



Nitrogen-Fixing Rhizobia Affect Multitrophic Interactions in the Field

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Abstract Virtually all plants employ direct and indirect defenses against herbivores. While it is known that plant defenses can be affected by below-ground symbiotic microbes under controlled conditions, studies showing these multitrophic interactions in nature are surprisingly scarce. Here we tested for effects of rhizobia on insect attraction and direct defense (cyanogenesis) in wild lima bean (*Phaseolus lunatus*) plants in Costa Rica. We performed bioassays with rhizobia-inoculated (R+) and rhizobia-free (R-) potted plants distributed among native lima bean communities at two spatially separated field sites (450 km apart) and in two field seasons. Without affecting overall plant size, rhizobia altered leaf chemistry (cyanogenesis and soluble leaf nitrogen) and ultimately insect communities visiting the plants. Natural herbivorous chrysomelid beetles were strongly attracted to R+ plants, while natural enemies, ants and parasitoid wasps, preferred R- plants resulting in a particularly high herbivore:carnivore ratio on R+ plants. This suggests that symbiotic microbes mediate trophic interactions by influencing

both direct and indirect plant defenses against herbivores. Our results show that rhizobia affect the plant defensive phenotype and have cascading effects on plant-insect interactions in nature.

Keywords Parasitoid wasp · volatile organic compounds (VOCs) · rhizobia · symbiosis · tritrophic interactions · *Phaseolus lunatus*

Introduction

Plant-associated rhizobial symbionts have been shown to not only fix atmospheric nitrogen supporting their host's primary metabolism but also to affect a wide variety of indirect and direct plant defenses (Summers and Mondor 2011; (Ballhorn et al. 2013c; Dean et al. 2014; Godschalx et al. 2015). Indirect plant defenses include extrafloral nectar (EFN) and the release of air-borne signaling molecules (VOCs; volatile organic compounds) that attract predators and parasitoids of herbivorous invertebrates (Turlings et al. 1990; Thaler 1999; Turlings and Wäckers 2004). Recruiting ants and parasitoid wasps typically reduces herbivore pressure for the plant either through ant aggression and predation (Kost and Heil 2008) or by killing the herbivore host in the course of internal parasitoid larva development (Turlings et al. 1990, 1993). By reducing plant consumer numbers, ants and parasitoid wasps contribute to structuring the herbivore community (Fowler and Macgarvin

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1985). Ants can alter feeding guild structure of aboveground herbivores, and parasitoid abundance and diversity can shape arthropod communities through exerting unique top-down effects on lower trophic levels (Letourneau et al. 2009). This dynamic is particularly influenced by host-specific parasitoids (Murdoch et al. 1985; Dehling et al. 2021), which recognize specific plant chemical signals that serve as olfactory cues and influence foraging selection patterns (Turlings et al. 1993; De Moraes et al. 1998). Indeed, plants that can take advantage of the signal-specific responses of wasps to parasitize their herbivores receive higher fitness benefits (Hoballah and Turlings 2001). Attracting and retaining ants and parasitoids can depend on the plants' quantitative and/or qualitative secretion of EFN and herbivore-induced release of VOCs (Thaler 1999; Röse et al. 2006). However, both EFN secretion and the emission of plant VOCs can be affected by plant-associated microbes such as rhizobia (Ballhorn et al. 2013c; Godschalx et al. 2015). Nitrogen-fixing rhizobia in root nodules consume photosynthates from the plant in exchange for the provision of fixed atmospheric nitrogen. Plant chemical composition, including nectar and VOC chemistry, shifts due to this exchange of resources between the plant and rhizobia provisioning nitrogen while serving as a strong carbon sink (Kaschuk et al. 2009; Pringle 2016). As a result, almost all VOCs, including compounds with known roles in plant defense such as methyl jasmonate and β -caryophyllene (Thaler 1999; Rasmann et al. 2005), are released in significantly lower amounts by rhizobia-inoculated plants relative to plants without the symbiosis (Ballhorn et al. 2013c). In contrast, specific compounds synthesized from products in the shikimate pathway, including salicylic acid and indole—a nitrogen-containing VOC, increased in plants with nitrogen-fixers (Ballhorn et al. 2013c). In controlled laboratory experiments, plant growth promoting rhizobacteria (PGPR) influenced olfactometer decisions of parasitoid wasps with insect hosts belonging to different herbivore guilds (Pineda et al. 2013; Pangesti et al. 2015b).

In addition to indirect defenses, root-associated microbes can have significant effects on chemical direct defenses. In a study involving lima bean, rhizobia, and herbivorous beetles (Mexican bean beetles, *Epilachna varivestis*), Thamer and co-workers (2011) showed significant effects of rhizobia on

the expression of cyanogenesis in leaves. The concentration of cyanogenic precursors (HCNp; cyanogenic potential) in leaves of rhizobial lima bean plants was elevated by a factor of 10 compared to rhizobia-free controls. Corresponding to elevated levels of cyanogenesis, leaf area consumption by Mexican bean beetles was significantly reduced, indicating strong effects of rhizobia on direct chemical defense in lima bean. In fact, rhizobial colonization had a quantitative effect on chemical defenses as the number of nodules correlated with increased cyanogenic potential (Godschalx et al. 2017).

While there is increasing information available on the effects of rhizobia in laboratory or greenhouse trials (Turlings and Wäckers 2004), evidence for the effect of rhizobia on multitrophic interactions in nature is scarce. To assess the relevance of microbially-mediated plant traits on multi-trophic interactions in nature, in the present study we determined whether herbivorous beetles, ants, and parasitoids in natural communities respond differently to plants with or without rhizobia. Building on our findings from previous laboratory experiments that predatory ants and herbivores interact with plants differently because of rhizobia-mediated chemistry, we hypothesized that maintaining symbiosis with rhizobia would also influence the plant's interactions with native populations of insects. We chose to quantify chrysomelid beetle, ant, and parasitoid wasp recruitment as these plant-insect interactions were the most frequent according to our observations at the Costa Rican field sites. We established experimental plant treatments with two levels of symbiosis, [rhizobia present: R+; rhizobia absent: R- (with or without nitrate-supplemented, depending on site), distributed these plants randomly among wild lima bean communities in the field, and counted arthropods by group repeatedly in the morning and evening in two separate field surveys. As most studies on predator responses to plant traits take place in controlled laboratory conditions (Turlings and Wäckers 2004), this study provides new insight into the natural arthropod responses faced with the effects of interacting biotic and abiotic factors inherent in natural field conditions.

Specifically, here we test whether the influence of rhizobia-mediated plant traits creates significant differences in arthropod responses to plants under field conditions. Given that rhizobia act as suppliers of plant available nitrogen (resulting in elevated

nitrogen-based direct defense such as cyanogenesis) and a strong carbon sink (reducing indirect defenses such as the secretion of EFN and – potentially - emission of C-based VOCs) we expected differential rhizobia-mediated effects on plant consumers, predators and parasitoids in nature.

Methods

Two separate field experiments manipulating rhizobia and quantifying associated insect communities were conducted in 2015 (Experiment 1) and 2018 (Experiment 2) in Costa Rica. Experiment 1 took place at the Estación Experimental Forestal Horizontes in the dry tropical forest Area de Conservación Guanacaste, Costa Rica (10.71171931, -85.57671547). Wild lima bean (Fabaceae: *Phaseolus lunatus* L.) plants were cultivated from seeds and allowed to germinate on a moist paper towel until developing a 5 mm radicle, before being transplanted in 10.4 cm square pots filled with previously sterilized local soil. For a period of 30 days, plants were watered with sterilized water to maintain rhizobia-free status before inoculation and cultivated on a covered outdoor patio with direct morning sunlight and rotated every week to exclude position effects. Experiment 2 was conducted in the vicinity of Puerto Jiménez, Puntarenas, Costa Rica (8.516433, -83.289941). Plants for this experiment were cultivated from seeds collected in 2017 from the local lima bean population. Seeds were germinated as described above in sterile Petri dishes on sterile moist filter paper. Plants were allowed to grow for 30 days to the same developmental stage as in Experiment 1 (with 4–8 secondary leaves). At this site we used natural farm soil that was sterilized using a benchtop autoclave (Benchmark BioClave™ 16 Research Autoclave, Benchmark Scientific, MA, USA).

Experimental Setup While plants are generally not rhizobia free in nature, there is strong variation in extent of colonization ranging from few small nodules to hundreds of nodules per plant, so our experimental manipulation was designed to cover this range of variation. In Experiment 1, plants were either inoculated with natural rhizobia (R+, $n=10$ plants) or maintained rhizobia-free (R-, $n=7$) and fertilized daily during the cultivation period with a $0.25 \text{ mmol} \cdot \text{L}^{-1} \text{ CaNO}_3$ solution made with sterilized

water, a concentration that adequately supplements plants for comparisons against those with nitrogen-fixing rhizobia (Kiers et al. 2006). R+ plants were inoculated with 50 mL of a water suspension of 15 homogenized nodules collected from extensively colonized wild lima bean roots at the Guanacaste field site. Nodule suspensions represent the identity and diversity of rhizobia at the natural site (Dove et al. 2020). At inoculation, lima bean seedlings had fully developed primary leaves (1 week after planting). Three weeks after inoculation, when plants developed three true leaves, potted plants were moved from the cultivation site to the field site which contained a local population of *P. lunatus* and distributed in clusters of two or three plants per treatment separated 30 cm apart within a $1 \times 2 \text{ m}$ plot among the underbrush along a roadside with direct morning sunlight that matched cultivation light conditions. The site was selected adjacent to a local lima bean population with plants at a similar developmental stage. To allow for acclimation, plants were watered daily for an additional week before collecting parasitoid survey data.

In Experiment 2, rhizobia-free plants (R-, $n=20$) were maintained without any additional nitrate due to the high nitrogen content of the local soil (average of $68 \text{ kg NO}_3 \text{ ha}^{-1}$). Similar to Experiment 1, rhizobia-inoculated plants (R+, $n=20$) were prepared by collecting nodules from local lima bean plants at the field site, grinding ~15 nodules and suspending in water before applying 50 mL per plant. Three weeks after inoculation, when plants developed eight true leaves, plants were moved to a nearby lima bean population consisting of >50 plants and distributed 50 cm apart within a $5 \times 10 \text{ m}$ plot.

Field Survey Experiment 1 was designed to quantify parasitoid recruitment to plants with and without rhizobia. Parasitoids per plant were counted during 30-minute sampling periods for four consecutive days in the morning and evening (7:30am and 4:30pm) to capture daily patterns in parasitoid activity. Observation periods were determined based on personal observations of the peak activity patterns of insects. Surveying position was rotated periodically for even visibility of all plants and all leaf surfaces. Wasps were only counted after landing on the plant surface, and approaches without contact were not included in the dataset. Individual wasps that landed twice on the same plant were only counted

separately if the wasp landed on a different plant in between landings. To document wasp identity, as many wasps as possible were photographed using an iPhone 6 with a Macro lens attachment (Photojojo, San Francisco, CA). Parasitoid wasps were identified by A. Diethelm. Specimens were identified from photographs using wing venation patterns, shape of body, coloration, and shape of the antennae along with number of flagella (Goulet and Huber 1993). Wasps were identified to family level where possible following the protocol outlined by Hanson and Gauld (1995) to classify the 22 morphologically distinct parasitoid wasps recruited to experimental plants in the 2015 field season.

Experiment 2 was designed to broaden the scope of the study by including natural herbivores and ant predators. At the Puerto Jiménez location, Banded Cucumber beetles (Chrysomelidae: *Diabrotica balteata*) were the most abundant insect herbivores. Banded Cucumber beetles, which occur in the Americas from the United States south to Colombia, Venezuela, and Cuba, are highly polyphagous herbivores utilizing host plants from several different families. Primary host plants of Banded Cucumber beetles include Cucurbitaceae crops such as cucumber, cantaloupe, squash, gourd, and pumpkin, as well as Fabaceae, including common bean (*Phaseolus vulgaris*) and lima bean (*P. lunatus*). Ants of the *Crematogaster crinosa* complex represented the most common predatory insect observed on lima bean. Beetles and ants were counted on each plant every morning (7:00 am) and evening (5:00 pm) for ten consecutive days.

Plant Trait Analyses Following the arthropod surveys in Experiment 1 and 2, plants were destructively harvested to quantify nodule number. Nodules were manually counted by gently sifting through soil and rinsing the root system while counting on a handheld counter. All nodules were included in the assessment, regardless of size and without any additional assessment of nodule activity. Abscised nodules that were deflated, perforated or mushy were not included in the final count. In Experiment 2, additional traits were measured to compare qualitative (R+/R-) and quantitative (nodule number) effects of rhizobia colonization on plant traits and insect communities. Above and belowground dry weight was determined by drying

the plant material in a field station oven (65 °C) for three days and weighing to the nearest 0.001 g (NewClassic MF, Mettler Toledo). Subsamples of each plant (1 young, fully unfolded leaf per plant) were used for quantification of leaf soluble protein content and cyanogenic potential (HCNp). These samples were dried in a desiccator on silica gel until constancy of weight before being vacuum sealed and analyzed in the Ballhorn lab at Portland State University.

Cyanogenic Potential (HCNp) Leaf HCNp was analyzed by complete enzymatic degradation of cyanogenic glycosides, and HCN released from cyanogenic precursors was spectrophotometrically quantified (at 585 nm) using the Spectroquant cyanide test (Merck, Darmstadt, Germany) following the method of Ballhorn et al. (2005). To release all cyanide contained within a leaf, supplemental β -glucosidase was added in excess (100 μ L, 20 nkat) to replenish internal β -glucosidase potentially degraded during the drying process and thus to ensure complete breakdown of cyanogenic precursors present in the leaf tissue. β -glucosidases were isolated from lima bean plants grown from seeds collected at the Puerto Jimenez site one year before the field survey (in 2017) and cultivated in the Portland State University research greenhouse. The isolation method for β -glucosidase followed Ballhorn et al. (2006). In short, to obtain β -glucosidase for quantification of HCNp, whole, fresh young lima bean leaves were weighed and homogenized in a 4-fold volume of 67 mmol L⁻¹ phosphate buffer adjusted to pH 6.4. The extract was filtered through cotton fabric and centrifuged at 20 000 g and 4 °C (RC5C, Sorvall). The protein-containing supernatant was concentrated by ammonium sulfate fractionation and filtered through membrane caps with a pore size of 10 000 kDa (Schleicher & Schuell BioScience GmbH, Dassel, Germany). The enzyme solution was tested for contamination with cyanide from leaf tissue prior use. No detectable levels of cyanide were found in β -glucosidase enzyme solution prepared from lima bean leaves.

Soluble protein Concentration in lima bean leaves was quantified according to Bradford (1976). Leaf material was homogenized in ice-cold sodium acetate buffer (pH 5.0). Leaf extracts were centrifuged at 13,000 g in a cooled (4 °C) centrifuge (Eppendorf

5810 R) and the supernatant was filtered over NAPTM columns containing SephadexTM G-25 DNA-Grade (GE Healthcare, Munich, Germany). Subsequently, 5 μL of the eluate were pipetted on microplates (96-well Microplates, F-bottom; Greiner Bio-One, Frickenhausen, Germany), and 250 μL of.

Bradford reagent (diluted with deionized water in the ratio 1 : 4) were added. The protein concentration of samples was spectrophotometrically quantified at 595 nm. Bovine serum albumin solutions (Merck) in the range between 10 and 600 mg mL^{-1} served as standard.

Statistical Analyses Plant traits were normally-distributed and analyzed with an ANOVA. Insect count data were analyzed as mean insects per plant per day/time per rhizobia treatment using a generalized linear model (GLM) with poisson-distributed residuals and Tukey's post hoc tests using the R package, *multcomp*. We further used a generalized linear mixed model fit by maximum likelihood (Laplace Approximation ['glmermod'] in the R package, *glmer*. For

these models, AIC values were compared between models holding time of day, rhizobia treatment, and insect type (ants or beetles) as fixed or interacting factors, with plant ID as random factor to account for the repeated sampling regime. Nodule number effects on plant traits or insect means were tested with a linear model or glm with poisson-distributed residuals. All statistical tests and data visualizations were performed in R Studio (R Core Team 2018), using packages *plyr*, *reshape*, and *ggplot2*. Datasets are deposited in Github (<https://github.com/agodschalx/MSdata2023>).

Results

Effects of Rhizobia on Plant Traits Leaf chemistry was significantly altered by rhizobia treatments in experiment 2. Soluble leaf protein increased by approximately 20% and cyanogenic potential (HCNp) by 30% in rhizobia-inoculated plants (R+; Fig. 1; ANOVA protein $F_{1,38}=152.4$, $p<0.001^{***}$; HCNp $F_{1,38}=63.27$, $p<0.001^{***}$), despite no significant differences in

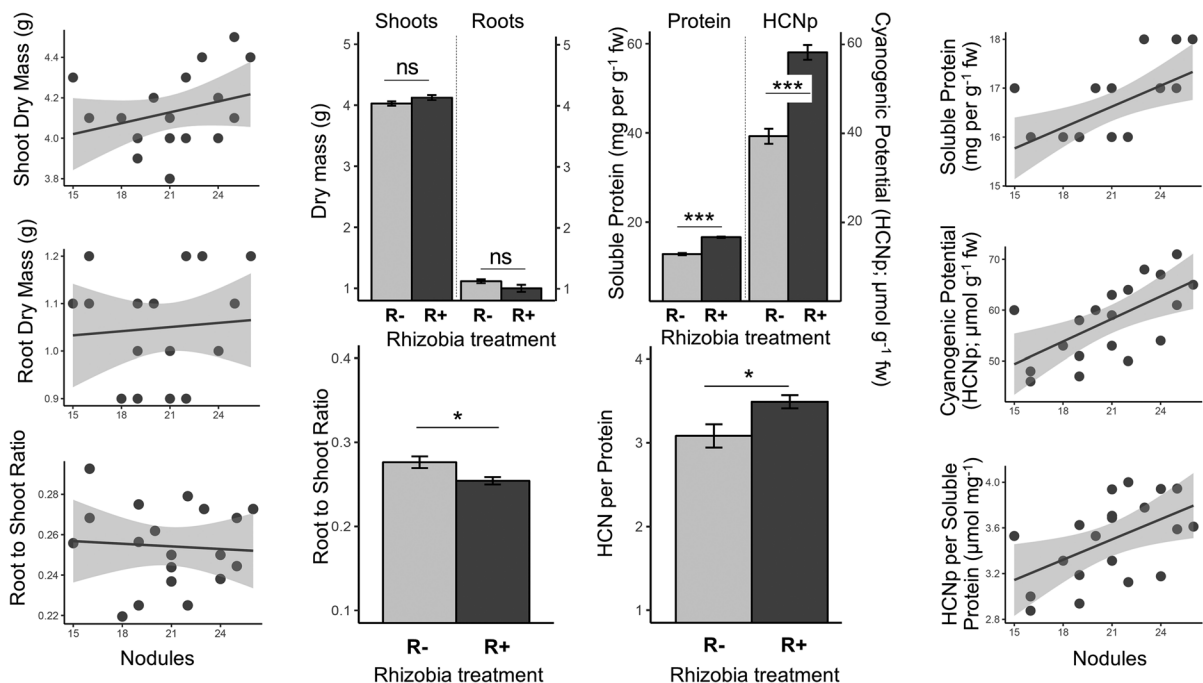
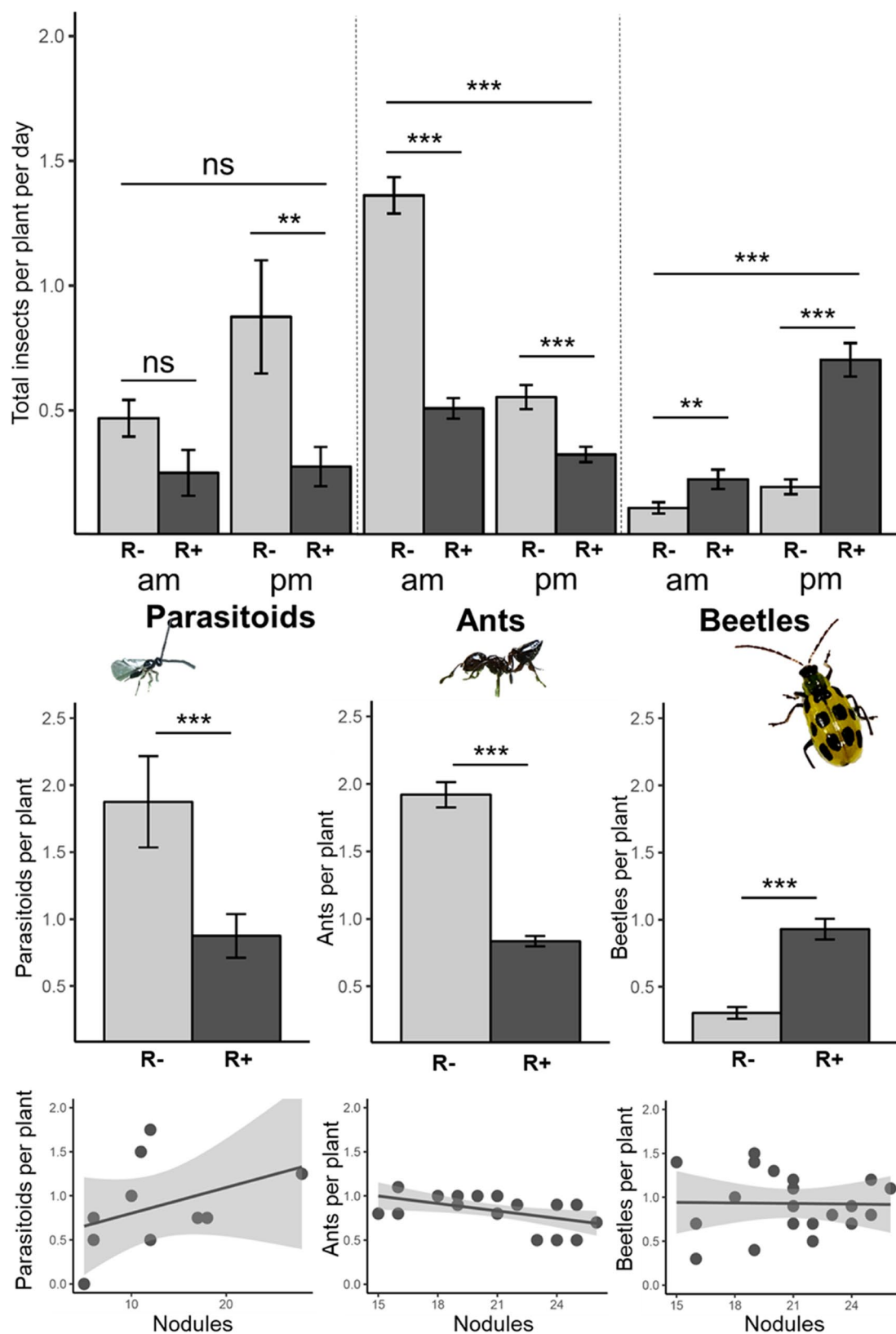


Fig. 1 Plant traits in the field with and without rhizobia inoculation. Dark gray bars represent rhizobia-inoculated plants; light gray bars show rhizobia-free plants. Significant differences of $p<0.001$, $p<0.01$, $p<0.05$, and $p>0.05$ are indicated by ***, **, *, and ns, respectively. Scatter plots show

quantitative relationships between nodule numbers and each trait and trait ratio. Protein was quantified as $\text{mg g}^{-1} \text{FW}$; HCNp was quantified as $\mu \text{g}^{-1} \text{FW}$. HCNp per protein was expressed as $\mu\text{mol mg}^{-1}$



◀**Fig. 2** Insect responses to rhizobia presence/absence (bar plots) and quantitative relationships with nodule number (scatter plots). Columns correspond to each insect type from left to right: parasitoids, ants, and beetles. Bars show the mean number of each insect present per day **a** per time of day, and **b** overall. Error bars represent standard error of the mean. Dark grey bars represent rhizobia-inoculated plants; light grey bars show rhizobia-free plants. Significant differences of $p < 0.001$, $p < 0.01$, $p < 0.05$, and $p > 0.05$ are indicated by ***, **, *, and ns, respectively. Scatter plots show **c** correlations between insects per day and nodule numbers from R+ treatment plants only. This figure is a composite of all of the insect results collected across both experiments. Parasitoid data collected in 2015 from 4 consecutive days at Guanacaste, Costa Rica; ant and beetle data from 2018 field season plants for 10 consecutive days at Puerto Jiménez, Puntarenas, Costa Rica

aboveground or belowground biomass accumulation (Fig. 1; ANOVA shoot dry mass $F_{1,38} = 3.119$, $p = 0.085$; root dry mass $F_{1,38} = 3.054$, $p = 0.089$). Root to shoot ratios were significantly higher in plants without rhizobia (Fig. 1; ANOVA root dry mass/shoot dry mass $F_{1,38} = 7.304$, $p = 0.010^*$). Within leaves, the cyanide:protein ratio, which critically affects the overall chemical defense against herbivores, was 14% higher in plants with rhizobia (Fig. 1; ANOVA HCNp/protein $F_{1,38} = 6.511$, $p = 0.015^*$). Quantitative rhizobia colonization, measured as nodule number, was not associated to above or belowground biomass (lm, slope = 0.017 $R^2 = 0.097$, $p = 0.197$), but influenced plant chemistry (Fig. 1, scatter plots), with a significant positive relationship between nodule number and HCNp (lm, slope = 1.481 $R^2 = 0.395$, $p = 0.003^{**}$) and protein (lm, slope = 0.142 $R^2 = 0.353$, $p = 0.006^{**}$). Despite this increase in both traits with increasing nodules, higher numbers of nodules also correlated positively with leaves producing a higher HCN content per protein (lm, slope = 0.059 $R^2 = 0.277$, $p = 0.017^*$), meaning that as plants form greater numbers of nodules, the ratio of cyanide per protein increases with each increase in nitrogen source. Overall, in Experiment 2, R+ plants were significantly larger and more nodulated (20.85 $\pm$ SEM 0.70 nodules) than in Experiment 1 (12.50 $\pm$ SEM 2.21 nodules).

Rhizobia-Treatment (R+/R-) Effects on Insect Behavior

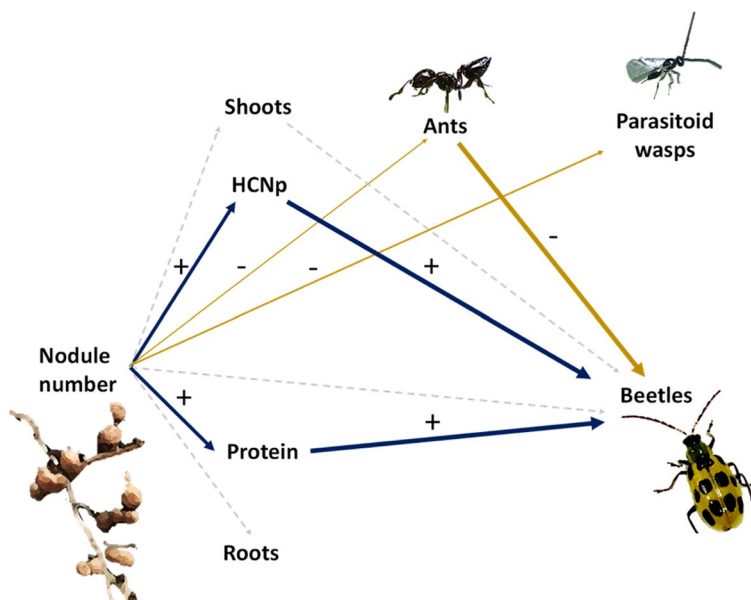
Chrysomelid beetles preferred plants with rhizobia over rhizobia-free plants in the field (Fig. 2b, *glmer*, $p < 0.001^{***}$), foraging on R+ plants twice as frequently as on R- plants. By contrast, ants and wasps, quantified

in separate experiments, were each about two-times more frequently observed on R- plants (Fig. 2b). In Experiment 1, R- control plants recruited more parasitoids (*glmer*, $p < 0.001^{***}$) than R+ plants, and in Experiment 2, R+ plants attracted more beetles (Fig. 2b, *glmer*, $p < 0.001^{***}$), but fewer ants (Fig. 2b *glm*, $p < 0.001^{***}$). Time of day significantly affected overall insect numbers per plant for ants and beetles (Fig. 2a; *glm*, $p < 0.001^{***}$), with more ants foraging in the morning and beetles foraging in the evening. Diurnal patterns of insects per plant depended on the rhizobia treatment. Parasitoid wasp attraction in the morning did not differ by rhizobia treatment. In the evening, the total number of parasitoid visitations to R- plants more than doubled that of R+ plants (*glm*, $p = 0.001^{**}$). Experiment 2 also showed significant interaction effects between rhizobia treatment and time of day on the presence of both ants and beetles with significantly more ants on R- plants in the morning, and significantly more beetle visits on R+ plants in the evenings (Fig. 2a, separate *glms*: am ants, pm ants, am beetles and pm beetles: $p < 0.001^{***}$; *glmer*, time $\times$ rhizobia $p = 0.021^*$, time $\times$ insect $p < 0.001^{***}$, rhizobia $\times$ insect $p < 0.001^{***}$). Rhizobia colonization (nodule number) differentially affected the insects observed in this study (Fig. 2c). Within R+ treatments, nodule number did not influence the number of beetles or parasitoids per plant (Fig. 2c, beetles: lm, slope = -0.021 $R^2 = 0.001$, $p = 0.933$), but nodules correlated negatively with ant abundances per plant (Fig. 2c, ants: lm, slope = -0.281, $R^2 = 0.263$, $p = 0.021^*$). Including zero values for nodule numbers of R- treatments, parasitoids and nodules correlated negatively as well (Fig. 2c, parasitoids: *glm*, $z = -2.095$, $p = 0.036^*$).

Plant Traits and Insect Responses Regardless of rhizobia presence, HCNp and protein content both were significantly correlated with beetle presence (HCNp lm, slope = 0.217 $R^2 = 0.387$, $p < 0.001^{***}$; protein lm, slope = 1.381 $R^2 = 0.506$, $p < 0.001^{***}$). Taken together, the ratio of HCN per protein was marginally associated with increased beetle presence (Fig. 3, lm, slope = 2.311 $R^2 = 0.088$, $p = 0.062$).

While both leaf chemical traits were correlated with beetle numbers, beetle attraction showed no relationship with aboveground shoot biomass. Conversely, smaller plants supported greater numbers of ants,

Fig. 3 Graphical summary of relationships between rhizobia nodulation, plant traits, and insect abundances. Line thickness indicates level of significance; dotted gray lines show no significance; solid thin lines show $p < 0.05$; medium lines show $p < 0.01$; thick lines show $p < 0.001$ (significance levels as shown in Figs. 1 and 2). Blue arrows and “+” symbols indicate positive correlations, yellow arrows and “-” symbols indicate negative correlations



especially within R+plants (Fig. 3). Ant presence was negatively correlated with shoot biomass overall (lm, slope=-10.677 $R^2=0.086$, $p=0.066$.), and within R+plant treatments only (Fig. 3, lm, slope=-0.611 $R^2=0.408$, $p=0.002^{**}$).

Although there is likely no direct interaction between ants and internal leaf compounds – other than resource allocation constraints with traits relevant for ants such as EFN – we noted that plants with higher numbers of ants had significantly lower protein content among plants across both treatments (lm, slope=-2.237 $R^2=0.576$, $p<0.001^{***}$). Cyanogenic potential—which is known to tradeoff with indirect defenses in lima bean (Ballhorn et al. 2008, 2013a)—also showed a negative correlation with ant presence overall (lm, slope=-0.423 $R^2=0.645$, $p<0.0001^{***}$). Within R+plants, but not within R-plants, ant presence was negatively correlated with HCNp (lm, slope=-0.017 $R^2=0.555$, $p<0.001^{***}$). Ants were found in higher numbers on plants with lowest HCNp per protein ratios overall (lm, slope=-6.341 $R^2=0.291$, $p<0.001^{***}$), and within R+treatment plants (Fig. 3, lm, slope=-0.298 $R^2=0.377$, $p=0.004^{**}$).

Discussion

Both nitrogen-fixing rhizobia and herbivore antagonists such as predators and parasitoids are key plant mutualists in most terrestrial ecosystems. Here we

investigated the interplay between plants and these above- and belowground symbionts in the tropical forests of Costa Rica, in dry forest in Guanacaste and a wet forest on the Osa Peninsula. We showed that natural communities of arthropods at these sites responded differently depending on whether plants were inoculated with rhizobia. Although plant-rhizobia and plant-predator interactions are well-studied, studies illuminating interactions among all three partners are scarce (but see Pineda et al. 2013; Pangesti et al. 2015a)—particularly under natural conditions.

Our field data revealed rhizobia colonization can shift the plant's local community of insects. We found greater numbers of herbivorous beetles alongside reduced ant and parasitoid wasp presence on wild lima bean plants with nitrogen-fixing rhizobia (R+) compared to nodule-free (R-) conspecifics. Insect community-level responses driven by root symbionts are not surprising given the critical role of rhizobia for legume physiology and chemistry (Godschalx et al. 2017). In overcoming nitrogen limitation, nitrogen-fixing symbionts enable plants to invest in protein rich tissues and direct protein-based defense pathways (Kempel et al. 2009; Thamer et al. 2011; Irmer et al. 2015). Beyond rhizobia-legume interactions, similar effects have been observed for other nitrogen-fixing plant-microbe systems such as rhizobia-*Parasponia* (Trinick and Hadobas 1990) but also *Frankia-Alnus* interactions (Ballhorn et al. 2017). Thus, diverse

microbes that provide symbiotically-fixed nitrogen, have the potential to alter foliar nutritive and defensive traits. In this study, the colonization by nodulating rhizobia increased both protein content and HCNp. The ratio of HCNp to protein is an important metric for overall defense against herbivores (Ballhorn et al. 2009, 2010) and, consistent with our previous work on the quantitative relationships between symbiosis and defense investment (Godschalx et al. 2017), we found that not only the amount of cyanogenic precursors was higher in plants inoculated with rhizobia, but that higher rhizobial colonization (numbers of nodules) further increased the cyanide per protein ratio. Given the increased defense to nutritive content ratio, the high numbers of chrysomelid beetles (*Diabrotica balteata*) on R+ plants in nature were surprising.

However, whether these herbivorous beetles preferred R+ plants due to (i) the increase in foliar protein, (ii) avoiding predator presence or, potentially, (iii) utilize elevated cyanogenesis as a cue for host plant location or mating-associated signaling is not known (Ballhorn et al. 2013b). Given the distinctly polyphagous feeding behavior of *D. balteata*, food nutritional quality or reduced presence of ants seem the most likely explanation, although these same plants show stronger direct defense. Nevertheless, highly polyphagous species are known to have elaborated detoxification pathways which include mechanisms to detoxify cyanide and cyanide-containing compounds (Ballhorn et al. 2009). In addition to the traits we measured, other variables may have been altered by rhizobia colonization and may play a role in plant-insect interactions. For example, foliar lignin content has been reported to be negatively affected by *Frankia* bacteria in the symbiosis with red alder (*Alnus rubra*) and to subsequently increase leaf susceptibility to Chrysomelid beetles in *Frankia*-colonized alder trees (Ballhorn et al. 2017). Similar effects may occur in the legume-rhizobia interaction but have not been tested in the present study. Alternatively, rather than responding to direct chemical defense and nutritive leaf traits directly, beetles could have been avoiding the high frequency of natural enemies that were patrolling R- plants.

Rhizobia-free lima bean plants are well-known to produce higher levels of several indirect defense traits such as extrafloral nectar (EFN) and volatile organic compounds (VOCs) (Summers and Mondor 2011; (Ballhorn et al. 2013c; Godschalx et al. 2015).

Although we did not measure these traits in the present study, we show the natural predator communities respond in line with the expected expression of these indirect defense traits, as more parasitoid wasps were observed on R- plants, and more ants patrolled R- plants compared with R+ plants. To explain predator responses based on known trends in indirect defense traits, here we consider two potential defense investment patterns that may help explain the arthropod community's response to plants with nitrogen-fixing rhizobia: first, carbon limitation, and second, a shift from indirect to direct defense investment.

Rhizobia as a Carbon Sink Nodule-housed symbionts consume between 16 and 30% of a plant's total photosynthates (Peoples et al. 1986; Kaschuk et al. 2009). In fact, microbial symbiont metabolism plays a largely underestimated role in terrestrial carbon cycles as sinks for organic carbon (Pringle 2016). If lima bean defense trait allocation is driven by resource availability, a symbiont consuming a portion of a plant's photosynthate budget should limit plants from being able to produce the same quantities of carbon-rich signals or rewards for predators. Although carbon-rich plant traits, such as EFN have been considered to be relatively "cheap" compared with nitrogen-demanding traits, removing photosynthetic area quantitatively reduces the amount of EFN plants produce, implying carbon limitations (Ballhorn et al. 2014). Among the volatiles compromised in R+ plants are jasmonates (Ballhorn et al. 2013c). Jasmonic acid (JA) induces octadecanoid pathway products that specifically attract parasitoids (Thaler 1999). Extrafloral nectar is a light- and jasmonate pathway-dependent trait (Radhika et al. 2010), so reduction in JA-induced traits in R+ treatments could explain the reduced amount of secreted EFN sugar (Godschalx et al. 2015) or nectary production (Summers and Mondor 2011), as well as the reduced ant attention in past and present studies. Additionally, increased herbivory leads to larger emission of VOCs, which further affect tritrophic interactions (Paré and Tumlinson 1999; Arimura et al. 2009). For volatile induction, JA-inducible traits serve as an anti-herbivore plant hormonal pathway, and likely provide reliable cues from plants suffering from herbivory to parasitoids indicating the presence of a food source. Parasitoids show no preference among plants with or without free-living rhizobacteria from aphid-infested *Arabidopsis* plants genetically modified to

inhibit JA production, whereas wild-type plants recruit fewer parasitoids when rhizobacteria were present (Pineda et al. 2013), consistent with our findings. Our data showing fewer parasitoids were attracted to plants with rhizobia could be an example of rhizobia directly reducing a plants' ability to produce the JA-inducible volatile products most important for attracting parasitoids as an indirect defense.

Rhizobia as Adaptive Defensive Partners An alternative to carbohydrate consumption being the driving force behind rhizobia's ability to influence plant resource allocation, investing highly in direct defenses (and less in indirect defenses) could be an adaptive defense strategy for a plant engaged in symbiosis with a microbial nitrogen-fixer. If symbiont-mediated physiology confers higher fitness to plants that invest in direct defenses over indirect defenses, such a symbiont defense pattern should be favored. Nodule formation is technically a microbial infection, and plants primed for pathogen resistance form fewer nodules (Ballhorn et al. 2014), indicating an interaction between plant-microbe symbiosis and defense pathways. Nodulation could therefore cause an increase in anti-pathogen defenses induced by salicylic acid, as well as volatiles known to interact with other plant-associated organisms, including indole. Indole and salicylic acid were the only measured VOCs to increase in rhizobia-inoculated wild lima bean plants in the previous study (Ballhorn et al. 2013c), leading us to design the present field study to test whether parasitoids would respond to rhizobial treatments differently.

Parasitoid larvae developed more rapidly and with a greater resulting mass on plants with arbuscular mycorrhizal fungi (Hempel et al. 2009). In another example but without microbes, nutritional status influenced parasitoid success as nitrogen-treated *Glycine max*-fed herbivores increased parasitoid larval mass, and quantitatively altered VOC emission, but adult wasps did not prefer these plants over nitrogen-deficient plants (Winter and Rostás 2010). To address specific plant-rhizobial effects on herbivores as a food source for parasitoids, a possible follow-up study would be to test whether or not herbivores fed on rhizobia-free plants are a better food source for developing wasps compared with herbivores consuming rhizobia-inoculated plant tissue.

Interestingly, in the present study, parasitoids significantly preferred rhizobia-free plants during the evening sampling time, while ants patrolled rhizobia-free plants more frequently in the morning. However, even though fewer ants were present on rhizobia-free plants in the evening, beetles continued to prefer foraging on symbiotic tissues, although in higher numbers when ant numbers were reduced. Predator patterns could be explained by indirect defense traits, EFN and VOCs, which follow a photoperiod-dependent circadian rhythm with peaks occurring late afternoon (Christensen et al. 2013). If volatile expression peaks coincide with parasitoid activity, this may explain why most of the wasp recruitment took place during our evening sampling time.

Parasitoid activity is also likely connected to circadian rhythms of herbivorous hosts. Due to the phylogenetically diverse group of parasitoids observed in our study, the specific herbivores each parasitoid utilizes as a host likely spans a diverse spread of feeding guilds. We observed a diversity of parasitoid wasps, including wasps from both the Chalcidoidea and Ichneumonoidea superfamilies, belonging to the Aphelinidae, Ichneumonidae and Braconidae families. Ranging from specialist to generalist predators, parasitoid activity may largely depend on oviposition patterns and active feeding times of Homoptera, Coleoptera, Lepidoptera, and other herbivorous hosts. Chewing insects damage leaf tissue to a greater extent than phloem feeders, which alone can influence plant VOC emission differences, but additionally, feeding mode can influence plant hormonal regulation (Bonaventure 2012) as well as subsequent volatile chemistry (Tan and Liu 2014). These different effects help explain why herbivore identity matters in understanding microbially-mediated parasitoid attraction. Our data showing fewer parasitoids on plants with rhizobia is consistent with soil rhizobacteria-associated plants infested with aphids (Pineda et al. 2013) but not leaf-chewing insects (Pangesti et al. 2015a). Future work is needed to characterize which specialist parasitoids are attracted towards or repelled from plants with symbiotic rhizobia, which would be an interesting visualization of the bottom-up trophic level effects of rhizobia with greater feeding guild resolution. Without information about taxonomic identity of wasps on either rhizobia treatment (R-, R+), it is noteworthy that we observed parasitoids across a relatively broad range of taxa responding consistently to plants in natural communities depending on rhizobia

symbiosis. Our results indicate that aboveground signaling mediated by belowground symbiosis can impact the third trophic level in nature. Therefore, symbiotic, nitrogen-fixing rhizobia contribute to an integrated ecological system in which bottom-up forces affecting the recruitment of predators subsequently influence top-down forces via plant defense.

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Data Availability Data are available at <https://github.com/agodschalx/MSdata2023>.

Declarations

Competing Interests The authors declare no competing interests.

Conflict of Interest The authors declare that they have no conflicts of interest.

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