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Environmental and Genetic Contributions to Symbiosis Traits in a Wild Legume

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by

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ABSTRACT OF THE DISSERTATION

Environmental and Genetic Contributions to Symbiosis Traits in a Wild Legume

by

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Plants can gain substantial growth benefits from microbial symbionts, but these benefits are threatened by ineffective symbionts that infect plants without providing a service. To minimize this threat, plants can preferentially associate with effective symbionts and avoid or punish ineffective symbionts. Although these 'host control traits' are central to our understanding of mutualism evolution, we know little about how much they genetically vary within plant species or how they perform in different environments. Here, I investigated variation in host control traits using the California native legume *Acmispon strigosus* and wild strains of its nitrogen-fixing symbiont, *Bradyrhizobium*.

In the first chapter, I tested for genetic variation in host control traits among six population sources of *A. strigosus* by inoculating plants with pure cultures of effective and ineffective *Bradyrhizobium.* In all hosts, the strain content of root nodules was biased toward the most effective *Bradyrhizobium* strain, but hosts varied genetically in mean nodule size as well as growth benefits. These patterns did not change under experimental nitrogen fertilization.

In the second chapter, I examined host genetic variation in nodule size in a new experimental setting, inoculating plants with soil slurries rather than pure *Bradyrhizobium* cultures. I found similar variation in nodule size and host benefits as I observed in chapter 1, indicating that host control trait variation was robust to the biotic complexity of inocula. Furthermore, plant growth benefits from soils were more strongly driven by plant genotype than soil source, further highlighting the importance of plant genotype.

In the third chapter, I investigated host and symbiont contributions to strain content of nodules by inoculating four plant genotypes with nine combinations of effective and ineffective rhizobia strains. I found significant variation among ineffective strains in the relative fitness they achieved in nodules. However, the dominant force shaping strain relative abundance was the nitrogen fixation phenotype, consistent with hosts being in control of this trait.

Overall, my dissertation research provides evidence of limited genetic variation in host control traits of *A. strigosus* in a variety of experimental settings.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GENERAL INTRODUCTION

Plants can gain substantial benefits from engaging in symbiosis with microbes (Douglas, 2010). However, the interacting partners can be in conflict over the magnitude of these benefits. Symbionts potentially benefit from using host resources for their own reproduction, rather than the service they are engaged to perform, creating an evolutionary conflict of interest that can destabilize mutualistic symbioses (Queller & Strassman, 2018). Microbes have an evolutionary advantage over their hosts in this conflict due to their short generation times and large population sizes, which enable 'cheating' mutations to arise and spread more quickly than in their plant hosts (Sachs *et al.*, 2004). Plants can exert 'host control' over their symbionts by preferentially associating with cooperative partners and/or punishing uncooperative partners (Kiers *et al.*, 2003; Javot *et al.*, 2007), but there is segregating variation for host control within plant species (Kiers *et al.*, 2007; Simonsen & Stinchcombe, 2014). We know little about the drivers of genetic variation in host control or the relationship of host control to plant benefits from symbiosis.

The legume-rhizobia symbiosis provides a tractable system for investigating these questions. Rhizobia are soil proteobacteria that can infect the roots of compatible legume hosts and form root nodules in which they fix atmospheric nitrogen (Oldroyd *et al.*, 2011). Fixed nitrogen is passed to the host and can greatly improve plant growth in nitrogen-limited conditions. The host supports the energetic cost of nitrogen fixation by providing nodules with photosynthates, which the rhizobia may use for nitrogen-fixation or their own future reproduction, thus creating a conflict of interest between the host and

symbiont. Rhizobial benefits from their host can be estimated as the size of the rhizobial population in nodules or the relative abundance of a particular strain in nodules when plants are infected by multiple strains (Sachs *et al.*, 2010a; Sachs *et al.*, 2010b). More crudely, rhizobial benefits can also be estimated as average nodule size, since this can correlate with the number of viable rhizobia in nodules. Root nodules eventually senesce and release a portion of the rhizobial population back into the soil (Muller *et al.*, 2001). Thus for legumes, host control involves regulation of nodule size and the fitness gains of rhizobia inside nodules.

In my dissertation research I investigated the symbiosis traits of a California native annual legume, *Acmispon strigosus*, which forms root nodules with *Bradyrhizobium* spp. (Sachs *et al.*, 2009). My first chapter examined how exogenous soil nitrogen affects host control over symbiosis. I used inbred *A. strigosus* plant lines from six natural field sites that span a soil nitrogen gradient ranging from ~2 ppm to 20 ppm nitrogen (i.e., extremely nitrogen-poor to nitrogen-rich, comparable to agricultural soils). Three field sites were low-nitrogen and three were high-nitrogen. I grew plants in sterile, zero-nitrogen sand and inoculated plants with three wild *Bradyrhizobium* strains (both individually and as a three-strain mixture) that naturally vary in their nitrogen fixing ability. First, I investigated whether *A. strigosus* shows intraspecific genetic variation in host control traits (nodule size, relative abundance of strains inside nodules). I was specifically interested in whether variation in host control was structured by the plants being from low-nitrogen or high-nitrogen field sites, since resource saturation is predicted to alter host dependence on and/or investment into symbiosis (Kiers *et al.*, 2007; Weese

et al., 2015; Shantz *et al.*, 2016). Second, I replicated the experiment in high-nitrogen growth conditions to test whether host control traits are altered physiologically when plants encounter high soil nitrogen.

My second chapter assessed the relative importance of the plant genotype for growth benefits from whole communities of soil microbes. If plant growth benefits from microbes are largely due to soil factors, such as legacy effects of previous soil conditioning or deterministic physicochemical properties, then plant growth benefits from microbes will not respond to selection on the plant, whether natural or artificial. Thus, this topic is of critical interest for both plant evolutionary ecologists and agronomists interested in breeding crops for increased benefits from microbes. First, I evaluated the relative contributions of plant genotype and soil source to plant benefits from microbes. I inoculated three *Acmispon* plant lines with six soil microbial communities (sourced from the same field sites used in chapter 1) and measured growth benefits from both live inocula and sterilized inocula, to isolate the effects of soil microbes. Plants were grown in sterile, zero-nitrogen sand to maximize plant demand for the rhizobial service of nitrogen fixation. Second, I tested whether plant genetic variation in symbiosis traits (uncovered in chapter 1) was reproducible when plants were exposed to more complex inocula. I inoculated each soil slurry onto inbred sympatric *A. strigosus* plant lines (i.e., from the same field site as the soil) and measured plant growth benefits and host control over nodule size. The goal of this chapter was to assess plant symbiosis traits under conditions of high biotic complexity to understand possible context-dependence of plant benefits or host control.

My third chapter investigated whether strain genotype can shape its abundance in nodules independently of host control acting on nitrogen-fixing activity. Although host control is an important and well-documented phenomenon, several studies have found strain genetic background to affect which strains occupy nodules in competitive conditions when multiple strains are present. In agriculture, for instance, highly beneficial rhizobial inocula often fail to occupy nodules of field-grown plants, which are instead occupied by rhizobia indigenous to the field soil. Thus, rhizobial adaptation to local soil conditions could be one prerequisite for being highly competitive for associations with hosts. Other studies have identified strains that fix nitrogen poorly but occupy a high proportion of nodules because they actively downregulate plant host defenses (Yuhashi *et al.*, 2000; Price *et al.*, 2015). In this chapter, I examined how much variation strains could contribute to their abundance in nodules. I grew four *A. strigosus* plant lines in sterile, zero-nitrogen calcine clay and inoculated plants with equal mixtures of effective (nitrogen-fixing) and ineffective (non-nitrogen-fixing) rhizobia. I used a total of six wild *Bradyrhizobium* strains, mixing three effective strains with each of three ineffective strains to generate nine co-inocula. I evaluated whether the abundance of each strain in nodules was always a function of its symbiotic effectiveness, or whether some genotypes were more competitive in nodules than others. If any ineffective strain genotypes exhibited competitiveness, I examined whether this resulted in a performance cost for co-inoculated plant hosts.

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CHAPTER 1

Host investment into symbiosis varies among genotypes of the legume *Acmispon strigosus*, but host sanctions are uniform

Abstract

Efficient host control predicts the extirpation of ineffective symbionts, but they are nonetheless widespread in nature. I tested three hypotheses for the maintenance of symbiotic variation in rhizobia that associate with a native legume: 1) partner mismatch between host and symbiont, such that symbiont effectiveness varies with host genotype, 2) resource satiation, whereby extrinsic sources of nutrients relax host control, and 3) variation in host control among host genotypes. I inoculated *Acmispon strigosus* from six populations with three *Bradyrhizobium* strains that vary in symbiotic effectiveness on sympatric hosts. I measured proxies of host and symbiont fitness in single- and co-inoculations under fertilization treatments of zero added nitrogen and near-growth-saturating nitrogen. I examined two components of host control: 'host investment' into nodule size during single- and co-inoculations, and 'host sanctions' against less effective strains during co-inoculations. The *Bradyrhizobium* strains displayed conserved growth effects on hosts, and host control did not decline under experimental fertilization. Host sanctions were robust in all hosts, but host lines from different populations varied significantly in measures of host investment in both single- and co-inoculation experiments. Variation in host investment could promote variation in symbiotic

effectiveness and prevent the extinction of ineffective *Bradyrhizobium* from natural populations.

Introduction

Plants can exhibit elegant host control traits that preferentially select beneficial over ineffective symbionts. For instance, yuccas and fig trees abort developing fruits that are overburdened by eggs of their specialized pollinators and preferentially allocate resources into fruits serviced by more effective pollinators (Pellmyr & Huth, 1994; Jandér *et al.*, 2012; Jandér & Herre, 2016). Barrel medics degrade arbuscules of mycorrhizae that do not deliver phosphorous (Javot *et al.*, 2007), and soybeans reduce growth of intracellular rhizobia that fail to fix nitrogen (Kiers *et al.*, 2003). Provided there are no other sources of selection on symbiotic services, host control traits are predicted to impose directional selection on symbiotic partners, reducing variation in the symbiotic services provided and favoring the fixation of beneficial genotypes (**Fig. 1.1a**; Denison, 2000; West *et al.*, 2002a,b; Foster & Kokko, 2006; Foster & Wenseleers, 2006). In nature, however, plant-associated symbionts commonly vary from beneficial to ineffective (Johnson *et al.*, 1997; Moawad *et al.*, 1998; Burdon *et al.*, 1999; Chen *et al.*, 2002; Carú *et al.*, 2003; Markham, 2008; Bromfield *et al.*, 2010; Sachs *et al.*, 2010a; Otero *et al.*, 2011; Granada *et al.*, 2014). Thus, there is a key gap in our knowledge: models predicting that host control traits purify populations of ineffective symbionts fail to explain the maintenance of variation in these diverse symbioses. An emerging framework predicts that both genetic and environmental sources of variation in host

control traits can promote the maintenance of symbiont variation, but tests of this framework remain scant.

Three main hypotheses can be identified for the maintenance of symbiont variation in interactions between plant hosts and microbes (**Fig. 1.1**). Under the partner mismatch hypothesis, symbionts that are ineffective or mediocre on one host genotype are maintained in a population because they are beneficial (i.e., effective) on other hosts due to specificity interactions (Bever, 1999; Burdon *et al.*, 1999; Heath & Tiffin, 2007; Heath, 2010; Barrett *et al.*, 2012). Even if all host genotypes exert host control traits over ineffective partners, partner mismatch would cause ineffective symbiont genotypes to be punished in a host-specific manner (**Fig. 1.1b**). Intuitively, partner mismatch is more likely when hosts interact with symbionts whose typical host is a different species (Thrall *et al.*, 2000). However, partner mismatch has also been observed among genotypes of the same host species (Burdon *et al.*, 1999; Heath, 2010). Thus, partner mismatch could be an important mechanism for maintaining variation in symbiont effectiveness at local scales.

Symbiont variation can also be maintained if host control itself varies. Variation in host control could occur physiologically (within a host genotype, depending on the external environment) or genetically (among host genotypes). In resource mutualisms like the legume-rhizobia symbiosis, physiological attenuation of host control traits could occur when plants encounter extrinsic sources of nutrients normally offered by symbionts. Under the resource satiation hypothesis, plants are predicted to switch to cheap mineral sources of nutrients when they are plentiful (Bronstein, 1994; West *et al.*,

2002b; Thrall *et al.*, 2007; Shantz *et al.*, 2016) and to downregulate costly pathways involved in symbiosis, including host control (**Fig. 1.1c**). Thus, spatial variation in soil nutrients could generate variation in host control over ineffective symbionts. There is evidence that resource satiation can lead hosts to downregulate symbiosis pathways in some systems, for instance when nitrogen fertilization causes legumes to form fewer root nodules with rhizobia (Streeter & Wong, 1988; Saturno *et al.*, 2017 and references therein). But other studies have found mixed effects (Heath *et al.*, 2010) or no effects of resource satiation on host control traits (Kiers *et al.*, 2006; Regus *et al.*, 2014; Grillo *et al.*, 2016). Thus, the role of resource satiation in the maintenance of variation in symbiont effectiveness requires further study.

The host variation hypothesis predicts that host control traits vary among host genotypes such that some host genotypes are more efficient at host control than others (**Fig. 1.1d**). Steidinger and Bever (2014) offered one model of how host genotypes differing in host control traits could coexist in a population through negative plant-soil feedbacks. Briefly, host genotypes with strong host control traits ('discriminators') are predicted to drive down the frequency of ineffective symbionts until only effective symbionts are regularly encountered. If host control is costly for hosts, genotypes with weak host control traits ('givers') are predicted to outperform discriminators when effective symbionts are abundant. However, givers would act as a refuge for ineffective symbionts and allow their frequency to rise, shifting selection to favor discriminator hosts. This dynamic equilibrium among host control strategies would also maintain populations of both ineffective and effective symbionts. This model and others (Foster &

Kokko, 2006) suggest that alternative host strategies could be driven by costs of host control. Some empirical studies have failed to find evidence of host control (Marco *et al.*, 2009; Gubry-Rangin *et al.*, 2010; Marco *et al*., 2015; Grillo *et al.*, 2016), consistent with the host variation hypothesis, but only a few studies have examined genetic variation of host control across populations of a species (Heath & Tiffin, 2009; Simonsen & Stinchcombe, 2014; Haney *et al.*, 2015). A common theme in the partner mismatch, resource satiation, and host variation hypotheses is that context-dependency of either symbiont effectiveness or host control traits could maintain variation in symbiont effectiveness.

Host control can be measured by host sanctions against ineffective symbionts and host investment into symbiotic structures. Host sanctions leads to differences in symbiont relative fitness when hosts are infected by multiple symbiont genotypes, such that the most effective symbiont achieves the greatest relative fitness (Denison, 2000; Kiers *et al.*, 2003; Sachs *et al.*, 2004). Host investment into symbiotic structures can also be a measure of host control if the resources that flow to symbionts affects symbiont fitness (e.g., previous work has found correlations between nodule size and rhizobia per nodule for individual rhizobial genotypes; Kiers *et al*., 2003; Heath & Tiffin, 2007). Co-inoculations permit the measurement of both host sanctions and host investment, but single-inoculations only permit the measurement of host investment, and inferring host control from single-inoculations requires comparing host investment into symbiont genotypes that vary in effectiveness on the same host genotype. This approach allows researchers to minimize the number of factors explaining symbiont fitness, but the no-

choice design can generate autocorrelation of host and symbiont fitness components due to fitness feedbacks (Oono *et al.*, 2009; Oono *et al.*, 2011; Kiers *et al.*, 2013). Thus, performing parallel single and co-inoculations generates a more thorough understanding of host control.

Here, I investigate mechanisms maintaining variation in effectiveness of *Bradyrhizobium* symbionts in a metapopulation of *Acmispon strigosus* hosts (formerly *Lotus strigosus*). *A. strigosus* is an annual legume native to the southwestern United States that associates with nitrogen fixing, root-nodulating rhizobia in the genus *Bradyrhizobium* (Sachs *et al.*, 2009). Like many legume species, *A. strigosus* initiates nodules with compatible rhizobial genotypes soon after germination. *A. strigosus* nodules grow rapidly and the *Bradyrhizobium* within nodules proliferate (Sachs *et al.*, 2010a). The nodules begin to senesce as the plant flowers and begins pod-filling, a stage at which nodule rhizobia are released back into the soil. At a well-studied population at Bodega Marine Reserve (BMR) in northern California, *A. strigosus* are nodulated by *Bradyrhizobium* strains that range from highly effective (e.g., *c*. 6-fold growth improvement of inoculated sympatric hosts relative to uninoculated controls) to ineffective (e.g., no growth improvement of inoculated sympatric hosts; Sachs *et al.*, 2010a). This striking variation is consistent with other surveys of symbiont effectiveness, which have uncovered both effective and ineffective symbionts (Burdon *et al.*, 1999; Rangin *et al.*, 2008; Bromfield *et al.*, 2010; Ehinger *et al.*, 2014). *A. strigosus* hosts from BMR also demonstrate efficient host control when inoculated with sympatric *Bradyrhizobium* that vary in effectiveness, forming nodules of reduced size with

ineffective strains (i.e., reduced host investment; Regus *et al*., 2015), and showing reduced *in planta* abundance of ineffective strains during co-inoculations with effective strains (i.e., host sanctions; Sachs *et al.*, 2010b; Regus *et al.*, 2014). The co-occurrence of ineffective symbionts and robust host control traits in the *Acmispon-Bradyrhizobium* system makes it powerful for testing hypotheses about the maintenance of variation in symbiont effectiveness.

I inoculated three *Bradyrhizobium* strains onto *A. strigosus* hosts from six populations and grew plants with and without mineral sources of nitrogen. The host populations were sampled from across a 10-fold range of soil nitrogen levels (2-20 ppm mineral nitrogen; Regus *et al.*, 2017). I sampled *A. strigosus* more deeply across populations than within populations (one to two seed sets from each population) to maximize the chance of sampling different genotypes from this species. I tested my hypotheses first in a single inoculation design, where each host was inoculated with a clonal culture of each strain, and also in a co-inoculation design, where each host was inoculated simultaneously with all three strains. In the single-inoculation experiment, I measured host benefits (relative growth, ^{15}N discrimination, and 'symbiotic efficiency' *sensu* Oono and Denison, 2010) and investment (mean nodule size) to infer symbiont effectiveness and host control, respectively. In the co-inoculation experiment, I quantified sanctions (the proportion of nodules occupied by the most effective strain, i.e. 'nodule occupancy') and investment (mean nodule size) to infer host control. I also compared plant relative growth from co-inoculation to the mean benefits from all single-inoculation treatments to examine whether host control is associated with increased host benefits

relative to the null expectation from single-inoculations (Heath & Tiffin, 2007). Under the partner mismatch hypothesis, I predicted that the effectiveness of each *Bradyrhizobium* strain would vary among *A. strigosus* hosts, such that the least effective strain on one host would be the intermediate or most effective strain on another host. Under the resource satiation hypothesis, I predicted that hosts would display weaker host control in fertilized than unfertilized conditions. Under the host variation hypothesis, I predicted that hosts would exhibit genetic differences in host control traits. My work contributes to an emerging theoretical framework to explain the maintenance of variation in populations of microbial mutualists (Sachs *et al.*, 2011a; Heath & Stinchcombe, 2013; Steidinger & Bever, 2014; Bever, 2015; Steidinger & Bever, 2016; Pahua *et al.*, 2018).

Materials and Methods

Bradyrhizobium strains

Bradyrhizobium isolates 05LoS21R6.43 (strain #18), 05LoS3.3 (strain #38), and 05LoS24R3.28 (strain #2) were isolated in 2005 from *A. strigosus* root surfaces or nodules at Bodega Marine Reserve ('BMR,' Sonoma Co., CA; Sachs *et al.*, 2009). Strains #18 and #38 provide different amounts of fixed nitrogen to sympatric BMR hosts, increasing shoot mass by *c.* 6-fold and 4-fold respectively, compared to uninoculated control hosts, whereas strain #2 forms nodules on sympatric hosts but does not enhance growth for these hosts (i.e., ineffective; Sachs *et al.*, 2010a; Regus *et al.*, 2015). In a survey of 1,292 *Bradyrhizobium* isolates across California, including the six field sites from which I sourced *A. strigosus* seeds for this experiment, the *glnII_recA* haplotypes

corresponding to strains #18, #38, and #2 were only recovered at BMR (Hollowell *et al.*, 2016a). This suggests that *A. strigosus* hosts from other field sites are not coevolved with these strains and improves the chance of uncovering partner mismatch in this system.

Acmispon strigosus host lines

A. strigosus seeds were collected from six field sites from the northern range (1 site; BMR) and southern range (5 sites) of this species in California between 2005 and 2012 (Calflora). Field sites varied in soil nitrogen levels (Regus *et al.*, 2014; Regus *et al.*, 2017): soil mineral N was low (2-4 ppm) at Anza-Borrego Desert State Park ('Anz') and Bodega Marine Reserve ('BMR'), intermediate (*c*. 7 ppm) at Griffith Park ('Gri') and Burns-Pinyon Ridge Reserve near Yucca Valley ('Yuc'), and high (11-20 ppm) at Bernard Field Station of the Claremont Colleges ('Cla') and University of California, Riverside ('UCR').

I raised plants from wild seeds in a glasshouse sprayed with insecticide to eliminate insect pollination, allowed plants to self, and collected seeds from individual plants to generate inbred lines. I selected two inbred seed sets derived from different wild seed ancestors per field site, but I used wild mixed seeds from Gri because there was poor seed production from those plants (**Table 1.1**). Hereafter, inbred host lines are referred to as Anz03, Anz11, BMR04, BMR07, Cla06, Cla10, UCR03, UCR10, Yuc02, and Yuc03, and the wild Gri seeds are referred to as Gri01.

I assessed genetic divergence among *A. strigosus* lines by sequencing the *nuclear ribosomal Internal Transcribed Spacer* (*nrITS*) and the nuclear gene *Cyclin Nucleotide*

Gated Channel 5 (*CNGC5*; **Table 1.2**). These loci have been used to resolve phylogenetic relationships within the legume tribe Loteae (*nrITS*, Allan & Porter, 2000) and the legume genus *Medicago* (*CNGC5*, Maureira-Butler *et al.*, 2008). For each inbred host line, I sequenced loci from at least two progeny of the wild seed ancestor defining the host line. To genotype the wild Gri01 seed set, I sequenced loci from at least eight plants grown from wild seeds. All analyzed nucleotide positions had at least 2x sequencing coverage. Sequence gaps were eliminated before pairwise distance analysis in MEGA7 (Kumar *et al*., 2016). Sequences are deposited in GenBank under accession numbers KX449152-KX449173.

Inoculation experiments

I raised axenic *A. strigosus* seedlings in sterilized quartzite sand in Ray-Leach SC10 conetainers (Stuewe & Sons, Corvallis, OR) following published protocols (Sachs *et al.*, 2009). Plants with true leaves (i.e., not just cotyledons) were acclimatized to glasshouse conditions (i.e., hardened) for eight days until inoculation on 4 March 2014. I grew *Bradyrhizobium* strains on modified arabinose gluconate (MAG) agar plates, washed cells off plates into liquid MAG, quantified cell titers by colorimetry, pelleted the cells, and resuspended them in sterile ddH_2O to generate inocula of 1×10^8 cells ml^{-1} (Sachs *et al.*, 2009). Plants were inoculated with 5 ml of clonal *Bradyrhizobium* cultures (single-inoculation experiment), 5 ml of a mixture comprising equal concentrations of each culture (co-inoculation experiment) or 5 ml sterile ddH_2O as a control (both experiments). Fertilization treatments consisted of weekly applications of 5 ml N-free

Jensen's solution ('unfertilized' plants) or 5 ml Jensen's with 0.5 g l^{-1} $K^{15}NO_3$ (2% ^{15}N by weight; 'fertilized' plants), beginning four days before inoculation (Sachs *et al.*, 2009).

The single-inoculation experiment included 288 plants (six host populations x two host lines x four inoculation treatments x two fertilization treatments x three plant replicates). The experiment was blocked by host line (Anz11, BMR07, Cla10, UCR10, and Yuc02 were placed on one greenhouse bench and Anz03, BMR04, Cla06, UCR03, and Yuc03 were placed on a second greenhouse bench, with Gri01 split evenly between the two benches). Size-matched plants from each host line were randomly assigned to each fertilization and inoculation treatment. Plant positions were randomized within blocks. The co-inoculation experiment included 240 plants (six host populations x two host lines x two inoculation treatments x two fertilization treatments x five plant replicates). Larger seedlings were used for the co-inoculation experiment so that competing *Bradyrhizobium* strains would have access to the larger root systems of these plants. The co-inoculation experiment was split into two blocks and plants were size-matched and randomly assigned to fertilization and inoculation treatments, as in the single-inoculation experiment.

Plant harvest and nodule culturing

The single- and co-inoculation experiments were harvested 51-57 days post-inoculation (dpi) and 48-55 dpi, respectively. Plants were removed from pots, washed free of sand, and dissected into root, shoot, and nodule portions. Nodules were counted

and photographed against graph paper to measure nodule area (ImageJ). Roots, shoots, and nodules not used for culturing were oven-dried (> four days, 60°C) and weighed. An empirically generated nodule area to mass equation was used to correct per-plant nodule dry masses for nodules removed for culturing:

$$Nodule\ dry\ mass\ (mg) = \frac{Nodule\ area\ (mm^2) - 0.9097853}{5.5258444}$$

Leaf tissue from singly-inoculated plants was assayed for ^{15}N content (one plant replicate per treatment; 96 samples; UC Davis Stable Isotope Facility).

Nodules for culturing were chosen from the upper and lower 50% of the nodule size distribution on the plant, avoiding senescent (green or brown) nodules. Nodules were surface-sterilized with bleach, rinsed, crushed, and spread onto two replicate MAG-agar plates in 10^{-3} and 10^{-5} dilutions (single-inoculation experiment) or onto three replicate plates to generate isolated colonies (co-inoculation experiment; Sachs *et al.* 2009). From the single-inoculation experiment, I cultured two nodules from one plant replicate of each host line and treatment (144 nodules total) and calculated number of rhizobial cells per nodule from at least two plates containing 3-800 colonies. From the co-inoculation experiment, I cultured four nodules from two plant replicates of each host line and treatment (192 nodules total). An average of 102 colonies per nodule were sub-cultured onto three separate MAG-agar plates containing i) 125 µg ml^{-1} streptomycin, ii) 100 µg ml^{-1} gentamycin, and iii) no antibiotic (positive control). Strain #2 is resistant to gentamycin and streptomycin, #18 is resistant to gentamycin, and #38 is sensitive to both. Colonies were scored after four days of growth at 29°C. Colonies with ambiguous scores

were sub-cultured again, and colonies with persistent ambiguous scores (0.4% of all colonies streaked) were excluded from calculations of nodule occupancy.

Estimating host benefits and host control over symbiosis

I estimated net host benefits from symbiosis as relative growth:

$$Relative\ growth = \frac{Total\ plant\ (root + shoot)\ DM_{I+}}{Total\ plant\ DM_{I-}}$$

where DM = dry mass in mg, I+ = inoculated, and I- = uninoculated. Relative growth greater than one indicates growth benefit from inoculation. To estimate host benefits in the context of their level of investment into nodules, I calculated symbiotic efficiency (*sensu* Oono and Denison, 2010):

$$Symbiotic\ efficiency = \frac{Total\ \ plant\ DM_{I+} - Total\ plant\ DM_{I-}}{Total\ nodule\ DM}$$

For unfertilized singly-inoculated plants, I measured ^{15}N discrimination (a proxy for nitrogen fixation), which is deviation from the atmospheric ^{15}N atom percentage due to isotopic fractionation by nitrogenase (i.e., $\delta^{15}N$; Unkovich, *et al*., 2008). For fertilized singly-inoculated plants, I calculated percent nitrogen derived from the atmosphere (percent Ndfa) from $\delta^{15}N$ values of size-matched plants:

$$\%Ndfa = 100 * \frac{\delta^{15}N_{F+I-} - \delta^{15}N_{F+I+}}{\delta^{15}N_{F+I-} - \delta^{15}N_{F-I+}}$$

where F+ = fertilized and F- = unfertilized. During single- and co-inoculations, I estimated host control using mean nodule size (total nodule dry mass divided by total nodule number). I also examined total nodule dry mass and total nodule number separately to understand how those traits contributed to variation in nodule size. During

co-inoculations, I estimated host control using nodule occupancy of the most effective strain (identified during single-inoculations). I estimated both 'inclusive' nodule occupancy (counting all nodules in which the most effective strain was found, including co-infected nodules) and 'exclusive' nodule occupancy (counting only nodules singly-infected by the most effective strain).

Data analysis

I used linear regressions to test whether mean nodule size significantly predicted number of rhizobial cells per nodule for each strain during single-inoculations. I used general linear mixed models (GLMMs) to test estimates of host benefit (relative growth, symbiotic efficiency, $\delta^{15}N$, and percent Ndfa) and host control (mean nodule size, total nodule dry mass, and total nodule number) for effects of host population, strain, fertilization, and interactions among those effects. Block was included as a random effect in all models. I removed non-significant interaction terms if this reduced corrected AIC (AICc) values by at least two units (**Table 1.3**). Significant differences among treatments were assessed using pairwise *t*-tests (Tukey's HSD) of least squares means, with significant interaction terms pre-empting significant main effects. Dependent variables were log-transformed if necessary to improve normality. I used the binomial test to evaluate whether nodule occupancy of the most effective strain deviated from the null expectation of 33%. To understand whether host control was associated with increased host benefits relative to the null expectation from single-inoculations (Heath & Tiffin, 2007), I tested relative growth from co-inoculations against mean relative growth from

single-inoculations for each host/fertilizer treatment using one-sample *t*-tests (single-inoculation means were calculated from *c.* 18 plants: three strains x two blocks x three plant replicates). Statistical analyses were performed in JMP Pro 13.0.0 (SAS Institute Inc., Cary, NC, USA) and Microsoft Excel (2016).

Results

Genotyping *A. strigosus* host lines

Sympatric host lines within BMR, Cla, UCR, and Yuc populations were identical at *nrITS* and *CNGC5* loci, but Anz03 and Anz11 differed at both loci. Host lines from different populations generally differed at both loci, but UCR and BMR hosts could not be differentiated using these loci (**Table 1.2**).

Nodulation of *Bradyrhizobium* strains on *Acmispon* host lines

In the single-inoculation experiment, strain #18 formed nodules on all but two inoculated plants (Anz11 and Cla10 host lines) and strain #38 formed nodules on all inoculated plants. Strain #2 formed nodules on most inoculated plants but failed to nodulate five of six Anz11 plants (the one nodulated plant bore only a single nodule) and one additional plant (Cla10). Inoculated plants that failed to form nodules were excluded from subsequent analyses. All co-inoculated plants formed nodules. There were no nodules on uninoculated plants.

Benefits of symbiosis during single-inoculations

Genetic effects on host benefits. Strain #18 was highly effective and #2 was ineffective on all unfertilized hosts, consistent with previous studies (Sachs *et al.*, 2010a). Of the three strains, #18 produced the greatest plant relative growth, symbiotic efficiency, and ^{15}N discrimination ($\delta^{15}N$ = -1.6‰), consistent with high rates of nitrogen fixation (**Fig. 1.2**; **Fig. 1.3**; **Table 1.4**). Strain #38 was intermediate in ^{15}N discrimination ($\delta^{15}N$ = -0.43‰), and strain #2 ($\delta^{15}N$ = 1.29‰) did not significantly differ from uninoculated plants ($\delta^{15}N$ = 1.76‰). Strain #38 was intermediate in effectiveness to strains #2 and #18 for BMR and Gri hosts but equally as effective as #18 for Anz, Cla, UCR, and Yuc hosts (**Fig. 1.2**). Of the five hosts, Cla hosts achieved the greatest relative growth and symbiotic efficiency from the effective strains (#18, #38) and Gri hosts achieved the least, although the remaining hosts did not significantly differ from these two extremes (**Fig. 1.2**; **Fig. 1.3**).

Fertilization effects on host benefits. Fertilization reduced plant relative growth with the effective strains and reduced the difference in their effectiveness (#18: 83% Ndfa; #38: 69% Ndfa), although both remained more effective than #2 (0.4% Ndfa; **Fig. 1.2**, **Table 1.5**). Fertilization reduced symbiotic efficiency for all hosts (**Fig. 1.3**).

Host control over symbiosis during single-inoculations

Regression analysis of number of rhizobia per nodule against nodule size. The number of rhizobia per nodule had a positive relationship with nodule size for strains #2 (adj. R^2 = 0.37, P = 0.0002, slope = 2.0 x 10^7 cells mm^{-2} nodule) and #18 (adj. R^2 = 0.12,

$P = 0.0126$, slope = 3.2×10^6 cells mm^{-2} nodule), but not for intermediate strain #38 (adj. $R^2 = 0.06$, $P = 0.0890$, slope = 7.8×10^5 cells mm^{-2} nodule; **Fig. 1.4**). The steeper regression line for strain #2 versus #18 corroborates previous findings that strain #2 achieves high population sizes in small nodules (Sachs *et al.*, 2010a). Thus, I used mean nodule size as a proxy of host control for strain #2 and #18, understanding that strain #2 had greater within-nodule population density than strain #18.

Genetic effects on host investment. Anz, BMR, and UCR hosts invested in rhizobia in a benefits-dependent way, making larger nodules for #18 than #2, with #38 intermediate (**Fig. 1.5**, **Table 1.6**). Cla, Gri, and Yuc hosts invested in rhizobia irrespective of benefits, showing no significant difference in nodule size among strains. Among hosts inoculated with strain #18, UCR and BMR hosts made the largest nodules and Yuc hosts made the smallest. Among hosts inoculated with strain #2, Gri hosts made the largest nodules and UCR hosts made the smallest. Hosts did not vary in nodule size for strain #38. Total nodule dry mass was greater for the effective strains (#18, #38) than ineffective strain #2 for hosts from each population (**Fig. 1.6a**, **Table 1.6**). For unfertilized plants, hosts from Cla, Gri, and Yuc formed more nodules with the effective strains (#18, #38) than ineffective strain #2, but hosts from Anz, BMR, and UCR did not significantly differ in total nodule number among strains (**Fig. 1.6b**, **Table 1.6**).

Fertilization effects on host investment. Fertilization reduced nodule size for strain #2 but did not affect nodule size for strains #18 or #38 (**Fig. 1.5**, **Table 1.6**). Fertilization increased total nodule dry mass for all strains, although strains #18 and #38 still had greater total nodule dry mass than ineffective strain #2 in fertilized conditions.

Fertilization increased total nodule dry mass for all hosts except Yuc (**Fig. 1.6a**, **Table 1.6**). Yuc hosts also formed more nodules with the effective strains (#18, #38) than ineffective strain #2 under fertilization, while the remaining hosts did not differ in total nodule number among strains (**Fig. 1.6b**, **Table 1.6**).

Benefits of symbiosis during co-inoculations

Co-inoculated Yuc hosts had lower relative growth than other hosts in unfertilized conditions (**Fig. 1.2**, **Table 1.4**). Relative growth of co-inoculated BMR, Gri, and UCR hosts exceeded their single-inoculation means, whereas Anz and Cla hosts had similar growth in both experiments. These patterns were not altered by fertilization. Relative growth of unfertilized Yuc hosts did not differ from the single-inoculation mean, but fertilized Yuc hosts gained more growth from co-inoculation than the single-inoculation mean (**Fig. 1.2**). Fertilization reduced the relative growth of all co-inoculated plants and erased the differences among hosts seen in unfertilized conditions. Symbiotic efficiency was greatest for Gri hosts and least for BMR hosts, although hosts from other populations did not significantly differ from those extremes (**Fig. 1.3**, **Table 1.4**). Fertilization reduced symbiotic efficiency.

Host control over symbiosis during co-inoculations

Host sanctions. Since the mean total nodule number on co-inoculated plants ranged from 40 (unfertilized plants) to 57 (fertilized plants), my nodule occupancy assays tested 7-10% of the nodules on selected plants. The most effective strain (#18) dominated

the majority of nodules on all tested hosts (**Fig. 1.7**). Of 19,312 colonies scored from nodules, 96.4%, 2.6%, and 1.0% were identified as strains #18, #38, and #2, respectively. Strain #38 was recovered from hosts of all six populations in the unfertilized treatment and from BMR, Anz, and Yuc hosts in the fertilized treatment. Strain #2 was recovered from UCR and Cla hosts in the unfertilized treatment and from UCR and Anz hosts in the fertilized treatment. Strains #18, #38, and #2 were identified in 170, 19, and six nodules, respectively, from the 177 nodules successfully sub-cultured from co-inoculated plants. Seventeen nodules were coinfected by more than one strain. For each host population x fertilization treatment combination, strain #18 was identified in nodules more often than expected by chance under a null nodule occupancy of 33% (binomial test, all $P < 0.0001$ for inclusive nodule occupancy and all $P < 0.0016$ for exclusive nodule occupancy).

Host investment. Mean nodule size varied significantly among hosts from different populations: UCR and BMR hosts produced the largest nodules and Yuc hosts produced the smallest, with the remaining populations intermediate (**Fig. 1.5**, **Table 1.6**). There was little variation among hosts for total nodule dry mass, but total nodule number was significantly greater for Yuc hosts than BMR, UCR, or Cla hosts (**Fig. 1.6**, **Table 1.6**). Fertilization increased mean nodule size, total nodule dry mass and total nodule number.

Discussion

To understand how variation in symbiont effectiveness is maintained, I tested for three kinds of refugia that could protect ineffective symbionts from host-mediated

purifying selection. I found no evidence of partner mismatch in my panel of three *Bradyrhizobium* strains and six population sources of *Acmispon strigosus*. Neither did I find evidence that resource satiation relaxed host control over the ineffective symbiont. However, hosts from different populations differed in host control traits, consistent with the host variation hypothesis.

Host variation hypothesis. Empirical evidence of host control exists for several legume species, including soybean (*Glycine max*; Kiers *et al.*, 2003), alfalfa and pea (*Medicago sativa* and *Pisum sativum*; Oono *et al.*, 2011), *Medicago lupulina* (Simonsen & Stinchcombe, 2014), and *A. strigosus* (Sachs *et al.*, 2010b). Here, I tested for host control as host sanctions during co-inoculations and host investment into nodule size during both co-inoculations and single-inoculations. Host genotypes from all six *A. strigosus* populations showed evidence of robust host sanctions, corroborating previous studies using mixed seed sources from BMR (Sachs *et al.*, 2010b; Regus *et al.*, 2014). In contrast, I found genetic variation for host investment into symbionts. Since population genetic structure of rhizobia can cause hosts to encounter different subsets of symbiont genotypes in nature, host variation in investment could affect symbiont relative fitness in situations where hosts encounter just one or a few strains, or one strain in large numerical excess to others (McInnes *et al.*, 2004; Hollowell *et al.*, 2016a). Thus, variation in host control operating at the level of host investment into nodules, but not at the level of host sanctions, could help maintain variation in symbiont effectiveness in the *Acmispon-Bradyrhizobium* system.

Strain #18 was generally the most effective strain for all hosts, inconsistent with the partner mismatch hypothesis, and the dominance of strain #18 in nodules of co-inoculated plants (with the near absence of less-effective strains) is consistent with robust host sanctions in plants from all host populations. However, strain #38 approached the effectiveness of strain #18 on some hosts, particularly in fertilized conditions. If symbiont nodule occupancy were strictly a function of sanctions acting on strain effectiveness, I would expect to see more evidence of strain #38 in nodules of co-inoculated plants. My data support the hypothesis that *in planta* symbiont fitness is a joint function of symbiont competitive ability and sanctions acting on symbiont effectiveness, consistent with other published data: Amarger (1981) showed that similarly effective strains co-inoculated onto *Medicago sativa* were not recovered from nodules in their inoculation ratio, but showed differential competitive ability *in planta*, and similar results exist for *Medicago truncatula* (Grillo *et al*., 2016). I found evidence that strain #38 had lower cell titers in nodules of singly-inoculated plants than other strains (**Fig. 1.4**), and previous work found that strain #38 had lower *in vitro* doubling rates than strains #2 or #18 (Sachs *et al.*, 2010b), possibly explaining its failure to significantly occupy nodules of co-inoculated plants. In contrast, the dominance of effective strain #18 over ineffective strain #2 in this study is consistent with sanctions, as previously reported (Sachs *et al.*, 2010b). The conservation of host sanctions across genotypes from different host populations and fertilization treatments suggests that this component of host control is fixed in *A. strigosus*.

I found significant variation among hosts from different populations in the degree to which they invested into nodules harboring the most effective strain (**Fig. 1.5**). Differences in symbiotic efficiency among hosts were modest compared to differences among strains and fertilization treatments (**Fig. 1.3**), suggesting that different hosts experience similar benefits from symbiosis per unit nodule mass. However, I found that plants from three host populations (UCR, BMR, Anz) showed 'scaled investment' during single-inoculations by increasing the size of nodules as benefits from symbiosis increased, whereas plants from Cla, Gri, and Yuc populations showed 'unscaled investment' (nodule size did not significantly change with changing benefits from symbiosis). Variation in nodule size was driven more by total nodule number than total nodule dry mass, such that the hosts forming the largest nodules with the most effective strain also formed relatively few nodules in total, potentially reflecting greater host control over the infection process. During co-inoculations, UCR and BMR hosts (but not Anz hosts) again formed larger nodules than other hosts and gained significantly more growth benefit than expected from the mean of single-inoculation treatments. This suggests that variation in host investment into nodules can influence host benefits even in a co-inoculation setting in which sanctions are invariant.

The drivers of variation in host investment are unclear. One possibility is that variation in host investment is driven by underlying variation in the magnitude of the benefit hosts gain from effective strains, which could create an appearance of host investment variation. However, Cla hosts gained an extraordinarily high amount of benefit from strains #18 and #38 (**Fig. 1.2**) and still displayed 'unscaled investment' in

terms of nodule size. Alternatively, the ability to differentially invest in symbionts based on effectiveness could be costly for hosts (Foster & Kokko, 2006; Steidinger and Bever, 2014), similar to the observation that R-gene-mediated plant defense against pathogens can reduce the growth of disease-free plants (Tian *et al.*, 2003). I found that uninoculated UCR and BMR hosts, which displayed 'scaled investment,' tended to have lower total plant dry mass than most 'unscaled investment' hosts, consistent with constitutive costs of host control (**Table 1.7**). However, Cla hosts also had relatively low plant dry mass and still displayed 'unscaled investment.' Furthermore, since ineffective strain #2 had greater population density in nodules compared to effective strain #18, similar-sized nodules occupied by different strains could still generate different fitness outcomes for the two strains. Thus, the drivers of variation in host investment into symbionts, and how this influences symbiont fitness in the soil, both merit further study.

Resource satiation hypothesis. Nitrogen fertilization has long been associated with reduced nodulation and biological nitrogen fixation in the agricultural sciences (Herridge & Rose, 2000; Van Kessel & Hartley, 2000; Wissuwa *et al.*, 2009). The energetic costs of building nodules and fueling the reduction of atmospheric nitrogen seem to provide an advantage to plants that exclusively use mineral sources of nutrients when they are plentiful. However, I did not find evidence for the resource satiation hypothesis in *A. strigosus*. Host sanctions were severe in both unfertilized and fertilized conditions, consistent with previous tests of sanctions (Kiers *et al.*, 2006; Regus *et al.*, 2014). Host investment during single-inoculations was unaffected by fertilization for the two effective strains but decreased with fertilization for ineffective strain #2, suggesting

that fertilization improved host control. A previous study of *A. strigosus* from BMR and UCR found that fertilization only reduced nodulation at levels that also caused high plant mortality (i.e., > 3.0 g l^{-1} KNO_3, compared to 0.5 g l^{-1} KNO_3 used here; Regus *et al.*, 2017). The fertilization-induced decline in strain #2 nodule size occurred well below the fertilization rate that causes host toxicity and probably represents adaptive host control. Furthermore, variation in host investment was not structured by the soil nitrogen regimes associated with those host populations, since the UCR and BMR hosts that displayed 'scaled investment' were from very high and low soil nitrogen regimes, respectively.

My results contrast with studies suggesting fertilization could erode host control. However, the best example of long-term nitrogen exposure reducing host control is confounded by crop breeding history, which generally does not target belowground traits and could allow host control traits to erode through drift (Kiers *et al*., 2007). A long-term study suggested that nitrogen fertilization can reduce the effectiveness of rhizobia associating with wild *Trifolium* (Weese *et al*., 2015), but hosts decreased in abundance during the study period, leading to fewer opportunities to interact with rhizobia and making it difficult to discern if hosts also reduced their selection for symbiont effectiveness (i.e., the resource satiation hypothesis). Here, I found evidence that hosts maintain robust host control in fertilized conditions, consistent with the alternative hypothesis that plant fitness in high-nitrogen soil is maximized when hosts only permit the best symbionts to proliferate *in planta*, enabling the plant's modest nitrogen needs to be met with a minimum of cost to plant carbon (Kiers *et al*., 2007). Thus, increased soil fertility may not contribute to the maintenance of variation in symbionts in natural

systems, to the extent that symbiont effectiveness depends on host control traits as opposed to host ecology.

Partner mismatch and other hypotheses. Although I tested three models for the maintenance of variation in symbiont effectiveness, there are other hypotheses I did not test. For instance, ineffective symbionts may be primarily adapted to the free-living portion of their lifecycle (i.e., in soil between cycles of plant infection), which could eventually lead to mutualism abandonment (Denison & Kiers, 2004; Sachs & Simms, 2006). Consistent with the idea that symbionts can 'specialize' in the free-living portion of their bipartite lifecycle, some *Bradyrhizobium* genotypes exhibit greater metabolic flexibility than other symbiont genotypes (Hollowell *et al.*, 2016b) and are also epidemic in distribution across a metapopulation of symbionts (Hollowell *et al.*, 2016a). *In vitro* evolution further shows that without host interaction, rhizobia can rapidly erode in their symbiotic effectiveness on hosts (Sachs *et al.*, 2011b). Partner mismatch operating at a coarser host taxonomic scale could also maintain variation in symbiont effectiveness: there is evidence that ineffective strain #2 used in this study forms relatively large nodules on another host species, *A. wrangelianus* (Pahua *et al.*, 2018). Finally, a reasonable null model for the maintenance of symbiont variation is mutation-selection balance, whereby mutation events constantly generate variation in symbiont benefits, and the less effective genotypes are slowly purged from symbiont populations due to having lower-than-average fitness (Van Dyken *et al.*, 2011). Further work is needed to examine rhizobial fitness in hosts and soils to discriminate among these other hypotheses.

Conclusions. Here, I used three *Bradyrhizobium* strains and host lines from six *A. strigosus* populations to test for context-dependency of host control, such that host control varies depending on availability of mineral nitrogen or the genotypes of the interacting partners. I found no evidence for the partner mismatch hypothesis, in which ineffective strains are maintained by being conditionally effective on other host genotypes. Instead, I found broad conservation of strain symbiotic effectiveness on hosts from across California. I found no evidence for the resource satiation hypothesis, in which hosts encountering high-nitrogen soils relax host control traits. Instead, I found that hosts significantly reduced investment into nodules occupied by the ineffective strain when they were fertilized, and co-inoculated hosts sanctioned the ineffective strain equally well in unfertilized and fertilized conditions, consistent with host control. My data support the host variation hypothesis, in which hosts vary genetically in host control and thus vary in the selection they impose on symbiont effectiveness. Host sanctions against ineffective symbionts were robust in hosts from all populations, but I found variation in host ability to preferentially invest in nodule size according to symbiont effectiveness, even when plants were also enacting sanctions (i.e., in the co-inoculation experiment). This study contributes to reports of variation in host control from two other legume species (soybean, Kiers *et al.*, 2007; *Medicago lupulina*, Simonsen & Stinchcombe, 2014), suggesting that this could be a consistent feature of legume species that engage in symbiosis. Differences in symbiont fitness produced by the combined action of invariant sanctions and variable investment could help maintain variation in the effectiveness of symbiont populations.

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Fig. 1.1. Three hypotheses for how variation in symbiotic effectiveness of rhizobia is maintained. (a) Mutualism theory and empirical studies predict that hosts will select against an ineffective strain (white) relative to an effective strain (grey) by forming relatively small nodules during single-inoculations and reducing nodule occupancy during co-inoculations. Over time, host-mediated selection is predicted to drive the ineffective strain extinct and reduce variation in symbiotic effectiveness among rhizobia, inconsistent with the high variation in effectiveness seen in soils worldwide. Panels (b)-(d) describe scenarios that reduce the fitness differential between the ineffective and effective strains by reducing their difference in nodule size (single-inoculations) or nodule occupancy (co-inoculations). (b) Under the partner mismatch scenario, the 'ineffective' strain is symbiotically effective on a different host genotype (Host B), enabling the 'ineffective' strain to form large nodules during single inoculations and achieve high nodule occupancy during co-inoculations. (c) Under the 'resource satiation' scenario, Host A relaxes host control traits when its nitrogen needs are met by the soil; although Host A does not benefit from the ineffective strain, relaxed host control enables the ineffective strain to form large nodules during single-inoculations and achieve high nodule occupancy during co-inoculations. (d) Under the 'host variation' scenario, the ineffective strain encounters a different host genotype (Host B) that fails to exert host control traits; although Host B does not benefit from the ineffective strain, it allows the ineffective strain to form large nodules during single-inocualtions and achieve high nodule occupancy during co-inoculations.

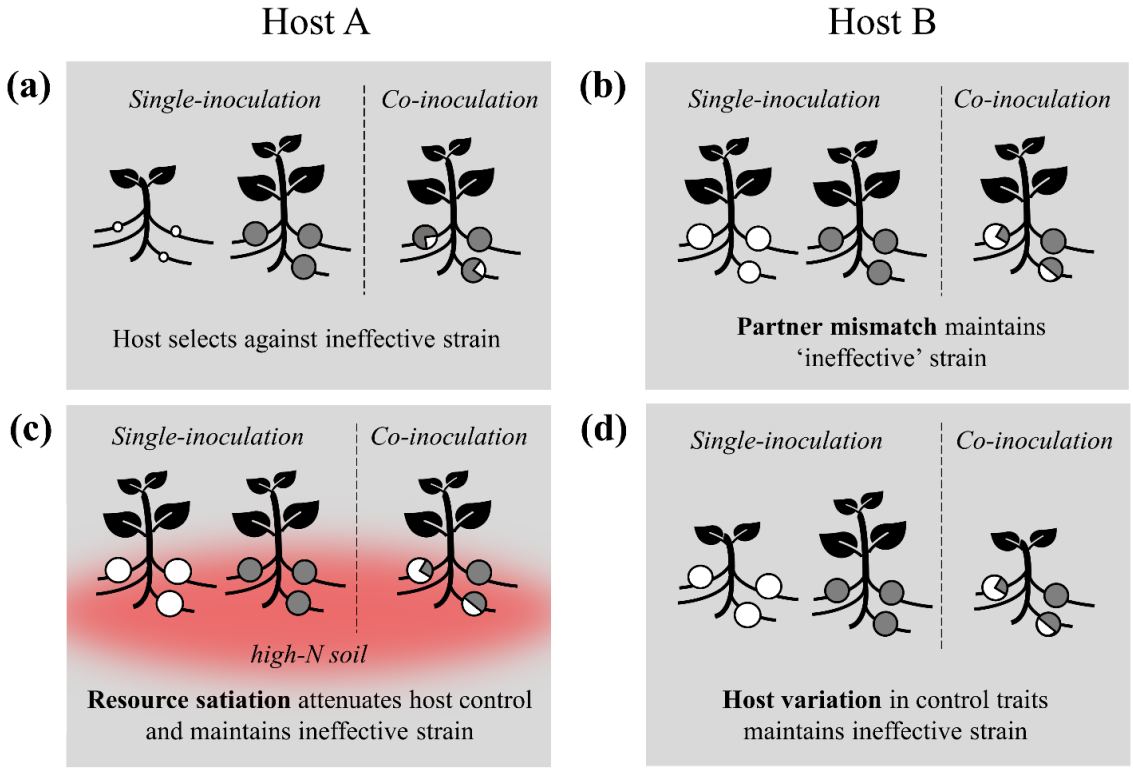


Fig. 1.2. Test of the partner mismatch hypothesis based on plant relative growth of *A. strigosus* from different populations in (a) unfertilized and (b) fertilized conditions. Relative growth was calculated as total plant dry mass (roots + shoots) of the inoculated plant divided by the total plant dry mass of its size-matched uninoculated control plant. Relative growth greater than one indicates growth benefit from symbiosis. Statistics were performed separately for singly- and co-inoculated plants. For singly-inoculated plants, different letters above strain treatments indicate significant differences among strain and fertilization treatments (strain x fertilization effect; **Table 1.4**). Daggers above a host population indicate that plant relative growth differed significantly among all three strains (#18 > #38 > #2); populations not marked with a dagger had significant growth differences only for strain #2 versus the other two strains (population x strain effect; **Table 1.4**). For co-inoculated plants, different letters indicate significant differences among population and fertilization treatments (population x fertilization effect; **Table 1.4**). Bold horizontal bars indicate the mean relative growth combining all three single-inoculation treatments for each host population in each fertilization treatment. Asterisks indicate that relative growth of co-inoculated plants significantly differed from the mean of single inoculation treatments in a one-sample *t*-test ($P < 0.05$). B = BMR (Bodega Marine Reserve), U = UCR (University of California, Riverside), C = Cla (Bernard Field Station of the Claremont Colleges), A = Anz (Anza-Borrego Desert State Park), G = Gri (Griffith Park), Y = Yuc (Burns-Pinyon Ridge Reserve near Yucca Valley). Bars represent +/- 1 SE.

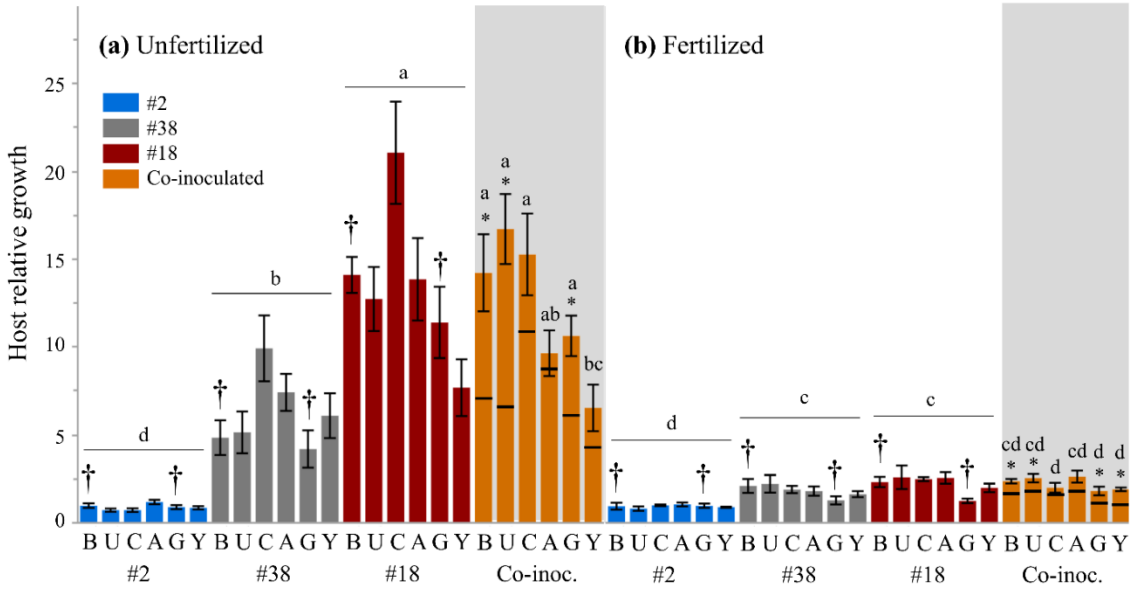


Fig. 1.3. Test of the partner mismatch hypothesis based on host symbiotic efficiency of *A. strigosus* from different populations in (a) unfertilized and (b) fertilized conditions. Symbiotic efficiency was calculated as mg total plant dry mass (roots + shoots) gained from symbiosis per mg total nodule dry mass. Statistics were performed separately for singly-inoculated and co-inoculated plants. Only main effects were significant, and different letters indicate the significant main effects of strain, fertilization, and host population (**Table 1.4**). Different letters above strain treatments indicate significant differences among strains; different letters above fertilization treatments indicate significant differences between fertilization treatments. Different letters above populations indicate significant differences among populations; populations without letters did not differ from either extreme (i.e., 'ab'). B = BMR (Bodega Marine Reserve), U = UCR (University of California, Riverside), C = Cla (Bernard Field Station of the Claremont Colleges), A = Anz (Anza-Borrego Desert State Park), G = Gri (Griffith Park), Y = Yuc (Burns-Pinyon Ridge Reserve near Yucca Valley). Bars represent +/- 1 SE.

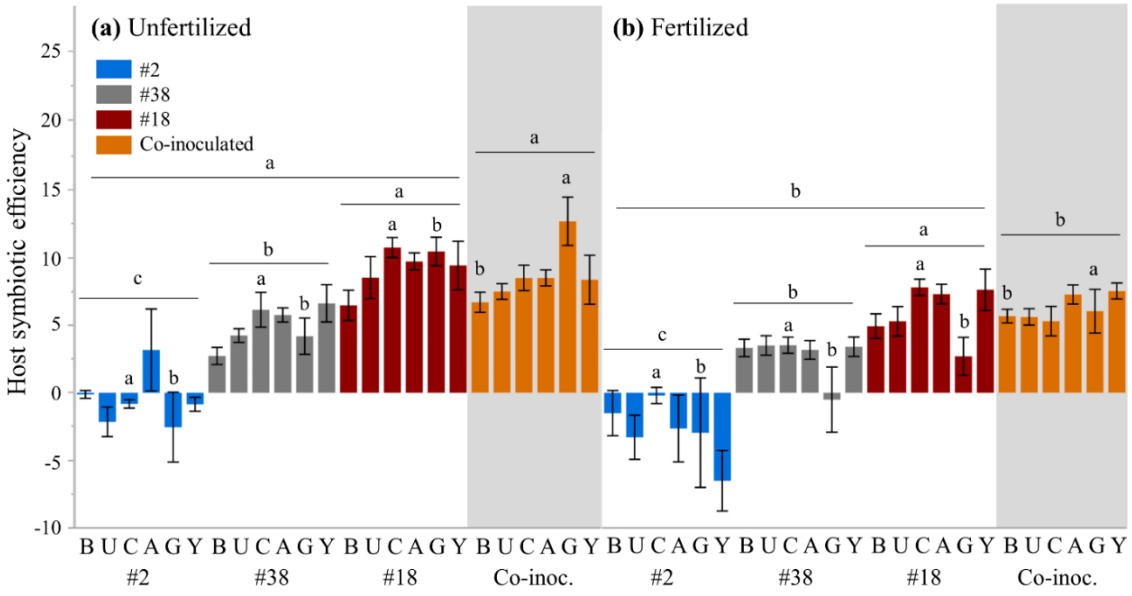


Fig. 1.4. Regression of rhizobia per nodule against nodule area for individual nodules cultured from *A. strigosus* singly-inoculated with each *Bradyrhizobium* strain, with data pooled among fertilization treatments and host lines. Strains #2, #38, and #18 are denoted by open gray circles, plus signs, and filled black circles, respectively. Regressions are significant for strain #2 and strain #18 but not for strain #38.

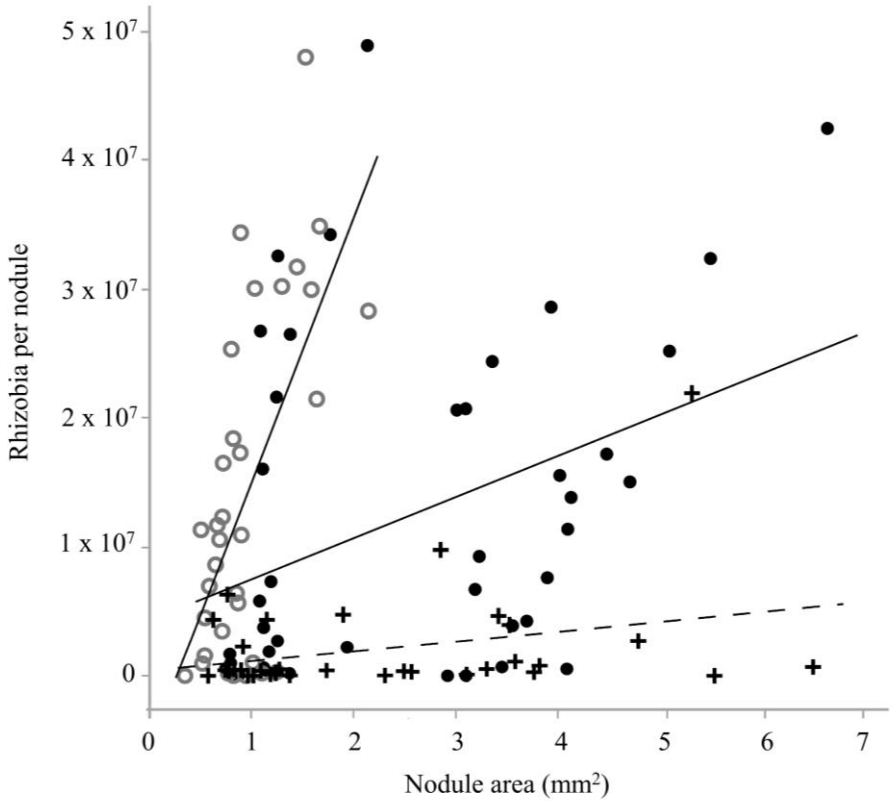


Fig. 1.5. Test of the resource satiation and host variation hypotheses using mean nodule size of *A. strigosus* from different populations in (a) unfertilized and (b) fertilized conditions. Mean nodule size was calculated as total nodule dry mass divided by total nodule number. Statistics were performed separately for singly-inoculated and co-inoculated plants. For singly-inoculated plants, different letters above strain treatments indicate significant differences among strain and fertilization treatments (strain x fertilization effect; **Table 1.6**). Daggers indicate host populations that produced significantly larger nodules with strain #18 than #2; populations not marked with a dagger did not significantly differ in nodule size for those strains (population x strain effect; **Table 1.6**). For co-inoculated plants, different letters above populations indicate significant differences among populations, whereas different letters above fertilization treatments indicate significant differences between fertilization treatments. B = BMR (Bodega Marine Reserve), U = UCR (University of California, Riverside), C = Cla (Bernard Field Station of the Claremont Colleges), A = Anz (Anza-Borrego Desert State Park), G = Gri (Griffith Park), Y = Yuc (Burns-Pinyon Ridge Reserve near Yucca Valley). Bars represent +/- 1 SE.

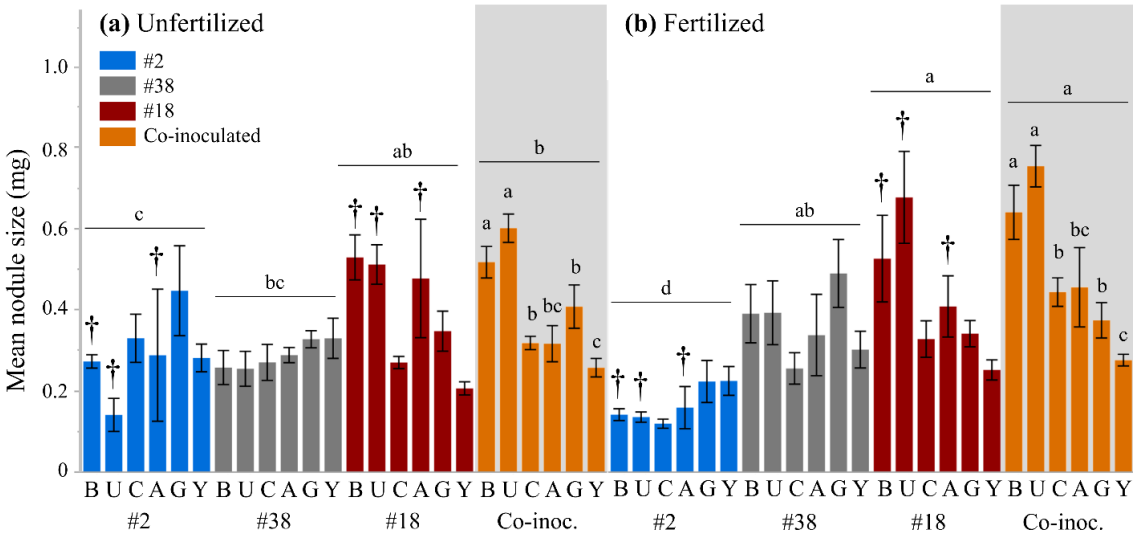


Fig. 1.6. Test of the resource satiation and host variation hypotheses using (a) total nodule dry mass and (b) total nodule number of *A. strigosus* lines from different populations in unfertilized and fertilized conditions. Statistics were performed separately for singly- and co-inoculated plants. (a) For total nodule dry mass of singly-inoculated plants, different letters above strain treatments indicate significant differences among strain and fertilization treatments (strain x fertilization effect; **Table 1.6**; note that the population x strain and the population x fertilization interactions were also significant). (b) For total nodule number of singly-inoculated plants, host populations denoted with daggers formed significantly fewer nodules with strain #2 than strains #38 and #18 (which did not differ) in the indicated fertilization treatment, whereas host populations without daggers did not differ in the number of nodules formed with the three strains in the indicated fertilization treatment (population x strain x fertilization effect; **Table 1.6**). (a,b) For co-inoculated plants, different letters indicate the significant main effects of host population and fertilization. Different letters above host populations indicate significant differences among populations; different letters above fertilization treatments indicate significant differences among fertilization treatments. B = BMR (Bodega Marine Reserve), U = UCR (University of California, Riverside), C = Cla (Bernard Field Station of the Claremont Colleges), A = Anz (Anza-Borrego Desert State Park), G = Gri (Griffith Park), Y = Yuc (Burns-Pinyon Ridge Reserve near Yucca Valley). Bars represent +/- 1 SE.

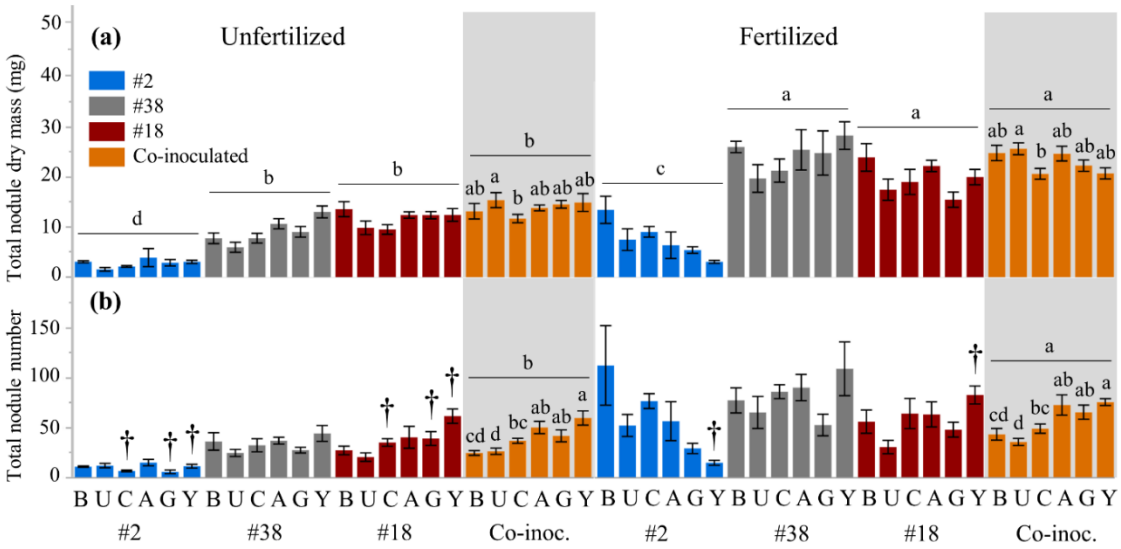
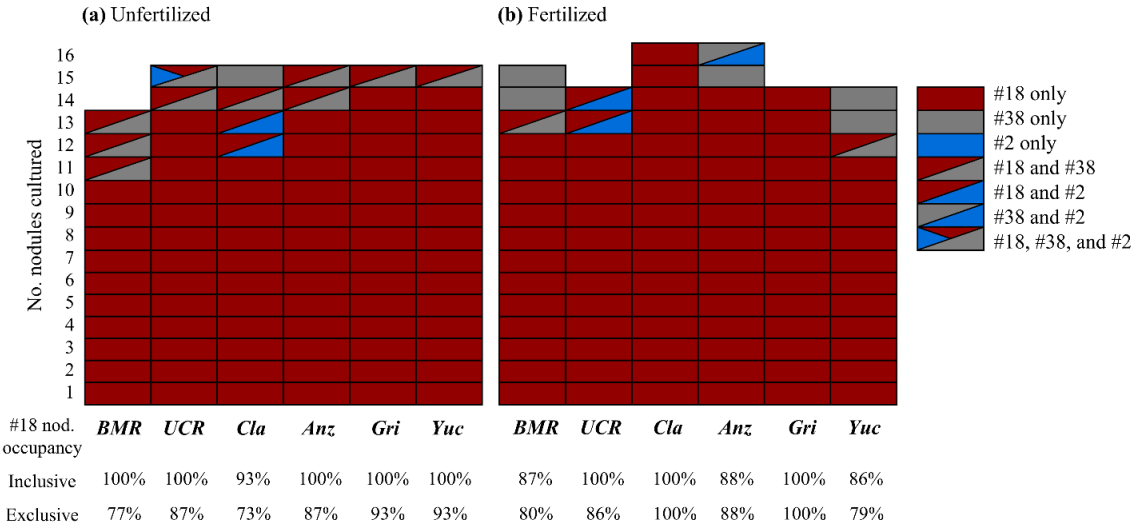


Fig. 1.7. Test of the resource satiation and host variation hypotheses using frequency of co-inoculated *Bradyrhizobium* strains in cultured nodules of *A. strigosus* in (a) unfertilized and (b) fertilized conditions. Up to 16 nodules were cultured from plants of each host population (four nodules x two plant replicates x two host lines). The strain occupancy of each nodule was determined by sub-culturing isolated colonies onto selective media. Nodule occupancy by effective strain #18 was calculated as the percentage of nodules that contained #18, whether or not other strains were also present (inclusive), and the percentage of nodules that contained only #18, without any other strains present (exclusive). BMR = Bodega Marine Reserve, UCR = University of California, Riverside, Cla = Bernard Field Station of the Claremont Colleges, Anz = Anza-Borrego Desert State Park, Gri = Griffith Park, Yuc = Burns-Pinyon Ridge Reserve near Yucca Valley.



#18 nod. occupancy	*BMR*	*UCR*	*Cla*	*Anz*	*Gri*	*Yuc*	*BMR*	*UCR*	*Cla*	*Anz*	*Gri*	*Yuc*
Inclusive	100%	100%	93%	100%	100%	100%	87%	100%	100%	88%	100%	86%
Exclusive	77%	87%	73%	87%	93%	93%	80%	86%	100%	88%	100%	79%

Table 1.1. Collection information for *A. strigosus* host lines.

A. strigosus host line	*A. strigosus* formal name	Collection year for wild seeds	Seed collection site (dec. deg.)	
			Lat.	Long.
Anz03	AcS046.Anz.m01.g1.r03	2011	33.2713	-116.4194
Anz11	AcS040.Anz.m01.g1.r06	2011	33.27068333	-116.4189333
BMR04	AcS074.BMR.u01.g1.r04	2011	38.31903333	-123.0642667
BMR07	AcS004.BMR.u01.g1.r01	2005	38.3193	-123.06365
Cla06	AcS047.Cla.m01.g1.r03	2011	34.11051667	-117.70845
Cla10	AcS047.Cla.m01.g1.r07	2011	34.11051667	-117.70845
Gri01	AcS075.Gri.u01.gwild	2012	34.12244444	-118.2930556
UCR03	AcS027.UCR.u01.g1.r01	2009	33.9659	-117.3227167
UCR10	AcS027.UCR.u01.g1.r03	2009	33.9659	-117.3227167
Yuc02	AcS052.Yuc.m01.g1.r01	2011	34.15315	-116.4751167
Yuc03	AcS052.Yuc.m01.g1.r02	2011	34.15315	-116.4751167

Table 1.2. Estimates of evolutionary divergence between *A. strigosus* lines for *nrITS* (466 nt, top) and *CNGC5* (441 nt, bottom). Positions containing gaps or missing data were deleted. Cell color intensity scales with the number of base substitutions per site between each pair of host lines. I genotyped 2-17 inbred progeny of the wild seed ancestor that defined each plant line, except for Gri01 (wild seed set), for which I genotyped 21 (*nrITS*) or 8 (*CNGC5*) plants grown from wild seeds.

nrITS	Anz03	Anz11	BMR04	BMR07	Cla06	Cla10	Gri01	UCR03	UCR10	Yuc02
Anz03										
Anz11	0.0021									
BMR04	0.0043	0.0065								
BMR07	0.0043	0.0065	0							
Cla06	0.0065	0.0086	0.0022	0.0022						
Cla10	0.0065	0.0086	0.0022	0.0022	0					
Gri01	0.0021	0.0043	0.0022	0.0022	0.0043	0.0043				
UCR03	0.0043	0.0065	0	0	0.0022	0.0022	0.0022			
UCR10	0.0043	0.0065	0	0	0.0022	0.0022	0.0022	0		
Yuc02	0	0.0021	0.0043	0.0043	0.0065	0.0065	0.0021	0.0043	0.0043	
Yuc03	0	0.0021	0.0043	0.0043	0.0065	0.0065	0.0021	0.0043	0.0043	0

CNGC5	Anz03	Anz11	BMR04	BMR07	Cla06	Cla10	Gri01	UCR03	UCR10	Yuc02
Anz03										
Anz11	0.0137									
BMR04	0.0373	0.0325								
BMR07	0.0373	0.0325	0							
Cla06	0.0325	0.0277	0.0091	0.0091						
Cla10	0.0325	0.0277	0.0091	0.0091	0					
Gri01	0.0349	0.0301	0.0301	0.0301	0.0254	0.0254				
UCR03	0.0373	0.0325	0	0	0.0091	0.0091	0.0301			
UCR10	0.0373	0.0325	0	0	0.0091	0.0091	0.0301	0		
Yuc02	0.0137	0	0.0325	0.0325	0.0277	0.0277	0.0301	0.0325	0.0325	
Yuc03	0.0137	0	0.0325	0.0325	0.0277	0.0277	0.0301	0.0325	0.0325	0

Table 1.3. Statistical model selection based on AICc (corrected Akaike's Information Criterion) of candidate models with different interaction terms. For each model, I tested all possible interactions among host population (P), strain (S), and fertilization (F) and then incrementally removed nonsignificant (non-bold) interactions. The AICc value of each chosen model (*) presented in **Table 1.4** and **Table 1.6** was at least 1.96 units lower than other candidate models. I performed a retrospective power analysis for the highest-order nonsignificant interaction term tested for each response variable (generally the PSF interaction). Retrospective power analysis calculates 'observed power' (OP), which is the chance of detecting a significant effect using the given sample size to test a population with parameters estimated from the sample (i.e., with a true effect size equal to the sample effect size, and residual error variance equal to the model RMSE). I excluded the random effect of block from each model to facilitate the power analysis. A dagger indicates the highest-order nonsignificant interaction effect included in the model, for which OP was calculated.

Model	Interactions included	AICc	†OP
Single-inoculation			
Log_{10}(Plant relative growth)			0.7998
	PS, PF, **SF**, PSF†	119.255	
	PS, PF, **SF**	58.9152	
	* **PS, SF**	22.8121	
Symbiotic efficiency			0.6059
	PS, PF, SF, PSF†	1136.991	
	PS, PF, SF	1129.95	
	PS, PF	1124.962	
	PS, SF	1126.007	
	PF, SF	1126.236	
	PS	1121.395	
	PF	1121.869	
	SF	1123.463	
	* none	1119.428	
Log_{10}(Delta $^{15}N + 3$)			0.2285
	PS†	171.1534	
	* none	24.9019	
Percent Ndfa			0.3315
	PS†	251.5754	
	* none	231.189	
Log_{10}(Mean nodule size)			0.5362
	PS, PF, **SF**, PSF†	164.1453	
	PS, PF, **SF**	99.0999	
	* **PS, SF**	65.87722	
Log_{10}(Total nodule dry mass)			0.6796
	PS, PF, SF, PSF†	157.8902	
	* **PS, PF, SF**	95.33293	
Log_{10}(Total nodule number)			
	* **PS, PF, SF, PSF**	221.708	

Table 1.3., continued.

Model	Interactions included	AICc	†OP
Co-inoculation			
Log_{10}(Plant relative growth)			
	* **PF**	62.68259	
Symbiotic efficiency			0.6853
	* PF†	635.1891	
	none	637.8435	
Log_{10}(Mean nodule size)			0.2341
	PF†	-26.9304	
	* none	-62.4644	
Total nodule dry mass			0.5034
	* PF†	680.2623	
	none	681.5416	
Log_{10}(Total nodule number)			0.1531
	PF†	-12.4138	
	* none	-48.6068	

Table 1.4. Models testing the partner mismatch hypothesis for singly-inoculated *A. strigosus* and variation in symbiotic host benefits for co-inoculated *A. strigosus*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Single-inoculation	**Log_{10}(Plant relative growth)**		**Symbiotic efficiency**		**[A]Log_{10}(Delta ^{15}N+3)**		**[B]Percent Ndfa**	
	n = 208, Adj. R^2 = 0.85		n = 207; Adj. R^2 = 0.59		n = 43; Adj. R^2 = 0.67		n = 34; Adj. R^2 = 0.95	
	df	***F***	**df**	***F***	**df**	***F***	**df**	***F***
Host population	5, 186.1	**5.5442*** **	5, 197.1	**2.7692***	5, 33.02	1.8395	5, 25	0.7117
Strain	2, 186.1	**313.372*** **	2, 197.1	**123.0521*** **	3, 33.17	**27.7131*** **	2, 25.1	**341.3825*** **
Fertilization	1, 186	**275.2759*** **	1, 197	**27.6379*** **	.	.	.	.
Pop x Strain	10, 186	**2.1776***	.	.	.	.	.	.
Strain x Fertilization	2, 186	**89.168*** **	.	.	.	.	.	.
Co-inoculation	**Log_{10}(Plant relative growth)**		**Symbiotic efficiency**					
	n = 119; Adj. R^2 = 0.72		n = 119; Adj. R^2 = 0.21					
	df	***F***	**df**	***F***				
Host population	5, 106	**5.0272*** **	5, 106	**2.4095***				
Fertilization	1, 106	**267.674*** **	1, 106	**16.5919*** **				
Pop x Fertilization	5, 106	**3.3398** **	5, 106	2.1596				

[A]The model used only unfertilized plants and also included uninoculated control plants such that the 'strain' effect included the control treatment; one outlier 5.5 SD above the mean (plant 25) was excluded; we added 3 to all raw values prior to log-transformation (this was the smallest integer that made all values positive).

[B]The model used only fertilized plants.

Table 1.5. Nitrogen content of *A. strigosus* during single-inoculations with *Bradyrhizobium.* BMR = Bodega Marine Reserve, UCR = University of California, Riverside, Cla = Bernard Field Station of the Claremont Colleges, Anz = Anza-Borrego Desert State Park, Gri = Griffith Park, Yuc = Burns-Pinyon Ridge Reserve near Yucca Valley.

	Tissue %N by weight (SE)				%N derived from fixation (SE)		
Fertilization, Host	Uninoculated	Strain #2	Strain #38	Strain #18	Strain #2	Strain #38	Strain #18
Unfertilized							
Anz	0.65 (0.05)	0.60 (.)	3.95 (0.25)	5.25 (0.15)	.	.	.
BMR	0.55 (0.05)	0.50 (0.0)	3.6 (0.20)	4.7 (1.0)	.	.	.
Cla	0.60 (0.0)	0.70 (.)	3.65 (1.05)	4.75 (0.15)	.	.	.
Gri	0.55 (0.05)	0.65 (0.15)	2.95 (0.25)	3.85 (0.15)	.	.	.
UCR	0.55 (0.05)	0.60 (0.0)	2.9 (.)	5.4 (0.3)	.	.	.
Yuc	0.60 (0.0)	0.65 (0.05)	4.3 (0.10)	4.35 (0.45)	.	.	.
Fertilized							
Anz	0.85 (0.05)	1.1 (.)	3.0 (0.5)	3.9 (1.0)	-0.43 (1.01)	54.80 (14.77)	83.73 (6.41)
BMR	1.2 (0.2)	1.45 (0.15)	3.55 (0.25)	3.25 (0.05)	-3.67 (2.26)	75.58 (9.89)	81.55 (1.54)
Cla	1.15 (0.15)	1.25 (0.15)	2.8 (0.5)	4.6 (0.8)	0.76 (1.85)	73.56 (1.83)	88.20 (1.43)
Gri	0.9 (0.1)	0.75 (0.15)	1.65 (0.05)	3.15 (0.45)	5.19 (2.71)	71.14 (5.91)	76.79 (5.94)
UCR	1.7 (0.7)	1.7 (0.2)	3.75 (0.55)	4.05 (0.25)	2.01 (.)	62.81 (.)	83.00 (7.52)
Yuc	1.1 (0.0)	1.0 (0.2)	3.85 (0.15)	3.55 (0.35)	0.22 (1.35)	74.48 (0.56)	82.32 (0.98)

Table 1.6. Models testing the resource satiation and host variation hypotheses for singly-inoculated and co-inoculated *Acmispon strigosus*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Single-inoculation	**Log_{10}(Mean nodule size)**		**Log_{10}(Total nodule dry mass)**		**Log_{10}(Total nodule number)**	
	n = 207; Adj. R^2 = 0.40		n = 207; Adj. R^2 = 0.74		n = 208; Adj. R^2 = 0.64	
	df	***F***	**df**	***F***	**df**	***F***
Host population	5, 185.1	**2.6963***	5, 179	**3.8622****	5, 171.1	**4.4561*****
Strain	2, 185.1	**36.8121*****	2, 180.6	**193.8363*****	2, 171.1	**52.2038*****
Fertilization	1, 185	0.9426	1, 180	**167.0683*****	1, 171	**139.025*****
Pop x Strain	10, 185.1	**4.9066*****	10, 179.6	**2.4856****	10, 171.1	**4.9277*****
Pop x Fertilization	.	.	5, 180.1	**3.5277****	5, 171	**3.0261***
Strain x Fertilization	2, 185	**8.3954*****	2, 180.3	**6.0356****	2, 171	**13.2344*****
Pop x Strain x Fert	.	.	.	.	10, 171	**2.1854***
Co-inoculation	**Log_{10}(Mean nodule size)**		**Total nodule dry mass**		**Log_{10}(Total nodule number)**	
	n = 120; Adj. R^2 = 0.46		n = 120; Adj. R^2 = 0.57		n = 120; Adj. R^2 = 0.46	
	df	***F***	**df**	***F***	**df**	***F***
Host population	5, 112	**20.0028*****	5, 107	**2.6878***	5, 112	**15.2086*****
Fertilization	1, 112	**7.3474****	1, 107	**158.7695*****	1, 112	**30.4258*****
Pop x Fertilization	.	.	5, 107	1.4717	.	.

Table 1.7. Mean total plant (root + shoot) dry mass (mg) of *A. strigosus* plants from different populations within each fertilization and *Bradyrhizobium* strain treatment. BMR = Bodega Marine Reserve, UCR = University of California, Riverside, Cla = Bernard Field Station of the Claremont Colleges, Anz = Anza-Borrego Desert State Park, Gri = Griffith Park, Yuc = Burns-Pinyon Ridge Reserve near Yucca Valley.

	Single-Inoculation Plant Mass, mg (SE)				Co-Inoculation Plant Mass, mg (SE)	
Fertilization, Host	Uninoculated	Strain #2	Strain #38	Strain #18	Uninoculated	Co-Inoculated
Unfertilized						
Anz	10.4 (1.3)	11.8 (1.3)	71.3 (6.5)	130.8 (8.8)	15.4 (1.6)	133.2 (9.6)
BMR	6.3 (0.5)	6.0 (0.7)	29.5 (5.6)	86.7 (5.0)	7.6 (0.8)	102.7 (13.6)
Cla	5.4 (0.5)	3.9 (0.4)	50.3 (7.0)	108.7 (13.6)	7.7 (0.7)	107.3 (11.1)
Gri	13.2 (0.9)	11.5 (1.3)	57.6 (14.9)	145.2 (18.5)	19.8 (1.5)	190.8 (11.6)
UCR	7.2 (1.0)	5.0 (0.6)	33.8 (7.0)	83.4 (5.5)	7.5 (0.7)	118.9 (9.7)
Yuc	18.5 (2.7)	15.5 (2.4)	99.1 (9.9)	128.1 (16.4)	40.7 (12.8)	170.7 (21.9)
Fertilized						
Anz	119.0 (10.3)	111.7 (9.7)	208.6 (26.1)	275.1 (13.4)	124.3 (10.2)	301.2 (15.6)
BMR	92.8 (10.7)	82.0 (15.4)	180.6 (17.4)	212.1 (32.0)	105.0 (9.2)	245.4 (17.8)
Cla	91.6 (7.5)	90.6 (7.0)	171.3 (19.8)	240.9 (14.4)	130.7 (12.5)	238.7 (16.2)
Gri	172.7 (13.6)	161.6 (12.4)	207.2 (25.7)	208.8 (16.1)	196.8 (12.7)	341.3 (34.7)
UCR	83.2 (17.5)	61.9 (13.9)	151.3 (17.8)	176.4 (25.8)	106.2 (10.8)	249.8 (13.4)
Yuc	164.6 (10.3)	146.4 (8.9)	262.9 (20.5)	316.3 (23.3)	177.8 (6.4)	336.2 (14.2)

CHAPTER 2

Plant genotype drives responsiveness to soil microbes across diverse soil environments

Abstract

Plants can gain major benefits from interactions with soil microbes, but these effects vary a great deal over environments, time, and individual hosts. To understand and manipulate these benefits, researchers must first resolve the degree to which this variation is mediated by plants or soil microbes. I measured the growth response of three legume plant lines (two *Acmispon strigosus* and one *A. heermannii*) to soil microbial communities sampled from six California field sites where *A. strigosus* occurs. I inoculated plants with either live or sterilized soil slurries to isolate the effect of soil microbes on plant growth. Each soil slurry was also used to inoculate two *A. strigosus* plant lines sourced from the same field site as the slurry, to examine variation in responsiveness of sympatric plant-soil assemblages. Plants were grown in zero-nitrogen substrate to maximize demand for nitrogen fixation by rhizobia. Soil microbial communities varied little in their ability to promote plant growth, but the most effective soil microbes were sourced from acidic soils with low cation-exchange capacity and potentially high conditioning by sympatric plants. Plant growth response to soil microbes varied strongly among *A. strigosus* plant lines, consistent with previous measures of plant genetic variation in responsiveness to pure cultures of rhizobia. Thus, I found support that variation in plant benefits from microbes is largely mediated by plant genotype. The

causes and consequences of plant genotypic variation in responsiveness merit further study.

Introduction

Soil microbes can substantially enhance plant performance, including traits such as growth and nutritional value (Bever *et al.*, 2013; Foyer *et al.*, 2016; X. Zhou *et al.*, 2017), as well as tolerance to pathogens (Yin, 2013; Sui *et al.*, 2018), herbivores (Badri *et al.*, 2013) and abiotic stress (Yang *et al.*, 2009; Shrivastava & Kumar, 2015; Gehring *et al.*, 2017). A key area of interest in plant science is the capacity and mechanisms of plants to adjust their growth in response to the complex communities of microbes they encounter (van der Heijden & Hartmann, 2016; Farid *et al.*, 2017; Sinclair & Nogueira, 2018). Furthermore, a new focus of agronomic research seeks to harness the benefits of plant-microbial interactions to improve the efficiency and sustainability of crop systems (Bakker *et al.*, 2012; Mueller & Sachs, 2015; Busby *et al.*, 2017; Kroll *et al.*, 2017). Whether plant benefits are dictated more by the plant genotype or the soil microbial community will inform the best approaches for harnessing these interactions. Experiments performed with low-diversity inoculations, in which plants only encounter one or a few microbial genotypes, suggest that the plant genotype strongly contributes to plant growth benefits from soil microbes (Haney *et al.*, 2015; Wintermans *et al.*, 2016). However, plant growth responses can also depend on functional traits of the soil microbial community, which can be shaped by top-down effects of plant conditioning

(Landa *et al.*, 2006; Panke-Buisse *et al.*, 2015) and/or bottom-up effects of soil abiotic traits (Rajkumar *et al.*, 2009; Aboudrar *et al.*, 2012); **Fig. 2.1**.

Plant genotypes can vary significantly in the growth benefits they gain from soil microbes (Wintermans *et al.*, 2016); (**Fig. 2.1**, right). One measure of these benefits is 'responsiveness,' defined as the ratio of plant performance with microbes to plant performance without microbes. In symbioses where microbes provide plants with a limiting nutrient (e.g., nitrogen fixation by rhizobia, or phosphorus acquisition by mycorrhizal fungi), responsiveness can depend on general plant nutrient-use traits (i.e., the ability of plants to convert nutrients into growth gains). Under this scenario, plants that vary in responsiveness may vary in their performance with and without microbes (i.e., the numerator and denominator of the responsiveness metric). This form of variation in responsiveness can be seen in many agricultural plants: improved nutrient use efficiency during crop breeding has generally reduced responsiveness to microbes, likely because growth of highly nutrient-use-efficient cultivars is already near-optimal and microbes can only contribute small benefits before plant growth is limited by a different nutrient (Kaeppler *et al.*, 2000; Hammond *et al.*, 2009; Galvan *et al.*, 2011; Martin-Robles *et al.*, 2018). However, responsiveness to microbes can also depend on the degree to which plants regulate microbial services, broadly termed 'host control' (Oono *et al.*, 2009; Sachs *et al.*, 2010b; Haney *et al.*, 2015). For instance, *Arabidopsis* lineages vary in growth benefits based on their genetic capacity to promote populations of beneficial *Pseudomonas* in the rhizosphere (Haney *et al.*, 2015). Similarly, legume genotypes have improved performance when they preferentially form nodules with nitrogen-fixing

rhizobia, as opposed to ineffective rhizobia, during mixed inoculations (Kiers *et al.*, 2007; Simonsen & Stinchcombe, 2014b). Importantly, segregating genetic variation in host control traits can impose selection on the mutualistic services provided by microbes (Heath & Tiffin, 2009), potentially conditioning the soil to have enhanced growth-promoting effects for subsequent plant generations (Denison, 2000; West *et al.*, 2002a; West *et al.*, 2002b; Berg & Smalla, 2009); (**Fig. 2.1**, red dashed arrow).

The composition of the soil microbial community can also affect the net growth benefits a plant receives from microbes (**Fig. 2.1**, left). Different soil microbial communities can have distinct effects on plant growth due to top-down effects of plant conditioning or bottom-up effects of soil abiotic parameters. For instance, long-term cultivation of wheat can build up disease-suppressive soil microbial communities (Landa *et al.*, 2006), and soil microbes conditioned by early- versus late-flowering plants can alter the flowering time of subsequent plants (Panke-Buisse *et al.*, 2015). Thus, plant conditioning can strongly shape the functional properties of the soil microbial community. Soil abiotic parameters can also generate bottom-up effects on the growth benefits plants gain from microbes. For example, serpentine soil conditions select for serpentine-tolerant microbes, which can improve the growth of plants under stressful serpentine conditions (Rajkumar *et al.*, 2009; Aboudrar *et al.*, 2012). In more benign soils, key soil parameters important for microbial community composition are pH (Lauber *et al.*, 2009; Rousk *et al.*, 2010; Li *et al.*, 2016; Fan *et al.*, 2018; Stefan *et al.*, 2018), soil cation exchange capacity (Ding *et al.*, 2017; Lynn *et al.*, 2017; Mapelli *et al.*, 2018), and soil nitrogen (Simonsen *et al.*, 2015; Weese *et al.*, 2015; Soman *et al.*, 2017).

Here, I use the symbiosis between legumes and their natural soil microbial communities to assess 1) the relative contributions of plant genotype and soil source to plant growth benefits from soil microbes and 2) potential drivers of plant-mediated and soil-mediated effects on plant growth (**Fig. 2.1**). I investigate whether plant growth response to microbes depends on soil parameters, including pH, soil cation exchange capacity, and soil nitrogen. I also consider whether soil effects are mediated by the abundance and diversity of focal beneficial taxa (Maherali & Klironomos, 2007; Bever *et al.*, 2013; van der Heijden & Hartmann, 2016) and the potential of local wild plants to condition soils. Although many studies have separately examined the effects of plant genotype and soil factors on plant responses to microbes, no studies to my knowledge have addressed them simultaneously.

I addressed this knowledge gap using the annual legume *Acmispon strigosus*, for which there is data on the soil microbial communities involved (i.e., *Bradyrhizobium* spp., Sachs *et al.*, 2009; Hollowell *et al.*, 2016a; Hollowell *et al.*, 2016b), the services microbes provide (i.e., nitrogen fixation; Sachs *et al.*, 2010a; Regus *et al.*, 2015), the structures in which microbes are housed (i.e., root nodules Regus *et al.*, 2017), and the nature of host control traits (i.e., control over nodule size and sanctions against ineffective rhizobia inside nodules; Sachs *et al.*, 2010b; Regus *et al.*, 2014; Quides *et al.*, 2017; Regus *et al.*, 2017; Wendlandt *et al.*, 2019). Since root nodules eventually senesce and release viable rhizobia back into the soil (Muller *et al.*, 2001), host control traits are the key predicted mechanism of soil conditioning. *A. strigosus* plant lines vary genetically in their growth benefits from individual *Bradyrhizobium* genotypes, and

plants also vary in host control over root nodule size (Wendlandt *et al*., 2019). However, using clonal inocula or simple *Bradyrhizobium* communities to assess plant benefits offers a reductionist view of how plants respond to complex microbial communities in varied abiotic conditions. Since *A. strigosus* thrives in soils that vary greatly in chemical characteristics (Regus *et al.*, 2017) and *Bradyrhizobium* diversity (Hollowell *et al.*, 2016a; Hollowell *et al.*, 2016b), these factors can also influence the benefits plants obtain from microbes in more natural settings.

I generated soil inocula from six *A. strigosus* field sites that vary in abiotic and biotic traits (Hollowell *et al.*, 2016a; Regus *et al.*, 2017). To isolate the effect of soil microbes on plant growth, I inoculated plants with either live or sterilized soil slurries. First, I conducted a full-factorial 'universal experiment' in which each soil inoculum was tested on axenic seedlings of three plant lines, allowing us to compare contributions of soil source and plant line to plant responsiveness. Plants were supplied with essential mineral nutrients but were not fertilized with nitrogen so that the rhizobial service of nitrogen fixation would be a limiting factor for plant growth in the live soil inocula treatments. I tested for variation in plant responsiveness to microbes due to plant traits (i.e., performance with microbes, performance without microbes, and size of root nodules) and due to soil traits (pH, cation exchange capacity, total nitrogen, the abundance and diversity of *Bradyrhizobium*, and the predicted level of conditioning by wild *Acmispon* hosts). Second, I conducted a 'sympatric experiment' in which each soil inoculum was tested on plant lines from the same population and for which I had data on plant responsiveness to simple *Bradyrhizobium* communities and investment into nodule

size (Wendlandt *et al.*, 2019). My goal was to test if these plant traits—previously measured with simple, characterized inocula—were robust under the more complex scenario of exposure to a whole soil microbial community. Thus, the sympatric experiment provided another opportunity to test the relative importance of the plant genotype versus the soil microbial community for shaping plant benefits from soil microbes. This work contributes to a more predictive understanding of plant performance in diverse microbial environments (Friesen *et al.*, 2011).

Materials and Methods

Acmispon plant lines

A. strigosus seeds were collected between 2005 and 2011 from ripe fruits at six natural field sites in California, including Anza-Borrego Desert State Park (Anz), Bodega Marine Reserve (BMR), Griffith Park (Gri), Pioneertown Mountains Preserve near Yucca Valley (Yuc), Bernard Field Station of the Claremont Colleges (Cla), and the Box Springs Reserve of the University of California, Riverside (UCR). Soils from these field sites were classified either as entisols (young mineral soils without distinct horizons; i.e., Anz, Yuc, Cla, and UCR), mollisols (fertile soils with organic-rich upper horizons; i.e., Gri), or unclassified 'dune land' (i.e., BMR; likely also an entisol based on sandy texture); (Soil Survey Staff, 2018). To generate inbred seed sets with minimal maternal effects, I raised plants from each field site in an insect-free glasshouse for one or two generations. I generated 14 *A. strigosus* inbred lines (2-3 per field site) and genotyped them at two loci including *nrITS* (Allan & Porter, 2000) and *CNGC5* (Maureira-Butler *et*

al., 2008). Each inbred plant line was derived from an independent wild seed except for the Yuc lines, which were derived in error from the same wild seed ancestor. Cla and Yuc plant lines could not be differentiated genetically within their respective field sites, whereas plant lines from the other field sites were genetically distinct from each other (**Table 2.1**). I also used outbred seeds of the California native perennial *A. heermannii* (S&S Seeds, Carpinteria, CA, USA), which is broadly sympatric to *A. strigosus* (Calflora) and interacts with a similar community of *Bradyrhizobium* (Sachs *et al*., 2009).

Preparation of soil inocula

I collected *c.* 20 soil cores (13 cm deep, 5.5 cm wide) from each of the above field sites between 27 February and 2 March 2015 (**Table 2.1**). Soil cores were spaced *c*. 1 m apart and taken within 20 cm of live *A. strigosus* plants to maximize the chance of sampling microbial communities conditioned by this plant species. The soil corer was sterilized between field sites by removing visible soil with a wet sponge, spraying the corer with ethanol, and flaming. Soil cores were transported back to the lab in new plastic bags and stored at room temperature until inoculum preparation on 8 March 2015. Bulked soil cores from each field site were passed through a flame-sterilized 2mm sieve, combined with 1 ml sterile water per gram of sieved soil, shaken vigorously to form a slurry, and filtered through eight layers of sterile cheesecloth (Unkovich, 1998). Soil slurries from UCR, Cla, and Gri initially clogged the filters, so I let the slurries stand *c.* 20 minutes and then transferred their supernatants to separate bottles. The liquid fractions of soil slurries from all six field sites were allowed to settle overnight at room

temperature. The top 60-80% of each fraction was transferred to a new bottle, mixed well, and split into two volumes: one was used as a 'live' soil inoculum, and the other was autoclave-sterilized, cooled to room temperature, and used as a 'sterilized' soil inoculum. Thus, I prepared 12 soil inocula (live and sterilized inocula from six field sites).

Inoculation experiments

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

Two inoculation experiments (the 'universal' and 'sympatric' experiments) were performed concurrently in the same glasshouse. In the universal experiment, three plant lines (Anz13.04, Cla12.04, and *A. heermannii*) were treated with all 12 soil inocula in a full-factorial design (three plant lines x 12 inocula x 10 replicates = 360 plants). The universal *A. strigosus* plant lines were chosen to represent divergent evolutionary histories: Anz13.04 and Cla12.04 were from very low and high soil nitrogen regimes,

respectively (Regus *et al.*, 2017). *A. heermannii* plants were used to assess variation in plant responsiveness not due to conditioning by wild conspecifics, since field sites were sampled only based on the presence of *A. strigosus*. In the sympatric experiment, two plant lines from each field site were treated with live and sterilized soil inocula from the same field site (two plant lines per locale x two inocula x six field sites x 10 plant replicates = 240 plants; see **Table 2.1** for specific plant lines used). Replication of treatments using Anz10.01, Anz13.04, and Gri01.13 plant lines was reduced due to high seedling mortality, leaving a total of 567 plants in the experiments.

Sets of size-matched axenic seedlings of each plant line were randomly assigned to inoculum treatments. Within blocks, plants in separate conetainers were clustered by inoculum treatment, with five plants per inoculum cluster (two sympatric plant lines, three universal plants). Plant positions within inoculum clusters were not randomized; *A. heermannii* was always the center plant of the cluster so that the four surrounding *A. strigosus* plants all experienced a similar micro-environment (i.e., on the edge of a cluster). Inoculum clusters were randomly assigned to grid positions within blocks with the constraint that live and sterilized soil inocula alternated to reduce the chance of cross-contamination among live soil inocula. Inoculum clusters were separated by at least 12 cm and inoculum positions were randomized independently for each block.

The overhead misters temporarily failed before harvest on 23 April 2015 (6.5 weeks post-inoculation, wpi) and many plants wilted. Dead shoot tissue was collected to prevent its being lost during transfer to the lab, and this tissue was pooled with shoots collected at the time of harvest (4-14 May 2015, 8-9.5 wpi). Twenty-two plants had shoot

portions collected early and five plants had their entire shoots collected early. At harvest, plants were removed from pots, washed free of sand, and dissected into root, shoot, and nodule portions. Nodules were counted and photographed. All tissues were oven-dried (>4 days, 60°C) and weighed.

Measurement of plant traits

I measured five parameters on plants treated with live soil inocula. i) Plant responsiveness to the soil microbial community was measured as the total plant dry mass (roots + shoots) of the live-inoculated plant divided by the total dry mass of its size-matched control plant treated with sterilized inoculum. Responsiveness greater than 1 indicates that live soil inoculum improved plant growth relative to the sterilized soil inoculum (i.e., that soil microbes had a net positive effect on plant biomass). The remaining four parameters focused on effects of symbiosis with rhizobia. ii) Red nodule frequency was quantified as the proportion of nodules visually scored as either red or pink as a proxy for symbiotic nitrogen fixation, since the red colored protein leghemoglobin is expressed in actively fixing nodules (Virtanen, 1947; Tajima *et al.*, 2007). Red nodule frequency was averaged across three independent blind observers who used a scoring guide to examine nodule photographs taken at the time of harvest (**Fig. 2.2**). iii) Mean nodule size was calculated as total dry mass of nodules divided by total number of nodules. This index is a proxy for average plant investment per rhizobial infection and can predict the number of viable rhizobia released from the nodule when it

senesces (Wendlandt *et al.*, 2019). I also analyzed iv) total nodule dry mass and v) total number of nodules separately to understand drivers of variation in mean nodule size.

Measurement of soil traits

To characterize soil abiotic metrics (pH, cation exchange capacity, and nitrogen), I sent samples of each sieved soil for standard nutrient analysis at A&L Western Laboratories (Modesto, CA, USA) on 30 March 2015. Samples of live and sterilized soil inocula were stored at 4˚C after preparation and sent for standard nutrient analysis at Soil and Plant Laboratory, Inc. (Anaheim, CA, USA) on 30 March 2015. To estimate the density of colony-forming units (CFU) in each live inoculum, samples were taken immediately after preparation and spread-plated in five dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) onto three replicate glucose-based rhizobium-defined medium agar plates containing cycloheximide (GRDM; (Sullivan *et al.*, 1996). GRDM provides specific growth conditions for rhizobia and related taxa, so these data provided a rough estimate of total rhizobial abundance. Colonies that formed within 11 days of plating were counted and plates containing 30-300 colonies were used to calculate CFU ml^{-1}. Undiluted sterilized inocula were plated onto three replica GRDM agar plates to check for microbial growth.

To characterize soil biotic metrics, I used CFU ml^{-1} of the inocula as well as published data on *Bradyrhizobium* haplotype richness, Simpson's diversity and evenness, and proportional abundance of *Bradyrhizobium canariense,* which is a widespread *Bradyrhizobium* species (Hollowell *et al.*, 2016a) that includes strains that are highly beneficial to *A. strigosus (Sachs et al.*, 2010b). Previous work on these six field sites did

not sample them evenly (Hollowell *et al.*, 2016a), so isolates from well-sampled field sites were randomly rarified to a constant number before analysis. To predict plant contributions to soil biotic metrics via soil conditioning, I used mean nodule size of plants treated with sympatric soil inocula (I also tested the component traits, total nodule mass and total nodule number, as proxies of soil conditioning).

Data analysis

Statistical analyses were performed in JMP Pro 13.0.0 (SAS Institute Inc., Cary, NC, USA). Dependent variables were log- or square root-transformed as needed to improve normality (see **Table 2.2** for specific transformations). Means discussed in the text are back-transformed (if applicable) from raw means and presented alongside a 95% confidence interval for the mean. My statistical approach used general linear mixed models (i.e., GLMM; Fit Model Platform; Standard Least Squares personality; REML method) with either mixed effects or all random effects (i.e., variance component models). Block was included as a random effect in all models. Significant differences among fixed effect levels were assessed with pairwise *t*-tests (Tukey's HSD) of least squares means.

I investigated nitrogen limitation of plant growth by examining 1) whether biomass of plants treated with sterilized inocula (i.e., nutrients only) varied with the nitrogen content of the inoculum, 2) whether plants that formed nodules had greater biomass than plants that failed to form nodules, and 3) whether the estimated amount of nitrogen fixation on the plant (i.e., red nodule frequency) predicted plant growth.

I tested for plant genetic differences in responsiveness to microbes (and investment into nodules) by examining 1) how much of the variation in these metrics on universal plant lines was explained by plant line versus soil source and their interaction in variance component models, and 2) how well the variation in these metrics on sympatric plant lines aligned with previous measurements using pure cultures of *Bradyrhizobium* (Wendlandt *et al.*, 2019). In that study, plant responsiveness to a mixture of three strains was greatest for BMR, UCR, and Cla plants and least for Gri and Yuc plants, and plant investment into individual nodules (i.e., mean nodule size) was greater for BMR and UCR hosts than plant lines from other field sites (Wendlandt *et al*., 2019). To understand plant-mediated drivers of responsiveness, I examined whether responsiveness was structured by plant performance with microbes, plant performance without microbes, or plant investment into nodules (i.e., mean nodule size).

I tested for effects of soil source on plant responsiveness to microbes. If responsiveness of universal plant lines varied among soil inocula (either globally or on specific plant lines), I tested whether plant responsiveness was correlated with various soil metrics or predicted levels of soil conditioning (see 'Measurement of soil traits'), similar to the approach by (Lynn *et al.*, 2017) for identifying relationships between soil parameters and microbial diversity. I used Spearman's rank-order correlation, since I did not necessarily predict a linear relationship between soil metrics and growth-promoting ability.

Results

Soil metrics vary among field sites

Abiotic soil parameters varied among the sampled field sites (**Table 2.3**). Soil pH ranged from moderately acid (pH 5.8, BMR) to moderately alkaline (pH 7.9, Anz), cation exchange capacity ranged from low (3.2 meq $100g^{-1}$, BMR) to moderately high (24.1 meq $100g^{-1}$, Gri; Hazelton & Murphy, 2016), and analysis of soil nitrogen indicated that BMR, Yuc, and Anz soils were nitrogen-poor (129-252 ppm total nitrogen) compared to Gri, Cla, and UCR soils (666-1491 ppm total nitrogen; **Table 2.3**), consistent with previous analyses (Regus *et al.*, 2017). Preparation of live inocula from sieved soil significantly reduced total nitrogen content by a mean of 90% (SD = 5%; paired-samples $t = 2.7421$, df = 5, $P = 0.0407$), but sterilization did not significantly change total nitrogen content relative to live inocula (paired-samples $t = -1.5854$, df = 5, $P = 0.1737$).

Live soil inocula from each of the six field sites formed abundant colonies on GRDM plates, whereas sterilized inocula failed to form colonies on GRDM plates (except for one colony from the BMR sterilized inoculum). CFU ml^{-1} varied significantly among the six live soil inocula ($F_{5,16} = 74.2176$, $P < 0.0001$), ranging from 6.4×10^5 CFU ml^{-1} (Anz) to 2.7×10^7 CFU ml^{-1} (Gri; **Table 2.3**). *Bradyrhizobium* diversity was greatest in Cla soil and lowest in Gri soil, and the focal beneficial taxon *B. canariense* was most abundant in UCR and BMR soils and least abundant in Gri soil (Hollowell *et al.*, 2016a; **Table 2.3**).

Nitrogen limited plant growth

No plants treated with sterilized inocula formed root nodules. The universal plant lines treated with sterilized inocula had the greatest plant biomass with inocula from high-nitrogen sites (Gri = 22.60 mg [95% confidence interval = 18.32-26.87 mg]; Cla = 22.16 mg [17.75-26.56 mg]; UCR = 21.52 mg [17.55-25.49 mg]) and the smallest biomass with inocula from low-nitrogen sites (Anz = 18.93 mg [15.05-22.82 mg]; Yuc = 18.67 mg [15.53-21.81 mg]; BMR = 17.64 mg [14.13-21.15 mg]), consistent with nitrogen being the growth-limiting nutrient in this experiment. Live soil inocula from BMR, Cla, Gri, UCR, and Yuc formed nodules on all inoculated plants and significantly improved plant growth relative to plants treated with sterilized inocula (responsiveness > 1x; all $P < 0.0001$). In contrast, live soil inoculum from Anz failed to nodulate plants except for one nodule on one Anz13.04 plant, and there were no detectable growth benefits from live Anz inoculum (responsiveness ~ 1x; $t = 0.4173$, df = 47, $P = 0.6784$). Plants that formed nodules had growth benefits proportional to the number of nodules predicted to be fixing nitrogen (i.e., red nodule frequency on nodulated plants was positively correlated with plant responsiveness to microbes; r = 0.61, $P < 0.0001$; **Fig. 2.3**). These data suggest that the experimental setup maximized plant nitrogen demand such that plant growth benefits from live inocula was primarily determined by symbiosis with nitrogen-fixing rhizobia. To focus on plant benefits from root nodulation by rhizobia, I excluded plants treated with the non-nodule-forming Anz inoculum from subsequent analyses.

Plant genotypes vary in responsiveness to microbes

In the universal experiment, 35.8% of the variance in plant responsiveness to microbes was driven by plant line, with smaller contributions from soil source (0.7%) and the plant line x soil source interaction (6.2%; **Table 2.2**). Mean responsiveness was greatest for Cla12.04 plants (9.32x [8.06-10.78x]), intermediate for *A. heermannii* (5.57x [4.98-6.23x]), and smallest for Anz13.04 plants (4.53x [3.91-5.25x]), resulting in a more than two-fold difference in mean growth benefits between the two *A. strigosus* lines (**Fig. 2.4a**). In the sympatric experiment, plant responsiveness varied significantly among field sites (**Fig. 2.4b**, **Table 2.2**) with the greatest growth benefits occurring for Cla (9.29x [7.57-11.20x]) and BMR (8.18x [5.82-10.93x]), followed by UCR (7.57x [5.46-10.03x]), Gri (4.99x [3.34-6.97x], and Yuc (4.64x [3.63-5.78x]). Variation in plant responsiveness was consistent with results from Wendlandt *et al*. (2019), suggesting that there is robust genetic variation within *A. strigosus* for responsiveness to soil microbes, specifically *Bradyrhizobium*.

I further examined whether variation in responsiveness was driven largely by plant performance in the presence or absence of microbes. I found that *A. strigosus* plant lines treated with live soil inocula did not differ in total dry biomass (Cla12.04 = 98.30 mg [89.21-107.40 mg]; Anz13.04 = 100.60 mg [90.13-111.07 mg]) or mean nodule size (Cla12.04 = 0.32 mg [0.29-0.35 mg]; Anz13.04 = 0.31 mg [0.27-0.35 mg]; **Fig. 2.5a**, **Table 2.2**). Conversely, plant total dry biomass differed between the *A. strigosus* lines when plants were treated with sterilized soil inocula (Anz13.04 = 21.24 mg [19.24-23.24 mg]; Cla12.04 = 10.45 mg [9.58-11.33 mg]; **Table 2.2**), indicating that variation in

responsiveness is largely driven due to some hosts having relatively poor performance in the absence of microbes. This was especially the case in the contrast between high responsiveness of the Cla12.04 line relative to the Anz13.04.

The perennial *A. heermannii* had greater total dry biomass than the annual *A. strigosus* lines in both the sterilized treatment (30.00 mg [27.94-32.06 mg]) and the live treatment (170.64 mg [156.52-184.76 mg]; **Table 2.2**). *A. heermannii* had similar nodule size to *A. strigosus* (0.29 mg [0.27-0.32 mg]; **Fig. 2.5a**, **Table 2.2**). Total nodule number and total nodule mass varied among the three lines but was consistent with species differences, with the perennial *A. heermannii* exceeding the annual *A. strigosus* lines for both total nodule mass (*A. heermannii* = 14.07 mg [12.72-15.41 mg]; Cla12.04 = 11.04 mg [9.89-12.18 mg]; Anz13.04 = 10.62 mg [9.38-11.87 mg]; **Fig. 2.6a**, **Table 2.2**) and total nodule number (*A. heermannii* = 45.2 nodules [40.4-50.6]; Cla12.04 = 32.4 nodules [29.3-35.8]; Anz13.04 = 31.5 nodules [28.7-34.5]; **Fig. 2.7a**, **Table 2.2**).

Soil conditioning is correlated with soil effects on plant benefits

In the universal experiment, plant benefits from soil microbes varied significantly among soil sources for only one plant line, Anz13.04 (**Fig. 2.4a**, **Table 2.2**). For Anz13.04 hosts, responsiveness was greatest with BMR inoculum (7.35x [5.33-10.15x]), followed by UCR (4.74x [3.38-6.65x]), Cla (4.15x [2.88-5.98x]), Yuc (3.73x [2.69-5.18x]), and Gri inocula (3.55x [2.64-4.76x]). I tested different soil metrics as predictors of Anz13.04 responsiveness. None of the abiotic metrics were significantly correlated

with plant benefits from microbes, but the biotic metrics, CFU per ml of the inocula, was negatively correlated with plant benefits (**Table 2.3**).

I then examined whether variation among soils in their growth-promoting effects was due to predicted soil conditioning of the different soils. In the sympatric experiment, the largest nodules were formed by BMR plants (0.46 mg [0.32-0.65 mg]) and UCR plants (0.41 mg [0.33-0.49 mg]), with smaller nodules formed by Cla (0.31 mg [0.26-0.37 mg]), Gri (0.29 mg [0.20-0.42 mg]), and Yuc plants (0.23 mg [0.18-0.29 mg]; **Fig. 2.5b**, **Table 2.2**), corroborating the genetic variation in nodule size measured by Wendlandt and colleagues (2019) and suggesting that BMR and UCR soils were more highly conditioned than the other soils. There was a significant positive correlation between plant responsiveness and predicted soil conditioning in terms of mean nodule size (**Table 2.3**). This correlation was not significant when I estimated soil conditioning as total nodule number or total nodule mass (**Table 2.3**). Variation among host lines in mean nodule size was primarily due to variation in total nodule number, which varied two-fold between the minimum (BMR = 21.0 nodules [17.1-24.8]) and maximum (Cla = 41.5 nodules [33.6-49.4]; **Fig. 2.7b**, **Table 2.2**), whereas total nodule mass varied only slightly between the minimum (Yuc = 8.97 mg [7.33-10.60 mg]) and maximum (BMR = 11.19 mg [8.49-13.88 mg]; **Fig. 2.6b**, **Table 2.2**).

Discussion

I found evidence for the primacy of genetically-determined plant traits in shaping plant interactions with soil microbes. Specifically, plant responsiveness to microbes and

investment into rhizobial symbionts varied robustly among genotypes of the legume *A. strigosus*, consistent with previous measurements where plants were inoculated with clonal *Bradyrhizobium* cultures (Wendlandt *et al.*, 2019). This pattern was surprising since plants with high responsiveness to microbes can become disproportionally targeted by pathogens or herbivores when they are grown in diverse biotic conditions (Simonsen & Stinchcombe, 2014a; Haney *et al.*, 2018), with the net effect of reducing observed plant genetic variation in responsiveness to microbes. Nevertheless, I uncovered segregating plant variation for both responsiveness to microbes and investment into nodules, consistent with my previous work (Wendlandt *et al.*, 2019) as well as studies using soybean (Kiers *et al.*, 2007) and *Arabidopsis* (Haney *et al.*, 2015). These results highlight the importance of the plant genotype in structuring plant benefits obtained from soil microbes and should prompt further research into the causes and consequences of plant responsiveness to soil microbes.

A dominant driver of variation in responsiveness in the *Acmispon-Bradyrhizobium* system was how plants performed in the absence of microbes. For instance, the highly responsive plant line Cla12.04 had worse performance than the poorly responsive plant line Anz13.04 in microbe-free conditions, but similar performance to Anz13.04 in microbe-rich conditions. Plants treated with sterilized inocula had almost no source of nitrogen except initial seed resources, so the two-fold difference in plant size between Anz13.04 and Cla12.04 was likely due to genetic differences in seed provisioning rather than nutrient use efficiency (mean individual seed mass (± 1 SD) produced by greenhouse-raised plants: Anz = 1.5 ± 0.3 mg; Cla = 0.8 ±

0.1 mg). However, the larger growth gains of Cla12.04 with live inocula, without notable differences from Anz13.04 in symbiosis traits, suggests that Cla responsiveness is tied to nutrient use efficiency rather than investment into symbiotic services. Cla plants also exceed Anz plants in their growth response to a purely mineral source of nitrogen (Wendlandt *et al.*, 2019), supporting the idea that Cla plants are very efficient for nutrient use with both mineral and symbiotic sources of nitrogen. My results corroborate reports of nutrient use efficiency driving variation in responsiveness to microbes in a variety of crop plants (Kaeppler *et al.*, 2000; Galvan *et al.*, 2011), which suggest that responsiveness to microbes is shaped mainly by plant traits like nutrient use efficiency that are fairly robust to changes in the soil microbiota, rather than traits specific to symbiosis. In the field of crop breeding, responsiveness to microbes is not considered a useful trait compared to plant performance, since crop plants almost always encounter compatible microbes (Sawers *et al.*, 2010). However, if genes underlying responsiveness are also important for nutrient use efficiency, introgression of such genes into highly productive cultivar backgrounds could potentially increase plant performance in field conditions.

My data raise the question of whether legume investment into rhizobia (measured here as mean nodule size) can improve the ability of soils to promote plant growth, due to larger nodules releasing more rhizobia back into the soil after nodules senesce. In natural populations, investment into nodules might increase a legume's inclusive fitness by giving kin a better chance of encountering a compatible symbiont at the sensitive early seedling stage. Theory in the evolutionary ecology of mutualisms supports the idea that

plants imposing selection for microbial services will improve the quality of those services in their associated microbial communities (Steidinger & Bever, 2014). Furthermore, variation in host control traits among plant genotypes is predicted to generate parallel variation in the growth-promoting ability of soil communities (Heath & Stinchcombe, 2013). Here, soils with the greatest ability to promote plant growth (i.e. BMR, UCR) were isolated from field sites where sympatric plants formed the largest nodules. Since I sampled soils near wild *A. strigosus* plants, the soil samples were likely conditioned by the root traits of plants genetically similar to those I used in the sympatric experiment, which could explain why BMR and UCR soils were highly beneficial for Anz13.04 plants. As an agricultural application, it may be worthwhile to breed legume cover crops for enhanced investment into nodules in order to boost rhizobial populations for subsequent legume crops. Although cover crops have long been used to replenish plant nutrients and preserve soil structure while fields lie fallow, only recently have researchers investigated how symbioses between cover crops and microbes influence beneficial soil microbial processes (Cui *et al.*, 2015; Zhou *et al.*, 2017; Manici *et al.*, 2018). Few studies have integrated cover crop legacy effects into the broader field of plant-soil feedbacks (Vukicevich *et al.*, 2016; Lepinay *et al.*, 2018), suggesting that this could be an exciting new avenue of research.

Another finding from this study was that variation in soil growth-promoting ability occurred for just one of the universal plant lines. Specifically, soil source only affected the growth of the *A. strigosus* plant line with the lowest average responsiveness to microbes. One explanation could be that soil effects are more visible on plant

genotypes with intrinsically less-efficient use of nutrients (i.e., on plants where there is still room for growth improvement before growth becomes limited by something else). This would explain why the highly responsive Cla12.04 line did not vary in responsiveness to different soils; responsiveness for Cla12.04 across all soil sources was already greater than the greatest responsiveness achieved by Anz13.04 hosts and might have been limited by availability of a different nutrient. In addition, the consistently moderate responsiveness of *A. heermannii* with all soil inocula could be a consequence of the soils having been sampled near wild *A. strigosus*, rather than *A. heermannii*. Although these species form nodules with similar *Bradyrhizobium* spp. (Sachs *et al.*, 2009), it is possible that they differ in their preference for particular rhizobial genotypes, as has been observed in *Acacia* (Vuong *et al.*, 2017).

I did not detect strong correlations between soil metrics and the ability of soils to promote plant growth. This could be due to low power to detect these effects, especially since one soil (Anz) failed to nodulate plants. Nevertheless, some interesting trends emerged and merit further study. Soils with the highest ability to promote plant growth (i.e., BMR, UCR) tended to be relatively acidic, and have moderate rhizobial content and low cation-exchange capacity (CEC). Rhizobial content may be the key trait here, since abundance and diversity of many soil bacteria is negatively associated with soil acidity (Lauber *et al.*, 2009; Rousk *et al.*, 2010) and low CEC (Lynn *et al.*, 2017). All other things being equal, low rhizobial content of soils could improve the efficiency of host selection for particular rhizobial genotypes, since this selection would take place in an environment with fewer competing pressures on microbial services. Intriguingly,

although acidic soils in general disfavor bacteria, *Bradyrhizobium* can achieve greater abundances in acidic than neutral soils (Fan *et al.*, 2018), potentially increasing the tractability of these soils to conditioning by the host plant. Lastly, there are mixed reports of how soil nitrogen affects soil microbial community structure and function. In some cases, no effects of soil nitrogen are found (Lynn *et al.*, 2017) or soil nitrogen has a positive effect on microbial populations (Li *et al.*, 2016). Fertilizer application of nitrogen can also change microbial community substrate use (Soman *et al.*, 2017) and the benefits that rhizobia provide plants (Simonsen *et al.*, 2015; Weese *et al.*, 2015). The high soil nitrogen I measured in soils from Gri and Cla (and UCR, to a lesser extent) was not associated with changes in soil growth-promoting ability, contrary to the results from (Weese *et al.*, 2015). Thus, my results confirm some aspects of previous work on how soil metrics are related to microbial community function, but further work is needed to elucidate these effects and disentangle them from effects of soil conditioning by plants.

Overall, plant genotype was a stronger determinant of plant benefits than the particular community of soil microbes a plant encountered, suggesting that plant benefits from microbes can respond to natural or human selection. Since plant responsiveness appears to be more tied to nutrient-use efficiency than investment into microbial services, selection for improved responsiveness to microbes could have broad effects on plant ecology by increasing plant performance even in environments lacking compatible symbionts. Compared to plant genotype, soil microbial communities from different field sites contributed relatively little variation to plant growth, but this variation was most correlated with the predicted strength of soil conditioning by wild plant hosts. Thus, even

variation in responsiveness that I attributed to soils may be an indirect function of the plant genotype. This research highlights the importance of plant genetically-determined traits in shaping plant responsiveness to soil microbes.

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Fig. 2.1. Conceptual model of how soil and plant traits independently or synergistically drive plant benefits from soil microbes. Plant benefits could be driven largely by characteristics of the soil (left), whereby the abiotic and biotic characteristics of the soil determine plant benefits. Plant benefits could also be driven largely by plant traits (right), such as nutrient use efficiency or host control over microbial services. Soil and plant drivers of plant growth benefits could be linked (red dashed arrow) if genetically-determined plant traits (i.e., host control traits), improve the growth-promoting capacity of the soil biota.

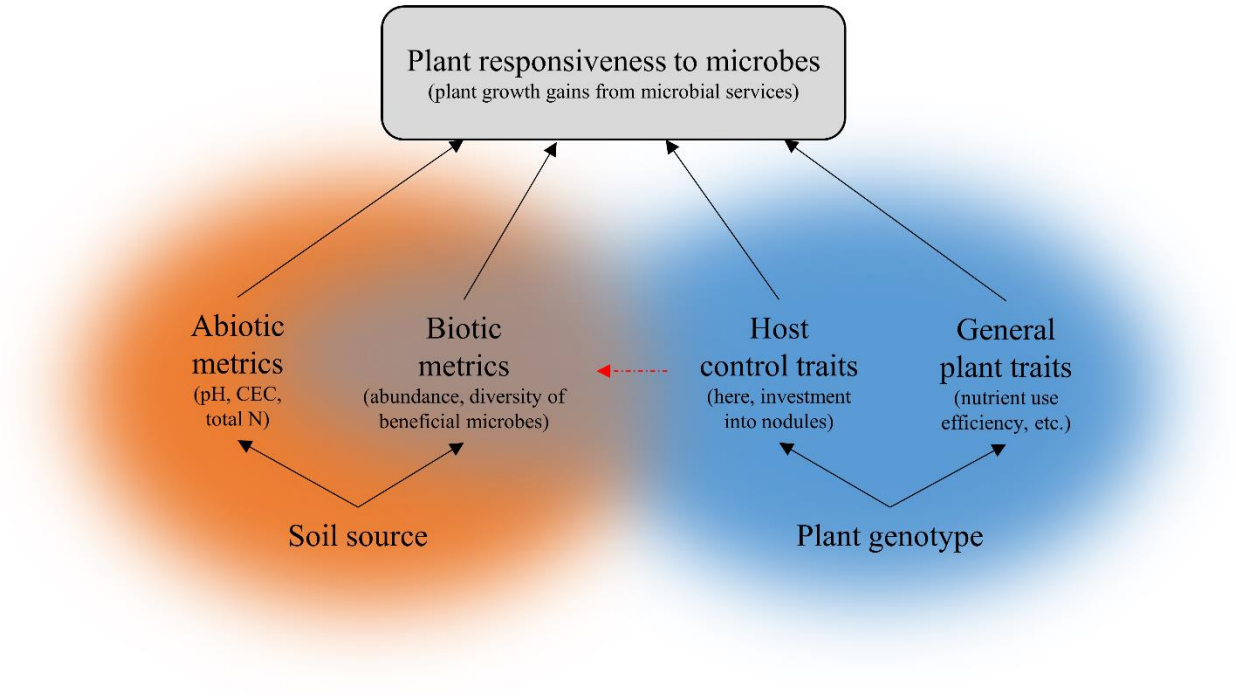

Fig. 2.2. Scoring guide used by three independent observers to assess nodule color from photographs taken at the time of plant harvest. Nodules scored as 'Red/Pink' were used to calculate red nodule frequency for each plant.

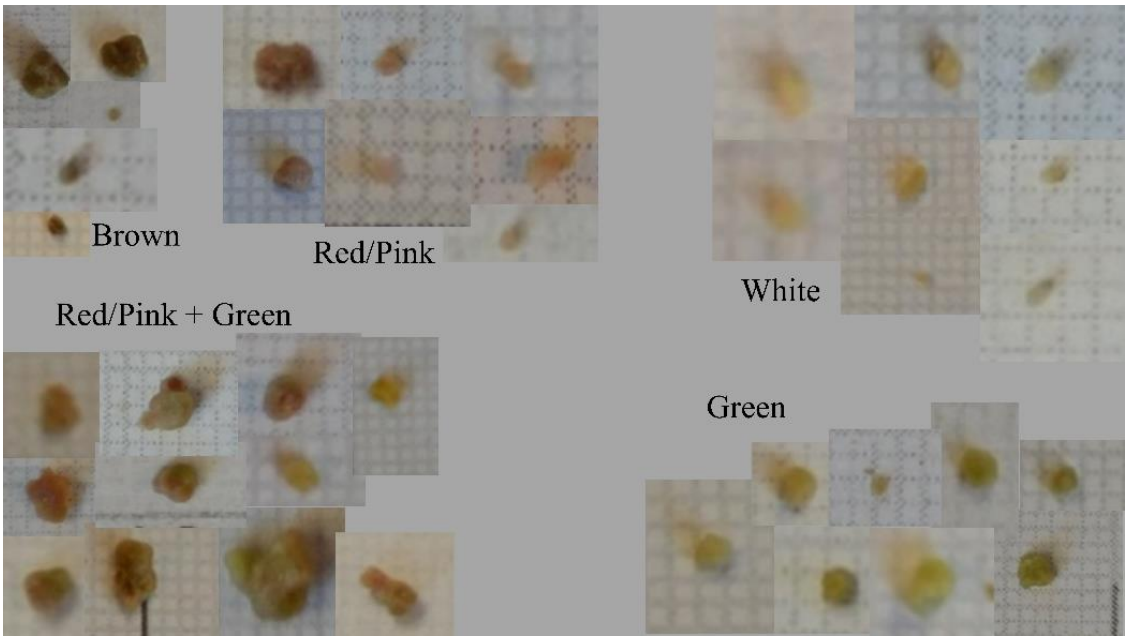

Fig. 2.3. Correlation between plant responsiveness to microbes and red nodule frequency (i.e., nodules scored as 'Red/Pink' in **Fig. 2.2**; r = 0.61, $P < 0.0001$), pooling all nodulated plants in the study. The frequency of nodules scored as 'Red/Pink + Green' (**Fig. 2.2**) was not significantly correlated with plant responsiveness (r = 0.06, $P = 0.3920$).

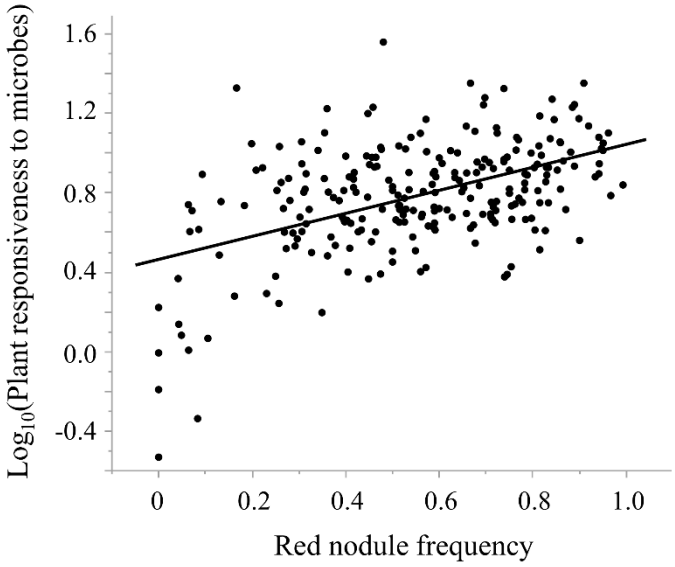

Fig. 2.4. Plant responsiveness to microbes for (a) universal plant lines and (b) sympatric *A. strigosus* plant lines treated with live soil inocula. Upper x-axis labels indicate soil inoculum source; lower x-axis labels indicate plant line. Box plots are color-coded by field site source of the plant line. Statistical analyses were performed separately for universal and sympatric plant lines. Different letters indicate significant differences (a) among soil source / plant line combinations and (b) among soil sources (**Table 2.2**).

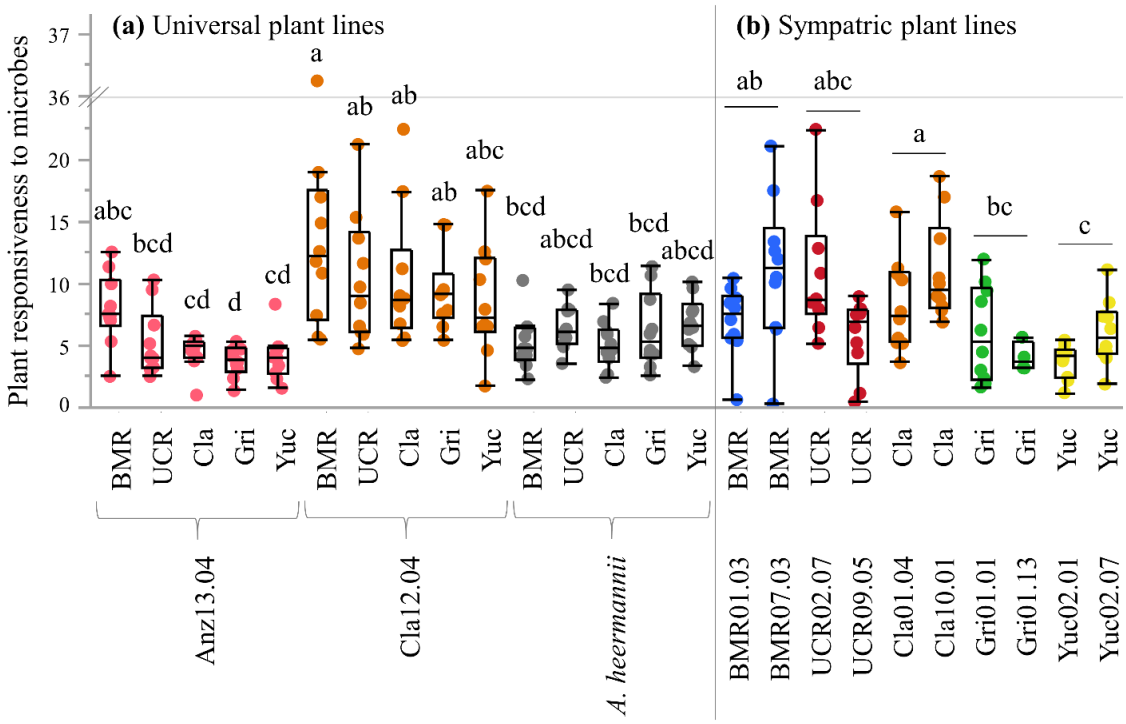

Fig. 2.5. Mean nodule size of (a) universal plant lines and (b) sympatric *A. strigosus* plant lines treated with live soil inocula. Upper x-axis labels indicate soil inoculum source; lower x-axis labels indicate plant line. Box plots are color-coded by field site source of the plant line. Statistical analyses were performed separately for universal and sympatric plant lines. Different letters indicate significant differences (a) among soil source / plant line combinations and (b) among soil sources (**Table 2.2**).

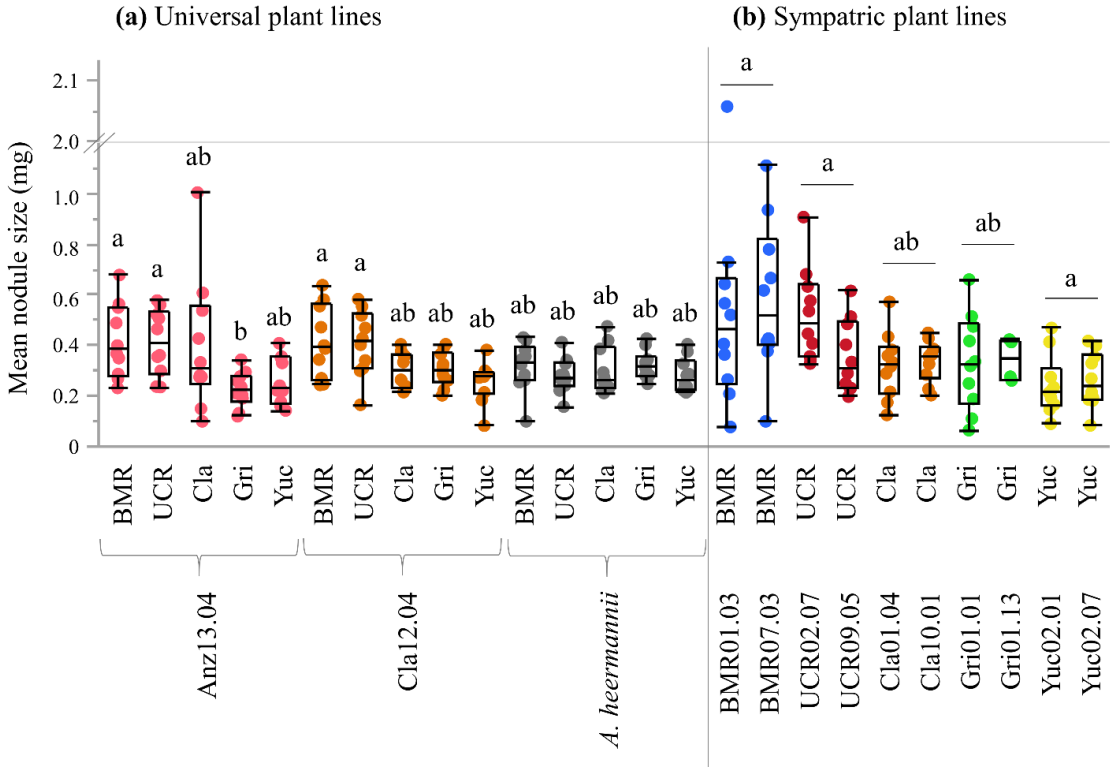

Fig. 2.6. Total nodule dry mass per plant of (a) universal plant lines and (b) sympatric *A. strigosus* plant lines treated with live soil inocula. Upper x-axis labels indicate soil inoculum source; lower x-axis labels indicate plant line. Box plots are color-coded by field site source of the plant line. Statistical analyses were performed separately for universal and sympatric plant lines. Different letters indicate significant differences (a) among plant lines and (b) among soil sources (**Table 2.2**).

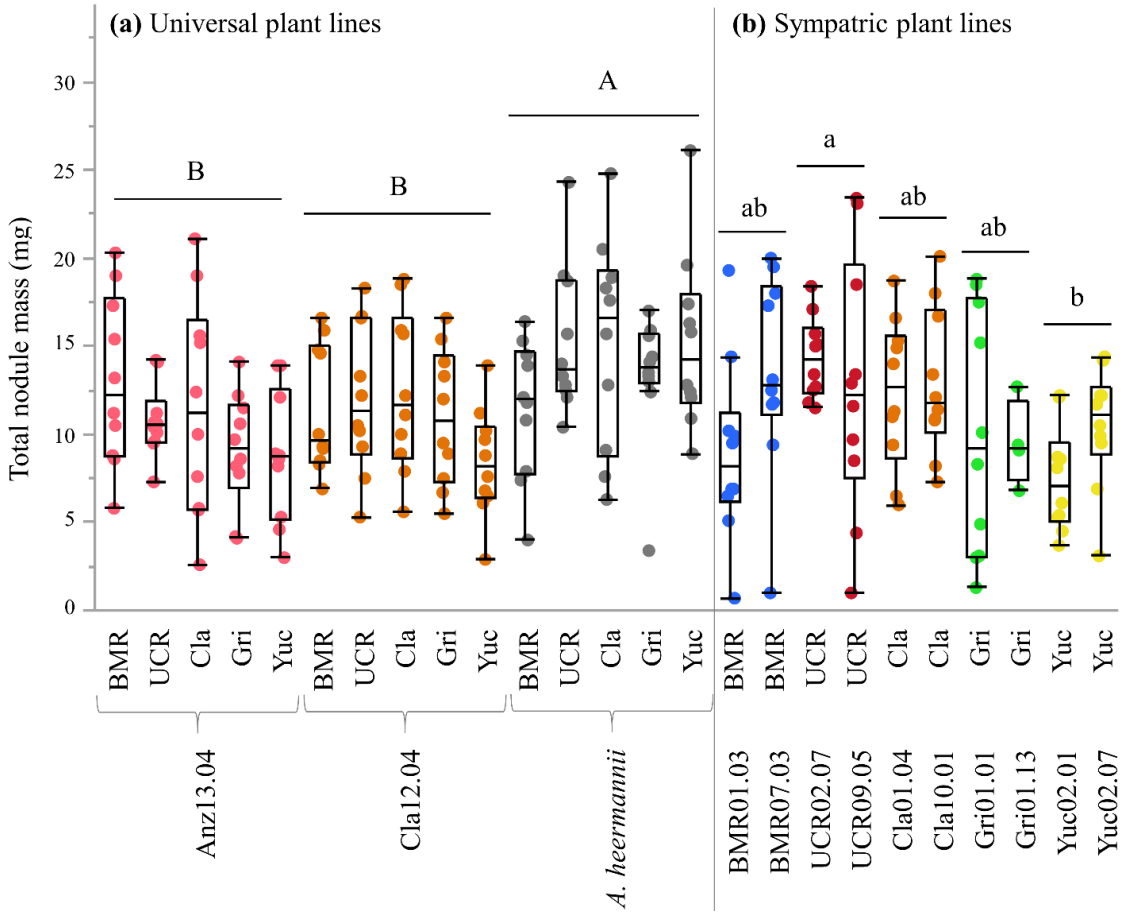

Fig. 2.7. Total nodule number per plant of (a) universal plant lines and (b) sympatric *A. strigosus* plant lines treated with live soil inocula. Upper x-axis labels indicate soil inoculum source; lower x-axis labels indicate plant line. Box plots are color-coded by field site source of the plant line. Statistical analyses were performed separately for universal and sympatric plant lines. Different letters indicate significant differences (a) among soil source / plant line combinations and (b) among soil sources (**Table 2.2**). Daggers correspond to 'abcd.'

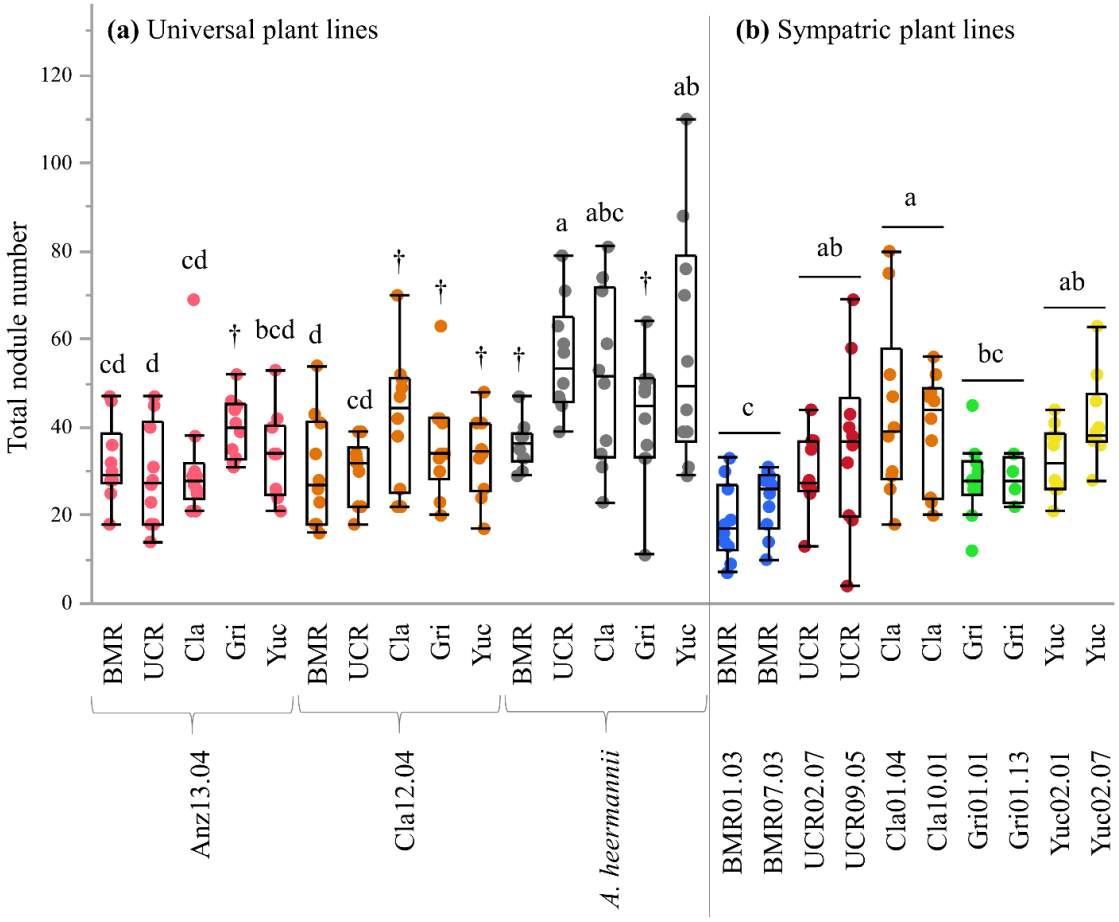

Table 2.1. Soil collection information and *A. strigosus* plant lines used in this study.

Field site	Soil collection date, time	Soil collection coordinates	Plant lines, experimental names	Plant lines, formal names	*nrITS* Accession	*CNGC5* Accession
BMR	1 Mar 2015, 0830 h	N 38.3193, W 123.06368	BMR01.03	AcS074.BMR.u01.g2.r01_03	MH201354	MH223487
			BMR07.03	AcS004.BMR.u01.g2.r01_03	KX449155	KX449166
UCR	2 Mar 2015, 1630 h	N 33.96591, W 117.32271	UCR02.07	AcS027.UCR.u01.g1.r10	MH201360	MH223492
			UCR09.05	AcS131.UCR.u01.g1.r05	MH201361	MH223493
Cla	2 Mar 2015, 0930 h	N 34.110555, W 117.708798	Cla10.01	AcS047.Cla.m01.g2.r07_01	KX449157	KX449168
			Cla01.04	AcS049.Cla.m01.g1.r04	MH201356	MH223488
			Cla12.04 (universal exp)	AcS047.Cla.m01.g2.r09_04	MH201358	MH223489
Anz	27 Feb 2015, 1130 h	N 33.2713, W 116.4194	Anz11.01	AcS040.Anz.m01.g2.r06_01	KX449153	KX449164
			Anz10.01	AcS039.Anz.m01.g2.r03_01	MH201351	MH223485
			Anz13.04 (universal exp)	AcS038.Anz.m01.g1.r11	MH201353	MH223486
Gri	2 Mar 2015, 1100 h	N 34.12197, W 118.309	Gri01.01	AcS075.Gri.u01.g1.r01	MH220053	MH223490
			Gri01.13	AcS075.Gri.u01.g1.r13	MH220054	MH223491
Yuc	27 Feb 2015, 1500 h	N 34.15315, W 116.47511	Yuc02.07	AcS052.Yuc.m01.g2.r01_07	KX449161	KX449172
			Yuc02.01	AcS052.Yuc.m01.g2.r01_01	KX449161	KX449172

Table 2.2. General linear mixed models (GLMMs) testing effects of plant line and soil source on traits of *Acmispon* plants.

	Plant responsiveness to microbes			**Total plant dry mass, mg (live inocula)**		**Total plant dry mass, mg (sterilized inocula)**		**Mean nodule size (mg)**			**Total nodule mass (mg)**			**Total nodule number**		
[a]**Universal experiment**	n = 150; Adj. R^2 = 0.37, log_{10}			n = 150; Adj. R^2 = 0.49, none		n = 140; Adj. R^2 = 0.80, none		n = 150; Adj. R^2 = 0.26, log_{10}			n = 150; Adj. R^2 = 0.29, none			n = 150; Adj. R^2 = 0.26, log_{10}		
	df	***F***	[b]***Var. comp.***	**df**	***F***	**df**	***F***	**df**	***F***	[b]***Var. comp.***	**df**	***F***	[b]***Var. comp.***	**df**	***F***	[b]***Var. comp.***
Plant line	2,126	**34.0519*****	35.79 %	2, 126	**59.0978*****	2, 117.8	**219.4066*****	2126	0.8337	0.00 %	2126	**11.085*****	13.108	2126	**17.5263*****	21.38 %
Soil source	4,126	**2.4862***	0.72 %	4, 126	1.8706	4, 116.3	**6.5177*****	4126	**5.5947*****	8.64 %	4126	1.7878	0	4126	1.7977	0.00 %
Plant line x Soil source	8,126	**2.1020***	6.17 %	8, 126	1.9907	8, 116.3	1.4351	8126	**2.4607***	8.50 %	8126	1.7924	5.577	8126	**2.2688***	7.71 %
Block *random*		ns	1.31 %		ns		ns		ns	7.98 %		ns	10.797		ns	1.56 %
[a]**Sympatric experiment**	[c]n = 94; Adj. R^2 = 0.25, square-root			n = 94; Adj. R^2 = 0.25, none		n = 93; Adj. R^2 = 0.69, log_{10}		n = 94; Adj. R^2 = 0.19, log_{10}			n = 94; Adj. R^2 = 0.18, none			n = 94; Adj. R^2 = 0.31, none		
	df	***F***		**df**	***F***	**df**	***F***	**df**	***F***		**df**	***F***		**df**	***F***	
Plant line (Soil source)	5,79.68	**3.1748***		5, 76.44	2.1253	5, 74.71	1.711	5,75.66	0.7913		5,75.56	1.4496		5,75.86	1.119	
Soil source	4,80.01	**5.6551*****		4, 76.58	**6.8146*****	4, 74.8	**36.0353*****	4,75.76	**5.0467****		4,75.65	**3.0314***		4,75.94	**8.7459*****	
Block *random*		***			ns		ns		ns			ns			ns	

[a] The header row for each model indicates total sample size, adjusted R^2 value for the model, and the transformation applied to the dataset. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

[b] Variance components were estimated for each effect in the model by treating all effects as random.

[c] The R^2-value for this model was negative when block was included, so we report the R^2 of the model when block was excluded. However, the F-statistics shown here and all post-hoc tests described in the Results reflect the original model with block included.

Table 2.3. Correlations between soil metrics and soil growth-promoting ability on a universal *A. strigosus* plant line (Anz13.04).

BMR	UCR	Cla	Anz	Gri	Yuc	Spearman's ρ	Metric
Soil growth-promoting ability for Anz13.04 plant line							
7.35	4.74	4.15	.	3.55	3.73	.	Mean responsiveness of Anz13.04 hosts, back-transformed (from $\log_{10}$)
[a] ***Soil abiotic metrics***							
5.8	6.2	7	7.9	6.4	7	-0.67	Soil pH
3.2	6.4	7.4	5.0	24.1	5.2	-0.70	Soil cation exchange capacity (meq per 100g)
2	32	29	9	21	7	-0.10	Soil NO3-N (ppm)
4.8	3.7	4.3	3.9	7.8	4.5	-0.40	Soil NH4-N (ppm)
122.2	630.3	1457.7	239.1	1396.2	231.5	-0.50	Soil organic N (ppm)
[b] ***Soil biotic metrics***							
2.6E+06	5.6E+06	2.6E+07	6.4E+05	2.7E+07	1.0E+07	-0.90*	Mean CFU per ml in live soil inoculum, back-transformed (from square-root)
11	9	12	.	7	4	0.60	*Bradyrhizobium* haplotype richness (S)
0.69	0.59	0.83	.	0.47	0.55	0.70	*Bradyrhizobium* haplotype diversity (Simpson's D1)
0.29	0.27	0.50	.	0.27	0.56	0.10	*Bradyrhizobium* haplotype evenness (Simpson's E)
0.95	0.97	0.74	.	0.10	0.85	0.80	*Bradyrhizobium canariense* proportional abundance
Predicted soil conditioning metrics							
0.46	0.41	0.31	.	0.29	0.23	0.90*	Mean nodule size (mg) of sympatric hosts, back-transformed (from $\log_{10}$)
11.19	13.49	12.63	.	9.91	8.97	0.60	Total nodule mass (mg) of sympatric hosts
20.95	32.90	41.50	.	28.07	37.00	-0.30	Total nodule number of sympatric hosts

Cell shading within a row is proportional to the cell value. Significance of the correlations between each soil metric and the responsiveness of Anz13.04 hosts is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

[a]Soil abiotic metrics were measured from the sieved soil samples used to prepare inocula.

[b]Soil biotic metrics were calculated from cultured inocula (mean CFU per ml) and a database of *Bradyrhizobium* isolates from each field site (Hollowell *et al*., 2016a); *Bradyrhizobium* haplotypes are concatenated sequences of two chromosomally-encoded loci (*glnII*, *recA*). Between 39 and 108 isolates were sampled from each of the five field sites whose soils formed nodules in the present study; we randomly rarified the Hollowell *et al*., 2016a data to a depth of 39 isolates per site before calculating indices of diversity per Morris *et al.* (2014).

CHAPTER 3

The house always wins: *Acmispon* hosts constrain fitness gains of ineffective *Bradyrhizobium* symbionts in mixed inoculations

Abstract

Plants can gain significant growth benefits from symbiosis with microbes, but these benefits are threatened by divergent fitness interests of host and symbiont. Symbiont fitness inside plant tissues represents a joint phenotype that the host and symbiont are predicted to push in opposite directions. Many studies have separately shown how hosts and symbionts can bias symbiont fitness in their own favor, but few studies have examined these sources of variation in the same experiment. Here, I used the legume-rhizobia symbiosis to assess host and symbiont contributions to symbiont fitness in the host. I co-inoculated four *A. strigosus* plant lines with nine combinations of effective and ineffective *Bradyrhizobium* strains and measured the relative fitness of these strains in nodules. Ineffective strains generally had low relative abundance in nodules, consistent with hosts controlling symbiont fitness. However, ineffective strains also varied genetically in their relative fitness in nodules, highlighting a role for symbiont competitiveness in shaping this joint phenotype. Variation in symbiont fitness during co-inoculations did not affect plant performance, suggesting that conflict over this joint phenotype is largely resolved in favor of the host.

Introduction

Eukaryotes gain substantial benefits from establishing mutualistic symbioses with microbes (Douglas, 2010). Microbial communities collectively possess a vast genetic repertoire that is unavailable to hosts and can generate key services such as nitrogen fixation (Masson-Boivin & Sachs, 2018), phosphorus acquisition (Smith & Read, 2008), antibiotic production (Currie *et al.*, 1999) and bioluminescence (Jones & Nishiguchi, 2004). In exchange, hosts shelter microbial partners from harsh external environments and provide reliable sources of energy that can greatly enhance microbial fitness. However, understanding the effects of symbiosis on microbial fitness is complicated by the fact that the microbes often reproduce within the host organism. The rate of reproduction of microbial symbionts inside host tissues represents a joint phenotype—a trait that is jointly influenced by the genes of both host and symbiont partners (Queller, 2014). This particular joint phenotype is subject to conflicts of interests in microbial symbioses (Queller & Strassman, 2018) since most host and microbe partners ultimately separate and reproduce independently, and thus have nonoverlapping fitness interests (Jones *et al.*, 2015; Douglas & Werren, 2016). From the perspective of host fitness, private 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 used for microbial reproduction (often at the expense of the mutualistic service they are engaged to perform). 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. Importantly, little is known about the amount of standing genetic

variation in the joint phenotype of symbiont fitness, or whether conflict is typically resolved towards the individual benefit of one partner or the other.

The legume-rhizobia mutualism is an ideal system in which to investigate the joint phenotype of symbiont fitness in host tissues. Rhizobia are free-living soil bacteria that infect the roots of compatible legume hosts and form root nodules in which the rhizobia fix nitrogen (Oldroyd *et al.*, 2011). Fixed nitrogen is passed to the host in exchange for photosynthates, and the nodules eventually senesce and release a portion of the rhizobial population back into the soil (Muller *et al.*, 2001). Joint phenotypes in this system include the benefits that plants gain from nodules (i.e., transfer of reactive nitrogen from the rhizobia to the host), as well as the number, size, and the *in planta* rhizobial population size of nodules, since these phenotypes can be strongly influenced by genotypes of both plant (Simonsen & Stinchcombe, 2014; Wendlandt *et al.*, 2019) and rhizobia (Sachs *et al.*, 2010b). I focus here on *in planta* rhizobial population size since this is most directly related to rhizobial fitness. The *in planta* fitness of rhizobia can be estimated as raw number of rhizobia that can be cultured from a nodule (when plants are inoculated with a single rhizobial strain), or the relative abundance of a focal strain in nodules (when plants are co-inoculated with multiple strains; Sachs *et al.*, 2010a; Sachs *et al.*, 2010b). In nature, nodule-forming rhizobia vary dramatically in symbiotic effectiveness, ranging from completely ineffective (i.e., non-nitrogen-fixing) to highly effective (i.e., improving host growth several fold; Burdon *et al.*, 1999; Sachs *et al.*, 2010a). Rhizobia are often under selection to provide less fixed nitrogen to their hosts (Porter & Simms, 2014; Gano-Cohen *et al.*, 2019), but legumes have mechanisms for

punishing low-quality symbionts (Kiers *et al.*, 2003; Sachs *et al.*, 2010b; Oono *et al.*, 2011). Given the intense conflict over fitness outcomes that occurs between legumes and rhizobia (Sachs *et al.*, 2018), it is critical to examine how phenotypically divergent symbionts and hosts contribute to symbiont fitness.

Host legumes can discriminate between effective and ineffective rhizobia in mixed inocula, and this discrimination can take the form of nodules being preferentially occupied by more effective strains (Heath & Tiffin, 2009; Gubry-Rangin *et al.*, 2010; Regus *et al.*, 2014), as well as plants forming larger nodules with more effective strains (Kiers *et al.*, 2003; Regus *et al.*, 2015; Wendlandt *et al.*, 2019). Since nodules can be co-infected by multiple symbiont genotypes, plants also punish ineffective rhizobia within co-infected nodules at the level of single infected plant cells (Regus *et al.*, 2017), which reduces the abundance of ineffective rhizobia within nodules (Sachs *et al.*, 2010b; Oono *et al.*, 2011; Regus *et al.*, 2014; Westhoek *et al.*, 2017). Despite the multiple lines of host defense against ineffective rhizobia (Sachs *et al.*, 2018), plants growing in natural conditions frequently associate with many genotypes of rhizobia simultaneously, rather than the single most cooperative genotype available (Burdon *et al.*, 1999; McInnes *et al.*, 2004). Moreover, in agricultural settings, crop legumes often fail to nodulate with highly effective rhizobia applied by farmers, and instead form nodules with moderately effective rhizobia indigenous to the field soil (Triplett & Sadowsky, 1992; Shiferaw *et al.*, 2004; Vlassak *et al.*, 2010; Yates *et al.*, 2011; Sinclair & Nogueira, 2018). Some of this variation in symbiont fitness has been 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). However, the other main source of variation is driven by rhizobia. Rhizobia compete intensely to colonize legumes, and 'competitiveness' traits in rhizobia often vary independently of symbiotic effectiveness (Triplett & Sadowsky, 1992). Symbiotic competitiveness can be a function of strain-strain antagonism or defense traits (Triplett & Sadowsky, 1992), saprophytic competence in the soil environment (Bottomley *et al.*, 1991; Hollowell *et al.*, 2016), nodulation speed (Kiers *et al.*, 2013; Hidalgo *et al.*, 2017) or downregulation of host regulatory mechanisms (Yuhashi *et al.*, 2000; Price *et al.*, 2015). Symbiont competitiveness could explain some of the evidence that low-quality symbionts in nature can occupy nodules, but few studies have precisely distinguished the role of strain competitiveness from the role of host variation in preference for effective symbionts (Bourion *et al.*, 2018).

Here, I investigated host and symbiont contributions to symbiont fitness using the association between the legume *Acmispon strigosus* and its *Bradyrhizobium* symbionts. *A. strigosus* is an annual herb that grows throughout the southwestern United States, where it forms nodules with diverse *Bradyrhizobium* that range from highly effective to ineffective. I performed greenhouse experiments in which I inoculated four genetically distinct host plant lines with nine combinations of effective and ineffective *Bradyrhizobium* strains (three strains each). I grew all plants with zero supplemental nitrogen to maximize host demand for the symbiont's mutualistic service. Using single inoculations, I characterized each strain's symbiotic effectiveness and *in planta* fitness on all four host lines. Using co-inoculations of effective and ineffective strains, I quantified relative fitness of rhizobia to see how much variation was contributed by the strain

genotypes (i.e., competitiveness) versus host genotypes (i.e., host control). Finally, I examined patterns of plant performance in the co-inoculation experiment, to test whether discrimination among effective and ineffective rhizobia had immediate effects on 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 variation in symbiont fitness affects host performance. This experimental design allowed me to 1) uncover variation in symbiont fitness contributed by symbiont and host genotypes, and 2) investigate the ability of each partner to bias fitness outcomes in their favor. This work contributes to a better understanding of how evolutionary conflicts of interest are resolved or continue to evolve in natural systems.

Materials and Methods

Bradyrhizobium strains

Six genetically unique *Bradyrhizobium* strains were used, including three strains that were previously categorized as effective (#49, #138, and CW09, mean relative growth for inoculated plants > 6.6x compared to uninoculated plants) and three strains that were previously categorized as ineffective (#2, #187, and CW01, mean relative growth < 1.8x) based upon tests on a single *A. strigosus* host line (Gano-Cohen *et al*., unpublished). Antibiotic resistance profiles were also previously characterized, allowing strains to be distinguished in mixed inocula by culturing on selective media (see **Table 3.1**).

Acmispon strigosus host lines

A. strigosus seeds were collected from four natural sites in California between 2009 and 2012: Bodega Marine Reserve (BMR), Griffith Park (Gri), University of California, Riverside (UCR), and Pioneertown Mountains Preserve near Yucca Valley (Yuc). Plants from these four population sources are genetically distinct at two loci (nrITS, CNGC5; **Table 3.2**) and vary in investment into 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, I anticipated that these plant lines might also show divergent patterns of host control over symbiont fitness *in planta*. Plants were raised from wild seeds in a glasshouse sprayed weekly with insecticide and allowed to self. I 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, I supplemented experimental plants with a replicate Gri line sourced from a different wild seed from the same collection location (**Table 3.2**).

Inoculation experiment

Axenic *A. strigosus* seedlings were raised in sterilized calcined clay (Turface Proleague Champion Brown; Profile Products LLC, Buffalo Grove, IL) in Ray-Leach SC10 conetainers (Stuewe & Sons, Corvallis, OR, USA) following published protocols (Sachs *et al.*, 2009). Plants were fertilized weekly with 1 ml nitrogen-free Jensen's (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 x 10^8 cells ml^{-1}. Plants were inoculated with 5 ml of clonal *Bradyrhizobium* cultures (single inoculations; 6 treatments), 5 ml of a 1:1 mixture of two clonal cultures (co-inoculations; 9 treatments comprising each pairwise combination of effective and ineffective strains) or 5 ml sterile ddH_2O as a control. Sets of size-matched plants from the same population were randomly assigned to inoculation treatments within each of the replica blocks, and plant positions within blocks were randomized. The two Gri lines were used interchangeably to represent the Gri population. In total, the experiment included four population sources of plants x sixteen inoculation treatments x eight replica blocks (512 plants total).

Dilutions of each inoculum were cultured to estimate CFU per ml and confirm that co-inocula represented equal mixtures of component strains (**Table 3.1**). Inocula were serially diluted and plated onto replicate MAG-agar plates; CFU per ml was estimated for each inoculum using only dilutions that yielded at least two replicate plates in the range of 30-300 colonies.

Plant harvest and nodule culturing

Two blocks of the experiment were 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 (> four days, 60°C) 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. In each case I cultured the block to which the larger seedlings were assigned at the start of the experiment. If plants in the selected block were flowering or producing pods, nodules were cultured from plants in the other harvested block. Nodules were chosen randomly for culturing after senescent (green or brown) nodules were removed from consideration. Nodules selected for culturing were immediately surface-sterilized with bleach, rinsed, and crushed to generate nodule extracts.

Among singly-inoculated plants, two nodules were cultured from one plant replicate each harvest week (2 nodules per harvest week x 4 harvest weeks x 4 hosts x 6 strains = 192 nodules total). Nodule extracts were spread onto two replicate MAG-agar plates in 10^{-3} and 10^{-5} dilutions (Sachs *et al.*, 2009), and the number of rhizobial colony-forming units (CFU) per nodule was calculated from at least two plates containing 3-800 colonies. Among co-inoculated plants, four nodules were cultured from one plant replicate each harvest week (4 nodules per harvest week x 4 harvest weeks x 4 hosts x 9 co-inocula = 576 nodules total). Nodule extracts were immediately spread onto three replicate MAG-agar plates, and colonies that formed on these plates were preferentially used as a source of colonies for sub-culturing. If few colonies formed on these plates, nodule extracts (stored at 4°C) were re-plated to generate additional colonies for sub-culturing. Approximately 100 colonies per nodule were sub-cultured onto two separate

MAG-agar plates, including one with an antibiotic (see **Table 3.1**) 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 3.1**). Colonies with ambiguous scores were sub-cultured again, and colonies with persistent ambiguous scores were excluded from further analyses.

Data analysis

Statistical analyses were performed in JMP Pro 13.0.0 (SAS Institute Inc., Cary, NC, USA). The statistical approach used generalized linear mixed models (i.e., GLMMs; Fit Model Platform; Standard Least Squares personality; REML method). 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 1% was subtracted). All models included a random effect of harvest week; models using just 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. Nonsignificant interactions were removed from the model if this reduced the corrected AIC (AICc) by at least 2 units, and the results from these trimmed models are reported (**Table 3.3**). 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 (this option allows contrasts to be performed among levels of one factor while

holding the levels of the other factor constant, thus focusing statistical power on just the comparisons of interest). Mean values discussed below are back-transformed (if applicable) from raw means and presented alongside 95% confidence intervals.

I characterized strain phenotypes in the single-inoculation experiment by examining symbiotic effectiveness and fitness payoffs from nodule formation on each host. Strains were categorized as 'effective' if the total dry plant biomass (roots + shoots) of inoculated plants was greater than the total dry biomass of uninoculated control plants. Strains were categorized as 'ineffective' if they failed to improve plant growth compared to uninoculated control plants. Fitness payoffs from nodule formation were measured as rhizobial (CFU) per nodule, averaged between the two replicate nodules cultured from each plant such that the plant was the unit of replication (n = 4). Fitness payoffs from nodule formation were tested for effects of strain genotype and host population.

I examined patterns of rhizobial relative fitness in the co-inoculation experiment by focusing on the ineffective strains (#2, #187, CW01), for which the null expectation was 50% (i.e., their relative abundance in the co-inocula). I calculated relative abundance of the ineffective strain on each plant (as a percentage). From the four replicate nodules cultured from the plant, I counted the total number of colonies identified as the ineffective strain and divided this by the total number of colonies scored, such that the plant was the unit of replication (n = 4). I tested ineffective strain relative abundance for effects of ineffective strain genotype, effective strain genotype, and host population.

I examined patterns of plant relative performance in the co-inoculation experiment to detect whether variation in ineffective strain relative fitness had immediate

effects on plant performance. I estimated plant relative performance in the co-inoculation experiment by dividing the total dry plant biomass of each co-inoculated plant by the total dry biomass of plants singly-inoculated with the effective strain in the co-inoculum. Relative performance less than 1 would indicate that plants performed worse during co-inoculations than with the effective strain alone, suggesting a cost to encountering the ineffective strain in the co-inoculum. I tested plant relative performance for effects of ineffective strain genotype, effective strain genotype, and host population.

Results

Characterizing strain phenotypes

Patterns of nodule formation

No host and strain combination consistently failed to form nodules, indicating that strains and hosts were compatible for nodule formation. Of 480 inoculated plants, only seven failed to form nodules. Four of these plants were inoculated with strain #2 (plant lines affected = Gri, UCR, Yuc) and three were UCR plants that died before harvest and showed no evidence of nodulation (inocula = uninoculated, #187, CW01-CW09 co-inoculum). None of the uninoculated control plants formed nodules.

Variation in symbiotic effectiveness

Total plant dry biomass exhibited a significant inoculum x host population effect (**Table 3.3**). Strains #49, #138, and CW09 were categorized as effective for all hosts, and strains #2 and #187 were categorized as ineffective for all hosts (**Fig. 3.1**). Strain CW01

exhibited host-dependent effectiveness, being effective on BMR and UCR hosts but ineffective on Gri and Yuc hosts (**Fig. 3.1**).

Rhizobial fitness payoff per nodule

Rhizobial fitness (i.e., colony-forming units per nodule, CFU) exhibited a significant strain x host population effect (**Table 3.3**). Most strains did not differ in CFU per nodule across different hosts, but strain CW09 had greater CFU per nodule on Yuc than Gri hosts (**Fig. 3.2**). Averaging across hosts, rhizobial fitness was greatest for strain #2 (6.1×10^6 [1.6×10^6-2.3×10^7] CFU per nodule), followed by #49 (4.5×10^6 [2.1-9.4×10^6] CFU per nodule), CW01 (1.8×10^6 [6.8×10^5-4.6×10^6] CFU per nodule), #187 (6.6×10^5 [2.8×10^5-1.6×10^6] CFU per nodule), CW09 (3.9×10^5 [1.3×10^5-1.2×10^6] CFU per nodule), and #138 (6.3×10^4 [2.6×10^4 – 1.5×10^5] CFU per nodule; **Fig. 3.2**). This indicates that ineffective strains experienced large fitness payoffs from nodule formation, similar to effective strains in single infections. Strains did not significantly vary in their fitness on Gri hosts, likely due to lack of replication for strain #2 on this host (**Fig. 3.2**). Overall, I obtained CFU per nodule data from 85/96 singly-inoculated plants from which I cultured nodules (representing data from 133/192 nodules cultured). The remaining nodules either failed to grow rhizobia on plate or had colony counts outside the acceptable range for quantifying CFU per nodule.

Ineffective strain relative abundance

I adjusted my analysis of the co-inoculation experiment to account for the finding that strain CW01 was only ineffective on two hosts (Gri, Yuc). Since plants co-inoculated with strain CW01 would not necessarily be expected to constrain the relative fitness of CW01 under the hypothesis that hosts control symbiont fitness, I used separate analyses for treatments using ineffective strains #2 and #187 and treatments using strain CW01.

For co-inoculation treatments using ineffective strains #2 and #187, ineffective strain percent abundance showed a significant effect of ineffective strain genotype (**Table 3.3**). Specifically, strain #2 achieved greater percent abundance in nodules (2.8% [1.4-5.0%]) than #187 (1.1% [0.5-1.9%]). No other effects were significant. Notably, both ineffective strains were at extremely low frequencies within nodules and below their inoculation percent abundance of 50%, consistent with hosts favoring effective strains in nodules (**Fig. 3.3a**). For co-inoculation treatments using strain CW01, percent abundance of CW01 in nodules showed a significant effect of effective strain genotype but not of host population (**Table 3.3**). CW01 achieved the greatest percent abundance 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%]; **Fig. 3.3b**).

Overall, I obtained rhizobial relative abundance data from 142/144 co-inoculated plants from which I cultured nodules (representing data from 478/576 nodules cultured). A total of 38,727 colonies were scored to generate nodule occupancy data. Most nodules (276/478) were sub-cultured at or above the desired depth of 100 colonies per nodule (median = 102 colonies per nodule). I re-assayed a subset of scored colonies to check the

reliability of the antibiotic assay. For the assay to screen strain CW01 from the three effective strains (using gentamycin; **Table 3.1**), I uncovered some inconsistency in the colony scores: colonies identified originally as one of the effective strains (49/138/CW09) changed score to CW01 (resistant to gentamycin) at a rate of ~50%, whereas colonies identified originally as CW01 almost always retained this score. Based on these data, CW01 could be even more abundant in nodules than I report (conservatively) here, using the original scores.

Relative performance of co-inoculated plants

For co-inoculation treatments using ineffective strains #2 and #187, plant relative performance exhibited a significant interaction effect of effective strain genotype x host population (**Table 3.3; Fig. 3.4a**). Plant relative performance varied significantly among effective strains for BMR hosts ($F_{2,172} = 10.2178$, $P < 0.0001$) and Gri hosts ($F_{2,172} = 3.1928$, $P = 0.0435$) but not UCR ($F_{2,172} = 2.0292$, $P = 0.1346$) or Yuc ($F_{2,172} = 0.1248$, $P = 0.8828$). For most co-inoculation treatments, plant relative performance was equal to or greater than plant performance with the effective strain alone. Mean relative performance for UCR and Yuc hosts was 0.89x (0.74-1.06x) and 1.03x (0.88-1.21x), respectively, indicating no detectable cost to encountering an ineffective strain (i.e., relative performance ~ 1x). For BMR hosts, relative performance was high when co-inocula contained CW09 (1.64x [1.02-2.66x]) or #138 (1.47x [0.87-2.50x]), and lower when co-inocula contained #49 (0.62x [0.49-0.77x]). For Gri hosts, relative performance was high when co-inocula contained #138 (1.09x [0.76-1.55x]) or #49 (0.92x [0.59-1.42x]), and

lower when co-inocula contained CW09 (0.61x [0.43-0.85x]). For co-inoculation treatments using strain CW01, plant relative performance exhibited a significant effect of host population (**Table 3.3, Fig. 3.4b**). However, pairwise comparisons among host populations found no significant differences in plant relative performance (BMR: 0.97x [0.62-1.50x]; UCR: 0.61x [0.48-0.78x]; Gri: 0.60x [0.41-0.86x]; Yuc: 1.03x [0.75-1.39x]). Thus, variation in plant performance during co-inoculations rarely dipped below performance with the effective strain alone, indicating few costs to plants encountering ineffective strains. When plant performance was lower than performance with effective strains alone, this was a function of effective strain genotype rather than ineffective strain genotype.

Discussion

I found that four population sources of *A. strigosus* exhibit robust host control over completely ineffective rhizobia. This corroborates a previous study that inoculated *A. strigosus* hosts with a mixture of three *Bradyrhizobium* strains and found that nodules of all hosts were dominated by the most effective strain (Wendlandt *et al.*, 2019). Here, rhizobia that were categorized as ineffective (strains #2, #187) had very low relative fitness in nodules of co-inoculated plants, consistent with hosts controlling this joint phenotype in their favor. In addition, I found no variation in strain fitness attributed to host genotype. This is notable given that hosts were sourced from diverse locations throughout the native range of *A. strigosus* (**Table 3.2**) and exhibit both genetic and phenotypic variation (see differences in plant biomass in **Fig. 3.1**). Furthermore, most

strain-host combinations were allopatric (**Table 3.2**), maximizing the chance of detecting incompatible interactions.

After accounting for host control acting against ineffective rhizobia, strain genotype drove variation in strain fitness in nodules. For the 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 nodules than strain #187. This result is notable because neither strain #2 nor strain #187 improved plant growth compared to uninoculated controls, so the higher relative fitness of strain #2 compared to #187 can be attributed to differential competitiveness. One intriguing possibility is that competitiveness in nodules during co-inoculations is related to *in planta* proliferative ability. For instance, during single inoculations strain #2 had greater CFU per nodule than strain #187. Similarly, the relative fitness of strain CW01 only varied significantly with the genotype of the effective strain with which it was co-inoculated. Strain CW01 had high relative fitness during co-inoculations with strain #138, and CW01 also exceeded #138 in CFU per nodule (during single inoculations) by an order of magnitude, potentially explaining its fitness advantage during co-inoculations. The competitive ability of strain CW01 was especially intriguing since it was ineffective on two hosts (i.e., Gri and Yuc) and was expected to be punished via host control on those plant lines. Other studies have identified specific genes or gene functions that confer competitiveness of ineffective strains, which could potentially explain the competitiveness of strain CW01. For example, *Sinorhizobium* bearing the *hrrP* locus fail to fix nitrogen but hyper-proliferate within nodule tissue compared to strains lacking this

locus (Price *et al*., 2015). Similarly, 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).

Despite the evidence that strain genotypes varied in their fitness in nodules, I 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 (**Fig. 3.4**). 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 relative 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. These data suggest that variation in strain fitness in nodules is so slight that it is not visible to the host in terms of its own growth performance.

It is difficult to reconcile robust host control (seen in co-inoculations, here) with other studies showing that ineffective symbionts can achieve high fitness in natural environments. Single inoculation studies have repeatedly identified trade-offs between symbiotic effectiveness and symbiont fitness (Porter & Simms, 2014; Gano-Cohen *et al.*,

2019), which creates positive selection for ineffective rhizobia. One possible way to reconcile these two kinds of data is to re-examine which experimental settings are biologically realistic for plants in nature. It is typical to think of co-inoculation experiments as more biologically realistic than single inoculations (Kiers *et al.*, 2013), since soil microbial communities are very diverse (Delgado-Baquerizo *et al.*, 2018) and plants are extremely likely to encounter more than one genotype of compatible rhizobia in their rhizosphere (McInnes *et al.*, 2004). At fine spatial scales, however, plant roots may encounter environments more similar to single inoculation experiments. For instance, spatial structure of symbionts in soils (Bever *et al.*, 2009; Wakelin *et al.*, 2018) can generate conditions where only one strain is present at an infection site on a host root. Even when multiple strains are present, differences among strains in nodulation speed could create *de facto* single inoculation environments where nodule occupancy is determined mainly by nodulation speed (Hidalgo *et al.*, 2017). Thus, it is possible that the well-mixed inocula used in co-inoculation experiments overestimate the power of host control to reduce the fitness of ineffective rhizobia in natural settings.

In conclusion, these data suggest that *A. strigosus* hosts have robust control over the fitness of ineffective *Bradyrhizobium* strains during co-inoculations, reducing the relative fitness of ineffective strains in favor of effective, nitrogen-fixing strains. The failure of host genotypes to contribute variation to strain fitness suggests that host control is extremely conserved in *A. strigosus*, potentially due to its importance for plant fitness, or due to high relatedness among populations of this species. The variation in relative fitness among ineffective strains shows that there is variation upon which selection could

act to generate more exploitative strains. However, the fact that variation in strain fitness had no consequences for plant performance suggests this variation is permitted by plant hosts, or at least invisible to them. I acknowledge that single inoculation environments can provide a very different view of which partner is winning this conflict. Overall, however, this work is consistent with plant hosts being under selection to keep their symbionts on a short leash.

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Fig. 3.1. Total plant dry mass (roots + shoots) of plants in each single-inoculation treatment, with uninoculated plants shown for reference, by plant population source. Note the different axes for different plant populations. Asterisks indicate significant differences between inoculated and uninoculated plants within the same plant population source (from Test Slices by Host within the significant host x inoculum interaction, using 'Uninoc' as the reference category). Blue = uninoculated treatment; Red = ineffective strains (#2, #187, CW01); Green = effective strains (#49, #138, CW09).

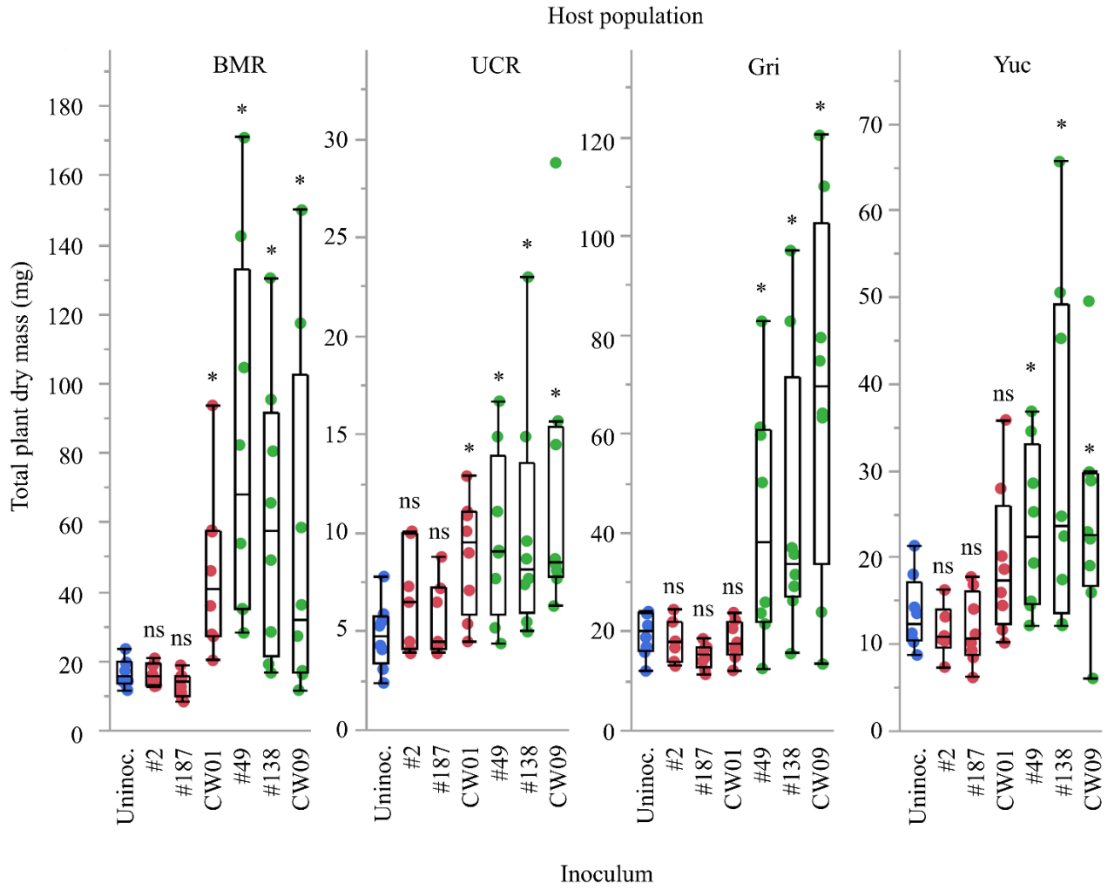

Fig. 3.2. Rhizobial colony-forming units (CFU) per nodule measured for each strain on each host during single inoculations. Each datapoint represents a mean of up to two nodules sampled from one plant replicate. Different letters indicate significant differences among hosts within a strain genotype.

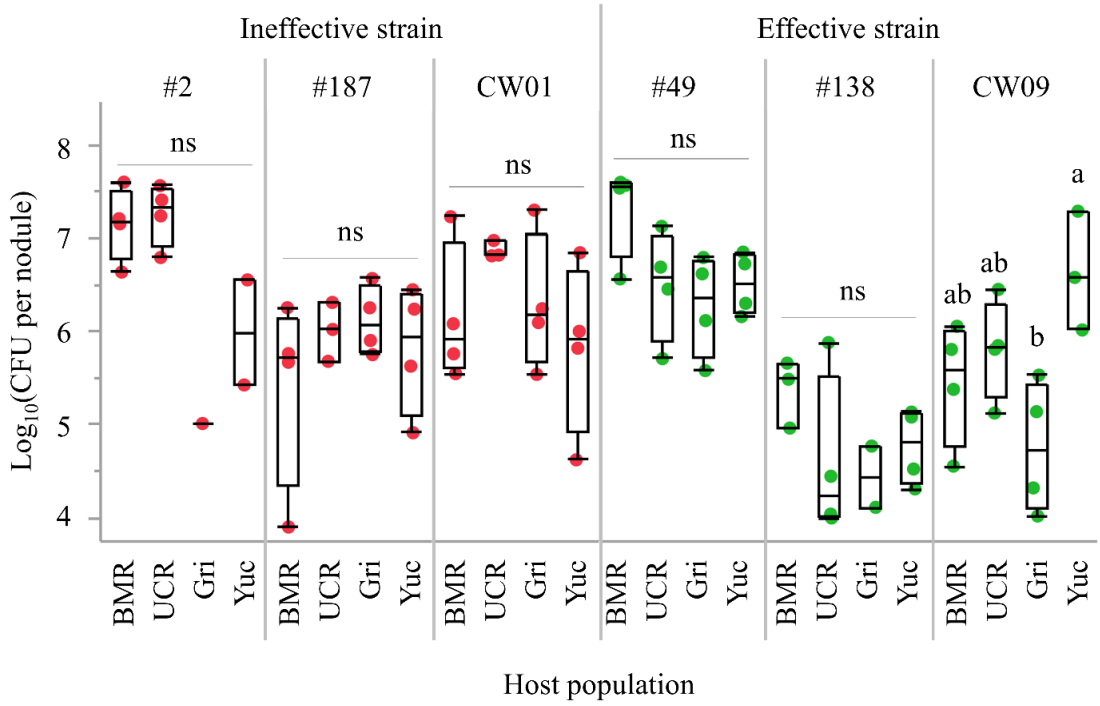

Fig. 3.3. Percent abundance of the ineffective strains (#2, #187, CW01) in nodules, during co-inoculations with each of the three effective strains (#49, CW09, #138). Each datapoint is consolidated data from up to four replicate nodules of one plant. I performed statistics separately for co-inocula containing 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 population in either model.

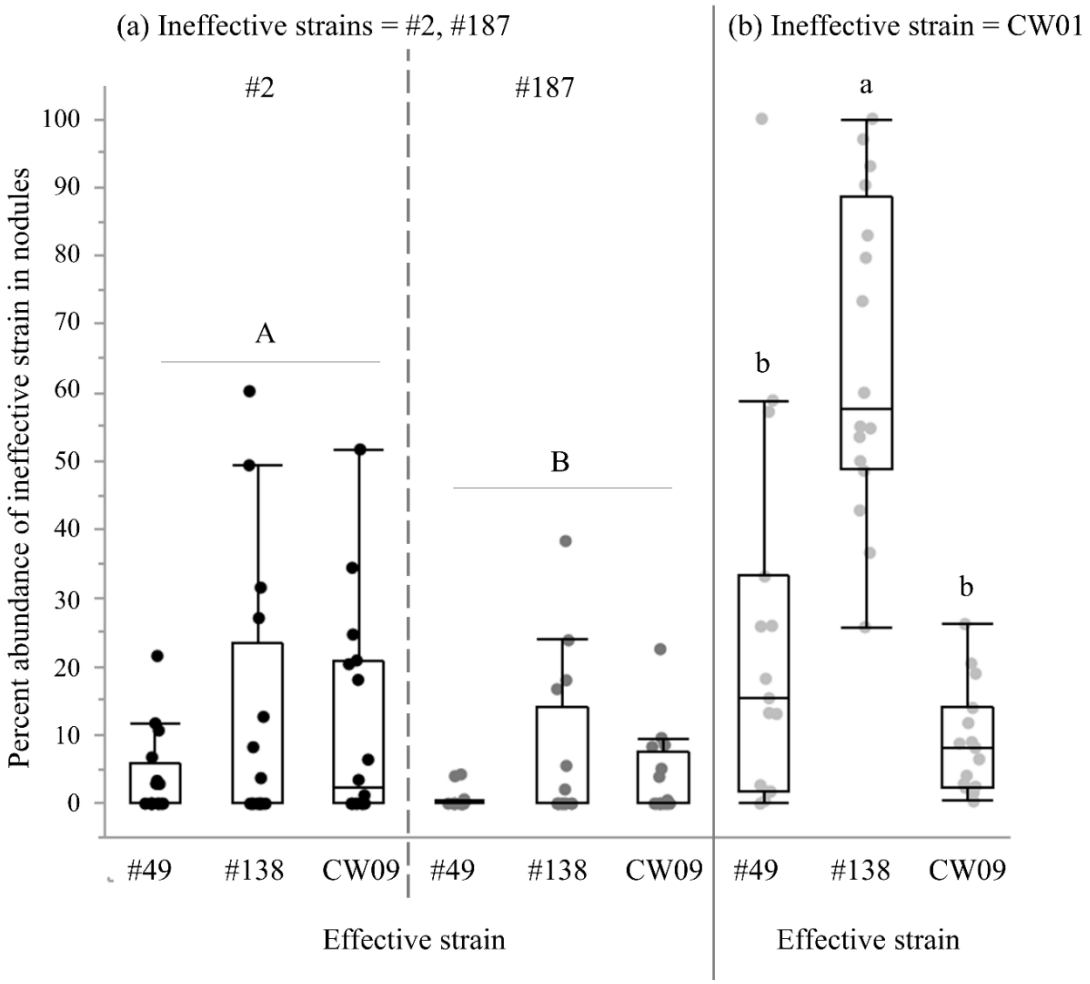

Fig. 3.4. Plant relative performance in co-inoculation compared to single inoculation with the effective strain. Statistics were performed separately on treatments including strains #2 and #187 (a) and treatments including strain CW01 (b). A reference line is drawn at relative performance = 1 to indicate whether co-inoculated plants performed better or worse than when singly inoculated with the effective strain in the co-inoculum.

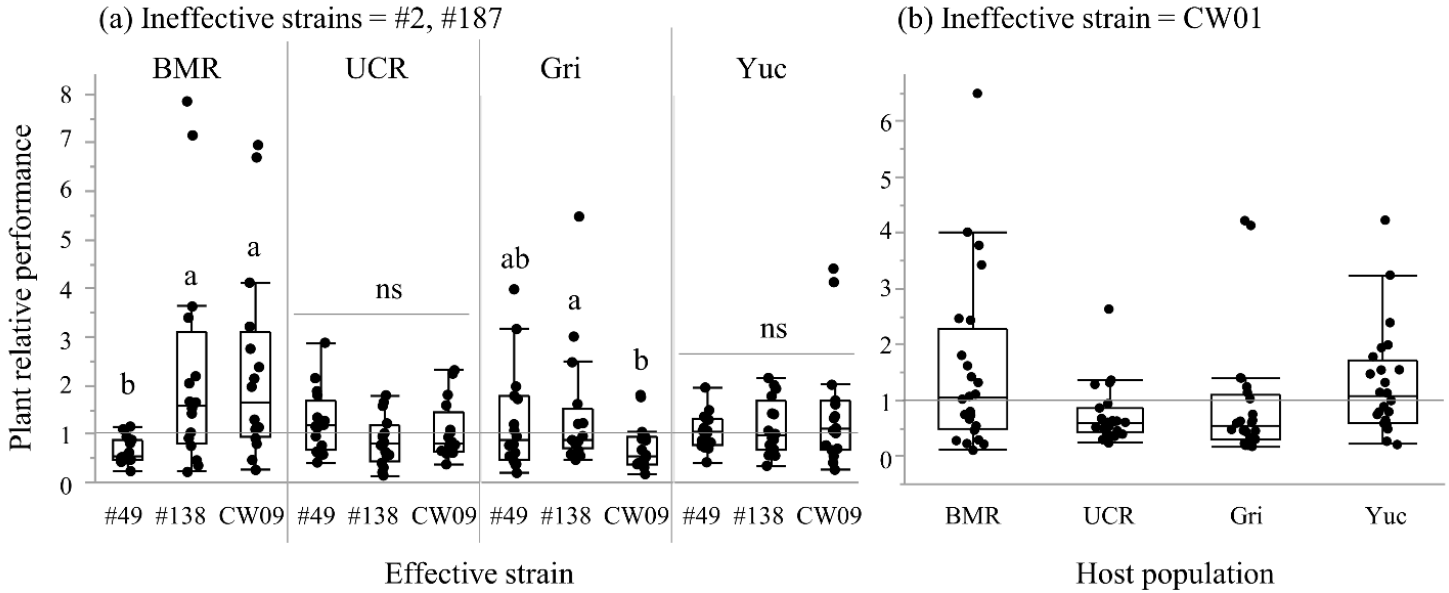

Table 3.1. Predicted effectiveness, collection information, genotype data, and antibiotic resistance profiles of *Bradyrhizobium* strains.

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

[a]Denotes field site where isolate was obtained (see Hollowell *et al.* , 2016b); BMR = Bodega Marine Reserve, Cla = Bernard Field Station of the Claremont Colleges, Dim = San Dimas, Gri = Griffith Park, UCR = University of California, Riverside

[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 3.2. Collection and genotyping information for *Acmispon strigosus* plant lines.

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

Table 3.3. General linear mixed models (GLMMs) 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. R^2***	***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 x 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. R^2***	***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 x 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. R^2***	***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. R^2***	***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

Table 3.3, continued.

3. Examining patterns of host relative performance			
Plant relative performance			
Data subset			
Co-inoculations, only treatments using strains #2, #187			
Transformation	***Adj. R^2***	***n***	
log_{10}	0.14	192	
Effect	***df***	***F***	***P***
Ineffective strain genotype	1, 172	0.9009	0.3439
Effective strain genotype	2, 172	0.8884	0.4132
Host population	3, 172	2.0085	0.1146
Effective strain x 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. R^2***	***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

GENERAL CONCLUSIONS

Host control traits are important for the evolutionary stability of plant-microbe mutualisms. Previous work has found segregating genetic variation in host control traits in wild species (Kiers *et al.*, 2007; Simonsen & Stinchcombe, 2014), which has interesting implications for the co-evolution of hosts and microbes. In my dissertation research, I investigated two forms of host control in the legume *Acmispon strigosus*, which engages in symbiosis with nitrogen-fixing *Bradyrhizobium* spp. One form of host control is plant regulation of the size of nodules (the structures in which rhizobia are housed), which can correlate with the total rhizobial content of nodules (Kiers *et al.*, 2003). Another form of host control is plant regulation of the strain content of nodules—the total rhizobial population size, or the relative abundance of a particular rhizobial strain in the nodule (Kiers *et al.*, 2003).

In the first chapter, I found that *A. strigosus* from six natural populations exhibited variation in mean nodule size when they were inoculated with either clonal effective *Bradyrhizobium* or a mixture of *Bradyrhizobium* strains. However, plants did not vary in host control over the strain content of nodules. Experimental nitrogen fertilization did not alter either of these patterns. This suggests the extent of variation in host control depends on the particular trait being measured, with less variation observed for traits more directly influencing symbiont fitness.

In the second chapter, I found that genetic variation among *A. strigosus* in mean nodule size was repeatable using inoculations of soil slurries rather than pure cultures of *Bradyrhizobium*. Furthermore, I found that most of the variation in plant growth from soil

inoculation was attributable to plant genotype, rather than source of the soil inoculum. This suggests that plant growth benefits from soil microbial communities are primarily determined by the plant genotype and can respond to selection.

In the third chapter, I examined the plant and rhizobial contributions to strain content of nodules and found that dominant force shaping this trait was symbiotic effectiveness, consistent with host control. Strain genotypes contributed some variation to their relative abundance in nodules, but this had no detectable consequences for plant performance during co-inoculations, suggesting that host control is near-optimal in *A. strigosus*.

Variation in host control has generated exciting theory on how mutualistic services could vary over time and space and with particular environmental parameters (Heath & Stinchcombe, 2013; Steidinger & Bever, 2014). My work contributes to this field by suggesting that host control over the most direct component of rhizobial fitness—strain content of nodules—is highly conserved across the geographic range of a California native legume (Chapters 1, 3). In contrast, host control over nodule size varied among genotypes of this host species (Chapters 1, 2), which could potentially generate differences in the magnitude of plant-soil feedbacks across the range of *A. strigosus*. The ecological consequences of nodule size variation in the legume-rhizobia symbiosis are certainly worthy of further study.

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