Journal of Evolutionary Biology, 2024, **XX**, 1–11
https://doi.org/10.1093/jeb/voae151
Advance access publication 11 December 2024
Research Article

JOURNAL OF
Evolutionary Biology
eseb

Host control by *Acmispon strigosus* constrains fitness gains of ineffective *Bradyrhizobium* symbionts in mixed infections

Camille E. Wendlandt[1], Julio Avelar-Barragan[1], Avissa J. Zomorrodian[2], Khadija Al-Moussawi[2], Stephanie S. Porter[3], Joel L. Sachs[1,2,4]

[1]Department of Botany & Plant Sciences, University of California, Riverside, CA, United States
[2]Department of Evolution, Ecology & Organismal Biology, University of California, Riverside, CA, United States
[3]School of Biological Sciences, Washington State University, Vancouver, WA, United States
[4]Institute of Integrative Genome Biology, University of California, Riverside, CA, United States

Handling editor: Rebekah Rogers, Associate editor: Loreta Brandao de Freitas

Corresponding author: Joel L. Sachs, Department of Botany & Plant Sciences, University of California, Riverside, 3401 Watkins Dr., Riverside, CA 92521, United States; Department of Evolution, Ecology & Organismal Biology, University of California, Riverside, 3401 Watkins Dr., Riverside, CA 92521, United States; Institute of Integrative Genome Biology, University of California, Riverside, 3401 Watkins Dr., Riverside, CA 92521, United States. Email: joels@ucr.edu

Abstract

Plant hosts can gain significant growth benefits from symbiosis with microbes, but these benefits could be threatened by divergent fitness interests among partners. Here, we measured fitness outcomes in symbiosis, by varying the genotypes of both microbes and hosts, to examine scenarios that might favour uncooperative symbionts. We studied associations between *Acmispon strigosus*, an annual legume native to California, and its nitrogen-fixing symbionts in the genus *Bradyrhizobium*. *Bradyrhizobium* symbionts form root nodules on compatible hosts, with strains varying from effective, fixing substantial nitrogen for the host, to ineffective strains that do not fix nitrogen and provide no benefit to host growth. We co-inoculated four *A. strigosus* plant lines with nine combinations of effective and ineffective *Bradyrhizobium* strains and measured the relative fitness of ineffective strains within individual nodules, as hosts must select against uncooperative symbionts to maintain benefits. In mixed infections, ineffective strains always had lower relative fitness in nodules compared to beneficial strains, consistent with efficient punishment of non-fixing rhizobia. However, ineffective strains exhibited genotypic variation in their fitness in nodules within individual nodules co-infected with a beneficial strain, suggesting a role for symbiont competitiveness in shaping this joint phenotype. Variation in symbiont fitness during co-inoculations did not measurably affect plant performance, suggesting that predicted conflict over the joint phenotype of rhizobia fitness has negligible effect on the host.

Keywords: conflict, fitness alignment, joint phenotype, mutualism, nitrogen fixation, sanctions

Introduction

Plants and animals gain substantial benefits from establishing mutualistic symbioses with microbes (Drew et al., 2021). Microbial communities collectively possess a vast genetic repertoire and can generate key services for hosts, such as nitrogen fixation, antibiotic production, and bioluminescence (Fronk & Sachs, 2022). In exchange, hosts shelter microbial partners from harsh external environments and provide reliable sources of energy that can enhance microbial survival and reproduction (Denison & Kiers, 2004). Because symbiotic microbes often reproduce within the host organism, their rate of reproduction can represent a joint phenotype—a trait that is influenced by the genes of both host and symbiont partners (Queller, 2014; Quides et al., 2021). In symbioses, there can be conflict over how much of the host's resources are invested into its microbial partners. From the perspective of host fitness, its resources should be used to fuel microbial services to a degree that optimizes host growth and reproduction. From the perspective of microbial fitness, host resources should be extracted to optimize microbial reproduction, potentially at the expense of providing mutualistic resources to the host (Douglas & Werren, 2016; Fronk & Sachs, 2022; Jones et al., 2015; Queller & Strassman, 2018). Natural selection on either partner can shift joint phenotypes toward one or the other partner's benefit, but only if there is sufficient genetic variation to enable such change. One important question is whether standing genetic variation, residing in both symbionts and hosts, often leads to interactions where uncooperative symbionts have superior fitness to cooperative ones, which could destabilize the mutualism.

The legume-rhizobia mutualism is a powerful system to investigate symbiont fitness and the host's capacity to affect it via selective rewards or punishment. Rhizobia are soil bacteria with the capacity to instigate the formation of root nodules on compatible legume hosts and to fix nitrogen for the host in exchange for photosynthates (Ledermann et al., 2021). Compatible rhizobia enter root cells, where a subset of cells differentiate into endosymbiotic bacteroids that can fix nitrogen (Ledermann et al., 2021). Nodules eventually senesce, usually when the plant host is setting seeds, thus

Received July 22, 2024; accepted December 3, 2024

releasing rhizobia back into the soil (Porter et al., 2024). In natural and managed settings, rhizobia vary in the magnitude of net benefits provided to hosts, ranging from effective, i.e., fixing substantial nitrogen and enhancing host growth, to ineffective, i.e., non-nitrogen-fixing, having neutral or even negative growth effects on hosts (Burdon et al., 1999; Chen et al., 2002; Collins et al., 2002; Denton et al., 2000; Gano-Cohen et al., 2020; Sachs et al., 2010a). Host legumes can selectively reward effective rhizobia and minimize investment in ineffective strains. In mixed inocula, this discrimination can take the form of nodules being preferentially formed by more effective strains (Gubry-Rangin et al., 2010; Heath & Tiffin, 2009; Regus et al., 2014), as well as plants forming larger nodules with more effective strains (Kiers et al., 2003; Sachs et al., 2010b; Regus et al., 2015; Wendlandt et al., 2019). Since nodules can be co-infected by multiple strains, some plants can also punish ineffective rhizobia at the level of individual plant cells by inducing cell-autonomous senescence of host cells infected with ineffective rhizobia (Regus et al., 2017), reducing the relative abundance of ineffective rhizobia within nodules (Oono et al., 2011; Regus et al., 2014; Sachs et al., 2010b, Westhoek et al., 2017). Finally, both the rhizosphere and nodules themselves can also contain a diversity of other (i.e., non-rhizobia) bacteria that can potentially alter strain competition or host control (Granada Agudelo et al., 2023; Han et al., 2020; Martínez-Hidalgo & Hirsch, 2017).

Despite multiple layers of host control against ineffective rhizobia, plants frequently associate with both effective and ineffective strains, rather than the single most cooperative genotype available (Montoya et al., 2023; Rahman et al., 2023). One potential explanation for this pattern is that the processes of nodulation and nitrogen fixation depend on both the plant and bacterial partners, resulting in some partner combinations that provide no benefit to the host. Some variation in symbiosis outcome is attributed to genetic variation among hosts in their preference for the most effective symbionts (Kiers et al., 2007; Simonsen & Stinchcombe, 2014; Wendlandt et al., 2019). Variation among rhizobia communities available in the soil can also influence the growth of legumes, irrespective of the host genotype (Burghardt & diCenzo, 2023; Han et al., 2020; Manci et al., 2023). Rhizobia compete intensely to colonize legumes, and competitiveness traits in rhizobia often vary independently of symbiotic effectiveness, promoting the nodulation of symbionts that provide little or no benefits to hosts (Burghardt & diCenzo, 2023). Joint control over symbiotic outcomes could lead to interspecific conflict, for instance if rhizobia gain benefits from providing less fixed nitrogen to their hosts (Gano-Cohen et al., 2019; Porter & Simms, 2014).

Here, we investigated symbiosis fitness outcomes in the association between the annual legume *Acmispon strigosus* and its *Bradyrhizobium* symbionts. *A. strigosus* is native to the southwestern United States and northwestern Mexico, where it forms nodules with diverse *Bradyrhizobium* that range from highly effective to ineffective (Gano-Cohen et al., 2019, 2020; Weisberg et al., 2022). We performed greenhouse experiments in which four host plant lines were inoculated with nine combinations of effective and ineffective *Bradyrhizobium* strains. Single-strain inoculations characterized each strain's symbiotic effectiveness and *in planta* fitness on all four host lines. Co-inoculations of effective and ineffective strains quantified relative fitness of rhizobia *in planta* to observe how much variation was contributed by the strain genotypes, host genotypes, and their interactions. Finally, we examined patterns of plant performance in the co-inoculation experiment to test whether ineffective strains negatively affected plant performance. This provides important context for possible variation in symbiont fitness since plants only have a selective incentive to evolve stricter host control if control over resource allocation to symbionts affects host performance. We hypothesize that interstrain competition among rhizobia can alter fitness outcomes during mixed infections, and predict that ineffective strains will have equal or superior fitness to beneficial strains in some strain combinations. We hypothesize that hosts will vary in their capacity to control mixed infections, predicting that some hosts will be more successful at punishing ineffective rhizobia, and that the fitness of hosts exposed to mixed infections will vary among host genotypes.

Materials and methods

Rhizobia strains

Diverse *Bradyrhizobium* strains were previously characterized on an inbred line of *A. strigosus* (AcS049) for nodulation, nitrogen fixation capacity, and growth benefits to hosts (Hollowell et al., 2016a,b; Gano-Cohen et al., 2020). Strains that fixed nitrogen and significantly improved host growth relative to uninoculated controls were classified as effective, whereas those that provided no significant growth benefit were classified as ineffective. Six focal strains were selected for experimental analysis of growth benefits to hosts and *in planta* fitness, including three effective strains (i.e., 49, 138, CW09) and three ineffective strains (i.e., 2, 187, CW01). Building on previous work, antibiotic resistance profiles were characterized, allowing strains to be distinguished in mixed inocula by culturing on selective media (Hollowell et al., 2015; Table 1).

Acmispon lines

Seeds of *A. strigosus* were collected from four natural sites in California between 2009 and 2012, including Bodega Marine Reserve (BMR), Griffith Park (Gri), University of California, Riverside (UCR), and Pioneertown Mountains Preserve near Yucca Valley (Yuc). Plants were raised from wild seeds in a glasshouse sprayed weekly with insecticide and plants were allowed to self-fertilize. We collected seeds from individual plants to generate full-sib inbred lines and selected one inbred line per field site to use here. Due to low germination of the Gri line, we supplemented experimental plants with a replicate Gri line sourced from a different wild seed from the same collection location (Table 2). Previous work demonstrated that these four host lines are genetically and phenotypically distinct, differing at two loci (nrITS, CNGC5; Table 2) and varying in mean nodule size, with BMR and UCR plants forming larger nodules than Gri and Yuc plants (Wendlandt et al., 2019). Since plant regulation of nodule size is one component of host control, we anticipated that these plant lines might also show divergent patterns of host control over symbiont fitness *in planta*.

Inoculation experiment

Axenic *A. strigosus* seedlings were germinated in sterilized calcined clay (Pro League; Turface Athletics, Buffalo Grove, IL, USA) in Ray-Leach SC10 pots (Stuewe & Sons, Corvallis, OR, USA). Plants were grown without supplemental nitrogen to maximize host demand for the symbiont's mutualistic service. Plants were fertilized weekly with 1 ml nitrogen-free Jensen's

Table 1. Predicted effectiveness, collection information, genotype data, and antibiotic resistance profiles of *Bradyrhizobium* strains used in this study.

Strain ID	Isolate ID	Year isolated	Site source[a]	CHR genotype[b]	SI genotype[c]	Strep100[d]	Chlor150[d]	Gent190[d]	Inoculum (CFU ml^{-1})[e]
Effective strains									
#49	05LoS23R7_12	2005	BMR	G03_R01	Z02_L75[f]	Sensitive	Resistant	Sensitive	1.06×10^8
#138	13LoS15_1	2013	Gri	G91_R225	Z12_L77[f]	Sensitive	Resistant	Sensitive	1.23×10^8
CW09	14LoS82_7	2014	Cla	G244_R01[f]	Z02_L04[f]	Sensitive	Resistant	Sensitive	1.77×10^8
Ineffective strains									
#2	05LoS24R3_28	2005	BMR	G14_R14	Z59_L74[f]	Resistant	.	.	1.26×10^8
#187	11LoS7_1	2011	Dim	G03_R01	Z37_L49	.	Sensitive	.	1.32×10^8
CW01	14LoS3_1	2014	UCR	G03_R01[f]	Z02_L04[f]	.	.	Resistant	1.27×10^8

Note. BMR = Bodega Marine Reserve, Cla = Bernard Field Station of the Claremont Colleges, Dim = San Dimas, Gri = Griffith Park, UCR = University of California, Riverside.
[a]Denotes field site where isolate was obtained (see Hollowell et al., 2016b).
[b]CHR genotype includes glnII (G) and recA (R) loci.
[c]SI genotype includes nodZ (Z) and nolL (L) loci.
[d]Strep100 = 100 ug ml^{-1} streptomycin; Chlor150 = 150 ug ml^{-1} chloramphenicol; Gent190 = 190 ug ml^{-1} gentamycin.
[e]Empirically determined concentration of the clonal inoculum.
[f]Loci were sequenced in 2016 by Kelsey Gano-Cohen.

Table 2. Collection and genotyping information for *Acmispon strigosus* plant lines used in this study.

Site	Formal name	Collection year	Greenhouse year	Sympatric strain(s)	nrITS accession	CNGC5 accession
BMR	AcS074.BMR.u01.g1.r04	2011	2012	#2, #49	KX449154	KX449165
Gri	AcS075.Gri.u01.g1.r01	2012	2014	#138	MH220053	MH223490
Gri	AcS075.Gri.u01.g1.r15	2012	2013	#138	.	.
UCR	AcS027.UCR.u01.g1.r10	2009	2014	CW01	MH201360	MH223492
Yuc	AcS052.Yuc.m01.g1.r02	2011	2012	.	KX449162	KX449173

Solution (Somasegaran & Hoben, 1994), increasing by 2 ml per week until reaching a total of 5 ml per week, which was maintained throughout the experiment. Plants with true leaves were transferred to the glasshouse and hardened for 1–3 weeks until inoculation on 21 February 2017. *Bradyrhizobium* strains were grown on modified arabinose gluconate (MAG) agar plates (Sachs et al., 2009), washed off plates into liquid MAG, quantified by colorimetry, pelleted, and resuspended in sterile ddH_2O to generate inocula of 1×10^8 cells ml^{-1}. Concentrations of single and mixed inocula were verified via serial dilution and plating onto replicate MAG-agar plates (Table 1).

The four lines of *A. strigosus* were each experimentally exposed to 16 inoculation treatments including six single inoculation treatments (three effective, three ineffective strains), nine co-inoculation treatments (comprising each pairwise combination of effective and ineffective strains) and an uninoculated control treatment. Axenic seedlings of each line were organized by size using true leaf counts and sets of size-matched plants from each plant line were randomly assigned to inoculation treatments within replica blocks, with plant positions within blocks being randomized. Plants were inoculated with either 5 ml of clonal *Bradyrhizobium* cultures (single inoculation treatments), 5 ml of a 1:1 mixture of two clonal cultures (co-inoculation treatments) or 5 ml sterile ddH_2O (uninoculated controls). In total, the experiment included four host lines × sixteen inoculation treatments × eight replica blocks (512 plants total).

Harvest

The plants were harvested over a 4-week span, with two blocks harvested each week at 6-, 7-, 8-, and 9-weeks post-inoculation (wpi). To minimize variation in plant size across harvest weeks, blocks were harvested in reverse order of initial seedling size assessed at the start of the experiment. Plants were removed from pots, washed free of sand, and dissected into root, shoot, and nodule portions. Roots and shoots were oven-dried (60 °C, >96 hr) and weighed. Nodules were counted and photographed on graph paper. At each harvest week, a subset of nodules was cultured from all plants in one experimental block. Non-senescent (i.e., ranging from pink to white, and lacking any green or brown coloration) nodules were selected at random for culturing, surface sterilized with bleach, rinsed, and crushed to generate nodule extracts.

Among singly inoculated plants, 192 nodules were individually cultured to assess relative strain frequency within individual nodules. This sample included 8 nodules sampled for each of the host line and strain combinations (i.e., 8 nodules × 4 host lines × 6 strains). The 8 replicate nodules were sampled from each of the four harvest weeks (two per week sampled from an individual plant, randomly selected from one block). Nodules were homogenized with sterile pestles, and the homogenates were spread onto two replicate MAG-agar plates (100 mm) with dilutions of 10^{-3} and 10^{-5} (Sachs et al., 2009), and the number of colony-forming units (CFU) per nodule was calculated from at least two plates containing from 3 to 800 colonies. Among co-inoculated plants, four nodules were cultured from one randomly selected plant replicate each harvest week (4 nodules per harvest week × 4 harvest weeks × 4 host lines × 9 co-inoculation treatments = 576 nodules total). Approximately 100 colonies per nodule were subcultured onto two separate MAG-agar plates, including one with an antibiotic (see Table 1) and another as positive

control (no antibiotic). Colony growth was scored after 4–10 days of growth at 29 °C, depending on the antibiotic (Table 1). Colonies with ambiguous scores were subcultured again, and colonies with persistent ambiguous scores were excluded from further analyses.

Data analysis

Generalized linear mixed models were used to test hypotheses using JMP Pro 13.0.0 (SAS Institute Inc., Cary, NC, USA). Dependent variables were $\log_{10}$-transformed as needed to improve normality. Proportional data was logit-transformed after applying a linear transformation to account for zeros and ones in the dataset (i.e., 1% was added to all datapoints except ones, from which 1% was subtracted). All models included a random effect of harvest week; models using plant biomass data (and not nodule culturing data) also included a random effect of block nested within harvest week. For each GLMM, all possible interactions among main effects of interest were initially tested. Non-significant interactions were removed from the model if this reduced the corrected AIC (AICc) by at least 2 units, and results from trimmed models were reported. Significant differences among levels of main effects were assessed with pairwise *t*-tests (Tukey's HSD) of least squares means. The Test Slices option was used to explore interaction effects when only specific contrasts were of interest. Mean values discussed below were backtransformed (if applicable) from raw means and presented alongside 95% confidence intervals.

Rhizobia strains were categorized as effective or ineffective depending on whether the total dry plant biomass of inoculated plants was significantly greater than that of the uninoculated control plants in the single inoculation experiment. Two different fitness proxies were estimated for rhizobia strains, including rhizobia population size per nodule (in the single inoculation experiment) and relative strain frequency within a nodule (i.e., proportion of CFU from a nodule of a particular strain in the co-inoculation experiment). Rhizobia population size (i.e., CFU) per nodule was averaged between replicate nodule cultures with the plant as the unit of replication ($n = 4$) and was tested for effects of strain genotype and host line. Nodule occupancy for a strain was quantified as its relative abundance (i.e., proportion of CFU/nodule) on each replicate plant ($n = 4$), for which the null expectation was 50% (i.e., relative abundance in the inoculum), and was tested for effects of the ineffective strain genotype, effective strain genotype, and host line.

Plant relative performance was examined in the co-inoculation experiment by dividing the total plant biomass of each co-inoculated plant by the biomass of plants singly inoculated with the effective strain. Relative performance less than one would indicate that plants performed worse during mixed inoculations than with the effective strain alone, suggesting a cost to encountering the ineffective strain. Relative performance of co-inoculated plants was tested for significant deviation from one based on whether the confidence interval overlapped with one. Plant performance was tested for effects of the effective strain genotype, ineffective strain genotype, and host line.

Results

The effectiveness of different rhizobia strains depended on the rhizobia strain × plant line combination (significant inoculum × plant line effect on plant biomass; Table 3). Strains 49, 138, and CW09 were effective for all plant lines, and strains 2 and 187 were ineffective for all plant lines (Figure 1). However, strain CW01 exhibited plant line-dependent effectiveness, being effective on BMR and UCR plants but ineffective on Gri and Yuc plants (Figure 1). Overall, more than 92% of single-strain inoculated plants (i.e., 215/232) formed nodules, and none of the uninoculated control plants formed nodules (Supplementary Table S1). The rhizobia population size per nodule data was obtained from 85/96 singly inoculated plants from which nodules were cultured (representing data from 133/192 nodules cultured). The remaining nodules either failed to generate colonies when plated or had colony counts outside the acceptable range for quantifying the rhizobia population size. Almost all co-inoculated plants also formed nodules (i.e., 287/288 plants; Supplementary Tables S1 and S2).

Rhizobia fitness in the single inoculation treatments varied strikingly among strains with almost 100-fold differences in mean population size per nodule. The fitness of rhizobia depended both on the rhizobia strain and the plant line (significant strain × plant line effect on rhizobia population size per nodule; Table 3). Averaging across plant lines, fitness was greatest for ineffective strain 2 (~6.1×10^6 rhizobia per nodule), followed by effective strain 49 (~4.5×10^6), context-dependent strain CW01 (~1.8×10^6), ineffective strain 187 (~6.6×10^5), effective strain CW09 (~3.9×10^5), and effective strain 138 (~6.3×10^4), indicating that effective strains did not experience greater fitness per nodule compared to ineffective strains in single infections (Figure 2). Most strains did not differ in rhizobia population size per nodule across plant lines, but strain CW09 had greater rhizobia population size per nodule on Yuc than Gri plant lines (Figure 2).

Data on relative strain frequency within a nodule were recovered from 142 of 144 co-inoculated plants, representing 478/576 cultured nodules and 38,727 scored colonies. Most nodules (276/478) were subcultured at or above the desired depth of 100 colonies per nodule (median = 102 colonies per nodule). For co-inoculation treatments using ineffective strains 2 and 187, both ineffective strains exhibited extremely low frequencies within nodules, significantly below the null expectation of 50%, consistent with hosts favouring effective strains in nodules (Figure 3A). Ineffective strain percent abundance varied significantly among ineffective strain genotypes (Table 3), with strain 2 achieving greater percent abundance in nodules (2.8% [1.4%–5.0%; i.e., 95% confidence intervals]) than 187 (1.1% [0.5%–1.9%]).

Relative strain frequency of CW01 within nodules varied significantly among co-inoculation treatments with effective strain genotypes but not among plant lines (i.e., including the plant population where it was effective; Table 3). CW01 achieved the greatest nodule occupancy when co-inoculated with 138 (74.5% [55.3%–87.3%]) and lower abundance when co-inoculated with 49 (17.5% [5.6%–43.2%]) or CW09 (7.7% [4.7%–12.3%]; Figure 3B). A subset of colonies was subcultured twice to check the reliability of the antibiotic assay. We found inconsistency in the assay distinguishing strain CW01 from the three effective strains: colonies identified originally as one of the effective strains (49, 138, CW09) were detected as CW01 at a rate of ~50% in the second assay, likely because of antibiotic resistance mutations (Table 1). However, colonies identified originally as CW01 were consistently identified as CW01 again in the second assay. Based on

Table 3. General linear mixed models testing *Acmispon* host and *Bradyrhizobium* strain contributions to (1) strain phenotypes in single inoculations, (2) strain relative fitness in co-inoculations, and (3) plant relative performance in co-inoculations.

1. Characterizing strain phenotypes			
Total plant dry mass, mg			
Data subset			
Single inoculations, all strains (including uninoculated controls)			
Transformation	***Adj. R2***	***n***	
$\log_{10}$	0.70	219	
Effect	***df***	***F***	***p***
Inoculum	6, 184.1	25.91747	<0.0001
Host population	3, 184.1	98.5084	<0.0001
Inoculum × Host population	18, 184.1	2.1968	0.0047
Block(Harvest week), *random*	.	.	0.2792
Harvest week, *random*	.	.	0.9408
Rhizobial CFU per nodule			
Data subset			
Single inoculations, all strains			
Transformation	***Adj. R2***	***n***	
$\log_{10}$	0.63	85	
Effect	***df***	***F***	***p***
Strain genotype	5, 58.25	16.2909	<0.0001
Host population	3, 58.5	5.0973	0.0033
Strain genotype × Host population	15, 58.24	2.9865	0.0014
Harvest week, *random*	.	.	0.4504
2. Examining patterns of rhizobial relative fitness			
Ineffective strain percent abundance in nodules			
Data subset			
Co-inoculations, only treatments using strains #2, #187			
Transformation	***Adj. R2***	***n***	
logit	0.16	96	
Effect	***df***	***F***	***p***
Ineffective strain genotype	1, 86	4.9818	0.0282
Effective strain genotype	2, 86	2.6501	0.0764
Host population	3, 86	0.5748	0.6331
Harvest week, *random*	.	.	0.3545
Ineffective strain percent abundance in nodules			
Data subset			
Co-inoculations, only treatments using strain CW01			
Transformation	***Adj. R2***	***n***	
logit	0.44	46	
Effect	***df***	***F***	***p***
Effective strain genotype	2, 37.19	18.1140	<0.0001
Host population	3, 37.17	0.4080	0.7482
Harvest week, *random*	.	.	0.7267
3. Examining patterns of host relative performance			
Plant relative performance			
Data subset			
Co-inoculations, only treatments using strains #2, #187			
Transformation	***Adj. R2***	***n***	
$\log_{10}$	0.14	192	
Effect	***df***	***F***	***p***
Ineffective strain genotype	1, 172	0.9009	0.3439

Table 3. Continued

3. Examining patterns of host relative performance			
Effective strain genotype	2, 172	0.8884	0.4132
Host population	3, 172	2.0085	0.1146
Effective strain × Host population	6, 172	4.8921	0.0001
Block(Harvest week), *random*	.	.	0.6444
Harvest week, *random*	.	.	0.9797
Plant relative performance			
Data subset			
Co-inoculations, only treatments using strain CW01			
Transformation	***Adj. R2***	***n***	
$\log_{10}$	−0.01	95	
Effect	***df***	***F***	***p***
Effective strain genotype	2, 82.28	0.4236	0.6561
Host population	3, 82.28	2.8051	0.0448
Block(Harvest week), *random*	.	.	0.3251
Harvest week, *random*	.	.	0.6967

these data, CW01 could be more abundant in nodules than we report (conservatively) here, using the original scores.

Co-inoculation treatments varied in their relative effects on plant performance, dependent both on host line and the strains present. Focussing on host lines, mean relative performance for co-inoculated UCR and Yuc plant lines was 0.89× (95% confidence interval; 0.74–1.06×) and 1.03× (0.88–1.21×), respectively, indicating no cost to encountering an ineffective strain. For the BMR and Gri plant lines, relative performance varied by strain combination. The BMR line had greater relative performance with co-inocula containing CW09 (1.64× [1.02–2.66×]) or 138 (1.47× [0.87–2.50×]), rather than 49 (0.62× [0.49–0.77×]). For Gri plants, relative performance was higher when co-inocula contained 138 (1.09× [0.76–1.55×]) or 49 (0.92× [0.59–1.42×]), rather than CW09 (0.61× [0.43–0.85×]). Focussing on rhizobia, the impact of ineffective strains 2 and 187 on the performance of co-inoculated plants differed among plant lines (i.e., significant interaction effect of effective strain genotype × plant line; Table 3; Figure 4A). The impact of strain CW01 also varied among plant lines (Table 3; Figure 4B). In cases where plant performance during co-inoculations was lower than performance with effective strains alone, this was a function of effective strain genotype rather than ineffective strain genotype. For instance, the relative performance of plants varied significantly among effective strains for BMR plants ($F_{2,172} = 10.2178$, $p < 0.0001$) and Gri plants ($F_{2,172} = 3.1928$, $p = 0.0435$) but not UCR ($F_{2,172} = 2.0292$, $p = 0.1346$) or Yuc ($F_{2,172} = 0.1248$, $p = 0.8828$) plants.

Discussion

Host legumes can constrain fitness rewards to rhizobia that do not provide sufficient benefit (Ledermann et al., 2021; Porter et al., 2024), but little is known about how consistent legume host control is, and whether it can be subverted by ineffective rhizobia. Here, we experimentally inoculated the legume host *Acmispon strigosus* with wild *Bradyrhizobium* strains that vary from highly beneficial to ineffective. Our experiments included both single-strain inoculations and two-strain co-inoculations to compare the relative fitness of plants and effective and ineffective rhizobia in both settings. We investigated the degree to which fitness outcomes are affected by the host and symbiont genotypes and their interactions.

Our work uncovered strong effects of host control when the host legumes were exposed to mixed inoculations of beneficial and non-fixing rhizobia. These inoculations resulted in dramatic fitness reductions of ineffective strains, irrespective of the host genotype. The failure of host genotypes to contribute variation to strain fitness in co-inoculation could have multiple explanations. One possibility is that *A. strigosus* populations are closely related, with little genetic differentiation among them. However, close host relatedness would be unexpected given that the source populations are hundreds of kilometres away from each other and that plant lines vary at two marker genes (Table 2). Another explanation is that host control is conserved, despite variation in other traits, perhaps due to its importance for plant fitness. The physiological bases of such host control are actively being researched and could involve host mediation of carbon or oxygen flux within nodules, selective senescence of nodules or rhizobia within them, or host mediation of antimicrobials within nodules (Ledermann et al., 2021; Porter et al., 2024). Beyond confirming the efficiency of host control, our results suggest two broad conclusions about the evolutionary maintenance of this mutualism in the face of uncooperative partners.

Strain fitness depends on the presence and identity of competitor strains

The first broad conclusion is that rhizobia fitness outcomes depend strongly on which strains are competing for host access. The most obvious effect was whether strain competitors were present at all. When hosts were clonally inoculated—preventing legume choice (Porter & Simms, 2014)—*in planta* fitness varied significantly among rhizobia genotypes. Inconsistent with host control, there was no fitness advantage for effective rhizobia in clonally infected hosts, in contrast to findings in related symbioses (Montoya et al., 2023). *In planta fitness* was consistently very high for the ineffective strain 2 across all hosts tested, comparable or higher than other strains, as shown in previous work (Pahua et al., 2018; Sachs et al., 2010a). The host line affected *in planta* fitness of

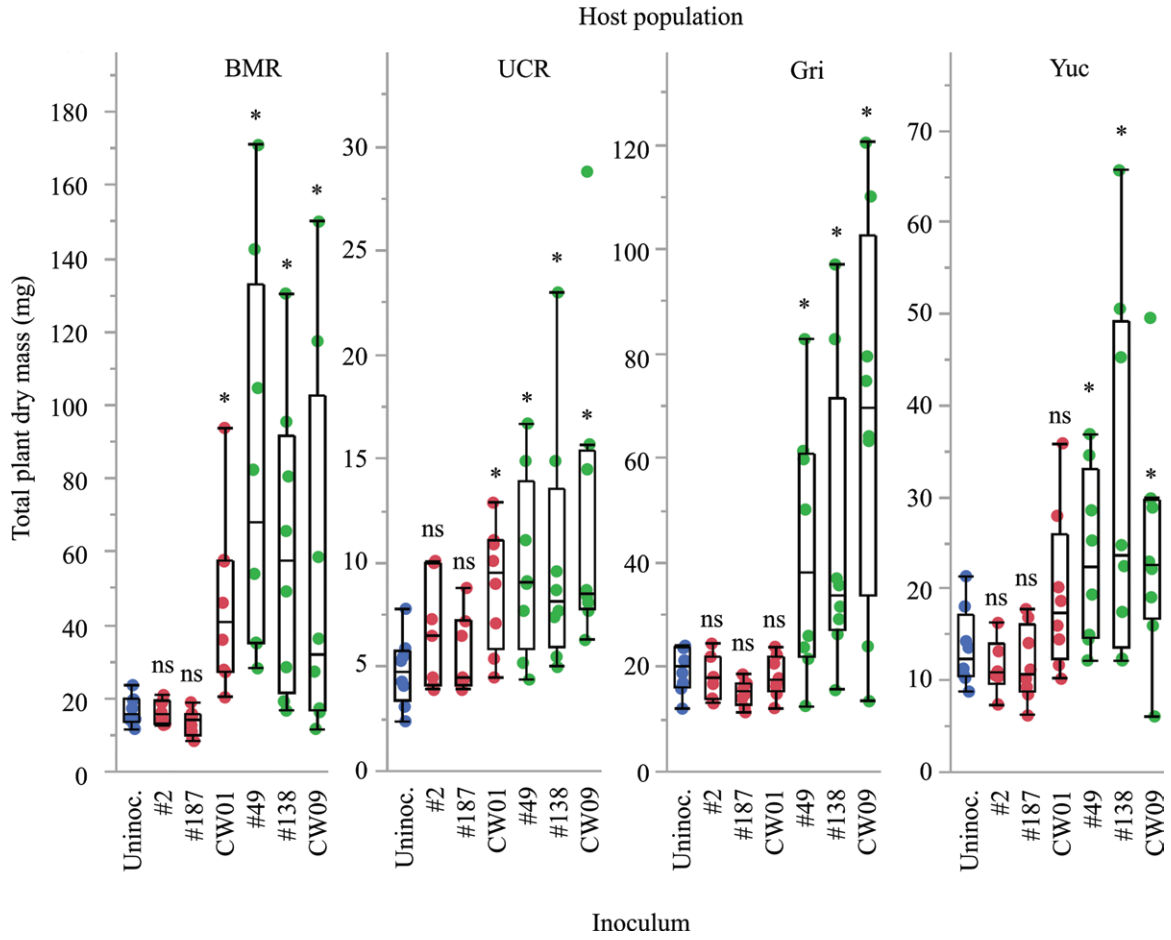

Figure 1. Plant biomass in the single inoculation treatments. Total plant dry mass (roots + shoots) of plants in each single inoculation treatment, with uninoculated plants shown for reference, by plant line. Note the different axes for different plant populations. Asterisks indicate significant differences between inoculated and uninoculated plants within the same plant line (from Test Slices by Host within the significant host × inoculum interaction, using "Uninoc" as the reference category). Uninoc. = uninoculated treatment; Ineffective strains = 2, 187, CW01; Effective strains = 49, 138, CW09.

rhizobia differently for different strains, as there were significant strain × host genotype interactions in the clonal inoculated hosts. However, these effects were rather modest as most strains did not vary significantly in fitness across the host lines tested. Importantly, strain fitness differences were detected under simplified experimental conditions, without other microbes present. Root nodules and surrounding rhizosphere can house a diversity of other bacteria, and these communities can affect the outcome of competition to infect the host (Granada Agudelo et al., 2023; Han et al., 2020; Martínez-Hidalgo & Hirsch, 2017) and the benefit of the rhizobia to the host (Kosmopoulos et al., 2024). Thus, the presence of competitor strains can have important impacts on the fitness of a focal rhizobia, in addition to its benefits to the host.

Parallel drivers of rhizobia *in planta* fitness were seen in the two-strain co-inoculations, though the fitness of ineffective rhizobia in a nodule was significantly reduced in the presence of effective rhizobia. Among the two consistently ineffective strains (2, 187), the only source of variation in their relative fitness in nodules was the genotype of the ineffective strain, with strain 2 achieving greater percent abundance in a nodule than strain 187. Since neither strain 2 nor strain 187 improved plant growth compared to uninoculated controls, the higher relative fitness of strain 2 compared to 187 could be attributed either to differential competitiveness or ability to evade host sanctioning. One intriguing possibility is that competitiveness in nodules during co-inoculations is related to *in planta* proliferative ability. Since strain 2 also had greater population size per nodule than strain 187 in the clonal infections, this suggests that its success occurs irrespective of effects of the other strain, similar to previous findings (Montoya et al., 2023). *Acmispon* plants are typically infected by multiple rhizobia genotypes (Sachs et al., 2009), but these data suggest that a rare, singly infected plant could provide a large fitness reward for ineffective strains.

Strain CW01 was especially intriguing since it was ineffective on two hosts (i.e., Gri and Yuc) and was expected to be sanctioned via host control on those plant lines. However, the relative fitness of CW01 within a nodule on co-inoculated plants did not vary among host lines, inconsistent with host control expectations. Instead, the relative fitness of CW01 varied based on the identity of its co-inoculated effective strain. Strain CW01 had high relative fitness during co-inoculations with strain 138, and CW01 also had greater CFU per nodule than 138 during single inoculations, potentially explaining its fitness advantage during co-inoculations. Other studies have identified specific genes that confer competitiveness to ineffective strains. For example, *Sinorhizobium* bearing the *hrrP* locus fail to fix nitrogen but hyper-proliferate within

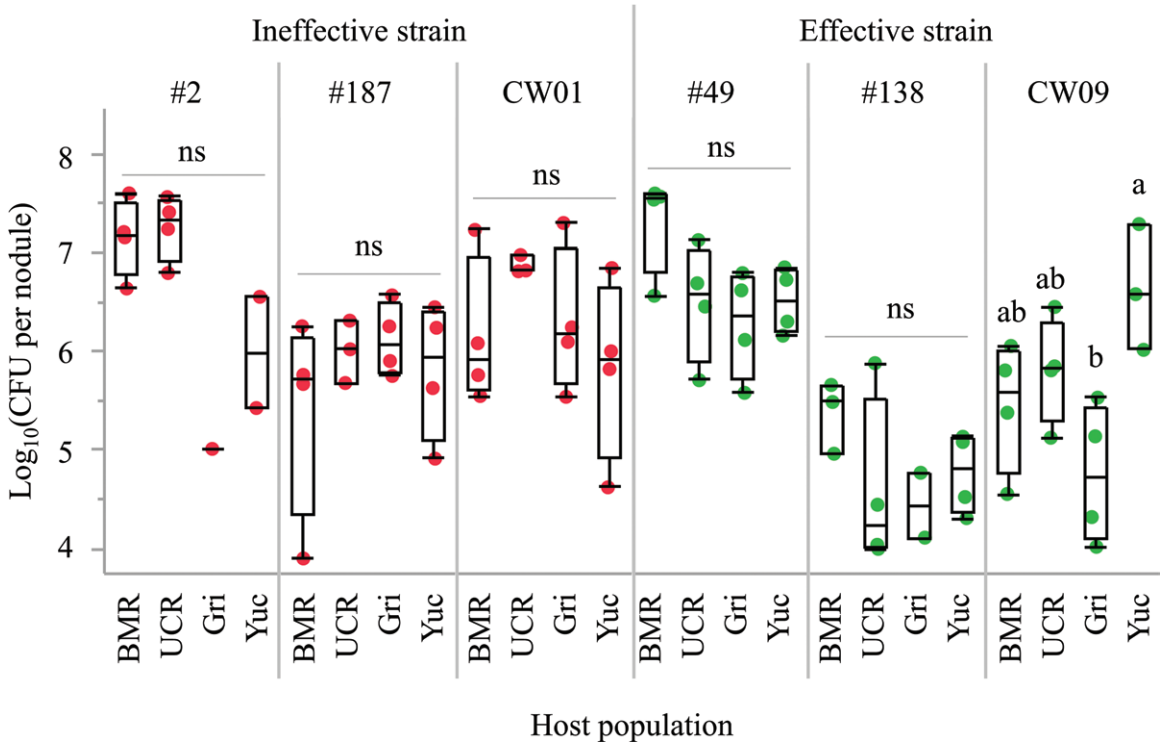

Figure 2. Rhizobia fitness in planta in single inoculation treatments. Rhizobial colony-forming units per nodule were measured for each strain on each host during single inoculations. Each datapoint represents a mean of up to two nodules sampled from one plant replicate. Different letters indicate significant differences among hosts within a strain genotype.

nodule tissue compared to strains lacking this locus (Price et al., 2015), although this effect can vary among *hrrP* alleles (Wendlandt et al., 2022). The production of rhizobitoxine by *Bradyrhizobium* strain USDA61 enables this strain to form many nodules, fix little nitrogen, and compete successfully against other strains for nodule occupancy (Yuhashi et al., 2000). These data suggest that natural selection can shape competitiveness traits in ineffective strains and might be critical for their maintenance. One intriguing possibility is that some ineffective strains are maintained in populations because of their fitness in the absence of competitor strains, as we observed for strain 2. Competitiveness traits in rhizobia are themselves a joint phenotype quantifiable as a symbiont GxG interaction or social genetic effect (Montoya et al., 2023; Rode et al., 2017).

Differences in relative fitness of ineffective strains do not translate to costs for plant hosts

A second broad conclusion is that the growth effects of mixed infections for the host are driven by the host genotype and any effective rhizobia strain present, irrespective of any competing ineffective strains. Despite the evidence that strain genotypes varied in their fitness in nodules—we found little evidence that this had consequences for plant performance. Even though ineffective strain 2 had greater relative fitness than ineffective strain 187 during co-inoculations, plants receiving strain 2 in a co-inoculum did not have reduced performance compared to plants receiving strain 187 in a co-inoculum. Instead, plant relative performance during co-inoculations varied little across treatments and tended to be at least as great as plant performance with single inoculations of effective strains (Figure 4). The only significant variation in plant relative performance in co-inoculations occurred for BMR and Gri hosts: BMR relative performance was greatest when co-inocula contained effective strains CW09 or 138, whereas Gri relative performance was greatest when co-inocula contained effective strain 138. Thus, plant performance in co-inoculations depended more on the identity of the effective strain in the co-inoculum, with some effective strains improving plant relative performance more than others. While previous work indicates that selection can favour host genotypes that exclude one particular ineffective strain (Simonsen & Stinchcombe, 2014), our examination of multiple ineffective rhizobia strains suggests that host control evolves in response to optimizing rewards from beneficial strains rather than excluding or sanctioning ineffective strains, similar to the conclusions of Batstone and colleagues (Batstone et al., 2017). For agriculture, and other applications of beneficial microbiota, these results suggest that choosing the right beneficial strain and a compatible host can go a long way to ensuring host success.

In conclusion, these data suggest that *A. strigosus* hosts have robust control over the fitness of ineffective *Bradyrhizobium* strains within individual nodules during co-inoculations, reducing the relative fitness of ineffective strains in favour of effective, nitrogen-fixing strains. The differences in relative fitness among ineffective strains show that there is variation upon which selection could act. However, the fact that ineffective strains had negligible consequences for plant performance suggests this variation is permitted by plant hosts, or at least invisible to them. We acknowledge that single inoculation environments provides a limited view of rhizobia fitness, since it restricts competition among strains as well as plant choice. Overall, however, this work is consistent with the idea that plant hosts keep their symbionts on a short leash (Foster et al., 2017).

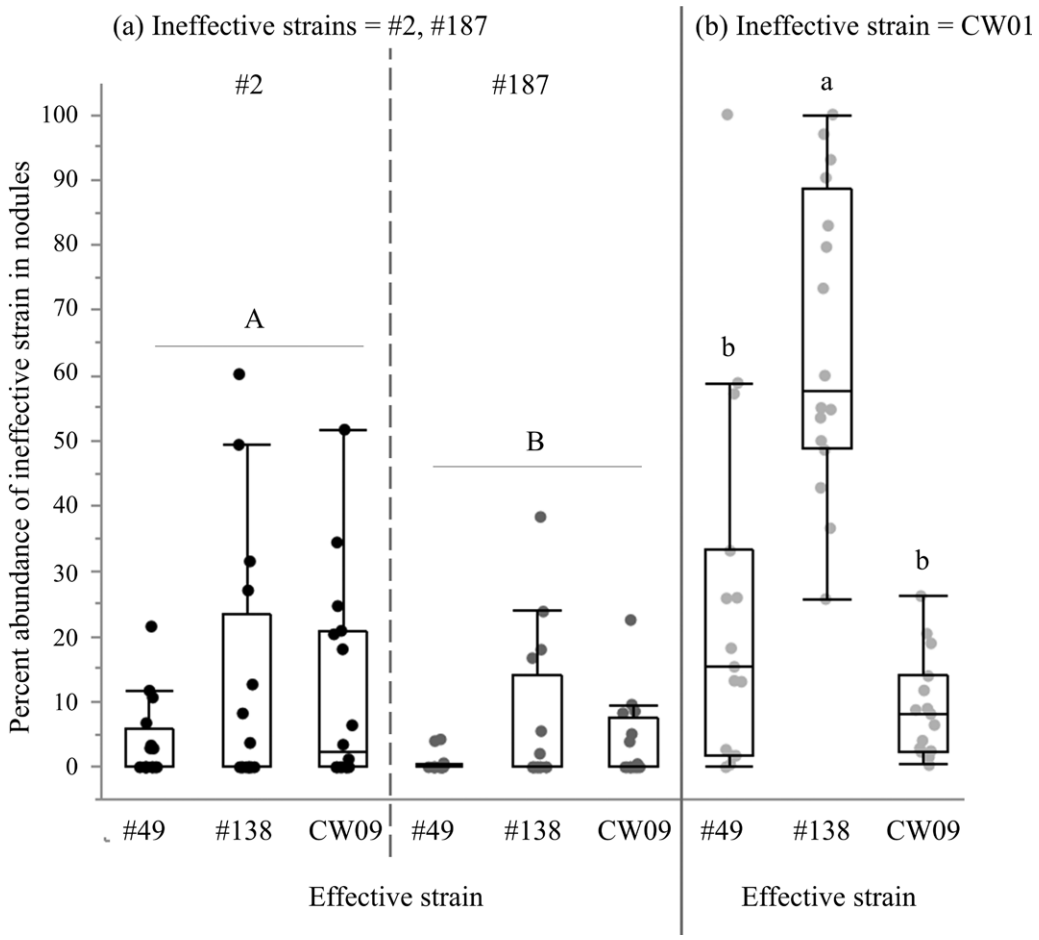

Figure 3. Percent occupancy of individual nodules by the consistently ineffective strains, 2 and 187 (a) and by the strain with host-dependent effectiveness, CW01 (b). Percent nodule occupancy of the ineffective strains (2, 187, CW01) in nodules, during co-inoculations with each of the three effective strains (49, CW09, 138). Each datapoint is consolidated data from up to four replicate nodules of one plant. We performed statistics separately for co-inocula containing strain CW01, since this strain had host-dependent effectiveness. Ineffective strain genotype had a significant main effect on relative abundance, with strain 2 achieving greater relative abundance than 187 (indicated with capital letters). Effective strain genotype had a significant main effect on the relative abundance of strain CW01, with CW01 competing best against 138 (indicated with lower-case letters). There was no effect of host line in either model.

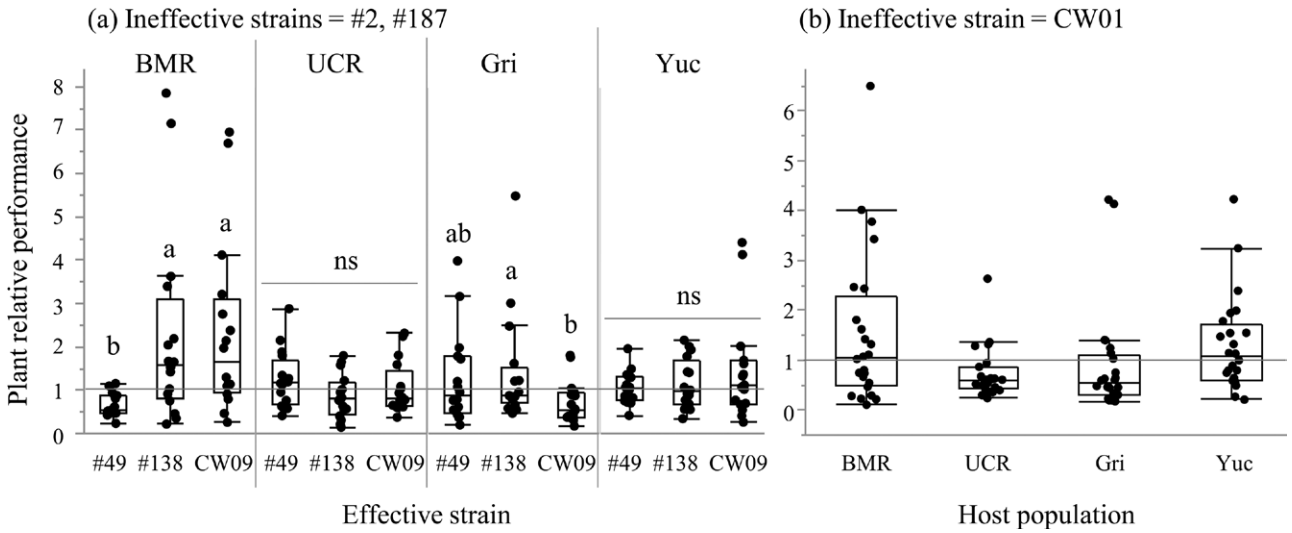

Figure 4. Plant relative growth in the co-inoculation treatments. Plant relative performance in co-inoculation compared to single inoculation with the effective strain. Statistics were performed separately on treatments including strains 2 and 187 (A) and treatments including strain CW01 (B). A reference line is drawn at relative performance = 1 to indicate whether co-inoculated plants performed better or worse than when singly inoculated with the effective strain in the co-inoculum.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material and also on the Dryad Digital Repository, at https://dx.doi.org/10.5061/dryad.2ngf1vj04

Author contributions

Camille Wendlandt (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Investigation [lead], Methodology [lead]), Julio Avelar-Barragan (Investigation [supporting]), Avissa J. Zomorrodian (Investigation [supporting]), Khadija Al-Moussawi (Investigation [supporting]), Stephanie Porter (Formal analysis [supporting], Methodology [equal], Writing—review & editing [equal]), and Joel Sachs (Conceptualization [lead], Funding acquisition [lead], Project administration [equal], Supervision [lead], Writing—review & editing [lead])

Funding

We gratefully acknowledge funding by the National Science Foundation of the USA, including award numbers 1150278 and 1738009 to J.L.S. We also gratefully acknowledge funding by the United States Department of Agriculture, including award number 2022-67019-36500 and Hatch Grant CA-R-EEOB-5200-H, both to J.L.S.

Conflicts of interest

None declared.

References

Batstone, R. T., Dutton, E. M., Wang, D., … Frederickson, M. E. (2017). The evolution of symbiont preference traits in the model legume *Medicago truncatula*. *New Phytologist*, *213*(4), 1850–1861. https://doi.org/10.1111/nph.14308

Burdon, J., Gibson, A., Searle, S. D., … Brockwell, J. (1999). Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian *Acacia*: Within-species interactions. *Journal of Applied Ecology*, *36*(3), 398–408.

Burghardt, L. T., & diCenzo, G. C. (2023). The evolutionary ecology of rhizobia: Multiple facets of competition before, during, and after symbiosis with legumes. *Current Opinion in Microbiology*, *72*, 102281. https://doi.org/10.1016/j.mib.2023.102281

Chen, L., Figueredo, A., Villani, H., … Hungria, M. (2002). Diversity and symbiotic effectiveness of rhizobia isolated from field-grown soybean nodules in Paraguay. *Biology and Fertility of Soils*, *35*(6), 448–457. https://doi.org/10.1007/s00374-002-0493-1

Collins, M., Thies, J., & Abbott, L. (2002). Diversity and symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* isolates from pasture soils in south-western Australia. *Soil Research*, *40*(8), 1319–1329.

Denison, R. F., & Kiers, E. T. (2004). Lifestyle alternatives for rhizobia: Mutualism, parasitism, and forgoing symbiosis. *FEMS Microbiology Letters*, *237*(2), 187–193. https://doi.org/10.1016/j.femsle.2004.07.013

Denton, M., Coventry, D., Bellotti, W., & Howieson, J. (2000). Distribution, abundance and symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* from alkaline pasture soils in South Australia. *Animal Production Science*, *40*(1), 25–35.

Douglas, A. E., & Werren, J. H. (2016). Holes in the hologenome: Why host-microbe symbioses are not holobionts. *mBio*, *7*(2), e02099. https://doi.org/10.1128/mBio.02099-15

Drew, G. C., Stevens, E. J., & King, K. C. (2021). Microbial evolution and transitions along the parasite–mutualist continuum. *Nature Reviews Microbiology*, *19*(10), 623–638. https://doi.org/10.1038/s41579-021-00550-7

Foster, K. R., Schluter, J., Coyte, K. Z., & Rakoff-Nahoum, S. (2017). The evolution of the host microbiome as an ecosystem on a leash. *Nature*, *548*(7665), 43–51. https://doi.org/10.1038/nature23292

Fronk, D. C., & Sachs, J. L. (2022). Symbiotic organs: The nexus of host-microbe evolution. *Trends in Ecology and Evolution*, *37*(7), 599–610. https://doi.org/10.1016/j.tree.2022.02.014

Gano-Cohen, K. A., Wendlandt, C. E., Al Moussawi, K., … Sachs, J. L. (2020). Recurrent mutualism breakdown events in a legume rhizobia metapopulation. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1919), 20192549. https://doi.org/10.1098/rspb.2019.2549

Gano-Cohen, K. A., Wendlandt, C. E., Stokes, P. J., … Sachs, J. L. (2019). Interspecific conflict and the evolution of ineffective rhizobia. *Ecology Letters*, *22*(6), 914–924.

Granada Agudelo, M., Ruiz, B., Capela, D., & Remigi, P. (2023). The role of microbial interactions on rhizobial fitness. *Frontiers in Plant Science*, *14*, 1277262. https://doi.org/10.3389/fpls.2023.1277262

Gubry-Rangin, C., Garcia, M., & Bena, G. (2010). Partner choice in *Medicago truncatula-Sinorhizobium* symbiosis. *Proceedings of the Royal Society B*, *277*(1690), 1947–1951.

Han, Q., Ma, Q., Chen, Y., … Li, X. (2020). Variation in rhizosphere microbial communities and its association with the symbiotic efficiency of rhizobia in soybean. *The ISME Journal*, *14*(8), 1915–1928. https://doi.org/10.1038/s41396-020-0648-9

Heath, K. D., & Tiffin, P. (2009). Stabilizing mechanisms in a legume-rhizobium mutualism. *Evolution*, *63*(3), 652–662. https://doi.org/10.1111/j.1558-5646.2008.00582.x

Hollowell, A. C., Gano, K. A., Lopez, G., … Sachs, J. L. (2015). Native California soils are selective reservoirs for multidrug-resistant bacteria. *Environmental Microbiology Reports*, *7*(3), 442–449. https://doi.org/10.1111/1758-2229.12269

Hollowell, A. C., Regus, J. U., Gano, K. A., … Sachs, J. L. (2016a). Epidemic spread of symbiotic and non–symbiotic Bradyrhizobium genotypes across California. *Microbial Ecology*, *71*(3), 700–710. https://doi.org/10.1007/s00248-015-0685-5

Hollowell, A. C., Regus, J. U., Turissini, D., … Sachs, J. L. (2016b). Metapopulation dominance and genomic-island acquisition of Bradyrhizobium with superior catabolic capabilities. *Proceedings of the Royal Society, 283*, 20160496. https://doi.org/10.1098/rspb.2016.0496

Jones, E. I., Afkhami, M. E., Akçay, E., … Friesen, M. L. (2015). Cheaters must prosper: Reconciling theoretical and empirical perspectives on cheating in mutualism. *Ecology Letters*, *18*(11), 1270–1284. https://doi.org/10.1111/ele.12507

Kiers, E. T., Hutton, M. G., & Denison, R. F. (2007). Human selection and the relaxation of legume defences against ineffective rhizobia. *Proceedings Biological Sciences*, *274*(1629), 3119–3126. https://doi.org/10.1098/rspb.2007.1187

Kiers, E. T., Rousseau, R. A., West, S. A., & Denison, R. F. (2003). Host sanctions and the legume–rhizobium mutualism. *Nature*, *425*(6953), 78–81. https://doi.org/10.1038/nature01931

Kosmopoulos, J. C., Batstone-Doyle, R. T., & Heath, K. D. (2024). Co-inoculation with novel nodule-inhabiting bacteria reduces the benefits of legume-rhizobium symbiosis. *Canadian Journal of Microbiology*, *70*(7), 275–288. https://doi.org/10.1139/cjm-2023-0209

Ledermann, R., Schulte Carolin, C. M., & Poole Philip, S. (2021). How rhizobia adapt to the nodule environment. *Journal of Bacteriology*, *203*(12), e00539–e00520.

Manci, M., Mercado, O. G., Camantigue, R. X., … Sachs, J. L. (2023). Live soil inocula, not host population or domestication status, is

the predominant driver of growth benefits to cowpea. *Plant and Soil*, *482*(1), 585–600.
Martínez-Hidalgo, P., & Hirsch, A. M. (2017). The nodule microbiome: N_2-fixing rhizobia do not live alone. *Phytobiomes Journal*, *1*(2), 70–82. https://doi.org/10.1094/pbiomes-12-16-0019-rvw
Montoya, A. P., Wendlandt, C. E., Benedict, A. B., … Porter, S. S. (2023). Hosts winnow symbionts with multiple layers of absolute and conditional discrimination mechanisms. *Proceedings Biological Sciences*, *290*(1990), 20222153. https://doi.org/10.1098/rspb.2022.2153
Oono, R., Anderson, C. G., & Denison, R. F. (2011). Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates. *Proceedings of the Royal Society B*, *278*(1718), 2698–2703.
Pahua, V. J., Stokes, P. J. N., Hollowell, A. C., … Sachs, J. L. (2018). Fitness variation among host species and the paradox of ineffective rhizobia. *Journal of Evolutionary Biology*, *31*(4), 599–610. https://doi.org/10.1111/jeb.13249
Porter, S. S., Dupin, S. E., Denison, R. F., … Sachs, J. L. (2024). Host-imposed control mechanisms in legume–rhizobia symbiosis. *Nature Microbiology*, *9*(8), 1929–1939. https://doi.org/10.1038/s41564-024-01762-2
Porter, S. S., & Simms, E. L. (2014). Selection for cheating across disparate environments in the legume-rhizobium mutualism. *Ecology Letters*, *17*(9), 1121–1129. https://doi.org/10.1111/ele.12318
Price, P. A., Tanner, H. R., Dillon, B. A., … Griffitts, J. S. (2015). Rhizobial peptidase HrrP cleaves host-encoded signaling peptides and mediates symbiotic compatibility. *Proceedings of the National Academy of Sciences*, *112*(49), 15244–15249. https://doi.org/10.1073/pnas.1417797112
Queller, D. C. (2014). Joint phenotypes, evolutionary conflict and the fundamental theorem of natural selection. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *369*(1642), 20130423. https://doi.org/10.1098/rstb.2013.0423
Queller, D. C., & Strassman, J. E. (2018). Evolutionary conflict. *Annual Review of Ecology, Evolution, and Systematics*, *49*, 73–93.
Quides, K. W., Salaheldine, F., Jariwala, R., & Sachs, J. L. (2021). Dysregulation of host-control causes interspecific conflict over host investment into symbiotic organs. *Evolution*, *75*(5), 1189–1200. https://doi.org/10.1111/evo.14173
Rahman, A., Manci, M., Nadon, C., … Sachs, J. L. (2023). Competitive interference among rhizobia reduces benefits to hosts. *Current Biology*, *33*(14), 2988–3001.e2984.
Regus, J. U., Gano, K. A., Hollowell, A. C., & Sachs, J. L. (2014). Efficiency of partner choice and sanctions in *Lotus* is not altered by nitrogen fertilization. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1781), 20132587. https://doi.org/10.1098/rspb.2013.2587
Regus, J. U., Gano, K. A., Hollowell, A. C., … Sachs, J. L. (2015). *Lotus* hosts delimit the mutualism-parasitism continuum of *Bradyrhizobium*. *Journal of Evolutionary Biology*, *28*(2), 447–456. https://doi.org/10.1111/jeb.12579
Regus, J. U., Quides, K. W., O'Neill, M. R., … Sachs, J. L. (2017). Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. *American Journal of Botany*, *104*(9), 1299–1312. https://doi.org/10.3732/ajb.1700165
Rode, N. O., Soroye, P., Kassen, R., & Rundle, H. D. (2017). Air-borne genotype by genotype indirect genetic effects are substantial in the filamentous fungus *Aspergillus nidulans*. *Heredity (Edinb)*, *119*(1), 1–7. https://doi.org/10.1038/hdy.2017.9
Sachs, J. L., Ehinger, M. O., & Simms, E. L. (2010a). Origins of cheating and loss of symbiosis in wild *Bradyrhizobium*. *Journal of Evolutionary Biology*, *23*(5), 1075–1089. https://doi.org/10.1111/j.1420-9101.2010.01980.x
Sachs, J. L., Kembel, S. W., Lau, A. H., & Simms, E. L. (2009). *In situ* phylogenetic structure and diversity of wild *Bradyrhizobium* communities. *Applied and Environmental Microbiology*, *75*(14), 4727–4735. https://doi.org/10.1128/AEM.00667-09
Sachs, J. L., Russell, J. E., Lii, Y. E., … Patil, A. S. (2010b). Host control over infection and proliferation of a cheater symbiont. *Journal of Evolutionary Biology*, *23*(9), 1919–1927. https://doi.org/10.1111/j.1420-9101.2010.02056.x
Simonsen, A. K., & Stinchcombe, J. R. (2014). Standing genetic variation in host preference for mutualist microbial symbionts. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1797), 20142036. https://doi.org/10.1098/rspb.2014.2036
Somasegaran, P., & Hoben, H. J. (1994). *Handbook for rhizobia: Methods in legume-Rhizobium technology*. Springer Verlag New York, Inc.
Weisberg, A. J., Rahman, A., Backus, D., … Sachs, J. L. (2022). Pangenome evolution reconciles robustness and instability of rhizobial symbiosis. *mBio*, *13*(3), e0007422. https://doi.org/10.1128/mbio.00074-22
Wendlandt, C. E., Regus, J. U., Gano-Cohen, K. A., … Sachs, J. L. (2019). Host investment into symbiosis varies among genotypes of the legume *Acmispon strigosus*, but host sanctions are uniform. *New Phytologist*, *221*(1), nph.15378.
Wendlandt, C. E., Roberts, M., Nguyen, K. T., … Porter, S. S. (2022). Negotiating mutualism: A locus for exploitation by rhizobia has a broad effect size distribution and context-dependent effects on legume hosts. *Journal of Evolutionary Biology*, *35*(6), 844–854. https://doi.org/10.1111/jeb.14011
Westhoek, A., Field, E., Rehling, F., … Turnbull, L. A. (2017). Policing the legume-Rhizobium symbiosis: A critical test of partner choice. *Scientific Reports*, *7*(1), 1419. https://doi.org/10.1038/s41598-017-01634-2
Yuhashi, K. I., Ichikawa, N., Ezura, H., … Minamisawa, K. (2000). Rhizobitoxine production by *Bradyrhizobium elkanii* enhances nodulation and competitiveness on *Macroptilium atropurpureum*. *Applied and Environmental Microbiology*, *66*(6), 2658–2663. https://doi.org/10.1128/aem.66.6.2658-2663.2000