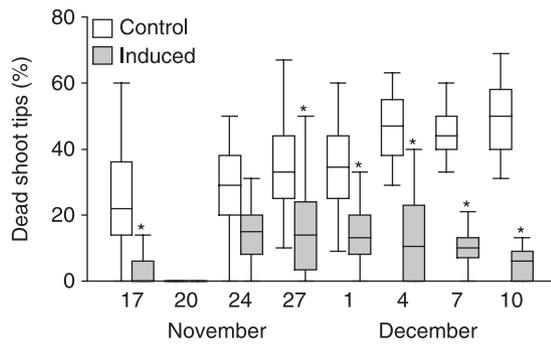


Fig. 4 Frequency of dead shoot tips (percentage of all tips present on an individual subcluster) during the term of the experiment. Treatment started *c.* 2 weeks before the first census. *Significantly lower percentage on treated than on untreated subclusters at a particular census date ($P < 0.05$, Wilcoxon pair test, sample sizes at the consecutive censuses are 20, 18, 18, 17, 16, 16 and 14).

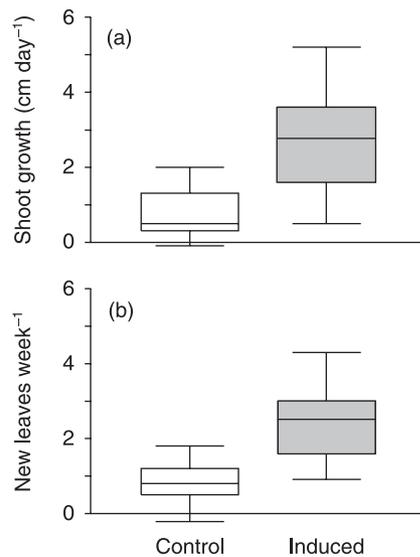


Fig. 5 Long-term effects of JA treatment on shoot growth (a) and production of new leaves (b).

significantly faster and produced more new leaves than tendrils belonging to control subclusters (Fig. 5). On average, tendril tips on control subclusters grew < 0.5 cm day⁻¹ during the last 12 days of the experiment, while those on induced subclusters grew *c.* 3 cm day⁻¹ during the same time span. Similarly, tendrils on control subclusters produced on average *c.* 1 new leaf week⁻¹, while those on induced subclusters produced almost 3 new leaves week⁻¹. For both parameters this difference was significant ($P < 0.05$ according to Wilcoxon pair tests, means derived from the two subclusters belonging to the same cluster, $n = 14$ clusters).

JA treatment also significantly affected the rate of herbivory. The percentage of missing leaf area as quantified from mature leaves collected at the end of the experiment was significantly lower on induced than on control subclusters for 11 of 14 clusters (Fig. 6).

Lima beans started to flower during the study period, thus allowing an estimation of putative effects of JA

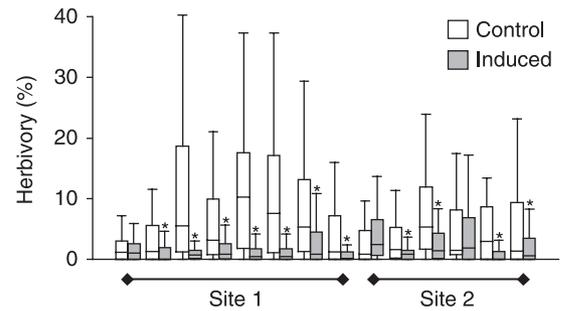


Fig. 6 Herbivory (percentage of missing leaf area) on JA-treated and control subclusters at the end of the experiment ($*P < 0.05$, Mann-Whitney *U*-tests). $n = 105$ leaflets (3 per leaf) for each bar shown.

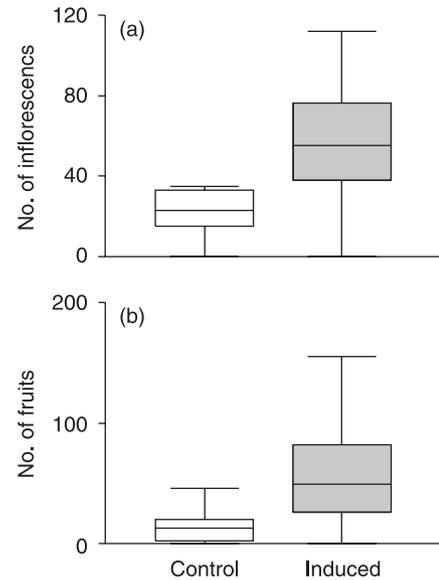


Fig. 7 Effect of JA treatment on the production of reproductive structures. Numbers of inflorescences (a) and numbers of fruits (b) were counted on each subcluster at the end of the experiment and averaged within treatments.

treatment on the production of reproductive parts. At the end of the experiment, induced subclusters bore more inflorescences and more fruits than the control subclusters (Fig. 7). On average, the number of inflorescences produced by treated subclusters was more than twice as high as on controls and the number of fruits over three times as high ($P < 0.05$ according to Wilcoxon pair test, $n = 14$).

Discussion

The main purpose of the present study was to investigate whether putative indirect defence traits (volatile organic compounds, HIPVs and extrafloral nectar, EFN) can be induced in a reproducible way under field conditions, and whether regular induction of jasmonic acid-mediated traits benefits naturally growing wild plants of Lima bean (*Phaseolus lunatus*). JA has repeatedly been demonstrated to elicit the release of volatile blends similar to those released by herbivore damaged

Lima bean (Hopke *et al.* 1994; Boland *et al.* 1995; Dicke *et al.* 1999; Ozawa *et al.* 2000), and it is also involved in the induction of EFN secretion by other species (Heil *et al.* 2001). Exogenous application of JA was therefore applied to Lima bean plants growing naturally in the coastal area of Oaxaca, Mexico.

Eleven of 14 regularly occurring HIPVs were released at significantly higher amounts from induced than from control subclusters (Fig. 2). Most major compounds released from JA-treated plants in the present study are known to be released by Lima bean plants in response to herbivore damage (Dicke 1994; Dicke *et al.* 1999). Although cultivated and wild forms of the same species might differ dramatically in composition and particularly amount of HIPVs released (Loughrin *et al.* 1995), blends as released by wild plants investigated in the present study were similar to those reported earlier for cultivated Lima beans (Dicke *et al.* 1990; Boland *et al.* 1995). Only one volatile, methylsalicylate, was released at significantly lower amounts from induced plant parts (Fig. 2). Salicylate is a known antagonist of JA and many studies have reported a down-regulation of JA or MeSA (and/or of compounds regulated by its presence) in plants producing or receiving the other phytohormone at high concentration (Doherty *et al.* 1988; Pena-Cortes *et al.* 1993; Doares *et al.* 1995; Baldwin *et al.* 1996). Therefore, it was possible to reproduce plant responses observed under laboratory conditions in wild plants.

Extrafloral nectar (EFN) secretion was also strongly induced by JA treatment (Fig. 3), and many more ants were observed to visit extrafloral nectaries on treated as compared with control tendrils (R. Krüger, personal communication). Nectar-secreting glands on both leaves and bracts were regularly visited by ants, but ants were never observed to enter flowers of Lima bean. According to the definition by Elias (1983) both leaf and inflorescence nectar-secreting glands investigated here can be referred to as extrafloral nectaries. Therefore, although most studies on defence in Lima bean have focused on the indirect as well as the direct role of induced HIPVs (Birkett *et al.* 2000; Dicke & van Loon 2000; Dicke *et al.* 2003b), attraction of ants to EFN also appears to play an important indirect defensive role.

The observation that treated subclusters exhibited a significant up-regulation of at least two putatively defensive traits (release of HIPVs and secretion of EFN) demonstrates that the experimental design successfully avoided an unintentional induction of controls by either applied JA or released HIPVs. Induction had strong beneficial effects on both vegetative and generative traits: treated parts bore fewer dead shoot tips (Fig. 4), grew faster (Fig. 5) and had less leaf area removed by herbivores (Fig. 6) than untreated parts of the same plants. Induced tendrils also produced more new leaves (Fig. 5), inflorescences and fruits (Fig. 7) than controls. Clusters with significant treatment effects on herbivory were equally distributed over both sites investigated, making strong biases by very localized site effects unlikely.

Several studies have reported fitness costs of induced resistance (Baldwin 1998; Heil *et al.* 2000b; Purrington 2000; Cipollini 2002; Zavala *et al.* 2004), resulting either from the allocation of limited resources to resistance traits or from so-called ecological costs, i.e. negative effects of resistance traits on some of the many other fitness-related interactions between plants and their environment (Heil 2002). For example, HIPVs have repeatedly been reported to attract herbivores and thereby incur ecological costs (Dicke & van Loon 2000). To be evolutionarily stable, the positive effects of induced resistance should, on average, exceed its negative effects. Costs of resistance are therefore usually quantified under enemy-free conditions, as this prevents the beneficial effects of resistance from being expressed (Heil & Baldwin 2002). In the present study, plants were exposed to the full spectrum of their enemies; plant traits at the end of the experiment reflect the net (integrated) effects of plant responses elicited by repeated JA application. Although costs of resistance induction may have affected the results, the benefits of eliciting resistance clearly exceeded its putative costs.

Although control subclusters produced both HIPVs (Fig. 2a) and EFN (Fig. 3) at low rates, treated subclusters produced both resistance compounds in much higher quantities (Figs 2b and 3). HIPV-release by Lima bean is generally assumed to be induced systemically (Dicke 1994; Dicke & Dijkman 2001). However, rates of HIPV release and EFN secretion by untreated subclusters did not differ from completely untreated plants (data not shown), making a systemic induction of untreated plant parts unlikely (any putative plant-internal systemic signal would have had to have been transported at least 80 cm between subclusters). Moreover, as all plants were subject to natural attack, untreated plant parts might well have been induced by damage caused, for instance, by herbivores. The data presented set a slight systemic induction of untreated plant parts to be separated from a natural (basal) induction. However, the significant increase in EFN secretion and HIPV production after JA-treatment demonstrates that both resistance traits showed a strongly localized response to treatment. Any induction of the control parts would have led to an under- rather than an overestimation of the effects of the JA treatment on the plant parameters measured. The paired experimental design almost completely excluded site effects and effects resulting from genetic differences among plants. The beneficial effects as observed in the present study thus can be directly linked to the regular application of JA.

Although definitive evidence on the causal relation between the induced responses and the observed beneficial effects is still missing, most direct effects of JA appear to suppress rather than induce growth (Creelman & Mullet 1997; Wasternack & Parthier 1997). It is thus very likely that the reduced detrimental effects of herbivores and the resulting beneficial effects observed on induced plants were, to a considerable part, the consequence of the defensive responses induced. The

production of HIPVs and the synthesis of EFN elicited by this treatment thus can be termed 'induced defences' *sensu* Karban & Baldwin (1997).

However, JA is not the only signal involved in the responses to herbivore attack (Dicke *et al.* 1999; Ozawa *et al.* 2000), and JA can also up-regulate other defensive traits. Direct chemical defences are often induced via the octadecanoid pathway (Karbon & Baldwin 1997) and can significantly benefit plants under natural growing conditions (Agrawal 1998; Baldwin 1998). A study on *Phaseolus vulgaris* found no evidence for induced direct resistance to spider mites (English-Loeb & Karban 1991) but, although such direct defences have not yet been described in Lima bean, they might have played a role. The natural history behind the observed defensive effects, e.g. which insects are herbivores and which carnivores and parasitoids, is not yet known. The factor responsible for the death of shoot tips that was reduced by JA treatment and the relative contribution of the induced responses to the overall defensive effect also remain unclear.

This study demonstrates the possibility of a reproducible induction of HIPVs and EFN in mature wild Lima bean plants growing at their natural site, and it is among the first studies demonstrating JA-mediated indirect defences under natural conditions (see Kessler & Baldwin 2001 for HIPVs and Heil *et al.* 2001 for EFN). EFN, which has for the first time been described as an indirect defensive response of Lima bean, has increased the complexity of the known defences in this species. Regular JA treatment had significant positive effects on plant traits such as growth, leaf production and reproduction, demonstrating the potential strength of the beneficial effects of induced resistances when plants are subject to natural herbivore pressure. The presence of two indirect defences in the same plant species could be used to elucidate the possibilities for plants benefiting from the attraction of various 'enemies of their enemies' (Price *et al.* 1980).

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