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Ants are less attracted to the extrafloral nectar of plants with symbiotic, nitrogen-fixing rhizobia

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Abstract. Plants simultaneously maintain mutualistic relationships with different partners that are connected through the same host, but do not interact directly. One or more participating mutualists may alter their host's phenotype, resulting in a shift in the host's ecological interactions with all other mutualists involved. Understanding the functional interplay of mutualists associated with the same host remains an important challenge in biology. Here, we show belowground nitrogen-fixing rhizobia on lima bean (*Phaseolus lunatus*) alter their host plant's defensive mutualism with aboveground ants. We induced extrafloral nectar (EFN), an indirect defense acting through ant attraction. We also measured various nutritive and defensive plant traits, biomass, and counted ants on rhizobial and rhizobia-free plants. Rhizobia increased plant protein as well as cyanogenesis, a direct chemical defense against herbivores, but decreased EFN. Ants were significantly more attracted to rhizobia-free plants, and our structural equation model shows a strong link between rhizobia and reduced EFN as well as between EFN and ants: the sole path to ant recruitment. The rhizobia-mediated effects on simultaneously expressed defensive plant traits indicate rhizobia can have significant bottom-up effects on higher trophic levels. Our results show belowground symbionts play a critical and underestimated role in determining aboveground mutualistic interactions.

Key words: ant plant; cyanogenesis; direct defense; extrafloral nectar; indirect defense; lima bean; multitrophic interactions; mutualism; nitrogen fixation; plant defense; plant resource allocation; rhizobia.

Introduction

Mutualistic relationships with microbes such as rhizobia, which fix atmospheric nitrogen (N_2), relieve the nitrogen limitation many plants experience in natural ecosystems. By making this otherwise inaccessible nitrogen available to their plant host, rhizobia are widely considered ecosystem engineers and keystone species: they have a disproportionately large impact on other organisms, including both plant antagonists and mutualists (Crooks 2002). For example, nitrogen fixation in root nodules critically impacts plant fitness, significantly altering resources available for plants to grow and defend themselves against herbivores by enhancing direct chemical defenses (Dean et al. 2009, Kempel et al. 2009, Thamer et al. 2011) or altering the indirect defenses plants use to recruit mutualistic invertebrates (Ballhorn et al. 2013*b*). Despite extensive knowledge on the benefits of rhizobia on parameters related to plant growth, the impacts of rhizobia on these diverse aboveground plant–insect interactions have only recently been explored. Our aim for the current study is to understand how rhizobia alter plant extrafloral nectar (EFN, nectar not involved in pollination) that serves as a plant indirect defense by attracting mutualist predatory ants.

EFN represents one of the most widely distributed indirect defenses in the plant kingdom, including angiosperms, gymnosperms, and ferns (Weber and Keeler 2013). EFN acts as a defense by attracting carnivorous arthropods, particularly ants; one of the most dominant insect groups in terrestrial ecosystems. Ants play a crucial role as defenders by attacking or evicting invertebrate herbivores from the plant, therefore, EFN is considered an effective indirect defense (McLain 1983, Ness et al. 2009). The plant kingdom contains a morphologically diverse suite of extrafloral nectaries with varying types of EFN secretion. Obligate ant plants constitutively produce EFN along with lipid-rich food bodies to nourish defending ants (Wagner 1997), whereas facultative ant-defended plants express EFN as an inducible defense only when damaged, conserving resources until the plant is under attack (Kost and Heil 2008). Lima bean (*Phaseolus lunatus*), the experimental plant used in this study, represents a facultative ant plant which increases the production of EFN when damaged. EFN is generally comprised of sugars derived from photosynthesis and therefore has

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historically been considered a metabolically cheap defense. However, some EFN contain a subset of amino acids to supplement the ants' diet as well as specific defensive components including alkaloids and tannins to deter nectar robbers and to exclude microorganisms, thereby increasing the plant's metabolic costs in nectar production (Heil 2011). EFN secretion is also dependent on soil nutrients and available photosynthetic leaf area, further indicating that EFN production comes at a cost to the plant (Mondor et al. 2006, Li et al. 2012, Ballhorn et al. 2014). Metabolic costs of EFN would imply secretion could be limited in plants with an additional carbon sink, such as rhizobial symbiosis.

The effect of rhizobia on EFN production has not been studied before. Lima bean is a particularly interesting system to use in understanding plant defense, because this model organism expresses multiple defenses in concert with EFN (Ballhorn et al. 2008). Lima bean releases volatile organic compounds (VOCs), which indirectly defend plant tissue by attracting parasitoid wasps that oviposit in the plant's enemies (Ballhorn et al. 2008, Kost and Heil 2008). Furthermore, lima bean shows various direct chemical defenses, including cyanogenesis, the wound-induced release of hydrogen cyanide from preformed precursors, as well as polyphenol oxidases effective against herbivores and fungal pathogens (Ballhorn et al. 2010).

Evidence for defense syndromes and tradeoffs between suites of traits is well documented in lima bean (Ballhorn et al. 2013*a*), making this system particularly suitable for examining the effects of rhizobia mutualism on multiple plant traits, as well as the impacts on higher trophic levels. In this study, we experimentally manipulated rhizobial colonization to measure effects of the microbial symbionts on ant recruitment to the plant hosts' extrafloral nectar. If the carbon costs of rhizobia symbiosis and EFN conflict, we expected a rhizobia-mediated decrease in EFN production, with a cascading influence on ant attraction.

MATERIALS AND METHODS

Study species

Wild lima bean (Fabaceae: *Phaseolus lunatus* L.) plants were grown from seeds collected in Mexico (15°55′31.80″ N, 97°9′4.68″ W, 8 m above sea level). Plants were grown in containers (one plant per pot) 12 cm in diameter in greenhouse mix 3 (SunGro Horticulture, Bellevue, Washington, USA), 175 g per pot. Potting soil was washed thoroughly three times to reduce substrate nutrients. Seeds with a nicked seed coat were germinated on moist paper towels and were subsequently planted 0.5 cm below soil surface. Plants were watered daily and moved monthly to avoid microclimatic effects. Plants were potted and cultivated at Portland State University (Portland, Oregon, USA) from 7 July to 4 October 2013 on the outdoor cultivation area of the research greenhouse to allow sun exposure. Plants were not protected from ambient herbivory in order to measure plant defense responses that more closely resemble plants in natural sites.

Experimental setup

Rhizobia inoculum was prepared by grinding three nodules for a total of 0.1 g fresh mass (Accession DJB1033, Ballhorn Lab, Portland State University) suspended in 1 L water (Ballhorn et al. 2013*a*). Once seedlings had two leaves, 100 mL rhizobia inoculum ($>10^7$ cells/mL) was poured on the soil at the base of each seedling representing R+ (with rhizobia) treatment plants ($n = 33$ plants); 100 mL sterile water was poured on the soil of each seedling representing R– (without rhizobia) treatment plants ($n = 20$ plants). Two weeks after inoculation, all R+ plants and two of the R– plants showed signs of early nodulation. At the end of experiments when plants were destructively harvested, these same plants had uniformly high nodulation, so the data for these two (R–) plants were added to the R+ treatment for a final sample size of 18 R– plants and 35 R+ plants.

Plant trait analyses

We selected young, intermediate, and mature leaves for all plant biochemical analyses to account for ontogenetic variability of plant traits. Young leaves were defined as the uppermost unfolded leaf on the vine, intermediate leaves were chosen two leaves below young leaves, and mature leaves were selected two leaves below the intermediate leaves.

EFN was quantified according to Kost and Heil (2008). To induce indirect defense, jasmonic acid (JA; 1 mmol/L aqueous solution) was applied to foliage by spraying, and 24 h later, young, intermediate, and mature leaves were mechanically damaged with a toothbrush. Twelve hours after mechanical damage, EFN volume was collected with microcapillaries (PCR micropipets 1–10 μL; Drummond Scientific, Broomall, Pennsylvania, USA). Sugar concentration in EFN was quantified with a Brix 0–18% refractometer (Fisher Scientific, Waltham, Massachusetts, USA). Leaves used for EFN collection were harvested and dried (IncuMax CV250 convection oven, Amerex Instruments, Mountain View, California, USA) at 72°C for 9 d. EFN values were calculated as mg sugar/g leaf dry mass.

The cyanogenic potential (HCNp; concentration of cyanogenic precursors) of leaves of similar developmental stages (next to leaves collected for EFN) was quantified using the Spectroquant cyanide test (Merck, Darmstadt, Germany; Ballhorn et al. 2008). In short, leaves were removed, weighed to the nearest 0.001 g (NewClassic MF, Mettler-Toledo, Greifensee, Switzerland), and three leaf punches were ground with a mortar and pestle at 4°C in 2 mL ice-cold Na_2HPO_4 buffer (67 mmol/L). Samples were analyzed for HCNp by complete enzymatic hydrolysis of cyanogenic precursors in gas-tight glass vessels (Thunberg vessels). Spectropho-

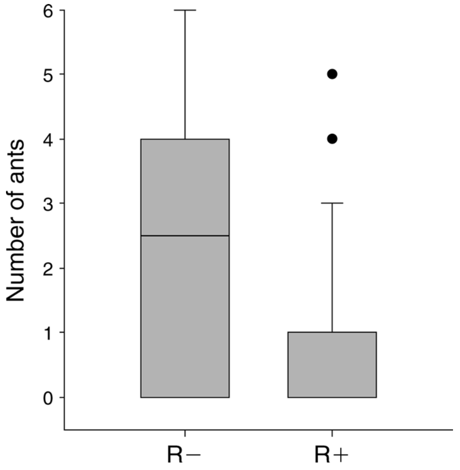

FIG. 1. Ant recruitment onto *Phaseolus lunatus* plants with and without rhizobia. Values are given as box plots of ants counted 8 h after extrafloral nectar (EFN) induction; center line indicates median values, ends of boxes indicate upper and lower quartiles. The error bars represent 90th percentile values (as the lower quartile reached zero, no 10th percentile values were possible), and the dots represent extreme values above the 90th percentile. Treatments R– and R+ represent rhizobia-free plants and rhizobia-inoculated plants, respectively.

tometric quantitative detection of HCN was carried out at 585 nm.

Total soluble protein (from the same leaf samples taken for HCNp analysis) was measured spectrophotometrically at 595 nm as a proxy for plant nutritive quality using the Bradford protein assay (Bradford 1976, Ballhorn et al. 2011). A calibration curve ranging from 50 to 1000 μg/mg was prepared with bovine serum albumin (Amresco, Solon, Ohio, USA). Total biomass was measured for above- and belowground fresh and dry mass. None of the plants flowered or set fruit over the experimental period.

Ant recruitment

To quantify ant attraction, induced and control plants were randomly distributed 1.5 m apart on a gravel area near the research greenhouse facility at Portland State University. Four dead twigs connected the soil with the foliage of each plant to facilitate exposure to naturally occurring pavement ants (*Tetramorium caespitum*), which were observed frequently throughout the gravel strip. Ants per plant were counted 8 h after EFN induction.

Statistical analyses

We used linear models (Type III sum of squares) to analyze the effects of rhizobia on plant biomass. Effects of rhizobia on leaf traits were analyzed using a split-plot linear mixed model with plant individual as random main plot factor and leaf age as subplot factor within plant individual. Number of ants was analyzed as a generalized linear model (GLM), assuming Poisson-distributed residuals with the loglink function (all analyses in SAS 9.2, Proc GLIMMIX; SAS Institute, Cary, North Carolina, USA). Tukey's HSD test was used for post hoc comparisons of means.

To examine the strength of indirect and direct interactions between rhizobia, plant traits, and ants, structural equation modeling (SEM) was used (SPSS Amos 22; IBM, Armonk, New York, USA), which allowed us to make conclusions about the relationship between all measured variables in a single analysis (Grace 2006). Rhizobia treatment was used as a binary exogenous variable; aboveground plant traits and number of ants were used as observed endogenous variables. For our initial conceptual model, we assumed that rhizobia affected all aboveground plant traits but had no direct effect on ants. We assumed possible effects of all leaf traits on ant abundance since not only extrafloral nectar but also leaf protein and HCNp could be suggested to influence ant attraction. However, we did not assume an effect of shoot biomass on ant abundance (all plants grew in pots of the same size, therefore larger plants did not represent larger targets). We further assumed possible covariation between all aboveground plant traits. Adequate model fits were confirmed by nonsignificant χ^2 tests ($P > 0.05$) and low root mean square error of approximation (<0.05). Stepwise removal of unimportant relationships was used to optimize the model based on these model fit indices (see Appendix B for the initial conceptual model and the model selection procedure). Data met the assumptions of SEM and did not deviate from normality and included no statistical outliers (SPSS Amos 22). Bivariate relationships between response variables are given in Appendix D.

RESULTS

Rhizobia-inoculated plants recruited fewer ants. Three times more ants were found on rhizobia-free plants (R–) as compared to plants with rhizobia symbiosis (R+; Fig. 1, GLM: $F_{1,51} = 19.10$, $P < 0.001$). We deciphered the net effect on ant recruitment in a SEM explaining 45% of the variation in ant attraction. The sole significant path to ant abundance was mediated through EFN, which was ultimately determined by rhizobia treatment (Fig. 2). Rhizobia directly reduced EFN with a path coefficient of –0.66, and this final model explained 44% of the variation in EFN. EFN did not significantly interact with any other plant trait. The direct link between rhizobia and EFN was not mediated by changes in biomass. Shoot biomass had a significant positive interaction with leaf protein and cyanogenic potential (HCNp). Rhizobia had strong direct positive effects on shoot biomass, protein, and HCNp, with path coefficients of 0.74, 0.62, and 0.60, respectively (Fig. 2). Protein and HCNp did not affect ant recruitment, as the standardized estimates were not significant ($P < 0.12$).

Analysis of variance results for individual plant traits confirmed the same relationships described in the SEM

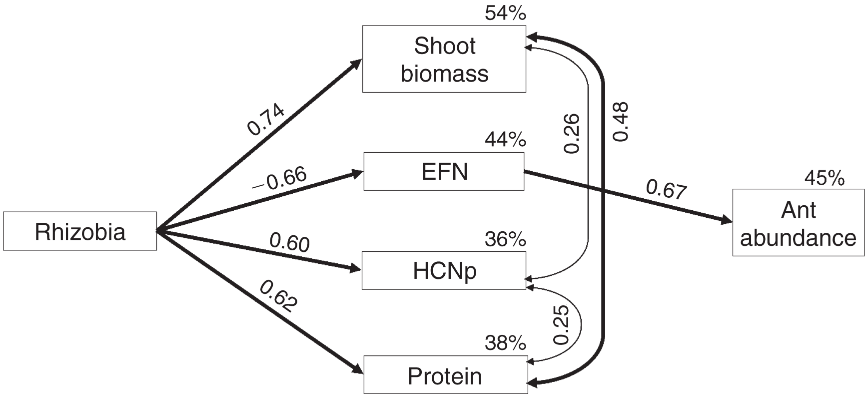

FIG. 2. Final reduced structural equation model for the relationship between rhizobia, aboveground *P. lunatus* plant traits (including cyanogenic potential, HCNp, and plant nutritive quality, expressed as total soluble protein), and ant abundance. Thin lines indicate marginally significant ($P < 0.1$) and thick lines indicate significant ($P < 0.05$) relationships. Numbers on arrows are standardized path coefficients. Percentages above endogenous variables indicate the variance explained by the model (R^2). The data did not significantly deviate from the model (see Appendix C for unstandardized estimates and model fit indices).

for the entire plant system with rhizobia treatments. Rhizobia symbiosis significantly affected all measured plant traits. R+ plants produced more aboveground and belowground biomass (Appendix A, all $P < 0.001$). R− plants had a shoot biomass of 2.29 ± 0.23 g (mean ± SE, dry mass), whereas shoot biomass increased more than threefold in R+ plants to 9.77 ± 0.70 g. Root biomass increased more than twofold in R+ plants (3.4 ± 0.33 g) relative to R− plants (1.45 ± 0.11 g). Rhizobia significantly increased leaf protein concentration (Fig. 3A, $F_{1,51} = 31.16$, $P < 0.001$), with the greatest difference between rhizobia treatments being in young leaves ($F_{2,102} = 24.96$, $P < 0.001$), but the interacting effects of rhizobia treatment and leaf age were not significant ($F_{2,102} = 2.15$, $P = 0.12$). HCNp followed a similar pattern to that of protein concentration; rhizobia increased cyanogenesis (Fig. 3B, $F_{1,51} = 29.13$, $P < 0.001$), and this chemical defense decreased as leaves aged ($F_{2,102} = 170.04$, $P < 0.001$). However, unlike protein content, HCNp was affected by the interacting effects of rhizobia and leaf age, with the younger, more productive leaf tissues more highly defended ($F_{2,102} = 8.73$, $P < 0.001$).

EFN secretion was highest in young leaves of R− plants (Fig. 3C). Rhizobia reduced EFN ($F_{1,51} = 39.43$, $P < 0.001$), and increasing leaf age lowered EFN ($F_{2,102} = 26.01$, $P < 0.001$). The interacting effects of rhizobia and leaf age were significant ($F_{2,102} = 16.21$, $P < 0.001$); EFN values were almost four times higher for R− young leaves compared to young leaves on R+ plants (Fig. 3C). In summary, young, actively growing leaves on plants with rhizobia produce less EFN than young leaves on plants without nitrogen-fixing symbionts, mediating effects on the aboveground ant–plant symbiosis.

DISCUSSION

Rhizobia are recognized keystone species, yet the impacts rhizobia have on aboveground interspecific interactions, particularly on mutualistic relationships, are still gradually being revealed. In this case, the rhizobia–legume mutualism and the ant–plant mutualism are in conflict in the same host-plant system. Both of these mutualisms are well studied and often serve as a model for understanding the evolutionary implications of mutualistic relationships (Kiers et al. 2003, Mayer et al. 2014). Yet, surprisingly, the effects of both mutualisms interacting with the same plant host were previously not studied. Multiple mutualism effects are known to play a role in the net outcome of a system including all partners interacting with the focal mutualist or host (Stanton 2003, Afkhami et al. 2014), although empirical data demonstrating these complex multispecies interactions are lacking. This study adds the novel finding that plants with rhizobial symbiosis secrete less EFN and recruit fewer ants.

Conflict among mutualisms within the same plant host are not surprising, given maintaining partner cooperation can be costly (Bronstein 2001, Werner et al. 2014). Any net outcome resulting in reduced fitness for the host plant can be considered a cost, including ecological costs. The most well-known ecological cost in ant–plant mutualisms is the potential to deter beneficial pollinators. A recent study shows bees pollinated fewer plants with ant decoys relative to plants with circular controls, indicating bees can perceive ant presence as a dangerous threat (Assunção et al. 2014). This conflict may depend on how well ants defend their plant host, usually with more aggressive behavior. In some cases, only the more aggressive ants have a negative impact on the plant–pollinator mutualism, posing a greater ecological cost with increasing mutualistic quality of ant partners (Ness 2006).

Rewarding mutualistic partners is intrinsically costly, as the relationship is generally established based on sharing resources. Extrafloral nectaries require large amounts of carbon when EFN production is induced,

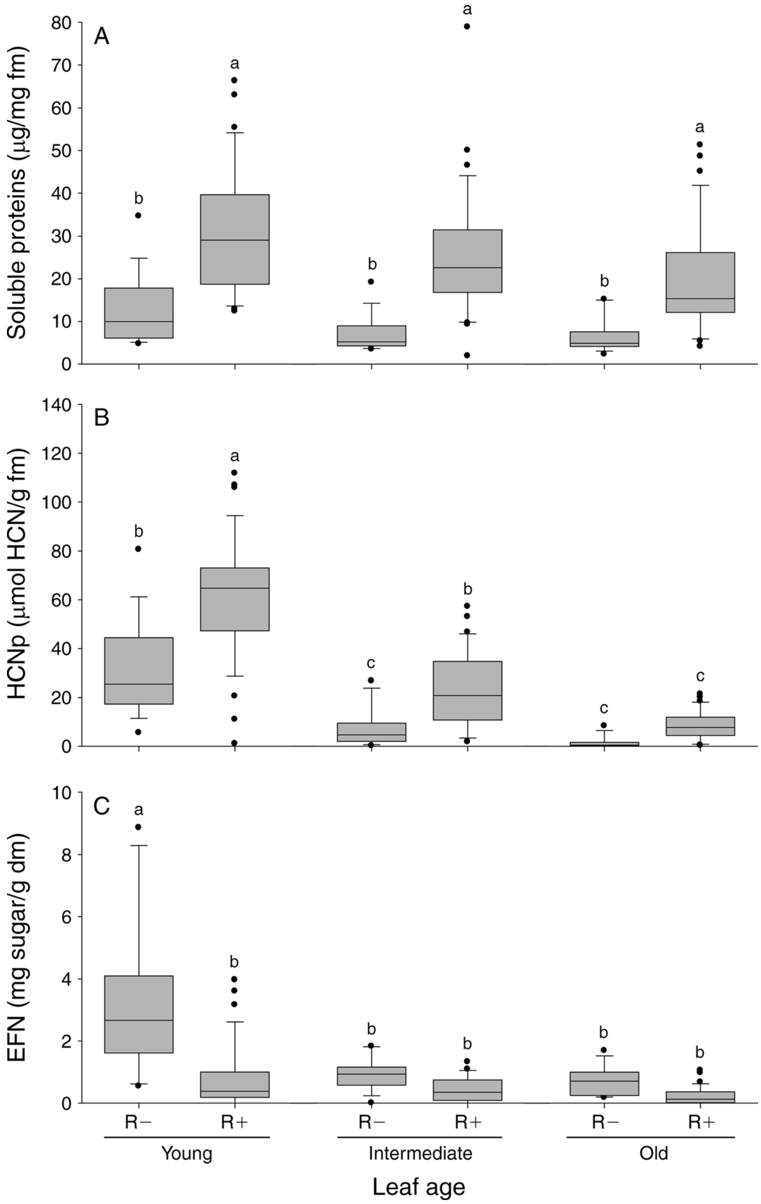

FIG. 3. *P. lunatus* plant traits with and without rhizobia for young, intermediate, and mature leaves. Rhizobia presence/absence is indicated by R+/R−. Different lowercase letters indicate significant differences following Tukey's post hoc tests ($P < 0.05$) from a split-plot linear mixed model with plant individual as random main plot factor and leaf age as subplot factor within plant individual. Values are presented as boxplots; center line indicates median values, ends of boxes indicate upper and lower quartiles. The error bars represent 10th and 90th percentile values, and the dots represent extreme values below or above the 10th and 90th percentiles. (A) Soluble protein concentration, measured in µg protein/mg leaf fresh mass (fm). (B) HCNp, measured in µmol/L hydrogen cyanide/g leaf fresh mass. (C) EFN secretion, measured in mg sugar/g leaf dry mass (dm).

but the question of how costly EFN production actually is to the plant remains unanswered. In general, inducible defenses are considered to have evolved as a mechanism to reduce the cost of defense by conserving resources until the plant is under attack. The inducible nature of EFN, as well as the cellular machinery involved in inducing EFN via jasmonic acid through the octadecanoid pathway, indicates that there is a cost to the plant in producing nectar for secretion (Mueller et al. 1993). EFN secretion is dependent on photosynthesis, as light regulates jasmonate signaling in the induction of EFN (Radhika et al. 2010), and photosynthetic area removal by caterpillar (Li et al. 2012) or by quantitative manipulation (Ballhorn et al. 2014) reduces the plant's capacity to produce EFN. Other constituents of EFN (e.g., antimicrobials, amino acids) are synthesized and lost along with the photosynthate investment once EFN is secreted (Heil 2011). There are no known mechanisms by which plants can reabsorb unconsumed EFN. This is one cost often overlooked in indirect defense investment: the external release of resources without recovery, versus the ability to catabolize and reallocate direct defense compounds. Our data contribute to this growing body of evidence showing EFN may not be as cheap to produce as was previously thought (O'Dowd 1979, Rudgers and Gardener 2004): plants with an additional carbon sink (rhizobia) produced less EFN.

The mechanisms leading to altered plant traits with rhizobia are reasonably driven by the symbiotic exchange of carbon-based photosynthates for organic forms of nitrogen. The benefits of nitrogen for plant nutrition are well understood (Brockwell et al. 1995), and our study reemphasizes that plant tissues contain more protein when plants are associated with rhizobia. Rhizobia also increase plant defensive traits involving large amounts of nitrogen, including cyanogenesis as observed in our study, which is in line with previous work (Thamer et al. 2011). The cost of maintaining nitrogen-fixing rhizobia nodules may also significantly alter plant physiology, as Peoples et al. (1986) reported about 20–30% of plant photosynthates are allocated to the microbial partner. Thus, high quantities of photosynthates are required to maintain rhizobial mutualism, and in environments that limit photosynthesis, the plant's obligation to rhizobia can lead to carbon starvation in other plant tissues (Tsikou et al. 2013). This same phenomenon occurs with arbuscular mycorrhizal fungi, another group of microbial symbionts requiring photosynthates from plant hosts, which reduced plant EFN production in a previous study (Laird and Addicott 2007). Therefore, a similar resource allocation constraint between two carbon sinks, rhizobia and EFN (Kiers et al. 2003, Mondor et al. 2006), may have driven the decrease in EFN in our study.

The observed decrease in EFN on plants with rhizobia is especially interesting given the simultaneous increase in aboveground biomass and protein content; traits indicating higher photosynthetic ability and carbon availability. However, biomass did not significantly interact with EFN in the structural equation models (Fig. 2), whereas biomass did directly link to both protein and cyanogenesis. An alternative interpretation of the reduced EFN on plants with rhizobia might be an interaction among plant traits. Cyanogenesis as a direct defense is innately more reliable than indirect defenses, which depend on insect behavior (Ballhorn et al. 2008). Rhizobia may provide enough nitrogen to enable plants to defend themselves via cyanogenesis, whereas nitrogen availability is limited for plants without rhizobia (Thamer et al. 2011), resulting in the observed low cyanogenic potential. In this case, plants may boost their defense system by secreting EFN. Loss of photosynthates to nectaries would not seem to offer additional benefit to a highly cyanogenic plant. The mechanisms regulating EFN production remain widely elusive (Heil 2011), and insight in this area would augment plant defense theory.

Plant defense vs. growth has been a study of interest without universal resolve for many years (Herms and Mattson 1992, Stamp 2003, Agrawal and Fishbein 2006, Heath et al. 2014), and several hypotheses seek to explain constraints leading to the observed patterns of plant defense (reviewed in Stamp 2003). Aligning these hypotheses into one congruent theory of defense may not be possible without incorporating all biotic influences, including mutualistic interactions (Heath et al. 2014). Our data suggest belowground symbiotic rhizobia critically impacted lima bean defense strategy, which consequentially affected ant recruitment. The implications of reduced ant attraction to plants with rhizobia have not yet been considered in empirical research. Ants drive many ecological processes, including nutrient cycling via effective foraging techniques (Verchot et al. 2003), and mutualistic interactions such as aphid farming (Schowalter 1981). As both players, rhizobia and ants, occur almost ubiquitously in terrestrial habitats, the effects of rhizobia on plant traits affecting ant behavior likely have a far-reaching impact. Thus, interactions among plant mutualists may have strong and widely overlooked impacts on plant ecology.

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SUPPLEMENTAL MATERIAL

Ecological Archives

Appendices A–D are available online: http://dx.doi.org/10.1890/14-1178.1.sm