Article

Current Biology

Competitive interference among rhizobia reduces benefits to hosts

Highlights

- Multiple strain inoculation caused hosts to receive reduced symbiotic benefit
- Coinoculated hosts were nodulated at lower rates, indicating strain interference
- A marginally beneficial strain was highly competitive for nodulation
- The dominance hierarchy suggests polygenic drivers of strain competition

Authors

Arafat Rahman, Max Manci, Cassandra Nadon, ..., Alexandra J. Weisberg, Jeff H. Chang, Joel L. Sachs

Correspondence

joels@ucr.edu

In brief

Rahman et al. experimentally compete a focal set of *Bradyrhizobium* strains on their native legume host. They characterize the fitness outcomes for symbionts and their hosts. Interstrain competition causes hosts to receive reduced benefit from symbiosis compared to clonal inoculation and can favor marginally beneficial strains.

Rahman et al., 2023, Current Biology *33*, 2988–3001
July 24, 2023
https://doi.org/10.1016/j.cub.2023.06.081

CellPress

CellPress

Current Biology

Article

Competitive interference among rhizobia reduces benefits to hosts

Arafat Rahman,[1,6] Max Manci,[2] Cassandra Nadon,[3] Ivan A. Perez,[3] Warisha F. Farsamin,[3] Matthew T. Lampe,[3] Tram H. Le,[3] Lorena Torres Martínez,[3,4] Alexandra J. Weisberg,[5] Jeff H. Chang,[5] and Joel L. Sachs[1,2,3,7,*]

[1]Institute of Integrative Genome Biology, University of California, Riverside, Riverside, CA 92521, USA
[2]Department of Microbiology & Plant Pathology, University of California, Riverside, Riverside, CA 92521, USA
[3]Department of Evolution Ecology & Organismal Biology, University of California, Riverside, Riverside, CA 92521, USA
[4]Department of Biology, St. Mary's College of Maryland, St. Mary's City, MD 20686, USA
[5]Department of Botany & Plant Pathology, Oregon State University, Corvallis, OR 97331, USA
[6]Twitter: @ac_arafat
[7]Lead contact
*Correspondence: joels@ucr.edu
https://doi.org/10.1016/j.cub.2023.06.081

SUMMARY

The capacity of beneficial microbes to compete for host infection—and the ability of hosts to discriminate among them—introduces evolutionary conflict that is predicted to destabilize mutualism. We investigated fitness outcomes in associations between legumes and their symbiotic rhizobia to characterize fitness impacts of microbial competition. Diverse *Bradyrhizobium* strains varying in their capacity to fix nitrogen symbiotically with a common host plant, *Acmispon strigosus*, were tested in full-factorial coinoculation experiments involving 28 pairwise strain combinations. We analyzed the effects of interstrain competition and host discrimination on symbiotic-interaction outcomes by relativizing fitness proxies to clonally infected and uninfected controls. More than one thousand root nodules of coinoculated plants were genotyped to quantify strain occupancy, and the *Bradyrhizobium* strain genome sequences were analyzed to uncover the genetic bases of interstrain competition outcomes. Strikingly, interstrain competition favored a fast-growing, minimally beneficial rhizobia strain. Host benefits were significantly diminished in coinoculation treatments relative to expectations from clonally inoculated controls, consistent with competitive interference among rhizobia that reduced both nodulation and plant growth. Competition traits appear polygenic, linked with inter-strain allelopathic interactions in the rhizosphere. This study confirms that competition among strains can destabilize mutualism by favoring microbes that are superior in colonizing host tissues but provide minimal benefits to host plants. Moreover, our findings help resolve the paradox that despite efficient host control post infection, legumes nonetheless encounter rhizobia that vary in their nitrogen fixation.

INTRODUCTION

Microbial mutualists provide terrestrial plants with diverse services,[1] but benefits provided by microbial partners are unreliable, causing unpredictable fitness outcomes for hosts.[2] For instance, interactions between plants and root-associated mycorrhizal fungi vary from highly beneficial to parasitic.[3,4] Epiphytic bacteria that fix nitrogen for tropical host plants also vary broadly in the amount of benefit provided to hosts.[5,6] During host colonization, microbial interactions can favor highly competitive strains irrespective of the level of benefit provided to hosts.[7,8] To optimize benefits from microbial associations, plants employ host control traits, including partner choice and sanctions that reward beneficial microbes and discriminate against strains that provide insufficient benefits.[9,10] The capacity of microbial partners to compete for host infection, and the ability of hosts to discriminate among them, can introduce an evolutionary conflict between microbe and host partners. The effects of this conflict on the services exchanged in plant microbial mutualism—and the evolutionary stability of these associations—remain poorly understood.

The legume-rhizobia association is an excellent model of mutualism where microbial strains compete to infect hosts. Rhizobia encompasses polyphyletic groups of proteobacteria with the capacity to induce root nodulation and fix nitrogen in legume hosts.[11] Partner quality, as measured by relative growth (RG) benefit the host gets from rhizobial strains, varies quantitatively due to differences in nitrogen fixation capability from those that fix substantial amounts to those that are ineffective and fail to provide any benefit for specific host plant partners.[12–17] Legumes exhibit a suite of host-control traits to minimize costs of ineffective infections. Host legumes exhibit partner choice, the ability to discriminate against incompatible and uncooperative partners, in this case by detecting molecular signals of nod factors and effector proteins deployed by type III secretion systems (T3SSs).[18] Moreover, when ineffective rhizobia gain access to nodules, legumes sanction them by reducing *in planta* proliferation of rhizobia.[19–22] Given the plant

Table 1. *Bradyrhizobium* strains and their key properties

Strain numbers[a]	Strain code[a]	Nod[b]	Fix[b]	T3SS[c]	Taxon[d]	Sampling site[a,b]
2	05LoS24R3_28	+	–	–	Novel XIII	Bodega Marin Reserve
186	11LoS6_2	+	–	+	N/A	San Dimas Reservoir
4	05LoS21R5_36	+	+	+	*B. canariense*	Bodega Marin Reserve
131	13LoS28_1	+	+	+	*B. canariense*	UC Riverside
187	11LoS7_1	+	–	–	*B. canariense*	San Dimas Reservoir
156	11LoS34_4	+	–	+	*B. canariense*	Burns Piñon Ridge Reserve
184	11LoS34_10	+	+	+	N/A	Burns Piñon Ridge Reserve
200	13LoS78_1	+	–	+	Novel IV	Pismo Dunes Natural Preserve

See also Data S2.

[a]Strain numbers and strain codes from Gano-Cohen et al.[17]

[b]Strains are categorized as to whether they consistently nodulate (i.e., NOD) and fix nitrogen (i.e., Fix) on the host species from which they were collected[17]

[c]Strains are categorized as to whether they encode necessary structural proteins to express a type III secretion system[51] (T3SS)

[d]Strains are categorized into species groups[52]

hosts' capacity to select for strains with high net benefit *in planta*, less beneficial strains are predicted to be selected against in the population.[23–29] Nonetheless, strains ineffective at fixing nitrogen are common both in natural and agricultural landscapes, suggesting that other forces counteract host control. These predicted forces include, but are not limited to, mutation-selection balance, stable coexistence of strategies, and selection mosaics shaped by G × G and G × E interactions.[2,30–32]

The legume-rhizobia mutualism generates the dominant natural input of nitrogen into terrestrial ecosystems[33] and has agronomic potential to reduce environmental damage caused by nitrogen fertilizer.[34–36] Attempts to inoculate legumes with "elite" rhizobia, strains that generate a high degree of benefits to plants in lab settings, often result in the inoculant strains being outcompeted by indigenous rhizobia that provide little or no benefits to the introduced host, a phenomenon referred to as the rhizobia competition problem.[37,38] However, bioinoculants made from rhizobia that are native to the applied soils can achieve better success than commercial inoculants,[39–41] indicating importance to characterize rhizobia genotypes for competitiveness and symbiotic growth benefit. Because diverse rhizobia are typically present in natural and field settings, clonal inoculation experiments do not predict performance of rhizobia where multiple strains compete for plant-derived nutrients.[42,43] Yet most experiments focus on inoculating clonal bacterial isolates on hosts as a means to evaluate their ability to form successful symbiosis.[44] New work addresses this limitation by incorporating multi-strain inoculations to test hypotheses of fitness outcomes in the legume-rhizobia symbiosis.[31,45]

Here, we characterized variation in competitive ability among rhizobia strains and related interstrain competition to the benefits that hosts derived from the symbiosis. Our objectives were to (1) compare fitness outcomes of rhizobia and hosts in single and coinoculation, (2) characterize the nodulation occupancy distribution of competing strains that vary in symbiotic benefit to the host, and (3) develop and test models that incorporate symbiotic benefit and nodulation capacity to predict mechanistic processes underlying competition. Experiments were conducted on *Acmispon strigosus* (formerly *Lotus strigosus*), an annual legume native to the southwestern US that is nodulated by diverse *Bradyrhizobium* spp.[17,46] We used eight focal *Bradyrhizobium* strains, isolated originally from *A. strigosus*, that range from beneficial to ineffective at nitrogen fixation symbiosis. In prior work, strains were phenotypically characterized on multiple genotypes of *A. strigosus* for nodulation and nitrogen fixation capacity.[17] Strains that fixed nitrogen and improved host growth were classified as effective (i.e., Fix+), whereas those that provided no growth benefit were classified as ineffective (i.e., Fix–). The strains had their genomes sequenced and the Fix– strains contain intact *nif*/*fix* genes, indicating that ineffectiveness is not a result of deletion or pseudogenization of these genes.[51] Because these *Bradyrhizobium* strains showed consistent response across multiple host genotypes,[17,47,48] a single inbred line of *A. strigosus* was used in this study. When coinoculated with beneficial and ineffective strains, *A. strigosus* can discriminate among them and sanction strains that do not fix nitrogen for the host, reducing the capacity of nonfixing rhizobial strains to proliferate within nodule tissues.[22,47,50] Strains were coinoculated onto *A. strigosus* in a full factorial pairwise experiment, which also included clonally inoculated and uninoculated treatments. Using Illumina amplicon sequencing, more than 1,100 nodules from coinoculated plants were genotyped to detect the occupying strains. Null models were developed from single-inoculation data to predict and then test effects of coinoculation treatments on host nodulation and growth. Understanding the role of competition among rhizobia to colonize legumes is critical for managing and improving sustainable agriculture and testing mutualism stability models.

RESULTS

Host benefit depends on clonal inoculation genotype

We first evaluated the effects of clonal inoculations on hosts to cross-validate prior results, establish baselines, and model effects of coinoculations on RG and nodulation response of plants. Eight strains were individually inoculated onto *A. strigosus* plants grown in a greenhouse and caused host responses that closely matched previous results[17,50] (Table 1; Figure 1; Data S1 and S2). Specifically, plants inoculated with the Fix+ strains 4, 131,

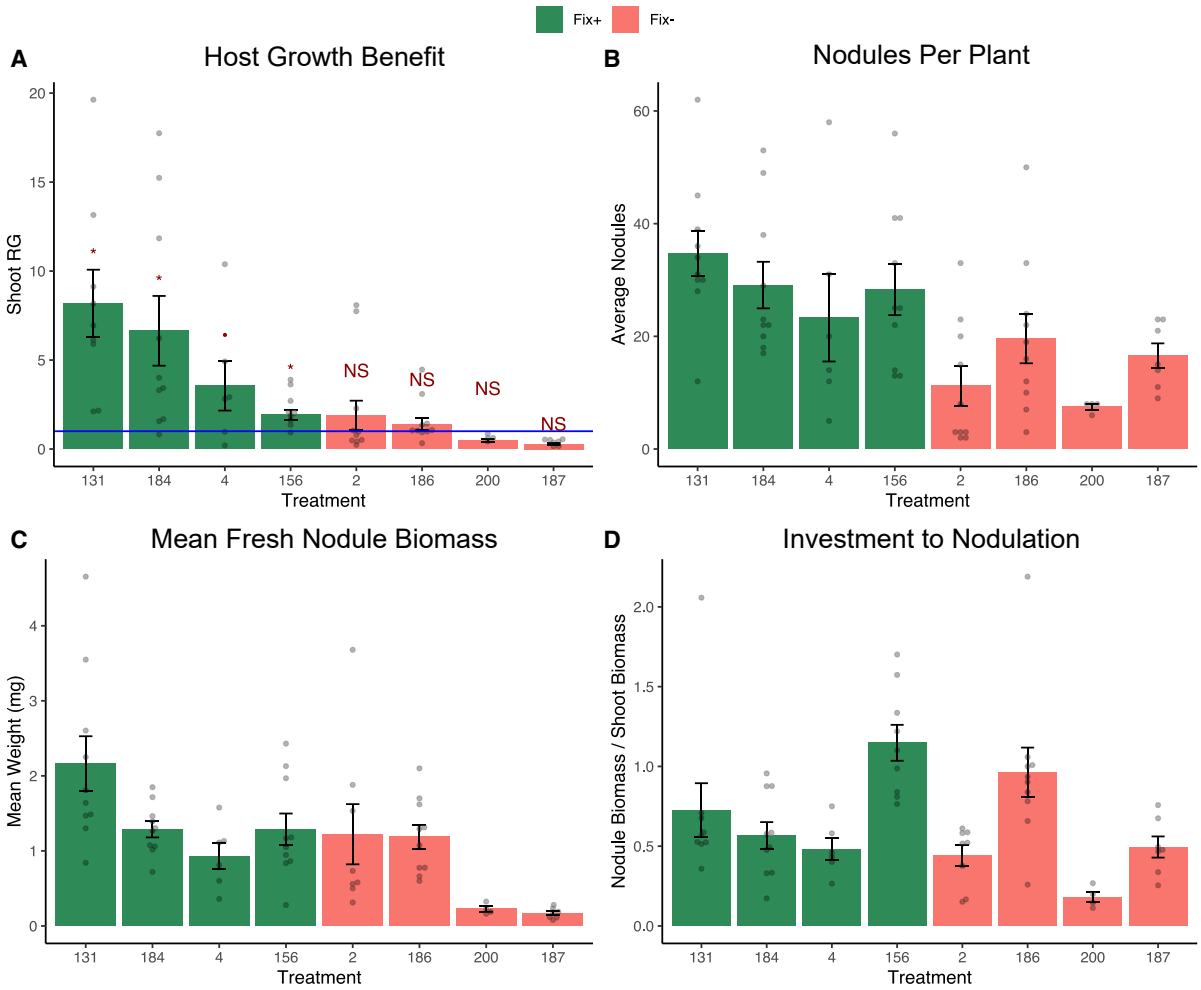


Figure 1. Plant response in clonal inoculation treatments

(A) Shoot relative growth (RG) indicates host growth benefit from inoculation relative to uninoculated controls. p values between 0.001 and 0.05 are indicated with single asterisk (*), between 0.05 and 0.1 with a period (.), and above 0.1 is NS (non-significant).

(B–D) Average number of nodules per plant (B), mean fresh nodule biomass (C), and investment to nodulation (D). Turquoise color represents Fix+ strains and red color represents Fix− strains. Error bars indicate SEM. The strains are ordered left to right based on being most to least beneficial based as determined by growth data in (A).

See also Data S2B and S2C.

156, and 184 had more biomass than uninoculated control plants (Data S2). Three of these strains elicited significant RG benefits, with the exception of marginal strain 4 (t = 1.84, df = 5, p < 0.0626; Data S2B; Figure 1A). Conversely, Fix− strains 2, 186, 187, and 200 were confirmed as ineffective, as host biomass values were not significantly greater than those of control plants (p > 0.05; Data S2B). Relative host growth response varied significantly among the clonal strain treatments (Table 2; $F_{(7,56)}$ = 11.24, p < 0.001). No significant random effect of inoculation batch was found on single or coinoculation results; hence, the design can be analyzed as a single experiment (Table S1).

Host nodulation response varied significantly among the clonal inoculation treatments, regardless of the Fix+/Fix− phenotype of strains (total nodules, $F_{(7,56)}$ = 7.009, p = <0.001; mean nodule biomass, $F_{(7,56)}$ = 18.092, p = <0.001; host investment [i.e., nodule proportion of total plant biomass], $F_{(7,56)}$ = 8.358, p = <0.001). Ineffective strains 2 and 200 elicited relatively low numbers of nodules (i.e., estimated marginal means of nodules <10 in most cases; Data S2C), while the remaining strains, including effective and ineffective ones, formed 16–35 nodules per plant (Figure 1B; Data S2C). Host growth response was positively correlated with nodule count (Pearson's product-moment correlation R^2 = 0.697, t = 8.24, df = 72, p < 0.001) and mean nodule weight (Pearson's product moment correlation R^2 = 0.63, t = 6.746, df = 70, p < 0.001). This pattern is consistent with host control over resource flow into nodules reflected by nodule size, where within-nodule fitness of ineffective strains is reduced by plants.[22]

Strain 156 provided low RG benefit (mean shoot RG = 1.94, p < 0.01; Figure 1; Data S2B) while inducing the highest host investment among the strains tested (estimated marginal mean

Table 2. Linear models testing the effects of treatment and days post-inoculation on host benefit and nodulation during single inoculation

	Log_{10}(shoot RG + 0.5)[a]			$\sqrt{Total\ nodules}$[a]			Log(mean nodule biomass + 0.1)[a]			Log_{10}(investment + 0.2)[a]		
Sample size[b] = 67	adj. R^2 = 0.53			adj. R^2 = 0.406			adj. R^2 = 0.66			adj. R^2 = 0.448		
Shapiro-Wilk test[c]	W = 0.97			W = 0.98			W = 0.98			W = 0.975		
	p = 0.165			p = 0.487			p = 0.553			p = 0.22		
ANCOVA	df	F	p	df	F	p	df	F	p	df	F	p
Intercept	1	2.103	0.15259	1	1.474	0.2295	1	3.1884	0.0795	1	0.022	0.8827
Treatment	7	11.24	8.576×10^{-09}	7	7.009	4.713×10^{-06}	7	18.092	2.542×10^{-12}	7	8.358	6.975×10^{-07}
DPI	1	6.641	0.01262	1	10.41	0.002	1	6.16	0.016	1	0.0164	0.8985
Residuals	56			58			56			54		

ANCOVA statistics test whether response variables significantly vary among single inoculation treatments using DPI as a covariate. Degrees of freedom (df), F-statistics, and associated p values are reported for each model. See also Data S2A and Table S1.

[a]Response variable transformations that have been used in linear models to fit single-inoculation data for different response variables (shoot RG, total nodules, mean nodule biomass, investment)

[b]Sample size indicates number of data points (n) used to fit the models. Adjusted R-squared values for each model are also reported.

[c]Shapiro-Wilk statistics testing normality of the residuals in the models. Test statistics, W and p value, are reported.

1.111; Data S2C). The host growth and nodulation response variables of strain 156 were consistent with previous findings that tested nodulation and growth benefit of this strain on multiple sympatric and allopatric host lines.[48]

Host benefit varied in coinoculation treatments

To examine the effect of strain-strain competition on mutualistic symbioses, we tested all 28 possible pairs of strain combinations. Host benefit, nodulation count, mean nodule biomass, and host investment all varied significantly among the 28 coinoculation treatments (host benefit, $F_{(27,200)} = 5.455$, $p < 0.001$; total nodules, $F_{27,202} = 2.63$, $p < 0.001$; mean nodule biomass, $F_{27,199} = 2.782$, $p < 0.001$; investment, $F_{27,197} = 3.41$, $p < 0.001$; Table 3; Figure 2). Like the clonal inoculation treatments, RG was positively correlated with nodule counts (Pearson's product-moment correlation $R^2 = 0.7619$, $t = 18.229$, $df = 240$, $p < 0.001$) and with mean nodule weight (Pearson's product moment correlation $R^2 = 0.39$, $t = 6.7537$, $df = 241$, $p < 0.001$).

Only 8 of the 22 coinoculation combinations with at least one Fix+ strain caused significant host growth relative to uninoculated controls (one-sample t test; $p < 0.05$; Table S2; Figure 2A). Five additional coinoculation treatments including at least one Fix+ strain provided growth benefits that were marginal (one-sample t test; $0.05 < p < 0.10$; Table S2). These marginal or no-benefit treatments included those for which at least one or both inoculant strains provided a significant benefit in their corresponding clonal inoculation treatments. Surprisingly, only 3 out of 6 Fix+/Fix+ (+/+) coinoculation treatments elicited significant RG >2.5 (Table S2). Other treatments that elicited significant host growth included 5 of 16 Fix+/Fix− (+/−) combinations (Table S2). None of the Fix−/Fix− (−/−) coinoculation combinations provided a significant host growth benefit.

When the coinoculated treatments were grouped and compared by +/+, +/−, and −/− combinations, plants that received at least one Fix+ strain grew 2-fold more and formed significantly more nodules than hosts that were coinoculated with two Fix− strains (Figure S1). There were significant differences between +/+ and −/− and +/− and −/− categories for RG benefit (Welch's t test; +/+ versus −/−, $p = 0.0035$; +/− versus −/−, $p = 0.0043$; Figure S1A). The number of nodules also significantly differed between +/− and −/−, likely because plants that received Fix+ were larger (Welch's t test; +/− versus −/−, $p = 0.019$; Figure S1B). There were no significant differences in fresh nodule biomass and host investment values among any of the +/+, +/−, and −/− categories (Figures S1C and S1D). However, for coinoculated treatments 156 + 200, 186 + 156, 187 + 156, 2 + 131, and 4 + 156, investment in nodulation was high (>1) (Table S3). These treatments were comprised by one Fix+ and one Fix− strain, and the plants received no significant growth benefit from inoculation (Figures 2D and 2E; Table S3).

Fix+ strains dominate over Fix– strains in nodule occupancy

We used amplicon sequencing of the *nifD* locus and a dual-indices barcoding method to genotype rhizobia in nodules from each plant in coinoculation treatments. Genotyping nodule-occupying bacteria of coinoculated plants revealed that in +/− combinations, effective strains dominated nodules relative to ineffective strains (Figure 3A). Non-random nodule occupancy was found for all coinoculated treatments (i.e., χ^2 test, rejecting the null of equal nodule occupancy among strains; Table S4). Non-random nodulation was also found for most coinoculated treatments when the inoculum ratio was used as a null (i.e., χ^2 test, rejecting the null for all treatments, except 4 + 131 and 4 + 156, where most of the nodules were coinfected; Table S4). Proportion of coinfected nodules was significantly higher in +/+ combinations (mean ± SE = 67.25 ± 10.08) compared to +/− (mean ± SE = 18.20 ± 3.94) or −/− (mean ± SE = 23.52 ± 8.58) combinations (Figure 3B). The dominant strain in each pair was determined by a majority occupancy of nodules (Figure 3C; Table S4). Mean nodule occupancy values were consistent with a linear dominance hierarchy, where 131 and 156 > 4 > 184 > 186 > 187 > 2 > 200 (the top four strains are

Table 3. Linear model testing effect of treatment and DPI on host benefit and nodulation during coinoculation

	Log_{10}(shoot RG + 0.5)[a]			$\sqrt{Total\ nodules}$[a]			Log(mean nodule biomass + 0.1) (mg)[a]			Log_{10}(investment + 0.2)[a]		
Sample size[b] = 231	adj. R^2 = 0.54			adj. R^2 = 0.40			adj. R^2 = 0.32			adj. R^2 = 0.29		
	W = 0.99			W = 0.988			W = 0.98			W = 0.97		
Shapiro-Wilk test[c]	p = 0.53			p = 0.063			p = 0.001			p = 0.00128		
ANCOVA	df	F	p	df	F	p	df	F	p	df	F	p
Intercept	1	48.502	4.647×10^{-11}	1	1.5763	0.2107	1	28.5467	2.490×10^{-07}	1	1.6847	0.195823
Elapsed DPI	1	164.206	$<2.2 \times 10^{-16}$	1	110.29	$<2.2 \times 10^{-16}$	1	62.34	1.906×10^{-13}	1	10.51	0.001389
Treatment	27	5.455	3.756×10^{-13}	27	2.63	6.513×10^{-05}	27	2.7821	2.466×10^{-05}	27	3.41	3.502×10^{-07}
Residuals	200			202			199			197		

ANCOVA statistics for testing whether response variables significantly vary among single inoculation treatments using DPI as covariate since plants were harvested continuously from 28 DPI. Degrees of freedom (df), F-statistics, and associated p values are reported for each model. See also Figure S1 and Tables S1–S3.

[a]Response variable transformations used in linear models to fit coinoculation data for different response variables (shoot RG, total nodules, mean nodule biomass, investment)

[b]Sample size indicates number of data points (n) used to fit the models. Adjusted R-squared values for each model are also reported.

[c]Shapiro-Wilk statistics testing normality of the residuals in the linear models. Test statistics, W and p value, are reported.

Fix+) (Figure 3D). The presence of a Fix+ strain increased mean nodule occupancy in coinoculation (Welch two-sample t test, t = 3.7941, df = 4.6801, p = 0.01437) as well as mean RG benefit (Welch two-sample t test, t = 4.0876, df = 3.7289, p = 0.01728); however, no significant effect was observed for mean nodule biomass (Welch two-sample t test, t = 1.766, df = 4.5128, p = 0.144) (Figures 3E and 3F). Strain 156 provided the lowest benefit among the four beneficial strains, but it dominated nodules relative to all ineffective strains and formed a high proportion of coinfected nodules when coinoculated with effective strains, except 131, from which it could not be genetically differentiated when assessed based on *nifD* alleles (Figures 3D–3F). As in the clonal inoculation results, strain 156 induced a pattern of host growth effects and nodulation that were consistent with a strategy of evading host sanctions despite providing marginal benefits.[49]

Nodule genotyping was validated by comparing Sanger and MiSeq sequence data for 90 samples. Both technologies yielded high-quality reads from 51 nodules, and both yielded complete genotype matches in 47% of them. On the other hand, in 41% of nodules sequenced, one technology detected both strains but the other detected only one of them, likely reflecting the differences in sensitivity between Sanger and MiSeq, while the remaining samples had no match (Table S5).

Coinoculated plants receive less host growth benefit than predicted from clonal inoculation

Based on data from the single-strain inoculation results, models were developed to infer expected values of symbiosis traits in coinoculated plants. Two models were developed and compared. Model I weighed symbiosis traits based on nodule occupancy and allowed us to test whether the observed overall plant growth benefit of each strain in coinoculation was significantly different from that expected based on the values from single-inoculation experiments. Model II normalized data based on the number of nodules formed and allowed us to tease apart some of the underlying mechanisms driving the overall reduction in plant growth in coinoculation experiments, including whether strains provided a lower degree of benefits, when controlling for the number of nodules formed. Twenty-seven coinoculated combinations were tested while the 156 + 131 combination was excluded. Under Model I, weighing for nodule occupancy, fourteen strain combinations induced significantly less host growth than expected based on clonal inoculation data (Equation 2). None of the coinoculated plants grew significantly more than expected (Figure 4A; Table S6). Similar patterns were observed for total number and area of nodules, in which observed trait values were almost always lower than the expected trait values (Figure 4B; Table S6). Among the 14 coinoculation treatments that produced a lower host growth response than the expected values, nine also had significantly lower observed nodulation relative to the values expected from the null model (Figures 4A and 4B). Only four of these coinoculation treatments also had significantly lower nodule areas than expected (Figure 4C).

Under Model II, normalizing based on nodule counts, the predicted RG had a better fit with the observed data (slope 0.93, $p < 0.001$; adjusted $R^2 = 0.6824$, $F_{1,232} = 501.7$, $p < 0.001$) compared to Model I (slope 0.334, $p < 0.001$; adjusted $R^2 = 0.063$, $F_{1,236} = 16.92$, $p < 0.001$). ANOVA revealed a significant difference between the expected shoot RG values in both models and the observed values. Based on post hoc Tukey HSD, the expected shoot RG values based on model I (i.e., nodule occupancy weighted method) were significantly greater than those observed (0.639 difference in mean, $p < 0.001$). Under model II, there was no significant difference between the expected and observed shoot RG values (0.139 difference in mean, $p = 0.117$). This suggests that the reduction in plant growth in coinoculation relative to single inoculation was not driven by a reduction in the per-nodule benefit each strain conferred to its host.

The deviation in growth of coinoculated plants from expected values was tightly correlated with nodule number differences (Pearson's product moment correlation $R^2 = 0.86$, $p < 0.001$; Figure S2). No autocorrelation was found in a Durbin-Watson test

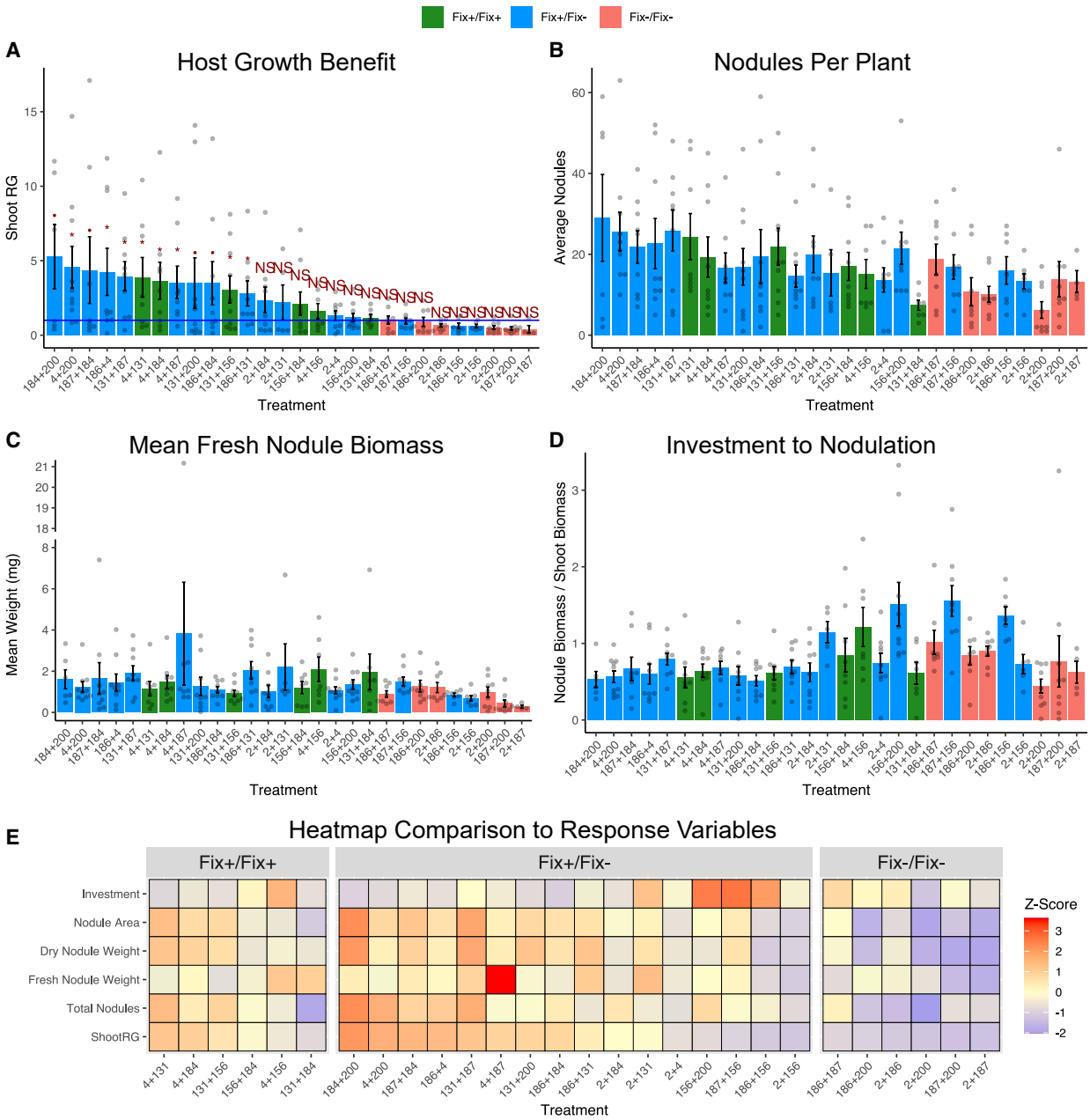


Figure 2. Plant growth and nodulation responses to coinoculation treatments

(A) Mean host relative growth is shown for each coinoculation treatment with the horizontal blue line indicating mean value of the uninoculated controls. p values between 0.001 and 0.05 are indicated with a single asterisk (*), between 0.05 and 0.1 with a period (.), and above 0.1 is NS (non-significant).

(B–D) Mean nodule counts (B), mean nodule biomass (C), and host investment into symbiosis (D).

Coinoculation treatments are organized from the most to the least beneficial. Bars indicate means and error bars indicate SEM.

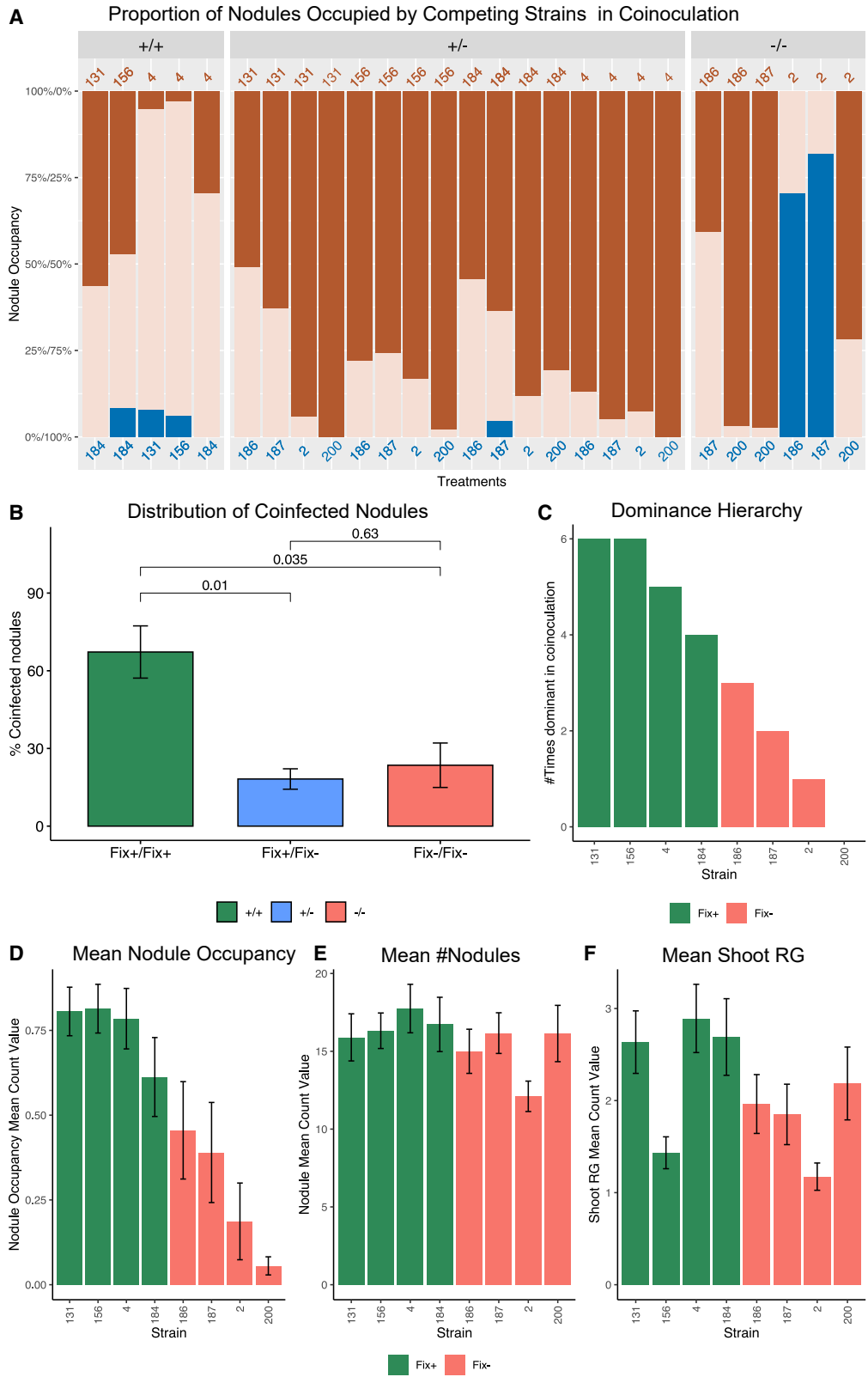
(E) A heatmap compares all response variables by standardizing the values into *Z* scores while breaking them in Fix+/Fix+, Fix+/Fix−, and Fix−/Fix− treatment groups. Bars are colored red when the coinoculation treatment was composed of two ineffective strains (Fix−/Fix−), blue for one effective and one ineffective strain (Fix+/Fix−), and green for two effective strains (Fix+/Fix+).

See also Figures S1 and S2 and Tables S2 and S3.

(D-W = 2.544, p = 0.122). In combination with the above data, we interpret this to mean that lower than expected benefits in coinoculation are associated with a reduction in nodule counts, consistent with inter-strain interference reducing both nodulation and net growth benefits.

Gene content differences suggest that competition traits are polygenic and linked with inter-strain interactions

To characterize why some inoculant strains were more competitive relative to others, the gene content of each strain was



(legend on next page)

compared. Genome sequences of the eight strains were analyzed for presence/absence variation (PAV) in 663 genes previously reported to be associated with competition.[30,95] Among the eight genomes, 130 genes exhibit PAV (Figure S3). PAV patterns of strains 156 and 131, the two most competitive strains, were compared to the other strains. Only one gene predicted to encode a hypothetical protein was found to be uniquely present in strain 131. No genes were uniquely present in strain 156 when strain 131 was excluded from the comparison. Compared to other Fix+ strains, strains 156 and 4 lacked genes associated with flagella-based motility (i.e., flagellin, probable flagellum biosynthesis repressor protein, putative flagellar synthesis-related protein, putative chemotaxis *MotC* protein, flagellar hook-associated protein 1, flagellar motor stator protein *MotA*, etc.; Figure S3). Strain 156 also contains several *virB* gene homologs associated with the type IV secretion system (T4SS) that are absent in other Fix+ strains (Figure S3). These T4SS genes are likely associated with conjugation of mobile genetic elements, such as integrative and conjugative elements and plasmids.[53,54] Finally, strain 156 contains several metabolic pathway genes that are not present in strain 131, including enzyme families of glucuronosyltransferase, glutamine synthetase, galactosyltransferase, phophoribosylformylglycinamidine cyclo-ligase, phosphoribosylglycinamide formyltransferase, and exopolyphosphatase (Figure S3).

Pangenome analysis of the eight focal genomes uncovered 39,676 gene families, the vast majority of which were defined as "cloud" genes (i.e., present in <15% of strains; Figure S4).[55] Strain 131 and strain 156 have 984 and 1173 unique genes, respectively, in comparison to the six other strains. However, of those, only 70 had a single-copy homolog present in both genomes compared to the remaining six strains. For strain 131, 85 unique genes with functional annotations were found but 61 are predicted to be associated with functions common among insertion sequence (IS) elements. Among the remaining unique genes of strain 131, putative encoded functions included efflux pumps (nickel and cobalt resistance protein *CnrB*, cation efflux system *CusA*) and antibiotic resistance protein (*AbaF*). For strain 156, 5 out of 43 annotated unique genes are IS elements, but no gene ontology-based functional enrichment was found for the rest of the genes. Strains 131 and 156 contain genes that encode for several light-activated proteins, including blue-light-activated and photosystem I assembly proteins, a category of genes that regulate root attachment during nodulation.[56] Sixteen unique genes encoding non-hypothetical proteins were found in both strain 131 and strain 156 (Data S3), including prophage integrase gene *IntA*, which is also a site-specific recombinase required for conjugative transfer of symbiotic and non-symbiotic ICEs.[57]

Competitive strains grow faster than less competitive strains

Four Fix+ strains were cultured in solid and liquid media in clonal and pairwise coinoculation to assess competition traits *in vitro*. During clonal growth in liquid minimal media, strain 156 had by far the fastest doubling time (8.82 h) and strain 4 was slowest (14.468 h), whereas strain 156 had the lowest carrying capacity (8.96×10^{10} cells) and strain 4 was the highest (9.04×10^{10} cells; Data S4A), differences that were significant among strain treatments (Data S4B).

In the mixed-strain experiments, carrying capacity had a significant treatment effect in the ANOVA followed by Tukey HSD post hoc test (Data S4B and S4C). Based on null predictions from clonal results, the mixed-strain treatment 4 + 184 was found to have significantly reduced carrying capacity ($t = -2.84$, $df = 4$, $p = 0.046$) and 156 + 184 ($t = 4.897$, $df = 4$, $p = 0.008$) was found to be significantly slower in doubling time (Data S4D).

In the solid media experiments that estimated population size, only strain 4 was significantly different from other treatments with a lower total population (2.25×10^8 cells/mL; Data S4E).

DISCUSSION

Our results suggest four main conclusions about interactions among rhizobia strains during the nodulation process. First, the linear dominance hierarchy that we uncovered indicates that competitive ability for nodulation is genetically determined and not altered by emergent effects of specific strain interactions, which could generate a nonlinear hierarchy or no hierarchy at all. It is likely that the hierarchy we uncovered does not perfectly reflect natural populations, given the high density of inocula we used in pairwise combinations and the otherwise sterile conditions, whereas in nature strains are likely to compete with a multitude of other rhizobia strains, and with other microbes as well. Nonetheless, dominance hierarchies were also uncovered in rhizobia communities that were inoculated onto *Acacia* hosts, although these varied depending on host species.[58] Population data are also consistent with dominance hierarchies; a genotypic meta-analysis of rhizobial populations reported that a handful of strains dominate nodules in host populations, with individual strains occupying more than 30% of those nodules.[59] Second, all four effective strains dominated nodule occupancy against ineffective rhizobia in terms of number of nodules inhabited, which provides a partial explanation of the observed dominance hierarchy. This dominance pattern of Fix+ strains over Fix− strains in coinoculation is observed irrespective of the strain they were coinoculated with, consistent with previous evidence of host sanctions that are robust to diverse strain identities.[60] Previous work also suggested that sanctions are robust to other

Figure 3. Nodule genotyping results

(A) Nodule occupancy is illustrated with bars indicating the proportion of each competitor strain within nodules that were coinoculated. Brown color indicates occupancy by the strain labeled at the top, blue indicates occupancy of the other strain labeled at the bottom, and tan indicates nodules infected by both strains. The coinoculated treatments are divided in +/+, +/−, and −/− groups based on the nitrogen-fixing capacity of each strain.

(B) Percent coinfected nodules in +/+, +/−, and −/− treatment groups.

(C) Strain dominance is quantified as the number of treatments where a strain had higher non-random nodule occupancy compared to the competing strain.

(D–F) Mean count value for number of nodules, relative growth, and nodule occupancy are indicated for each strain among all coinoculated treatments, respectively. Error bars indicate SEM.

See also Tables S4 and S5.

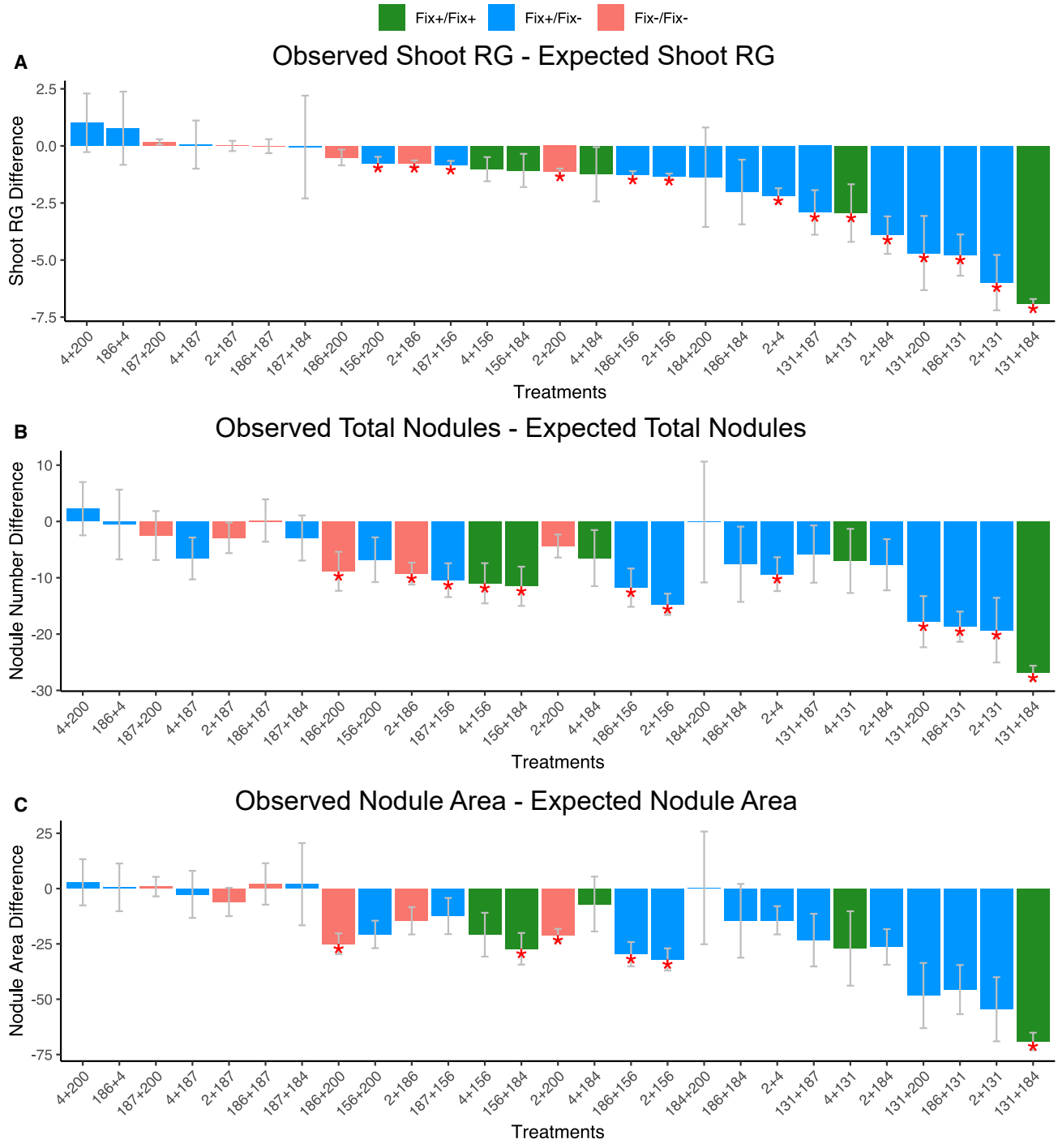


Figure 4. Observed data in coinoculated treatments relative to expectations based on the clonal inoculations

Relative growth (A), total nodules (B), and nodule area (C) are each displayed with the relevant expected values subtracted from them. Negative values indicate that the observed values were less than the expected ones. Asterisks indicates significant difference between observed and expected values (i.e., $p < 0.05$). Error bars indicate SEM. See also Figure S2 and Table S6.

sources of variation, showing that ineffective rhizobia are sanctioned independent of the level of extrinsic fertilization in the soil[61,62] or the host genotype.[48,63] This type of host control can be conditional, as data from peas suggest that host sanctions depend on the magnitude of nitrogen fixation in competing strains, where intermediate fixers are tolerated only if a better strain is not available.[64] Our data suggest some degree of conditionality. For instance, when plants were coinoculated with two

Fix+ strains, prevalence of coinfected nodules is significantly high compared to treatments with one or two Fix− strains in co-inoculation. The prevalence of coinfected nodules is also high compared to other published work in similar or different host-rhizobia systems (i.e., *Bradyrhiozbium-Acmispon*[47] or *Rhizobium leguminosarum/Trifolium*[65]). Third, while evidence suggests that hosts were able to selectively favor beneficial versus ineffective strains for nodule occupancy, the host control among Fix+ strains—which varied quantitatively in their nitrogen fixation benefits—does not suggest discrimination against marginally beneficial strains. In particular, strain 156, the fastest growing strain *in vitro*, occupied a higher number of single-infected nodules when competed against strain 184 and formed >90% coinfected nodules against strain 4. Although we cannot differentiate strain 156 from 131, and we cannot determine the relative occupancy of each strain in coinfected nodules, taking account of its very low host benefit in clonal inoculation and coinoculation indicates plant hosts can be less effective at sanctioning this strain in the presence of other effective strains.[48] In the broader context, such phenotypes can be viewed as an extreme end in a continuum of benefits to mutualist partners, where a strain can evade host sanctions despite providing minimal host benefit.[3,28,49,66] These results suggest that hosts and symbionts may be in conflict over the magnitude of resources exchanged, whereby some symbionts that provide minimal host benefits nonetheless receive greater host investment compared to more beneficial symbionts, thereby having a fitness advantage over other strains.[67,68] Fourth, and perhaps most urgent for the application of rhizobia in agriculture, we found that in pairwise coinoculation, hosts received significantly less benefit from rhizobia than expectations based on the clonal inoculations. A significant reduction was found in ~50% of strain combinations (Figure 4A), and in no cases did coinoculated hosts receive significantly greater than expected benefits. However, this study only tested pairwise combinations, whereas in agricultural or natural settings more strains are naturally present and participate in the symbiosis, and it remains to be seen how competition in a community of rhizobia impacts symbiotic benefit potential compared to clonal inoculation. These data, combined with the parallel reduction in the number of nodules formed in coinoculated plants, suggest that strain interactions reduce both the number of nodules formed and the net benefit received by hosts.

Strain interactions throughout the host infection process can favor competitiveness and erode the net benefit of symbiosis.[7,8] To predict effects of rhizobia in mixed-strain populations, we developed null models of host benefit parameterized with empirical data from clonal infections as well as the genotypic data on nodule occupancy in coinoculation. In a majority of coinoculated treatments, the observed RG was significantly lower than expected (Figure 4A). Strikingly, the coinoculation treatment of 131 + 184 generated no significant host growth benefits (Figure 2A), although these were the two highest benefiting strains in the clonal setting (Figure 1A). A similar trend was also found for nodulation by these two strains, where coinoculated plants formed among the fewest nodules in any coinoculation treatment (Figure 2B). More generally, the lower nodulation in coinoculation compared to clonal treatments, irrespective of nitrogen-fixation effectiveness, suggests that interference among strains is reducing nodulation. Rhizobia strain interactions exhibit antagonism *in vitro*.[69,70] Native *Bradyrhizobium* and *Rhizobium* inhibit growth of other strains in culture and in coinoculation on hosts, where bacteriocin-producing strains are found occupying more nodules relative to non-producing strains.[71] The potential for strains to be in conflict should be taken into consideration when preparing high-performing bioinocula to improve agricultural yield. A variety of interstrain competitiveness traits have been identified, including diverse bacteriocins, altered mobility, and metabolic capabilities of strains to utilize complex hydrocarbon chains.[72] Our work suggests that competitiveness is determined by the rhizobia genotype and is highly polygenic, shaped by functions such as conjugation and integration (Figure S3; Data S3). The ability to acquire novel genomic elements could allow strains to acquire loci that affect root attachment,[56] as well as antibiotic and resistance functions that can modulate inter-strain allelopathy (i.e., *cnrB*, *cusA*, and *abaF*). These data are also consistent with traits of competitiveness for nodulation and efficiency of nitrogen fixation being independent.[43] The *in vitro* experiments revealed that in some strain combinations, both doubling time and carrying capacity of rhizobia can be reduced in mixed populations relative to clonal ones (Data S4). Also, the lack of difference of observed per-nodule benefit in coinoculation from expected values based on model II indicates that the per-nodule growth benefit from coinoculation does not vary significantly from expected performance based on single inoculation. Overall, these data suggest that reduced host growth and reduced nodulation in coinoculated plants are largely driven by competitive interference among strains that occurs during the initial colonization of host roots—likely before nodule formation.

A long-standing goal in symbiosis research is to resolve the degree to which fitness is aligned between partners.[8,32,44,45,48,67,73,74] A meta-analysis observed that the fitness interests of rhizobia and plant hosts are aligned.[44] However, analyses were based largely on sets of single inoculation experiments, which cannot reliably predict performance of rhizobia in natural settings where multiple strains simultaneously compete for plant-derived nutrients.[42] In a handful of experiments that compared both clonal and a community inoculation, inoculation of single bacterial strains to a host plant was useful to evaluate the genotype's ability to form successful symbiosis but could not predict competitiveness with other strains.[43] Results from our coinoculation experiments indicate that beneficial strains are consistently more competitive than ineffective ones, but among beneficial strains, the dominance cannot always be confirmed due to a high number of coinfected nodules and technical limitations to determine relative nodule occupancy in coinfected nodules. However, the higher number of coinfected nodules among Fix+/Fix+ treatments reinforces the idea that host control has a threshold of benefit above which plants cannot effectively defend against strains that provide marginal benefits. Only in 4 of the 27 treatment combinations were nodules found to be singly infected by each strain alongside the presence of coinfected nodules, whereas in the remaining treatments, nodules were either singly infected by the dominant strain or coinfected by both strains (Figure 3A). This indicates that although all strains have the ability to form nodules on their own during single inoculation, only the dominant strain was able to form single-infected nodules in most of the cases, whereas the competitor strain was only

able to coinfect nodules. Formation of a high number of mixed nodules with no negative effect on plant growth has been reported where low or non-beneficial strains of *Sinorhizobium meliloti* can evade sanctions by *Medicago sativa* in the presence of a highly beneficial strain.[75] When focusing only on the strains that were effective on *A. strigosus*, all of them in coinoculation showed higher dominance over ineffective ones (Figure 3C) and elicited high nodule occupancy (Figure 3D). Although single-strain inoculation is less ecologically relevant since it excludes competitive interactions among strains, our results show that it is still predictive of the per-nodule growth benefit that a strain provides to its host in coinoculation.

Host control by legumes engages rhizobia at two stages of the infection process. The first stage, partner choice, involves flavonoid signals that hosts release, promoting responsive signals in the rhizobia, including nod factors and effectors.[76] This signal exchange winnows the pool of microbes that gain access to the root surface and selects against nodulation by incompatible strains.[77,78] Signal exchange may also involve plant immunity and rhizobial effectors secreted by the T3SS.[79,80] However, this initial stage is limited in its efficacy, as many ineffective strains can and do gain access to host nodules; even strains that cannot nodulate themselves can coinfect nodules alongside nodulating strains.[17] When legumes encounter rhizobia mutants that vary markedly in their capacity to fix nitrogen, but do not otherwise differ genetically from the parental strain, the plant host cannot differentiate among them prior to nodulation.[21,81,82] The second stage of host control, sanctioning, which occurs within nodules, is efficient at punishing non-fixing strains.[10,19,21–23,47] In our data, strains 2 and 200 elicited the lowest nodule counts in single inoculation, as well as lowest mean nodule occupancy in coinoculation, whereas strains 186 and 187 have the opposite pattern (Figures 1B and 3C), which reflects a difference in the competitiveness level of Fix− strains as well as a variation in host control. Hosts can sanction nonperforming symbionts, but the threshold that triggers this mechanism is unknown, and there may be a cost to this action. Therefore, it may not be beneficial for the host to sanction every non- or low-performing rhizobia.[41] Our findings help resolve the paradox that despite efficient host control post infection, legumes nonetheless encounter strains that generate only moderate host benefit compared to what is possible from single infection in symbiosis.[2] Despite efficiency of host control after nodulation, the host appears to have limited ability to overcome the reduction in growth benefits associated with competitive strain interference in the rhizosphere.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Rhizobia and plant genotypes
- METHOD DETAILS
  - Inoculation experiment
  - Plant harvest and nodule genotyping
  - Genomic analyses
  - *In vitro* experiments
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Data analyses

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.06.081.

ACKNOWLEDGMENTS

We acknowledge the Nelson lab for their help, as well as Holly Clark, Clay Clark, and Matthew Collin from the Genomics Core at the Institute of Integrative Genome Biology (IIGB), University of California, Riverside, for help and suggestions on the MiSeq sequencing. We also acknowledge the help of critical reviewers, whose comments led to substantial improvement in the manuscript. This work was funded by NSF DEB 1738009 and USDA Hatch Grant CA-R-EEOB-5200-H to J.L.S. and NSF DEB 1738028 to J.H.C.

AUTHOR CONTRIBUTIONS

A.R., M.M., A.J.W., J.H.C., and J.L.S. planned and designed the research. A.R., M.M., C.N., I.A.P., W.F.F., M.T.L., T.H.L., and L.T.M. conducted the experiments. A.R., M.M., A.J.W., L.T.M, J.H.C., and J.L.S. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community. One or more of the authors of this paper received support from a program designed to increase minority representation in their field of research. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

Received: January 23, 2023
Revised: March 31, 2023
Accepted: June 29, 2023
Published: July 24, 2023; corrected online: August 3, 2023

REFERENCES

1. Friesen, M.L., Porter, S.S., Stark, S.C., von Wettberg, E.J., Sachs, J.L., and Martinez-Romero, E. (2011). Microbially mediated plant functional traits. Annu. Rev. Ecol. Evol. Syst. *42*, 23–46.
2. Heath, K.D., and Stinchcombe, J.R. (2014). Explaining mutualism variation: a new evolutionary paradox? Evolution *68*, 309–317.
3. Johnson, N.C., Graham, J.H., and Smith, F.A. (1997). Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytol. *135*, 575–585.
4. Hoeksema, J.D., Chaudhary, V.B., Gehring, C.A., Johnson, N.C., Karst, J., Koide, R.T., Pringle, A., Zabinski, C., Bever, J.D., Moore, J.C., et al. (2010). A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. Ecol. Lett. *13*, 394–407.
5. Bentley, B.L. (1987). Nitrogen fixation by epiphylls in a tropical rainforest. Ann. Mo. Bot. Gard. *74*, 234.

6. Fürnkranz, M., Wanek, W., Richter, A., Abell, G., Rasche, F., and Sessitsch, A. (2008). Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica. ISME J. *2*, 561–570.
7. Frank, S.A. (1996). Host-symbiont conflict over the mixing of symbiotic lineages. Proc. Biol. Sci. *263*, 339–344.
8. Sachs, J.L., Quides, K.W., and Wendlandt, C.E. (2018). Legumes versus rhizobia: a model for ongoing conflict in symbiosis. New Phytol. *219*, 1199–1206.
9. Kiers, E.T., Duhamel, M., Beesetty, Y., Mensah, J.A., Franken, O., Verbruggen, E., Fellbaum, C.R., Kowalchuk, G.A., Hart, M.M., Bago, A., et al. (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. Science *333*, 880–882.
10. Kiers, E.T., Rousseau, R.A., West, S.A., and Denison, R.F. (2003). Host sanctions and the legume-rhizobium mutualism. Nature *425*, 78–81.
11. Sawada, H., Kuykendall, L.D., and Young, J.M. (2003). Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. J. Gen. Appl. Microbiol. *49*, 155–179.
12. Burdon, J.J., Gibson, A.H., Searle, S.D., Woods, M.J., and Brockwell, J. (1999). Variation in the effectiveness of symbiotic associations between native rhizobia and temperate AustralianAcacia: within-species interactions. J. Appl. Ecol. *36*, 398–408.
13. Denton, M.D., Coventry, D.R., Bellotti, W.D., and Howieson, J.G. (2000). Distribution, abundance and symbiotic effectiveness of Rhizobium leguminosarum bv. trifolii from alkaline pasture soils in South Australia. Aust. J. Exp. Agric. *40*, 25–35.
14. Chen, L., Figueredo, A., Villani, H., Michajluk, J., and Hungria, M. (2002). Diversity and symbiotic effectiveness of rhizobia isolated from field-grown soybean nodules in Paraguay. Biol. Fertil. Soils *35*, 448–457.
15. Collins, M.T., Thies, J.E., and Abbott, L.K. (2002). Diversity and symbiotic effectiveness of Rhizobium leguminosarum bv. trifolii isolates from pasture soils in south-western Australia. Soil Res. *40*, 1319–1329.
16. Ehinger, M., Mohr, T.J., Starcevich, J.B., Sachs, J.L., Porter, S.S., and Simms, E.L. (2014). Specialization-generalization trade-off in a Bradyrhizobium symbiosis with wild legume hosts. BMC Ecol. *14*, 8.
17. Gano-Cohen, K.A., Wendlandt, C.E., Al Moussawi, K., Stokes, P.J., Quides, K.W., Weisberg, A.J., Chang, J.H., and Sachs, J.L. (2020). Recurrent mutualism breakdown events in a legume rhizobia metapopulation. Proc. Biol. Sci. *287*, 20192549, https://doi.org/10.1098/rspb.2019.2549.
18. Poole, P., Ramachandran, V., and Terpolilli, J. (2018). Rhizobia: from saprophytes to endosymbionts. Nat. Rev. Microbiol. *16*, 291–303.
19. Oono, R., Denison, R.F., and Kiers, E.T. (2009). Controlling the reproductive fate of rhizobia: how universal are legume sanctions? New Phytol. *183*, 967–979.
20. Oono, R., Anderson, C.G., and Denison, R.F. (2011). Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates. Proc. Biol. Sci. *278*, 2698–2703.
21. Westhoek, A., Field, E., Rehling, F., Mulley, G., Webb, I., Poole, P.S., and Turnbull, L.A. (2017). Policing the legume-Rhizobium symbiosis: a critical test of partner choice. Sci. Rep. *7*, 1419.
22. Regus, J.U., Quides, K.W., O'Neill, M.R., Suzuki, R., Savory, E.A., Chang, J.H., and Sachs, J.L. (2017). Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. Am. J. Bot. *104*, 1299–1312.
23. Denison, R.F. (2000). Legume sanctions and the evolution of symbiotic cooperation by rhizobia. Am. Nat. *156*, 567–576.
24. Simms, E.L., and Taylor, D.L. (2002). Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. Integr. Comp. Biol. *42*, 369–380.
25. West, S.A., Kiers, E.T., Simms, E.L., and Denison, R.F. (2002). Sanctions and mutualism stability: why do rhizobia fix nitrogen? Proc. Biol. Sci. *269*, 685–694.
26. West, S.A., Kiers, E.T., Pen, I., and Denison, R.F. (2002). Sanctions and mutualism stability: when should less beneficial mutualists be tolerated? J. Evol. Biol. *15*, 830–837.
27. Sachs, J.L., Mueller, U.G., Wilcox, T.P., and Bull, J.J. (2004). The evolution of cooperation. Q. Rev. Biol. *79*, 135–160.
28. Foster, K.R., and Wenseleers, T. (2006). A general model for the evolution of mutualisms. J. Evol. Biol. *19*, 1283–1293.
29. Kiers, E.T., and Denison, R.F. (2008). Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. Annu. Rev. Ecol. Evol. Syst. *39*, 215–236.
30. Mendoza-Suárez, M., Andersen, S.U., Poole, P.S., and Sánchez-Cañizares, C. (2021). Competition, nodule occupancy, and persistence of inoculant strains: key factors in the rhizobium-legume symbioses. Front. Plant Sci. *12*, 690567.
31. Batstone, R.T., Burghardt, L.T., and Heath, K.D. (2022). Phenotypic and genomic signatures of interspecies cooperation and conflict in naturally occurring isolates of a model plant symbiont. Proc. Biol. Sci. *289*, 20220477.
32. Heath, K.D. (2010). Intergenomic epistasis and coevolutionary constraint in plants and rhizobia. Evolution *64*, 1446–1458.
33. Cleveland, C.C., Townsend, A.R., Schimel, D.S., Fisher, H., Howarth, R.W., Hedin, L.O., Perakis, S.S., Latty, E.F., Von Fischer, J.C., Elseroad, A., and Wasson, M.F. (1999). Global patterns of terrestrial biological nitrogen (N2) fixation in natural ecosystems. Global Biogeochem. Cycles *13*, 623–645. https://doi.org/10.1029/1999gb900014.
34. Ohyama, T. (2017). The role of legume-rhizobium symbiosis in sustainable agriculture. In Legume Nitrogen Fixation in Soils with Low Phosphorus Availability: Adaptation and Regulatory Implication, S. Sulieman, and L.-S.P. Tran, eds. (Springer International Publishing), pp. 1–20.
35. Foyer, C.H., Nguyen, H., and Lam, H.-M. (2019). Legumes-the art and science of environmentally sustainable agriculture. Plant Cell Environ. *42*, 1–5.
36. Choudhury, A.T.M.A., and Kennedy, I.R. (2005). Nitrogen fertilizer losses from rice soils and control of environmental pollution problems. Commun. Soil Sci. Plant Anal. *36*, 1625–1639.
37. Irisarri, P., Cardozo, G., Tartaglia, C., Reyno, R., Gutiérrez, P., Lattanzi, F.A., Rebuffo, M., and Monza, J. (2019). Selection of competitive and efficient rhizobia strains for white clover. Front. Microbiol. *10*, 768. https://doi.org/10.3389/fmicb.2019.00768.
38. Yates, R.J., Howieson, J.G., Reeve, W.G., and O'Hara, G.W. (2011). A re-appraisal of the biology and terminology describing rhizobial strain success in nodule occupancy of legumes in agriculture. Plant Soil *348*, 255–267.
39. Ouma, E.W., Asango, A.M., Maingi, J., and Njeru, E.M. (2016). Elucidating the potential of native rhizobial isolates to improve biological nitrogen fixation and growth of common bean and soybean in smallholder farming systems of Kenya. Int. J. Agron. *2016*, 1–7.
40. Koskey, G., Mburu, S.W., Njeru, E.M., Kimiti, J.M., Ombori, O., and Maingi, J.M. (2017). Potential of native rhizobia in enhancing nitrogen fixation and yields of climbing beans (Phaseolus vulgaris L.) in contrasting environments of eastern Kenya. Front. Plant Sci. *8*, 443.
41. Goyal, R.K., Mattoo, A.K., and Schmidt, M.A. (2021). Rhizobial-host interactions and symbiotic nitrogen fixation in legume crops toward agriculture sustainability. Front. Microbiol. *12*, 669404.
42. Kiers, E.T., Ratcliff, W.C., and Denison, R.F. (2013). Single-strain inoculation may create spurious correlations between legume fitness and rhizobial fitness. New Phytol. *198*, 4–6.
43. Bourion, V., Heulin-Gotty, K., Aubert, V., Tisseyre, P., Chabert-Martinello, M., Pervent, M., Delaitre, C., Vile, D., Siol, M., Duc, G., et al. (2017). Co-inoculation of a pea core-collection with diverse rhizobial strains shows competitiveness for nodulation and efficiency of nitrogen fixation are distinct traits in the interaction. Front. Plant Sci. *8*, 2249.

44. Friesen, M.L. (2012). Widespread fitness alignment in the legume–rhizobium symbiosis. New Phytol. *194*, 1096–1111.

45. Burghardt, L.T., Epstein, B., Guhlin, J., Nelson, M.S., Taylor, M.R., Young, N.D., Sadowsky, M.J., and Tiffin, P. (2018). Select and resequence reveals relative fitness of bacteria in symbiotic and free-living environments. Proc. Natl. Acad. Sci. USA *115*, 2425–2430.

46. Hollowell, A.C., Regus, J.U., Gano, K.A., Bantay, R., Centeno, D., Pham, J., Lyu, J.Y., Moore, D., Bernardo, A., Lopez, G., et al. (2016). Epidemic spread of symbiotic and non-symbiotic Bradyrhizobium genotypes across California. Microb. Ecol. *71*, 700–710.

47. Wendlandt, C.E., Regus, J.U., Gano-Cohen, K.A., Hollowell, A.C., Quides, K.W., Lyu, J.Y., Adinata, E.S., and Sachs, J.L. (2019). Host investment into symbiosis varies among genotypes of the legume Acmispon strigosus, but host sanctions are uniform. New Phytol. *221*, 446–458.

48. Gano-Cohen, K.A., Wendlandt, C.E., Stokes, P.J., Blanton, M.A., Quides, K.W., Zomorrodian, A., Adinata, E.S., and Sachs, J.L. (2019). Interspecific conflict and the evolution of ineffective rhizobia. Ecol. Lett. *22*, 914–924.

49. Jones, E.I., Afkhami, M.E., Akçay, E., Bronstein, J.L., Bshary, R., Frederickson, M.E., Heath, K.D., Hoeksema, J.D., Ness, J.H., Pankey, M.S., et al. (2015). Cheaters must prosper: reconciling theoretical and empirical perspectives on cheating in mutualism. Ecol. Lett. *18*, 1270–1284.

50. Sachs, J.L., Ehinger, M.O., and Simms, E.L. (2010). Origins of cheating and loss of symbiosis in wild Bradyrhizobium. J. Evol. Biol. *23*, 1075–1089.

51. Weisberg, A.J., Rahman, A., Backus, D., Tyavanagimatt, P., Chang, J.H., and Sachs, J.L. (2022). Pangenome evolution reconciles robustness and instability of rhizobial symbiosis. mBio *13*, e0007422.

52. Hollowell, A.C., Regus, J.U., Turissini, D., Gano-Cohen, K.A., Bantay, R., Bernardo, A., Moore, D., Pham, J., and Sachs, J.L. (2016). Metapopulation dominance and genomic-island acquisition of Bradyrhizobium with superior catabolic capabilities. Proc. Biol. Sci. *283*, 20160496. https://doi.org/10.1098/rspb.2016.0496.

53. Costa, T.R.D., Harb, L., Khara, P., Zeng, L., Hu, B., and Christie, P.J. (2021). Type IV secretion systems: advances in structure, function, and activation. Mol. Microbiol. *115*, 436–452.

54. Grohmann, E., Christie, P.J., Waksman, G., and Backert, S. (2018). Type IV secretion in Gram-negative and Gram-positive bacteria. Mol. Microbiol. *107*, 455–471.

55. Sitto, F., and Battistuzzi, F.U. (2020). Estimating pangenomes with Roary. Mol. Biol. Evol. *37*, 933–939.

56. Bonomi, H.R., Posadas, D.M., Paris, G., Carrica, M.d.C., Frederickson, M., Pietrasanta, L.I., Bogomolni, R.A., Zorreguieta, A., Goldbaum, F.A., and Goldbaum, F.A. (2012). Light regulates attachment, exopolysaccharide production, and nodulation in Rhizobium leguminosarum through a LOV-histidine kinase photoreceptor. Proc. Natl. Acad. Sci. USA *109*, 12135–12140.

57. Hernández-Tamayo, R., Sohlenkamp, C., Puente, J.L., Brom, S., and Romero, D. (2013). Characterization of IntA, a bidirectional site-specific recombinase required for conjugative transfer of the symbiotic plasmid of Rhizobium etli CFN42. J. Bacteriol. *195*, 4668–4677.

58. Vuong, H.B., Thrall, P.H., and Barrett, L.G. (2017). Host species and environmental variation can influence rhizobial community composition. J. Ecol. *105*, 540–548.

59. Mcinnes, A. (2004). Structure and diversity among rhizobial strains, populations and communities? A review. Soil Biol. Biochem. *36*, 1295–1308.

60. Sachs, J.L., Russell, J.E., Lii, Y.E., Black, K.C., Lopez, G., and Patil, A.S. (2010). Host control over infection and proliferation of a cheater symbiont. J. Evol. Biol. *23*, 1919–1927.

61. Kiers, E.T., Rousseau, R.A., and Denison, R.F. (2006). Measured sanctions: legume hosts detect quantitative variation in rhizobium cooperation and punish accordingly. Evol. Ecol. Res. *8*, 1077–1086.

62. Regus, J.U., Gano, K.A., Hollowell, A.C., and Sachs, J.L. (2014). Efficiency of partner choice and sanctions in Lotus is not altered by nitrogen fertilization. Proc. Biol. Sci. *281*, 20132587.

63. Ortiz-Barbosa, G.S., Torres-Martínez, L., Manci, A., Neal, S., Soubra, T., Khairi, F., Trinh, J., Cardenas, P., and Sachs, J.L. (2022). No disruption of rhizobial symbiosis during early stages of cowpea domestication. Evolution *76*, 496–511.

64. Westhoek, A., Clark, L.J., Culbert, M., Dalchau, N., Griffiths, M., Jorrin, B., Karunakaran, R., Ledermann, R., Tkacz, A., Webb, I., et al. (2021). Conditional sanctioning in a legume-Rhizobium mutualism. Proc. Natl. Acad. Sci. USA *118*, e2025760118.

65. Mendoza-Suárez, M.A., Geddes, B.A., Sánchez-Cañizares, C., Ramírez-González, R.H., Kirchhelle, C., Jorrin, B., and Poole, P.S. (2020). Optimizing Rhizobium-legume symbioses by simultaneous measurement of rhizobial competitiveness and N2 fixation in nodules. Proc. Natl. Acad. Sci. USA *117*, 9822–9831.

66. Johnson, N.C., and Graham, J.H. (2013). The continuum concept remains a useful framework for studying mycorrhizal functioning. Plant Soil *363*, 411–419.

67. Porter, S.S., and Simms, E.L. (2014). Selection for cheating across disparate environments in the legume-rhizobium mutualism. Ecol. Lett. *17*, 1121–1129.

68. Price, P.A., Tanner, H.R., Dillon, B.A., Shabab, M., Walker, G.C., and Griffitts, J.S. (2015). Rhizobial peptidase HrrP cleaves host-encoded signaling peptides and mediates symbiotic compatibility. Proc. Natl. Acad. Sci. USA *112*, 15244–15249.

69. Schwinghamer, E.A. (1971). Antagonism between strains of Rhizobium trifolii in culture. Soil Biol. Biochem. *3*, 355–363.

70. Maan, P.K., and Garcha, S. (2018). Bacteriocins from Gram-negative Rhizobium spp. Adv. Bio. Res. *9*, 36–43.

71. Goel, A.K., Sindhu, S.S., and Dadarwal, K.R. (1999). Bacteriocin-producing native rhizobia of green gram (Vigna radiata) having competitive advantage in nodule occupancy. Microbiol. Res. *154*, 43–48.

72. Wielbo, J. (2012). Rhizobial communities in symbiosis with legumes: genetic diversity, competition and interactions with host plants. Open Life Sci. *7*, 363–372.

73. Kimbrel, J.A., Thomas, W.J., Jiang, Y., Creason, A.L., Thireault, C.A., Sachs, J.L., and Chang, J.H. (2013). Mutualistic co-evolution of type III effector genes in Sinorhizobium fredii and Bradyrhizobium japonicum. PLoS Pathog. *9*, e1003204.

74. Quides, K.W., Salaheldine, F., Jariwala, R., and Sachs, J.L. (2021). Dysregulation of host-control causes interspecific conflict over host investment into symbiotic organs. Evolution *75*, 1189–1200.

75. Checcucci, A., Azzarello, E., Bazzicalupo, M., Galardini, M., Lagomarsino, A., Mancuso, S., Marti, L., Marzano, M.C., Mocali, S., Squartini, A., et al. (2016). Mixed nodule infection in Sinorhizobium meliloti–Medicago sativa symbiosis suggest the presence of cheating behavior. Front. Plant Sci. *7*, 835.

76. Wang, D., Yang, S., Tang, F., and Zhu, H. (2012). Symbiosis specificity in the legume: rhizobial mutualism. Cell Microbiol. *14*, 334–342.

77. Walker, L., Lagunas, B., and Gifford, M.L. (2020). Determinants of host range specificity in legume-rhizobia symbiosis. Front. Microbiol. *11*, 585749.

78. Wang, Q., Liu, J., and Zhu, H. (2018). Genetic and molecular mechanisms underlying symbiotic specificity in legume-Rhizobium interactions. Front. Plant Sci. *9*, 313. https://doi.org/10.3389/fpls.2018.00313.

79. Yasuda, M., Miwa, H., Masuda, S., Takebayashi, Y., Sakakibara, H., and Okazaki, S. (2016). Effector-triggered immunity determines host genotype-specific incompatibility in legume-Rhizobium symbiosis. Plant Cell Physiol. *57*, 1791–1800.

80. Nelson, M.S., and Sadowsky, M.J. (2015). Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes. Front. Plant Sci. *6*, 491.

81. Amarger, N. (1981). Competition for nodule formation between effective and ineffective strains of Rhizobium meliloti. Soil Biol. Biochem. *13*, 475–480.

82. Hahn, M. (1986). Competitiveness of a nif− Bradyrhizobium japonicum mutant against the wild-type strain. FEMS Microbiol. Lett. *33*, 143–148.

83. Regus, J.U., Wendlandt, C.E., Bantay, R.M., Gano-Cohen, K.A., Gleason, N.J., Hollowell, A.C., O'Neill, M.R., Shahin, K.K., and Sachs, J.L. (2017). Nitrogen deposition decreases the benefits of symbiosis in a native legume. Plant Soil *414*, 159–170.

84. Somasegaran, P., and Hoben, H.J. (1994). Verifying the nitrogen-fixing potential of glasshouse-selected soybean rhizobia in the field environment. In Handbook for Rhizobia (Springer), pp. 189–197.

85. Sachs, J.L., Kembel, S.W., Lau, A.H., and Simms, E.L. (2009). In situ phylogenetic structure and diversity of wild Bradyrhizobium communities. Appl. Environ. Microbiol. *75*, 4727–4735.

86. Sachs, J.L., Kembel, S.W., Lau, A.H., and Simms, E.L. (2009). In situ phylogenetic structure and diversity of wild *Bradyrhizobium* communities. Appl. Environ. Microbiol. *75*, 4727–4735.

87. Cruaud, P., Rasplus, J.-Y., Rodriguez, L.J., and Cruaud, A. (2017). High-throughput sequencing of multiple amplicons for barcoding and integrative taxonomy. Sci. Rep. *7*, 41948. https://doi.org/10.1038/srep41948.

88. Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T.G., Fookes, M., Falush, D., Keane, J.A., and Parkhill, J. (2015). Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics *31*, 3691–3693.

89. Fadrosh, D.W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R.M., and Ravel, J. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome *2*, 6.

90. Somasegaran, P., and Hoben, H.J. (2012). Handbook for Rhizobia: Methods in Legume-Rhizobium Technology (Springer Science & Business Media).

91. Mi, H., Muruganujan, A., Huang, X., Ebert, D., Mills, C., Guo, X., and Thomas, P.D. (2019). Protocol update for large-scale genome and gene function analysis with the PANTHER classification system (v.14.0). Nat. Protoc. *14*, 703–721.

92. Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., and Schloss, P.D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. *79*, 5112–5120.

93. Somervuo, P., Koskinen, P., Mei, P., Holm, L., Auvinen, P., and Paulin, L. (2018). BARCOSEL: a tool for selecting an optimal barcode set for high-throughput sequencing. BMC Bioinf. *19*, 257. https://doi.org/10.1186/s12859-018-2262-7.

94. Griekspoor, A., and Groothuis, T. (2006). 4peaks. mekentosj.com.

95. Wheatley, R.M., Ford, B.L., Li, L., Aroney, S.T.N., Knights, H.E., Ledermann, R., East, A.K., Ramachandran, V.K., and Poole, P.S. (2020). Lifestyle adaptations of Rhizobium from rhizosphere to symbiosis. Proc. Natl. Acad. Sci. USA *117*, 23823–23834.

96. UniProt Consortium (2021). UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Res. *49*, D480–D489.

97. Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T.L. (2009). BLAST+: architecture and applications. BMC Bioinf. *10*, 421.

98. Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. Bioinformatics *30*, 2068–2069.

99. Ratzke, C., Barrere, J., and Gore, J. (2020). Strength of species interactions determines biodiversity and stability in microbial communities. Nat. Ecol. Evol. *4*, 376–383.

100. Sexton, J.T., and Tabor, J.J. (2020). Multiplexing cell-cell communication. Mol. Syst. Biol. *16*, e9618.

101. Bartlett, J.W. (2018). Covariate adjustment and estimation of mean response in randomised trials. Pharm. Stat. *17*, 648–666. https://doi.org/10.1002/pst.1880.

102. R Core Team (2013). R: A language and environment for statistical computing. http://www. R-project. org/.

103. Wendlandt, C.E., Gano-Cohen, K.A., Stokes, P.J.N., Jonnala, B.N.R., Zomorrodian, A.J., Al-Moussawi, K., and Sachs, J.L. (2022). Wild legumes maintain beneficial soil rhizobia populations despite decades of nitrogen deposition. Oecologia *198*, 419–430.

104. Pahua, V.J., Stokes, P.J.N., Hollowell, A.C., Regus, J.U., Gano-Cohen, K.A., Wendlandt, C.E., Quides, K.W., Lyu, J.Y., and Sachs, J.L. (2018). Fitness variation among host species and the paradox of ineffective rhizobia. J. Evol. Biol. *31*, 599–610.

105. Holm, S. (1979). A simple sequentially rejective multiple test procedure. Scand. Stat. Theory Appl. *6*, 65–70.

106. Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

107. Magoč, T., and Salzberg, S.L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics *27*, 2957–2963.

108. Savin, N.E., and White, K.J. (1977). The Durbin-Watson test for serial correlation with extreme sample sizes or many regressors. Econometrica *45*, 1989.

109. Sprouffske, K., and Wagner, A. (2016). Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. BMC Bioinf. *17*, 172.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Bradyrhizobium sp.	Lab stock	See Table 1
Chemicals, peptides, and recombinant proteins		
Conetainer pots	Steuwe and Sons, Corvallis, Oregon, USA	Item # SC10R
Calcined clay (Turface Pro League)	Turface Athletics, Buffalo Grove, Illinois, USA	Catalog # BFEL5040P
Nitrogen-free Jensen's fertilizer	Sachs et al.[85]	N/A
Modified Arabinose Gluconate (MAG)	Sachs et al.[85]	NRRL Medium # 46, USDA
Rhizobium defined medium (RDM)	Sachs et al.[85]	Catalog # 30627030, PlantMedia
Critical commercial assays		
HighPrep PCR cleanup	MagBio, USA	Catalog #AC-60050
Deposited data		
Harvest data	This paper	GitHub: https://github.com/acarafat/competition_experiment
Experimental models: Organisms/strains		
Acmispon strigosus	Lab stock	Line # AcS049
Oligonucleotides		
PCR1 *nifD* sequence specific primers	This study	See STAR Methods
PCR2 dual index primer	Cruaud et al.[87]	N/A
Software and algorithms		
ImageJ	https://ImageJ.org	Version 1.50i
R	https://r-project.org/	Version 4.1.3
Python	https://python.org/	Version 3.8
FastQC	https://github.com/s-andrews/FastQC	Version 0.12.1
FLASH	https://github.com/ebiggers/flash	Version 1.2.11

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Joel L. Sachs (joels@ucr.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The data are openly available at GitHub on https://github.com/acarafat/competition_experiment. Any other data reported in this paper will be shared by the lead contact upon request.
- All original code has been deposited at GitHub and is publicly available as of the date of publication on https://github.com/acarafat/competition_experiment.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Rhizobia and plant genotypes

Eight *Bradyrhizobium* strains were selected that varied quantitatively in the magnitude of growth benefits provided to hosts, including four strains that were categorized as effective because they elicited significant growth benefits in inoculated hosts relative to

uninoculated control hosts (i.e., Fix+; #'s 4, 131, 156, 184) and four strains that were categorized as ineffective because they did not cause significant host growth benefits (i.e., Fix-; #'s 2, 186, 187, 200)[17] (Table 1). The host line *A. strigosus* AcS049 was selected for greenhouse experiments, and was initially sampled from the Bernard Field Station, Claremont, CA.[62,83] Plants were raised from a wild seed progenitor, and were allowed to self for at least two generations in a greenhouse before being used here.[47]

METHOD DETAILS

Inoculation experiment

Seeds were surface sterilized in 5% NaOCl for 3 min, rinsed in autoclaved reverse-osmosis water (RO-H_2O) for 7 min, nick scarified, and sowed into sterilized SC10R Ray Leach Conetainer pots (diameter 3.81 cm, depth 20.96 cm, volume 164 mL, Steuwe and Sons, Corvallis, Oregon, USA) filled with sterilized calcined clay (Turface Pro League, Turface Athletics, Buffalo Grove, Illinois, USA) which offers negligible nutrients. Once true leaves formed, seedlings were moved to a greenhouse and fertilized weekly with 1 mL nitrogen-free Jensen's fertilizer.[84] Fertilization volume was increased weekly by 1 mL until a maximum of 5 mL was reached, which continued until harvest. After 4 days of hardening to greenhouse conditions under 50% shade, plants were inoculated. Rhizobia inocula were prepared by streaking single colonies onto plates of Modified Arabinose Gluconate medium (MAG[86]), scraping grown cells, adjusting cell concentration based on turbidimetric readings, and washing cells in RO-H_2O. A Klett-Summerson 800-3 photoelectric colorimeter was used (American Laboratory Trading, San Diego, California, USA) to get turbidimetric reading of the culture on a KlettTH scale which is proportional to optical density.

The full factorial coinoculation experiment included plants that were treated with each of eight clonal strains, 28 pairwise strain combinations, and uninoculated controls. Plants received 5 mL cultures at concentrations of 1×10^8 cells/mL. This protocol was selected based on previous inoculation experiments showing consistent nodulation of *A. strigosus* at this concentration of *Bradyrhizobium*.[17,47,86] For coinoculations, the concentration of cultures from each strain were adjusted before combining to reach a total concentration of 1×10^8 cells/mL (Data S2). Control plants received 5 mL of autoclaved RO-H_2O. Each treatment group included 10 plant replicates, and locations for plants were randomized across all treatments. A total of 370 plants were used during the inoculation experiment (10 replicates × 37 treatments [8 single-inoculation, 28 coinoculation, 1 control]). To verify concentrations, each clonal inoculum was quantitatively plated.[90] Plants were watered daily with 10 min of misting. Inoculation occurred in two batches on April 5 and 11, 2019. Each batch received inoculation in five replicates of all treatments.

Plant harvest and nodule genotyping

Plants were harvested starting 28 days post inoculation (dpi) from May 6 - June 19, 2019. Plants were harvested continuously, and individual plants were randomly selected for harvest. Plants were removed from the soil, shoots and roots were photographed, and nodules were dissected and counted. From photographs, nodule area was measured using ImageJ (v1.50i). Ten nodules from each coinoculated plant were randomly selected for rhizobia genotyping and preserved at -80°C. Roots, shoots, and remaining nodules were separated and dried in a convection oven at 60°C to quantify dry biomass. For plants selected for nodule genotyping with fewer than ten nodules, all nodules were genotyped. A total of 63 plants were removed from the dataset, 35 because of algal contamination or multiple plants growing in the same pot, 24 plants that died before harvest, and 4 were removed due to human error during data-collection process (Data S1). No pattern was observed in plant mortality or in algal contamination across treatments.

A pooled dual-indexed amplicon sequencing approach was used to genotype nodules. Nodules were thawed, surface sterilized in bleach for 30 s, rinsed in autoclaved RO-H_2O, and using a sterile pestle ground to a slurry in 200 μL RO-H_2O. The nodule slurry was directly used for a PCR reaction since within nodules DNA from rhizobia exists in high concentrations. Two PCR steps were used to prepare the library.[87] Primer pairs used in the first PCR were designed to amplify the *nifD* gene (target sequence for forward primer 5′-GAAAAGGATATCGTSTTCGGC-3′ and reverse primer 5′-GTCRCCRCCGATGTTRTARTC-3′) and also included sequences of the standard Illumina sequencing primers and a 0 to 3 bp "heterogeneity spacer".[89] The *nifD* gene contains SNPs that differentiate 27 of the 28 strain combinations (except strains #131 and #156 for which the sequences were identical). The primer pairs used in the second step added adapter sequences and eight-nucleotide index sequences sampled from an index-list using BARCOSEL.[92,93]

In the first PCR, reactions contained nodule slurry (2μL), 5x Q5 buffer (2μL), dNTPs (2mM, 1μL), Q5 Polymerase (0.1μL), forward and reverse primers (2mM, 0.5μL), and molecular grade water (3.9μL). DNA concentration between samples were not controlled since presence-absence of each strain is being measured based on minimal read cutoffs, thereby DNA concentration variation is unlikely to play an important role. PCR conditions were 98°C for 30 s for initial denaturation, then 98°C for 10 s and 74°C for 30 s for both extension and annealing for 35 cycles, and a final extension in 72°C cycle for 2 min. In the second PCR, amplicons were dual indexed with multiple identifiers for each sample.[87] The same PCR conditions were used as in the first PCR step. Negative controls, where only DNA grade water instead of nodule slurry was added, were included in each PCR batch. After the second PCR, all amplicons were pooled. The pooled library was cleaned using the HighPrep PCR cleanup (MagBio, USA). An Agilent Bioanalyzer 2100 with the DNA High Sensitivity kit (Agilent Technologies, USA) was used to check for quality and quantity of the library along with a qPCR reaction targeting the adapter sequences (New England BioLabs library quantification kit for Illumina). A PhiX control library was combined with the amplicon library (3.9%). The library was sequenced (2 × 300 bp) on a MiSeq flowcell, using a 600 cycle V3 MiSeq sequencing kit.

To cross-validate MiSeq genotyping, 90 samples from *nifD* PCR products were genotyped using Sanger sequencing (Table S5). The 4Peaks software package was used to examine single and dual peaks in electropherograms to identify SNPs that differ between strains.[94]

Genomic analyses

Genome content of the eight strains was investigated for presence-absence patterns of previously reported competition-associated genes, including 535 genes associated with nodulation competition in *Rhizobium leguminosarum*[95] and 128 genes associated with rhizosphere colonization, interstrain interference, or establishment of effective symbiosis and plant-growth promotion across multiple rhizobial taxa.[30] Gene sequences were downloaded from UniProt, and tBLASTn was used to search for presence of homologs in the eight genome sequences.[96,97] A filter of e-value < 0.004 and BLAST coverage > 80% was used to summarize the BLAST output. Heatplot was used to visualize gene presence-absence patterns using R (version 4.1.3) (Figure S3).

Pangenome analysis was performed to associate gene presence patterns with nodulation success. Prokka (version 1.14.6) was used to annotate the genomes and Roary (3.11.2) was used for pangenome analysis[88,98] (Figure S4). PantherDB.org was used to test for statistical overrepresentation of gene sets unique to competitive strains with a *Bradyrhizobium diazoefficiens* genome[91] (Data S3).

In vitro experiments

The Fix+ strains had higher nodule occupancy in the coinoculation experiment against Fix- strains, but some Fix+ strain combinations resulted in low host benefit compared to the single inoculation experiment. To quantify growth and cell-cell interactions, growth rate and competition were assayed for the four Fix+ strains using liquid and solid media experiments[99,100] (Data S4). For liquid experiments, two independent flask cultures of each strain were prepared in MAG media, grown cells were washed, and 10^8 cells per replicate were used to seed 30 mL liquid cultures in minimal Rhizobium Defined Medium (RDM).[85] To quantify growth rate and strain interaction effects, treatments were replicated 5-fold and were either clonal (i.e., initiated with 10^8 cells each from two different flasks with the same strain) or mixed (i.e., initiated with 10^8 cells each from flasks with different strains) and all pairwise strain combinations were tested. To initially allow cell-cell interactions, cultures were incubated at 29°C and shaken at 60 RPM for 18 h, increased to 100 RPM for 6 h, then to 180 RPM for the rest of the experiment. Colorimeter readings quantified doubling time and carrying capacity over 150 h. The same replicates and strain combinations were repeated for a solid media experiment, wherein 10^8 cells per replicate culture were spread-plated onto 23 mL solid RDM plates and incubated at 29°C for 8 days. After incubation, cells were scraped from plates and quantified for population size using a Klett-Summerson 800-3 colorimeter as described above.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analyses

Statistical analyses were carried out using R (version 4.1.3).[102] Host growth response to inoculation was estimated by dividing the shoot biomass values of inoculated plants by the shoot biomass values of uninoculated control plants[103] (Data S2; Table S2). Nodule count and total nodule mass were used to estimate rhizobial fitness at the plant level.[104] Nodules from each plant which were selected for genotyping were used to measure mean nodule weight after drying and then multiplied by total nodule numbers to get the nodule biomass estimation. Host investment into symbiosis was quantified as nodule biomass value divided by the shoot biomass value of each inoculated plant.[63] Data transformation was carried out to achieve normality and heteroscedasticity. Linear models were used to investigate variation in host growth response and nodulation traits. For each response variable, two linear models testing effects of treatments, with or without inoculation batch as a random-effect variable, were compared using a log-likelihood test for a significant random effect (Table S1). Analysis of covariance (ANCOVA) was carried out with type III sum of square errors to test effects of clonal and coinoculation treatments on the response variables (relative growth, total nodules, mean nodule weight, host investment) with dpi as a covariate. Significant differences among treatments were assessed using Tukey's HSD tests. Marginal means were estimated which are useful to compare among treatments since they adjust for the covariate and other factors[101] (Data S2; Table S3). Pearson's product-moment correlation coefficient was computed to assess the linear relationship of nodule count and mean nodule weight with host growth response separately. Coinoculation treatments were categorized as '+/+', '+/-', and '-/-' depending on whether Fix+ (strain 4, 131, 156, 184) and or Fix- (strain 2, 186, 187, 200) strains were paired together within or across groupings. Two sample t-tests were carried out between the coinoculation categories for relative growth, total nodules, mean fresh nodule biomass and investment, using Holm–Bonferroni correction for multiple comparisons[105] (Figure S1).

Nodule occupancy in the coinoculation treatments was inferred by analyzing MiSeq reads. Briefly, quality of the demultiplexed fastq files were assessed with FastQC.[106] FLASH v1.2.11[107] was used to merge forward and reverse reads in the fastq files, using 10-100 bp overlap range, 0.3 mismatch ratio, and a 28 Phred score cutoff. For each read in each sample, unique SNPs were compared with each reference sequence.[52] A custom R script (GitHub: https://github.com/acarafat/competition_experiment) was used to assign strain occupancy in the nodules, wherein reads were categorized to a strain if there were > 80% match to the unique SNPs and $\geq$ 10 reads matching the strain. If both coinoculated strains met this criterion, the nodule was classified as being coinfected. For each coinoculation treatment, all genotyped nodules were aggregated to calculate an average strain occupancy. In total, reads from 1125 nodules were analyzed. Nodule occupancy values for each coinoculation treatment (i.e., strains A + B) were tested using a goodness of fit $\chi 2$ test against a null model of nodule occupancy wherein an equal number of nodules were expected to be

randomly infected by A only, B only, and A + B (i.e., 1:1:1)[47] (Table S4). Another χ^2 analysis was carried out to test whether the number of nodules occupied by each strain in co-inoculation treatments significantly differed from the empirically estimated inoculum ratio (i.e., quantitative culturing results; Table S1) wherein coinfected nodules were counted as being 50% infected by each strain (Table S4). To cross-validate sequencing data, MiSeq and Sanger genotyping results were compared and categorized as a 'match' (identical), 'partial match' (where one approach shows a single genotype but other shows coinfection), or 'mismatch' (where each approach identifies a different genotype) (Table S5). In Sanger sequencing trace files, presence of two chromatogram peaks in multiple known SNP sites was used to identify coinfected nodules. The distribution of coinfected nodules was compared by classifying coinoculated treatment groups in three categories: Fix+/Fix+, Fix+/Fix-, and Fix-/Fix- combinations and a Wilcoxon rank-sum test was used to compare the mean percentage of coinfected nodules in each category followed by adjustment of p values for multiple testing using Holm-Bonferroni method.[105]

Data from the relevant clonal inoculation treatments weighted by nodule occupancy of each participating strain were used to develop null models (Model I and Model II) to infer expected values of symbiosis traits in coinoculated plants. Nodule occupancy for a focal strain 'A' was calculated as the total fraction of nodules that were wholly and partially occupied by the strain (i.e., f_A).

$$Nodule\ occupancy, f_A = \frac{\#Nodules\ occupied\ by\ A}{\#Total\ Nodules} + \frac{\#Nodules\ coinfected}{2 \times \#Total\ Nodules} \quad \text{(Equation 1)}$$

To predict the growth effects of two coinoculated strains, A & B (i.e., *Expected relative growth*$_{AB}$), we summed the growth effect of each relevant strain (in clonal inoculation) weighted by its relative nodule occupancy from the nodule genotyping results (i,e., f_A and f_B). This was the first approach used to predict expected value and hereby referred to as Model I. The same approach was also used to predict expected values for nodule counts and mean nodule biomass:

$$Expected\ Value_{AB} = Value_A \times f_A + Value_B \times f_B \quad \text{(Equation 2)}$$

One-sample t-tests were used to test whether observed trait values were significantly different from the expected values (Table S6). However, for the observed difference in relative growth, this test does not resolve if the deviation from expected is due to lower nodulation or lower benefit provided by rhizobia in infected nodules compared to clonal inoculation. To test whether the observed difference from expected was due to lower per nodule symbiotic benefit provided by the rhizobia or not, another expected relative growth was calculated. For this second approach (Model II), average per nodule growth benefit of each strain is calculated for single inoculation. The number of nodules formed by each strain in coinoculation, if they were infected by a single strain (i.e., no coinfected nodules), was calculated by multiplying the frequency of each strain from nodule occupancy data by the total number of nodules formed in coinoculation. Finally, per nodule expected growth benefit was calculated by multiplying the strain means for per nodule host benefit in single inoculation by the number of nodules formed with each strain in coinoculation and summing the resulting values for the two strains. A linear regression model was used to compare both expected values (i.e., Model I, nodule occupancy weighted relative growth and Model II, nodule-normalized relative growth) with the observed value, and a one-way ANOVA was used to compare observed relative growth with expected values predicted by these two models, followed by a Tukey's HSD test.

A Pearson correlation between residuals of nodule number versus nodule area and relative growth was used to evaluate association between observed and expected relative growth, nodule number, and nodule area (Figure S2). A Durbin-Watson test was used to check for autocorrelation in the residuals.[108] To understand the general performance of all coinoculation treatments that contain a specific strain, mean values for the number of nodules, relative growth, and nodule occupancy were calculated for each strain across all coinoculated treatment combinations using the following formula:

$$Mean\ Count\ Value, MCV_A = \frac{\left(\sum_{A \neq B} Count\ in\ Treatment_{AB}\right)}{(n - 1)} \quad \text{(Equation 3)}$$

A Welch two-sample t-test was used to test for significant differences in mean count values of nodule occupancy, relative growth, and number of nodules between Fix+ and Fix- traits.

For the *in vitro* analyses, growth curve data from strains grown in a liquid medium were analyzed using the Growthcurver package (v0.3.1) in R which fits a logistic equation based on point estimates of bacterial population size[109] (Data S4). Using these data, doubling time and carrying capacity were estimated. ANOVA and post-hoc Tukey's HSD tests were used to investigate differences between treatments in each clonal and competition experiment. Doubling time and carrying capacity of each strain was used to make null predictions for the mixed strain combinations by calculating mean values for strain pairs. One-sample t-tests were used to compare observed means in competition and predicted values for each variable.