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PLANT-MICROBE-ANIMAL INTERACTIONS – ORIGINAL RESEARCH

Wild legumes maintain beneficial soil rhizobia populations despite decades of nitrogen deposition

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Abstract

Natural landscapes are increasingly impacted by nitrogen enrichment from aquatic and airborne pollution sources. Nitrogen enrichment in the environment can eliminate the net benefits that plants gain from nitrogen-fixing microbes such as rhizobia, potentially altering host-mediated selection on nitrogen fixation. However, we know little about the long-term effects of nitrogen enrichment on this critical microbial service. Here, we sampled populations of the legume *Acmispon strigosus* and its associated soil microbial communities from sites spanning an anthropogenic nitrogen deposition gradient. We measured the net growth benefits plants obtained from their local soil microbial communities and quantified plant investment into nodules that house nitrogen-fixing rhizobia. We found that plant growth benefits from sympatric soil microbes did not vary in response to local soil nitrogen levels, and instead varied mainly among plant lines. Soil nitrogen levels positively predicted the number of nodules formed on sympatric plant hosts, although this was likely due to plant genotypic variation in nodule formation, rather than variation among soil microbial communities. The capacity of all the tested soil microbial communities to improve plant growth is consistent with plant populations imposing strong selection on rhizobial nitrogen fixation despite elevated soil nitrogen levels, suggesting that host control traits in *A. strigosus* are stable under long-term nutrient enrichment.

Keywords Host control · Mutualism · Nutrient enrichment · Soil microbes · Symbiosis

Introduction

Terrestrial plants invest substantial amounts of fixed carbon into their roots to acquire growth limiting nutrients like nitrogen and phosphorus (Lynch and Ho 2005). To enhance access to nitrogen, legumes have evolved a root nodule symbiosis with rhizobia, which are soil bacteria that convert atmospheric dinitrogen into a reduced form that plants can use for growth (Sprent et al. 1987). Free-living rhizobia infect the roots of legumes, fix atmospheric nitrogen inside root nodules, and return to a saprotrophic lifestyle in the soil after nodule senescence (Denison and Kiers 2004). Legumes incur carbon costs to fuel the reduction of nitrogen and the metabolism of the nodule rhizobia population (White et al. 2007; Quides et al. 2021), but symbiosis usually provides a net growth benefit to plants in nitrogen-limited soils. However, human activity over the last century has introduced copious amounts of nitrogen into soils via agricultural runoff (Wang and Li 2019) and deposition of airborne nitrogen (Egerton-Warburton et al. 2001; Fenn et al. 2010). Elevated soil nitrogen, even at modest levels, can eliminate any growth benefits that legumes gain from associating with rhizobia, rendering the symbiosis superfluous to the plant (Regus et al. 2017a). Environmental changes in soil nitrogen could alter how hosts and microbial mutualists coevolve, with potentially drastic consequences for global nutrient cycling and agricultural sustainability (Six 2009; Kiers et al. 2010).

Legume hosts are thought to be the predominant selective force favoring the nitrogen-fixing ability of rhizobia populations (West et al. 2002b). Genotypes of rhizobia vary

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substantially in the magnitude of services they provide to hosts, ranging from highly beneficial, nitrogen-fixing strains to ineffective rhizobia that fail to provide fixed nitrogen (Sachs et al. 2010a; Gano-Cohen et al. 2020). Legumes have evolved 'host control' mechanisms to preferentially invest in genetically compatible and beneficial rhizobia during nodule organogenesis ('partner choice') and to punish ineffective strains after nodule formation has occurred ('host sanctions'; Kiers et al. 2003; Heath and Tiffin 2009; Sachs et al. 2010b; Oono et al. 2011; Regus et al. 2017b). As a consequence of host control, beneficial rhizobia can receive tremendous fitness rewards from forming nodules on legumes, leading to the enrichment of nitrogen-fixing strains in the soil (Miethling et al. 2000). Selection that legumes impose on rhizobia could be altered under conditions of enriched soil nitrogen, but it is unclear whether selection for nitrogen fixation would become degraded or enhanced. Degraded selection by hosts is predicted if legumes minimize nodule formation under enriched nitrogen. Elevated levels of soil nitrogen can often inhibit the formation of new root nodules (Nishida and Suzaki 2018; Dupin et al. 2019) since mineral forms of nitrogen can be cheaper for plants to acquire than symbiotically fixed nitrogen (Pfau et al. 2018). In parallel, elevated soil nitrogen could cause hosts to physiologically downregulate or evolve weakened host control, particularly if these traits are costly for hosts to maintain (Kiers et al. 2007; Porter and Sachs 2020). Over generations, fewer interactions between legume hosts and rhizobia and/or weakened host control could reduce the selective importance of nitrogen fixation to rhizobia (Denison and Kiers 2004; Oono et al. 2020), thus favoring rhizobia with degraded nitrogen fixation ability (Sachs et al. 2011). Conversely, enhanced selection for nitrogen fixation is predicted if legumes can adjust host control to more severely punish rhizobia that do not provide net benefits (Kiers et al. 2006). In this scenario, hosts would only reward rhizobia that provide net benefit in the presence of soil nitrogen, thus punishing many beneficial strains and only rewarding rhizobia with very high levels of nitrogen fixation ability (Kiers et al. 2006).

Nutrient enrichment might also influence the nitrogen-fixing ability of rhizobia by altering the outcomes of competition between rhizobia and other microbes in the rhizosphere. Rhizobia compete against other microbes to colonize the surface of the root, and this competition can interfere with the ability of rhizobia to form nodules, depressing plant growth benefits from symbiosis (Gano-Cohen et al. 2016). Nutrient enrichment of soil can alter soil microbial community diversity (Cotton 2018; Huang et al. 2019; Bledsoe et al. 2020) and function (Schmidt et al. 2017; Williams et al. 2017; Yan et al. 2017). Nutrient enrichment can also alter the success of rhizobia competing for colonization sites on the roots. Simonsen et al. (2015) found that one season of soil fertilization increased the nitrogen-fixing ability of rhizobia on a test legume. In contrast, Weese et al. (2015) uncovered the opposite trend after two decades of experimental soil nutrient enrichment, with rhizobia showing lower nitrogen fixation ability in enriched plots compared to control plots. However, the decline in effectiveness of rhizobia would have been strongly influenced by a loss of interaction opportunities with the legume host plants, which showed severe population declines in the enriched plots (Weese et al. 2015). No study has yet measured how soil nitrogen affects plant benefits from symbiosis in the context of their native soil microbial communities. Therefore, a major knowledge gap is to understand how nitrogen enrichment affects the services that rhizobia provide to hosts in natural systems where hosts and symbionts can both respond to nutrient enrichment and can coevolve.

Here, we investigate the impacts of long-term soil nitrogen enrichment on the mutualism between natural rhizobia populations and the wild legume host *Acmispon strigosus*. We sample soils from six *A. strigosus* populations along a 750 km nitrogen deposition gradient that has persisted for several decades (Egerton-Warburton et al. 2001) and generated a corresponding gradient of soil nitrogen (Regus et al. 2017a). Previous work has examined variation in host and rhizobia traits across this gradient. Isotopic analysis of field-collected seeds indicate that wild *A. strigosus* gains ~ 85% of its nitrogen from symbiosis at the low end of the nitrogen deposition gradient (4 ppm mineral nitrogen) and only ~ 67% from symbiosis at the high end of the gradient (20 ppm mineral nitrogen (Regus et al. 2017a), consistent with legumes shifting from symbiotic to mineral sources of nitrogen when the latter is readily available (Gano-Cohen et al. 2019, 2020). Host control over ineffective rhizobia was tested for plant lines sourced from across the gradient, but host control was not found to differ between *A. strigosus* plant lines from contrasting soil nitrogen regimes (Wendlandt et al. 2019). Both beneficial and ineffective rhizobium strains have been isolated from soils across the nitrogen deposition gradient, but it is unclear whether soil nitrogen is a driver of this variation (Sachs et al. 2010a; Gano-Cohen et al. 2020). We performed greenhouse inoculations of sterile-grown *A. strigosus* seedlings with soil slurries from their sites of origin and measured plant growth benefits from inoculation and host investment into root nodules. We raised plants without added nitrogen to maximize the detection of rhizobial effects in this soil microbial community context. We also inoculated each soil slurry onto three control plant lines (two *A. strigosus* and one *A. heermannii*) to test for the generality of soil effects (i.e., to identify whether variation in rhizobial nitrogen fixation is driven mainly by differences among soil microbial communities or by differences among host plant lines). Our work contributes to an understanding of how human-influenced landscapes can affect the evolution of mutualisms.

Materials and methods

Acmispon plant lines and growth conditions

We used two *Acmispon* species in our experiments: *A. strigosus* (strigose lotus, formerly *Lotus strigosus*) and *A. heermannii* (Heermann's lotus, formerly *Lotus heermannii*), two species that are closely related (Allan and Porter 2000), broadly sympatric, and interact with similar communities of *Bradyrhizobium* (Sachs et al. 2009). *A. strigosus* seeds were collected between 2005 and 2011 from ripe fruits at six natural field sites in California (supplementary material, Fig. S1): Anza-Borrego Desert State Park (Anz), Bodega Marine Reserve (BMR), Griffith Park (Gri), Pioneertown Mountains Preserve near Yucca Valley (Yuc), Bernard Field Station of the Claremont Colleges (Cla), and the campus of the University of California, Riverside (UCR). These field sites span a nitrogen deposition gradient ranging from 1.84 kg ha^{-1} yr^{-1} at BMR to 8.67 kg ha^{-1} yr^{-1} at UCR (Regus et al. 2017a). Soils from these field sites are classified either as entisols (young mineral soils without distinct horizons; i.e., Anz, Yuc, Cla, and UCR), mollisols (fertile soils with organic-rich upper horizons; i.e., Gri), or unclassified "dune land" (i.e., BMR; likely also an entisol based on sandy texture; (Soil Survey Staff).

To generate inbred lines and minimize maternal effects, plants from each field site were raised in an insect-free glasshouse for one to two generations. We generated 14 *A. strigosus* inbred lines (2–3 lines per field site) and genotyped them at two loci: *nrITS* (Allan and Porter 2000) and *CNGC5* (Maureira-Butler et al. 2008). Each inbred plant line was derived from an independent wild seed except for the Yuc lines, which were derived in error from the same wild ancestor. Cla and Yuc plant lines could not be differentiated genetically within their respective field sites, whereas plant lines from the other field sites were genetically distinct from each other (supplementary material, Table S1). Although we only sampled a small portion of the genetic variation within plant populations, we were primarily interested in variation among populations, and so we focused our sampling efforts on breadth instead of depth. We used mixed seeds of the California native perennial *A. heermannii* (S&S Seeds, Carpinteria, California, USA).

For the greenhouse experiment, axenic seedlings of each plant line were germinated in an environmental chamber and transferred to sterilized Ray-Leach SC10 'conetainers' (Stuewe & Sons, Corvallis, Oregon, USA) filled with sterilized quartzite sand following published protocols (Sachs et al. 2009). Plants were fertilized weekly with nitrogen-free Jensen's solution (Somasegaran and Hoben 1994), starting with 1.0 mL and increasing by 2.0 mL per week until reaching 5.0 mL, which was used for the duration of the experiment. Plants with true leaves were moved to a glasshouse to harden for 11 days until inoculation. Each plant was treated with 5.0 mL soil inoculum on 9 March 2015 by slowly dripping the inoculum around the base of the plant.

Preparation of soil inocula

We collected approximately 20 soil cores (13 cm deep, 5.5 cm wide) from each of the above field sites between 27 February and 2 March 2015 (supplementary material, Table S1). Soil cores were spaced 1 m apart and taken within 20 cm of live *A. strigosus* plants to maximize the chance of sampling microbial communities interacting with this plant species. The soil corer was sterilized between field sites by removing visible soil with a wet sponge, spraying the corer with ethanol, and flaming. Soil cores were transported back to the lab in new plastic bags and stored at room temperature until inoculum preparation on 8 March 2015. Bulked soil cores from each field site were passed through a flame-sterilized 2 mm sieve, combined with 1 mL sterile water per gram of sieved soil, shaken vigorously to form a slurry, and filtered through eight layers of sterile cheesecloth (Unkovich and Pate 1998). Soil slurries from UCR, Cla, and Gri initially clogged the filters, so we let the slurries stand approximately 20 min and then transferred their supernatants to separate bottles. The liquid fractions of soil slurries from all six field sites were allowed to settle overnight at room temperature. The top 60–80% of each fraction was transferred to a new bottle, mixed well, and split into two volumes. One of the volumes was used as a 'live' soil inoculum, and the other was autoclave-sterilized, cooled to room temperature, and used as a 'sterilized' soil inoculum, to control for the nutrient profile of the soil but without microbes. Thus, we prepared 12 soil inocula (live and sterilized inocula from six field sites). Both live and sterilized soil inocula had high turbidity, so we mixed them well before dripping them onto plants. To estimate the colony-forming units (CFU) in each live soil inoculum, samples were taken immediately after preparation and spread-plated in five dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) onto three replicate agar plates containing a glucose based rhizobia defined medium, with cycloheximide (GRDM; Sullivan et al. 1996). GRDM provides specific growth conditions for rhizobia and related taxa, so these data provided a rough estimate of total rhizobial abundance (Sachs et al. 2009). Colonies that formed within 10 days of plating were counted, and plates containing 30–300 colonies were used to calculate CFU mL^{-1}. Undiluted sterilized inocula were plated onto three replica GRDM agar plates to check for successful sterilization of the microbial community. We performed standard nutrient analysis for each sieved soil at A&L Western Laboratories (Modesto, California, USA). Live and sterilized soil inocula were analyzed

for nitrogen content at Soil and Plant Laboratory, Inc. (Anaheim, California, USA).

Experimental design

We performed a greenhouse experiment to test for variation in plant responses to soil microbes across six field sites that vary in soil nitrogen. Thus, field site was the unit of replication in this context. A soil slurry from each field site was used to inoculate a set of sympatric or common plant hosts (the Sympatric Host Experiment and Common Host Experiment, respectively). All inocula were applied in either live or sterilized form (12 inocula total) so that the contribution of soil microbiota to plant responses could be distinguished from abiotic factors in the soil. For the Sympatric Host Experiment, we used two *A. strigosus* plant lines from the same field site as each soil inoculum, for a total of 12 plant lines (2 plant lines per field site × 6 field sites). For the Common Host Experiment, we used three plant lines: two *A. strigosus* plant lines from opposite soil nitrogen regimes (Anz13.04 and Cla12.04; Regus et al. 2017a) and the related outgroup plant (*A. heermannii*). *A. heermannii* was included to detect if *Acmispon* responses to inocula were generally similar among related species.

Within each experimental block, we spatially clustered plants that received identical inocula (for a diagram, see supplementary material, Fig. S2). Plants receiving the same inocula were spaced with ~ 4 cm between plants, and clusters receiving different inocula were spaced farther apart (~ 12 cm between clusters) to maximize the number of plants in the experiment while minimizing cross-contamination among different inocula. Each of the 12 inocula was applied to one cluster of plants per block. We randomly assigned each of the six live inocula to non-adjacent clusters and each of the six sterilized inocula to the remaining clusters, generating a checkerboard pattern of live and sterilized inocula to reduce the chance of cross-contamination among live inocula. Each inoculum cluster contained five plants: three plants belonged to the Universal Host Experiment (comprising one plant each of *A. strigosus* Cla12.04, *A. strigosus* Anz13.04, and *A. heermannii*), and two plants belonged to the Sympatric Host Experiment (i.e., two *A. strigosus* lines sourced from the same field site as the inoculum; see supplementary material, Table S1). We did not randomize plant positions within inoculum clusters: *A. heermannii* was always the center plant of the cluster so that the four surrounding *A. strigosus* plants all experienced the same microenvironment (i.e., the edge of a cluster). We decided to give *A. strigosus* all the same microenvironment to maximize our ability to detect differences in their responses to inocula, since we were more interested in making comparisons among *A. strigosus* plants than in comparing *A. strigosus* to *A. heermannii*. In total, we planned for 600 plants in the greenhouse experiment (5 plants per inoculum cluster × 12 inoculum clusters per block × 10 blocks), with 360 plants in the Common Host Experiment and 240 plants in the Sympatric Host Experiment. Due to early seedling mortality for three plant lines (Anz10.01, Anz13.04, and Gri01.13), these experiments actually contained 348 plants and 219 plants, respectively.

The overhead misters temporarily failed on 23 April 2015 (6.5 weeks post-inoculation, wpi) and many plants wilted. Dead shoot tissue was collected to prevent its being lost during transfer to the lab, and this tissue was pooled with shoots collected at the time of harvest (4–14 May 2015, 8–9.5 wpi). Twenty-two plants had shoot portions collected early and five plants had their entire shoots collected early; affected plants were distributed among several treatments in both experiments (12 plants from 4/12 inoculation treatments in the sympatric host experiment; 15 plants from 8/12 inoculation treatments in the common host experiment). At harvest, plants were removed from pots, washed free of sand, and dissected into root, shoot, and nodule portions. Nodules were counted and photographed. Roots, shoots, and nodule fractions of each plant were oven-dried (> four days, 60 °C) and weighed.

Measurement of plant traits

We measured total plant dry mass (the sum of dry root mass and dry shoot mass) and root:shoot ratio (dry root mass divided by dry shoot mass) on all experimental plants. Root mass was calculated after removal of any nodules, which permits total plant mass and root:shoot ratio to be compared to measurements on non-nodulated plants (Dupin et al. 2019; Cirocco et al. 2021). For plants treated with live soil inocula, we also measured (i) relative growth response to the soil microbial community (total dry mass of the live-inoculated plant divided by total dry mass of the sterilized-inoculated plant from the same block), (ii) red nodule frequency (proportion of root nodules visually scored as either red or pink as a proxy for symbiotic nitrogen fixation), since the red colored protein leghemoglobin is expressed in actively fixing nodules (Virtanen 1947; Tajima et al. 2007), (iii) total nodule dry mass, (iv) total nodule count per plant, and (v) mean nodule dry mass per plant (total nodule dry mass divided by total nodule count). For relative growth, a value greater than 1 indicates that live soil inocula improved plant growth relative to sterilized soil inocula. For red nodule frequency, values were averaged across three independent blind observers who used a scoring guide to examine nodule photographs taken at the time of harvest (supplementary material, Fig. S3). For all plant responses, individual plants were the experimental unit of analysis.

Data analysis

Statistical analyses were performed in R v.4.1.2 (R Core Team 2018). To summarize variation in soil nitrogen levels among field sites, we performed principal components analysis (PCA) on measures of soil nitrogen from the field sites, using data gathered in 2013 (dry N deposition rate, mineral N, and total N; (Regus et al. 2017a) and 2015 (nitrate–N, ammonium-N, and organic-N; this study). Using data from two years and multiple forms of nitrogen gives us a robust estimate of the soil nitrogen regime at each field site. To summarize variation in other soil properties among field sites, we performed PCA of eight non-nitrogen general soil traits: pH, cation exchange capacity (CEC), Na, and the major plant nutrients P, K, Ca, S, and Mg, all measured from soils collected in 2015. From two measures of P from each soil, we used the Weak Bray value for soils with pH ≤ 7.3 and the Olsen value for soil with pH > 7.3, due to differences in the accuracy of these tests at different pH values. In total, we generated one nitrogen PC1 value and one non-nitrogen PC1 value from each field site, and we used these values as alternative covariates in models of plant responses to soil inocula.

We analyzed greenhouse data using general linear mixed models (GLMM) implemented with lme4 v.1.1-27.1 (Bates et al. 2015). We used Gaussian error distributions for all responses and checked the appropriateness of error distributions in DHARMa v.0.4.4 (Hartig 2019). Total plant mass was log-transformed to improve normality of the data. For the Sympatric Host Experiment, we modeled plant responses as PC1 + Inoculum type + PC1:Inoculum type + (1|Host line) + (1|Block). For the Common Host Experiment, we modeled plant responses as PC1 + Inoculum type + Host line + PC1:Inoculum type + PC1:Host line + Inoculum type:Host line + PC1:Inoculum type:Host line + (1|Block). 'PC1' was the first principal component from the nitrogen (or non-nitrogen) PCA and corresponded to the field site of the soil inoculum. 'Inoculum type' indicated whether the inoculum was live or sterilized. We did not include the Inoculum type term(s) in any models for responses that only applied to plants receiving live inocula (e.g., relative growth, red nodule frequency, total nodule count, total nodule mass, and mean nodule size).

We tested the significance of model terms using likelihood ratio tests. Specifically, we used the drop1 function in the R package 'stats' to measure the change in model likelihood between a model containing the term of interest and a reduced model lacking the term of interest. For the Sympatric Host Experiment, for instance, we tested the PC1:Inoculum type interaction by comparing the full model PC1 + Inoculum type + PC1:Inoculum type + (1|Host line) + (1|Block) to a reduced model lacking the interaction term. We tested the PC1 and Inoculum type main effects by comparing the model PC1 + Inoculum type + (1|Host line) + (1|Block) to a reduced model lacking either the PC1 term or the Inoculum type term, respectively. For each model term we tested, the degrees of freedom for the likelihood ratio chi squared statistic was equal to the difference in degrees of freedom of the two models being compared.

Overall, our analysis included 28 statistical models (7 response variables × 2 covariate options (N_{PC1} or $NonN_{PC1}$) × 2 experiments). To determine if the 27 plants harvested early had undue influence on the results, we performed all statistical tests with and without these plants. In 27/28 models, the significance of model terms was not influenced by plants harvested early (one exception is noted in the Results).

Results

Principal components analysis of soil traits

Our soil analyses corroborated previous work that showed a striking nitrogen deposition gradient across California (Fenn et al. 2010; Regus et al. 2017a). PC1 for soil nitrogen (hereafter, N_{PC1}) explained 68% of the variance in the soil nitrogen dataset. The top loadings for N_{PC1} were dry nitrogen deposition (2013 data; ranging 0.34–7.67 kg ha^{-1} $year^{-1}$) and soil nitrate–N (2015 data; ranging 2–32 ppm). The six *A. strigosus* field sites were ordered as follows by N_{PC1}: BMR < Anz < Yuc < Gri < UCR < Cla (Fig. 1a), consistent with the soil nitrogen differences among sites previously reported using measurements from only 2013 (Regus et al. 2017a). PC1 for non-nitrogen soil traits (hereafter, $NonN_{PC1}$) explained 43% of the variance in the non-nitrogen soil trait dataset. The top loading for $NonN_{PC1}$ was CEC (ranging 3.2–24.1 meq per 100 g), followed by Ca (ranging 302–1502 ppm) and Mg (ranging 58–1700 ppm). The six field sites were ordered as follows by $NonN_{PC1}$: BMR < Yuc < UCR < Anz < Cla < Gri (Fig. 1b). $NonN_{PC2}$ explained 34% of the variance in non-nitrogen soil traits (Fig. 1b), with top loadings of phosphorus, potassium, and sulfate. Since $NonN_{PC2}$ explained almost as much variance in soil data as $NonN_{PC1}$, we also ran statistical models using $NonN_{PC2}$ as a covariate (supplementary material, Table S2, Table S3).

Sympatric host experiment

All live soil inocula were rich in culturable bacteria with *Bradyrhizobium*-like characteristics (creamy colonies, forming 6–10 days after inoculation). Estimated cell densities in each field soil ranged from 6.7×10^5 CFU mL^{-1} (Anz) to 2.7×10^7 CFU mL^{-1} (Gri). Sterilized inocula failed to grow on media, except for one colony from the BMR sterilized inoculum. In the sympatric host experiment, plants treated

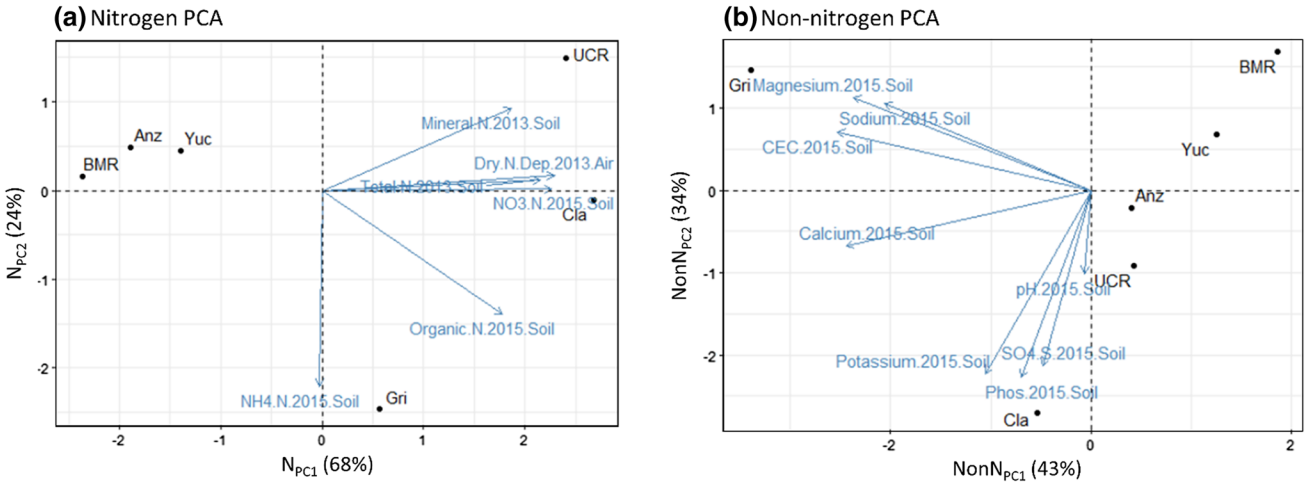

Fig. 1 Principal components analysis for (**a**) soil nitrogen and (**b**) non-nitrogen general soil traits at six *Acmispon strigosus* field sites. The percentage of variation explained by the first two principal components is indicated on the axes. The variable loadings in the PCA are indicated with blue arrows. We used N_{PC1}, $NonN_{PC1}$, and $NonN_{PC2}$ as alternative covariates in our models of soil microbial effects on plant traits

Table 1 Likelihood ratio test χ^2 statistics for fixed effects in GLMMs modeling traits of *Acmispon strigosus* inoculated with sympatric soils in the Sympatric Host Experiment

Model, response	Model terms		
	P	I	P:I
	df = 1	df = 1	df = 1
$Nitrogen_{PC1}$ models			
Log(Total plant mass)	2.16	248.06***	2.97†
Root:shoot ratio	0.21	187.17***	0.18
Plant relative growth	0.68		
Red nodule frequency	2.40		
Total nodule mass	3.59†		
Total nodule count	4.00*		
Mean nodule size	0.78		
$NonNitrogen_{PC1}$ models			
Log(Total plant mass)	8.90*	248.33***	0.19
Root:shoot ratio	10.45*	188.17***	2.84†
Plant relative growth	0.65		
Red nodule frequency	0.18		
Total nodule mass	0.047		
Total nodule count	0.13		
Mean nodule size	1.54		

P = PC1 for either soil nitrogen or non-nitrogen soil traits. *I* = inoculum type (live vs. sterilized). For log(Total plant mass) and Root:shoot ratio, $n = 187$ plants. For other responses, $n = 93$–94 plants. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, † $P < 0.1$

with live soil inocula had greater total dry mass and lower root:shoot ratio than plants treated with sterilized soil inocula (Table 1; Figs. 2a, 3a), consistent with soil microbes enhancing plant growth and reducing relative allocation to roots under these experimental conditions.

If soil nitrogen regime influenced the growth benefits plants gain from soil microbial communities, we expected to see a significant effect of N_{PC1} on relative growth of plants treated with live soil inocula. Instead, soil nitrogen (N_{PC1}) did not predict the effectiveness of soil microbes for sympatric plant hosts (Table 1; supplementary material, Fig. S4). Other supporting evidence would be a significant interaction effect of N_{PC1} x inoculum type on total plant mass, since this would mean the effect of nitrogen on plant mass is different for live and sterilized inocula, which could be attributed to the microbial content of the live inocula. However, there was no significant effect of N_{PC1} x inoculum type (live vs. sterilized soil) for total plant mass (Table 1; Fig. 2a), indicating that the effect of soil microbes did not depend on soil nitrogen content. N_{PC1} had a significant positive effect on nodule count of sympatric plants, which was driven by low nodule count for the plant/soil combination at the lowest soil nitrogen regime (i.e., BMR) and higher nodule counts in the other plant/soil combinations (Table 1; Fig. 4).

Non-nitrogen soil traits ($NonN_{PC1}$) positively predicted total dry mass and negatively predicted root:shoot ratio of sympatric plants (Table 1). The effect of $NonN_{PC1}$ did not differ between live and sterilized soil inocula (no significant effect of $NonN_{PC1}$ x inoculum type), indicating that differences in the abiotic (i.e., nutrient) content of soil inocula drove the effect of $NonN_{PC1}$ on plant total dry mass and root:shoot ratio, irrespective of differences in the microbial communities. $NonN_{PC1}$ did not predict any other plant responses to sympatric soil inocula (Table 1; supplementary material, Fig. S5-S7). There was a significant effect of

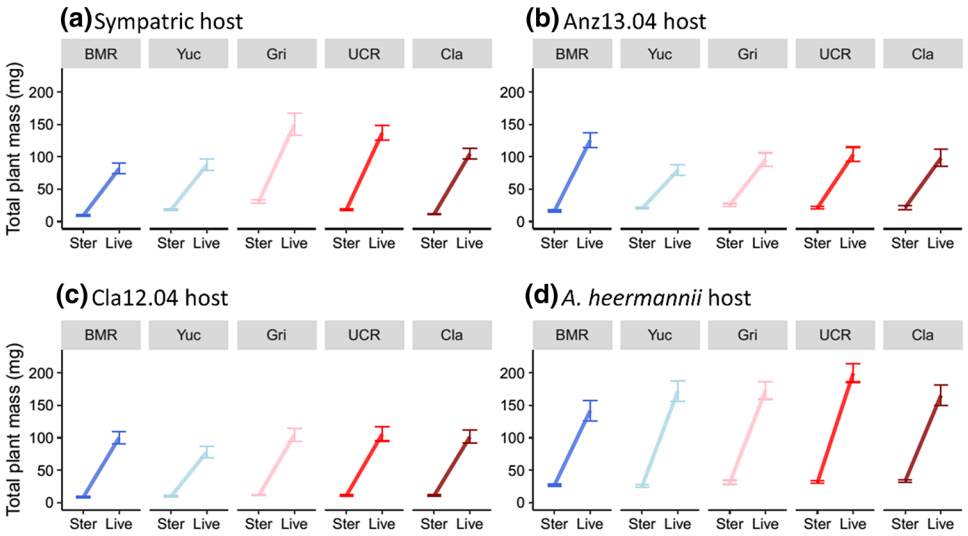

Fig. 2 Effects of sterilized (ster) and live soil inocula on total plant mass of (**a**) sympatric *Acmispon strigosus*, (**b**) *A. strigosus* Anz13.04, (**c**) *A. strigosus* Cla12.04, and (**d**) *A. heermannii* for five soils (BMR, Yuc, Gri, UCR, Cla). Soils are arranged in order of increasing soil nitrogen (i.e., N_{PC1} values; see Fig. 1). Bars represent ± 1 SE

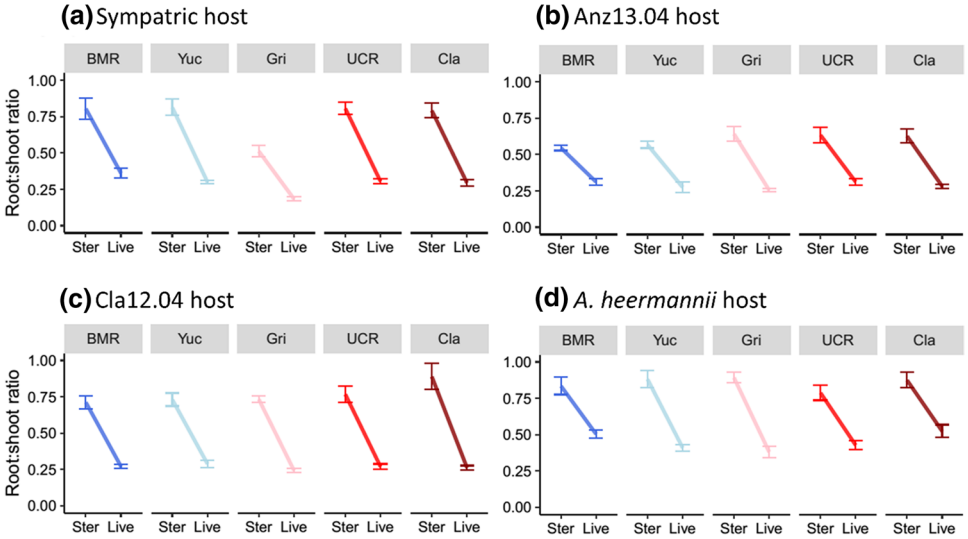

Fig. 3 Effects of sterilized (ster) and live soil inocula on root:shoot ratio of (**a**) sympatric *Acmispon strigosus*, (**b**) *A. strigosus* Anz13.04, (**c**) *A. strigosus* Cla12.04, and (**d**) *A. heermannii* for five soils (BMR, Yuc, Gri, UCR, Cla). Soils are arranged in order of increasing soil nitrogen (i.e., N_{PC1} values; see Fig. 1). Bars represent ± 1 SE

$NonN_{PC2}$ x inoculum type on total plant mass (supplementary material, Table S2), with $NonN_{PC2}$ having a positive effect on total plant mass in the sterilized inoculum treatments, but a negative effect in the live inoculum treatments. Similarly, $NonN_{PC2}$ had negative effects on red nodule frequency and total nodule count of live-inoculated plants (supplementary material, Table S2).

Common Host Experiment: effects of soil traits

N_{PC1} positively predicted total plant mass as a main effect, but there was no significant effect of N_{PC1} x inoculum type (live vs. sterilized), indicating that differential responses to inocula were due to abiotic differences present in both live and sterilized soil (Table 2; Fig. 2b–d). $NonN_{PC1}$ also positively predicted total plant mass as a main effect (Table 2). $NonN_{PC1}$ interacted with inoculum type (live vs. sterilized) in determining root:shoot ratio, suggesting that general soil traits (here, Ca, Mg, and CEC) modify the impact of soil microbes on plant allocation to roots (Table 2). N_{PC1} and $NonN_{PC1}$ did not predict any other plant responses to soil inocula (Table 2; supplementary material, Fig. S4-S7). When we performed analyses without the plants harvested early, however, we detected a significant effect of the $NonN_{PC1}$ x host line interaction on red nodule frequency ($\chi^2 = 6.483$, df = 2, $P = 0.039$). There was also significant effect of the three-way interaction ($NonN_{PC2}$ x inoculum type x host line) on root:shoot ratio and a negative effect of $NonN_{PC2}$ on total nodule mass (supplementary material, Table S3).

Common Host Experiment: effects of plant line

The three plant lines in the common host experiment (two *A. strigosus*, one *A. heermanii*) differed in their responses to

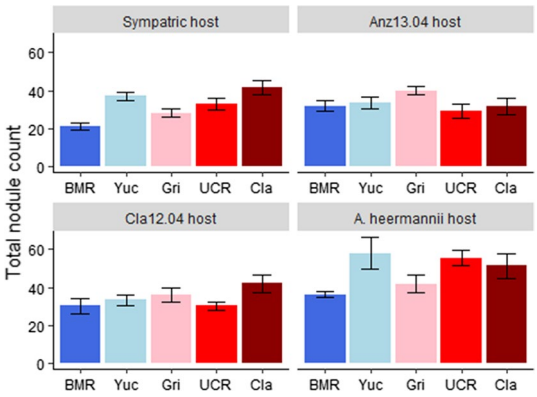

Fig. 4 Variation in total nodule count for four types of *Acmispon* plant hosts (sympatric *A. strigosus*, *A. strigosus* Anz13.04, *A. strigosus* Cla12.04, and *A. heermannii*) in response to inoculation with five soils (BMR, Yuc, Gri, UCR, Cla). Soils are arranged in order of increasing soil nitrogen (i.e., N_{PC1} values; see Fig. 1). Bars represent ± 1 SE

live soil inocula (Table 2). *A. strigosus* Cla12.04 had greater relative growth and red nodule frequency (10.6 ± 0.8 relative growth; 0.69 ± 0.03 red nodule frequency) than either *A. heermannii* (6.0 ± 0.3 relative growth; 0.51 ± 0.03 red nodule frequency) or *A. strigosus* Anz13.04 (5.1 ± 0.4 relative growth; 0.49 ± 0.03 red nodule frequency; Table 2; supplementary material, Fig. S4, Fig. S5). *A. heermannii* had greater nodule mass and total nodule count (14.1 ± 0.7 mg nodules; 48.6 ± 2.7 nodule count) than either *A. strigosus* Cla12.04 (11.0 ± 0.6 mg nodules; 34.3 ± 1.7 nodule count) or *A. strigosus* Anz13.04 (10.6 ± 0.6 mg nodules; 33.1 ± 1.5 nodule count; Table 2; supplementary material, Fig. S6, Fig. 4).

Discussion

No effect of soil nitrogen history on plant benefits from microbes

We find no association between soil nitrogen enrichment and the benefits that wild legumes gain from rhizobial symbionts, even though elevated soil nitrogen is predicted to alter the host control traits that enforce cooperation by nitrogen-fixing symbionts (West et al. 2002a; Kiers et al. 2006). Our results align with empirical work suggesting that legume host control is maintained, rather than weakened or enhanced, under nitrogen fertilization (Regus et al. 2014; Grillo et al. 2016; Wendlandt et al. 2019). In one study showing that nitrogen fertilization compromised host control over nodule size, weakened host control did not translate into a projected fitness benefit for rhizobia (Oono et al. 2020). Moreover, host control is robust in *A. strigosus* plant lines from high soil nitrogen regimes, and the capacity of *A. strigosus* to sanction ineffective symbionts in nodules is not affected by the degree of nitrogen enrichment of their home

Table 2 Likelihood ratio test χ^2 statistics for fixed effects in GLMMs modeling traits of three *Acmispon* plant hosts inoculated with five soils in the Common Host Experiment

Model, response	Model terms						
	P df = 1	I df = 1	H df = 2	P:I df = 1	P:H df = 2	I:H df = 2	P:I:H df = 2
$Nitrogen_{PC1}$ models							
log(Total plant mass)	9.60*	577.07***	185.39***	1.48	2.92	57.08***	1.36
Root:shoot ratio	2.35	361.99***	100.044***	3.22†	1.93	24.90***	3.81
Plant relative growth	2.71†		50.91***		1.11		
Red nodule frequency	0.43		45.50***		3.26		
Total nodule mass	3.16†		20.02***		2.31		
Total nodule count	2.23		33.58***		2.56		
Mean nodule size	0.20		3.31		0.97		
$NonNitrogen_{PC1}$ models							
log(Total plant mass)	6.77*	573.36***	184.82***	2.97†	0.61	56.39***	3.26
Root:shoot ratio	0.0015	359.39***	100.33***	6.69*	0.33	25.85***	0.52
Plant relative growth	3.81†		51.25***		2.28		
Red nodule frequency	0.71		45.65***		4.87†		
Total nodule mass	0.13		19.62***		1.80		
Total nodule count	1.13		33.36***		2.72		
Mean nodule size	3.78†		3.39		5.40†		

P = PC1 for either soil nitrogen or non-nitrogen soil traits. *I* = inoculum type (live vs. sterilized). *H* = host line (*A. strigosus* Anz13.04, *A. strigosus* Cla12.04, or *A. heermannii*). For log(Total plant mass) and Root:shoot ratio, $n = 290$ plants. For other responses, $n = 149$–150 plants. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, † $P < 0.1$

soil (Wendlandt et al. 2019). Since host control is predicted to be the main driver of rhizobial effectiveness (West et al. 2002b), the simplest explanation for our findings is that host control traits in *A. strigosus* have remained effective at selecting for rhizobial nitrogen fixation, even after decades of soil nitrogen enrichment from atmospheric deposition.

The hypothesis that elevated soil nitrogen will alter host control traits assumes that exogenous nitrogen changes the cost:benefit ratio of symbiosis for plants by giving them a less-costly alternative to symbiotic nitrogen fixation. As an alternative hypothesis, we propose that elevated soil nitrogen primarily affects symbiotic investment, rather than partner choice or host sanctions. If plants reduce investment into symbiosis when they have access to plentiful soil nitrogen, this could prevent symbiosis from becoming too costly, thereby maintaining selection for partner choice and sanctions, which are the main forces selecting for cooperation by rhizobia. There is evidence that plants with access to soil nitrogen reduce their investment in symbiosis, by either reducing nodule growth (Regus et al. 2015) or reducing nodule nitrogen fixation activity (Gan et al. 2004; Naudin et al. 2011). Indeed, decreased nitrogen fixation by nodules is a common response to many plant abiotic stressors (Valentine et al. 2011), showing that symbiotic investment by plants is highly plastic. A key prediction of our hypothesis is that the cost:benefit ratio of symbiosis for legumes can remain stable over a wide range of soil nitrogen levels, due to plants adjusting their investment into symbiosis. It would be useful for future research to test this hypothesis by explicitly measuring these symbiotic costs and benefits. It would also be interesting to study whether symbiotic investment can evolve, which could constrain or enhance the ability of plants to keep the cost:benefit ratio of symbiosis stable. Overall, we hypothesize that legumes are physiologically equipped to maintain a stable cost:benefit ratio of symbiosis across a wide range of soil nitrogen conditions, which prevents elevated soil nitrogen from imposing selection to alter the strength of host control traits.

Although our work suggests that legume host control is not influenced by elevated soil nitrogen, plant populations still show significant genotypic variation in the efficiency of host control over rhizobia (Simonsen and Stinchcombe 2014; Wendlandt et al. 2019), and the drivers of this variation are poorly understood. Previous work on soybean suggested that crop breeding in high nitrogen soils has caused a decline of sanctioning ability in many newly released cultivars relative to older cultivars (Kiers et al. 2007). However, changes in sanctions in soy could have been driven by other domestication-related phenomena, such as trade-offs with other agronomic traits or genetic drift due to low effective population sizes. Whether these other domestication-related changes have affected symbiosis is an area of active research (Liu et al. 2020; Porter and Sachs 2020). In wild legumes, variation in host control could be maintained across populations by spatial differences in local coevolutionary dynamics with rhizobia, such that some legume populations experience selection for stricter sanctions due to high local abundance of exploitative rhizobia, whereas other legume populations experience relaxed selection for sanctions because exploiters are rare (Steidinger and Bever 2014).

Previous work finding evidence of nitrogen driving changes in mutualism benefits for plants has considered only effects of nitrogen addition on the plant host (Kiers et al. 2007) or on the rhizobia (Simonsen et al. 2015; Weese et al. 2015). In our study system, however, both plant hosts and soil microbial communities could have evolved in response to soil nitrogen enrichment, potentially generating context-dependency in the benefits plants gain from soil microbes (van Cauwenberghe et al. 2016). For instance, soil nitrogen could predict the growth benefits microbes provide to sympatric hosts (due to coevolution between local hosts and microbes) but fail to predict the benefits microbes provide to allopatric hosts (here, plants in the common host experiment). However, we did not find evidence for context-dependency in the benefits plants gain from soil mutualists. All nodule-forming soil inocula produced very similar growth effects on individual host lines, suggesting that elevated soil nitrogen levels have not altered nitrogen fixation by rhizobia, whether through direct selection on rhizobia populations, direct selection on legume host control traits, or coevolutionary selection on sympatric hosts and microbes.

Plant genotypic variation in symbiosis traits

We show that *Acmispon strigosus* plant lines vary in their ability to gain growth benefits from soil microbes. One *A. strigosus* line (Cla12.04) gained about twice the relative growth benefits from microbes as the other *A. strigosus* line (Anz13.04), despite the two lines forming similar numbers of root nodules. Past work on *A. strigosus* also found plant genotypic variation in benefits from microbes (Wendlandt et al. 2019), although in response to clonal rhizobium cultures rather than soil slurries. Thus, the effect of *A. strigosus* lines on the benefits obtained from microbes, particularly rhizobia, need not depend on a particular rhizobium genotype or mixture of genotypes. Other researchers have found evidence of plant genotypic variation in response to beneficial microbes outside the legume-rhizobia system, suggesting that the traits contributing to superior benefits from microbes could be functionally diverse (Wintermans et al. 2016).

Acmispon strigosus plant lines also vary in the number of root nodules formed with rhizobia. Soil nitrogen regime predicted the nodule count of plants inoculated with sympatric soils, with high-nitrogen soil inocula forming more nodules than low-nitrogen soil inocula. The positive relationship

between soil nitrogen regime and sympatric host nodule count was driven by the low number of nodules formed by the BMR plant/soil combination at the lowest soil nitrogen regime. Two pieces of evidence suggest that the low number of nodules formed by BMR soil on BMR plants is due to the plant host genotype, rather than the microbial community. First, BMR plants inoculated with a beneficial *Bradyrhizobium* strain form fewer and larger nodules than other *A. strigosus* plant lines receiving the same inoculum (Wendlandt et al. 2019), providing independent evidence of plant genotypic variation in nodule-forming capacity. Second, the three plant lines in the common host experiment did not form low numbers of nodules with BMR soil, relative to other soil inocula, suggesting that BMR soil has a sufficient rhizobia population to form many nodules and is comparable in nodule-forming capacity to other soils in this study. Plant genotypic variation in nodule number could create variation in the intensity of plant selection on rhizobia populations, which could contribute to a geographic mosaic of coevolution between legumes and rhizobia (Thompson 2005; van Cauwenberghe et al. 2016).

Physical soil drivers of microbial effectiveness for plants

Although we found no evidence of soil nitrogen influencing plant benefits from microbes, non-nitrogen soil traits ($NonN_{PC1}$ and $NonN_{PC2}$) significantly influenced the effect of microbes on several plant traits, indicating that we had power to detect any effects of nitrogen enrichment on microbial services. Furthermore, the effects of soil traits other than nitrogen on soil microbial effectiveness could shed light on constraints microbes face when experiencing selection by plant hosts versus the soil environment. For instance, the soil properties captured by $NonN_{PC2}$ appear to reduce the ability of soil microbes to promote plant growth, by reducing total nodule count, nodule mass, and the proportion of nodules that are fixing nitrogen. $NonN_{PC2}$ was mainly influenced by soil phosphorus, indicating that this nutrient could be a key determinant of plant benefits from soil microbes. Soil microbes and non-nitrogen soil traits also showed interactive effects on root:shoot ratio, which reflects the relative allocation of plants to belowground growth. Plants typically increase their root:shoot ratio when they are limited by nitrogen and/or phosphorus (Poorter and Sack 2012) and decrease root:shoot ratio when they have more access to these nutrients. Both $NonN_{PC1}$ and live soil inoculations reduced the root:shoot ratio of plants, consistent with these treatments increasing plant access to nutrients. Furthermore, soil microbes accounted for some of the relationship between non-nitrogen soil traits and root:shoot ratio, since plants treated with live and sterilized inocula differed in how $NonN_{PC1}$ affected root:shoot ratio. Since $NonN_{PC1}$ mainly reflects differences in soil cation exchange capacity (the nutrient-holding capacity of soil), this suggests that soils with higher CEC (here, Gri soil), contain microbial populations that are better able to supply plants with nutrients than microbes from other soils, potentially by increasing the abundance and diversity of soil microbes (Lynn et al. 2017; Sen and Sengupta 2018). It will be interesting for future work to more deeply explore the mechanisms linking physicochemical soil traits to the benefits plants gain from soil microbial communities.

Conclusions and future directions

We find that *Acmispon* plant benefits from soil microbial communities are not predicted by long-term differences in soil nitrogen levels but do show strong structuring by plant genotype. We confirm previous measurements of soil nitrogen differences among the field sites we sampled (Regus et al. 2017a), increasing the evidence that nitrogen deposition causes sustained increases in soil nitrogen in natural areas. The whole soil inoculation approach we used captures net effects of nitrogen on microbial effectiveness for plants, integrating over many potential sources of variation in microbial effectiveness such as rhizobia population size, rhizobia genotypic composition, and the composition and functional traits of the broader soil microbial community, which would be valuable areas of inquiry for future research. Understanding the genetic basis of plant genotypic variation in symbiosis traits should also be a priority as genomics and high-throughput phenotyping become more accessible resources. Such work may shed light on the mechanisms by which plants regulate investment into symbiotic services in a changing environment.

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Declarations

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