Basic and Applied Ecology

Increased availability of extrafloral nectar reduces herbivory in Lima bean plants (*Phaseolus lunatus*, Fabaceae)

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Summary

Lima bean (*Phaseolus lunatus*) features two inducible indirect defences to protect itself against herbivores. Besides the emission of plant volatiles, extrafloral nectar is secreted to attract carnivorous arthropods to herbivore-damaged plants. The activation of both putative defences efficiently protects Lima beans from leaf damage. In a field experiment in Mexico, we studied whether extrafloral nectar alone can benefit the Lima bean under natural conditions. An artificial blend mimicking natural nectar both qualitatively and quantitatively was repeatedly applied to Lima bean tendrils. Ants, wasps and flies were significantly more abundant on treated tendrils than on untreated controls already after 1 week (i.e. after two treatment applications). Sticky traps were used to assess the functional groups of flying insects attracted to the Lima beans. After 24 h, 71% of all trapped flies and 98% of all wasps belonged to families comprising either parasitoid or predatory species. This observation suggests that also some of the flying visitors have played a role as putative defenders of Lima beans. Most of the trapped flies belonged to the families Dolichopodidae and Phoridae (each ca. one third of all individuals). Two thirds of the wasps belonged to Chalcidoidea (68%). All ant species that had been collected manually belonged to generalist genera with *Camponotus novogranadensis* and *Cephalotes minutus* being most regularly encountered on study tendrils. An additional experiment, where both 'nectar' and 'control' tendrils were treated with artificial nectar, revealed that ants responded with an increased abundance on tendrils that had experienced the 'nectar' treatment before.

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After 25 days, the treated tendrils showed a significantly reduced herbivory as compared to controls. The mere presence of increased amounts of extrafloral nectar thus can benefit the Lima bean under natural conditions.

Zusammenfassung

Die Limabohne (*Phaseolus lunatus*) verfügt über zwei induzierbare, indirekte Verteidigungsformen zur Abwehr von Herbivoren. Neben der Emission volatiler Verbindungen ist die Limabohne zusätzlich dazu in der Lage, extrafloralen Nektar zu sezernieren. Beides dient der Anlockung von Fraßfeinden zu den von Herbivoren befallenen Pflanzen. In einem Freilandexperiment in Mexiko wurde untersucht, ob die Limabohne unter natürlichen Bedingungen von der Sekretion extrafloralen Nektars profitiert. Hierzu wurde ein künstliches Nektargemisch wiederholt auf Limabohnen-ranken aufgetragen, welches natürlichen Nektar quantitativ und qualitativ imitierte. Bereits nach einer Woche (d.h. nach zwei Behandlungen) war die Abundanz von Ameisen, Fliegen und Wespen auf behandelten Ranken signifikant höher als auf unbehandelten Kontrollranken. Zur Erfassung der zur Limabohne angelockten fliegender Insekten sowie deren Zugehörigkeit zu funktionellen Gruppen wurden die Versuchsranken mit Klebefallen bestückt. Mehr als zwei Drittel der nach 24 h gefangenen Fliegen und 98% aller Wespen gehörten parasitisch oder räuberisch lebenden Fliegen- bzw. Wespen-Familien an. Diese Beobachtung legt nahe, dass nicht nur Ameisen, sondern auch einige der gefangenen fliegenden Besucher eine Rolle als potentielle Verteidiger der Limabohne gespielt haben könnten. Von den gefangen Fliegen gehörten die meisten den Familien Dolichopodidae und Phoridae (je ca. ein Drittel aller gefangenen Individuen) an, wogegen die Chalcidoidea zwei Drittel (68%) der gefangenen Wespen ausmachten. Unter den durch Handaufsammlung gefangenen Ameisen gehörten *Camponotus novogranadensis* und *Cephalotes minutus* zu den am häufigsten auf behandelten Ranken angetroffen Arten. Ein zusätzliches Experiment, in dem das künstliche Nektargemisch sowohl auf 'Nektar'- als auch auf 'Kontroll'-Ranken aufgebracht wurde, ergab, dass die Ameisen mit einer erhöhten Abundanz auf solchen Ranken reagierten, die bereits vorher die, Nektar'-Behandlung erfahren hatten.

Nach 25 Tagen zeigten behandelte Ranken signifikant weniger Blattfraß im Vergleich zu unbehandelten Kontrollranken. Die bloße Erhöhung der Menge an extrafloralem Nektar reichte offensichtlich dazu aus, unter natürlichen Bedingungen wachsenden Limabohnen einen Vorteil zu verschaffen.

Introduction

Plants have developed a large number of different strategies to defend themselves against herbivores. Such strategies can be divided into direct or indirect defences. Direct defences per definition directly exert negative impacts on herbivores. They comprise, e.g., spines, thorns, trichomes, waxes and a large diversity of secondary plant metabolites. On the other hand, indirect defences are plant characteristics that include higher trophic levels such as parasitoids or predators of the herbivores, which then fulfil the defensive function (Price et al., 1980).

Extrafloral nectaries are nectar-secreting organs that are not directly involved in pollination, yet play a vital role in maintaining other mutually beneficial relationships among plants and animals (Elias, 1983). In general, extrafloral nectar (EFN) is believed to function as an indirect defensive mechanism. It is mainly composed of mono- and disaccharides and amino acids (Ruffner & Clark, 1986; Galetto & Bernardello, 1992; Heil, Fiala, Baumann, & Linsenmair, 2000). Besides ants, which constitute the main portion, other insects such as ichneumonid and braconid wasps (Bugg, Ellis, & Carlson, 1989; Stapel, Cortesero, DeMoraes, Tumlinson, & Lewis, 1997; Gentry, 2003) or mosquitoes (Foster, 1995) utilize this food source as well, resulting in varying degrees of anti-herbivore protection. The presence of ants, for example, has been repeatedly shown to enhance the protection of entire plants or plant parts (Bentley, 1977a; Heil et al., 2001; Sobrinho, Schoereder, Rodrigues, & Collevatti, 2002), whereas other visitors may act as commensals or even parasites (O'Dowd, 1979; Heil, Hilpert, Krüger, & Linsenmair, 2004).

Studies investigating the role of extrafloral nectar as an indirect defence face considerable methodological problems. In the majority of studies

this issue was addressed by excluding ants using sticky barriers such as Tangletrap® (Stephenson, 1982; Labeyrie et al., 2001; Cuautle & Rico-Gray, 2003). The drawback of this attempt is that crawling herbivores are excluded as well (Mackay & Whalen, 1998; Freitas, Galetto, Bernardello, & Paoli, 2000) and that this systematic error finally can lead to an underestimation of the 'true' effect. The second way to approach this issue is using plant-derived elicitors such as jasmonic acid (JA) that induce extrafloral nectar flow (Heil et al., 2001) or to induce EFN secretion by natural herbivory (Ness, 2003). Such induction phenomena often act via the octadecanoid pathway, which is well known to not only regulate the flow of extrafloral nectar, but also to mediate many other induced direct or indirect defences, such as, e.g., the change of chemical constituents in attacked plant tissues, or the production of volatile organic compounds (VOCs) to attract natural enemies of the herbivores (Karban & Baldwin, 1997). Consequently, the studies conducted so far suffered from different methodological problems: Exclusion experiments run the risk of accidentally barring crawling herbivores and thus underestimating the defensive effect, those inducing EFN secretion may overestimate it due to undesigned inductions of additional direct or indirect defences.

A possibility that circumvents all the above-mentioned difficulties is the artificial increase of extrafloral nectar by exogenous application of sugar solution. Amongst others (Bentley, 1976; Tempel, 1983), this approach was followed up by Jacob and Evans (1998), who sprayed alfalfa plants (*Medicago sativa*) with an aqueous solution of sucrose. Two days later, significantly higher numbers of adult parasitoids as well an increase in the parasitation rate of herbivorous weevils were observed in plots sprayed with sugar solution as compared to control plots, which had been sprayed with water only.

Phaseolus lunatus L. (Lima bean, Fabaceae) is a common model plant used in genetic, biochemical or ecological studies. Besides the emission of VOCs that follows herbivore attack and attracts carnivorous animals (Dicke, Sabelis, Takabayashi, Bruin, & Posthumus, 1990; Dicke, Vanbaarlen, Wessels, & Dijkman, 1993; Dicke, 1994), the Lima bean features extrafloral nectaries (Heil, 2004). Although several aspects of VOC emission of Lima beans have been studied intensively in laboratory or greenhouse experiments, almost nothing is known on the ecological function of its extrafloral nectaries and of VOC-emission in nature. A previous study demonstrated that regularly treating wild Lima bean with JA and thereby inducing both the

production of extrafloral nectar and VOCs benefits the plants at their natural growing site (Heil, 2004). However, it remained unclear which insects were involved in this interaction on both the side of herbivores and defenders. Moreover, it was not clear whether extrafloral nectar alone could also exert a defensive effect.

The aim of the present study was to follow the approach of Jacob and Evans (1998), yet applying artificial nectar directly onto or nearby extrafloral nectaries in an amount and quality that mimicked the natural occurrence of extrafloral nectar. The following questions were addressed: (i) What kinds of insects are attracted to the artificial nectar applied to Lima beans? (ii) Does extrafloral nectar alone exhibit a beneficial effect for the Lima bean at its natural growing site? and (iii) Does the presence of ants on Lima bean tendrils depend on their previous foraging experience (i.e. do they appear in increased numbers where more nectar can be expected)?

Material and methods

Study site and species

The study was conducted in the coastal area of the state of Oaxaca, Mexico, a location close to the centre of the genetic diversity of wild Lima beans (Salgado, P., & Debouck, 1995). Two sites, some 15 km northwest of Puerto Escondido, were selected. These two sites were about 3 km apart from each other and 1–2 km away from the Pacific Ocean. Both study sites represented waysides along dirt roads leading to extensively used pastures or plantations, with Site 1 featuring denser vegetation and Site 2 being much more exposed to direct sunlight. The two sites are identical with those of a previous study (Heil, 2004). The climate in the study area is characterized by one main rainy season from June to October, which follows a bimodal distribution peaking in July and September. Annual rainfall averages between 1000 and 1400 mm and the mean annual temperature is 28 °C (Sträßner 1999).

Wild Lima bean (*Phaseolus lunatus* L., Fabaceae) grows as a twining vine or herbaceous bush, entwining trees and shrubs along open sites such as forest edges or along roadsides. The Lima bean grows in part as a perennial plant, although the aboveground parts usually die during the dry season. In June or July the plants start to germinate or bud, 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 (Heil, 2004). As many other Fabaceae, the Lima bean bears extrafloral nectaries. These are located on its bracts, or arranged pairwise at the stipules of the trifoliate leaves as well as at the petioles of the individual leaflets (Heil, 2004). Voucher specimens are deposited at Herbario MEXU (UNAM, Mexico City, Mexico) and in the personal collection of M. Heil.

Experimental design

In the beginning, 23 pairs of Lima bean tendrils were selected. Eleven of these pairs were located at Site 1 and 12 at Site 2. Due to the tangled growth of Lima bean it was not possible to distinguish single plant individuals. Therefore, paired tendrils either belonged to one single plant individual or to two adjacent plants with a maximum distance of 2 m. Tendrils of each pair were stretched along two strings for further growth. One tendril per pair was randomly assigned to treatment 'nectar', the other one served as an untreated control. 'Nectar treatment' means an aqueous solution of artificial nectar was applied directly on the extrafloral nectaries using an Eppendorf pipette. 'Nectar' tendrils were treated every 3–4 days from 27.10.2003 until 18.11.2003, resulting in six application events. During this time, the initial number of pairs was reduced to a final sample size of 17 due to human impact.

Artificial nectar

To mimic the extrafloral nectar production of the Lima beans qualitatively and quantitatively, both the secretion rate and the chemical composition of extrafloral nectar had to be assessed. Lima bean plants growing at the aforementioned study sites had been induced by spraying with 1 mmol aqueous JA-solution (Heil, 2004). After 24 h, the nectar production rate of these plants was quantified as amounts of secreted soluble solids, by quantifying the nectar volume with micro capillaries and the nectar concentration with a refractometer (Heil et al., 2000; Heil et al., 2001). The nectar secretion rate was then related to the secreting leaves and their respective dry weight. The estimated secretion rate for a strongly induced Lima bean leaf averaged about 1.5 mg soluble solids g^{-1} leaf dry mass $24 h^{-1}$ as compared to 0.02 mg g^{-1} $24 h^{-1}$ for an uninduced control leaf (Heil, 2004).

The subsequent analysis of EFN focused on sugars and amino acids, as they comprise the most important components of extrafloral nectar (Baker,

Opler, & Baker, 1978; Ruffner et al., 1986; Galetto et al., 1992). Other potential nectar constituents such as proteins, vitamins or lipids were not considered. To analyse sugars and amino acids as their trimethylsilylated derivatives, 50 µl N-Methyl-N-(trimethylsilyl)-trifluoracetamide (MSTFA) was added to a 2 µl nectar sample and heated for 1 h to 60 °C. The samples were diluted with dichloromethane in a ratio of 1:100. Sugars from commercial sources (SIGMA) served as standards: 2 µl of an aqueous solution (100 mg ml^{-1}) was treated as described above.

Samples were analysed on a GC-Trace mass spectrometer (Thermo Finnigan) using a DB 5 column (15 m × 0.25 mm × 0.25 µm; Alltech, Unterhaching, Germany). Sugars were separated with the following program: 120 °C for 2 min isothermal, then at 7 °C min^{-1} to 250 °C, temperature of the injection port of 220 °C and temperature of the GC-interface at 280 °C. Simultaneous analysis of sugars and amino acids required a modified program: 40 °C for 2 min isothermal, then at 10 °C min^{-1} to 120 °C, after that at 7 °C min^{-1} to 250 °C and a GC operating with a split-ratio of 1:10. This analysis identified glucose, fructose and sucrose as the main components of the extrafloral nectar blend, with amino acids being below the detection limit (i.e. < 1.3 pmol/ µl). The blend of artificial nectar, which was adjusted to the cumulated secretion rate of one leaf for 3 days, consisted of 4.01 g sucrose l^{-1}, and 24.24 g l^{-1} of each glucose and fructose. Every 3–4 days, 40 µl of this blend were applied directly to the extrafloral nectaries of every leaf of the 'nectar' tendrils.

Insect counts

The first census of the arthropods on the 23 selected pairs of tendrils was performed immediately before the first treatment (27.10.03 for Site 2 and 29.10.03 for Site 1). Both censuses were performed at the same time of day. Number of ants, wasps or flies present on the plants were recorded, as they represented the most abundant groups.

Two additional censuses were performed in the course of the experiment, to test the influence of the 'nectar' treatment on the insect community. The first census started at day 7 of the experiment (7.11.03) at Site 1. Ten pairs of tendrils were selected, which had experienced two treatments until then. The first census was performed prior to the nectar application at 8:00 am. Thereafter all 'nectar' tendrils were treated and insects on all pairs were counted repeatedly every 2 or 3 h until

midnight. Two additional censuses were performed at 9:00 and 10:00 am on the following 2 days, resulting in a total of 14 monitorings. The same was done with 10 pairs of plants at Site 2. This second series started at day 18 after the onset of the experiment (14.11.03) and consisted of a total of 13 censuses.

Defensive effect

The herbivory rate in percent leaf loss was chosen as a fitness-relevant parameter. This parameter was quantified by assigning leaves to one of the following ranges of missing leaf area: 0%, 1–5%, 6–10%, 11–25%, 26–50% and 51–100%.

At the beginning of the experiment (24.10.03), the herbivory rate for all leaves present on the studied tendrils was assessed as a reference. After 25 days, the remaining tendrils were checked again: The herbivory rate of all leaves was estimated when the total leaf number per tendril was <25, otherwise 25 leaves were randomly selected and the herbivory rate assessed. Finally, average values were calculated for the two estimations and the difference between these two was used to measure the development of leaf damage.

To test the accuracy of this method, the missing leaf area of 74 randomly chosen leaves was both estimated as mentioned above and quantified as follows: First, leaves were dried between sheets of paper for transport (leaves shrink less than 5%
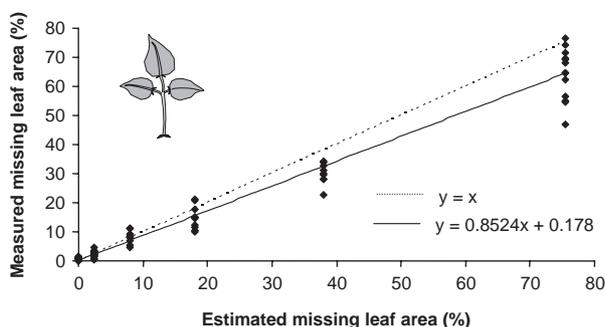
during this procedure). The leaves then were scanned together with a reference area of $1\,cm^2$ (hp scanjet 8200 scanner). The resulting digital pictures were analyzed with the graphics software package Corel photo paint version 8.232. By outlining the area of both a given trifoliate leaf and the reference area, the corresponding leaf area could be determined, as indicated by the number of pixels. The missing leaf area was outlined accordingly. In cases of complete leaflets missing, the corresponding area was estimated since both the areas of the left and the central leaflet ($r = 0.93$, $P < 0.001$, $n = 58$, $y = 0.8397x + 20786$) as well as the two lateral leaflets showed a strong correlation ($r = 0.95$, $P < 0.001$, $n = 56$, $y = 0.8361x + 13361$). Finally, the percentage of missing leaf area was computed from missing and total area. The close linear relationship between the measured and estimated missing leaf areas (Fig. 1) allowed the simplified determination of the missing leaf area by estimation.

Insect collection

Ants, wasps and flies were the insect groups most regularly encountered on experimental Lima bean tendrils. To study their affiliation to functional groups, specimens of all three groups were collected. Ants were sampled at each of the two study sites from randomly chosen Lima bean clusters that were directly neighbouring the study tendrils. All ants that showed up within approximately 45 min were collected manually with tweezers. Flies and wasps were collected with sticky traps that consisted of $100\,cm^2$ pieces of green plastic foil that had been coated with a thin layer of a trapping adhesive (Tangletrap®). Two such sticky traps were attached with plastic strings to each of 14 'nectar' and 'control' tendrils that were equally distributed between the two study sites. After 24 h of exposure, the traps were re-collected and the insects transferred to 75% ethanol. Ants were determined to species level whenever possible by Manfred Verhaagh, Staatl. Museum für Naturkunde Karlsruhe. Flies and wasps were identified to family level using keys and information provided by Arnett (2000) and Schaefer et al. (1994) for insect families and Noyes (2003) for chalcid families. On the basis of the natural history information provided by Honomichl, Bellmann, Jacobs, and Renner (1996) for flies and wasps, Rettenmeyer (1961) and Disney (1994) for phorid flies as well as Rico-Gray (1993) and Brown (2000) for ants, the collected insects were assigned to the following guilds according to nutritional or functional aspects: Detrivore (D),

Figure 1. Comparison between estimated and measured leaf area missing of 74 leaves of *Phaseolus lunatus*. Leaf area was estimated by assigning leaves to the closest of six different classes of missing leaf area (0%, 1–5%, 6–10%, 11–25%, 26–50% and 51–100%), of which the corresponding mid-points are displayed. For exact measurements digitized pictures of leaves were analysed by relating pixel numbers of outlined leaves to a reference area. A regression was calculated (solid line) to relate the estimated to the measured leaf area ($r = 0.981$, $P < 0.001$, $n = 74$). The axial bisector (dashed line) shows the theoretically perfect correlation.

Frugivore (F), Herbivore (H), Parasitoid (P), Predator (R) or utilization of other plant-derived resources including floral or extrafloral nectar, pollen and honeydew (S).

Nectar experiment

To assess whether increased ant numbers on the nectar-treated tendrils resulted from the actual amount of artificial nectar alone, or whether the frequency of ant foragers had been adjusted to their previous foraging experience, nine pairs of tendrils (three at Site 1 and six at Site 2) were selected and both the 'nectar' and the 'control' tendril treated with artificial nectar. The abundance of ants, wasps and flies was assessed in advance, and every 30–45 min after the treatment, resulting in 9–11 countings per tendril pair.

Statistical analysis

The approach of using adjacent pairs of 'nectar' and 'control' tendrils allowed the use of the Wilcoxon test for matched pairs whenever single or averaged values between the two treatments had to be compared. The influence of the factor 'site' was verified by applying Mann-Whitney U-tests to the census data of different insect groups which were assessed before the onset of the experiment (i.e. 27.10.03 for Site 2 and 29.10.03 for Site 1). Data was pooled for tendrils that afterwards were assigned to the two treatments since no treatment had been administered at that time. Tests for the factor 'time' were done by comparing insect numbers assessed before the start of the experiment, with one comparable census (i.e. same time of day) 7 days (Site 1) or 18 days (Site 2) after the start of the experiment. All statistical evaluations were done using SPSS 10.0.

Results

Effects of the 'nectar' treatment on insect abundance

The application of artificial nectar resulted in a significant increase in the abundance of ants, wasps and flies on nectar tendrils compared to control tendrils already after 7 days (i.e. after two treatments, Fig. 2). The second assessment of insect abundance starting from day 18 revealed an even more pronounced increase in the number of ants. However, no statistically significant differ-

ence between 'nectar' and 'control' tendrils could be detected for wasps and flies during the second census. The three focal insect groups showed a strong diurnal activity pattern with a subsequent decline during the night for wasps and flies, but no strong relation to the time of day for ant activity (Fig. 2).

Insect censuses represented not only two different dates (i.e. 7–9.11.03 and 14–16.11.03), but they were also performed at different sites. Therefore, a priori differences of insect abundance (factor 'site') and the changes of insect abundance in the course of the experiment (factor 'time') were assessed. Differences between sites were determined for the abundances of ants, wasps and flies present on study tendrils at the onset of the experiment. As no treatment had been administered at that time, insect abundances on the tendrils which were later subjected to the treatments 'nectar' and 'control' were pooled. The corresponding analysis included 23 tendrils ($n = 11$ at Site 1 versus $n = 12$ at Site 2) and revealed that abundances of wasps and flies were significantly higher at Site 1 as compared to Site 2 (Mann-Whitney U-tests in both cases $P < 0.001$), whereas no such difference between sites could be detected for ants (Mann-Whitney U-test $P = 0.515$).

The putative influence of the factor 'time' was determined by comparing insect abundances on 'control' tendrils before the onset of the experiment with those found seven (Site 1) or 18 days (Site 2) later on the same untreated tendrils. No statistically significant influence of the factor 'time' could be detected for the abundance of any of the focal insect groups ($P > 0.05$ according to Wilcoxon pair tests with $n = 10$ for both sites). Hence the abundance of ants was neither influenced by the factors 'site' nor 'time', whereas the abundance of wasps and flies was heavily influenced by a priori 'site'-effects.

As ants responded strongest to the application of artificial extrafloral nectar, an additional experiment was conducted to clarify whether their increased presence on nectar tendrils depended on their previous foraging experience? In this case, the presence of ants would be higher on tendrils on which increased amounts of nectar had been available before (i.e. 'nectar' tendrils). Artificial nectar was applied to both the 'nectar' tendrils, which so far had been treated six times every 2–3 days, and to the 'control' tendrils. The subsequent monitoring of ant activity on both classes of tendrils revealed a very similar pattern of ant response: Ant activity peaked 1.3 h after nectar application, thereafter declined (Fig. 3). However,
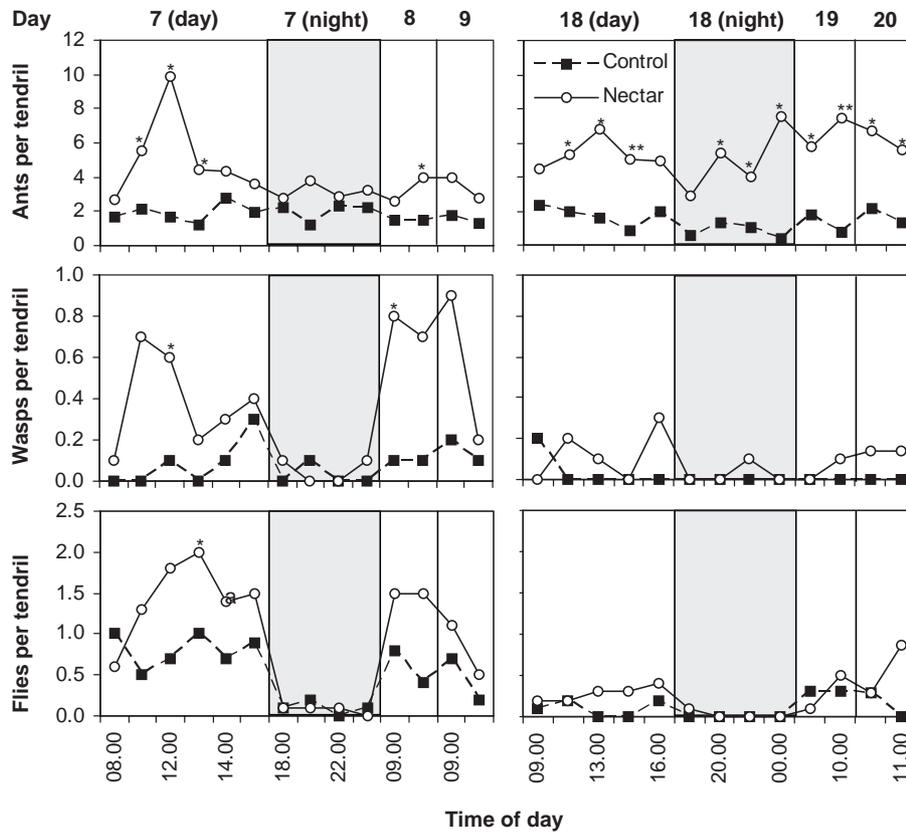
Figure 2. Temporal pattern of the three most abundant insect groups on tendrils treated with artificial nectar and untreated controls, recorded at Site 1 (left) and Site 2 (right). The first census at each site shows insect numbers before the application of artificial nectar; the following censuses represent insect abundance thereafter. Insect numbers for 10 pairs of plants at a time were averaged for each census. Censuses were performed at three consecutive days starting at Site 1 on day seven after the beginning of the experiment and on Site 2 on day 18. Significant differences between corresponding pairs of tendrils are indicated as $*P < 0.05$, or $** \leqslant 0.01$; Wilcoxon pair test.
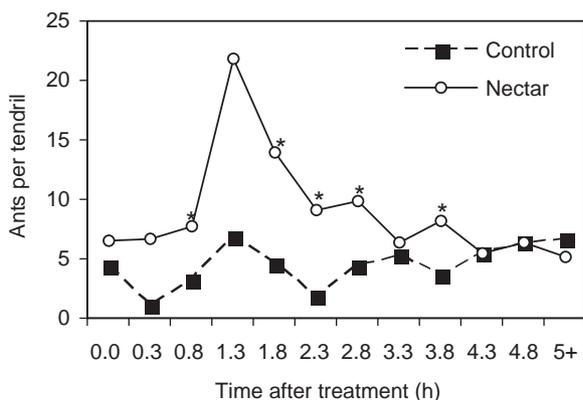

Figure 3. Temporal pattern of the presence of ants on nine pairs of tendrils (three from Site 1 and six from Site 2) after treating both 'control' and 'nectar' tendrils with artificial nectar. Ant numbers were averaged for each census. Significant differences between corresponding pairs of tendrils are indicated as $*P < 0.05$; Wilcoxon pair test.

the intensity of ant response was significantly lower on 'control' compared to 'nectar' tendrils.

Visitors of the Lima bean

The sticky trap experiment confirmed the observation that flies and wasps played an important role as visitors of the Lima bean: 44% of all insects trapped were dipterans and 26% hymenopterans (Table 1). Other groups trapped were mainly Coleoptera (11%), Auchenorrhyncha (9%) or Araneida (6%). Although there was no significant difference detectable between control and nectar tendrils for any of the trapped arthropod groups (Wilcoxon signed rank test $P > 0.05$), a more detailed analysis of the functional groups indicated a large proportion of putative beneficial insects visiting the Lima bean. Among the flies, Dolichopodidae (31%) and Phoridae (27%) were the most abundant families (Table 1). The Dolichopodidae

Table 1. Wasps and flies trapped on experimental clusters with sticky traps and ants collected manually from Lima bean clusters adjacent to the experimental clusters.

Order	Taxon	Number [ind.]	Guild					
			R	P	S	F	H	D
Diptera	Dolichopodidae	47	l/a		a			
	Phoridae	40	l	a	a			
	Tachinidae	11		l	a			
	Chloropidae	9	l	l	a		l	
	Culicidae	6			a			l
	Drosophilidae	4				l/a		
	Platystomatidae	3			a			l
	Agromyzidae	2			a		l/a	
	Canacidae	2	?					
	Lauxaniidae	2			a			a
	Muscidae	2	l		a			l
	Psychodidae	2			a			l
	Scenopinidae	2	l		a			l
	Syrphidae	2	l		a			l
	Others	14						
Hymenoptera								
Wasps	Chalcidoidea	60	l/a	a				
	Braconidae	8	l/a	a	a			
	Ichneumonidae	3	l/a	a	a			
	Dryinidae	2	l/a	a				
	Sphecidae	2	l/a					
	Others	5						
Ants	*Camponotus novogranadensis*	n. d.	l		a			
	Camponotus (Myrmobrachys) sp.	n. d.	l		a			
	Cephalotes minutus	n. d.	l		a			
	Crematogaster sp.	n. d.	l		a	a		
	Monomorium sp.	n. d.	l		a	a		
	Paratrechina longicornis	n. d.	l		a			
	Pseudomyrmex sp. 1	n. d.	l		a			
	Pseudomyrmex sp. 2	n. d.	l		a			

Numbers of wasps and flies refer to the total number of insects trapped and was not determined for ants (n. d.). The observed flies, wasps and ants were assigned to the following guilds according to nutritional or functional aspects: Detrivore (D), Frugivore (F), Herbivore (H), Parasitoid (P), Predator/ Entomophaga (R), or utilization of other plant-derived resources including floral or extrafloral nectar, pollen and honeydew (S). The affiliation to a guild is further differentiated by the developmental stage of each taxon (l = larval, a = adult) that features the respective trait.

are known to prey on smaller insects or insect larvae that live in the vegetation along coastal areas. The family Phoridae covers many predatory or parasitoid species of mainly Formicidae, but also of Orthoptera or Blattodea. In total, 71% of all flies trapped belonged to fly families that comprise species with predatory and parasitoid life habits. Among the Hymenoptera this trend became even more conspicuous: Except one single individual of the Cephidae (Symphyta), all trapped Hymenoptera (98%) showed either parasitoid or predatory life habits. Here, members of the superfamily Chalcidoidea were the most abundant group, which alone already contributed 68% to all hymenopterans trapped. These parasitoid wasps belonged to 14 different families with the Pteromalidae being the most abundant (27%).

Among ants, *Camponotus novogranadensis*, *Cephalotes minutus* and *Crematogaster* sp. were most regularly encountered on study tendrils (C. Kost, personal observation). The remaining species could only be observed occasionally and thus seem to be of minor importance. All ants collected were generalists, which apart from their preference for sugar sources such as extrafloral nectar, are well known to prey on various arthropods (Table 1).
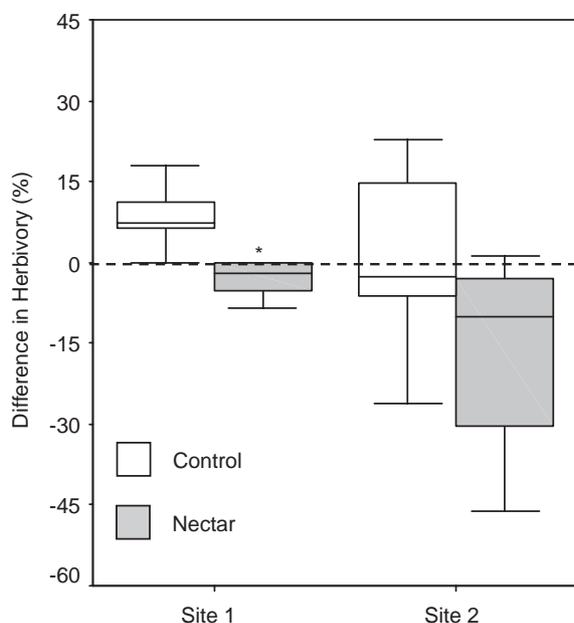
Figure 4. Effect of the application of artificial nectar on herbivory of *Phaseolus lunatus* at the two study sites. Herbivory is presented as relative differences (day 0/day 34) in the estimated herbivory rate (%) for 17 pairs of Lima bean tendrils ($n = 10$ pairs for Site 1 and $n = 7$ pairs for Site 2, $*P<0.05$; Wilcoxon pair test).

Effect of the 'nectar' treatment on the herbivory rate

The application of artificial nectar resulted in a significant reduction of the herbivory rate in treated versus control tendrils (Fig. 4, $P = 0.01$; Wilcoxon pair test for pooled pairs). However, this protective effect differed between the two sites (Fig. 4): It was very obvious at Site 1 (Wilcoxon pair test: $P = 0.047$), whereas the same trend but no significant difference could be detected for Site 2 (Wilcoxon pair test: $P = 0.091$).

Discussion

The main purpose of the present study was to unravel whether the secretion of extrafloral nectar benefits the Lima bean at its natural growing site. Many studies on this topic have already been conducted before, but most were hampered by methodological problems such as (i) unintended exclusion of crawling herbivores, (ii) not taking into account putative influences of flying plant defenders, and (iii) simultaneous induction of other defensive mechanisms. In order to focus on defensive effects of EFN only, artificial nectar that mimicked the nectar secretion of induced Lima

bean plants in terms of amount and chemical composition was applied to Lima bean plants.

The application of artificial nectar resulted in a significantly increased abundance of ants, wasps and flies on treated compared to control tendrils after only 7 days (Fig. 2). Most of the wasp and fly families encountered belonged to families that exclusively or at least partially comprise parasitoid or predatory species (Table 1). On this account, the observed defensive effect may not only be due to the increased presence of ants, but could also be exerted by flying defenders that are attracted to the extrafloral nectaries. The following findings may also point into this direction: A comparison of insect abundances between the two sites before the start of the experiment revealed significantly higher frequencies of wasps and flies at Site 1 than at Site 2, whereas no such difference between sites could be detected for ants. At the end of the experiment, Site 1 showed a strongly reduced herbivory level, while at Site 2 only a trend towards a reduced herbivory level of treated tendrils could be observed (Fig. 4). The protective effect was thus strong at the site where, besides ants, also wasps and flies showed increased activities, but it was weak at the site where mainly ants were attendant to fulfil a defensive function. Obviously, flying defenders added significantly to the overall defensive effect. Which of these two groups (flies and wasps) contributed most to the observed defensive effect?

It seems likely that the attracted wasps contributed more to this effect than the attracted flies. All flies belonging to the family of Dolichopodidae were relatively small and thus may not have been able to prey on larger herbivores. Furthermore, the Phoridae are known not only to parasitize herbivorous species (Rettenmeyer, 1961) but also ants (Disney, 1994). An increased abundance of phorid flies might therefore also have led to a reduction of the 'true' defensive effect.

Most of the studies regarding the defensive effect of EFN focused on ants, without taking into account influences of other putative plant defenders such as spiders (Ruhren & Handel, 1999) or parasitoids (Pemberton & Lee, 1996). Besides this study, only one study is available to date where the defensive roles of several groups of plant defenders have been studied simultaneously: Cuautle and Rico-Gray (2003) showed that either ants or wasps exerted beneficial effects on the extrafloral nectary bearing plant *Turnera ulmifolia* when selectively excluded; when both insect groups had access, however, their effects were not additive.

Extrafloral nectaries secrete small amounts of nectar throughout the day. Nectar can be secreted

in diurnal patterns that are characterized by short peaks (Heil et al., 2000; Raine, Willmer, & Stone, 2002) or at relatively constant rates throughout day and night (Bentley, 1976, 1977b). The exact mode of nectar secretion is not known for *Phaseolus lunatus*. An application of artificial nectar at intervals of 3–4 days, however, does most likely not match the natural situation. Yet, the attracted insects may have responded to the increased sugar amounts itself rather than being visually attracted to the relatively large droplets of sugar solution, since already 10–15 min after nectar application the solvent water was completely evaporated leaving behind only the sugars.

Even though the applied nectar was consumed very rapidly (C. Kost, personal observation), the abundance of ants, wasps and flies was sustainably increased during the time of application: Even at the third day following nectar application, all three insect groups still showed increased abundances (Fig. 2).

As ants were most dominant, an experiment was performed to verify, whether this sustainable increase in ant numbers on 'nectar' tendrils was due to their ability to respond with a modification of their foraging behaviour based on previous experiences. The 'nectar experiment', in which artificial nectar was applied to both 'nectar' and 'control' tendrils at the end of the experiment, revealed a similar pattern of ant response for both types of tendrils (Fig. 3). However, the intensity of the response was significantly increased for tendrils that had experienced the nectar treatment before. Obviously, the ants responded positively by preferentially foraging on tendrils that usually were characterized by an increased availability of extrafloral nectar.

This effect could be explained by increased time periods the ants spend on plants that supply EFN more regularly, with increased visitation frequencies of foraging ants, or with a more efficient recruitment to these tendrils. Experimental findings seem to support the second alternative: The time foraging ants spend at a food source depends on flow rates experienced previously and decreases with increasing nectar flow rates (Schilman & Roces, 2003). Increased flow rates lead at the same time to an increase of the volume collected. The ecological function of such a foraging behaviour may be a defence-by-exploitation strategy. Resident ants avoid competition against other nectar consumers by systematic and frequent visitation, thus leading to an exploitation of the food source (Dreisig, 2000). Such an increased visitation frequency advances the probability of ant attendance, thereby facilitating the protective effect. This strategy seems to be characteristic not only for the species tested in the studies mentioned before, but for all generalistic ants (F. Roces, personal communication).

The 'control' tendrils were characterized by a certain level of 'background' EFN secretion. The secretion rate of 18 Lima bean plants that were untreated and adjacent to the experimental tendrils at Site 1 ranged about ca. 0.36 mg soluble solids g^{-1} $24 h^{-1}$ (C. Kost, unpublished data). Adding the constitutive secretion of EFN to the amount of applied artificial nectar results in ca. 1.06 mg soluble solids g^{-1} $24 h^{-1}$ of nectar being available on 'nectar' tendrils. Earlier measurements (2002) at the same site revealed a secretion rate of ca. 0.02 mg soluble solids g^{-1} $24 h^{-1}$ for untreated and ca. 1.5 mg soluble solids g^{-1} $24 h^{-1}$ for Lima bean plants induced with JA (Heil, 2004). This comparison illustrates that the total amount of nectar, which was available on 'nectar' tendrils, did not exceed the physiological limits of nectar production in Lima beans. Moreover, since also 'control' tendrils produced certain amounts of EFN, the protective effect of EFN secretion that became obvious at the end of the experiment was systematically underestimated and thus provides a conservative measure of this defensive trait.

In the present study Lima bean plants benefited from EFN secretion in nature. Since not only ants but also flying insects were attracted to the artificial nectar, these may also have contributed to the observed protective effect. Further studies are required to elucidate which of the observed flying insects are beneficial to the Lima bean and which ones even cause ecological costs by consuming EFN without exerting a beneficial effect.

Acknowledgements

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