

Rhizobium alters inducible defenses in broad bean, *Vicia faba*

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ABSTRACT

Conversion of inorganic nitrogen by mutualistic nitrogen-fixing bacteria is essential for plant growth and reproduction, as well as the development of chemical and mechanical defenses. It is unclear, however, how these bacteria alter co-occurring symbioses at higher trophic levels; e.g., extrafloral nectary (EFN) induction, in response to herbivory, to attract defensive mutualists. We hypothesized that plants colonized by nitrogen-fixing bacteria would mount a larger inducible, defensive response than plants lacking symbioses, as defensive traits are costly. We predicted that bean plants, *Vicia faba* L., harboring *Rhizobium leguminosarum* bv. *viciae* Frank would produce more EFNs upon leaf damage, than plants lacking the symbionts, as EFN induction in *V. faba* is resource-dependent. Here we report that *V. faba* colonized by *R. leguminosarum* produced similar numbers of EFNs as did plants without symbionts. Plants with symbionts, however, produced significantly fewer EFNs over 1 week in response to leaf damage, than those without leaf damage. As such, nitrogen-fixing bacteria may not always benefit the host plant, but rather, the utility of these bacteria may be dependent on the prevailing ecological conditions.

Keywords: Extrafloral Nectary; Inducible Defense; Mutualism; Phenotypic Plasticity; Rhizobia

1. INTRODUCTION

Nitrogen-fixing bacteria—host plant mutualisms have been studied for decades [1,2]. Fixed nitrogen, the conversion of atmospheric nitrogen to ammonium in plant root nodules, is used for plant growth and reproduction as well as chemical defenses [3,4]. Despite their ubiquity, these symbioses are very host specific; only certain spe-

cies or strains of bacteria can colonize host plants [5,6]. Broad bean, *Vicia faba* L., roots are one of the most discriminating mutualists, being colonized only by *Rhizobium leguminosarum* bv. *viciae* Frank [5,7,8]. This nitrogen-fixing bacteria increases plant size, vigor, and seed set [9]. Whether this symbiont alters broad bean ecological interactions with higher trophic levels, through altered defensive capabilities, however, is unknown.

Defensive mutualisms between plants and predatory arthropods are common. Over 93 plant families produce sugar-producing structures, called extrafloral nectaries (EFNs) [10], which are highly attractive to predatory ants [11,12]. Plants increasing nectar secretion rates generally attract more ants, have lower rates of herbivory, and increased survivorship [12-15]. Increased nectar production may also be inducible depending upon environmental conditions [13,16]. In broad bean, EFNs are located on the stipules at the base of each leaf petiole [17,18]. Attractive to ants [18,19], broad bean plants increase EFN numbers when herbivory increases [17, 20]. As broad bean EFNs are visually conspicuous it may be adaptive to produce a more striking visual display rather than to augment nectar from existing nectaries [17].

The induction of extrafloral nectar [13,16,21] and nectaries [17,20,22] are believed to be costly as, in several cases, these responses are resource dependent [14, 20]. Resultantly, many plants have evolved phenotypically plastic responses to augment these defensive traits only when risk of herbivory increases [23,24]. As nitrogen-fixing bacteria provide plants with additional nitrogen, broad bean plants with rhizobia may have the ability to produce additional defensive structures (e.g., EFNs) in response to leaf damage.

As defensive traits have frequently been shown to be costly, we hypothesized that plants with nutritional symbionts would produce a larger inducible response than plants lacking nutritional symbionts. More specifically, we predicted that broad bean plants, *V. faba*, harboring the nitrogen-fixing bacteria, *R. leguminosarum* bv. *viciae*,

would produce more EFNs in response to leaf damage, than plants lacking these mutualists.

2. MATERIALS AND METHODS

2.1. Experiment

To carry out this study broad bean seeds were sprouted in deionized water. Water was changed every 24 hours, for 5 days. After 5 days, sprouted beans ($n = 60$) were used for the experiment. Thirty sprouts were each inoculated with 1g of Royal Peat Inoculant; containing 2×10^8 viable cells of *R. leguminosarum* bv. *viciae* per gram (Becker Underwood, Ames, Iowa 50010). We chose to use a commercially available inoculant, containing *R. leguminosarum* bv. *viciae*, so that others could easily repeat our experimental protocol. Each seed was dipped into deionized water and coated with inoculant. The remainder of the 1g of inoculant was sprinkled on the soil directly under the sprout. The remaining 30 sprouts were each dipped in water but not inoculated prior to planting. Sprouted seeds were planted in Sta-Green All-Purpose Potting Mix (NPK 0.14:0.11:0.08; Spectrum Brands, Atlanta, GA 30328) in 1L pots (ITML Horticultural Products, Brantford, ON Canada N3T 5M8). Unsterilized soil was used for this experiment so as not to kill any beneficial microbes in the soil, which could alter the experimental results. Plants were grown under greenhouse conditions (32°C - 41°C, 27% - 95% rh, natural lighting) in a computer-generated random order (JMP 5.1) [25].

Treatments were randomized using a random number generator (JMP 5.1) [25]. Pots were then labeled with the experimental treatments: RD (*Rhizobium*, leaf damage; $n = 15$), RND (*Rhizobium*, no leaf damage; $n = 15$), NRD (no *Rhizobium*, leaf damage; $n = 15$) and NRND (no *Rhizobium*, no leaf damage; $n = 15$). Plants were watered every second day. Once plants reached a height of approximately 10cm, they were staked to help the plant grow upright. After the fourth week, when plants were approximately 60 cm tall (mean = 56 ± 9 cm), initial plant traits were recorded and treatments were conducted.

We recorded the plant traits: plant height, number of fully expanded leaves, number of partially expanded leaves, and number of EFNs. While each broad bean leaf pair generally has 0 or 2 EFNs on the associated stipules, broad beans can alter the total number of EFNs on a plant by clustering the stipules, and hence the EFNs, on the apical meristem prior to the leaves unfolding [20]. After recording these traits, leaf damage treatments were administered to the appropriate plants. For replicates with leaf damage, the outer one-third of each of the plants fully and partially expanded leaf pairs were excised

using floral scissors. To ensure that compounds were not transferred between plants, the scissors were cleaned with an alcohol swab after excising tissue from each plant.

After allowing the plants to grow for 1 additional week, plant traits were again recorded. A few plants, distributed across treatments, broke before final traits could be assessed; these plants were not included in any analyses. We calculated the degree of change in all of the traits by subtracting the initial trait values from the final trait values. We also destructively sampled the plants to assess: total numbers of root nodules, total root nodule weight, shoot dry weight, and root dry weight (minus nodules). Once total nodule numbers were recorded, nodules were excised from the root, placed in aluminum weighing dishes, and placed in a drying oven (55°C \pm 2°C) for 2 weeks. Roots and shoots were placed in separate brown paper bags and dried similarly. Root nodule, shoot, and root weights were determined using a microbalance (ExplorerPro, Ohaus Corporation, Pine Brook, NJ 07058).

2.2. Statistical Analyses

For a robust statistical analysis we conducted similar two-factor ANCOVAs, pre- and post-leaf damage treatments. By conducting similar analyses we could confirm that significant differences did not exist in the treatment groups prior to leaf damage. Independent variables in both analyses were: *Rhizobium* (no vs. yes), leaf damage (no vs. yes), and the first-order interaction *Rhizobium* \times leaf damage. Covariates in the pre-treatment model were: number of partially expanded leaves and number of fully expanded leaves. Covariates in the post-treatment model were: change in the number of partially expanded leaves and change in the number of fully expanded leaves, over 1 week. The dependent variables were: the number of EFNs per plant (pre-treatment model) or change in number of EFNs over 1 week (post-treatment model).

To more accurately quantify the effects of rhizobia on EFN induction we conducted two multiple regression analyses exclusively on the *Rhizobium* treatment group (*i.e.*, there were almost no root nodules on plants in the “no *Rhizobium*” group). In the first regression, the independent variables were: leaf damage (no vs. yes), change in the number of partially expanded leaves over 1 week, change in the number of fully expanded leaves over 1 week, and total number of root nodules. In the second regression, total weight of root nodules was incorporated as a variable, instead of root nodule number. For both multiple regressions the dependent variable was the change in number of EFNs over 1 week.

To better understand the effects of rhizobia on overall plant growth, we conducted 2, two-way ANOVAs. Inde-

pendent variables in the analyses were: *Rhizobium* (no vs. yes), leaf damage (no vs. yes), and the first-order interaction *Rhizobium* × leaf damage. Dependent variables were: shoot dry weight, and root dry weight (minus nodules).

All data was analyzed using JMP 5.1 [25]. Post-hoc treatment effects were determined with Tukey’s Hon-estly Significant Difference (HSD) test.

3. RESULTS

Prior to leaf damage, there was no difference in the number of EFNs on inoculated vs. uninoculated plants. There was also no effect of leaf damage, or any interaction between our main variables, showing that we did not have an *a priori* differences in our treatment groups, prior to leaf excision treatments (Table 1). There were however, positive relationships between numbers of partially expanded leaves and fully expanded leaves with EFN numbers (Table 1). As EFNs are located on the leaf stipules, a high correlation between leaf pairs and EFN numbers is not unexpected.

Post-leaf damage, colonization with mutualistic nitrogen-fixing bacteria did alter EFN numbers in broad bean plants. Overall, inoculated plants did not produce more EFNs over 1 week compared to uninoculated plants (Table 1). Plants also did not produce more EFNs in response to leaf damage (Table 1). There was, however, a significant interaction between *Rhizobium* and leaf damage (Table 1; Figure 1). Plants without nitrogen-fixing bacteria produced approximately the same number of EFNs whether or not they suffered leaf damage. Contrary to our hypothesis, however, plants with *Rhizobium* produced fewer EFNs after experiencing leaf damage, compared to inoculated plants without leaf damage (Figure 1). Like in pre-treatment plants, there was a significant relationship between numbers of partially

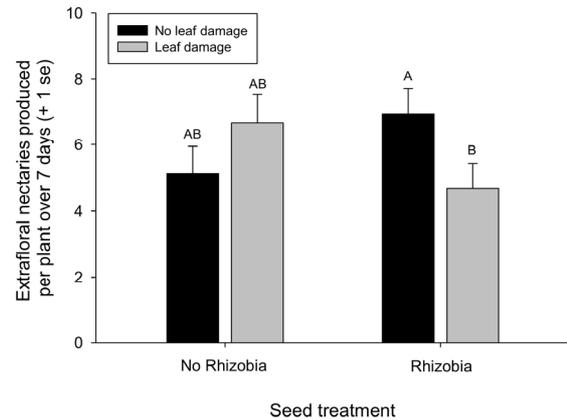


Figure 1. Extrafloral nectaries produced by inoculated vs. uninoculated broad bean, *Vicia faba*, plants over 7 days when given mechanical leaf damage. Rhizobia × leaf damage interaction; $F_{1,41} = 5.41$, $P = 0.025$. Data are presented as Least Squared Means. Columns with different letters are significantly different; Tukey’s HSD ($P < 0.05$).

expanded leaves and EFN numbers. Unlike the pre-treatment analysis, however, the relationship dissipated between numbers of fully expanded leaves and EFN numbers (Table 1).

Looking more closely at the *Rhizobium* plants, there was a trend towards the total number of root nodules, but not total weight of nodules, influencing EFN production. In the first regression analysis, total number of root nodules was marginally non-significant ($F_{1,21} = 3.31$, $P = 0.083$; Figure 2) in being inversely related to EFN production after factoring out the effects of leaf damage ($F_{1,21} = 6.47$, $P = 0.019$), change in partially expanded leaf pairs ($F_{1,21} = 1.99$, $P = 0.17$), and fully expanded leaf pairs ($F_{1,21} = 0.31$, $P = 0.59$). The second regression analysis showed that the total weight of root nodules was not significantly related to EFN production ($F_{1,21} = 1.94$, $P = 0.18$) even when accounting for leaf damage ($F_{1,21} =$

Table 1. Effects of Rhizobia and (or) leaf damage on the numbers of extrafloral nectaries, in broad bean, before and after leaf excision.

Variable	Pre-treatment		Post-treatment	
	F	P	F	P
Rhizobia (df = 1, 41)	0.010	0.92	0.013	0.91
Leaf damage (df = 1, 41)	0.30	0.59	0.201	0.66
Rhizobia × Leaf damage (df = 1, 41)	1.56	0.22	5.60	0.023
*Partially developed leaf pairs (df = 1, 41)	8.03	0.0071	7.08	0.011
*Fully developed leaf pairs (df = 1, 41)	7.96	0.0073	2.02	0.16

*Post-treatment analyses used the “change in partially developed leaf pairs” over 7 days, following leaf damage treatments (see text for further details).

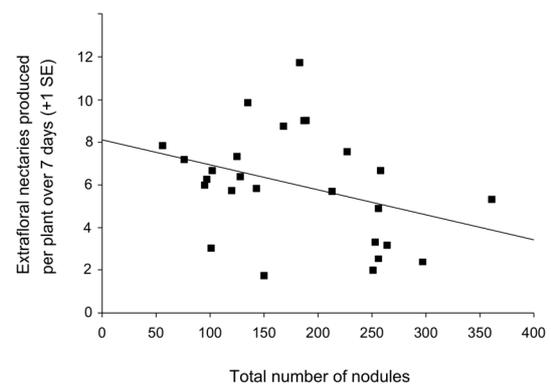


Figure 2. The effect of *Rhizobium leguminosarum* cv. *viciae* colonization on EFN production in broad bean plants. There was a trend towards reduced EFN induction responses as root colonization rates increased ($t_{1,21} = -1.82$, $P = 0.083$).

4.87, $P = 0.039$), change in partially expanded leaf pairs ($F_{1,21} = 2.59$, $P = 0.12$), and fully expanded leaf pairs ($F_{1,21} = 0.51$, $P = 0.48$).

In regards to overall plant growth, dry shoot biomass was not significantly altered by rhizobia colonization (mean \pm 1se; no rhizobia, 1.13 ± 0.04 ; rhizobia, 1.13 ± 0.04 ; $F_{1,44} = 0.0060$, $P = 0.94$). There were lower shoot weights in plants that experienced leaf damage (mean \pm 1se; no damage, $1.19\text{g} \pm 0.04$; damage, $1.07\text{g} \pm 0.04$; $F_{1,44} = 3.98$, $P = 0.052$), as leaf tissue had been removed from these plants. There was no significant interaction between rhizobia and leaf damage on dry shoot weights ($F_{1,44} = 0.0113$, $P = 0.92$). Dry root weights were significantly lower when colonized by rhizobia (mean \pm 1se; no rhizobia, 0.57 ± 0.04 ; rhizobia, 0.47 ± 0.03 ; $F_{1,44} = 4.42$, $P = 0.041$), but there was no effect of leaf damage on shoot weights (no damage, $0.55\text{g} \pm 0.04$; damage, $0.49\text{g} \pm 0.04$; $F_{1,44} = 1.50$, $P = 0.23$), and there was no interaction between these main variables ($F_{1,44} = 0.0058$, $P = 0.94$).

4. DISCUSSION

Despite their ubiquity, nitrogen-fixing bacteria – host plant mutualisms are exceedingly specific interactions [26-29]. *Vicia faba*, for example, is one of the most discriminating of the legume hosts towards different strains of *R. leguminosarum* [5]. Adding additional complexity, symbioses are frequently nested within each other [30], thus changes in one mutualism may indirectly alter the functioning of other mutualisms. Here we showed that the presence of nitrogen-fixing bacteria directly altered the induction of EFNs in broad bean plants. The direction of this response, however, ran counter to our hypothesis and prediction; plants with rhizobia produced fewer EFNs when damaged.

Without rhizobia, plants did not produce more EFNs when damaged, compared to those that were undamaged. This might seem contrary to prior experiments, where damaged plants produce more EFNs compared to undamaged plants [17,20,22]. It must be noted, however, that EFN induction has also been shown to be resource dependent [20]. It is likely that the plants in our experiments, grown in soil with extremely low fertilizer levels (NPK 0.14:0.11:0.08), did not have the resources to produce additional EFNs when herbivory increased. Extrafloral nectary [20] and food body induction [14] are both influenced by nutrient availability.

Overall, plants with rhizobia did not produce more EFNs than did plants without nitrogen fixing bacteria. Plants experiencing leaf damage, however, actually had decreased EFN numbers compared to undamaged plants. Furthermore, there were indications that the number of EFNs decreased as root nodule numbers increased. In

the rhizobia—plant mutualism, symbiotic bacteria consume plant photosynthate in exchange for increased nitrogen availability [31,32]. A negative relationship between EFN induction and total number of root nodules indicates a possible tradeoff in resource allocation between symbiont nutrition and host plant defense [33]. As plants have only a finite photosynthetic capability, the development of rhizobial colonies may come at the expense of EFN induction. Such tradeoffs may be common between plants and their mutualistic partners, especially under relatively nutrient-poor conditions [33].

So why did damaged plants with rhizobia produce significantly fewer EFNs than undamaged plants with rhizobia? We advance several, non-mutually exclusive hypotheses to explain these enigmatic results. First, rhizobia may have contributed to alternate forms of defense, e.g., increased production of phenolics [3,4]. Upon leaf damage, it is possible that broad bean plants increase secondary compound production at the expense of other forms of defense (e.g., EFN production). Second, rhizobia may enable plants to produce additional EFNs, but reduced leaf tissue resulting from leaf damage may have prevented increased expression due to reduced photosynthetic capacities [34]. Third, as all plants were grown in a common greenhouse environment, it is possible that plants received volatile compounds from damaged conspecifics [35,36]. These compounds may have induced EFN formation in the undamaged rhizobial plants, as EFN induction is resource dependent [20]. More research needs to be conducted to determine the mechanism underlying this induction, and lack thereof.

Plants may respond very differently when inoculated with different rhizobia strains [5,6,28]. A more effective symbiosis might promote EFN induction, even under adverse circumstances (*i.e.*, when herbivory is intense). Conversely, ineffective symbioses may lead to decreased or nearly non-existent nitrogen fixation, leading to a possible parasitic relationship between the two parties [37, 38]; but see [39] for an alternate viewpoint]. For example, bacteria are not needed if nutrient rich soil is already providing the necessary components for plant growth. Under this scenario, rhizobia do not carry out nitrogen fixation, but form small, ineffective nodules [1].

While a very different mutualism, it is important to note that arbuscular mycorrhizal fungi also significantly decreased EFN numbers in *V. faba*, even when plant growth increased [40]. Unlike nitrogen-fixing bacteria, mycorrhizal fungi promote phosphorous uptake, in the absence of other phosphorous sources, leading to augmented plant growth [41,42]. As damaged broad bean plants increase EFN numbers when supplemented with a balanced fertilizer [20], it is uncertain which element promotes EFN induction.

While many questions remain to be answered about

the nitrogen-fixing bacteria—host plant mutualism, it is clear that rhizobia alter EFN induction responses in damaged broad bean, *V. faba*, plants. Thus, belowground mutualisms have the potential to alter aboveground symbioses [43,44]. Corroborating other studies, nitrogen-fixing bacteria colonizing the roots of host plants may span the continuum from mutualistic to parasitic [45-48], depending on current ecological conditions, resulting in altered trophic functioning.

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