

Quantitative Effects of Leaf Area Removal on Indirect Defense of Lima Bean (*Phaseolus lunatus*) in Nature

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Abstract Plants employ a diverse array of defensive traits against multiple enemies. While many plant defenses are well-studied, quantitative feedback effects of leaf area loss on the expression of defensive traits remain little understood. Extrafloral nectar (EFN; an indirect defense acting via the attraction of carnivorous arthropods) is generally considered ‘cheap’ as it is composed mainly of photosynthates. However, to what extent EFN secretion is related to the amount of intact photosynthetic leaf area is unknown. In this study, we measured the production of EFN, ant attraction, and herbivore damage in response to a gradient of leaf area removal in wild lima bean (*Phaseolus lunatus*) under natural conditions in southern Mexico. EFN production and ant recruitment were significantly decreased with increasing leaf area removal. Consequently, EFN production was inversely correlated with leaf area loss, which suggests that EFN is metabolically more expensive than previously thought. Further, we found increased herbivory in plants with reduced EFN secretion indicating additive negative feedback effects of leaf area loss. Our study is one of the first showing a quantitative negative impact of leaf damage on EFN secretion—one of the most widely distributed defensive traits in the plant kingdom.

Keywords Extrafloral nectar · Herbivory · Ants · Allocation constraints

Introduction

Plants employ multiple defense mechanisms against herbivore attack. Indirect defenses attract antagonists of herbivores or

pathogens, thus reducing plant damage. Extrafloral nectar (EFN) is an effective indirect defense that attracts ants, parasitoids, and generalist predators such as wasps and spiders (Kost and Heil 2006). Extrafloral nectar has been observed in 745 genera comprising angiosperms, gymnosperms, and ferns, and may be more ancient than floral nectar (Weber and Keeler 2012). Primarily composed of carbohydrates, EFN is derived directly from photosynthesis. The amount of sucrose in EFN accounts only for a small percentage of total plant photosynthates. Thus, EFN often is considered a relatively inexpensive defense. However, recent research by Li et al. (2012) shows that caterpillar feeding on aspen reduces EFN secretion. This reduction is due possibly to loss of photosynthetic tissue, which may limit the capacity of EFN secretion. Yet, quantitative information on the association between available photosynthetic leaf area and EFN production is scarce, and the question arises whether EFN—which is secreted in amounts of several milligrams of sugars per g leaf weight over 24 h—is actually that cheap for the plant.

In this study, we used wild lima bean (*Phaseolus lunatus*) to test the hypothesis that inducible EFN secretion is quantitatively impacted by leaf area loss. Further, we quantified ant attraction and herbivory to examine ecological costs of leaf area removal. Lima bean is a common model for research on plant defenses including both indirect and direct defenses (Ballhorn et al. 2013; Kost and Heil 2006).

Methods and Materials

Plant Material Wild lima bean plants (Fabaceae: *Phaseolus lunatus*) were grown from seeds derived from natural lima bean populations in southern Mexico (Oaxaca). Plants were cultivated in plastic pots (15 cm diam) filled with soil and maintained in close proximity (<500 m) to natural lima bean populations in August 2009. Wooden sticks served as climbing support, and anti-aphid net and a ring of Tangle Trap® around the top of the pots protected from herbivores. Plants were watered daily with tap water. At the time of the

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experiments, plant size was 50–60 cm, and plants had developed 9 to 11 leaves.

Mechanical Damage of Plants and Experimental Setup Three levels of damage were applied to fully unfolded leaves, while the control group remained undamaged ($N=12$ plants per treatment). Leaf area was removed with a razor blade reducing total leaf area per plant by approx. 17, 33, and 50 %, respectively (Fig. 1). Clipping was applied in the early morning (5:00–6:00 am) when ambient relative humidity was high (>90 %) reducing stress due to treatment. We never observed any dead leaf area along the cutting edge. Extrafloral nectaries on lima bean leaves are located at the base of trifoliate leaves, and individual leaflets and remained unaffected by the treatments (Fig. 1).

Plants were positioned in 12 blocks ($N=4$ plants per block) consisting of one plant per treatment group each. Spaces between blocks were 5 m. Within blocks, plants were positioned 60 cm apart at random order.

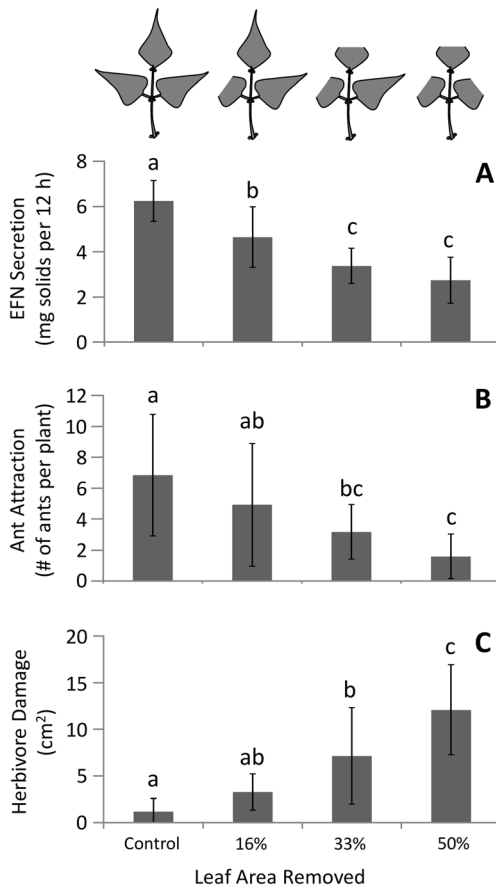


Fig. 1 Quantitative effects of leaf area removal on extrafloral nectar (EFN) secretion, ant attraction, and herbivory. EFN secretion (a) of lima bean (*Phaseolus lunatus*) plants with different levels of leaf clipping (Level 1: 17 %; Level 2: 33 %; Level 3: 50 %) was measured, ant attraction (b) as well as herbivory (c) on the damaged plants was recorded in the field. Small-typed letters above columns indicate significant differences between groups [according to post-hoc analysis (Tukey's HSD $P<0.05$)] after one-way ANOVA ($N=12$ plants per treatment group)

Induction and Quantification of Extrafloral Nectar Twenty-four hours after experimental reduction of leaf area, all plants were thoroughly sprayed with water to remove all EFN secreted in response to damage and to set the initial EFN load on plants to 0. When dry, all plants were sprayed with an aqueous solution of 1 mmol L⁻¹ jasmonic acid (JA) between 08:00 and 09:00 a.m. Leaves were sprayed with JA solutions until completely moistened (20 ml) and allowed to dry (10 min) before being sprayed a second time (20 ml). Twenty-four hours after the priming of experimental plants with JA, plants were washed with water again to remove any nectar produced in the meantime, and they were subsequently damaged with a pestle consisting of 12 blunt steel needles (each with a diameter of 0.8 mm; overall diam of the pestle 7 mm) to induce EFN production. Secreted extrafloral nectar was collected 12 hr after mechanical damage was applied. To reduce potential uncontrolled variation of EFN production depending on leaf age, one fully developed leaf per plant inserting five positions down the apex of the shoot was sampled. Extrafloral nectar production was quantified according to Kost and Heil (2006) as amounts of soluble solids by using microcapillaries (PCR Micropipets 1–10 μ l; Drummond) for determination of volume and a brix refractometer for determination of sugar concentration. Plants used for collection of extrafloral nectar were protected by anti-aphid nets against flying nectar consumers (bees and wasps), and were protected against nectar-consuming ants with Tangle Trap[®]. Any vegetation providing potential bridges for ants was carefully removed.

Ant Attraction An identically treated set of plants was openly exposed to natural insect communities after EFN induction. Access of ants was facilitated by 4 dead twigs connecting the soil and ambient vegetation with the foliage of experimental plants. Ants on the experimental plants were counted once 6 h after EFN induction.

Herbivore Damage Twelve hours after mechanical induction of EFN secretion, all leaves per plant were harvested and digitally photographed (Canon 5D) on a scale to quantify missing leaf area using the analySIS software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Results and Discussion

Extrafloral nectar production significantly decreased when leaf area was experimentally removed (one-way ANOVA, $F_{3,47}=27.008$; $P<0.001$; Fig. 1a). Along with reduced EFN production, ant attraction significantly decreased on plants with reduced leaf area ($F_{3,47}=6.764$; $P=0.001$; Fig. 1b), whereas herbivore damage on these plants significantly increased ($F_{3,47}=19.765$; $P<0.001$, Fig. 1c). Extrafloral nectar

production and ant attraction were positively correlated (Pearson's correlation, $r=0.504$, $P<0.001$). EFN was negatively correlated with herbivore damage ($r=-0.536$, $P<0.001$), as was ant attraction ($r=-0.479$, $P<0.001$). Attracted ants almost entirely belonged to one species (*Dorimyrmex* spec.) (pers. observation); herbivore damage mostly was incurred by Chrysomelid beetles (*Gynandrobrotica guerreoensis* and *Cerotoma ruficornis*) (Ballhorn et al. 2009).

It is commonly assumed that extrafloral nectar is a relatively inexpensive plant defense (Rudgers and Gardener 2004; O'Dowd 1979). This is based partially on the assumptions that photosynthesis is rarely limited under sufficient light and nutrient availability and that EFN is mostly composed of photosynthates. However, in line with the results of Li et al. (2012) on reduced EFN production in herbivore damaged aspen leaves, our study supports their hypothesis that EFN production may be impaired by photosynthate availability. This is further consistent with the results of a study by Rutter and Rausher (2004) on *Chamaecrista fasciculata* (Fabaceae), reporting costs associated with EFN production in the presence of increasing herbivory.

Among chemical indirect defensive traits, EFN sugars are relatively expensive in terms of carbon and may be down-regulated as photosynthetic capacity becomes limited (Radhika et al. 2008). Compared to carbon-based volatile organic compounds, which are usually released in the range of micrograms per gram leaf mass and 24 hr, the loss of carbon due to EFN production is higher by a factor of 1000. Nevertheless, compared to other chemical defenses of lima bean such as cyanogenesis (the release of hydrogen cyanide from cyanogenic precursors in response to cell damage) the production of EFN is likely to be less costly. Cyanogenic precursors are considered expensive, as nitrogen containing amino acids are required for their synthesis resulting in allocation constraints with protein biosynthesis. On the other hand, in contrast to EFN, cyanogenic precursors can be recycled by the plant in case of nitrogen demand. Once secreted, EFN represents an inaccessible carbon source for the plant, as there are no known mechanisms by which plants can reabsorb unconsumed nectar, increasing the cost of this defense.

The apparent widely underestimated costs of EFN may have important ecological and evolutionary consequences

for plant defense strategies. Future studies are needed in order to understand the selective pressures on EFN as a defensive trait across various ecological conditions, in particular under combinations of pathogen and herbivore attack and variable abiotic conditions in nature. It is known that EFN co-varies with other defensive traits (Ballhorn 2011; Ballhorn et al. 2013), and our study provides the first quantitative evidence that EFN decreases with loss of leaf area, implying EFN is more costly than previously assumed.

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