PROCEEDINGS B

royalsocietypublishing.org/journal/rspb

Research

Cite this article: Fronk DC, Ortiz-Barbosa GS, Macedo F, Lee J, Wun K, Sachs JL. 2025 Nitrogen fertilization nullifies host sanctions against non-fixing rhizobia and drives divestment from symbiosis in *Lotus japonicus*. *Proc. R. Soc. B* **292**: 20251055.
https://doi.org/10.1098/rspb.2025.1055

Received: 21 April 2025
Accepted: 11 June 2025

Subject Category:
Ecology

Subject Areas:
evolution, ecology, plant science

Keywords:
fertilization, host control mechanisms, nitrogen fixation, nutritional mutualism, robustness, rhizobia

Author for correspondence:
Joel L. Sachs
e-mail: joels@ucr.edu

Electronic supplementary material is available online at.

THE ROYAL SOCIETY PUBLISHING

Nitrogen fertilization nullifies host sanctions against non-fixing rhizobia and drives divestment from symbiosis in *Lotus japonicus*

David Charles Fronk, Gabriel Santiago Ortiz-Barbosa, Fatima Macedo, Jason Lee, Kaylie Wun and Joel L. Sachs

Department of Evolution, Ecology and Organismal Biology, University of California Riverside, Riverside, CA 92521, USA

JLS, 0000-0002-0221-9247

Plants and animals house microbes that provide critical nutrients, but little is known about host control over microbial cooperation when resources are also accessed from the environment. Changes in nutrient access can challenge the host's ability to detect and selectively reward beneficial partners, destabilizing symbiosis. Legumes acquire nitrogen from soil and from symbiosis with rhizobia, but it is unclear if extrinsic sources of nitrogen interfere with host control systems. We inoculated the legume *Lotus japonicus* with rhizobia-bearing nitrogen fixation or nitrogen metabolism knockouts, and factorially varied molecular sources of nitrogen fertilizer. *Lotus* hosts selectively rewarded beneficial rhizobia and sanctioned non-fixing strains when extrinsic nitrogen was unavailable. Host benefits were undiminished when inoculated with rhizobia-bearing nitrogen metabolism knockouts, suggesting redundancies in nitrogen provisioning systems. However, under nitrogen fertilization, hosts did not discriminate between fixing and non-fixing rhizobia. Fertilized hosts formed miniaturized nodules housing limited rhizobia, divesting from symbiosis. Thus, sanctioning mechanisms rely on the detection of nitrogen fixation differences among rhizobia strains and can break down in nitrogen-rich environments. Nonetheless, divestment from symbiosis offers legumes robust host control, minimizing investment into rhizobia strains, irrespective of their capacity to provide benefit, when symbiosis services are not needed.

1. Introduction

In symbiotic associations with diverse microbes, plants and animals gain growth-limiting nutrients that they cannot otherwise reliably access [1–3]. Nutritional mutualisms appear of particular importance to plants. Most land plants associate with mycorrhizal fungi that can provide phosphorus to host plants, as well as other benefits [4]. Legumes associate with rhizobia, a polyphyletic group of proteobacteria that instigate nodule formation on roots or stems and fix nitrogen for the host [5,6]. Nodulation by rhizobia can provide legumes with substantial growth benefits, especially in habitats where nitrogen would be unreliable and growth-limiting if only accessed from the soil. However, rhizobia vary in the amount of nitrogen that they fix for hosts, ranging from highly beneficial to ineffective, i.e. non-fixing [7–9]. Thus, the net benefits that hosts receive from nutritional symbioses are unpredictable and can be affected by the intrinsic capacities of the microbe to

provide nutrients and by nutrient availability in the environment, which can reduce the net benefit of symbiosis [8,10–14].

Plant and animal hosts have evolved systems to regulate investment into symbiosis depending on net benefits [15,16]. Host control in the legume rhizobia symbiosis can occur at two stages. Prior to nodule organogenesis, hosts regulate which symbiotic partners can infect them based on genetic compatibility. Species-specific flavonoids secreted by legume hosts attract compatible rhizobia, which in turn release Nod factors that allow them to infect host tissue and induce nodule formation [17,18]. After root nodules are formed, legumes preferentially enhance the growth of nodules containing beneficial rhizobia, while sanctioning non-beneficial symbionts through cellular control over nodule senescence [13,19,20]. Host control mechanisms by legumes are thought to optimize host fitness by minimizing investment into uncooperative symbionts compared with beneficial strains [20–24].

In nutrient-rich environments, plants can access nutrients from symbiotic or mineral sources. Mycorrhizal fungi can provide phosphorus to hosts, but in phosphorus-enriched soils, these benefits are reduced or eliminated [25,26]. In parallel, legumes can access nitrogen from rhizobia or from the soil. Substantial plant metabolic resources are required to support root nodules with rhizobia that fix nitrogen [27–29], generating an opportunity cost for investing in symbiotic nitrogen when soil nitrogen is available [19,30–32]. Many legumes can minimize investment into symbiosis when mineral nitrogen is available, by forming fewer or smaller nodules, reducing metabolic support to nodules or downregulating nitrogen fixation, though the effects on rhizobia fitness are poorly understood [13,27,29,33–39]. What is more, some research has suggested that host sanctioning of non-fixing rhizobia can occur in nitrogen-rich soils, and hence that hosts can differentiate biologically fixed nitrogen (i.e. from symbionts) from mineral nitrogen (i.e. from soil; [35,40,41]). However, a limitation is that these experiments used different genotypes of rhizobia that vary in nitrogen fixation and thus cannot differentiate the host's capacity to discriminate among rhizobia genotypes versus host detection of nitrogen fixation differences among them. How host legumes might recognize different sources of nitrogen remains unknown, and there is disagreement over which molecular forms of nitrogen are transferred from symbionts to hosts, which might be differentiated from sources in the soil [29,42]. Thus, two related knowledge gaps must be addressed. First is whether legumes can sanction non-fixing rhizobia when they are fertilized with nitrogen, given that the host might be unable to differentiate symbiotic versus mineral sources of nitrogen. Second is whether rhizobia transfer organic forms of nitrogen to hosts, such as alanine or aspartate, as this is a predicted mechanism to provide nitrogen to the plant that can be differentiated from other sources in the soil [29]. Providing a recognizable signal of its cooperation could benefit the symbiont, as this is predicted to promote directed reciprocation by the host to cooperative strains [16].

Here, we conducted two parallel experiments to address these knowledge gaps. First, in a fertilization experiment, we factorially exposed *Lotus japonicus* plants to different molecular forms of nitrogen fertilizer and inoculations of fixing and non-fixing strains of their symbiont, *Mesorhizobium loti*. The inorganic nitrogen sources, including ammonium sulfate and potassium nitrate, were used to simulate nitrogen-rich soils [43], whereas the organic sources alanine and aspartate provided nitrogen molecules predicted to be excreted *in planta* by rhizobia [44–46]. Rhizobia treatments included clonal inoculation with a beneficial strain of *M. loti*, a near-isogenic non-fixing mutant, a mixture of both strains, or sterile water as a control, allowing examination of host control over investment into symbiosis and discrimination against non-fixing rhizobia in clonal and mixed strain settings. We hypothesize that under nitrogen fertilization, *Lotus* hosts cannot detect nitrogen fixation differences between otherwise isogenic rhizobia. We predict that sanctions, the capacity of the host to selectively reduce the fitness of non-fixing rhizobia, would be nullified under these conditions.

Second, in a gene knockout experiment, *M. loti* with single gene inactivating mutations for nitrogen metabolism was inoculated on *L. japonicus* to test for metabolic pathways that are required for rhizobia to fix nitrogen for hosts. Mutant strains were selected with knockouts of enzymes specific to alanine and aspartate metabolism, whose transfer to the host is thought to be critical for host uptake of fixed nitrogen, based on experimental inoculation studies [44–46], as well as metabolic and gene expression data [42,47]. We hypothesized that genes for alanine and aspartate metabolism are required for nitrogen transfer to hosts. We predicted that strains with knockout mutations for these pathways would provide no benefits to hosts. Both experiments examined the importance of alanine and aspartate as sources of nitrogen predicted to be transferred from symbiont to host that the host might respond to differently from other forms of nitrogen—as tested in the fertilization experiment—or that might eliminate symbiotic benefits to the host if their metabolic pathways are inactivated—as tested in the knockout experiment.

2. Material and methods

(a) Sole nitrogen source fertilization experiment

The *L. japonicus* ecotype Miyakojima MG-20 was used, inbred at the University of California Riverside to generate seeds following published protocols [48]. The *M. loti* genotype MAFF303099 (hereafter MAFF) is a highly effective nitrogen-fixing strain on *L. japonicus*. MAFF was modified to express dsRed, a red fluorescent protein visible under natural light [49]. The near-isogenic strain STM6 (strain ID 17T02d02) was employed as a non-nitrogen-fixing mutant. STM6 was generated by a signature-tagged transposon inserted in the *nifD* gene [50] and was subsequently modified to express green fluorescent protein (GFP) [48]. Both modified strains efficiently form nodules on *L. japonicus*, with MAFF providing substantial benefits to host growth while STM6 provides none [48]. MAFF and STM6 were grown on agar plates with a modified arabinose gluconate (MAG) medium [51]. In mixed cultures, MAFF and STM6 can be easily differentiated by colony colour. Other than differences in nitrogen fixation and colony colour, previous work uncovered no differences between the strains in terms of their *in vitro* growth rate or capacity to induce nodule formation under clonal conditions [48].

Lotus japonicus plants thrive under organic and inorganic sources of extrinsic nitrogen (i.e. alanine, aspartic acid, ammonium sulfate, potassium nitrate), and plants fertilized with these nitrogen sources reduce investment in nodules infected with MAFF [29]. In this experiment, *L. japonicus* was exposed to fertilization treatments and rhizobia that vary in nitrogen fixation to quantify effects on rhizobia fitness and examine how sanctions traits respond under fertilization. *Lotus japonicus* seedlings were exposed to clonal treatments of nitrogen-fixing MAFF and non-fixing STM6 and mixed infections of both strains. Rhizobia cell proliferation, dry nodule biomass and nitrogen fixation were quantified to examine rhizobia fitness, and fitness effects *in planta* under different treatments.

Lotus japonicus seeds were surface-sterilized in bleach (5% sodium hypochlorite), rinsed in sterile water, nick-scarified and planted under axenic conditions in bleach-sterilized Ray-Leach SC10 pots (Stuewe & Sons, Corvallis, OR, USA) using an equal mix of coarse sand on top of a layer of fine calcined clay that was autoclave-sterilized (Pro League, Quickdry; Turface Athletics, Buffalo Grove, IL, USA). In a factorial experiment, plants were inoculated on 21 December 2021 with one of four treatments including MAFF, STM6 an equal mix of both strains and sterile ddH_2O as a control. Inoculations were 5 ml and included 5×10^8 cells, except for the water controls. High cell concentrations are needed to assure consistent nodulation in sterile coarse soils, likely because of low bacterial survival during inoculation. For mixed inoculation treatments, 2.5×10^8 cells of each strain were combined, and the mixed inoculum was plated to empirically confirm the ratio of MAFF to STM6. All plants were supplemented weekly with 5 ml of Broughton & Dilworth solution, including key nutrients but lacking nitrogen [52]. Hosts were fertilized with one of four sources of nitrogen dissolved in the Broughton & Dilworth solution, including alanine ($C_3H_7NO_2$, 2.64 g l^{-1}), ammonium sulfate ($(NH_4)_2SO_4$, 3.92 g l^{-1}), aspartic acid ($C_4H_7NO_4$, 3.95 g l^{-1}) or potassium nitrate (KNO_3, 3 g l^{-1}). Concentrations were set to equalize the nitrogen moiety at levels previously shown to optimize host growth in the absence of rhizobia inoculation, thus attempting to isolate the growth effect of nitrogen [29,40]. Fertilization occurred weekly, starting 1 day before inoculation (i.e. 20 December 2021) with 2 ml of fertilizer per plant and increasing by 1 ml per week until a maximum of 5 ml was reached three weeks later. This final level of fertilizer was maintained until the plants were harvested.

Fourteen experimental blocks each contained a single plant replicate per each of the 20 treatments, including four inoculation treatments (i.e. MAFF, STM6, MAFF+STM6, water) crossed with five fertilization treatments (i.e. alanine, ammonium, aspartate, nitrate, no added nitrogen), totalling 280 plants. To minimize within-block variance, seedlings were assigned to blocks based on the size at the time of inoculation. Treatments within blocks were assigned randomly. Two blocks were harvested at three weeks post-inoculation, the time when nodules first become visible on *L. japonicus* roots. Two additional blocks were harvested five weeks post-inoculation, when most nodules have typically established. Data from early harvests were used to quantify rhizobia nodulation and growth effects over time in relation to fertilization treatments. The remaining 10 blocks were harvested at week 7 post-inoculation to quantify dry root and shoot biomass, nodule count and dry nodule biomass, to quantify rhizobia fitness *in planta* (i.e. rhizobia population size in nodules) and to test for evidence of host sanctions (i.e. over-representation of the nitrogen-fixing strain MAFF in the nodules of coinoculated plants). The mixed inoculum was plated to estimate the initial ratio of MAFF : STM6 by distinguishing colony colour.

During harvests, plants were removed from pots, washed free of soil and dissected into root, shoot and nodule portions. Nodules were counted and photographed. Within each inoculated treatment, four plants were randomly selected for quantitative culturing, wherein two nodules each were randomly sampled, surface-sterilized in bleach for 30 s, followed by three rinses in autoclave-sterilized ddH_2O. Nodules were then crushed in 200 µl of sterile ddH_2O and the slurry was serially diluted to 0.5×10^{-6} and 0.5×10^{-8} on MAG-agar plates. Colony counts were averaged across at least two plate replicates per nodule to estimate rhizobia population size within each nodule. The ratio of MAFF : STM6 colonies was estimated from nodules plated from coinoculated plants and compared with the MAFF : STM6 ratio in the initial inoculum. Roots, shoots and remaining nodules were weighed for dry biomass after being oven-dried (>3 days, 60°C). Ten plants were removed from the analysis, five owing to mortality and five owing to contamination.

Leaf $\partial^{15}N$ 'atom per cent difference' was estimated as the percentage of ^{15}N atoms over total nitrogen in each sample, and the values were used as a measure of nitrogen fixation [40,53]. Only plants harvested at seven weeks post-inoculation were used. Individual leaves from each plant were oven-dried, weighed and placed in tin capsules for isotopic analysis at the UC Davis Stable Isotope Facility.

(b) Nitrogen metabolism knockout experiment

Rhizobia strains with individual gene knockouts were inoculated onto *L. japonicus* MG-20 plants to test for changes in nodulation or host benefits. *Mesorhizobium loti* strains were selected from a large collection of single-gene mutants, focusing on knockouts of genes whose loss is predicted to disrupt the symbiotic transfer of nitrogen from rhizobia to the host. No nitrogen fertilizer was used, so we could examine the effects of these mutations when hosts have no other nitrogen source. Five *M. loti* strains were tested, including MAFF as a control, and four near-isogenic, signature-tagged mutants [50]. *Mesorhizobium loti* 03T03g02 has a knockout for a periplasmic amino acid ABC transporter (mll3861; [54]), a protein similar to one in *Rhizobium* that transfers alanine or aspartate to the host [44]. *Mesorhizobium loti* 27T02f13 has a knockout for L-2,4-diaminobutyric acid transaminase (mlr5943), an enzyme involved in alanine synthesis that is upregulated in nodules compared to growth on media, suggesting the importance of nitrogen transfer to the host during symbiosis [55]. The last two mutants carry knockouts for paralogs of aspartate aminotransferase, which produces aspartate from oxaloacetate, predicted to be a mechanism to generate nitrogen usable to the host. *Mesorhizobium loti* 8T11d03 carries a knockout for mlr5883, encoded on the symbiosis island, and whose expression is upregulated *in planta* during symbiosis [56], whereas *M. loti* 20T04d01 has a knockout for the other copy,

mlr2541, which is encoded on the bacterial chromosome, and is constitutively expressed [57]. None of the mutants has been previously tested on *L. japonicus*.

Fifteen replicate plants per treatment were singly inoculated with one of the mutant strains, with MAFF, or with sterile water, to examine nodulation traits and growth effects, totalling 90 plants. Plants were prepared, inoculated and maintained as outlined in the fertilization experiment, with the modification of only receiving the nitrogen-free Broughton & Dilworth (micronutrient) solution. Plants were harvested seven weeks after inoculation to quantify dry root and shoot biomass, nodule count, and dry nodule biomass.

(c) Symbiosis trait measurements

Host biomass was used to estimate host growth in different treatments. Nodulation was used to estimate host investment into symbiosis, quantified as the number of nodules formed, their mean biomass or total nodule biomass per plant. Host investment into symbiosis was also calculated by dividing the dry nodule biomass of each plant by the total plant biomass [58]. Rhizobia population size in nodules, a proxy of rhizobia fitness *in planta*, was calculated by quantitative culturing of nodules of individual plants. The ratio of MAFF : STM6 colonies cultured from coinoculated plants was used to analyse the relative fitness of the beneficial strain and to test for sanctions. Nitrogen fixation was measured using the abundance of ^{15}N in the plant shoots from different treatments.

(d) Data analysis

Statistical analyses were conducted using linear mixed models in JMP (v. 16.0.0). All data distributions were tested for normality using the Shapiro–Wilk test, as this is a prerequisite for parametric statistics. Data were transformed when necessary to achieve normal distributions to satisfy this requirement. In some cases, log or square root transformations were used. Full models, transformations and random versus fixed factors are all listed in the electronic supplement. Tukey's tests were used to test for significant differences among groups in a *post hoc* manner, after observing a significant effect. The $\partial^{15}N$ of each sample was calculated by comparing ^{15}N abundance expressed as parts per thousand with atmospheric N_2. The values were then used to compare values of $\partial^{15}N$ between treatments by subtracting the mean sample atom% ^{15}N of uninoculated plants from the sample atom% ^{15}N of inoculated plants. When plants incorporate fixed nitrogen, leaf tissues exhibit a decrease in ^{15}N (compared with uninfected plants) owing to isotopic fractionation by rhizobia [40]. To test for sanctions, a χ-squared test was used to test whether MAFF was over-represented in nodules of coinoculated plants, relative to the proportion of MAFF in the inoculum.

3. Results

(a) Nitrogen fertilization caused hosts to reduce fitness of rhizobia in a strain-specific manner

Inoculation and fertilization each independently caused significant effects on *L. japonicus* growth and nodulation (electronic supplementary material, tables S1 and S2). When plants were inoculated and fertilized, all benefits of symbiosis were eliminated, such that inoculation with neither MAFF nor STM6 caused any growth effects on *L. japonicus* (figure 1 and table 1; electronic supplementary material, tables S3 and S4). For plants inoculated with MAFF, fertilization caused significant reductions in total nodule biomass ($F_{4,42} = 9.95$; $p < 0.0001$), mean nodule biomass ($F_{4,42} = 9.57$; $p < 0.0001$), nodule count ($F_{4,42} = 9.57$; $p < 0.0001$) and within-nodule rhizobia populations of MAFF ($F_{4,35} = 9.20$, $p < 0.0001$) and MAFF+STM6 ($F_{4,31} = 3.97$; $p = 0.0103$; figures 2 and 3; table 2; electronic supplementary material, tables S5–S7). Overall, fertilized plants inoculated with MAFF reduced investment into symbiosis to levels similar to unfertilized plants inoculated with STM6 (electronic supplementary material, table S5). These findings are the first to demonstrate that fertilization not only leads to reduced investment in nodule tissue by *L. japonicus* plants but also is associated with substantial fitness reduction of the rhizobia, to the same minimal fitness experienced by non-fixing symbionts (electronic supplementary material, tables S4, S6 and S8).

Conversely, in plants inoculated with only STM6, nitrogen fertilization had no significant effect on total nodule biomass ($F_{4,40} = 1.63$; $p = 0.1864$), mean nodule biomass ($F_{4,40} = 0.17$; $p = 0.9505$), nodule count ($F_{4,40} = 1.21$; $p = 0.3233$) or rhizobia population size within nodules ($F_{4,27} = 2.10$; $p = 0.109$; figure 3, table 2; electronic supplementary material, tables S7 and S8). These findings demonstrate that reduced investment occurred in a strain-specific manner, most likely responding to the presence or absence of nitrogen fixation since that is the only relevant difference between MAFF and STM6 other than the expression of red versus green fluorescent proteins [48].

The control treatments confirmed previous work on *L. japonicus* [29,48], specifically that MAFF provided significant growth benefits to unfertilized plants, STM6 did not, and all nitrogen sources caused substantial growth benefit to uninoculated plants (figure 1; electronic supplementary material, tables S1–S5 and figure S1). All plants inoculated with rhizobia formed nodules, irrespective of fertilization treatment (electronic supplementary material, table S5 and figure S2). Harvest data from three and five weeks post-inoculation uncovered significant effects of fertilization on host biomass but no effect of nitrogen treatment on initial nodulation (electronic supplementary material, tables S9 and S10).

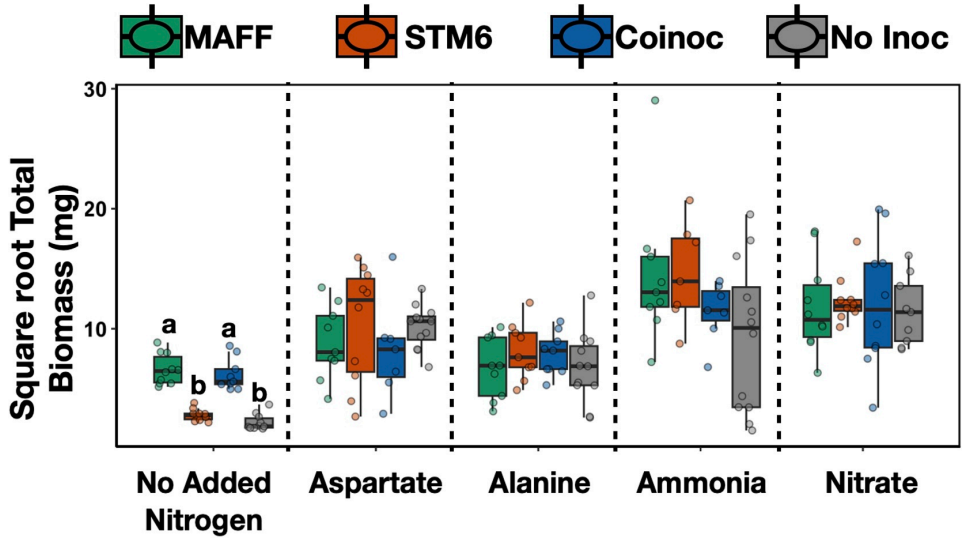

Figure 1. Effects of inoculation treatments on the growth of *L. japonicus* plants fertilized with different nitrogen sources. Plants were inoculated with MAFF, STM6, MAFF+STM6 (Coinoc) or with water (No Inoc). Values for total plant biomass are square root transformed. Connecting letter reports indicate significant differences between inoculation treatments within each nitrogen source treatment group. Points represent individual plant replicates. The upper and lower limits of the box correspond to the first and third quartiles. The upper whiskers extend from the box to the largest value.

Table 1. Linear mixed models testing effects of inoculation and fertilization on symbiosis traits.

	host growth			nodule investment							
	sqrt total biomass			log nodule number			sqrt nodule biomass			log mean nodule biomass		
fixed effect	*F*	d.f.	*p*-value	*F*	d.f.	*p*-value	*F*	d.f.	*p*-value	*F*	d.f.	*p*-value
inocula	2.625	3	0.0523	1.310	2	0.2738	0.5539	2	0.5762	0.9531	2	0.3886
nitrogen source	33.94	4	<0.0001	7.003	4	<0.0001	9.012	4	<0.0001	8.385	4	<0.0001
inocula × nitrogen source	2.021	12	0.0254	1.393	8	0.2067	2.923	8	0.0052	2.616	8	0.0115

random effect	variance ratio	Wald *p*-value	variance ratio	Wald *p*-value	variance ratio	Wald *p*-value	variance ratio	Wald *p*-value
block	0.0047	0.8762	−0.0462	0.0008	−0.058	<0.0001	−0.0043	0.8972

(b) Nitrogen fixation was reduced in a subset of fertilizer treatments

No difference was found in nitrogen fixation among unfertilized plants inoculated with MAFF versus those coinoculated with MAFF+STM6 (electronic supplementary material, table S11). Among plants inoculated with MAFF (i.e. also including coinoculated plants), fertilization with alanine or nitrate caused significant reductions in nitrogen fixation, as both of these fertilizer treatments caused higher $\Delta^{15}N$ values than the unfertilized treatment (alanine, $\Delta^{15}N = -0.708 \pm 2.011$; $p = 0.0261$; nitrate, $\Delta^{15}N = 0.095 \pm 1.067\%$; $p = 0.0001$; figure 4; electronic supplementary material, tables S11 and S12). Conversely, no significant reduction of nitrogen fixation was evident for the aspartate or ammonia treatments, respectively ($\Delta^{15}N = -1.842 \pm 2.916\%$; $p = 0.7768$; $\Delta^{15}N = -1.050 \pm 1.755\%$; $p = 0.1414$). The fact that nitrogen fixation was significantly reduced under a subset of fertilizer treatments suggests that the host can detect and respond to different molecular sources of nitrogen.

The control treatments confirmed the nitrogen fixation phenotypes of each strain. Unfertilized plants inoculated with MAFF had mean $\Delta^{15}N$ levels of approxmately −2.5, indicating efficient nitrogen fixation [40,53], irrespective of being singly infected (MAFF) or coinfected (MAFF+STM6; electronic supplementary material, tables S12 and S13). Plants receiving water or STM6 had $\Delta^{15}N$ levels near zero, indicating no evidence of nitrogen fixation (electronic supplementary material, table S12).

(c) Nitrogen fertilization nullified host sanctions against non-fixing rhizobia

Fertilized plants that received mixed inoculations of MAFF and STM6 produced nodules without a significant over-representation of MAFF (i.e. 1.20×; table 3; electronic supplementary material, table S6 and figure S3); hence there was no evidence of sanctions against the non-fixing STM6 mutant. This was in contrast to the unfertilized controls, wherein plants that received mixed inoculations were predominantly infected by MAFF (i.e. 2.20×), a statistically significant over-representation relative to the initial inoculum proportion (i.e. >2×; $\chi^2 = 5.115$; $p = 0.024$). Fertilization had a disproportionate effect on MAFF. The unfertilized plants with mixed inoculations supported significantly larger populations of MAFF in nodules than in the fertilized treatments (i.e. mean rhizobia per nodule; $t = 3.93$, $p = 0.0002$; figure 3; electronic supplementary material, table S6). No such

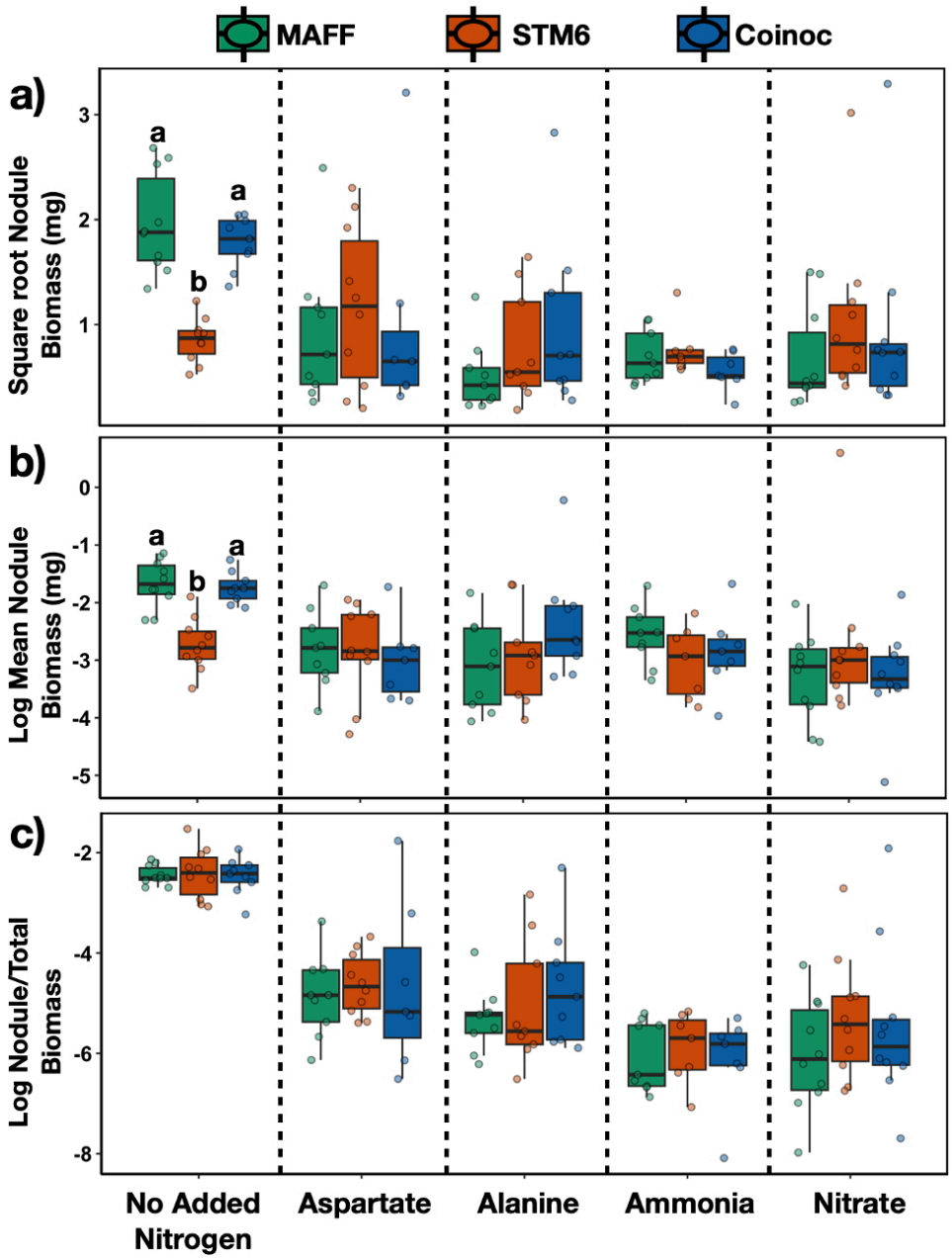

Figure 2. Effects of inoculation treatments on nodulation of *L. japonicus* plants fertilized with different nitrogen sources. Plants were inoculated with MAFF, STM6, MAFF+STM6 (Coinoc) or water (not shown here). Values for nodule biomass are square root transformed (a) and values for mean nodule biomass (b) and nodule over total plant biomass are log transformed (c). Connecting letter reports indicate significant differences among inoculation treatments within each nitrogen source treatment group. Points represent individual plant replicates. The lower and upper hinges correspond to the first and third quartiles. The upper whiskers extend from the box to the largest value.

difference was uncovered for the mean population size of STM6 in nodules of coinoculated plants ($t = 0.88$, $p = 0.3853$; electronic supplementary material, table S6). These data suggest that fertilization led to a reduction in rewards to the nitrogen-fixing strain MAFF, with no changes in response to the non-fixing STM6.

(d) No effects on symbiosis were observed for gene knockouts of nitrogen metabolism

Plants inoculated with the mutant strains all showed significant increases in host biomass compared with uninoculated controls, and no significant differences in host growth effects or nodulation were observed between any of the mutant strains and the wild-type MAFF (electronic supplementary material, tables S14–S16).

4. Discussion

Plants invest significant resources to support nutritional mutualisms, producing novel organs on roots or shoots that house and feed microbes, and providing metabolic support that allows microbes to thrive within plant tissues [59,60]. Substantial investment into microbial mutualism creates a vulnerability for hosts. Because returns on investment from microbial partners

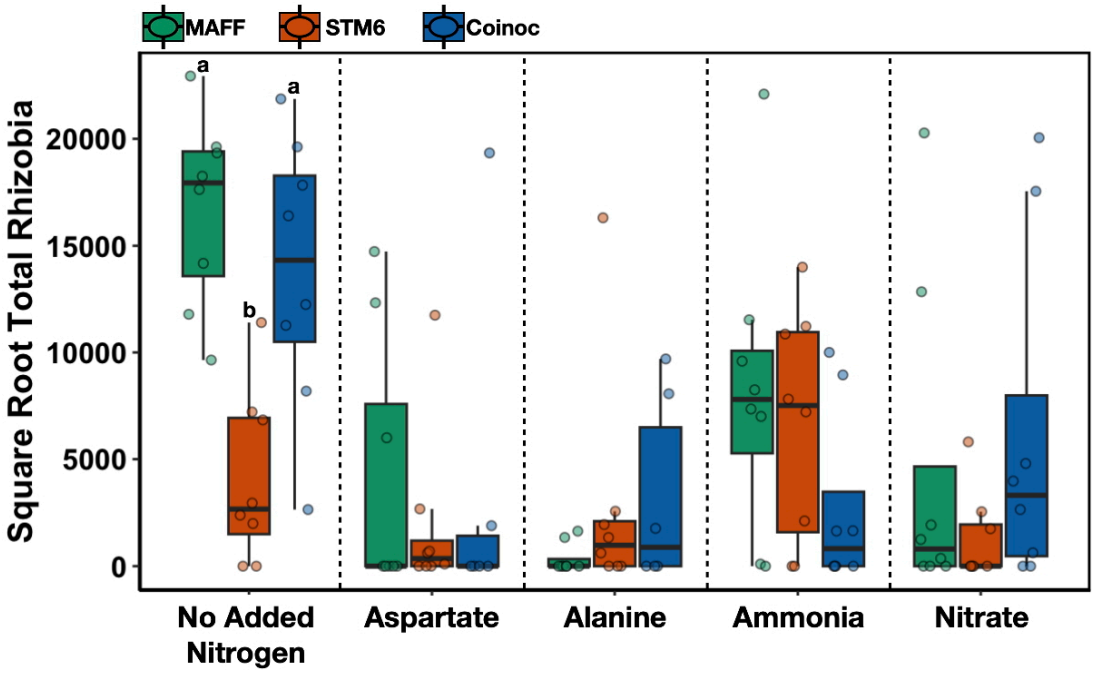

Figure 3. Effects of inoculation treatments on the population size of rhizobia in nodules of *L. japonicus* under different nitrogen source treatments. Plants were inoculated with MAFF, STM6, MAFF+STM6 (Coinoc) or water (not shown here). Rhizobia population size is square root transformed. Connecting letter reports indicate significant differences between inoculation treatments within each nitrogen source treatment group. Points represent individual plant replicates. The lower and upper hinges correspond to the first and third quartiles. The upper whiskers extend from the box to the largest value.

Table 2. Rhizobia population size of MAFF and STM6 in nodules. Replicates indicate the number of nodules in each treatment that had rhizobia population sizes below detection limits, indicated by no growth (NG), and the total number of nodules cultured in each treatment. Mean (μ) and standard error (σ) values are listed for each of the rhizobia population sizes.

		MAFF colony count		STM6 colony count		total colony count		no. replicates	
N-source	inoculum	μ	σ	μ	σ	μ	σ	NG	total
alanine	MAFF	5.60×10^5	1.06×10^6	—	—	5.60×10^5	1.06×10^6	6	8
	STM6	—	—	4.55×10^7	1.08×10^8	4.55×10^7	1.08×10^8	3	8
	coinoculant	1.57×10^7	3.84×10^7	1.14×10^7	2.63×10^7	2.70×10^7	4.17×10^7	3	6
aspartate	MAFF	5.06×10^7	8.53×10^7	—	—	5.06×10^7	8.53×10^7	5	8
	STM6	—	—	1.52×10^7	4.32×10^7	1.52×10^7	4.32×10^7	3	8
	coinoculant	6.23×10^7	1.53×10^8	5.97×10^5	1.46×10^6	6.29×10^7	1.52×10^8	4	6
ammonia	MAFF	1.11×10^8	1.59×10^8	—	—	1.11×10^8	1.59×10^8	1	8
	STM6	—	—	6.97×10^7	7.15×10^7	6.97×10^7	7.15×10^7	2	8
	coinoculant	4.13×10^6	9.17×10^6	1.91×10^7	3.36×10^7	2.32×10^7	4.16×10^7	4	8
nitrate	MAFF	7.27×10^7	1.48×10^8	—	—	7.27×10^7	1.48×10^8	3	8
	STM6	—	—	5.41×10^6	1.17×10^7	5.41×10^6	1.17×10^7	5	8
	coinoculant	4.02×10^7	1.08×10^8	5.43×10^7	1.41×10^8	9.45×10^7	1.63×10^8	2	8
no added nitrogen	MAFF	2.95×10^8	1.43×10^8	—	—	2.95×10^8	1.43×10^8	0	8
	STM6	—	—	3.09×10^7	4.52×10^7	3.09×10^7	4.52×10^7	2	8
	coinoculant	2.03×10^8	1.75×10^8	2.20×10^7	3.80×10^7	2.25×10^8	1.64×10^8	0	8

are variable, and cheaper alternative sources of nutrition are sometimes available in the environment, hosts must calibrate investment into symbiosis in a context-dependent manner, to avoid costly associations. In symbioses with mycorrhizae, host plants can reduce investment in fungal partners that generate lower benefit [4], but whether divestment occurs dependent on local nutrient access is less clear [61]. Our experiments manipulated the *Lotus–Mesorhizobium* symbiosis, varying microbial partner quality and extrinsic fertilization to examine net benefits from and host control over the association. The results suggest four main findings about the costs and benefits of host investment into microbial symbiosis and the limits of host control systems over microbes.

First, and most strikingly, we found that host sanctions mechanisms were nullified under nitrogen fertilization. The fertilized *L. japonicus* plants failed to differentiate rewards directed to nitrogen-fixing versus non-fixing symbionts in nodules, irrespective

Table 3. Percentage of MAFF colonies in the nodules of coinoculated plants. $MAFF\%_{end}$ is the mean percentage of nodule rhizobia represented by MAFF. $MAFF_{end}/MAFF_{inocula}$ is the ratio of MAFF% colonies in initial inoculum over that from nodules. χ^2 goodness of fit and *p*-value are used to test which treatments had significantly different MAFF/STM6 ratios from initial ratios.

N-source	$MAFF\%_{end}$ (%)	$\frac{MAFF_{end}}{MAFF_{inocula}}$	nodules with CFU/nodules cultured	χ^2 goodness of fit	*p*-value
nitrogen treatments combined[a]	55.54	1.357	15/28	0.528	0.467
no added nitrogen	90.23	2.204	8/8	5.115	0.024

[a]Statistical test groups all nitrogen fertilizer treatments (see electronic supplementary material, table S6, for separate tests).

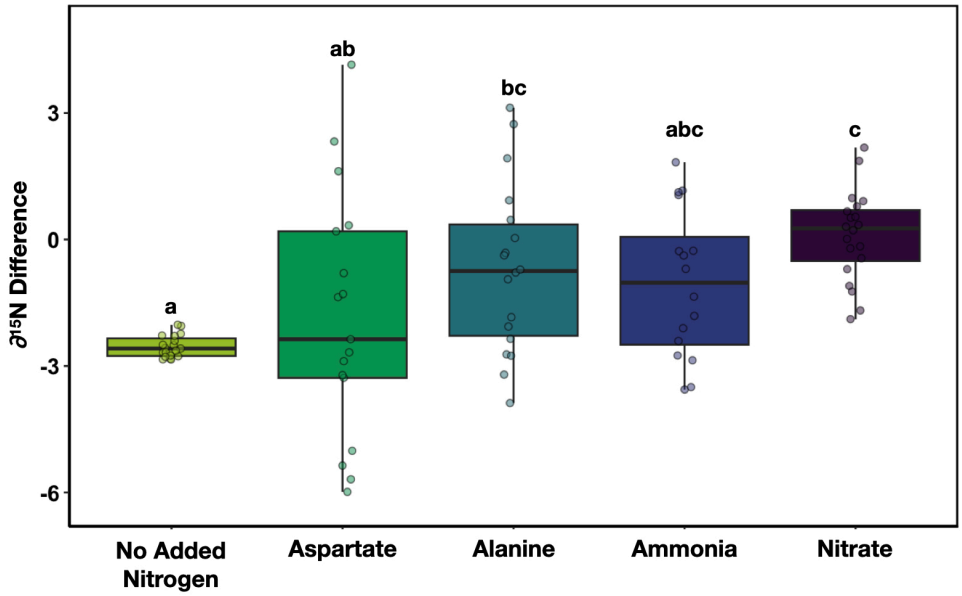

Figure 4. Leaf 'atom% difference' ($\Delta^{15}N$) is indicated for the different fertilization treatments, for plants inoculated with nitrogen-fixing rhizobia (data shown combined results from plants inoculated with MAFF and MAFF+STM6). Connecting letter reports indicate significant differences between nitrogen source tested within the pooled data. Points represent individual plant replicates. The lower and upper hinges correspond to the first and third quartiles. The upper whiskers extend from the hinge to the largest value no further than 1.5 interquartile range.

of nitrogen source, eliminating the selective advantage of nitrogen fixation by MAFF (figures 2 and 3; electronic supplementary material, tables S4 and S6). This contrasted with the findings in unfertilized plants, where efficient host sanctions were in evidence: the non-fixing strain STM6 suffered significantly lower fitness compared with MAFF, both in clonally infected plants (electronic supplementary material, table S6) and in coinoculated plants (table 3). Sanctions have been demonstrated in a broad variety of legumes, including alfalfa, cowpea, deerweeds, lotus, lupins, peas and soybeans, but in all these cases in unfertilized plants [20,48,58,62–64]. Two previous studies found evidence that fertilized legumes could sanction non-fixing rhizobia strains [35,40], but neither employed a near-isogenic mutant as was used here, so those hosts might have detected signalling or compatibility differences between the strains rather than the amount of nitrogen fixed. Sanctions are predicted to be critical to maintain costly mutualistic traits like symbiotic nitrogen fixation [16,65]. Our results suggest that sanctioning mechanisms can be compromised in nitrogen-rich environments. This might occur because of the detection limits of the host, because sanctions incur costs to the host that are not worth expressing in nitrogen-rich settings, or for some other reason.

Second, we found that, under fertilization, *L. japonicus* divested from symbiosis in a way that was strain-specific, only reducing the fitness of the nitrogen-fixing strain MAFF. All metrics of nodulation for MAFF were strikingly reduced under fertilization, compared with hosts that received no extrinsic nitrogen (figures 2 and 3; electronic supplementary material, tables S5 and S6). Conversely, plants inoculated with the non-fixing strain STM6 had no evidence of reduced symbiont fitness (table 2; electronic supplementary material, tables S5 and S6). One explanation for this pattern is that investment in STM6 is already at a minimal level, with little potential for the host to reduce investment further. Another idea is that divestment has evolved to minimize the costs of nitrogen fixation rather than the production or support of nodule tissues, which might have minimal costs relative to nitrogen fixation [30,66]. For instance, nitrogen fixation itself was downregulated under fertilization, in some cases to levels where little or no nitrogen was getting fixed (figure 4; electronic supplementary material, table S12), as has been observed in other legumes, including *Pisum sativum*, *Vigna unguiculata* and *Panicum virgatum* [39,67]. However, the maintenance of substantial nodule tissue in our study, in scenarios where no detectable benefit was derived from them, is difficult to explain. This might indicate that a base level of host investment is unavoidable, that mechanisms of downregulation of nodule organogenesis are costly or imperfect, or that the host benefits from retaining nodules in case the local environment changes. The exact costs of and control mechanisms over nodulation and nitrogen fixation require further exploration, but our results suggest hosts can respond to some costs more readily than others.

Third, we confirmed previous work showing that nitrogen fertilization can meet and exceed the benefits that hosts get from nitrogen fixation by rhizobia. *Lotus japonicus* was fertilized with concentrations of nitrogen previously found to optimize host growth in the absence of symbionts [29,40]. Under these conditions, fertilized plants without symbionts most often grew better than unfertilized hosts inoculated with the beneficial symbiont, MAFF (figure 1). These results suggest that it is cheaper for hosts to obtain nitrogen directly from the soil rather than invest substantial plant resources to support the formation and maintenance of root nodules [28,68]. When analysing the costs and benefits of microbial mutualism, we must consider multiple variables, including the types of nutrients and microbial mutualists that are available to hosts, as each can alter host performance and net benefits or costs of the association. The data here illustrate that even while most rhizobia are beneficial, they might incur a net cost to hosts in nitrogen-rich soils.

Finally, our results suggest that both partners in the legume–rhizobia mutualism have evolved redundancies in their systems of providing and receiving nitrogen. Evidence of the former is that rhizobia-bearing knockouts for individual nitrogen metabolism genes exhibited no deficits in their nodulation or growth effects on hosts, compared with wild-type MAFF (electronic supplementary material, tables S14–S16). This was surprising given that mutants were selected for pathways evident for their function in providing symbiotic nitrogen to host cells, including genes for alanine and aspartate metabolism, as well as a transmembrane complex predicted to transport these amino acids to the host, though none of these phenotypes had been experimentally tested on *L. japonicus* [54,55,57,69]. The lack of effects of these gene knockouts could be because rhizobia bear gene duplicates for some genes critical for mutualism services, such that lost function in one copy is compensated by others [45], or because multiple sources of nitrogen are excreted from bacteroids into the host cytoplasm, potentially including alanine, aspartate and ammonia, providing redundancy in the nitrogen sources transferred to hosts [30,31,69]. Evidence of the latter, that legumes bear redundancies in the forms of nitrogen they can receive, is that *L. japonicus* hosts were able to thrive under a diversity of nitrogen sources, including organic and inorganic sources from the soil as well as nitrogen fixed by rhizobia. This evidence of bidirectional redundancies in mutualism services, and plasticity of nutrients provided and received, supports the principle of robustness, the theory that biological systems are optimized for maintenance of core functions in the face of genetic and environmental perturbations [70,71].

In conclusion, our results suggest that host control traits of legumes, including sanctions and divestment from symbiosis, diverge in their response to fertilization. While sanctions were compromised under fertilization, hosts divested from rhizobia in this setting, forming smaller nodules (electronic supplementary material, table S5), with reduced population sizes of rhizobia in them (table 2). Other studies complement this work and suggest that, even under long-term exposure to nitrogen fertilization, nitrogen-fixing rhizobia are maintained in populations, presumably via host control. For instance, in *Acmispon strigosus* populations across an anthropogenic nitrogen deposition gradient in California, studies found no correlation between soil nitrogen concentration and the net benefit that rhizobia provide to hosts in those locations [41,72]. In parallel, hosts in nitrogen-rich habitats were found to be highly efficient in their capacity to sanction rhizobia, suggesting that host control traits are maintained even when rhizobia provide negligible benefits [11,13]. A contrast to these findings is a long-term fertilization experiment wherein rhizobia with reduced nitrogen fixation capacity were found to be enriched under fertilization [73]. However, this might have been driven by the greatly reduced frequency of legumes in the fertilized plots, rather than decreased control in those hosts. Surprisingly little is known about the drivers that shape changes in mutualism services of rhizobia. Our study highlights host mechanisms but cannot rule out the role of environmental forces, outside of the host, that shape how rhizobia evolve over the long term.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Data are available online at Dryad [74]. Supplementary material is available online.

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. D.C.F.: conceptualization, data curation, formal analysis, investigation, writing—original draft; G.S.O.-B.: conceptualization, data curation; F.M.: investigation; J.L.: investigation; K.W.: investigation; J.L.S.: conceptualization, data curation, funding acquisition, investigation, methodology, project administration, resources, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed herein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This work was funded by grants to J.L.S., including a Delfino Agriculture Technology Grant, an NSF DEB Award no. 1738009, an NIFA-USDA Award 2022-67019-36500, and a USDA Hatch Grant CA-R-EEOB-5200-H.

Acknowledgements. We thank LegumeBase at the University of Miyazaki Japan, from which we acquired MG-20 seeds and our mutant strains of *Mesorhizobium loti*. We thank Audrey Burr, Jeff Chang, Spencer Peterman, and two anonymous reviewers for helpful comments and constructive criticism on the manuscript.

References

1. Delaux PM, Schornack S. 2021 Plant evolution driven by interactions with symbiotic and pathogenic microbes. *Science* **371**, 796–807. (doi:10.1126/science.aba6605)
2. Friesen ML, Porter SS, Stark SC, von Wettberg EJ, Sachs JL, Martinez-Romero E. 2011 Microbially mediated plant functional traits. *Annu. Rev. Ecol. Evol. Syst*. **42**, 23–46. (doi:10.1146/annurev-ecolsys-102710-145039)
3. Douglas AE. 2010 *The symbiotic habit*. Princeton, NJ: Princeton University Press.
4. Kiers ET *et al*. 2011 Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* **333**, 880–882. (doi:10.1126/science.1208473)
5. Sawada H, Kuykendall LD, Young JM. 2003 Changing concepts in the systematics of bacterial nitrogen-fixing symbionts. *J. Appl. Microbiol.* **49**, 155–179. (doi:10.2323/jgam.49.155)
6. Wang ET. 2019 Symbiosis between rhizobia and legumes. In *Ecology and evolution of rhizobia, principles and applications* (eds E Wang, CF Tian, WF Chen, JPW Young, WX Chen). Singapore: Springer Singapore. (doi:10.1007/978-981-32-9555-1_1)

7. Burdon JJ, Gibson AH, Searle SD, Woods MJ, Brockwell J. 1999 Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian *Acacia*: within-species interactions. *J. Appl. Ecol*. **36**, 398–408. (doi:10.1046/j.1365-2664.1999.00409.x)
8. Porter SS, Simms EL. 2014 Selection for cheating across disparate environments in the legume-rhizobium mutualism. *Ecol. Lett*. **17**, 1121–1129. (doi:10.1111/ele.12318)
9. Sachs JL, Ehinger MO, Simms EL. 2010 Origins of cheating and loss of symbiosis in wild *Bradyrhizobium*. *J. Evol. Biol*. **23**, 1075–1089. (doi:10.1111/j.1420-9101.2010.01980.x)
10. Heath KD, Tiffin P. 2007 Context dependence in the coevolution of plant and rhizobial mutualists. *Proc. R. Soc. B* **274**, 1905–1912. (doi:10.1098/rspb.2007.0495)
11. Wendlandt CE, Regus JU, Gano-Cohen KA, Hollowell AC, Quides KW, Lyu JY, Adinata ES, Sachs JL. 2019 Host investment into symbiosis varies among genotypes of the legume *Acmispon strigosus*, but host sanctions are uniform. *New Phytol.* **221**, 446–458. (doi:10.1111/nph.15378)
12. Gano-Cohen KA, Wendlandt CE, Al Moussawi K, Stokes PJ, Quides KW, Weisberg AJ, Chang JH, Sachs JL. 2020 Recurrent mutualism breakdown events in a legume rhizobia metapopulation. *Proc. R. Soc. B* **287**, 20192549. (doi:10.1098/rspb.2019.2549)
13. Regus JU, Wendlandt CE, Bantay RM, Gano-Cohen KA, Gleason NJ, Hollowell AC, O'Neill MR, Shahin KK, Sachs JL. 2017 Nitrogen deposition decreases the benefits of symbiosis in a native legume. *Plant Soil* **414**, 159–170. (doi:10.1007/s11104-016-3114-8)
14. Batstone RT, Peters MAE, Simonsen AK, Stinchcombe JR, Frederickson ME. 2020 Environmental variation impacts trait expression and selection in the legume–rhizobium symbiosis. *Am. J. Bot*. **107**, 195–208. (doi:10.1002/ajb2.1432)
15. Sachs JL, Skophammer RG, Regus JU. 2011 Evolutionary transitions in bacterial symbiosis. *Proc. Natl Acad. Sci. USA* **108**, 10800–10807. (doi:10.1073/pnas.1100304108)
16. Sachs JL, Mueller UG, Wilcox TP, Bull JJ. 2004 The evolution of cooperation. *Q. Rev. Biol.* **79**, 135–160. (doi:10.1086/383541)
17. Geurts R, Bisseling T. 2002 *Rhizobium* Nod factor perception and signalling. *Plant Cell* **14**, S239–S249. (doi:10.1105/tpc.002451)
18. Subramanian S, Stacey G, Yu O. 2007 Distinct, crucial roles of flavonoids during legume nodulation. *Trends Plant Sci.* **12**, 282–285. (doi:10.1016/j.tplants.2007.06.006)
19. Sachs JL, Quides KW, Wendlandt CE. 2018 Legumes versus rhizobia: a model for ongoing conflict in symbiosis. *New Phytol.* **219**, 1199–1206. (doi:10.1111/nph.15222)
20. Kiers ET, Rousseau RA, West SA, Denison RF. 2003 Host sanctions and the legume–*Rhizobium* mutualism. *Nature* **425**, 78–81. (doi:10.1038/nature01931)
21. Ferguson BJ, Mens C, Hastwell AH, Zhang M, Su H, Jones CH, Chu X, Gresshoff PM. 2019 Legume nodulation: the host controls the party. *Plant Cell Environ.* **42**, 41–51. (doi:10.1111/pce.13348)
22. Westhoek A, Field E, Rehling F, Mulley G, Webb I, Poole PS, Turnbull LA. 2017 Policing the legume-*Rhizobium* symbiosis: a critical test of partner choice. *Scient. Rep.* **7**, 1419. (doi:10.1038/s41598-017-01634-2)
23. Daimon H, Yoshioka M. 2008 Responses of root nodule formation and nitrogen fixation activity to nitrate in a split-root system in peanut (*Arachis hypogaea* L.). *J. Agron. Crop Sci*. **187**, 89–95. (doi:10.1046/j.1439-037x.2001.00505.x)
24. Jeudy C *et al*. 2010 Adaptation of *Medicago truncatula* to nitrogen limitation is modulated via local and systemic nodule developmental responses. *New Phytol*. **185**, 817–828. (doi:10.1111/j.1469-8137.2009.03103.x)
25. Chen E, Liao H, Chen B, Peng S. 2020 Arbuscular mycorrhizal fungi are a double-edged sword in plant invasion controlled by phosphorus concentration. *New Phytol.* **226**, 295–300. (doi:10.1111/nph.16359)
26. Smith FA, Smith SE. 2011 What is the significance of the arbuscular mycorrhizal colonisation of many economically important crop plants? *Plant Soil* **348**, 63–79. (doi:10.1007/s11104-011-0865-0)
27. Heath KD, Stock AJ, Stinchcombe JR. 2010 Mutualism variation in the nodulation response to nitrate. *J. Evol. Biol*. **23**, 2494–2500. (doi:10.1111/j.1420-9101.2010.02092.x)
28. Nishimura R *et al*. 2002 HAR1 mediates systemic regulation of symbiotic organ development. *Nature* **420**, 426–429. (doi:10.1038/nature01231)
29. Ortiz-Barbosa GS, Torres-Martínez L, Rothschild J, Sachs JL. 2023 *Lotus japonicus* regulates root nodulation and nitrogen fixation dependent on the molecular form of nitrogen fertilizer. *Plant Soil* **483**, 533–545. (doi:10.1007/s11104-022-05762-1)
30. diCenzo GC, Tesi M, Pfau T, Mengoni A, Fondi M. 2020 Genome-scale metabolic reconstruction of the symbiosis between a leguminous plant and a nitrogen-fixing bacterium. *Nat. Commun*. **11**, 2574. (doi:10.1038/s41467-020-16484-2)
31. Pfau T, Christian N, Masakapalli SK, Sweetlove LJ, Poolman MG, Ebenhöh O. 2018 The intertwined metabolism during symbiotic nitrogen fixation elucidated by metabolic modelling. *Scient. Rep.* **8**, 12504. (doi:10.1038/s41598-018-30884-x)
32. Sulieman S, Tran LSP. 2013 Asparagine: an amide of particular distinction in the regulation of symbiotic nitrogen fixation of legumes. *Crit. Rev. Biotechnol*. **33**, 309–327. (doi:10.3109/07388551.2012.695770)
33. Hussain A, Jiang Q, Broughton WJ, Gresshoff PM. 1999 *Lotus japonicus* nodulates and fixes nitrogen with the broad host range *Rhizobium* sp. NGR234. *Plant Cell Physiol.* **40**, 894–899. (doi:10.1093/oxfordjournals.pcp.a029619)
34. Keller KR, Lau JA. 2018 When mutualisms matter: rhizobia effects on plant communities depend on host plant population and soil nitrogen availability. *J. Ecol*. **106**, 1046–1056. (doi:10.1111/1365-2745.12938)
35. Kiers ET, Rousseau RA, Denison RF. 2006 Measured sanctions: legume hosts detect quantitative variation in rhizobium cooperation and punish accordingly. *Evol. Ecol. Res*. **8**, 1077–1086.
36. Nanjareddy K, Blanco L, Arthikala M, Affantrange XA, Sánchez F, Lara M. 2014 Nitrate regulates rhizobial and mycorrhizal symbiosis in common bean (*Phaseolus vulgaris* L.). *J. Integr. Plant Biol.* **56**, 281–298. (doi:10.1111/jipb.12156)
37. Oono R, Muller KE, Ho R, Jimenez Salinas A, Denison RF. 2020 How do less-expensive nitrogen alternatives affect legume sanctions on rhizobia? *Ecol. Evol*. **10**, 10645–10656. (doi:10.1002/ece3.6718)
38. Regus JU, Gano KA, Hollowell AC, Sofish V, Sachs JL. 2015 Lotus hosts delimit the mutualism–parasitism continuum of Bradyrhizobium. *J. Evol. Biol*. **28**, 447–456. (doi:10.1111/jeb.12579)
39. Voisin AS, Salon C, Munier-Jolain NG, Ney B. 2002 Effect of mineral nitrogen on nitrogen nutrition and biomass partitioning between the shoot and roots of pea (*Pisum sativum* L.). *Plant Soil* **242**, 251–262. (doi:10.1023/A:1016214223900)
40. Regus JU, Gano KA, Hollowell AC, Sachs JL. 2014 Efficiency of partner choice and sanctions in *Lotus* is not altered by nitrogen fertilization. *Proc. R. Soc. B* **281**, 20132587. (doi:10.1098/rspb.2013.2587)
41. Wendlandt CE, Gano-Cohen KA, Stokes PJN, Jonnala BNR, Zomorrodian AJ, Al-Moussawi K, Sachs JL. 2022 Wild legumes maintain beneficial soil rhizobia populations despite decades of nitrogen deposition. *Oecologia* **198**, 419–430. (doi:10.1007/s00442-022-05116-9)
42. Waters JK, Mawhinney TP, Emerich DW. 2020 Nitrogen assimilation and transport by *ex planta* nitrogen-fixing *Bradyrhizobium diazoefficiens* bacteroids is modulated by oxygen, bacteroid density and L-malate. *Int. J. Mol. Sci*. **21**, 7542. (doi:10.3390/ijms21207542)
43. Galloway JN *et al*. 2004 Nitrogen cycles: past, present, and future. *Biogeochemistry* **70**, 153–226. (doi:10.1007/s10533-004-0370-0)

44. Lodwig EM, Hosie AHF, Bourdès A, Findlay K, Allaway D, Karunakaran R, Downie JA, Poole PS. 2003 Amino-acid cycling drives nitrogen fixation in the legume–*Rhizobium* symbiosis. *Nature* **422**, 722–726. (doi:10.1038/nature01527)
45. Rastogi VK, Watson RJ. 1991 Aspartate aminotransferase activity is required for aspartate catabolism and symbiotic nitrogen fixation in *Rhizobium meliloti*. *J. Bacteriol*. **173**, 2879–2887. (doi:10.1128/jb.173.9.2879-2887.1991)
46. Waters JK, Hughes BL, Purcell LC, Gerhardt KO, Mawhinney TP, Emerich DW. 1998 Alanine, not ammonia, is excreted from N_2-fixing soybean nodule bacteroids. *Proc. Natl Acad. Sci. USA* **95**, 12038–12042. (doi:10.1073/pnas.95.20.12038)
47. Tatsukami Y, Nambu M, Morisaka H, Kuroda K, Ueda M. 2013 Disclosure of the differences of *Mesorhizobium loti* under the free-living and symbiotic conditions by comparative proteome analysis without bacteroid isolation. *BMC Microbiol*. **13**, 180. (doi:10.1186/1471-2180-13-180)
48. Quides KW, Stomackin GM, Lee HH, Chang JH, Sachs JL. 2017 *Lotus japonicus* alters *in planta* fitness of *Mesorhizobium loti* dependent on symbiotic nitrogen fixation. *PLoS One* **12**, e0185568. (doi:10.1371/journal.pone.0185568)
49. Maekawa T, Maekawa-Yoshikawa M, Takeda N, Imaizumi-Anraku H, Murooka Y, Hayashi M. 2009 Gibberellin controls the nodulation signaling pathway in *Lotus japonicus*. *Plant J.* **58**, 183–194. (doi:10.1111/j.1365-313x.2008.03774.x)
50. Shimoda Y *et al*. 2008 Construction of signature-tagged mutant library in *Mesorhizobium loti* as a powerful tool for functional genomics. *DNA Res*. **15**, 297–308. (doi:10.1093/dnares/dsn017)
51. Sachs JL, Kembel SW, Lau AH, Simms EL. 2009 *In situ* phylogenetic structure and diversity of wild *Bradyrhizobium* communities. *Appl. Environ. Microbiol*. **75**, 4727–4735. (doi:10.1128/aem.00667-09)
52. Somasegaran P, Hoben J. 1994 *Handbook for rhizobia*. New York, NY: Springer-Verlag.
53. Unkovich M, Herridge DH, Peoples MB, Boddey RM, Cadisch G, Giller K, Alves B, Chalk PM. 2008 *Measuring plant-associated nitrogen fixation in agricultural systems*, p. 258. Canberra: ACIAR Monograph No.136. Australian Centre forInternational Agricultural Research.
54. Kajiwara H, Kaneko T, Ishizaka M, Tajima S, Kouchi H. 2003 Protein profile of symbiotic bacteria *Mesorhizobium loti* MAFF303099 in mid-growth phase. *Biosci. Biotechnol. Biochem*. **67**, 2668–2673. (doi:10.1271/bbb.67.2668)
55. Uchiumi T *et al*. 2004 Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. *J. Bacteriol*. **186**, 2439–2448. (doi:10.1128/jb.186.8.2439-2448.2004)
56. Kaneko T *et al*. 2000 Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti* (supplement). *DNA Res*. **7**, 381–406. (doi:10.1093/dnares/7.6.381)
57. Nambu M, Tatsukami Y, Morisaka H, Kuroda K, Ueda M. 2015 Quantitative time-course proteome analysis of *Mesorhizobium loti* during nodule maturation. *J. Proteom.* **125**, 112–120. (doi:10.1016/j.jprot.2015.04.034)
58. Ortiz-Barbosa GS, Torres-Martínez L, Manci A, Neal S, Soubra T, Khairi F, Trinh J, Cardenas P, Sachs JL. 2022 No disruption of rhizobial symbiosis during early stages of cowpea domestication. *Evolution* **76**, 496–511. (doi:10.1111/evo.14424)
59. Holland BL, Matthews ML, Bota P, Sweetlove LJ, Long SP, diCenzo GC. 2023 A genome-scale metabolic reconstruction of soybean and *Bradyrhizobium diazoefficiens* reveals the cost–benefit of nitrogen fixation. *New Phytol.* **240**, 744–756. (doi:10.1111/nph.19203)
60. Fronk DC, Sachs JL. 2022 Symbiotic organs: the nexus of host–microbe evolution. *Trends Ecol. Evol*. **37**, 599–610. (doi:10.1016/j.tree.2022.02.014)
61. Bogar LM. 2024 Modified source–sink dynamics govern resource exchange in ectomycorrhizal symbiosis. *New Phytol.* **242**, 1523–1528. (doi:10.1111/nph.19259)
62. Oono R, Anderson CG, Denison RF. 2011 Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates. *Proc. R. Soc. B* **278**, 2698–2703. (doi:10.1098/rspb.2010.2193)
63. Sachs JL, Russell JE, Lii YE, Black KC, Lopez G, Patil AS. 2010 Host control over infection and proliferation of a cheater symbiont. *J. Evol. Biol*. **23**, 1919–1927. (doi:10.1111/j.1420-9101.2010.02056.x)
64. Simms EL, Taylor DL, Povich J, Shefferson RP, Sachs JL, Urbina M, Tausczik Y. 2006 An empirical test of partner choice mechanisms in a wild legume–rhizobium interaction. *Proc. R. Soc. B* **273**, 77–81. (doi:10.1098/rspb.2005.3292)
65. West SA, Kiers ET, Simms EL, Denison RF. 2002 Sanctions and mutualism stability: why do rhizobia fix nitrogen? *Proc. R. Soc. Lond. B* **269**, 685–694. (doi:10.1098/rspb.2001.1878)
66. Schwab ST *et al*. 2023 Effective rhizobia enhance legume growth during subsequent drought despite water costs associated with nitrogen fixation. *Plant Soil* **492**, 157–175. (doi:10.1007/s11104-023-06164-7)
67. Eaglesham ARJ, Hassouna S, Seegers R. 1983 Fertilizer-N effects on N_2 fixation by cowpea and soybean 1. *Agron. J.* **75**, 61–66. (doi:10.2134/agronj1983.00021962007500010016x)
68. Krusell L *et al*. 2002 Shoot control of root development and nodulation is mediated by a receptor-like kinase. *Nature* **420**, 422–426. (doi:10.1038/nature01207)
69. Lodwig E, Poole P. 2003 Metabolism of *Rhizobium* bacteroids. *Crit. Rev. Plant Sci*. **22**, 37–78. (doi:10.1080/713610850)
70. Kitano H. 2004 Biological robustness. *Nat. Rev. Genet*. **5**, 826–837. (doi:10.1038/nrg1471)
71. Weisberg AJ, Rahman A, Backus D, Tyavanagimatt P, Chang JH, Sachs JL. 2022 Pangenome evolution reconciles robustness and instability of rhizobial symbiosis. *mBio* **13**, e00074-22. (doi:10.1128/mbio.00074-22)
72. Gano-Cohen KA, Wendlandt CE, Stokes PJ, Blanton MA, Quides KW, Zomorrodian A, Adinata ES, Sachs JL. 2019 Interspecific conflict and the evolution of ineffective rhizobia. *Ecol. Lett*. **22**, 914–924. (doi:10.1111/ele.13247)
73. Weese DJ, Heath KD, Dentinger BTM, Lau JA. 2015 Long-term nitrogen addition causes the evolution of less-cooperative mutualists. *Evolution* **69**, 631–642. (doi:10.1111/evo.12594)
74. Fronk D *et al*. 2025 Data from: Nitrogen fertilization nullifies host sanctions against non-fixing rhizobia and drives divestment from symbiosis in *Lotus japonicus*. Dryad Digital Repository. (doi:10.5061/dryad.xksn02vtc)