FACTORS AFFECTING RESOURCE ALLOCATION IN THE LEGUME-RHIZOBIA SYMBIOSIS

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ABSTRACT

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The legume-rhizobia symbiosis is an interaction in which nitrogen fixing bacteria called rhizobia colonize plant roots and supply fixed nitrogen to the plant in exchange for photosynthetically fixed carbon. This interaction has global impacts on the nitrogen cycle and offers an alternative to environmentally damaging synthetic nitrogen fertilizers. Resource allocation between plants and rhizobia is shaped by several factors, including the abiotic environment and various levels of host-symbiont specificity. The first two chapters of this dissertation explore the effects of the abiotic environment. First, a theoretical ecological model was adapted to the legume-rhizobia symbiosis and parameterized with a series of detailed measurements of plant and nodule biomass, carbon and nitrogen content, and plant photosynthesis. These results were compared to model predictions, which illustrated that the model assumption of fair trade was invalid, that plants have more bargaining power than rhizobia, and that plant bargaining power is highest when soil nitrogen is lowest. In the second chapter, the effects of factorial manipulation of soil nitrogen and light availability on resource trade between legumes and rhizobia was assessed. The results revealed that plants adjusted their resource acquisition strategy to take up the most limiting resource, and that both nitrogen and light affect allocation to rhizobia, but not their symbiotic effectiveness per unit of resource received. Finally, the third project assessed the level of specificity between host plant and rhizobial symbiont by comparing the effectiveness of rhizobia isolated from the same plant species to that of rhizobia isolated from different plant species. The results were contrary to

ecological theory predicting positive plant-soil feedbacks between legumes and rhizobia. The effects of environmental context dependence and plant-rhizobia specificity are vital for understanding the role of rhizobia in natural and agricultural ecosystems as well as the future development of effective rhizobial crop inoculants.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AON	Autoregulation of nodulation
EPS	exopolysaccharide
GS-GOGAT	glutamate synthase-glutamine oxoglutarate aminotransferase
lhk1	*Lotus* histidine kinase 1
LRR-RLK	leucine-rich receptor-like protein kinase
Nfr5	Nod factor receptor 5
NOP	nodulation outer proteins
PBU	peribacteroid unit
PHB	poly-β-hydroxybutyrate
R/FR	red/far red
TCA	tricarboxylic acid

CHAPTER 1

LITERATURE REVIEW

Introduction

Nutritional mutualisms, or interactions in which participants trade nutrients for mutual benefit, are ancient and widespread. Mutualisms have led to innovations such as eukaryotic cells and the colonization of land by plants (Bronstein, 2015). Nutritional plant-microbe mutualisms play an important role in global nutrient cycling. For example, mycorrhizal fungi colonize plant roots and use their hyphal networks to supply phosphorus and other minerals in exchange for roughly five billion tons of photosynthetically fixed carbon globally each year (Bago *et al.*, 2000). Similarly, rhizobia, soil bacteria that colonize plant roots, fix more than forty million tons of atmospheric nitrogen and exchange it for host carbon (Udvardi & Poole, 2013). There has been a large body of research exploring why mutualisms have persisted over evolutionary time despite the large benefits mutualists stand to gain if they defect (Sachs *et al.*, 2004; Ghoul *et al.*, 2014; Bronstein, 2015). However, the theoretical threat of cheaters has rarely been backed up by empirical evidence of cheaters prospering in contemporary mutualisms (Jones *et al.*, 2015), and phylogenetic analysis demonstrates that the evolution of parasites from within mutualistic clades occurs rarely (Sachs *et al.*, 2011). In wild populations, parasites of mutualisms often come from outside the focal interaction, as illustrated by non-fixing rhizobia that originated from non-symbiotic lineages (Sachs *et al.*, 2010a), and mutualistic symbioses are highly stable over evolutionary time (Werner *et al.*, 2015). Together, these observations suggest the operation of mechanisms, potentially operating at multiple scales, that result in the evolutionary robustness of

mutualisms as a whole. Further complicating our understanding of mutualisms is the observation that interactions may be mutualistic under some contexts but parasitic under others; this context dependence is often linked to the availability of external resources (Johnson *et al.*, 1997; Chamberlain *et al.*, 2014). Plant-microbe nutritional mutualisms are amenable systems for addressing these issues because resource fluxes can be tracked in both directions and there are existing models at multiple biological scales in addition to relevant empirical datasets (Clark *et al.*, 2017). Critical empirical work addressing these issues is lacking (Friesen & Heath, 2013), and larger questions about the regulation of mutualism and its evolutionary dynamics remain.

Legumes: the plant side

This dissertation will focus on the interactions between legumes and nitrogen-fixing bacteria known as rhizobia. Legumes belong to the family Fabaceae (Leguminosae), which consists of approximately 19,000 species across 750 genera, making it the third largest family of flowering plants (Andrews & Andrews, 2017; Sprent *et al.*, 2017). Fabaceae had traditionally been divided into three subfamilies (Caesalpinioideae, Mimosoideae and Papilionoideae), but recent taxonomic work has suggested that it is more appropriate to divide Fabaceae into six subfamilies (Duparquetioideae, Cercidoideae, Detarioideae, Dialioideae, Caesalpinioideae [this now includes the mimosoid clade], and Papilionoideae (Azani *et al.*, 2017)). The Duparquetioideae, Cercidoideae, Detarioideae and Dialioideae do not associate with rhizobia and thus will not be discussed further (Sprent *et al.*, 2017). Caesalpinioideae, which now includes the mimosoid clade, consists of woody and herbaceous plants mainly found in the tropics. Some members nodulate, particularly in the mimosoid clade, but not all do (Azani *et al.*, 2017). The plant species used in this work all come from Papilionoideae, a cosmopolitan subfamily that

includes approximately 14,000 species. The only non-legumes that are able to nodulate with rhizobia are in the genus Parasponia, part of the family Cannabaceae (Trinick, 1973). Legumes are estimated to have evolved nodulation 65 million years ago, but Parasponia seems to have evolved nodulation much more recently (Op den Camp *et al.*, 2011).

Rhizobia: the bacteria side

Rhizobia is the generally accepted term for a large, phylogenetically diverse group of α- and β- proteobacteria that are able to form nitrogen fixing symbioses with legumes (Mus *et al.*, 2016). Rhizobia of the α-proteobacteria (α-rhizobia) are all members of Rhizobiales, while rhizobia of the β-proteobacteria (β-rhizobia) belong to Burkholderiales (Shamseldin *et al.*, 2017). The same genus or species of bacteria may contain both nodulating and non-nodulating strains, and non-nondulating strains may vastly outnumber nodulating strains (Laguerre *et al.*, 1993; VanInsberghe *et al.*, 2015; Hollowell *et al.*, 2016). These non-nodulating strains may reduce the benefits plants gain from nodulating strains because of competition on the root surface (Gano-Cohen *et al.*, 2016). Rhizobia tend to have large genomes (ranging from 5.4-9.2 Mb) that are highly plastic, using large numbers of transport and regulatory genes to be able to survive in highly variable soil environments (MacLean *et al.*, 2007). The genomes of rhizobia are very disparate, and analysis of gene content suggests that there is not a single shared genetic program that allows for plant-microbe symbioses: genes that are involved in symbiosis frequently have close homologs in non-symbiotic relatives, or they are very narrowly distributed within the rhizobial phylogeny (Amadou *et al.*, 2008; Masson-Boivin *et al.*, 2009). All rhizobia employ the molybdenum-dependent nitrogenase, which requires very low oxygen levels and has a high

energy requirement of 16 moles of ATP per mole of nitrogen fixed, conditions that plants meet with high carbon supply and leghemoglobin (Masson-Boivin *et al.*, 2009).

The largest genus in terms of number of strains isolated thus far is *Bradyrhizobium*, which nodulates the most diverse set of legume genera as well as *Parasponia* (Parker, 2015). Because *Bradyrhizobium* nodulates such a broad host range, and also nodulate most of the early diverging nodulating legumes, it has been suggested that *Bradyrhizobium* was the original legume symbiont (Parker, 2015; Sprent *et al.*, 2017). *Rhizobium* and *Ensifer* (*Sinorhizobium*) are also common α-rhizobia that are found in soil in a wide variety of environments across many continents (Peix *et al.*, 2015; Sprent *et al.*, 2017).

The mechanisms of legume-rhizobia interaction

An important defining characteristic of different types of legumes is whether they form determinate or indeterminate nodules. Determinate nodules are formed by tropical legumes, such as *Phaseolus vulgaris* (common bean) and *Glycine max* (soybean). Determinate nodules have a transient meristem and grow for a certain time period (usually a few weeks) and then stop, resulting in round nodules (Udvardi & Poole, 2013). Inside a determinate nodule, rhizobia differentiate into a homogenous group of bacteroids that are similar to free living rhizobia. Typically several bacteroids are found in each peribacteroid unit (PBU), which is a group of bacteroids surrounded by the plant membrane (Denison, 2000). Bacteroids in determinate nodules can accumulate poly-β-hydroxybutyrate (PHB), why may allow them to hoard resources from their host (Lodwig & Poole, 2003). Determinate nodules convert fixed NH_3 to ureides for export through the xylem to the rest of the plant (Baral *et al.*, 2016).

In contrast, the legumes used in these studies all form indeterminate nodules. In indeterminate nodules, the nodule maintains an active meristem and continues to grow throughout its life cycle (Udvardi & Poole, 2013). This results in a nodule that is divided into various development zones: the meristem, the invasion zone where rhizobia move from infection threads to plant cells, the interzone where rhizobia are differentiated, the nitrogen fixation zone, and the senescence zone where the bacteroids are degraded and nitrogen fixation stops (Suzaki *et al.*, 2015). *Medicago truncatula* and *Trifolium* species are members of the Inverted Repeat Lacking Clade (IRLC), which has lost one of two 25-kb inverted repeats in the chloroplast genome (Sprent *et al.*, 2017). IRLC legumes are unique in their rhizobial specificity and the degree to which they control their rhizobial partners. IRLC legumes produce nodule cysteine rich (NCR) peptides that induce terminal bacteroid differentiation in their rhizobia while also increasing rhizobial membrane permeability and inducing extreme endoreduplication (Mergaert *et al.*, 2006; Van de Velde *et al.*, 2010). This means that the rhizobia that fix nitrogen in nodules of IRLC legumes cannot reproduce; only the undifferentiated rhizobia remaining in the infection threads are able to reproduce (Denison, 2000). In addition, the swollen bacteroids induced by NCR peptides appear to be more efficient at nitrogen fixation than those in determinate systems (Oono & Denison, 2010). Swollen bacteroids have evolved independently at least five times, suggesting that this trait is beneficial (Oono & Denison, 2010). However, this theory is difficult to test because a given plant species can only form one type of nodule, and few rhizobia nodulate plants that produce swollen and nonswollen bacteroids (Oono & Denison, 2010; Terpolilli *et al.*, 2012). PHB accumulation has not been detected in swollen bacteroids (Paau *et al.*, 1980; Lodwig & Poole, 2003), although free living rhizobia and rhizobia in the infection thread can accumulate PHB (Paau *et al.*, 1980; Tombolini & Nuti, 1989). In symbiosis, PHB is synthesized but is

presumably used as quickly as it is made, perhaps to fuel bacteroid differentiation (Lodwig *et al.*, 2005, Trainer & Charles, 2006). In addition, indeterminate nodules export fixed nitrogen as asparagine rather than the ureides used by determinate nodules (Poole *et al.*, 2018).

The initiation of nodules requires a complicated signaling interaction between legumes and rhizobia. Rhizobia are chemotactically attracted to plant roots (Miller *et al.*, 2007). When the rhizobia get close enough to the roots, they detect flavonoids (polyhydroxy polyphenol secondary metabolites) released by the plant. If the plant-rhizobia combination is compatible, the flavonoids bind to the nodD receptor protein, which induces the production of rhizobia nod factors (Peck *et al.*, 2006). Nod factors are signaling molecules with a chitooligosaccharide backbone consisting of several N-acetylglucosamine subunits (Long, 1996). These subunits are decorated with various chemical groups, and the structure of the nod factors determines its effect on the host plant (Roche *et al.*, 1991; Oldroyd, 2013). If the interaction is compatible, the nod factor is recognized by a receptor-like kinase with extracellular LysM domains (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). When nod factor is recognized by LysM receptor-like kinases, the receptor interacts with a number of proteins to induce calcium spiking (Ehrhardt *et al.*, 1996; Kosuta *et al.*, 2008). The calcium-activated kinase CCaMK perceives calcium oscillations first in epidermal cells and later in cortical cells, and activates transcription factors to change gene expression and lead to nodule organogenesis (Mitra & Long, 2004; Lévy *et al.*, 2004; Guinel, 2015). A root hair curls around a single rhizobial cell or microcolony, forming a shepherd's hook shape and trapping the rhizobia, which continue to divide (Oldroyd, 2013). The infection thread is an invagination of the plant cell, and grows toward the nodule meristem that is developing de novo (Gage, 2004). Once the infection thread reaches the nodule, the rhizobia are released into infection droplets, which are surrounded by plant membrane tissue (Garg & Renseigne, 2007).

These organelle-like structures are known as symbiosomes, and rhizobia inside them differentiate into bacteroids and begin nitrogen fixation plant cells to begin nitrogen fixation (Mortier *et al.*, 2012).

Once rhizobia are inside the nodule, plants create an environment conducive to nitrogen fixation. Plants use leghemoglobin proteins to keep free oxygen levels low in nodules and prevent inactivation of nitrogenase (Ott *et al.*, 2005). Plants feed their rhizobia by transporting sucrose from the shoot to the root, then converting it to malate before the dicarboxylate transporter brings it to the bacteroids (Lodwig & Poole, 2003; Yurgel & Kahn, 2004). In the bacteroids, the carbon source feeds the tricarboxylic (TCA) cycle, which provides electrons and ATP for the energetically intensive process of nitrogen fixation (Poole & Allaway, 2000; Udvardi & Poole, 2013). Most rhizobia lack *nifV* and thus cannot produce homocitrate, a part of the Fe-molybdenum cofactor that is necessary for nitrogenase function (Andrews *et al.*, 2009). The plant is able to synthesize homocitrate to allow the rhizobia to fix nitrogen inside a nodule (Hakoyama *et al.*, 2009). Plants can also use the environment of the nodule to control rhizobia. Bacteroids become auxotrophs and require branched chain amino acids from the plant to fix nitrogen (Lodwig *et al.*, 2003; Prell & Poole, 2006; Prell *et al.*, 2010). The ammonia produced by nitrogenase is transported to the infected plant cell and assimilated through GS-GOGAT (Udvardi & Day, 1997). The form in which nitrogen is transported depends on the type of nodule: plants that form indeterminate nodules transport amides (glutamine and asparagine) (Prell & Poole, 2006), while plants that form determinate nodules transport nitrogen as ureides (Baral *et al.*, 2016).

As discussed above, for the nodule to be formed, plant flavonoids need to be recognized by nodD in the rhizobia and the rhizobial nod factors need to be recognized by plant lysM

receptors. Tropical legumes are typically much more promiscuous than their temperate counterparts, but it is not entirely clear what implications this has for signaling between tropical legumes and their rhizobia (Lira *et al.*, 2015). There are many levels of specificity in the legume-rhizobia symbiosis: some rhizobia may be able to initiate nodulation but fail to form successful infection threads (Simsek *et al.*, 2007), or form nodules but fail to fix nitrogen (Yates *et al.*, 2005). The factors underlying these levels of specificity are not entirely clear. Some rhizobia use Type III secretion systems to release nodulation outer proteins (NOPs) that may suppress the plant immune response or modulate plant cytoskeletal rearrangement during nodule formation (Deakin & Broughton, 2009). Another important factor in nodulation is exopolysaccharides (EPS), which form part of the rhizobial cell surface. Defects in EPS production can inhibit nodule formation during infection thread growth and at the stage of nitrogen fixation (Finan *et al.*, 1985; Leigh *et al.*, 1985; Simsek *et al.*, 2007).

Ecological and evolutionary theory

Since a single plant generally interacts with a number of rhizobial strains in the soil, the legume-rhizobia mutualism may be subject to the tragedy of the commons (Hardin, 1968). That is, an individual rhizobium benefits from collective nitrogen fixation (which fuels plant photosynthesis), but that rhizobium would benefit more from taking its resources away from nitrogen fixation and instead focusing them on its own reproduction (Denison, 2000; West *et al.*, 2002; Denison *et al.*, 2003; Kiers & Denison, 2008). Rhizobia behaving in this way are commonly referred to as cheaters. The exact definition of cheating has been a controversial topic (Frederickson, 2013; Jones *et al.*, 2015), but the current accepted definition is behavior that increases the fitness of the cheater above average fitness in that population, while decreasing the

fitness of the cheater's partner below average fitness in the partner population (Jones *et al.*, 2015). Evolutionary theory predicts that cheating rhizobia would take over rhizobial populations (Denison *et al.*, 2003), but empirical studies have found a large diversity of nitrogen fixation abilities in rhizobial populations (Burdon *et al.*, 1999; Thrall *et al.*, 2000). This leads to two questions: first, why do cheaters not take over rhizobial populations? And second, why do poorly performing rhizobia exist, if plant controls on cheaters are so effective?

For question one, several mechanisms have been suggested for how plants control cheaters. The first method is partner choice, which is a pre-infection mechanism in which plants avoid interacting with poor-quality rhizobia (Bull & Rice, 1991; Simms & Taylor, 2002; Kiers & Denison, 2008). Partner choice requires plants to be able to determine a rhizobium's nitrogen fixation level before nodulation, presumably through some sort of signal exchange (Bull & Rice, 1991; Archetti *et al.*, 2011). Partner choice would also require less investment than other methods of mutualism stabilization, since the plant does not have to waste energy initiating nodules with low quality partners (Simms & Taylor, 2002). However, effective partner choice would require rhizobia to offer honest signals about their nitrogen fixation level (Kiers & Denison, 2008). Cooperation theory suggests that low quality rhizobium would be benefited by sending dishonest signals about its level of cooperation (Kiers & Denison, 2008; Oono *et al.*, 2009; Padje *et al.*, 2016). Given that rhizobia are also expected to be at an advantage in evolutionary arms races due to their shorter generation time, it seems unlikely that these signals would be consistently reliable (Kiers & Denison, 2008; Oono *et al.*, 2009; Padje *et al.*, 2016). However, if signals have a high mutation rate and there is linkage disequilibrium between signal genes and quality genes, partner choice can maintain cooperation (Jansen & van Baalen, 2006). Empirical testing of partner choice in legumes has produced mixed results, and the situation is

further complicated by differing definitions of partner choice and sanctions (Frederickson, 2013). Partner choice is notably difficult to test because natural strains that vary in effectiveness likely vary in many other ways that could affect nodulation competitiveness, and an isogenic mutant that has been made non-fixing would be expected to retain the wild-type signal for effectiveness. Partner choice (as demonstrated by preferentially nodulating with the more beneficial strain in a multi-strain inoculation) has been demonstrated in *Medicago truncatula* (Heath & Tiffin, 2009; Gubry-Rangin *et al.*, 2010), *Lotus strigosus* (Sachs *et al.*, 2010b), and *Trifolium purpureum* and *Trifolium polymorphum* (Yates *et al.*, 2005, 2008). However, a different study in *M. truncatula* showed no evidence of partner choice between effective and ineffective mutants (Amarger, 1981), a trend that was also detected in *Pisum sativa* (pea) (Westhoek *et al.*, 2017).

In contrast to partner choice, sanctions are a post-infection method of host control of rhizobia. Sanctions refer to the practice of restricting allocation to poor-performing rhizobia (Kiers *et al.*, 2003; Kiers & Denison, 2008). Alternatively, this concept may be viewed in terms of preferential allocation, where plants reward high-performing rhizobia with more resources (Kiers & van der Heijden, 2006). Sanctions are predicted to require more energy than partner choice because the plant has to expend resource initiating nodules that may contain ineffective rhizobia (Kiers & Denison, 2008). However, sanctions are expected to be more reliable because they avoid the problem of dishonest signaling (Westhoek *et al.*, 2017). Sanctions do not appear to be universal, as sanctions have been identified in pea (Westhoek *et al.*, 2017), soybean (Kiers *et al.*, 2003), and *Lotus japonicus* (Regus *et al.*, 2014, 2015), but not in *Medicago truncatula* (Heath & Tiffin, 2009; Gubry-Rangin *et al.*, 2010; Grillo *et al.*, 2016). A major question in this area is the scale at which plants are able to discriminate between different rhizobial strains--on the whole root level, single nodule, or somewhere in between.

A final mechanism that may prevent the spread of noncooperative rhizobia is partner fidelity feedback. Partner fidelity feedback means that the benefits a symbiont will receive from its partner in the future depend on its investment in the present, because there are positive feedbacks between host and partner fitness (Bull & Rice, 1991; Sachs *et al.*, 2004). This mechanism requires spatial structuring of the population or vertical transmission of symbionts that makes an individual or its relatives more likely to interact repeatedly over time (Yamamura, 1996; Doebeli & Knowlton, 1998). Meta-analysis has provided empirical support for partner-fidelity feedback by showing a positive correlation between plant performance and nodule number and biomass (Friesen, 2012).

This leaves the question of why poor-performing rhizobia are able to persist. Effective host control mechanisms are predicted to produce rhizobial populations that consist entirely of cooperators (West *et al.*, 2002; Sachs *et al.*, 2004; Foster & Wenseleers, 2006). However, ineffective rhizobia are abundant in many environments (Burdon *et al.*, 1999; Denton *et al.*, 2002; Sachs *et al.*, 2010a). One possible explanation is the existence of mixed nodules. While most nodules consist of a rhizobial population that originated from a single cell trapped in a curling root hair, mixed nodules that are initiated by multiple cells of different strains are possible, with 2 to 74% of nodules containing two different strains under lab testing (Denison, 2000; Gage, 2002; Westhoek *et al.*, 2017). Depending on the scale at which sanctions operate, mixed nodules may allow noncooperative rhizobia to escape host control (Denison, 2000; Kiers & Denison, 2008; Steidinger & Bever, 2016). However, recent evidence suggests that at least some plants can specifically sanction noncooperative rhizobia in mixed nodules with cooperative rhizobia (Regus *et al.*, 2017; Daubech *et al.*, 2017). In addition, the mechanisms used by plants

to control poor performing rhizobia do not appear to be universal-different plant species use different mechanisms (Pahua *et al.*, 2018).

The abiotic environment and the legume-rhizobia interaction

Legumes have two options for acquiring nitrogen: direct uptake from the soil and trade with rhizobia. Plants can take up either inorganic nitrogen in the form of nitrate or ammonium, or organic nitrogen in the form of amino acids and peptides (Mohd-Radzman *et al.*, 2013). Nitrate tends to be the dominant nitrogen source in aerated soils, while ammonium is more prevalent in acidic and anaerobic soils (Miller & Cramer, 2005). While organic nitrogen sources may play important roles in boreal ecosystems, they have received relatively little attention (Näsholm *et al.*, 2009) and will not be addressed further. Ammonium and nitrate tend to receive more attention because they are typically present in agricultural systems at much higher levels than any other nitrogen source (Miller & Cramer, 2005). Nitrate is the dominant form of nitrogen in most soils, and plants generally prefer nitrate as compared to ammonium because excess nitrate can be stored, while excessive ammonium levels can lead to toxicity for the plant (Glass *et al.*, 2002). Since nitrogen fixation is energetically expensive (Voisin *et al.*, 2003), plants tend to reduce nodulation at high soil nitrogen levels and favor direct nitrogen acquisition instead (Voisin *et al.*, 2002).

High concentrations of nitrogen (above 3 mM), whether as nitrate or ammonium, tend to uniformly inhibit nodulation (Silsbury *et al.*, 1986; Gan *et al.*, 2004; Fei & Vessey, 2009; Dan & Brix, 2009; Mohd-Radzman *et al.*, 2013). Lower concentrations of nitrogen generally promote nodulation (e.g. Weber *et al.*, 2007), though the opposite effect has been shown in other studies (Dan & Brix, 2009). Not all studies assess both total nodule number and specific nodulation, or

nodulation per unit of root biomass, so a treatment that increases root biomass and does not change specific nodulation could report an increase in total nodule number simply due to increased plant size. In *Pisum sativum*, both total and specific nodulation were increased by low concentrations of ammonium (Gulden & Vessey, 1997), while in *Glycine max*, low ammonium concentrations increase total nodulation but decrease specific nodulation (Gulden & Vessey, 1998; Gan *et al.*, 2004).

Nitrate and ammonium have different regulatory effects on root and nodule development, but when plants are exposed to both forms of nitrogen, the effects of nitrate outweigh those of ammonium (Bollman & Vessey, 2006). Ammonium effects on nodulation are less well studied than those of nitrate (Forde & Clarkson, 1999; Gan *et al.*, 2004). Ammonium appears to inhibit nodulation at a relatively early stage in the signaling pathway by inhibiting root hair curling (Barbulova *et al.*, 2007). In contrast, nitrate inhibits nodulation downstream of root hair curling (Barbulova *et al.*, 2007). Nitrate appears to alter flavonoid metabolism, plant defense responses, and the redox state (van Noorden *et al.*, 2016). Nitrate is also believed to play a role in autoregulation of nodulation (AON), a process plants use to control total nodule number (Mortier *et al.*, 2012). The mechanisms of AON have not been fully elucidated, but AON is believed to occur when a signal is produced in the root in response to nodulation (Kinkema *et al.*, 2006). This signal is transported to the shoot, where it induces the production of another signaling molecule that is transported back to the root, where it inhibits the formation of more nodules (Downie, 2014). Evidence suggests that CLE peptides are the root-produced signal, and that they are recognized by leucine-rich repeat receptor-like kinases (LRR-RLKs) in the shoot (Miyazawa *et al.*, 2010; Krusell *et al.*, 2011; Okamoto *et al.*, 2013; Araya *et al.*, 2016; Nishida *et al.*, 2016). These receptors induce the production of cytokinin that is then transported to the roots (Sasaki *et*

al., 2014), where it downregulates nodulation through *TML* and *NIN* genes (Takahara *et al.*, 2013; Soyano *et al.*, 2015). Mutants that are deficient in AON are also frequently nitrate insensitive, suggesting that nitrate signaling and AON may be interconnected (Schnabel *et al.*; Wopereis *et al.*, 2000; Magori *et al.*, 2009). However, nitrate still negatively affected nodulation in AON-deficient mutants (Jeudy *et al.*, 2010; Okamoto & Kawaguchi, 2015), showing that nitrate affects nodulation in ways other than just the AON pathway (Nishida & Suzaki, 2018).

While the effects of nitrogen on nodulation have been well characterized, there is less literature on the effects of light on the legume-rhizobia symbiosis. Nitrogen fixation by rhizobia is an energy-intensive process, and can require up to 28% of the plant's total supply of photosynthate (Kaschuk *et al.*, 2009). It has been suggested that rhizobia can increase the plant's photosynthesis rate because they form such a strong carbon sink (Kaschuk *et al.*, 2009). An interaction between *Phaseolus lunatus* and *Rhizobium* that increased plant biomass and seed production relative to uninoculated plants in high light (600 $\mu molm^{-2}s^{-1}$) decreased plant biomass and seed production relative to uninoculated plants in low light conditions (300 $\mu molm^{-2}s^{-1}$) (Ballhorn *et al.*, 2016). Nodule biomass was unaffected by light level in this study (Ballhorn *et al.*, 2016). *Bradyrhizobium japonicus* increased *Glycine max* shoot biomass and did not affect root biomass at high light (300 $\mu molm^{-2}s^{-1}$), while it decreased root biomass and did not affect shoot biomass at low light (50 $\mu molm^{-2}s^{-1}$) (Lau *et al.*, 2012). In this system, decreasing light sharply decreased nodule biomass (Lau *et al.*, 2012). Light effects have been more thoroughly studied in forest ecosystems due to the importance and prevalence of shading in those ecosystems. In these systems, light has been shown to change responses to mycorrhizal colonization (Gehring, 2003) and to qualitatively change plant-soil feedback, or the process by which plants alter the composition of the microbial community, which then changes the fitness

benefits the plant receives from that community (Smith & Reynolds, 2015). The effects of light on plant-mycorrhizae interactions have been more thoroughly studied. Short term (six day) shading significantly disrupted phosphate transportation in the *Medicago truncatula-Rhizophagus irregularis* symbiosis, while long term shading (38 days) at 35% or below of full greenhouse daylight (~5% of natural sunlight) decreased plant growth in *R. irregularis*-inoculated plants compared to control plants (Konvalinkova *et al.*, 2015). Decreasing light levels from 1300 $\mu molm^{-2}s^{-1}$ to 660 $\mu molm^{-2}s^{-1}$ reduced the ability of *Allium vineale* to preferentially allocate carbon to more effective mycorrhizae (Zheng *et al.*, 2015).

The molecular pathways that translate changes in light into changes in nodulation are not well understood. The effects of light on a soybean variety defective in AON suggests that increased light and CO2 levels increase nodulation in a manner independent of the AON pathway (Hansen *et al.*, 1990; Bacanamwo & Harper, 1997). Chlorophyll absorbs red light but does not absorb far red light. Thus, plants can use phytochrome to detect the red (R) to far red (FR) ratio to determine whether there are other plants nearby competing for light. *Lotus japonicus phyB* mutants show reduced nodulation, and wild-type plants respond to high R/FR through JA signaling (Suzuki *et al.*, 2011). In the *phyB* mutant, nodulation is restricted due to reduced JA-Ile production and reduced transport of JA-Ile from the shoot to the root (Shigeyama *et al.*, 2012). Similarly, the R/FR ratio regulates mycorrhizal colonization through JA signaling (Nagata *et al.*, 2015). Light availability alters levels of flavonoids in legumes (Zavala *et al.*, 2015), which due to their importance in signaling with rhizobia (Zhang *et al.*, 2009), may alter rhizobial colonization levels (Gundel *et al.*, 2014).

The biotic environment and the legume-rhizobia interaction

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts that can supply phosphorus, nitrogen, and other resources to plants in exchange for photosynthetic carbon (Parniske, 2008). Dual inoculation with AMF and rhizobia is predicted to have synergistic effects on plant and microbial performance, since phosphate is a common limiting nutrient for both plant growth and rhizobial nitrogen fixation (Augusto *et al.*, 2013). Indeed, a number of studies have illustrated increased nitrogen fixation and plant growth in the presence of AMF compared to singly-inoculated plants (Carling, 1978; Khan *et al.*, 1995; Chalk *et al.*, 2006; Wang *et al.*, 2011). However, this synergy does not appear to be universal. A meta-analysis detected no synergistic effects between AMF and rhizobia, though the authors caution this may be due to lack of control for soil nutrient levels, which play important roles in both individual symbioses and their synergies (Larimer *et al.*, 2010; Wang *et al.*, 2011; Ossler *et al.*, 2015). In some cases, it seems that dual inoculation with rhizobia and AMF may overtax the limited plant C supply, leading to reduced symbiont function (Bethlenfalvay *et al.*, 1985; Brown & Bethlenfalvay, 1987) or shifting the plant to carbon limitation rather than nutrient limitation, particularly in lower light greenhouse or growth chamber studies (Bethlenfalvay *et al.*, 1983).

The allocation of carbon between rhizobia and AMF in tripartite symbiosis has not been thoroughly explored. It is believed that rhizobia co-opted mycorrhizal signaling for nodule establishment, and the two still share a common symbiosis signaling pathway [cite]. Despite this overlap, there is no evidence for genetic covariance between the establishment and regulation of rhizobial and mycorrhizal symbioses (Ossler & Heath, 2018). A preliminary transcriptomic analysis rhizobia-AMF interactions in *Medicago truncatula* found complex patterns of changes in differentially expressed genes in singly and dually-inoculated plants (Afkhami &

Stinchcombe, 2016). Mycorrhizal colonization displays autoregulation patterns similar to autoregulation of nodulation: once a threshold of colonization is reached, the plant uses systemic signaling to suppress further colonization (Vierheilig, 2004). In split-root systems, pre-colonization of one half of the root system with AMF or rhizobia suppresses colonization by the other symbiont on the other half of the root system (Catford *et al.*, 2003, 2006; Meixner *et al.*, 2005). Rhizobial inhibition of mycorrhizal colonization as well as mycorrhizal autoregulation are deficient in autoregulation of nodulation (AON) mutants, suggesting that autoregulation of both symbionts and inhibition of one symbiont by the other may use the AON pathway (Meixner *et al.*, 2005; Sakamoto *et al.*, 2013).

Rhizobia are only a small part of a large, complex community of microbes in the soil. Plants exert strong effects on the community of microbes associated with their root surface, or rhizosphere (Turner *et al.*, 2013). This community has a very different composition from and is much less diverse than the microbial community in bulk soil, likely due to plant selection (Turner *et al.*, 2013; Tkacz *et al.*, 2015). The effects of rhizobial symbiosis on the makeup of the rest of the microbiome has not been thoroughly studied. There is evidence that the nodulation signaling pathway plays a role in microbiome structuring. *Lotus japonicus* Nod factor receptor5 *(nfr5),* Nodule inception *(nin)* and *Lotus* histidine kinase1 *(lhk1)* exhibited extremely different root and rhizosphere microbiomes compared to wildtype plants (Zgadzaj *et al.*, 2016). These differences were maintained even under nitrogen levels that eliminated nodulation in wildtype plants, suggesting that these effects are due to the changes to symbiosis signaling and not just the loss of symbiosis (Zgadzaj *et al.*, 2016). In addition, the H_2 produced by nodules as a byproduct of nitrogen fixation can affect the makeup of the microbiome and may select for plant-beneficial bacteria (Dong *et al.*, 2003; Maimaiti *et al.*, 2007).

Other members of the plant microbiome may play important roles in mediating the legume-rhizobia symbiosis. There are a number of examples of non-rhizobial "helper strains" improving rhizobial colonization and plant growth benefit when co-inoculated with rhizobia (Sturz *et al.*, 1997; Bai *et al.*, 2002; Egamberdieva *et al.*, 2010). The mechanisms by which these "helper strains" improve the rhizobial symbiosis are not entirely known, but may include cellulase (Ibáñez *et al.*, 2009), auxin (Ibáñez *et al.*, 2009; Mishra *et al.*, 2009), and siderophore production (Maymon *et al.*, 2015), and bacterial quorum sensing (Miao *et al.*, 2018). Similarly, soil bacteria may improve mycorrhizal colonization in a number of ways, including increasing the receptiveness of the host plant to colonization, improving soil conditions for fungal growth, and promoting spore germination and survival (Artursson *et al.*, 2006; Pivato *et al.*, 2009; Hassani *et al.*, 2018).

On a larger scale, legume-rhizobia interactions can also play a role in plant-plant interactions. Rhizobial symbionts may offer plants an opportunity for niche differentiation (Bever, 1999; Parker, 1999), which can promote coexistence and biodiversity (Chesson, 2000). Variation in rhizobial quality can increase the fitness advantage of competing with kin as opposed to nonkin for legumes (Simonsen *et al.*, 2014). Legumes may derive more nitrogen from fixation when they are competing with non-leguminous plants (Karpenstein-Machan & Stuelpnagel, 2000; Hodge & Fitter, 2013). Differentiating their nitrogen uptake strategy to nitrogen fixation and away from direct soil uptake may free up resources for the growth of non-nitrogen fixing plants (Temperton *et al.*, 2007; von Felten *et al.*, 2009). Interacting with microbial symbionts such as rhizobia and AMF has been shown to increase plant diversity in model grassland communities (van der Heijden *et al.*, 2015). However, these effects are likely context-dependent, as symbionts do not affect competition and coexistence in fertilized

ecosystems (Ren *et al.*, 2017). In addition, in some ecosystems with diverse rhizobial populations, mutualism may actually lead to competitive exclusion rather than coexistence of multiple plant lineages (Wilkinson & Parker, 1996; Keller, 2014). Rhizobia can also affect the ability of invasive species to disrupt existing ecosystems: the degree of association with rhizobia is positively correlated with invasion success in *Acacia*, likely due to the benefits of high levels of nitrogen fixation (Rodríguez-Echeverría *et al.*, 2009). Conversely, interactions between plants can also affect how legumes interact with their rhizobia. Intercropping maize with faba bean increases yield and nodulation because maize root exudates increase faba bean flavonoid biosynthesis, leading to increased nodulation and nitrogen fixation (Li *et al.*, 2016). Allelopathic chemicals released by other plants can disrupt the legume-rhizobia symbiosis of another plant (Alsaadawi & Rice, 1982; Portales-Reyes *et al.*, 2015).

Conclusions

The nitrogen fixing symbiosis between legumes and rhizobia is an ancient relationship with important implications for the functioning of agricultural and natural ecosystems. This resource trade has frequently been conceptualized as a biological market, with accompanying assumptions about how trade negotiations are conducted (Grman *et al.*, 2012; Clark *et al.*, 2017). Unfortunately, this theoretical work has rarely been parameterized with empirical data (Clark *et al.*, 2017). To address this gap, chapter 1 describes empirical model parameterization that tests the assumption of fair trade between legume and rhizobia, one of the central assumptions of theoretical biological market models. Importantly, this work assesses how soil nitrogen availability affects trade negotiations between legume and rhizobia. In chapter 2, this assessment of the effects of nutrient availability is extended to explore the effects of manipulating the

availability of both traded resources. While the effects of nitrogen on nodulation have been extensively studied, the effects of carbon availability are less well understood, and there has been almost no exploration of their interactive effects. Thus, chapter 3 will explore how factorial manipulation of nitrogen and light affect the legume-rhizobia symbiosis. In chapter 4, the focus shifts from the effects of the external environment to how the identity of the legume and rhizobia affect the costs and benefits of trade. This research provides insight into how both external and intrinsic factors affect the balance of trade between legumes and rhizobia and will provide insights into the evolutionary dynamics of the symbiosis and its role in natural and agricultural ecosystems.

REFERENCES

REFERENCES

Afkhami ME, Stinchcombe JR. **2016**. Multiple mutualist effects on genomewide expression in the tripartite association between Medicago truncatula, nitrogen-fixing bacteria and mycorrhizal fungi. *Molecular Ecology* **25**: 4946–4962.

Alsaadawi IS, Rice EL. **1982**. Allelopathic effects ofPolygonum aviculare L. II. Isolation, characterization, and biological activities of phytotoxins. *Journal of Chemical Ecology* **8**: 1011–1023.

Amadou C, Pascal G, Mangenot S, Glew M, Bontemps C, Capela D, Carrère S, Cruveiller S, Dossat C, Lajus A, *et al.* 2008. Genome sequence of the beta-rhizobium Cupriavidus taiwanensis and comparative genomics of rhizobia. *Genome research* **18**: 1472–83.

Amarger N. **1981**. Competition for nodule formation between effective and ineffective strains of Rhizobium meliloti. *Soil Biology and Biochemistry* **13**: 475–480.

Andrews M, Andrews ME. **2017**. Specificity in Legume-Rhizobia Symbioses. *International Journal of Molecular Sciences* **18**: 705.

Andrews M, Lea PJ, Raven JA, Azevedo RA. **2009**. Nitrogen use efficiency. 3. Nitrogen fixation: genes and costs. *Annals of Applied Biology* **155**: 1–13.

Araya T, von Wirén N, Takahashi H. **2016**. CLE peptide signaling and nitrogen interactions in plant root development. *Plant Molecular Biology* **91**: 607–615.

Archetti M, Ubeda F, Fudenberg D, Green J, Pierce NE, Yu DW. **2011**. Let the right one in: a microeconomic approach to partner choice in mutualisms. *The American naturalist* **177**: 75–85.

Artursson V, Finlay RD, Jansson JK. **2006**. Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environmental Microbiology* **8**: 1–10.

Augusto L, Delerue F, Gallet-Budynek A, Achat DL. **2013**. Global assessment of limitation to symbiotic nitrogen fixation by phosphorus availability in terrestrial ecosystems using a meta-analysis approach. *Global Biogeochemical Cycles* **27**: 804–815.

Azani N, Babineau M, Bailey CD, Banks H, Barbosa A, Pinto RB, Boatwright J, Borges L, Brown G, Bruneau A, *et al.* 2017. A new subfamily classification of the Leguminosae based on a taxonomically comprehensive phylogeny – The Legume Phylogeny Working Group (LPWG). *Taxon* **66**: 44–77.

Bacanamwo M, Harper JE. **1997**. Response of a hypernodulating soybean mutant to increased photosynthate supply. *Plant Science* **124**: 119–129.

Bago B, Pfeffer P, Shachar-Hill Y. **2000**. Carbon Metabolism and Transport in Arbuscular Mycorrhizas. *Plant Physiology* **124**: 949–958.

Bai Y, D'Aoust F, Smith DL, Driscoll BT. **2002**. Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. *Canadian Journal of Microbiology* **48**: 230–238.

Ballhorn DJ, Schädler M, Elias JD, Millar JA, Kautz S, Smith S, Read D, Harley J, Sprent J, Carney K, *et al.* 2016. Friend or Foe—Light Availability Determines the Relationship between Mycorrhizal Fungi, Rhizobia and Lima Bean (Phaseolus lunatus L.) (R Aroca, Ed.). *PLOS ONE* **11**: e0154116.

Baral B, Teixeira da Silva JA, Izaguirre-Mayoral ML. **2016**. Early signaling, synthesis, transport and metabolism of ureides. *Journal of Plant Physiology* **193**: 97–109.

Barbulova A, Rogato A, D'Apuzzo E, Omrane S, Chiurazzi M. **2007**. Differential Effects of Combined N Sources on Early Steps of the Nod Factor–Dependent Transduction Pathway in Lotus japonicus. *Molecular Plant-Microbe Interactions* **20**: 994–1003.

Bethlenfalvay GJ, Bayne HG, Pacovsky RS. **1983**. Parasitic and mutualistic associations between a mycorrhizal fungus and soybean: The effect of phosphorus on host plant-endophyte interactions. *Physiologia Plantarum* **57**: 543–548.

Bethlenfalvay GJ, Brown MS, Stafford AE. **1985**. Glycine-Glomus-Rhizobium Symbiosis: II. Antagonistic Effects between Mycorrhizal Colonization and Nodulation. *Plant physiology* **79**: 1054–8.

Bever JD. **1999**. Dynamics within mutualism and the maintenance of diversity: inference from a model of interguild frequency dependence. *Ecology Letters* **2**: 52–61.

Bollman MI, Vessey JK. **2006**. Differential effects of nitrate and ammonium supply on nodule initiation, development, and distribution on roots of pea (Pisum sativum). *Canadian Journal of Botany* **84**: 893–903.

Bronstein JL (Ed.). **2015**. *Mutualism*. Oxford: Oxford University Press.

Brown MS, Bethlenfalvay GJ. **1987**. Glycine-Glomus-Rhizobium Symbiosis : VI. Photosynthesis in Nodulated, Mycorrhizal, or N- and P-Fertilized Soybean Plants. *Plant physiology* **85**: 120–123.

Bull JJ, Rice WR. **1991**. Distinguishing mechanisms for the evolution of co-operation. *Journal of Theoretical Biology* **149**: 63–74.

Burdon JJ, Gibson AH, Searle SD, Woods MJ, Brockwell J. **1999**. Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian Acacia: within-species interactions. *Journal of Applied Ecology* **36**: 398–408.

Carling DE. **1978**. Effects of a Vesicular-Arbuscular Mycorrhizal Fungus on Nitrate Reductase and Nitrogenase Activities in Nodulating and Non-Nodulating Soybeans. *Phytopathology* **68**: 1590.

Catford JG, Staehelin C, Larose G, Piché Y, Vierheilig H. **2006**. Systemically suppressed isoflavonoids and their stimulating effects on nodulation and mycorrhization in alfalfa split-root systems. *Plant and Soil* **285**: 257–266.

Catford J-G, Staehelin C, Lerat S, Piché Y, Vierheilig H. **2003**. Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with Nod factors. *Journal of Experimental Botany* **54**: 1481–1487.

Chalk PM, Souza R de F, Urquiaga S, Alves BJR, Boddey RM. **2006**. The role of arbuscular mycorrhiza in legume symbiotic performance. *Soil Biology and Biochemistry* **38**: 2944–2951.

Chamberlain SA, Bronstein JL, Rudgers JA. **2014**. How context dependent are species interactions? (R Etienne, Ed.). *Ecology Letters* **17**: 881–890.

Chesson P. **2000**. Mechanisms of Maintenance of Species Diversity. *Annual Review of Ecology and Systematics* **31**: 343–366.

Clark TJ, Friel CA, Grman E, Shachar-Hill Y, Friesen ML. **2017**. Modelling nutritional mutualisms: challenges and opportunities for data integration (M van Baalen, Ed.). *Ecology Letters* **20**: 1203–1215.

Dan TH, Brix H. **2009**. Growth responses of the perennial legume Sesbania sesban to NH4 and NO3 nutrition and effects on root nodulation. *Aquatic Botany* **91**: 238–244.

Daubech B, Remigi P, Doin de Moura G, Marchetti M, Pouzet C, Auriac M-C, Gokhale CS, Masson-Boivin C, Capela D. **2017**. Spatio-temporal control of mutualism in legumes helps spread symbiotic nitrogen fixation. *eLife* **6**.

Deakin WJ, Broughton WJ. **2009**. Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. *Nature Reviews Microbiology* **7**: 312.

Denison RF. **2000**. Legume Sanctions and the Evolution of Symbiotic Cooperation by Rhizobia. *The American Naturalist* **156**: 567–576.

Denison RF, Bledsoe C, Kahn M, O'Gara F, Simms EL, Thomashow LS. **2003**. Cooperation in the rhizosphere and the 'free rider' problem. *Ecology* **84**: 838–845.

Denton MD, Coventry DR, Murphy PJ, Howieson JG, Bellotti WD. **2002**. Competition between inoculant and naturalised Rhizobium leguminosarum bv. trifolii for nodulation of annual clovers in alkaline soils. *Australian Journal of Agricultural Research* **53**: 1019.

Doebeli M, Knowlton N. **1998**. The evolution of interspecific mutualisms. *Proceedings of the*

National Academy of Sciences of the United States of America **95**: 8676–80.

Dong Z, Wu L, Kettlewell B, Caldwell CD, Layzell DB. **2003**. Hydrogen fertilization of soils - is this a benefit of legumes in rotation? *Plant, Cell and Environment* **26**: 1875–1879.

Downie JA. **2014**. Legume nodulation. *Current Biology* **24**: R184–R190.

Egamberdieva D, Berg G, Lindström K, Räsänen LA. **2010**. Co-inoculation of Pseudomonas spp. with Rhizobium improves growth and symbiotic performance of fodder galega (Galega orientalis Lam.). *European Journal of Soil Biology* **46**: 269–272.

Ehrhardt DW, Wais R, Long SR. **1996**. Calcium Spiking in Plant Root Hairs Responding to Rhizobium Nodulation Signals. *Cell* **85**: 673–681.

Fei H, Vessey JK. **2009**. Stimulation of nodulation in Medicago truncatula by low concentrations of ammonium: quantitative reverse transcription PCR analysis of selected genes. *Physiologia Plantarum* **135**: 317–330.

von Felten S, Hector A, Buchmann N, Niklaus PA, Schmid B, Scherer-Lorenzen M. **2009**. Belowground nitrogen partitioning in experimental grassland plant communities of varying species richness. *Ecology* **90**: 1389–1399.

Finan TM, Hirsch AM, Leigh JA, Johansen E, Kuldau GA, Deegan S, Walker GC, Signer ER. **1985**. Symbiotic mutants of rhizobium meliloti that uncouple plant from bacterial differentiation. *Cell* **40**: 869–877.

Forde BG, Clarkson DT. **1999**. Nitrate and Ammonium Nutrition of Plants: Physiological and Molecular Perspectives. *Advances in Botanical Research* **30**: 1–90.

Foster KR, Wenseleers T. **2006**. A general model for the evolution of mutualisms. *Journal of Evolutionary Biology* **19**: 1283–1293.

Frederickson ME. **2013**. Rethinking Mutualism Stability: Cheaters and the Evolution of Sanctions. *The Quarterly Review of Biology* **88**: 269–295.

Friesen ML. **2012**. Widespread fitness alignment in the legume-rhizobium symbiosis. *The New phytologist* **194**: 1096–111.

Friesen ML, Heath KD. **2013**. One hundred years of solitude: integrating single-strain inoculations with community perspectives in the legume-rhizobium symbiosis. *New Phytologist* **198**: 7–9.

Gage DJ. **2002**. Analysis of infection thread development using Gfp- and DsRed-expressing Sinorhizobium meliloti. *Journal of bacteriology* **184**: 7042–6.

Gage DJ. **2004**. Infection and Invasion of Roots by Symbiotic, Nitrogen-Fixing Rhizobia during

Nodulation of Temperate Legumes. *Microbiology and Molecular Biology Reviews* **68**: 280–300.

Gan Y, Stulen I, van Keulen H, Kuiper PJC. **2004**. Low concentrations of nitrate and ammonium stimulate nodulation and N2 fixation while inhibiting specific nodulation (nodule DW g−1 root dry weight) and specific N2 fixation (N2 fixed g−1 root dry weight) in soybean. *Plant and Soil* **258**: 281–292.

Gano-Cohen KA, Stokes PJ, Blanton MA, Wendlandt CE, Hollowell AC, Regus JU, Kim D, Patel S, Pahua VJ, Sachs JL. **2016**. Nonnodulating Bradyrhizobium spp. Modulate the Benefits of Legume-Rhizobium Mutualism. *Applied and environmental microbiology* **82**: 5259–68.

Garg N, Renseigne N. **2007**. Symbiotic nitrogen fixation in legume nodules: process and signaling. A review. *Sustain. Dev* **27**: 59–68.

Gehring CA. **2003**. Growth responses to arbuscular mycorrhizae by rain forest seedlings vary with light intensity and tree species. *Plant Ecology* **167**: 127–139.

Ghoul M, Griffin AS, West SA. **2014**. TOWARD AN EVOLUTIONARY DEFINITION OF CHEATING. *Evolution* **68**: 318–331.

Glass ADM, Britto DT, Kaiser BN, Kinghorn JR, Kronzucker HJ, Kumar A, Okamoto M, Rawat S, Siddiqi MY, Unkles SE, *et al.* 2002. The regulation of nitrate and ammonium transport systems in plants. *Journal of Experimental Botany* **53**: 855–864.

Grillo MA, Stinchcombe JR, Heath KD. **2016**. Nitrogen addition does not influence pre-infection partner choice in the legume-rhizobium symbiosis. *American journal of botany* **103**: 1763–1770.

Grman E, Robinson TMP, Klausmeier CA. **2012**. Ecological specialization and trade affect the outcome of negotiations in mutualism. *The American naturalist* **179**: 567–81.

Gubry-Rangin C, Garcia M, Béna G. **2010**. Partner choice in Medicago truncatula-Sinorhizobium symbiosis. *Proceedings. Biological sciences / The Royal Society* **277**: 1947–51.

Guinel FC. **2015**. Ethylene, a Hormone at the Center-Stage of Nodulation. *FRONTIERS IN PLANT SCIENCE* **6**: 1121–1121.

Gulden RH, Vessey JK. **1997**. The stimulating effect of ammonium on nodulation in Pisum sativum L. is not long lived once ammonium supply is discontinued. *Plant and Soil* **195**: 195–205.

Gulden RH, Vessey JK. **1998**. Low concentrations of ammonium inhibit specific nodulation (nodule number g-1 root DW) in soybean (Glycine max [L.] Merr.). *Plant and Soil* **198**: 127–136.

Gundel PE, Pierik R, Mommer L, Ballaré CL. **2014**. Competing neighbors: light perception and root function. *Oecologia* **176**: 1–10.

Hakoyama T, Niimi K, Watanabe H, Tabata R, Matsubara J, Sato S, Nakamura Y, Tabata S, Jichun L, Matsumoto T, *et al.* 2009. Host plant genome overcomes the lack of a bacterial gene for symbiotic nitrogen fixation. *Nature* **462**: 514–7.

Hansen AP, Gresshoff PM, Pate JS, Day DA. **1990**. Interactions between Irradiance Levels, Nodulation and Nitrogenase Activity of Soybean cv. Bragg and a Supernodulating Mutant. *Journal of Plant Physiology* **136**: 172–179

Hardin G. **1968**. The tragedy of the commons. *Science* **162**: 1243–1248.

Hassani MA, Durán P, Hacquard S. **2018**. Microbial interactions within the plant holobiont. *Microbiome 2018 6:1* **6**: 58.

Heath KD, Tiffin P. **2009**. Stabilizing mechanisms in a legume-rhizobium mutualism. *Evolution; international journal of organic evolution* **63**: 652–62.

van der Heijden MG, Bruin S de, Luckerhoff L, van Logtestijn RS, Schlaeppi K. **2015**. A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity, plant nutrition and seedling recruitment. *The ISME journal* **10**: 389–399.

Hodge A, Fitter AH. **2013**. Microbial mediation of plant competition and community structure (D Robinson, Ed.). *Functional Ecology* **27**: 865–875.

Hollowell AC, Regus JU, Turissini D, Gano-Cohen KA, Bantay R, Bernardo A, Moore D, Pham J, Sachs JL. **2016**. Metapopulation dominance and genomic-island acquisition of Bradyrhizobium with superior catabolic capabilities. *Proceedings of the Royal Society of London B: Biological Sciences* **283**.

Ibáñez F, Angelini J, Taurian T, Tonelli ML, Fabra A. **2009**. Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. *Systematic and Applied Microbiology* **32**: 49–55.

Jansen VAA, van Baalen M. **2006**. Altruism through beard chromodynamics. *Nature* **440**: 663–666.

Jeudy C, Ruffel S, Freixes S, Tillard P, Santoni AL, Morel S, Journet E-P, Duc G, Gojon A, Lepetit M, *et al.* 2010. Adaptation of Medicago truncatula to nitrogen limitation is modulated via local and systemic nodule developmental responses. *New Phytologist* **185**: 817–828.

Johnson NC, Graham JH, Smith FA. **1997**. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytologist* **135**: 575–585.

Jones EI, Afkhami ME, Akçay E, Bronstein JL, Bshary R, Frederickson ME, Heath KD,

Hoeksema JD, Ness JH, Pankey MS, *et al.* 2015. Cheaters must prosper: reconciling theoretical and empirical perspectives on cheating in mutualism (N van Dam, Ed.). *Ecology Letters* **18**: 1270–1284.

Karpenstein-Machan M, Stuelpnagel R. **2000**. Biomass yield and nitrogen fixation of legumes monocropped and intercropped with rye and rotation effects on a subsequent maize crop. *Plant and Soil* **218/2**: 215–232.

Kaschuk G, Kuyper TW, Leffelaar PA, Hungria M, Giller KE. **2009**. Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? *Soil Biology and Biochemistry* **41**: 1233–1244.

Keller KR. **2014**. Mutualistic rhizobia reduce plant diversity and alter community composition. *Oecologia* **176**: 1101–1109.

Khan MK, Sakamoto K, Yoshida T. **1995**. Dual inoculation of peanut with *Glomus* sp. and *Bradyrhizobium* sp. enhanced the symbiotic nitrogen fixation as assessed by ^{15}N-Technique. *Soil Science and Plant Nutrition* **41**: 769–779.

Kiers ET, Denison RF. **2008**. Sanctions, Cooperation, and the Stability of Plant-Rhizosphere Mutualisms. *Annual Review of Ecology, Evolution, and Systematics* **39**: 215–236.

Kiers ET, van der Heijden MGA. **2006**. Mutualistic stability in the the arbuscular mycorrhizal symbiosis: exploring hypotheses of evolutionary cooperation. *Ecology* **87**: 1627–1636.

Kiers ET, Rousseau RA, West SA, Denison RF. **2003**. Host sanctions and the legume–rhizobium mutualism. *Nature* **425**: 78–81.

Kinkema M, Scott PT, Gresshoff PM. **2006**. Legume nodulation: successful symbiosis through short- and long-distance signalling. *Functional Plant Biology* **33**: 707.

KonvalinkovÃ¡ T, Pachel D, JanouÅ¡kovÃ¡ M, Gryndler M, Jansa J. **2015**. Duration and intensity of shade differentially affects mycorrhizal growth- and phosphorus uptake responses of Medicago truncatula. *Frontiers in Plant Science* **6**: 65.

Kosuta S, Hazledine S, Sun J, Miwa H, Morris RJ, Downie JA, Oldroyd GED. **2008**. Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. *Proceedings of the National Academy of Sciences* **105**: 9823–9828.

Krusell L, Sato N, Fukuhara I, Koch BE V., Grossmann C, Okamoto S, Oka-Kira E, Otsubo Y, Aubert G, Nakagawa T, *et al.* 2011. The Clavata2 genes of pea and Lotus japonicus affect autoregulation of nodulation. *The Plant Journal* **65**: 861–871.

Laguerre G, Bardin M, Amarger N. **1993**. Isolation from soil of symbiotic and nonsymbiotic *Rhizobium leguminosarum* by DNA hybridization. *Canadian Journal of Microbiology* **39**: 1142–1149.

Larimer AL, Bever JD, Clay K. **2010**. The interactive effects of plant microbial symbionts: a review and meta-analysis. *Symbiosis* **51**: 139–148.

Lau JA, Bowling EJ, Gentry LE, Glasser PA, Monarch EA, Olesen WM, Waxmonsky J, Young RT. **2012**. Direct and interactive effects of light and nutrients on the legume-rhizobia mutualism. *Acta Oecologica* **39**: 80–86.

Leigh JA, Signer ER, Walker GC. **1985**. Exopolysaccharide-deficient mutants of Rhizobium meliloti that form ineffective nodules. *Proceedings of the National Academy of Sciences of the United States of America* **82**: 6231–5.

Lévy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet E-P, Ané J-M, Lauber E, Bisseling T, *et al.* 2004. A putative Ca2+ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science (New York, N.Y.)* **303**: 1361–4.

Li B, Li Y-Y, Wu H-M, Zhang F-F, Li C-J, Li X-X, Lambers H, Li L. **2016**. Root exudates drive interspecific facilitation by enhancing nodulation and N2 fixation. *Proceedings of the National Academy of Sciences of the United States of America* **113**: 6496–501.

Lira MA, Nascimento LRS, Fracetto GGM. **2015**. Legume-rhizobia signal exchange: promiscuity and environmental effects. *Frontiers in Microbiology* **6**: 945.

Lodwig EM, Hosie AHF, Bourdès A, Findlay K, Allaway D, Karunakaran R, Downie JA, Poole PS. **2003**. Amino-acid cycling drives nitrogen fixation in the legume–Rhizobium symbiosis. *Nature* **422**: 722–726.

Lodwig EM, Leonard M, Marroqui S, Wheeler TR, Findlay K, Downie JA, Poole PS. **2005**. Role of Polyhydroxybutyrate and Glycogen as Carbon Storage Compounds in Pea and Bean Bacteroids. *Molecular Plant-Microbe Interactions* **18**: 67–74.

Lodwig E, Poole P. **2003**. Metabolism of Rhizobium Bacteroids. *Critical Reviews in Plant Sciences* **22**: 37–78.

Long SR. **1996**. Rhizobium symbiosis: nod factors in perspective. *The Plant cell* **8**: 1885–98.

MacLean AM, Finan TM, Sadowsky MJ. **2007**. Genomes of the symbiotic nitrogen-fixing bacteria of legumes. *Plant physiology* **144**: 615–22.

Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, *et al.* 2003. A receptor kinase gene of the LysM type is involved in legumeperception of rhizobial signals. *Nature* **425**: 637–640.

Magori S, Oka-Kira E, Shibata S, Umehara Y, Kouchi H, Hase Y, Tanaka A, Sato S, Tabata S, Kawaguchi M. **2009**. *TOO MUCH LOVE* , a Root Regulator Associated with the Long-Distance Control of Nodulation in *Lotus japonicus*. *Molecular Plant-Microbe Interactions* **22**: 259–268.

Maimaiti J, Zhang Y, Yang J, Cen Y-P, Layzell DB, Peoples M, Dong Z. **2007**. Isolation and characterization of hydrogen-oxidizing bacteria induced following exposure of soil to hydrogen gas and their impact on plant growth. *Environmental Microbiology* **9**: 435–444.

Masson-Boivin C, Giraud E, Perret X, Batut J. **2009**. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? *Trends in Microbiology* **17**: 458–466.

Maymon M, Martínez-Hidalgo P, Tran SS, Ice T, Craemer K, Anbarchian T, Sung T, Hwang LH, Chou M, Fujishige NA, *et al.* 2015. Mining the phytomicrobiome to understand how bacterial coinoculations enhance plant growth. *Frontiers in Plant Science* **6**: 784.

Meixner C, Ludwig-Müller J, Miersch O, Gresshoff P, Staehelin C, Vierheilig H. **2005**. Lack of mycorrhizal autoregulation and phytohormonal changes in the supernodulating soybean mutant nts1007. *Planta* **222**: 709–715.

Mergaert P, Uchiumi T, Alunni B, Evanno G, Cheron A, Catrice O, Mausset A-E, Barloy-Hubler F, Galibert F, Kondorosi A, *et al.* 2006. Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium-legume symbiosis. *Proceedings of the National Academy of Sciences* **103**: 5230–5235.

Miao J, Zhang N, Liu H, Wang H, Zhong Z, Zhu J. **2018**. Soil commensal rhizobia promote Rhizobium etli nodulation efficiency through CinR-mediated quorum sensing. *Archives of Microbiology*: 1–10.

Miller AJ, Cramer MD. **2005**. Root nitrogen acquisition and assimilation. In: Springer, Dordrecht, 1–36.

Miller LD, Yost CK, Hynes MF, Alexandre G. **2007**. The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae* is essential for competitive nodulation. *Molecular Microbiology* **63**: 348–362.

Mishra PK, Mishra S, Selvakumar G, Kundu S, Shankar Gupta H. **2009**. Enhanced soybean (*Glycine max* L.) plant growth and nodulation by *Bradyrhizobium japonicum*- SB1 in presence of *Bacillus thuringiensis*- KR1. *Acta Agriculturae Scandinavica, Section B - Plant Soil Science* **59**: 189–196.

Mitra RM, Long SR. **2004**. Plant and bacterial symbiotic mutants define three transcriptionally distinct stages in the development of the Medicago truncatula/Sinorhizobium meliloti symbiosis. *Plant physiology* **134**: 595–604.

Miyazawa H, Oka-Kira E, Sato N, Takahashi H, Wu G-J, Sato S, Hayashi M, Betsuyaku S, Nakazono M, Tabata S, *et al.* 2010. The receptor-like kinase KLAVIER mediates systemic regulation of nodulation and non-symbiotic shoot development in Lotus japonicus. *Development (Cambridge, England)* **137**: 4317–25.

Mohd-Radzman NA, Djordjevic MA, Imin N. **2013**. Nitrogen modulation of legume root

architecture signaling pathways involves phytohormones and small regulatory molecules. *Frontiers in Plant Science* **4**: 385.

Mortier V, Holsters M, Goormachtig S. **2012**. Never too many? How legumes control nodule numbers. *Plant, Cell & Environment* **35**: 245–258.

Mus F, Crook MB, Garcia K, Costas AG, Geddes BA, Kouri ED, Paramasivan P, Ryu MH, Oldroyd GED, Poole PS, *et al.* 2016. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. *Applied and Environmental Microbiology* **82**: 3698–3710.

Nagata M, Yamamoto N, Shigeyama T, Terasawa Y, Anai T, Sakai T, Inada S, Arima S, Hashiguchi M, Akashi R, *et al.* 2015. Red/Far Red Light Controls Arbuscular Mycorrhizal Colonization via Jasmonic Acid and Strigolactone Signaling. *Plant and Cell Physiology* **56**: pcv135.

Näsholm T, Kielland K, Ganeteg U. **2009**. Uptake of organic nitrogen by plants. *New Phytologist* **182**: 31–48.

Nishida H, Handa Y, Tanaka S, Suzaki T, Kawaguchi M. **2016**. Expression of the CLE-RS3 gene suppresses root nodulation in Lotus japonicus. *Journal of Plant Research* **129**: 909–919.

Nishida H, Suzaki T. **2018**. Nitrate-mediated control of root nodule symbiosis. *Current Opinion in Plant Biology* **44**: 129–136.

van Noorden G, Verbeek R, Dinh Q, Jin J, Green A, Ng J, Mathesius U. **2016**. Molecular Signals Controlling the Inhibition of Nodulation by Nitrate in Medicago truncatula. *International Journal of Molecular Sciences* **17**: 1060.

Okamoto S, Kawaguchi M. **2015**. Shoot HAR1 mediates nitrate inhibition of nodulation in *Lotus japonicus*. *Plant Signaling & Behavior* **10**: e1000138.

Okamoto S, Shinohara H, Mori T, Matsubayashi Y, Kawaguchi M. **2013**. Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. *Nature Communications* **4**: 2191.

Oldroyd GED. **2013**. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature reviews. Microbiology* **11**: 252–63.

Oono R, Denison RF. **2010**. Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant physiology* **154**: 1541–8.

Oono R, Denison RF, Kiers ET. **2009**. Controlling the reproductive fate of rhizobia: how universal are legume sanctions? *The New phytologist* **183**: 967–79.

Op den Camp R, Streng A, De Mita S, Cao Q, Polone E, Liu W, Ammiraju JSS, Kudrna D, Wing R, Untergasser A, *et al.* 2011. LysM-type mycorrhizal receptor recruited for rhizobium

symbiosis in nonlegume Parasponia. *Science (New York, N.Y.)* **331**: 909–12.

Ossler JN, Heath KD. **2018**. Shared Genes but Not Shared Genetic Variation: Legume Colonization by Two Belowground Symbionts. *The American Naturalist*: 000–000.

Ossler JN, Zielinski CA, Heath KD. **2015**. Tripartite mutualism: facilitation or trade-offs between rhizobial and mycorrhizal symbionts of legume hosts. *American journal of botany* **102**: 1332–41.

Ott T, van Dongen JT, Gu¨nther C, Krusell L, Desbrosses G, Vigeolas H, Bock V, Czechowski T, Geigenberger P, Udvardi MK. **2005**. Symbiotic Leghemoglobins Are Crucial for Nitrogen Fixation in Legume Root Nodules but Not for General Plant Growth and Development. *Current Biology* **15**: 531–535.

Paau AS, Bloch CB, Brill WJ. **1980**. Developmental fate of Rhizobium meliloti bacteroids in alfalfa nodules. *Journal of bacteriology* **143**: 1480–90.

Padje A van't, Whiteside MD, Kiers ET. **2016**. Signals and cues in the evolution of plant–microbe communication. *Current Opinion in Plant Biology* **32**: 47–52.

Pahua VJ, Stokes PJN, Hollowell AC, Regus JU, Gano-Cohen KA, Wendlandt CE, Quides KW, Lyu JY, Sachs JL. **2018**. Fitness variation among host species and the paradox of ineffective rhizobia. *Journal of Evolutionary Biology*.

Parker MA. **1999**. Mutualism in Metapopulations of Legumes and Rhizobia. *The American naturalist* **153**: S48–S60.

Parker MA. **2015**. The Spread of Bradyrhizobium Lineages Across Host Legume Clades: from Abarema to Zygia. *Microbial Ecology* **69**: 630–640.

Parniske M. **2008**. Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature reviews. Microbiology* **6**: 763–75.

Peck MC, Fisher RF, Long SR. **2006**. Diverse flavonoids stimulate NodD1 binding to nod gene promoters in Sinorhizobium meliloti. *Journal of bacteriology* **188**: 5417–27.

Peix A, Ramírez-Bahena MH, Velázquez E, Bedmar EJ. **2015**. Bacterial Associations with Legumes. *Critical Reviews in Plant Sciences* **34**: 17–42.

Pivato B, Offre P, Marchelli S, Barbonaglia B, Mougel C, Lemanceau P, Berta G. **2009**. Bacterial effects on arbuscular mycorrhizal fungi and mycorrhiza development as influenced by the bacteria, fungi, and host plant. *Mycorrhiza* **19**: 81–90.

Poole P, Allaway D. **2000**. Carbon and nitrogen metabolism in Rhizobium. *Advances in microbial physiology* **43**: 117–63.

Poole P, Ramachandran V, Terpolilli J. **2018**. Rhizobia: from saprophytes to endosymbionts. *Nature Reviews Microbiology*.

Portales-Reyes C, Van Doornik T, Schultheis EH, Suwa T. **2015**. A novel impact of a novel weapon: allelochemicals in Alliaria petiolata disrupt the legume-rhizobia mutualism. *Biological Invasions* **17**: 2779–2791.

Prell J, Bourdès A, Kumar S, Lodwig E, Hosie A, Kinghorn S, White J, Poole P. **2010**. Role of Symbiotic Auxotrophy in the Rhizobium-Legume Symbioses (J Rutherford, Ed.). *PLoS ONE* **5**: e13933.

Prell J, Poole P. **2006**. Metabolic changes of rhizobia in legume nodules. *Trends in Microbiology* **14**: 161–168.

Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Grønlund M, Sato S, Nakamura Y, Tabata S, Sandal N, *et al.* 2003. Plant recognition of symbiotic bacteria requires **Regus JU, Gano KA, Hollowell AC, Sachs JL**. **2014**. Efficiency of partner choice and sanctions in Lotus is not altered by nitrogen fertilization. *Proceedings. Biological sciences / The Royal Society* **281**: 20132587.

Regus JU, Gano KA, Hollowell AC, Sofish V, Sachs JL. **2015**. Lotus hosts delimit the mutualism-parasitism continuum of Bradyrhizobium. *Journal of Evolutionary Biology* **28**: 447–456.

Regus JU, Quides KW, O'Neill MR, Suzuki R, Savory EA, Chang JH, Sachs JL. **2017**. Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. *American Journal of Botany* **104**: 1299–1312.

Ren H, Gao T, Hu J, Yang G. **2017**. The effects of arbuscular mycorrhizal fungi and root interaction on the competition between *Trifolium repens* and *Lolium perenne*. *PeerJ* **5**: e4183.

Roche P, Debellé F, Maillet F, Lerouge P, Faucher C, Truchet G, Dénarié J, Promé J-C. **1991**. Molecular basis of symbiotic host specificity in rhizobium meliloti: nodH and nodPQ genes encode the sulfation of lipo-oligosaccharide signals. *Cell* **67**: 1131–1143.

Rodríguez-Echeverría S, Crisóstomo JA, Nabais C, Freitas H. **2009**. Belowground mutualists and the invasive ability of Acacia longifolia in coastal dunes of Portugal. *Biological Invasions* **11**: 651–661.

Sachs JL, Ehinger MO, Simms EL. **2010a**. Origins of cheating and loss of symbiosis in wild Bradyrhizobium. *Journal of Evolutionary Biology* **23**: 1075–1089.

Sachs JL, Mueller UG, Wilcox TP, Bull JJ. **2004**. The Evolution of Cooperation. *The Quarterly Review of Biology* **79**: 135–160.

Sachs JL, Russell JE, Lii YE, Black KC, Lopez G, Patil AS. **2010b**. Host control over

infection and proliferation of a cheater symbiont. *Journal of evolutionary biology* **23**: 1919–27.

Sachs JL, Skophammer RG, Regus JU. **2011**. Evolutionary transitions in bacterial symbiosis.
Sakamoto K, Ogiwara N, Kaji T. **2013**. Involvement of autoregulation in the interaction between rhizobial nodulation and AM fungal colonization in soybean roots. *Biology and Fertility of Soils* **49**: 1141–1152.

Sasaki T, Suzaki T, Soyano T, Kojima M, Sakakibara H, Kawaguchi M. **2014**. Shoot-derived cytokinins systemically regulate root nodulation. *Nature Communications* **5**: 4983.

Schnabel E, Journet E-P, De Carvalho-Niebel F, Duc G, Frugoli J. The Medicago truncatula SUNN gene encodes a CLV1-like leucine-rich repeat receptor kinase that regulates nodule number and root length.

Shamseldin A, Abdelkhalek A, Sadowsky MJ. **2017**. Recent changes to the classification of symbiotic, nitrogen-fixing, legume-associating bacteria: a review. *Symbiosis* **71**: 91–109.

Shigeyama T, Tominaga A, Arima S, Sakai T, Inada S, Jikumaru Y, Kamiya Y, Uchiumi T, Abe M, Hashiguchi M, *et al.* 2012. Additional cause for reduced JA-Ile in the root of a Lotus japonicus phyB mutant. *Plant Signaling & Behavior* **7**: 746–748.

Silsbury J, Catchpoole D, Wallace W, Silsbury J, Catchpoole D, Wallace W. **1986**. Effects of Nitrate and Ammonium on Nitrogenase (C_2H_2 Reduction) Activity of Swards of Subterranean Clover, *Trifolium subterraneum* L. *Australian Journal of Plant Physiology* **13**: 257.

Simms EL, Taylor DL. **2002**. Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. *Integrative and comparative biology* **42**: 369–80.

Simonsen AK, Stinchcombe JR, Bronstein J, Boucher D, James S, Keeler K, Bronstein J, Sachs J, Simms E, Trivers R, *et al.* 2014. Standing genetic variation in host preference for mutualist microbial symbionts. *Proceedings. Biological sciences / The Royal Society* **281**: 214–217.

Simsek S, Ojanen-Reuhs T, Stephens SB, Reuhs BL. **2007**. Strain-ecotype specificity in Sinorhizobium meliloti-Medicago truncatula symbiosis is correlated to succinoglycan oligosaccharide structure. *Journal of bacteriology* **189**: 7733–40.

Smith LM, Reynolds HL. **2015**. Plant–soil feedbacks shift from negative to positive with decreasing light in forest understory species. *Ecology* **96**: 2523–2532.

Soyano T, Shimoda Y, Hayashi M. **2015**. NODULE INCEPTION Antagonistically Regulates Gene Expression with Nitrate in Lotus japonicus. *Plant and Cell Physiology* **56**: 368–376.

Steidinger BS, Bever JD. **2016**. Host discrimination in modular mutualisms: a theoretical framework for meta-populations of mutualists and exploiters. *Proceedings. Biological sciences* **283**: 20152428.

Sturz A V., Christie BR, Matheson BG, Nowak J. **1997**. Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils* **25**: 13–19.

Suzaki T, Yoro E, Kawaguchi M. **2015**. Chapter Three – Leguminous Plants: Inventors of Root Nodules to Accommodate Symbiotic Bacteria. *International Review of Cell and Molecular Biology* **316**: 111–158.

Suzuki A, Suriyagoda L, Shigeyama T, Tominaga A, Sasaki M, Hiratsuka Y, Yoshinaga A, Arima S, Agarie S, Sakai T, *et al.* 2011. Lotus japonicus nodulation is photomorphogenetically controlled by sensing the red/far red (R/FR) ratio through jasmonic acid (JA) signaling. *Proceedings of the National Academy of Sciences* **108**: 16837–16842.

Takahara M, Magori S, Soyano T, Okamoto S, Yoshida C, Yano K, Sato S, Tabata S, Yamaguchi K, Shigenobu S, *et al.* 2013. TOO MUCH LOVE, a Novel Kelch Repeat-Containing F-box Protein, Functions in the Long-Distance Regulation of the Legume–Rhizobium Symbiosis. *Plant and Cell Physiology* **54**: 433–447.

Temperton VM, Mwangi PN, Scherer-Lorenzen M, Schmid B, Buchmann N. **2007**. Positive interactions between nitrogen-fixing legumes and four different neighbouring species in a biodiversity experiment. *Oecologia* **151**: 190–205.

Terpolilli JJ, Hood GA, Poole PS. **2012**. Chapter 5 – What Determines the Efficiency of N2-Fixing Rhizobium-Legume Symbioses? In: Advances in Microbial Physiology. 325–389.

Thrall PH, Burdon JJ, Woods MJ. **2000**. Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian legumes: interactions within and between genera. *Journal of Applied Ecology* **37**: 52–65.

Tkacz A, Cheema J, Chandra G, Grant A, Poole PS. **2015**. Stability and succession of the rhizosphere microbiota depends upon plant type and soil composition. *The ISME Journal* **9**: 2349–2359.

Tombolini R, Nuti MP. **1989**. Poly(β-hydroxyalkanoate) biosynthesis and accumulation by different Rhizobium species. *FEMS Microbiology Letters* **60**: 299–304.

Trainer MA, Charles TC. **2006**. The role of PHB metabolism in the symbiosis of rhizobia with legumes. *Applied Microbiology and Biotechnology* **71**: 377–386.

Trinick MJ. **1973**. Symbiosis between Rhizobium and the Non-legume, Trema aspera. *Nature* **244**: 459–460.

Turner TR, James EK, Poole PS, Gilbert J, Meyer F, Jansson J, Gordon J, Pace N, Tiedje J, Ley R, *et al.* 2013. The plant microbiome. *Genome Biology* **14**: 209.

Udvardi MK, Day DA. **1997**. METABOLITE TRANSPORT ACROSS SYMBIOTIC

MEMBRANES OF LEGUME NODULES. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 493–523.

Udvardi M, Poole PS. **2013**. Transport and metabolism in legume-rhizobia symbioses. *Annual review of plant biology* **64**: 781–805.

VanInsberghe D, Maas KR, Cardenas E, Strachan CR, Hallam SJ, Mohn WW. **2015**. Non-symbiotic Bradyrhizobium ecotypes dominate North American forest soils. *The ISME Journal* **9**: 2435–2441.

Van de Velde W, Zehirov G, Szatmari A, Debreczeny M, Ishihara H, Kevei Z, Farkas A, Mikulass K, Nagy A, Tiricz H, *et al.* 2010. Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science (New York, N.Y.)* **327**: 1122–6.

Vierheilig H. **2004**. Further root colonization by arbuscular mycorrhizal fungi in already mycorrhizal plants is suppressed after a critical level of root colonization. *Journal of Plant Physiology* **161**: 339–341.

Voisin AS, Salon C, Jeudy C, Warembourg FR. **2003**. Root and Nodule Growth in Pisum sativum L. in Relation to Photosynthesis: Analysis Using 13C-labelling. *Annals of Botany* **92**: 557–563.

Voisin A-S, Salon C, Munier-Jolain NG, Ney B. **2002**. Effect of mineral nitrogen on nitrogen nutrition and biomass partitioning between the shoot and roots of pea (Pisum sativum L.). *Plant and Soil* **242**: 251–262.

Wang X, Pan Q, Chen F, Yan X, Liao H. **2011**. Effects of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on soybean growth as related to root architecture and availability of N and P. *Mycorrhiza* **21**: 173–181.

Weber J, Tham FY, Galiana A, Prin Y, Ducousso M, Lee SK. **2007**. Effects of nitrogen source on the growth and nodulation of Acacia mangium in aeroponic culture. *Journal of Tropical Forest Science* **19**: 103–112.

Werner GDA, Cornwell WK, Cornelissen JHC, Kiers ET. **2015**. Evolutionary signals of symbiotic persistence in the legume-rhizobia mutualism. *Proceedings of the National Academy of Sciences of the United States of America* **112**: 10262–9.

West SA, Kiers ET, Simms EL, Denison RF. **2002**. Sanctions and mutualism stability: why do rhizobia fix nitrogen? *Proceedings. Biological sciences / The Royal Society* **269**: 685–94.

Westhoek A, Field E, Rheling F, Mulley G, Webb I, Poole P, Turnbull L. **2017**. Policing the legume-Rhizobium symbiosis: a critical test of partner choice. *Scientific Reports* **7**: 1419.

Wilkinson HH, Parker MA. **1996**. Symbiotic specialization and the potential for genotypic coexistence in a plant-bacterial mutualism. *Oecologia* **108**: 361–367.

Wopereis J, Pajuelo E, Dazzo FB, Jiang Q, Gresshoff PM, de Bruijn FJ, Stougaard J, Szczyglowski K. **2000**. Short root mutant of Lotus japonicus with a dramatically altered symbiotic phenotype. *The Plant Journal* **23**: 97–114.

Yamamura N. **1996**. Evolution of mutualistic symbiosis: A differential equation model. *Researches on Population Ecology* **38**: 211–218.

Yates RJ, Howieson JG, Real D, Reeve WG, Vivas-Marfisi A, O'Hara GW. **2005**. Evidence of selection for effective nodulation in the Trifolium spp. saymbiosis with Rhizobium leguminosarum biovar trifolii. *Australian Journal of Experimental Agriculture* **45**: 189.

Yates RJ, Howieson JG, Reeve WG, Brau L, Speijers J, Nandasena K, Real D, Sezmis E, O'Hara GW. **2008**. Host–strain mediated selection for an effective nitrogen-fixing symbiosis between Trifolium spp. and Rhizobium leguminosarum biovar trifolii. *Soil Biology and Biochemistry* **40**: 822–833.

Yurgel SN, Kahn ML. **2004**. Dicarboxylate transport by rhizobia. *FEMS Microbiology Reviews* **28**: 489–501.

Zavala JA, Mazza CA, Dillon FM, Chludil HD, Ballare CL. **2015**. Soybean resistance to stink bugs (N ezara viridula and P iezodorus guildinii) increases with exposure to solar UV-B radiation and correlates with isoflavonoid content in pods under field conditions. *Plant, Cell & Environment* **38**: 920–928.

Zgadzaj R, Garrido-Oter R, Jensen DB, Koprivova A, Schulze-Lefert P, Radutoiu S. **2016**. Root nodule symbiosis in Lotus japonicus drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America* **113**: E7996–E8005.

Zhang J, Subramanian S, Stacey G, Yu O. **2009**. Flavones and flavonols play distinct critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti*. *The Plant Journal* **57**: 171–183.

Zheng C, Ji B, Zhang J, Zhang F, Bever JD. **2015**. Shading decreases plant carbon preferential allocation towards the most beneficial mycorrhizal mutualist. *The New phytologist* **205**: 361–8.

CHAPTER 2

UNFAIR TRADE UNDERGROUND REVEALED BY INTEGRATING DATA WITH NASH BARGAINING MODELS

Preface

This project began as a group proposal written for PLB801 in Fall 2013. Our original group included myself, Teresa Clark (TC), Chad Zirbel, Klara Scharnagl, and Pengfei Cao. We developed a proposal that united our interests, spanned scales from molecular biology and ecology, and included a modeling component. Maren Friesen (MF) and Yair Shachar-Hill (YSH) were interested in the ideas we proposed, and TC and I decided to move forward with a subset of the proposal, focusing on testing and modeling carbon-nitrogen trade in legume-rhizobia mutualisms. We participated in regular meetings with MF, YSH, and Emily Grman (who created the model on which this project is based) to conceptualize the questions we were asking and the goals of the study. I led the growth system optimization for our system, and we chose to measure 4- to 6-week-old uninoculated and nodulated plants grown with 8, 24, 40, or 80 mg/L N. These conditions resulted in reproducible growth rates, large effects on growth and nodulation, and relatively steady soil nitrogen availability.

Once the growth conditions were established, we conducted a number of experiments to collect the biomass, C:N elemental compositions, and photosynthesis data required to parameterize the model. TC and I were equally involved in growing the experimental plants and measuring the model parameters. I also led measurements (e.g., root scans to analyze root architecture) that were less connected to the model and thus were not included in this manuscript. When the data had been collected, I was responsible for statistical analyses and visualization of

the data, while TC carried out the modeling work. TC and I co-wrote the many drafts of the manuscript found here. The content of the text and figures was based on the consensus decisions of the larger group.

Abstract

Mutually beneficial resource exchange is foundational to global biogeochemical cycles and plant and animal nutrition. However, there is inherent potential conflict in mutualisms, as each organism benefits more when the exchange ratio ("price") minimizes its own costs and maximizes its benefits. Understanding the bargaining power that each partner has in these interactions is key to our ability to predict the exchange ratio and thus the functionality of the cell, organism, community, and ecosystem. We tested whether partners have symmetric ("fair") or asymmetric ("unfair") bargaining power in the legume-rhizobia nitrogen fixing symbiosis using measurements of carbon and nitrogen dynamics in a mathematical modeling framework derived from economic theory. A model of symmetric bargaining power was not consistent with our data. Instead, our data indicate that the growth benefit to the plant has greater weight in determining trade dynamics than the benefit to the bacteria. Quantitative estimates of the relative power of the plant reveal that the plant's influence rises as soil nitrogen availability decreases and trade benefits to both partners increase. Our finding that legumes have more bargaining power than rhizobia at lower nitrogen availabilities highlights the importance of context-dependence for the evolution of mutualism with increasing nutrient deposition.

Introduction

Mutualistic relationships abound in nature. They are rooted in the exchange of resources or services between different partners whose distinct capabilities allow them to perform better together than either could alone. Mutualisms involving the exchange of carbon for mineral nutrients between plants and microbes are ancient interactions that have shaped the evolution of land plants and play central roles in ecosystem functioning worldwide (Bronstein, 2015). Plants participating in these nutritional mutualisms with microbes such as mycorrhizal fungi or nitrogen-fixing rhizobium prokaryotes must optimize their allocation of photosynthate between taking up nutrients directly and trading for them with mutualists (Bloom *et al.*, 1985). Indeed, plants exhibit considerable plasticity in partitioning carbon among shoots, roots, and mutualistic partners in response to environmental cues (Harris *et al.*, 1985; Wang *et al.*, 2011). This optimal allocation is determined by soil nutrient availability and the cost:benefit ratio of acquiring the nutrient through trade. However, it is in each partner's best interest to influence the carbon-for-nutrient exchange ratio ("price") to maximize the benefit to itself (Akçay & Roughgarden, 2007; Grman *et al.*, 2012), conditions that should lead to a power struggle over the price. Considerable effort has been devoted to applying economic principles to analyzing nutrient exchange, stability, and other aspects of mutualisms (Weyl *et al.*, 2010; Werner *et al.*, 2014; Clark *et al.*, 2017), but there is a major gap: we do not understand how the exchange ratio and the quantity traded between plant and microbe are determined.

This question has been explored using mathematical models in which partners have disparate abilities to acquire resources and divergent resource requirements (Akçay & Roughgarden, 2007; Grman *et al.*, 2012; Franklin *et al.*, 2014). In these models, mutualistic partners negotiate based on the principles of the Nash bargaining solution, an axiomatically

derived result describing the expected distribution of benefits after bargaining between self-interested partners that are able to regulate their participation in trade in response to the benefits they receive from trade (Nash, 1950; Binmore *et al.*, 1986; Akçay & Roughgarden, 2007).

A central assumption underlying the Nash bargaining solution is symmetry in bargaining power between the partners, where the bargaining power of a partner is defined as the weight given to the benefit received by that partner in the determination of trade dynamics. The best indicator of symbiotic benefit is reproductive fitness in the field, but biomass is conventionally used as a proxy for fitness (Younginger at el 2017). Consequently, the symmetric Nash product is the product of the partners' growth gains from trade:

$$(gP_{trade} - gP_{notrade})\,(gR_{trade} - gR_{noTrade}) \qquad (1)$$

where gP_{trade} and gR_{trade} are the plant and rhizobial growth rates with trade, and $gP_{noTrade}$ and $gR_{noTrade}$ are the respective growth rates without trade. An extension of this framework allows for unequal power between partners through the asymmetric Nash product:

$$(gP_{trade} - gP_{notrade})^{\beta}\,(gR_{trade} - gR_{noTrade})^{1-\beta} \qquad (2)$$

which arises when bargaining power differs between partners (Binmore *et al.*, 1986). β is a scaling exponent that assigns different weights to the gains from trade by the plant and microbe. Increases in β correlate with increases in plant bargaining power relative to the microbial symbiont, and bargaining is symmetrical when β = 0.5 (Binmore *et al.*, 1986).

Previous modeling analyses of nutrient exchange symbioses have been limited by the

absence of quantitative experimental studies in which all the major relevant parameters were measured in a single study across a range of environmental conditions and used explicitly within a mathematical framework (Clark *et al.*, 2017). To address this knowledge gap, we measured biomass distributions, nitrogen uptake and exchange rates, photosynthetic carbon assimilation fluxes, and carbon and nitrogen compositions in the model *Medicago truncatula-Ensifer medicae* (legume-rhizobia) symbiosis under conditions ranging from low to high nitrogen availability. We used the measurements to quantitatively test whether trade in a mutualism follows the predictions of the Nash bargaining solution under different conditions of resource availability. To do this, we refined and parameterized a mechanistic model of resource trade between a plant and microbe (Figure 2.1; Grman *et al.*, 2012). This model assumes that the growth of each partner is limited by its ability to obtain carbon and/or a mineral nutrient and determines the exchange ratio to be the one that maximizes the product of partner benefits from trade, consistent with the Nash bargaining solution (Nash, 1950; Akçay & Roughgarden, 2007; Grman *et al.*, 2012). The exchange ratio is then used to predict per capita partner growth rates and allocation to growth versus trade. We tested the assumption of symmetric bargaining by comparing the accuracy of predictions made by the model with experimental measurements. The experimental results were inconsistent with predictions based on the symmetric Nash bargaining solution, so we explored how predictions based on asymmetries in relative bargaining power aligned with experimental measurements and determined the value of β for which model fit was greatest.

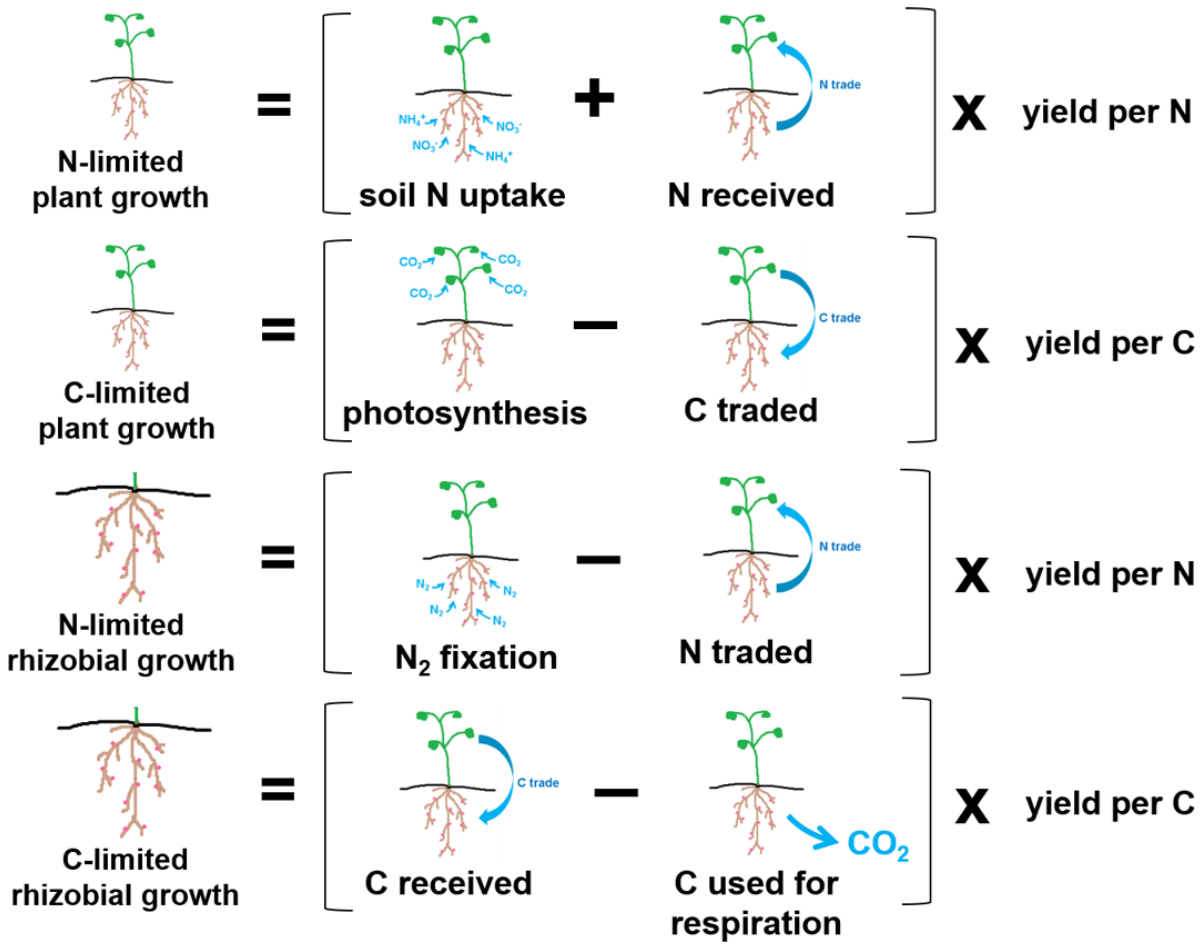

Figure 2.1 | Pictorial representation of the legume-rhizobia nutrient exchange model. Plant and rhizobial growth are limited by the ability to obtain carbon (C) or nitrogen (N). In general, the growth rates are the rates of biomass carbon increase that complement via yield parameters the net amount of carbon or nitrogen obtained directly and/or from trade. Carbon and/or nitrogen can be lost during trade or respiration. More information about model construction is provided in the Methods.

Materials and Methods

Experimental methods

SC10 Cone-Tainer pots (Steuwe and Sons Inc., Corvallis, OR, USA) were plugged with ⅜" diameter cotton wicks leading to opaque 50 mL reservoirs and filled with medium grain vermiculite. Pots were wetted with 25 mL deionized water, covered, and autoclaved for 45 minutes. After 24 hours, pots were wetted with a further 25 mL of deionized water and autoclaved again for 45 minutes. 24 hours later, the pots were wetted with 25 mL of Fahraeus nutrient solution (Fahraeus, 1957) supplemented with 8, 24, 40, or 80 mg L^{-1} N in the form of NH_4NO_3.

Medicago truncatula A17 seeds (Young *et al.*, 2011) were scarified with 600 grit sandpaper, sterilized in commercial bleach (8.25% $NaHClPO_3$) for 3 minutes, and rinsed at least 6 times with sterile deionized water. Following 3 hours of incubation in sterile deionized water at room temperature, the seeds were re-sterilized in 0.825% $NaHClPO_3$ for 30 seconds and rinsed at least 6 times with sterile deionized water. Seeds were then incubated in sterile deionized water for 48 hours at 4°C. The water was replaced approximately every twelve hours during this incubation. The seedlings were then transferred to sterile petri dishes, sealed with Parafilm, and germinated at room temperature for 48 hours. Seedlings with 1 cm or longer radicles were aseptically transplanted into prepared pots. After planting, the plants were fully randomized and grown at 22°C with a 16 hour day/8 hour night cycle at approximately 250 µmol m^{-2} s^{-1}. Seedlings were misted daily with sterile deionized water for the first week. Throughout the growth period, plants were continuously supplied via their reservoirs with Fahraeus nutrient solution.

Ensifer medicae WSM419 (Reeve *et al.*, 2010) was grown for 48 hours in tryptone-yeast broth at 30°C with rotary shaking at 200 RPM. The OD600 of the culture was measured to estimate cell density. Half of the week-old plants were inoculated with 1 mL of 10^6 CFU mL^{-1} inoculum in ½ x phosphate buffered saline (+ rhizobia treatment), while the other half were mock-inoculated with sterile buffer (- rhizobia treatment). At least 7 plants per nitrogen level and inoculation status were harvested after 4 or 6 weeks of growth for biomass measurements (in total, 64 or 101 plants were harvested after 4 or 6 weeks, respectively, half of which were nodulated). Roots and shoots were separated, and roots were carefully washed in deionized water to remove vermiculite. Washed roots were checked for nodulation, and nodules were removed and counted. All tissue was dried at 60°C for at least one week. Of these plants, 3 plants per nitrogen level, inoculation status, and age were analyzed for carbon and nitrogen elemental compositions of roots, shoots, and nodules (48 plants in total). Dried plant tissues were ground using a NutriBullet, LLC household blender followed by a Retsch MM301 Mixer Mill. 2-5 mg dried tissue was weighed, packaged in tin capsules, and analyzed by the Robertson lab at Michigan State University's Kellogg Biological Station using a Costech ECS4010 analyzer.

To measure direct nitrogen uptake with ^{15}N enrichment, 24 plants (3 per nitrogen level and inoculation status) were grown as described above until 4.5 weeks, whereupon the pots and reservoirs were flushed with 500 mL N-free Fahraeus solution to remove soluble unlabeled nitrogen. The plants were then watered with 25 mL of Fahraeus solution containing the appropriate concentration of $^{15}NH_4{}^{15}NO_3$, supplied with this solution via their reservoirs for 1 week and harvested. Plants were harvested, weighed, dried, ground, and packaged into tin capsules as described above. The nitrogen content and ^{15}N abundance were then analyzed by the Stable Isotope Lab at Utah State University using a Europa Scientific SL-2020 system.

Photosynthesis rates were measured on 5-week-old nodulated and uninoculated plants (4-6 plants per nitrogen level and inoculation status; 41 plants total) using whole-plant (6”x6”x12”) photosynthesis chambers connected to LI-COR LI-6400 apparatuses, illuminated with LED lights, and provided a constant airflow of 1000 µmol s^{-1} with 400 µmol CO_2 per mol of air (Figure 2.7a,b). CO_2 from below ground was excluded using modeling clay. Assay conditions (e.g., light, humidity, temperature) matched the growth conditions. Steady-state photosynthetic rates were measured for at least 90 minutes after a pre-equilibration period in the chambers with the light on for at least 1 hour (Figure 2.7c). The final steady state CO_2 assimilation rate (µmol CO_2 sec^{-1}), was converted to a daily rate (mg C day^{-1}), assuming a constant rate throughout the 16h light period. After measuring photosynthesis rates, the plants were harvested, dried, and weighed as described above.

To test the effects of soil nitrogen and rhizobia on root and shoot biomass, we used a linear model ANOVA with Type II sum of squares (aov and car packages, R 3.3.1) with soil nitrogen, rhizobia, and the nitrogen by rhizobia interaction as fixed effects. Since we detected significant main effects of nitrogen and rhizobia, we conducted *post-hoc* testing with the Tukey test at a significance level of 0.05 (lsmeans package, R 3.3.1) to determine whether group means were significantly different.

Model construction

The legume-rhizobia model (Figure 2.1) was derived from the model of Grman *et al.* (2012) that assumes plants adjust carbon allocation to roots or shoots on a faster timescale than the carbon-for-nutrient exchange ratio is negotiated between the partners. Refinements to model structure equations were made using Wolfram Mathematica 11.3. Model predictions include

plant and rhizobial specific growth rates (gP and gR, respectively) as functions of biomass carbon gain, the proportion of plant carbon allocated to roots (a_{NP}) or shoots, and the carbon-for-nitrogen exchange ratio (T). The model assumes organismal growth is limited by the ability to obtain nitrogen and/or carbon for biomass production. Consequently, for each partner, the predicted growth rate is the minimum growth predicted when nitrogen (gP_{Nlim}, gR_{Nlim}) or carbon (gP_{Clim}, gR_{Clim}) is limiting:

$$gP = \min(gP_{Clim}, gP_{Nlim})$$

$$gR = \min(gR_{Clim}, gR_{Nlim}).$$

When nitrogen is limiting, the organismal growth rates are represented as

$$gP_{Nlim} = (f_{np} + X/(p\ T))*y_{np}$$

$$gR_{Nlim} = (f_{nr} - X/(r\ T))*y_{nr},$$

,

where X is the rate of carbon traded from the plant to the rhizobia and, for the plant and rhizobia, respectively, p and r are the organismal carbon contents, f_{np} and f_{nr} are the rates of nitrogen uptake per organismal carbon content, and y_{np} and y_{nr} are the carbon biomass yields per unit nitrogen. Briefly in the vernacular, the growth rates are the rates of biomass carbon increase that complement (via the yield parameters) the amount of nitrogen obtained directly and/or from trade. Comparably, when carbon is limiting, the organismal growth rates are represented as

$$gP_{Clim} = f_{cp} - X/p$$

$$gR_{Clim} = X/r,$$

,

where f_{cp} is the rate of photosynthetic carbon uptake per organismal carbon content. Yield parameters are not necessary here because the growth rates are in units of biomass carbon gain.

Respiratory costs associated with nitrogen fixation were added to the model by reducing the rhizobial growth rate by the rate of nitrogen fixation (f_{nf}) multiplied by the biochemical stoichiometric trade constraints of 2.57 g C g^{-1} N (Phillips, 1980). Consequently, the rhizobial growth rate equations become

$$gR_{Nlim} = (f_{nr} - X/(r\ T))*y_{nr} - 2.57*f_{nr}$$

$$gR_{Clim} = X/r - 2.57*f_{nr}.$$

.

Plant respiratory costs are not as well-defined (Wardlaw, 1990) and thus were incorporated via the plant carbon uptake (f_{cp}) measurement as described above.

In addition to serving as model predictions, the growth rate equations provide the foundation for how the model predicts the rate of carbon trade, root-to-shoot allocation, and the carbon-for-nitrogen exchange ratio. Because rhizobia in nodules are unable to take up external carbon, we assumed the modeled trade is rhizobia-limited. Consequently, the rhizobial partner trades away all surplus nitrogen (i.e., nitrogen unnecessary for growth) in exchange for carbon from the plant. The traded nitrogen and that received by root uptake are used for plant growth and thus determine the plant surplus carbon (via the yield parameter y_{np}) that is traded to the rhizobia (i.e., X is the plant surplus carbon). As described in Grman *et al.* (2012), the model predicts that the optimal root-to-shoot allocation is when, after trade, the total carbon uptake by

the shoots complements (via the yield parameter) the total nitrogen uptake by the roots. Partner negotiations for the carbon-for-nitrogen exchange ratio are predicted to be consistent with economic modeling methods. When bargaining is symmetric, as in the Grman *et al.* (2012) model, the negotiated ratio is assumed to result in the Nash bargaining solution, in which the Nash product (Equation 1) is maximized. If bargaining is asymmetric, then negotiations should result in maximizing the asymmetric Nash product (Equation 2).

Computational methods

The model derivation (Grman et al. 2012) does not rely on the assumption of extended steady state because the solutions are instantaneous for any given set of input values for the plant (*Medicago truncatula*) and symbiont (*Ensifer medicae*). However, we first confirmed that over the period in which measurements were made (4-6 weeks), the plant per capita growth rate was near linear (Figure 2.7), thus its growth and nutrient uptake rates should change slowly compared to the negotiations. Measurements were either taken at 5 weeks of age or averaged between measurements at 4 and 6 weeks to represent 5-week-old systems. Goodness of fits for model predictions and parameters were assessed using 90% confidence intervals from the results of modeling 50 pseudo datasets per growth condition and age (Methods S2). The pseudo datasets were generated by Monte Carlo sampling of the experimental measurements; i.e., for each measurement, we generated random pseudo data points with normal distribution around the measured average with the measured standard deviation. To be consistent with biological reality, we assumed that biomasses and nutrient uptake rates could not be negative, so any randomly generated negative values were rounded up to zero. Each pseudo set contained one point for every measurement and were used as model inputs to generate one set of model predictions. This

generated 50 sets of model predictions per nitrogen level, which were assessed using confidence intervals. This statistical method allowed us to evaluate how variations in measurements affected model predictions.

Because the model tracked how carbon was obtained and allocated, most model inputs (Table 2.1) and predictions (Table 2.2) were expressed per unit of biomass carbon content. Carbon and nitrogen contents were calculated using biomass and elemental composition measurements of carbon and nitrogen, respectively (Table 2.3-2.4). Both contents were used to calculate the carbon per nitrogen organismal yield parameters, and allocation to root biomass was calculated as the root carbon content divided by whole plant carbon content. Per capita growth rates were calculated as the carbon content gained between 4 and 6 weeks of age, divided by 5-week carbon content. For the plant, photosynthesis and soil nitrogen uptake rates were expressed per shoot carbon and root carbon, respectively, while rhizobial nitrogen fixation was per nodule carbon. Please note: when the abundance of nodule carbon is low (e.g., at 80 mg L^{-1} N), this requires dividing by a small number which amplifies the associated uncertainties.

Nitrogen elemental composition and ^{15}N enrichment measurements were used to differentiate between the rates of soil nitrogen uptake and nitrogen trade in nodulated plants. As described above, nodulated and uninoculated plants were labeled by flushing the soil with nitrogen-free media and then watering for 1 week with $^{15}NH_4{}^{15}NO_3$. Labeling revealed that 96.7% of the variation in nitrogen uptake per total biomass was due to the abundance of nitrogen in the nutrient solution ($p < 0.001$; Figure 2.8a) and at 24, 40, and 80 mg L^{-1} N, there was no significant difference in ^{15}N uptake per total plant biomass between nodulated and uninoculated plants. Consequently, at these nitrogen levels, we concluded that the rate of soil nitrogen uptake in nodulated plants is equivalent to the rate of biomass nitrogen uptake in uninoculated plants,

and the remaining biomass nitrogen content in nodulated plants can be attributed to trade. When analyzing the model with pseudo datasets, this was implemented by generating pseudo data for the uninoculated plants as well as nodulated. At 8 mg L^{-1} N, the average ^{15}N uptake rate was 2.4-fold greater in uninoculated plants than in nodulated plants. This average difference was incorporated into the model analysis by dividing the corresponding (per pseudo replicate) uninoculated nitrogen uptake rate by 2.4.

A minimum of 2.57 g C is biochemically required to produce the ATP and reductant necessary for rhizobia to fix 1 g N (Phillips, 1980). Using this biochemical minimum and the amount of nitrogen traded to the plant, we obtained a minimum for carbon to be traded for and consumed by rhizobial respiration. In addition to respiratory costs, the rhizobia used carbon for biomass production. We experimentally derived the quantity of biomass carbon using biomass and carbon composition measurements. Consequently, the predicted carbon-for-nitrogen exchange ratio was experimentally estimated as the amount of carbon traded to the rhizobia for biomass and respiration, divided by the amount of nitrogen traded to the plant. These estimates should be regarded as conservative (low) because they do not include any additional respiration costs for growth or maintenance; however, we found that modest increases (e.g., 3.6 mg C mg^{-1} N, Ryle et al 1984) beyond the minimum had little effect on model predictions.

Measurements from uninoculated plants were used to test the effect of including plant respiration in the model because the presence of nodules prevented accurate measurement of below-ground (i.e., root) respiration in nodulated plants. It has been shown that when relative plant growth is constant (as in the near linear legume-rhizobia system), the rate of plant respiration per unit of biomass is also constant (Lambers *et al.*, 1983). Furthermore, the rate of whole-plant respiration has been found to be a linear function of plant biomass and rate of gross

photosynthesis. Therefore, we assumed that the rate of respiration per unit plant biomass was the same for plants with and without rhizobia. We found that in uninoculated plants, there was a significant correlation ($R^2 = 0.346$, $p < 0.001$) between biomass carbon content and the proportion of photosynthetic carbon used for biomass (Figure 2.8b). We used this relationship to estimate the amount of carbon used for plant respiration in nodulated plants. Plant respiratory costs were included in the model by reducing the measured photosynthesis rate by the estimated respiration rate.

Overall model fits were quantified using the sum of squared differences between predicted and measured allocation to root biomass, plant growth, and rhizobial growth. The carbon-for-nitrogen exchange ratio predictions were not used in this assessment because the ratio was experimentally estimated (as described above) but not directly measured. The best fit value of β was identified as the one yielding the smallest normalized sum of squares. The sum of squares for each β in a pseudo dataset was normalized by dividing by the average sum of squares for that pseudo dataset. This allowed fit comparisons to be between the β values without bias from differences between pseudo datasets.

Results

Changes in soil nitrogen alter carbon and nitrogen uptake and the benefits of the legume-rhizobia mutualism

We determined the values of model input parameters (Table 2.1) for the *Medicago truncatula-Ensifer medicae* (legume-rhizobia) symbiosis by measuring biomasses, nutrient uptake rates, and elemental compositions, and of the resulting model predictions, including growth rates, root-to-shoot allocation, and the carbon-for-nitrogen exchange ratio (Table 2.2).

The collective nodules of a single plant were used as a proxy for the rhizobial partner because, although nodules contain plant cells as well as rhizobia, the whole nodule represents the plant's investment toward trade. In addition, nodule biomass correlates well with rhizobial abundance (Ratcliff et al 2011), thus larger nodules indicate greater plant investment as well as greater rhizobial benefits from trade.

Plants were grown in the presence or absence of rhizobia and fertilized with 8, 24, 40, or 80 mg L^{-1} N (Figure 2.2a; Valladares *et al.*, 2002). Total plant biomass was significantly increased by soil nitrogen ($p < 0.001$). Inoculation with rhizobia significantly increased total plant biomass at 8, 24, and 40 mg L^{-1} N, but not at 80 mg L^{-1} N (Figure 2.2b), showing that rhizobia enhanced plant growth at low and intermediate nitrogen levels. Nodule biomass significantly decreased with increasing soil nitrogen ($p < 0.001$, Fig 2.2c). Together, these findings confirm that mutualistic relationships are more beneficial for both partners at lower nitrogen availabilities and that nitrogen is a key limiting resource in these experiments (Regus, *et al.*, 2017).

Plants interacting with rhizobia have two options for acquiring nitrogen: taking it up directly from the soil or trading for it with rhizobia. These two nitrogen acquisition routes are rarely differentiated experimentally, but this differentiation is essential to quantifying symbiotic nutrient exchange. To do so, we measured direct nitrogen uptake using ^{15}N enriched nutrient solution and compared it to the total increase in plant nitrogen content. Our findings revealed that fixed nitrogen from trade adds to but does not replace nitrogen obtained by direct uptake. On a per-plant basis, plants with rhizobia obtained at least as much nitrogen by direct uptake as those without rhizobia at all nitrogen levels (Figure 2.3a). Furthermore, in comparison to uninoculated plants, the rate of direct nitrogen uptake per root carbon was slightly lower in nodulated plants at

the lowest soil nitrogen, modestly greater at 24 and 40 mg L^{-1} N, and the same at 80 mg L^{-1} N (Figure 2.4b).

In addition to increasing plant growth rates and supplementing nitrogen acquisition, the presence of rhizobia significantly altered the pattern of carbon allocation within the plant (Voisin *et al.*, 2002; Goh *et al.*, 2016). Increasing soil nitrogen significantly decreased root:shoot ratio in uncolonized plants (Figure 2.4a; Rufty *et al.*, 1984; Paponov *et al.*, 2000), while the root:shoot ratio of nodulated plants did not vary with soil nitrogen (Figure 2.4a). This shows that plants adjust their relative allocation of biomass between roots and shoots depending on their ability to take up nitrogen directly from the soil or trade for it with rhizobia. However, root:shoot ratios are an incomplete measure of carbon allocation because they do not account for allocation to mutualists, respiration, exudates, and other carbon sinks (Wardlaw, 1990). To determine how organ allocation relates to the total carbon budget, we measured total plant carbon content and whole-plant photosynthetic carbon uptake. As expected from their larger shoot biomasses, we measured higher rates of carbon uptake per plant in nodulated plants grown at 8, 24, and 40 mg L^{-1} N than in uninoculated plants (Figure 2.3b, Figure 2.4c). Plant respiration ranged from 11-65% of total photosynthetic carbon, and carbon allocation to nodules ranged from 2-10% (Figure 2.3b). The magnitude of respiratory costs highlights the importance of accounting for respiration in models of plant carbon budgets (Table 2.2); the strong dependence of respiration rates on nitrogen levels points to the value of measuring them directly.

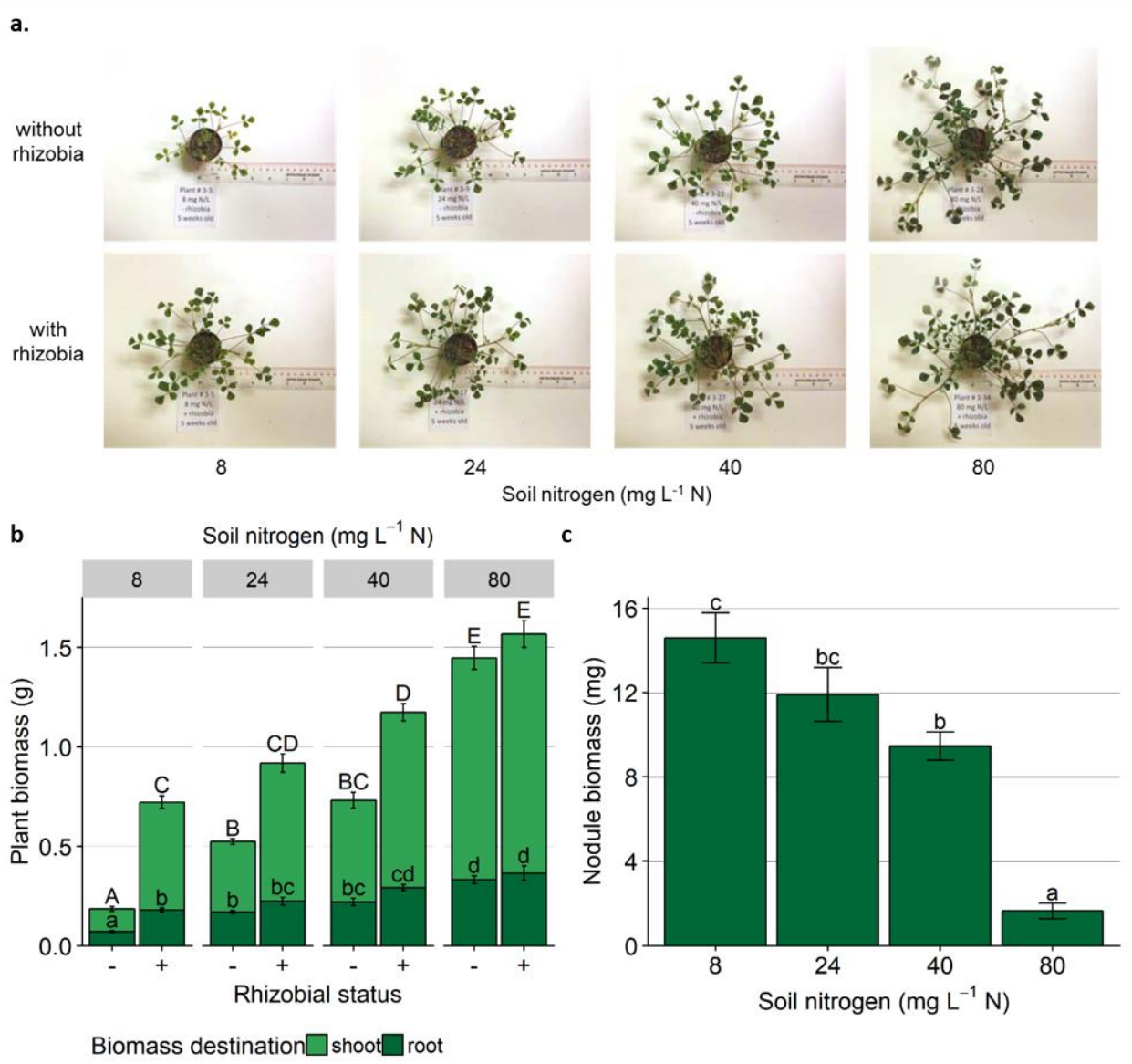

Figure 2.2 | Both partners benefit more from trade at lower nitrogen availabilities. (a) Images of 5-week-old nodulated and uninoculated *M. truncatula* at 8, 24, 40, and 80 mg L^{-1} N. (b,c) Average 5-week root (b, dark green), shoot (b, light green), and nodule (c) biomasses. Error bars indicate standard error (n = 7-15), and bars with the same letter within the same panel do not differ significantly after *post hoc* testing with the Tukey test ($p < 0.05$). (b) Capital letters refer to shoot biomass and lowercase letters refer to root biomass.

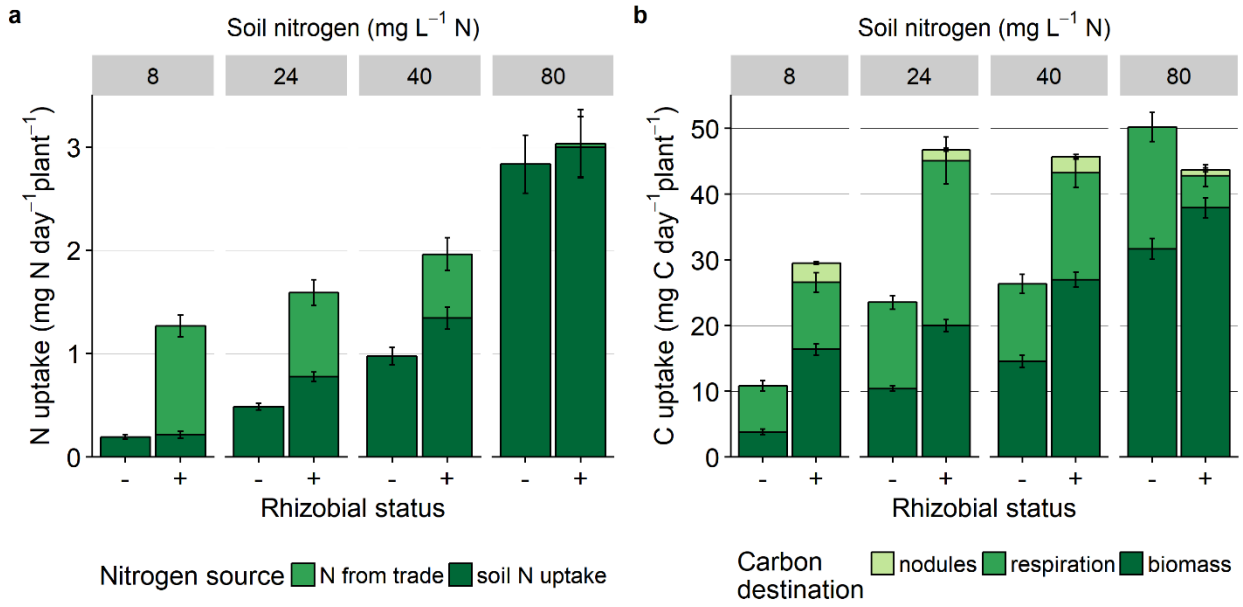

Figure 2.3 | The effect of rhizobial inoculation on plant carbon and nitrogen budgets as functions of nitrogen availability. Whole-plant nitrogen (a) and carbon (b) uptake and allocation rates by nodulated (+) and uninoculated (-) *M. truncatula* at 8, 24, 40, and 80 mg L^{-1} N. (a) Direct nitrogen uptake by roots (dark green) and nitrogen received from trade with rhizobia (medium green). (b) Photosynthetic carbon allocated to plant biomass (dark green), plant respiration (medium green), and nodule growth or respiration (light green). Values and error bars represent the average and 90% confidence intervals, respectively, of 50 pseudo datasets generated by Monte Carlo sampling.

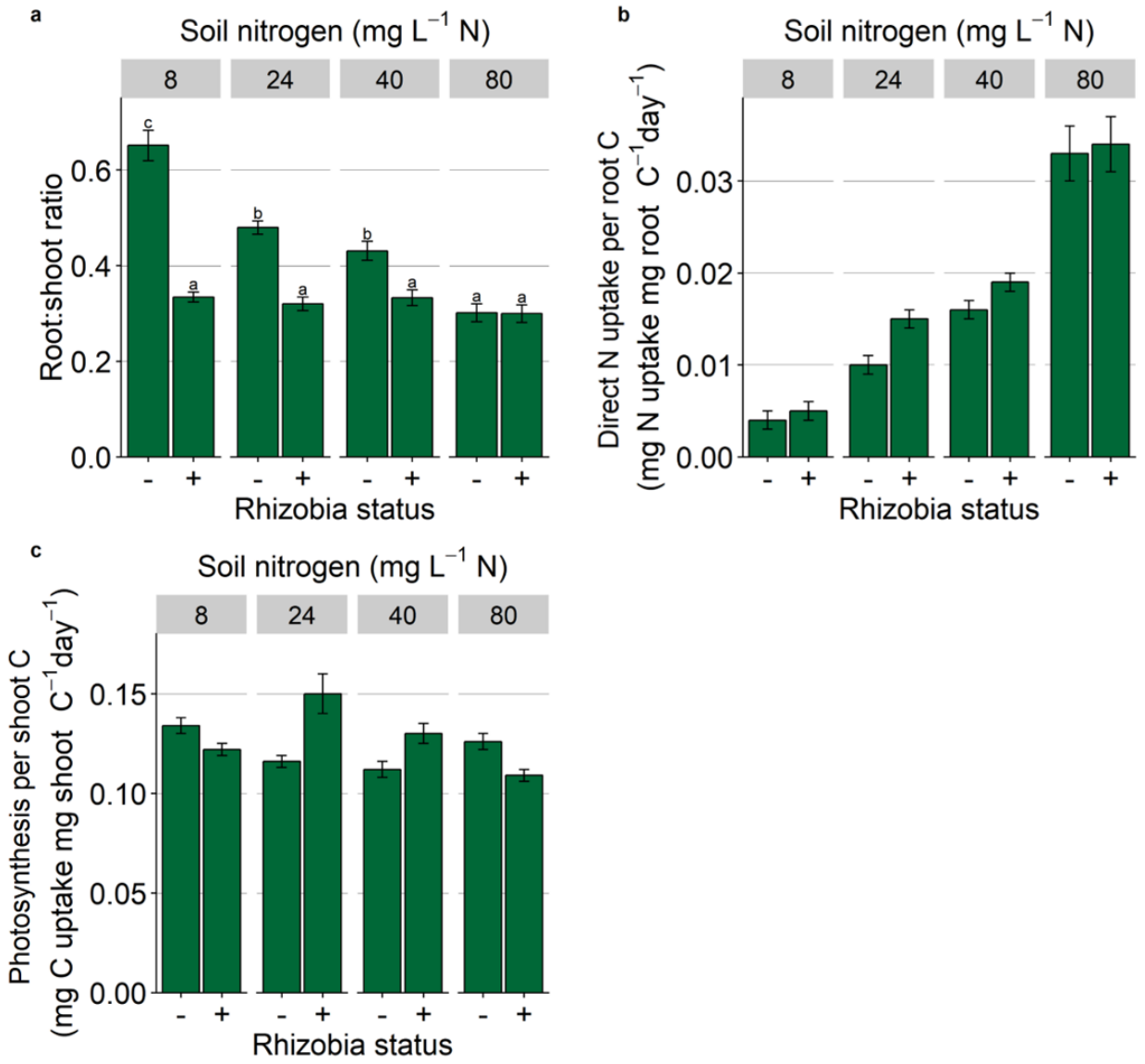

Figure 2.4 | Plant carbon allocation to nutrient uptake. Root-to-shoot ratio (a), direct nitrogen uptake per root carbon (b), and photosynthesis per shoot carbon (c) of 5-week-old nodulated (+) and uninoculated (-) plants at 8, 24, 40, and 80 mg L^{-1} N. a, Values and error bars represent the average and standard error, respectively, of 7-15 biological replicates. Bars with the same letter do not differ significantly after *post hoc* testing with the Tukey test ($p > 0.05$). b, c, Values and error bars represent the average and 90% confidence intervals, respectively, of 50 pseudo datasets generated by Monte Carlo sampling.

The benefits and exchange ratio of legume-rhizobia trade are better explained by asymmetric bargaining

We used the measurements described above to predict the carbon-for-nitrogen exchange ratio consistent with the Nash bargaining solution in the legume-rhizobium model. Using the biochemical stoichiometric trade constraints for nitrogen fixation of at least 2.57 mg C mg^{-1} N (Phillips, 1980) and measured volumes of nitrogen traded and nodule biomass carbon contents (Figure 2.2 and 2.3), we estimate that the nodules received 2.72-3.25 mg C mg^{-1} N for growth and respiration (Figure 2.5a). The model-predicted exchange ratios approached these estimates at the highest nitrogen level, but at the lowest, the predicted exchange ratio was 2.5-fold higher than experimentally estimated (Figure 2.5a). These estimates are consistent with measurements on the soybean-rhizobia mutualism, where nodules received 3.6 mg C mg^{-1} N (Ryle *et al.*, 1984). In addition to comparing experimentally estimated and model-predicted exchange ratios, we also examined how the exchange ratio influences partner benefits from trade, and thus how well the model predicts partner growth rates and the proportion of plant carbon allocated to roots or shoots. Using the predicted exchange ratio, the symmetric bargaining model predicted plant growth and carbon allocation to roots within 50% of measured (Figure 2.5b,c), but predicted nodule growth to be 3 to 7-fold higher than measured rates (Figure 2.5d). These discrepancies could not be resolved by modifications in respiratory cost interpretations, growth rate definitions (specific versus organismal), assuming that only 50% of the nodule biomass represents the rhizobial partner (Table AI-2), or assumptions concerning the timescale of adjusting root-to-shoot allocation (Grman *et al.*, 2012), and thus suggest that this legume-rhizobia mutualism fails to meet fundamental conditions of the symmetric Nash bargaining solution (Equation 1; Nash, 1950).

Consequently, we investigated asymmetric bargaining using asymmetric Nash products in our model (Equation 2) and found that it could better explain the observed growth rates, nutrient allocations and trade across the range of growth conditions. We determined how values of β between 0 and 1 affected model fit for the carbon-for-nitrogen exchange ratio and other model predictions. We found that across the soil nitrogen levels, the best fit value of β was 0.70 (Figure 2.6). Using this estimate, model predictions of nodule growth at 8, 24, and 40 mg L^{-1} N were improved up to 2-fold, but there was little improvement in plant growth, root:shoot allocation, or 80 mg L^{-1} N predictions (Figure 2.5). Next, we let β vary among the nitrogen levels and found that the best fit value of β increased from 0.57 to 0.86 as soil nitrogen decreased (Figure 2.6, Table 2.2). The 90% confidence intervals of β for 8 and 80 mg L^{-1} N were inconsistent with the value of 0.70 obtained assuming a constant value of β across nitrogen levels. Importantly, β for 8, 24, and 40 mg L^{-1} N indicate asymmetric trade (i.e., $\beta \neq 0.50$), and model fit was dramatically improved compared to symmetric predictions (Figure 2.5). For example, at 8 mg L^{-1} N, nodule growth was predicted within 3% of measured values (Figure 2.5d), and overall agreement between predicted and measured growth rate and root:shoot allocation predictions was increased 7-fold (Table 2.2**)**. For all soil nitrogen levels, plant growth and root allocation predictions were improved, and the exchange ratio was predicted within 25% of experimental estimates (Figure 2.5b,c). Together, these findings indicate that plant bargaining power rises as its investment in trade and the benefits to both partners increase.

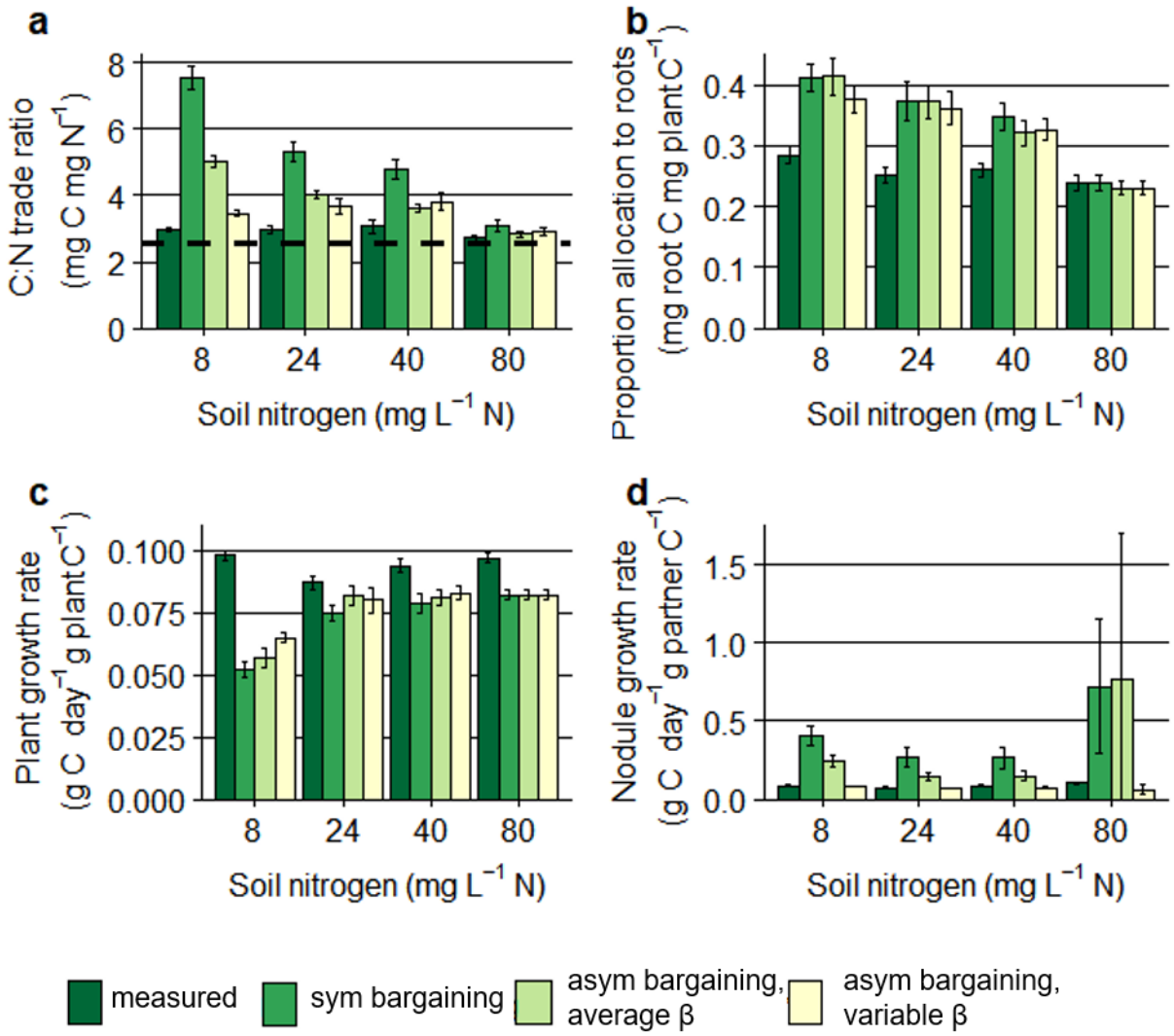

Figure 2.5 | Model fit to experimentally estimated data is improved by allowing asymmetric bargaining power. Empirically estimated (measured) and model predicted carbon-for-nitrogen exchange ratios (a), percent allocations toward roots (b), plant growth rates (c), and nodulerhizobial growth rates (d) of 5-week-old nodulated *M. truncatula* at 8, 24, 40, and 80 mg L^{-1} N. The symmetric (sym) bargaining predictions correspond to those predicted using symmetric Nash products, while the asymmetric (asym) bargaining predictions correspond to using asymmetric Nash products with either β fitted across the soil nitrogen levels (average β) or β fitted to individual soil nitrogen levels (variable β). The dotted line in (a) represents the biochemical minimum value of the exchange ratio. Values and error bars represent the average and 90% confidence intervals, respectively, of 50 pseudo datasets generated by Monte Carlo sampling.

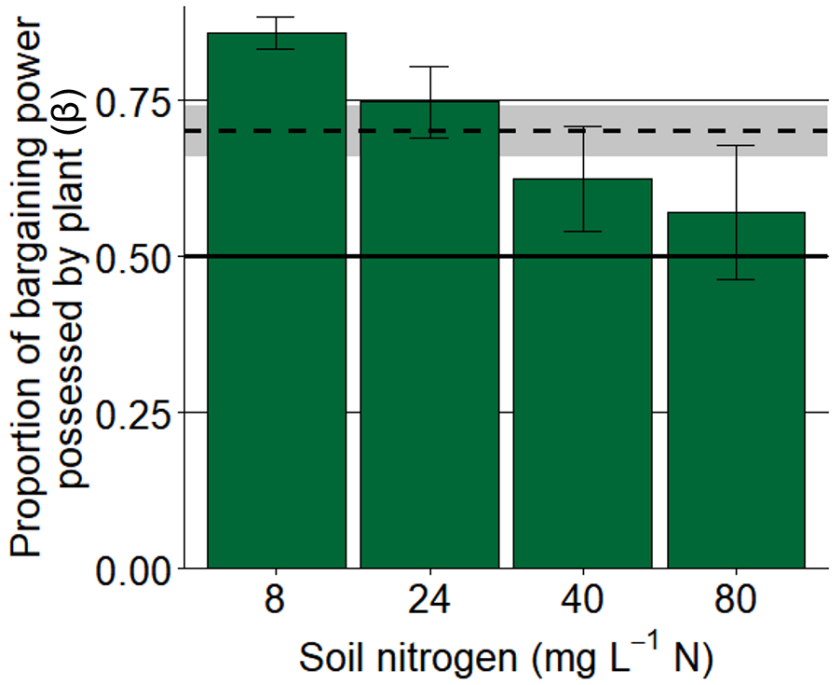

Figure 2.6 | Plant bargaining power as a function of nitrogen availability. Best fit average β (dotted line; shaded area indicates the 90% confidence interval) and the best fit variable β at 8, 24, 40, and 80 mg L^{-1} N (bars; error bars indicate 90% confidence intervals of 50 pseudo datasets). Best fit β were those associated with the smallest sum of squared differences between predicted (using asymmetric Nash products, Equation 2) and measured allocation to root biomass, plant growth, and nodule growth in 5-week-old nodulated *M. truncatula*. The solid line represents symmetric bargaining.

Discussion

Potential mechanisms underlying asymmetric bargaining

Our measurements of nutrient uptake, per capita growth, and partner composition in a legume-rhizobia system were more consistent with the plant having greater bargaining power than its symbiotic partner, than with a model of fair trade. Asymmetries in bargaining power can arise by three mechanisms that may be operating in the legume-rhizobia mutualism. First, one partner can have a lower effective "discount rate" (the rate at which benefits lose value to that partner if negotiations are prolonged), thus conferring greater bargaining power to the partner

able to endure longer negotiations (Kawamori, 2014). In the legume-rhizobium mutualism, the plant's longer lifespan and potentially larger nutrient reserves could serve to provide a lower discount rate. Second, group bargaining dynamics can affect bargaining power. In "pure bargaining" situations, groups of individuals negotiating as a single partner have less apparent bargaining power than independent partners because group benefits would be divided among group members after negotiation (Chae & Heidhues, 2001). In this case, if the bacteroids that comprise a nodule (Udvardi & Poole, 2013) negotiate as a group (e.g., at the nodule level), then the rhizobial partner would have less apparent bargaining power because the carbon received by the nodule would be divided among the bacteroids. A third mechanism that can increase an individual's bargaining power is the ability to simultaneously negotiate with multiple trade partners (Chakraborty *et al.*, 2009; Chakraborty, 2011). In this case, the presence of multiple nodules on a plant and/or multiple bacteroids within a nodule could lead to bargaining power asymmetry. This mechanism could be particularly important if bacteroids within nodules bargain independently with the plant, which is consistent with the plant being able to interact differently with different bacteroids within a nodule (Daubech *et al.*, 2017; Regus, JU *et al.*, 2017). Each of these mechanisms can explain why the plant has more bargaining power than the rhizobia, but alone, they do not explain why plant bargaining power appears to be higher when nitrogen is scarce (e.g., 8 mg L^{-1} N). At lower soil nitrogen levels, the plant would be expected to have fewer nitrogen reserves and nitrogen received from trade comprises a larger proportion of the plant's nitrogen budget (Figure 2.3a), which could increase its discount rate and lower its bargaining power. However, we found that as soil nitrogen decreases, total nodule biomass (Figure 2.2) increases. This apparent increase in the number of bacteroids (i.e., trade partners) could lead to increased plant bargaining power even as the plant's reliance on the rhizobia

increases. Studies in other systems would be important for assessing the prevalence of asymmetric bargaining, and manipulating microbial numbers would allow the influence of partner number to be further investigated.

Relating trade conflict to the concept of cheating

Our finding that the plant host has a high degree of control over the carbon-for-nitrogen exchange ratio when soil nitrogen is limiting has important implications for conceptualizing conflict and cheating within mutualisms. Cheaters have been the focus of much empirical and theoretical work as they have the potential to lead to mutualism collapse, yet there has been debate in the literature regarding how to identify cheaters (Frederickson, 2013). A recent synthesis defines cheating as increasing one's own relative fitness while decreasing that of the partner (Jones *et al.*, 2015). However, cheating is not synonymous with conflict, and in fact Jones et al. (2015) show that under both fitness conflict and fitness alignment cheating genotypes may be present within the population. Within the economic model that we use in our study, there is a fundamental conflict between plant and symbiont over the exchange ratio (Schwartz & Hoeksema, 1998; Grman *et al.*, 2012). Our finding of asymmetric bargaining can be interpreted as evidence of this conflict because it demonstrates that partners may not benefit equally from trade, even if trade is mutually beneficial. However, given that the exchange ratio seems to be determined almost entirely by the plant, this control could in effect force the fitness interests of the symbiont to align with the host. Consequently, we predict that with multiple genotypes of varying fixation abilities there would be a strong signal of fitness alignment—even in the face of underlying conflict.

Limitations and future work

The nutrient-exchange model employed connects nutrient uptake rates to partner growth by predicting the allocation strategies that maximize growth, given the organismal stoichiometric compositions. More sophisticated models that include detailed chemical reactions can predict the metabolic processes involved (Resendis et al 2011, Zhao et al 2012), but they also rely on observed stoichiometries and maximizing growth rates. Consequently, one limitation of our approach is that nutrient stoichiometries and rates are treated as constants, which is not necessarily consistent with biological systems (Näsholm et al 2009, Wolf et al. 2017). We addressed this challenge by independently measuring model parameters and predictions at all four nitrogen levels. Another limitation is that stoichiometric models do not account for other benefits of mutualisms, such as rhizobia acting as biocontrol agents to protect the plant from fungal pathogens (Das et al 2017) or association with rhizobia leading to the induction of defense signaling pathways (Dean et al 2014). We sought to minimize the influence of these other benefits by protecting the plants from pathogens and keeping them well-watered and under stable conditions.

To our knowledge, the symmetry of bargaining power has not yet been directly tested in other plant-microbe mutualisms, but researchers have proposed several mechanisms that influence the power dynamics, such as symbionts conferring greater competitive power to more cooperative plants (Bücking et al 2016), partner strategies for preventing imbalances in benefits received from trade (Kiers et al 2011), and outside negotiator options leading to joint control of the mutualism (Ackay and Simms 2011). Further work in other plant-microbe symbioses is needed before it can be determined if asymmetric bargaining power is a broad feature of nutritional symbioses and how it relates to these mechanisms.

Conclusion

Our finding of experimental support for a model of asymmetric ("unfair") bargaining power between legumes and rhizobia versus a model of "fair" trade highlights the power of integrating quantitative models with data in the study of mutualisms (Clark *et al.*, 2017), which we believe and will be broadly applicable to other systems. This work improves our understanding of the drivers of quantitative variation in symbiotic nitrogen fixation, a process that makes a major contribution to the global nitrogen cycle (Fowler *et al.,* 2013) and is critical for agricultural sustainability (Herridge *et al.*, 2008). Our finding of a reduction in plant bargaining power at higher nitrogen levels combined with the frequently high rates of fertilizer application to legume crops underscores the potential evolutionary danger of relaxed host control under anthropogenic inputs (Kiers *et al.*, 2007; Weese *et al.*, 2015). The ability to estimate bargaining power using this approach will allow bargaining strength to be compared with measurements of natural selection. We also believe these estimates contribute to investigating the genetic, metabolic, and physiological mechanisms underlying the regulation of resource exchange and could facilitate future efforts to breed crops that can maintain their own beneficial microbiomes (Busby *et al.*, 2017).

APPENDIX

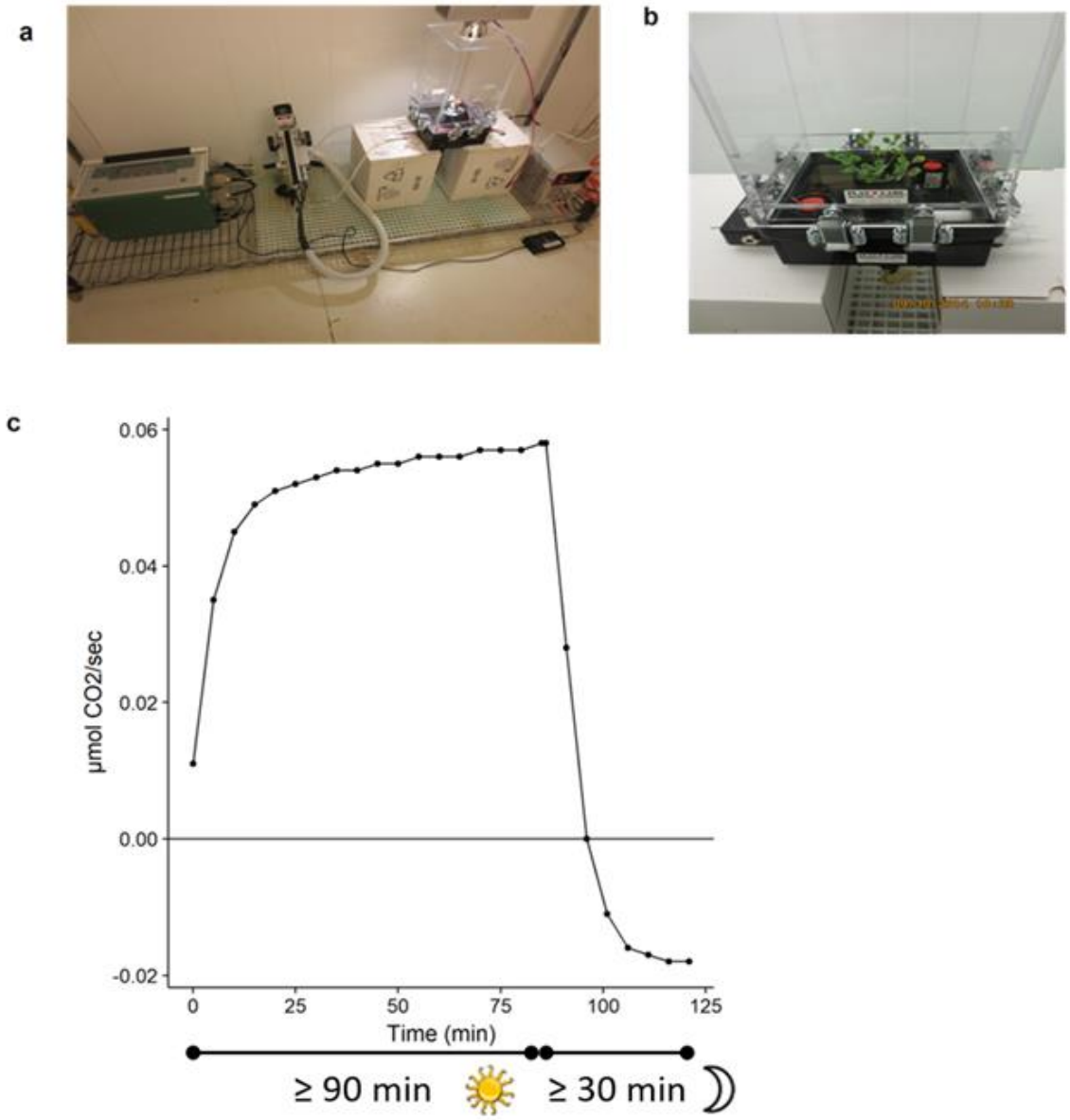

Figure 2.7 | Whole-plant photosynthesis chambers were used to measure carbon uptake rates. (a) Chambers were connected to LI-COR LI-6400 apparatuses, illuminated with LED lights, and used in a growth chamber to ensure assay conditions matched growth conditions. (b) Chambers included fans to promote air circulation. (c) Steady-state photosynthesis was measured by collecting readings every 5 min for at least 90 min in the light. Respiration was estimated with dark measurements. Data shown is from a nodulated plant grown at 40 mg L^{-1} N, but is representative of all measured plants.

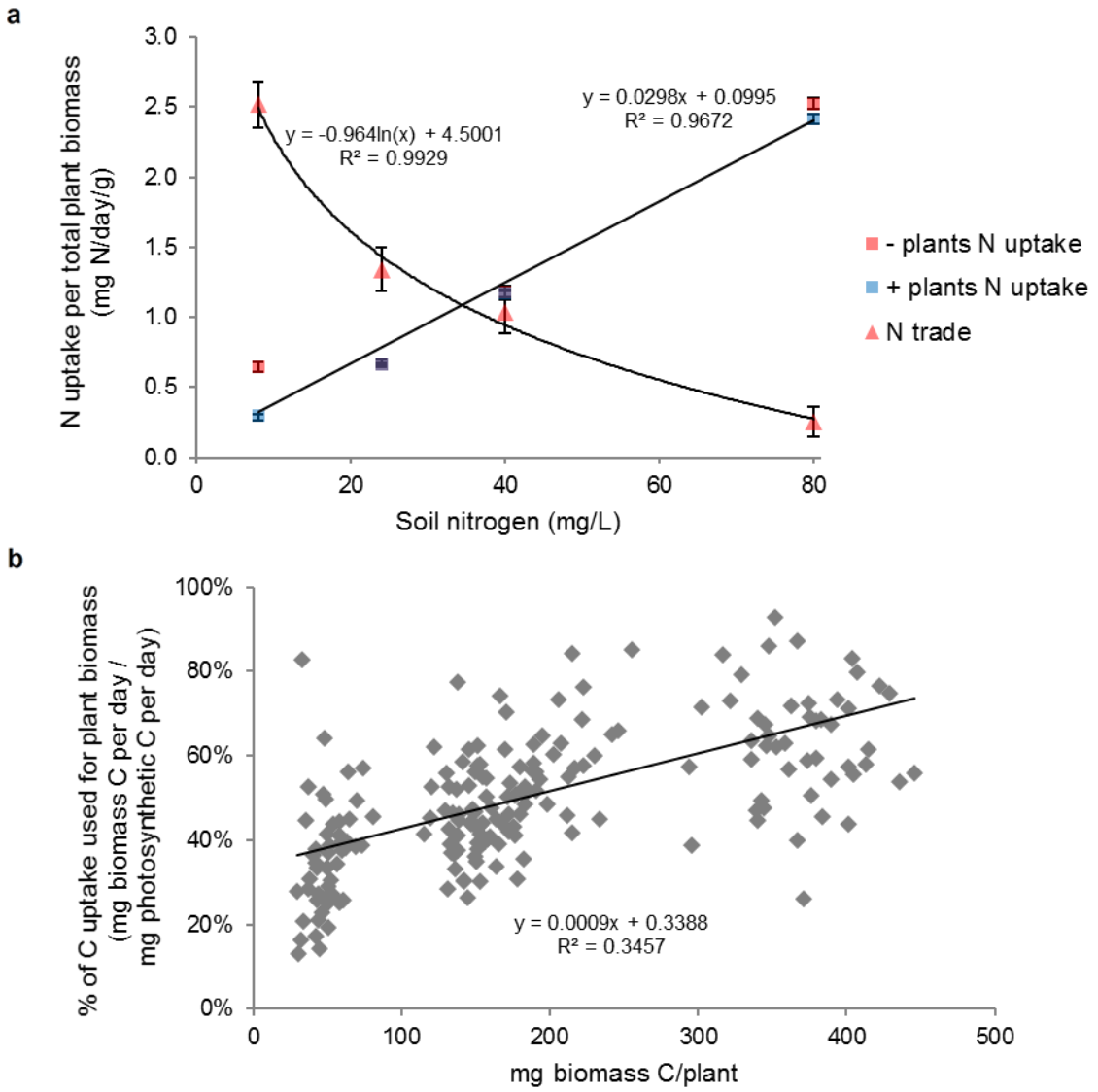

Figure 2.8 | Regression analyses used to calculate model parameters. (a) Direct nitrogen uptake (squares) and trade (triangles) rates per total plant biomass were experimentally estimated for uninoculated (blue) and/or nodulated (red) plants at 8, 24, 40, and 80 mg L^{-1} N. Linear or exponential regression was used to analyze the correlation between direct uptake or trade, respectively, and nitrogen availability. Values and error bars represent average and 90% confidence intervals, respectively, of 50 pseudo datasets generated by Monte Carlo sampling. (b) The percent of photosynthetic carbon uptake used for biomass is directly correlated with total carbon content in uninoculated plants. This correlation was analyzed with linear regression and used to estimate respiration rates in nodulated plants. Values represent 50 pseudo datasets per nitrogen treatment. (a,b) The linear equations were used in model analysis as described in the Methods.

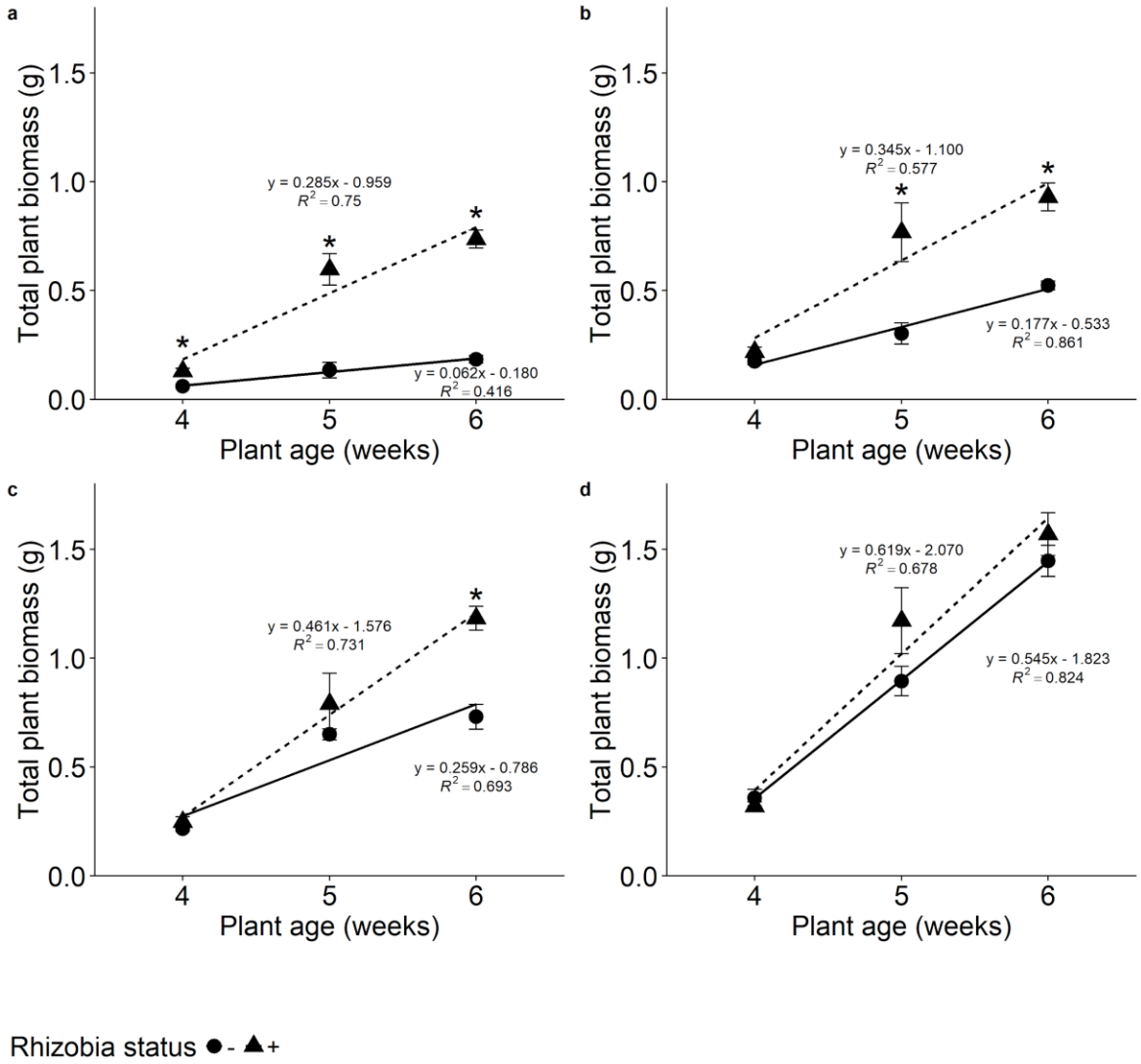

Figure 2.9 | Plant growth is near linear between 4 and 6 weeks. Nodulated (triangles, dashed lines) and uninoculated (circles, solid lines) plant dry weights were measured at 8 (a), 24 (b), 40 (c), and 80 (d) mg L^{-1} N. Growth rates were analyzed using linear regression. All rates were found to have $p < 0.001$, and equations and R^2 values as indicated. Error bars represent standard error (n = 7-15). Plant ages with asterisks indicate significant differences between inoculated and uninoculated plants at that age; plant ages without asterisks do not significantly differ after *post hoc* testing with the Tukey test ($p < 0.05$).

Table 2.1 | Model input parameters and measured values. Parameters were measured and used in model construction as described in the Methods. Values correspond to the average ± 90% confidence interval by Monte Carlo sampling with 50 pseudo datasets.

Parameter	Biological interpretation	Units	8 mg/L N	24 mg/L N	40 mg/L N	80 mg/L N
p	plant size	mg C	172.87 ± 6.15	231.54 ± 8.22	285.49 ± 9.97	389.92 ± 13.61
r	rhizobial partner size	mg C	3.91 ± 0.21	3.45 ± 0.19	2.58 ± 0.12	0.42 ± 0.05
ynp	plant carbon biomass yield per unit N	mg C/mg N	11.94 ± 0.20	11.48 ± 0.12	11.17 ± 0.23	10.39 ± 0.05
ynr	nodule carbon biomass yield per unit N	mg C/mg N	6.06 ± 0.04	6.17 ± 0.05	6.01 ± 0.03	6.19 ± 0.06
fcp'	photosynthetic carbon uptake rate	mg C/shoot C /day	0.12 ± 0.00	0.15 ± 0.01	0.13 ± 0.01	0.11 ± 0.00
fnp'	soil nitrogen uptake rate	mg N/root C /day	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
fnr'	nitrogen fixation rate	mg N/nod C/day	0.29 ± 0.03	0.27 ± 0.04	0.36 ± 0.07	3.05 ± 3.56

Table 2.2 | Model predictions and corresponding values. Experimentally estimated or model predicted carbon-for-nitrogen exchange ratio (T; mg C mg^{-1} N), proportion of plant carbon allocated to roots (aNP; g root C g^{-1} plant C), plant per capita growth rate (gP; g C day^{-1} g^{-1} plant C), rhizobial per capita growth rate (gR; g C day^{-1} g^{-1} rhizobial partner C), and proportion of bargaining power possessed by the plant (β). Model predictions include those predicted with or without organismal respiration, use the plant's collective nodules as a proxy for the rhizobial partner or assume only 50% of the nodule biomass represents the rhizobial partner, and assume symmetric power (i.e., β = 0.50), average asymmetric power across the observed soil nitrogen range (i.e., β = 0.70), or asymmetric power which varies with soil nitrogen. Values correspond to the average ± 90% confidence interval by Monte Carlo sampling with 50 pseudo datasets (see Methods for further details).

8 mg/L N	**T**	**aNP**	**gP**	**gR**	**β**
experimentally estimated	2.98 ± 0.07	0.29 ± 0.02	0.10 ± 0.00	0.08 ± 0.01	-
symmetric, without respiration	4.93 ± 0.16	0.62 ± 0.02	0.08 ± 0.00	0.86 ± 0.11	0.50
symmetric, with respiration	7.57 ± 0.34	0.41 ± 0.02	0.05 ± 0.00	0.41 ± 0.07	0.50
symmetric, with respiration, assume 50% rhizobia	9.74 ± 3.79	0.43 ± 0.04	0.05 ± 0.00	0.75 ± 0.10	0.50
average β, without respiration	2.23 ± 0.05	0.60 ± 0.02	0.09 ± 0.00	0.52 ± 0.07	0.70
average β, with respiration	5.04 ± 0.19	0.41 ± 0.03	0.06 ± 0.00	0.24 ± 0.04	0.70
variable β, without respiration	0.35 ± 0.04	0.57 ± 0.02	0.11 ± 0.00	0.08 ± 0.00	0.94 ± 0.01
variable β, with respiration	3.46 ± 0.09	0.38 ± 0.02	0.06 ± 0.00	0.08 ± 0.00	0.86 ± 0.03
variable β, with respiration, assume 50% rhizobia	3.05 ± 0.08	0.38 ± 0.02	0.07 ± 0.00	0.09 ± 0.00	0.92 ± 0.01

24 mg/L N	**T**	**aNP**	**gP**	**gR**	**β**
experimentally estimated	2.97 ± 0.09	0.25 ± 0.01	0.09 ± 0.00	0.07 ± 0.01	-
symmetric, without respiration	3.65 ± 0.15	0.53 ± 0.03	0.11 ± 0.01	0.55 ± 0.08	0.50
symmetric, with respiration	5.32 ± 0.30	0.37 ± 0.03	0.07 ± 0.00	0.27 ± 0.06	0.50
symmetric, with respiration, assume 50% rhizobia	5.25 ± 0.20	0.38 ± 0.02	0.08 ± 0.00	0.48 ± 0.08	0.50
average β, without respiration	1.76 ± 0.06	0.53 ± 0.03	0.12 ± 0.01	0.33 ± 0.05	0.70

Table 2.2 (cont'd)

variable β, with respiration	3.69 ± 0.23	0.36 ± 0.03	0.08 ± 0.00	0.07 ± 0.00	0.75 ± 0.06
variable β, with respiration, assume 50% rhizobia	3.18 ± 0.17	0.37 ± 0.03	0.08 ± 0.00	0.06 ± 0.01	0.83 ± 0.05

40 mg/L N	**T**	**aNP**	**gP**	**gR**	**β**
experimentally estimated	3.06 ± 0.19	0.26 ± 0.01	0.09 ± 0.00	0.08 ± 0.01	-
symmetric, without respiration	3.03 ± 0.15	0.46 ± 0.02	0.11 ± 0.00	0.63 ± 0.13	0.50
symmetric, with respiration	4.80 ± 0.29	0.35 ± 0.02	0.08 ± 0.00	0.26 ± 0.07	0.50
symmetric, with respiration, assume 50% rhizobia	4.46 ± 0.19	0.32 ± 0.01	0.08 ± 0.00	0.51 ± 0.11	0.50
average β, without respiration	1.51 ± 0.06	0.45 ± 0.02	0.11 ± 0.00	0.38 ± 0.08	0.70
average β, with respiration	3.61 ± 0.11	0.32 ± 0.02	0.08 ± 0.00	0.15 ± 0.03	0.70
variable β, without respiration	1.62 ± 0.77	0.45 ± 0.02	0.11 ± 0.00	0.08 ± 0.01	0.84 ± 0.06
variable β, with respiration	3.80 ± 0.26	0.33 ± 0.02	0.08 ± 0.00	0.08 ± 0.01	0.62 ± 0.08
variable β, with respiration, assume 50% rhizobia	3.28 ± 0.22	0.33 ± 0.02	0.08 ± 0.00	0.08 ± 0.01	0.77 ± 0.06

80 mg/L N	**T**	**aNP**	**gP**	**gR**	**β**
experimentally estimated	2.72 ± 0.07	0.24 ± 0.01	0.10 ± 0.00	0.10 ± 0.01	-
symmetric, without respiration	1.83 ± 0.10	0.31 ± 0.01	0.11 ± 0.00	2.72 ± 1.10	0.50
symmetric, with respiration	3.09 ± 0.15	0.24 ± 0.01	0.08 ± 0.00	0.72 ± 0.43	0.50
symmetric, with respiration, assume 50% rhizobia	2.86 ± 0.12	0.22 ± 0.01	0.08 ± 0.00	1.22 ± 0.57	0.50
average β, without respiration	0.98 ± 0.05	0.31 ± 0.01	0.11 ± 0.00	1.63 ± 0.66	0.70
average β, with respiration	2.83 ± 0.08	0.23 ± 0.01	0.08 ± 0.00	0.77 ± 0.93	0.70
variable β, without respiration	2.24 ± 0.54	0.31 ± 0.01	0.11 ± 0.00	0.10 ± 0.02	0.37 ± 0.11

Table 2.2 (cont'd)

variable β, with respiration	2.91 ± 0.12	0.23 ± 0.01	0.08 ± 0.00	0.06 ± 0.03	0.57 ± 0.11
variable β, with respiration, assume 50% rhizobia	2.89 ± 0.12	0.23 ± 0.01	0.08 ± 0.00	0.05 ± 0.02	0.63 ± 0.11

Table 2.3 | Plant carbon and nitrogen elemental compositions. Carbon and nitrogen elemental compositions were measured in 4- and 6-week-old uninoculated (-) and nodulated (+) plants at 8, 24, 40, and 80 mg L^{-1} N. Values are average ± standard deviation.

Age (wk)	[N] (mg/L)	Rhizobia	Root %C (%wt)	Shoot %C (%wt)	Root %N (%wt)	Shoot %N (%wt)	Plant Yield (mg C mg^{-1} N)
4	8	-	45.89 ± 1.90	39.18 ± 0.58	2.19 ± 0.14	3.46 ± 0.53	13.76 ± 1.69
4	24	-	42.89 ± 2.40	40.49 ± 0.76	2.31 ± 0.19	3.34 ± 0.13	13.74 ± 0.69
4	40	-	43.96 ± 0.84	39.80 ± 0.18	2.31 ± 0.26	3.45 ± 0.28	13.24 ± 1.20
4	80	-	44.19 ± 1.89	39.06 ± 1.06	2.71 ± 0.27	4.57 ± 0.55	10.00 ± 1.65
4	8	+	44.59 ± 1.85	41.06 ± 0.13	2.34 ± 0.23	4.41 ± 0.19	10.69 ± 0.46
4	24	+	44.13 ± 0.74	40.72 ± 1.42	2.40 ± 0.13	4.26 ± 0.08	10.86 ± 0.36
4	40	+	43.51 ± 0.49	39.81 ± 0.43	2.82 ± 0.08	4.38 ± 0.31	10.21 ± 0.59
4	80	+	45.63 ± 0.38	39.34 ± 1.21	3.23 ± 0.24	4.75 ± 0.35	9.23 ± 0.52
6	8	-	43.43 ± 1.41	38.40 ± 0.78	2.00 ± 0.36	2.60 ± 0.52	17.16 ± 3.12
6	24	-	44.32 ± 0.48	39.17 ± 0.43	1.99 ± 0.26	2.52 ± 0.09	17.48 ± 1.14
6	40	-	41.93 ± 4.68	39.45 ± 0.65	2.08 ± 0.21	2.89 ± 0.22	15.21 ± 1.47
6	80	-	44.43 ± 2.32	39.56 ± 1.02	2.99 ± 0.15	4.26 ± 0.83	10.25 ± 1.64
6	8	+	43.02 ± 3.15	39.30 ± 1.54	2.28 ± 0.09	3.47 ± 0.51	12.77 ± 1.90
6	24	+	42.10 ± 1.73	39.45 ± 0.56	2.16 ± 0.41	3.67 ± 0.31	12.06 ± 1.02
6	40	+	41.51 ± 2.64	39.98 ± 0.29	2.42 ± 0.21	3.65 ± 0.59	12.24 ± 1.68
6	80	+	42.86 ± 0.48	41.12 ± 0.10	2.98 ± 0.18	3.79 ± 0.06	11.49 ± 0.17

Table 2.4 | Nodule carbon and nitrogen elemental compositions. Carbon and nitrogen elemental compositions were measured in 4- and 6-week-old nodulated plant systems at 8, 24, 40, and 80 mg L^{-1} N. Values are average ± standard deviation.

Age (wk)	[N] (mg/L)	Nodule %C (%wt)	Nodule %N (%wt)	Nodule Yield (mg C mg^{-1} N)
4	8	44.30 ± 0.52	8.10 ± 0.11	5.47 ± 0.01
4	24	45.26 ± 0.06	7.85 ± 0.48	5.78 ± 0.36
4	40	43.02 ± 1.14	7.50 ± 0.42	5.74 ± 0.27
4	80	43.15 ± 0.00	7.17 ± 0.00	6.02 ± 0.00
6	8	43.53 ± 1.14	6.52 ± 0.36	6.69 ± 0.36
6	24	44.56 ± 1.10	6.72 ± 0.24	6.64 ± 0.15
6	40	43.57 ± 1.07	6.89 ± 0.24	6.32 ± 0.09
6	80	40.47 ± 0.92	6.43 ± 0.68	6.32 ± 0.52

REFERENCES

REFERENCES

Akçay E, Roughgarden J. 2007. Negotiation of mutualism: rhizobia and legumes. *Proceedings of the Royal Society of London B: Biological Sciences* **274**(1606): 25-32.

Binmore K, Rubinstein A, Wolinsky A. 1986. The Nash bargaining solution in economic modelling. *The RAND Journal of Economics*: 176-188.

Bloom AJ, Chapin III FS, Mooney HA. 1985. Resource limitation in plants-an economic analogy. *Annual review of Ecology and Systematics* **16**(1): 363-392.

Bronstein JL. 2015. *Mutualism*: Oxford University Press, USA.

Busby PE, Soman C, Wagner MR, Friesen ML, Kremer J, Bennett A, Morsy M, Eisen JA, Leach JE, Dangl JL. 2017. Research priorities for harnessing plant microbiomes in sustainable agriculture.*PLoS biology* **15**(3): e2001793.

Chae S, Heidhues P. 2001. Nash bargaining solution with coalitions and the joint bargaining paradox:WZB Discussion Paper.

Chakraborty T. 2011. *Bargaining and pricing in networked economic systems.* University of Pennsylvania.

Chakraborty T, Kearns M, Khanna S 2009. Network bargaining: algorithms and structural results. *Proceedings of the 10th ACM conference on Electronic commerce*: ACM. 159-168.

Clark TJ, Friel CA, Grman E, Shachar-Hill Y, Friesen ML. 2017. Modelling nutritional mutualisms:challenges and opportunities for data integration. *Ecology letters* **20**(9): 1203-1215.

Das, K., Prasanna, R., & Saxena, A. K. (2017). Rhizobia: a potential biocontrol agent for soilborne fungal pathogens. **Folia microbiologica, 62(5), 425-435.**

Daubech B, Remigi P, de Moura GD, Marchetti M, Pouzet C, Auriac M-C, Gokhale CS, Masson-Boivin C, Capela D. 2017. Spatio-temporal control of mutualism in legumes helps spread symbiotic nitrogen fixation. *Elife* **6**.

Dean, J. M., Mescher, M. C., & De Moraes, C. M. (2014). Plant dependence on rhizobia for nitrogen influences induced plant defenses and herbivore performance. International journal of molecular sciences**, 15(1), 1466-1480.**

Fahraeus G. 1957. The Infection of Clover Root Hairs by Nodule Bacteria Studied by a Simple Glass Slide Technique. *Microbiology* **16**: 374–381.

Franklin O, Näsholm T, Högberg P, Högberg MN. 2014. Forests trapped in nitrogen limitation–an ecological market perspective on ectomycorrhizal symbiosis. *New phytologist* **203**(2): 657-666.

Frederickson ME. 2013. Rethinking mutualism stability: cheaters and the evolution of sanctions. *The Quarterly review of biology* **88**(4): 269-295.

Ghoul, M., Griffin, A. S., & West, S. A. (2014). Toward an evolutionary definition of cheating. *Evolution*, ***68*(**2), 318-331

Goh CH, Nicotra AB, Mathesius U. 2016. The presence of nodules on legume root systems can alter phenotypic plasticity in response to internal nitrogen independent of nitrogen fixation. *Plant, cell & environment* **39**(4): 883-896.

Grman E, Robinson TM, Klausmeier CA. 2012. Ecological specialization and trade affect the outcome of negotiations in mutualism. *The American Naturalist* **179**(5): 567-581.

Harris D, Pacovsky R, Paul E. 1985. Carbon economy of soybean–Rhizobium–Glomus associations. *New phytologist* **101**(3): 427-440.

Herridge DF, Peoples MB, Boddey RM. 2008. Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil* **311**: 1–18.

Jones EI, Afkhami ME, Akçay E, Bronstein JL, Bshary R, Frederickson ME, Heath KD, Hoeksema JD, Ness JH, Pankey MS, et al. 2015. Cheaters must prosper: reconciling theoretical and empirical perspectives on cheating in mutualism. *Ecology letters* **18**(11): 1270-1284.

Kawamori T. 2014. A noncooperative foundation of the asymmetric Nash bargaining solution. *Journal of Mathematical Economics* **52**: 12-15.

Kiers ET, Hutton MG, Denison RF. 2007. Human selection and the relaxation of legume defences against ineffective rhizobia. *Proceedings of the Royal Society of London B: Biological Sciences* **274**(1629): 3119-3126.

Lambers H, Szaniawski RK, Visser R. 1983. Respiration for growth, maintenance and ion uptake. An evaluation of concepts, methods, values and their significance. *Physiologia plantarum* **58**(4): 556-563.

Nash JF. 1950. The bargaining problem. *Econometrica: Journal of the Econometric Society*: 155-162.

Näsholm, T., Kielland, K., & Ganeteg, U. (2009). Uptake of organic nitrogen by plants. *New phytologist*, **182**(1), 31-48**.**

Paponov I, Posepanov O, Lebedinskai S, Koshkin E. 2000. Growth and biomass allocation, with varying nitrogen availability, of near-isogenic pea lines with differing foliage structure. *Annals of botany* **85**(4): 563-569.

Phillips DA. 1980. Efficiency of symbiotic nitrogen fixation in legumes. *Annual Review of Plant Physiology* **31**(1): 29-49.

Ratcliff, W. C., Underbakke, K., & Denison, R. F. 2011. Measuring the fitness of symbiotic rhizobia. *Symbiosis*, **55**(2), 85-90.

Reeve W, Chain P, O'Hara G, Ardley J, Nandesena K, Bräu L, Tiwari R, Malfatti S, Kiss H, Lapidus A, et al. 2010. Complete genome sequence of the Medicago microsymbiont Ensifer (Sinorhizobium) medicae strain WSM419. *Standards in genomic sciences* **2**(1): 77.

Regus J, Wendlandt C, Bantay R, Gano-Cohen K, Gleason N, Hollowell A, O'Neill M, Shahin K, Sachs J. 2017. Nitrogen deposition decreases the benefits of symbiosis in a native legume. *Plant and Soil* **414**(1-2): 159-170.

Regus JU, Quides KW, O'Neill MR, Suzuki R, Savory EA, Chang JH, Sachs JL. 2017. Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. *American Journal of Botany* **104**(9): 1299-1312.

Resendis-Antonio, O., Hernández, M., Salazar, E., Contreras, S., Batallar, G. M., Mora, Y., & Encarnación, S. 2011. Systems biology of bacterial nitrogen fixation: high-throughput technology and its integrative description with constraint-based modeling. *BMC systems biology*, ***5***(1), 120.

Rufty TW, Raper CD, Huber SC. 1984. Alterations in internal partitioning of carbon in soybean plants in response to nitrogen stress. *Canadian Journal of Botany* **62**(3): 501-508.

Ryle G, Arnott R, Powell C, Gordon A. 1984. N2 fixation and the respiratory costs of nodules, nitrogenase activity, and nodule growth and maintenance in Fiskeby soyabean. *Journal of Experimental Botany* **35**(8): 1156-1165.

Schwartz MW, Hoeksema JD. 1998. Specialization and resource trade: biological markets as a model of mutualisms. *Ecology* **79**(3): 1029-1038.

Simms, E. L., & Taylor, D. L. (2002). Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. *Integrative and Comparative Biology*, **42**(2), 369-380.

Udvardi M, Poole PS. 2013. Transport and metabolism in legume-rhizobia symbioses. *Annual review of plant biology* **64**: 781-805.

Valladares F, Villar-Salvador P, Domínguez S, Fernández-Pascual M, Peñuelas JL, Pugnaire FI. 2002. Enhancing the early performance of the leguminous shrub Retama sphaerocarpa (L.) Boiss.: fertilisation versus Rhizobium inoculation. *Plant and Soil* **240**(2): 253-

262.

Voisin A-S, Salon C, Munier-Jolain NG, Ney B. 2002. Effect of mineral nitrogen on nitrogen nutrition and biomass partitioning between the shoot and roots of pea (Pisum sativum L.). *Plant and Soil* **242**(2): 251-262.

Wang X, Pan Q, Chen F, Yan X, Liao H. 2011. Effects of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on soybean growth as related to root architecture and availability of N and P. *Mycorrhiza* **21**(3): 173-181.

Wardlaw IF. 1990. Tansley Review No. 27 The control of carbon partitioning in plants. *New phytologist* **116**(3): 341-381.

Weese DJ, Heath KD, Dentinger B, Lau JA. 2015. Long-term nitrogen addition causes the evolution of less-cooperative mutualists. *Evolution* **69**(3): 631-642.

Werner GD, Strassmann JE, Ivens AB, Engelmoer DJ, Verbruggen E, Queller DC, Noë R, Johnson NC, Hammerstein P, Kiers ET. 2014. Evolution of microbial markets. *Proceedings of the National Academy of Sciences* **111**(4): 1237-1244.

Weyl EG, Frederickson ME, Douglas WY, Pierce NE. 2010. Economic contract theory tests models of mutualism. *Proceedings of the National Academy of Sciences* **107**(36): 15712-15716.

Wolf, A. A., Funk, J. L., & Menge, D. N. (2017). The symbionts made me do it: legumes are not hardwired for high nitrogen concentrations but incorporate more nitrogen when inoculated. *New Phytologist*, **213**(2), 690-699.

Young ND, Debellé F, Oldroyd GE, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KF, Gouzy J, Schoof H, et al. 2011. The Medicago genome provides insight into the evolution of rhizobial symbioses. *Nature* **480**(7378): 520.

Younginger, B. S., Sirová, D., Cruzan, M. B., & Ballhorn, D. J. 2017. Is biomass a reliable estimate of plant fitness?. *Applications in plant sciences*, ***5***(2), 1600094.

Zhao, H., Li, M., Fang, K., Chen, W., & Wang, J. 2012. In silico insights into the symbiotic nitrogen fixation in Sinorhizobium meliloti via metabolic reconstruction. *PLoS One*, ***7***(2), e31287.

CHAPTER 3

LEGUMES MODULATE ALLOCATION TO RHIZOBIAL NITROGEN FIXATION IN RESPONSE TO FACTORIAL RESOURCE MANIPULATION

Abstract

The costs and benefits that define gain from trade in resource mutualisms depend on resource availability. Optimal partitioning theory predicts that allocation to direct uptake versus trade will be determined by both the relative benefit of the resource acquired through trade, and the relative cost of the resource being traded away. The costs and benefits of carbon:nitrogen exchange in the legume-rhizobia symbiosis have been examined in depth with regards to mineral nitrogen availability. However, the effects of varying carbon costs have rarely been considered and we currently lack definitive empirical examples of legumes modulating symbiosis in response to carbon availability. Here, we report plant growth and symbiosis investment in the model legume *Medicago truncatula* and its symbiont *Ensifer medicae* across varying nitrogen and light environments. We demonstrate that plants modulate their allocation to roots and nodules as their return on investment varies according to external nitrogen and carbon availability. We find empirical evidence that plant allocation to nodules responds to carbon availability, but that this depends upon the nitrogen environment. In particular, at low nitrogen–where rhizobia provided the majority of nitrogen for plant growth–relative nodule allocation increased when carbon limitation was alleviated with high light levels. This context-dependent modulation of resource allocation to rhizobia prevents this interaction from becoming parasitic even in low-light, high-nitrogen environments.

Introduction

Interactions between plants and microbial symbionts are major drivers of global nutrient cycles and play vital roles in the productivity of natural and agricultural ecosystems (van der Heijden *et al.*, 2008). Microbial symbionts can supply plants with both nitrogen and phosphorus, essential nutrients that commonly limit plant growth (Erisman *et al.*, 2013). Similarly, soil bacteria known as rhizobia can colonize plant roots and induce the formation of nodules, inside of which the rhizobia fix an estimated 40 million tons of plant-inaccessible nitrogen from the atmosphere in exchange for photosynthetic carbon (Udvardi & Poole, 2013). While these interactions are generally regarded as mutualistic, with the symbiosis increasing fitness for both partners (Bronstein, 2015), theory predicts that symbioses exist along a gradient from mutualism to parasitism depending on the environmental context (Bronstein, 1994, 2001; Johnson *et al.*, 1997; Neuhauser & Fargione, 2004).

One key factor that may shift an interaction along the mutualism-parasitism continuum is the availability of the traded resources in the environment. Symbionts are predicted to shift from mutualism towards parasitism when the resource they supply is abundant in the soil, and thus the benefit to the host of obtaining it from the symbiont is reduced (Bronstein, 1994; Neuhauser & Fargione, 2004). With high levels of nitrogen fertilizer use and nitrogen deposition across ecosystems (Foley *et al.*, 2005), there is concern that nitrogen-fixing symbioses between plants and microbes such as rhizobia may break down. Even a single growing season of fertilizer application is sufficient to change the composition of the rhizobia population in soil (Simonsen *et al.*, 2015). Long-term nitrogen addition experiments have shown that prolonged nitrogen fertilization leads to the evolution of less effective rhizobia in the *Trifolium*-rhizobium symbiosis (Weese *et al.*, 2015). This partner quality decline may be linked to evolutionary differentiation in

the symbiotic plasmid (Klinger *et al.*, 2016). However, in the presence of externally supplied nitrogen the plant may be able to minimize the costs associated with less beneficial rhizobia by reducing or eliminating its allocation of resources to the microbes.

The way a plant allocates resources to microbial symbionts such as rhizobia can be considered in the framework of biological market theory (Schwartz & Hoeksema, 1998). The plant can allocate its resources in two distinct ways: it can increase root biomass to take up nitrogen directly from the soil or it can increase photosynthesis to acquire carbon to trade for nitrogen with rhizobia. The cost-benefit analysis of trade versus direct uptake depends on the availability of both traded resources. Optimal partitioning theory predicts that the plant will allocate biomass to the part of the plant that acquires the resource that is most limiting to the plant (Thornley, 1972; Bloom *et al.*, 1985). In this case, each partner will specialize in acquiring the resource for which it has a comparative advantage and trade to acquire the other resources, and both partners will acquire more total resources than they would in isolation (Schwartz & Hoeksema, 1998). When trade is beneficial, an organism increases its own potential fitness by engaging in trade and mutualisms can readily evolve, essentially as by-product mutualisms. However, if the resource being traded away is not available in excess, or if the resource being traded for is abundant in the environment and/or cheap to obtain, the fitness gain from trade may become negative (Johnson *et al.*, 1997; Schwartz & Hoeksema, 1998). In this context, optimal partitioning theory predicts that under high mineral nitrogen levels the plant will downregulate allocation to symbiosis–in extreme cases perhaps terminating the relationship entirely if they are able to. However, a plant's optimal allocation to nitrogen uptake will also depend critically upon both the carbon cost of each uptake strategy as well as the carbon available to the plant.

There is a large body of empirical evidence for shifts in allocation and context-dependent benefits in response to nutrient availability for the nutrient supplied by the microbe in plant-microbe nutritional symbioses, but very few studies have investigated the effects of factorially manipulating both traded resources. The negative effects of soil nitrate, the most commonly available form of soil nitrogen, on nodulation has long been reported in the literature (Streeter & Wong, 1988; Lucinski *et al.*, 2002; Glyan'ko *et al.*, 2009), though the magnitude of the effect of nitrate on nodulation may be strongly affected by genotype-by-genotype interactions (Heath *et al.*, 2010).

In contrast to the preponderance of literature exploring the effects of mineral nitrogen on legume-rhizobia interactions, relatively little is known about the effects of light on these interactions. The studies that do exist have found wildly varying results. Three species of *Desmodium* exhibit reduced plant biomass and total nodule number with shade, but no corresponding change in the ratio of nodule biomass to root biomass and plant nitrogen content (Houx *et al.*, 2009). *Trifolium repens* reduced total nodule biomass in shaded conditions, but this was mostly explained by reduced root biomass (Chu & Robertson, 1974). Various studies in soybean have shown that shading increases nodule biomass and decreases efficiency (Santos *et al.*, 1997), that shading decreases nodule biomass and increases efficiency (Araujo *et al.*, 2018), and that shading decreases nodule biomass but does not affect efficiency (Hansen *et al.*, 1990).

The literature regarding interactions between nitrogen and carbon availability is even sparser and currently limited to determinate legume-rhizobium symbioses in which the rhizobia are non-terminally differentiated as bacteroids (Denison, 2000). Lau *et al.*, (2012) manipulated N-P-K fertilizer and light levels and found that *Bradyrhizobium japonicum* nodulation on *Glycine max* (soybean) was significantly decreased by low light levels, but this study did not

detect an effect of fertilizer on nodulation. *Bradyrhizobium* increased plant biomass in the low nutrient, high light conditions, but had no effect on aboveground plant biomass in low nutrient, low light conditions or in any high nutrient conditions (Lau *et al.*, 2012), making it difficult to interpret this study in the framework of market theory. Similarly, Regus *et al.*, (2015) manipulated KNO_3 application and light regime (by season of growth in the greenhouse) in the *Lotus strigosus-Bradyrhizobium* symbiosis. They found that nitrogen fertilization eliminated plant growth benefits from rhizobia in the fall when there light levels were lower, while nitrogen fertilization reduced but did not eliminated plant growth benefits from rhizobia in the winter when light levels were higher (Regus *et al.*, 2015). Nitrogen decreased nodule biomass in both seasons, while it decreased nodule number in fall but not winter, consistent with the idea that low light makes carbon more expensive and thus reduces the overall investment in symbiosis (Regus *et al.*, 2015). These results suggest that there may be interactions between the availability of carbon and nitrogen, though it is impossible to rule out other environmental factors that varied between seasons. Thus, these initial results highlight the need for a highly controlled analysis of the interactions between carbon and nitrogen availability and their effects on plant investment in trade with rhizobia.

In this study, we examined changes in plant biomass allocation and trade with rhizobia in response to variation in both light level and soil mineral nitrogen. We used the model legume *Medicago truncatula* and its rhizobial partner *Ensifer medicae* WSM419 grown under controlled conditions with factorial light and nitrogen resource manipulation. We hypothesized that plants would allocate resources optimally according to optimal partitioning theory, leading to three predictions: 1) plants will allocate more resources to acquiring the limiting nutrient (nitrogen or carbon) as a function of external inputs, 2) plants will allocate relatively more resources to

nodules than to roots when soil nitrogen is low and thus the return on investment for root allocation is reduced relative to high soil nitrogen, and 3) when soil nitrogen is low, plants at high light will invest highly in nodules but carbon scarcity will modulate this allocation because when carbon is expensive the return on investment from roots will be higher than that from nodules.

Materials and Methods

Pot preparation

We filled SC10 Cone-Tainers (Steuwe and Sons Inc., Corvallis, OR, USA) with medium vermiculite. The pots had a ⅜" cotton wick leading to an opaque 50 mL reservoir to ensure constant access to liquid and nutrients. Prior to planting, we added 25 mL of deionized water to each pot, autoclaved once for 60 minutes, then fertilized with 25 mL of Fahraeus solution with 8 or 80 mg/L N (as NH_4NO_3). We then autoclaved the pots twice more for 60 minutes with approximately 24 hours between each run.

Seedling preparation

We scarified seeds of the model legume *Medicago truncatula* genotype A17 (Young *et al.*, 2011) with 600 grit sandpaper and sterilized them in full strength commercial bleach (8.25% $NaHClPO_3$) for 3 minutes, followed by 6 rinses with sterile deionized water. We incubated the seeds in sterile deionized water for 3 hours at room temperature, then re-sterilized imbibed seeds in 10% bleach for 30 seconds. After 6 rinses with sterile deionized water, seeds were incubated in sterile deionized water for 48 hours at 4°C. We changed the water once every twelve hours. After 48 hours, we transferred seeds to sterile petri dishes sealed with Parafilm and germinated them at room temperature in the dark for 48 hours. Seedlings with radicles at least 1 cm long

were transplanted into prepared pots. After planting, the plants were fully randomized and grown at 25°C with a 16 hour day/8 hour night cycle at approximately 150 µmol $m^{-2}s^{-1}$ to encourage uniform seedling establishment. Seedlings were misted daily with sterile deionized water to keep the radicles moist. After five days, plants were transferred to a high light growth chamber where half of the plants received full irradiation (400 µmol $m^{-2}s^{-1}$) and half were shaded with Sun Mesh Sunblock shade cloth to 200 µmol $m^{-2}s^{-1}$. Full irradiance plants were grown in one rack while shaded plants were grown in a separate rack. All plants were grown with equal spacing that prevented shading by other plants. All plants were grown at 25°C with a 16 hour day/8 hour night cycle.

Rhizobia preparation

Cultures of *Ensifer medicae* WSM419 (Reeve *et al.*, 2010) were grown for 48 hours in ½ TY at 30°C shaking at 200 RPM. Cell density was determined by measuring the OD600 of the culture using a NanoDrop. After 3 days of acclimation to the new growth chamber conditions, rhizobia inoculated plants received 1 mL of 10^7 CFU/mL suspended in ½x phosphate buffered saline (PBS) and rhizobia-free plants received 1 mL of sterile ½x PBS. Sterility of the mock inoculum and cell count of the rhizobial inoculum were checked using spot plating and serial dilution on TY agar.

Plant growth and harvest

Plants were watered from below with 25 mL of sterile Fahraeus nutrient solution with 8 or 80 mg/L nitrogen (as NH_4NO_3) whenever the reservoirs ran dry (at least twice a week). After 4 weeks of growth, plants were harvested. Nodules were plucked and counted and tissue was

dried at 60°C for one week. We measured root, shoot, and nodule dry weight and nodule number. No uninoculated plants developed nodules, indicating that there was no rhizobial contamination of uninoculated plants.

Statistical analysis

To test the effects of soil nitrogen and light availability on nodule biomass and the ratio of nodule biomass to root biomass, we used a linear model ANOVA with Type II sum of squares (car package, R 3.4.3) with nitrogen and light main effects and nitrogen by light interactions as fixed effects. Nodule biomass and the nodule:root ratio were log transformed to improve normality. We tested the effects of nitrogen and light on nodule number using a generalized linear model with a poisson distribution and ANOVA with Type II sum of squares (car package, R 3.4.3).

To test the relationship between nodule biomass and shoot biomass across nitrogen and light conditions, we used a linear model ANOVA with Type II sum of squares (car package, R 3.4.3) with nitrogen, light, nodule biomass, and all their interactions as fixed effects. Shoot biomass and nodule biomass were log transformed to improve normality.

To test the effects of nitrogen, light, and rhizobial inoculation on shoot biomass, root biomass, and the root:shoot ratio, we used a linear model ANOVA with Type II sum of squares (car package, R 3.4.3) with light, soil nitrogen, and rhizobial status and all interactions as fixed effects. Shoot biomass and root biomass were ln-transformed to improve normality. In all cases, to determine whether group means were significantly different, we conducted post-hoc testing with the Tukey test at a significance level of 0.05 (emmeans package, R 3.4.3).

Results

Both light and nitrogen affect total investment in rhizobia

High light increased nodule number by 32% overall (p = 4.93e-05), while high soil nitrogen decreased nodule number by 77% overall (p < 2e-16; Table 3.1). We did not detect a significant interactive effect of light and nitrogen on nodule number (p = 0.275; Table 3.1). This was surprising since high light significantly increases nodule number by 35% at low soil nitrogen (p = 0.0002) but non-significantly by 11% at high soil nitrogen (p = 0.906; Figure 3.1a). It seems likely that this statistical anomaly is caused by the relatively small effect size of light overall.

We detected a significant interaction between the effects of light and nitrogen on nodule biomass (p = 6.54e-04; Table 3.1). High soil nitrogen significantly reduced nodule biomass by 95% regardless of light level (p < 0.001 at both light levels; Figure 3.1b). However, at low soil nitrogen, high light increased nodule biomass by 103% (p < 0.001), while high light did not significantly increase nodule biomass at high soil nitrogen (p = 0.889; Figure 3.1b).

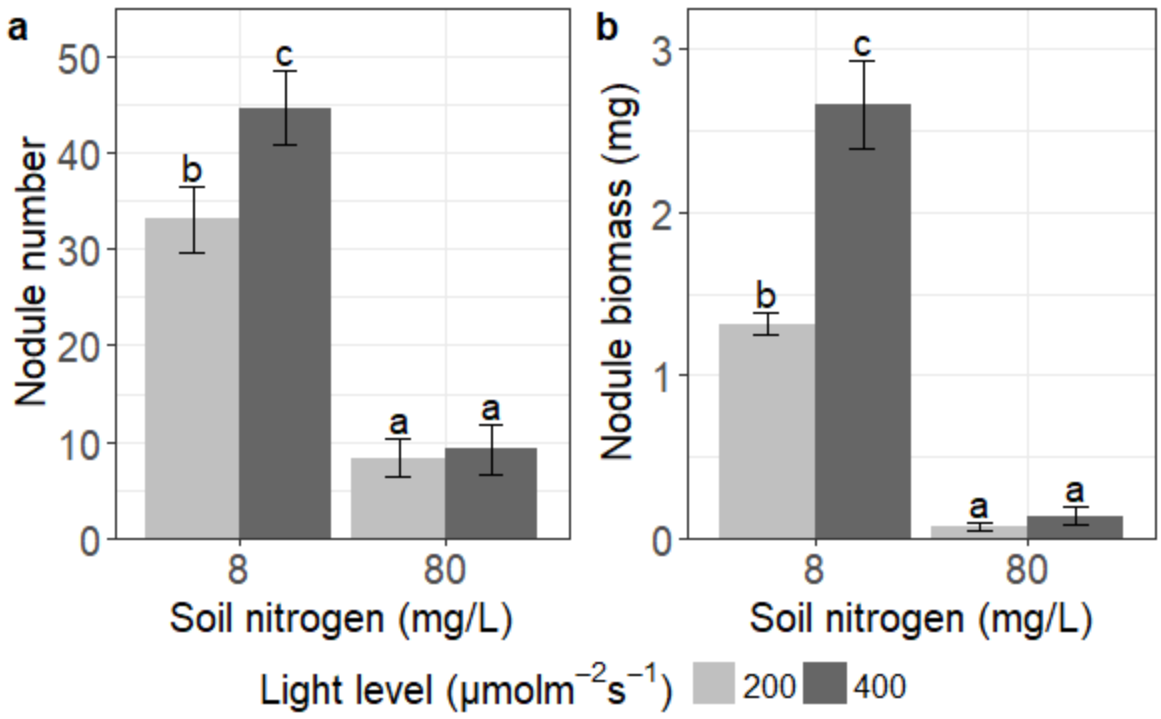

Figure 3.1 | Effects of light and nitrogen on total nodulation. Nodule biomass (a) and nodule number (b) in inoculated *M. truncatula* plants in response to changing soil nitrogen and light availability levels. Error bars represent +/- one standard error. Note that nodule biomass was ln-transformed to improve normality in the ANOVA, but is represented here without transformation for ease of interpretation. Bars with the same letter within an individual panel do not significantly differ after Tukey post hoc testing ($p > 0.05$).

Table 3.1 | ANOVAs summarizing the effects of light and nitrogen on nodulation. Bold indicates statistically significant effects ($p < 0.05$).

	Nodule number		Nodule biomass	
	χ^2	p	$F_{(1,34)}$	p
Light (L)	**16.5**	**4.93e-05**	**25.1**	**1.69e-05**
Nitrogen (N)	**375**	**< 2e-16**	**342**	**< 2.2e-16**
L*N	1.19	0.275	**14.1**	**6.54e-04**

Both light and nitrogen affect investment in rhizobia versus trade, but not return on investment

High light decreased specific nodulation (nodule number per mg of root biomass) by 31% overall ($p = 0.005$; Table 3.2), though we could not detect significant differences in specific nodulation when all pairwise combinations were tested during Tukey testing ($p = 0.355$ at low nitrogen and $p = 0.061$ at high nitrogen; Figure 3.2a). High nitrogen decreased specific nodulation by 88% overall ($p < 2.2e016$; Table 3.2, Figure 3.2a).

We detected a significant interaction between the effects of light and nitrogen on nodule:root biomass (mg nodule biomass per mg root biomass) ($p = 0.022$; Table 3.1). High nitrogen significantly decreased nodule:root biomass by approximately 97% regardless of light level ($p < 0.001$ for both light levels; Figure 3.1b). High light increased nodule:root biomass by 16% at low nitrogen ($p = 0.021$) but did not have a significant effect on nodule:root biomass at high nitrogen ($p = 0.977$; Figure 3.1b)

We detected a significant interaction between the effects of nitrogen and nodule biomass on shoot biomass gain from rhizobia, or shoot biomass minus mean biomass of control plants per condition ($p = 0.004$; Table 3.3). We did not detect a significant main effect of light, nitrogen, an interaction between the effects of light and nitrogen, or a three-way interaction between light, nitrogen, and nodule biomass (Table 3.3). The significant nitrogen x nodule biomass term indicated that the slope of the linear regression of shoot biomass gain on nodule biomass differed based on nitrogen treatment (Figure 3.2c). However, neither slope was significantly different from zero ($p = 0.796$ for low nitrogen and 0.393 for high nitrogen).

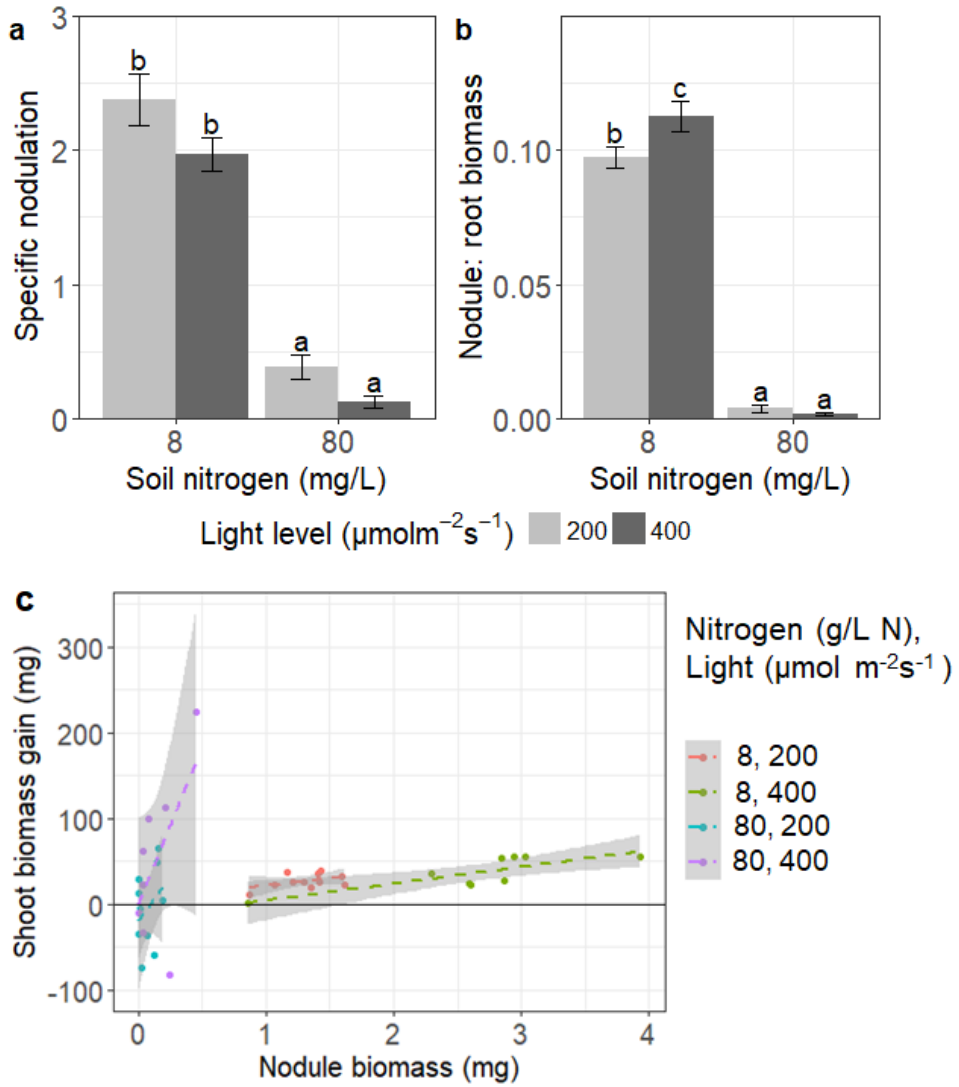

Figure 3.2 | Effects of light and nitrogen on investment in trade versus direct uptake and return on investment in trade. Specific nodulation (nodule number per mg root biomass) (a), nodule:root biomass (mg nodule biomass per mg root biomass) (b), and efficiency (shoot biomass gain, or shoot biomass minus mean shoot biomass for control plants of each treatment group versus nodule biomass: mg shoot gained per mg invested in rhizobia). Dashed lines indicate nonsignificant slopes.

Table 3.2 | ANOVAs summarizing the effects of light and nitrogen on nodulation scaled by root biomass. Bold indicates statistically significant effects ($p < 0.05$).

	Specific nodulation		Nodule:root biomass	
	$F_{(1,34)}$	p	$F_{(1,34)}$	p
Light (L)	**9.05**	**0.005**	3.80	0.060
Nitrogen (N)	**318**	**< 2e-16**	**858**	**< 2.2e-16**
N*L	0.560	0.459	**5.77**	**0.022**

Table 3.3 | ANCOVA summarizing the effects of light and nitrogen on the relationship between nodule biomass and shoot biomass gain. Bold indicates statistically significant effects ($p < 0.05$).

	Shoot biomass gain	
	$F_{(1,30)}$	p
Light (L)	0.910	0.348
Nitrogen (N)	2.05	0.163
Nodule biomass (NB)	2.67	0.112
L*N	0.540	0.469
L*NB	0.056	0.814
N*NB	**9.62**	**0.004**
L*N*NB	0.446	0.509

Nitrogen, light, and rhizobia affect relative allocation between root and shoot

High light increases root:shoot ratio by 16% overall (p = 0,004; Table 3.4), though we did not detect a significant effect of light when all pairwise comparisons were tested during Tukey testing (Figure 3.3). We detected a significant interaction between the effects of nitrogen and rhizobia on root:shoot ratio (p = 1.29e-04; Table 3.4). Rhizobia decreased root:shoot ratio 44% at low soil nitrogen ($p < 0.001$) but had no effect at high soil nitrogen (Figure 3.3). Similarly, increasing soil nitrogen decreased root:shoot ratio by 39% in the absence of rhizobia ($p < 0.001$), but had no significant effect in the presence of rhizobia (Figure 3.3).

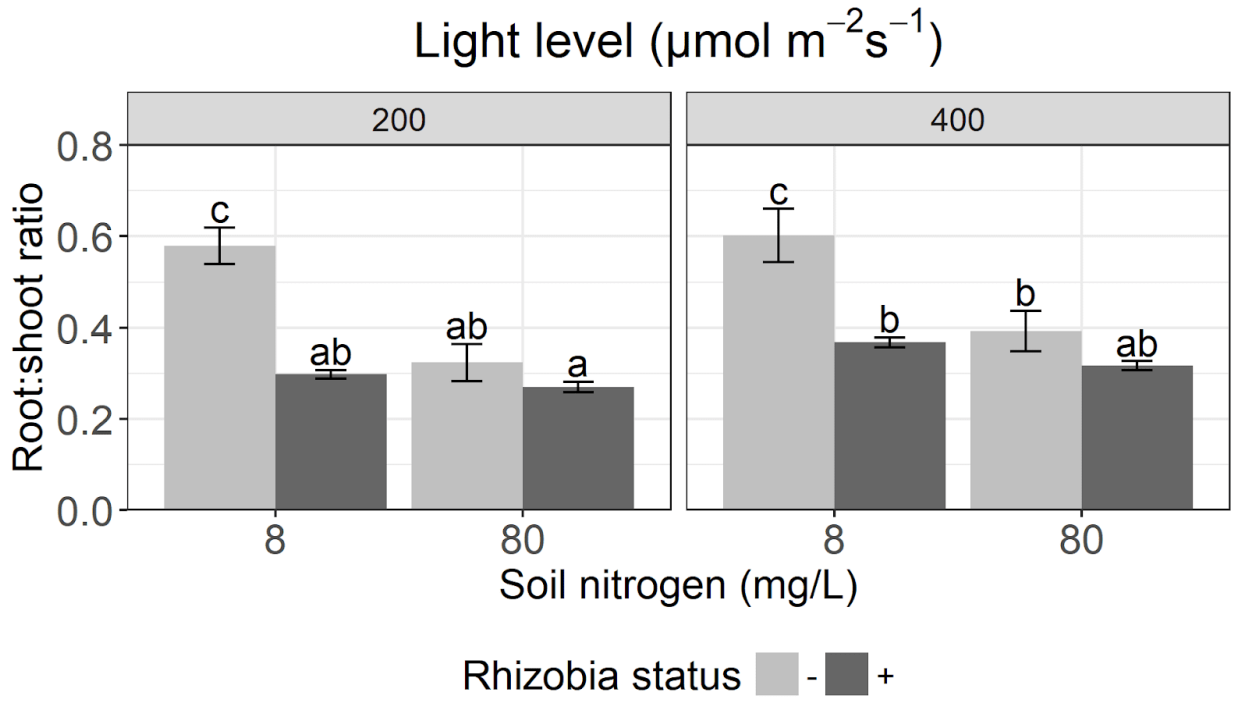

Figure 3.3 | Effects of light, nitrogen, and rhizobia on root:shoot ratio. Error bars represent +/- one standard error. Note that root:shoot ratio was ln-transformed to improve normality in the ANOVA, but is represented here without transformation for ease of interpretation. Bars with the same letter do not significantly differ after post hoc testing with the Tukey test ($p > 0.05$).

Table 3.4 | ANOVA summarizing the effects of light, nitrogen, and rhizobia on root:shoot ratio. Bold indicates statistically significant effects ($p < 0.05$).

	Root:shoot ratio	
	$F_{(1, 67)}$	p
Light (L)	**8.85**	**0.004**
Nitrogen (N)	**42.2**	**1.18e-08**
Rhizobia (R)	**58.4**	**1.07e-10**
L*N	0.399	0.530
L*R	0.872	0.354
N*R	**16.5**	**1.29e-04**
L*N*R	1.24	0.270

Light, nitrogen, and rhizobia modulate plant performance

We detected significant interactions between the effects of light and nitrogen on shoot biomass ($p = 0.006$, Table 3.5). Light increased shoot biomass by 35% at low soil nitrogen and by 149% at high soil nitrogen (Figure 3.4a). Similarly, increasing soil nitrogen increased shoot biomass by 183% at low light and by 423% at high light (Figure 3.4a). We also detected significant interactions between the effects of soil nitrogen and rhizobia ($p = 3.50e\text{-}05$, Table 3.5). Rhizobia increased shoot biomass by 135% at low soil nitrogen but had no significant effect at high soil nitrogen (Figure 3.4a). Increasing soil nitrogen increased shoot biomass by 581% in the absence of rhizobia but only 207% in the presence of rhizobia (Figure 3.4a).

We detected similar trends for root biomass. We detected a significant interaction between the effects of nitrogen and light ($p = 0.001$, Table 5). Increasing light increased root biomass by 62% at low soil nitrogen and by 192% at high soil nitrogen (Figure 3.4b). Increasing soil nitrogen increased root biomass by 121% at low light and by 299% at high light (Figure 3.4b). We also detected significant interactions between the effects of rhizobia and nitrogen on root biomass ($p = 0.011$, Table 3.5). Rhizobia did not have a significant effect on root biomass, but it changed the magnitude of the effect of soil nitrogen: increasing soil nitrogen increased root biomass by 307% in the absence of rhizobia, but only by 175% in the presence of rhizobia (Figure 3.4b).

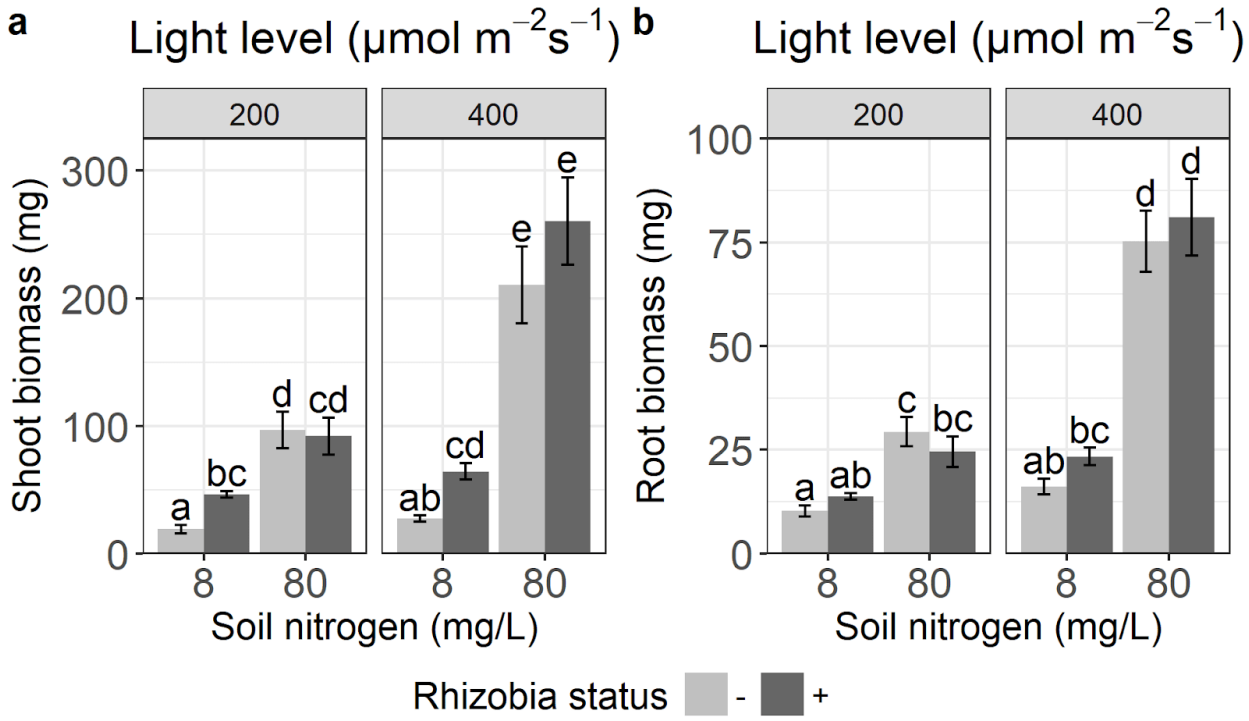

Figure 3.4 | Effects of light, nitrogen, and rhizobia on shoot and root biomass. a) Shoot biomass and b) root biomass in *M. truncatula* plants in response to changing soil nitrogen, light availability, and the presence or absence of rhizobia. Error bars represent +/- one standard error. Note that root and shoot biomass were ln-transformed to improve normality in the ANOVA, but are represented here without transformation for ease of interpretation. Bars with the same letter within an individual panel do not significantly differ after post hoc testing with the Tukey test ($p > 0.05$).

Table 3.5 | ANOVAs summarizing the effects of light, nitrogen, and rhizobia on shoot and root biomass. Bold indicates statistically significant effects ($p < 0.05$).

	Shoot biomass		Root biomass	
	$F_{(1, 67)}$	p	$F_{(1, 67)}$	p
Light (L)	**41.6**	**1.43e-08**	**71.5**	**3.60e-12**
Nitrogen (N)	**194**	**< 2.2e-16**	**132**	**< 2.2e-16**
Rhizobia (R)	**27.9**	**1.45e-06**	2.68	0.106
L*N	**8.19**	**0.006**	**11.5**	**0.001**
L*R	0.204	0.653	0.945	0.334
N*R	**19.7**	**3.50e-05**	**6.80**	**0.011**
L*N*R	1.69	0.199	0.646	0.424

Discussion

As predicted, shifting the resource environment altered the plant's allocation of resources between their rhizobial trading partners and the acquisition of external nutrients. In the biological market framework, increasing soil nitrogen is expected to reduce allocation to trade with rhizobia because the plant is able to obtain a larger proportion of its nitrogen needs through direct nitrogen uptake, which is less energetically costly than nitrogen fixation (Voisin *et al.*, 2002). This is exactly what we observe in response to increased soil nitrogen: plants sharply decreased both their total investment in nodulation (Figure 3.1) and their relative investment to nodules versus roots (Figure 3.2). This means that increasing soil nitrogen decreases both nodule initiation and the amount of biomass being allocated to existing nodules. In contrast, *Lotus japonicus* has been shown to regulate nodulation in response to nitrogen mainly through changes in nodule size (Regus *et al.*, 2015), and soybean has been shown to regulate nodulation in response to nitrogen mainly through changes in nodule number (Lau *et al.*, 2012). The effects of soil nitrogen on nodulation in *M. truncatula* have been shown to vary depending on genotype x genotype interactions between host and rhizobia, suggesting that these effects are highly specific (Heath *et al.*, 2010).

In contrast to trends in the abundance of soil nitrogen, increasing light availability increases the plant's potential for carbon fixation and thus presumably its carbon supply. This should result in increasing investment into nitrogen acquisition, though the breakdown between direct nitrogen update and trade would be based on the relative cost of each, which is determined by the soil nitrogen level (Schwartz & Hoeksema, 1998). We found that increasing light availability increased both total nodule number and total nodule biomass, and this effect was much more pronounced at low soil nitrogen—the only condition in which rhizobia significantly increased shoot biomass (Figure 3.1, Figure 3.4). In addition, light increased the nodule:root

biomass ratio only at low soil nitrogen (Figure 3.2). This suggests that plants are investing relatively more biomass into existing nodules with increasing light, but only under nitrogen conditions in which the rhizobia are beneficial to the plant. In contrast, light decreased specific nodulation regardless of nitrogen level (Figure 3.2), which indicates that the increase in total nodule number with increasing light (Figure 3.1) is being driven by an increase in root biomass that overcomes a reduced level of nodule initiation per unit of root biomass. In total, these complex effects of light suggest, since the magnitude and direction of the effect of light is different for nodule number and nodule biomass, light may be acting through multiple pathways to regulate nodulation. Furthermore, these results highlight the importance of examining nodulation relative to root biomass when other experimental treatments are expected to alter plant size. While unscaled nodule number and biomass are important predictors of rhizobial fitness (Ratcliff *et al.*, 2012), assessing only unscaled nodulation may lead to misleading conclusions about plant allocation. The wide variation in the effects of light on nodulation reported in the literature may be due to variation in genotype x genotype responses to light, similar to the varying effects of nitrogen detected by Heath *et al.*, 2010, but it may also be explained by differences in the type of nodulation measures reported (i.e. nodule:root biomass in Houx *et al.*, 2009 but unscaled nodule biomass in Santos *et al.*, 1997).

The benefit that plants get from trade with rhizobia is the product of their investment in rhizobia and the return on investment, or efficiency of the nodules. We measured efficiency as the increase in shoot biomass relative to the control, divided by nodule biomass. We did not detect a significant effect of nodule biomass on shoot biomass gain, but there was generally a positive relationship between the two (Figure 3.2). Figure 3.2 also suggests that nodules have a much higher efficiency at high nitrogen than at low, but this is likely an artefact of the

visualization. When the same plot was visualized for the four-week-old plants from Chapter 1, where only soil nitrogen was varied, the 80 mg/L N plants appeared to have a much higher slope than low nitrogen plants (Figure 3.5), but there was no difference in the carbon:nitrogen trade ratio for those plants (Figure 2.5). Thus, these apparent trends are most likely caused by much larger variation in shoot biomass combined with very low nodule biomass at high soil nitrogen, rather than any real relationship.

Regardless, there is no support for an effect of light on nodule efficiency as was reported in soybean (Santos *et al.*, 1997; Araujo *et al.*, 2018). There are several possible explanations for this discrepancy, including differences in the magnitude of the change in light, the way efficiency was measured, and the fact that soybeans form determinate nodules while *M. truncatula* forms indeterminate nodules (Oono *et al.*, 2009). We were only able to measure efficiency as shoot biomass gain per unit of nodule biomass, but Santos *et al.*, (1997) and Araujo *et al.*, (2018) measured it as mg of nitrogen fixed per mg of nodule. Neither measure is perfect because nodule biomass does not account for all of the carbon allocated to rhizobia (Rainbird *et al.*, 1984), and the amount of nitrogen fixed may not directly translate into plant fitness benefit if the biomass yield per nitrogen or relative nitrogen allocation changes. However, combining our results here with the results from Chapter 1 that the carbon:nitrogen exchange ratio does not change with changing soil nitrogen, it appears that the efficiency of nodules in *M. truncatula* is not affected by nutrient conditions. This suggests that *M. truncatula* has the regulation of rhizobial nitrogen fixation very tightly controlled and is always operating it at maximum efficiency, likely due to the energy intensity of nitrogen fixation (Silsbury, 1977; Andrews *et al.*, 2009).

The final aspect of plant biomass allocation is the balance of biomass between the roots (for nitrogen acquisition) and shoots (for carbon acquisition). When we assessed these allocation patterns by measuring the root:shoot ratio, we found strong interactions between the effects of nitrogen and rhizobia. Nitrogen only affected root:shoot ratio in uninoculated plants, and rhizobia only affected root:shoot ratio at low nitrogen (Figure 3.3). Low nitrogen inoculated plants, high nitrogen uninoculated plants, and high nitrogen inoculated plants all had statistically indistinguishable root:shoot ratios (Figure 3.3), even though the low nitrogen plants were significantly smaller than the high nitrogen plants (Figure 3.4). Thus, the rhizobial effect on root:shoot ratio is not fully explained by changes in plant nitrogen status. This suggests that plants are changing their allocation strategy to acquire more carbon to trade for nitrogen instead of directly acquiring nitrogen with root biomass. Goh *et al.*, (2016) used a non-fixing *nodD* mutant to show that rhizobial effects on root:shoot ratio in *M. truncatula* appear to depend only on nodule initiation, not on nitrogen fixation by the rhizobia. Thus, this trend may show that rhizobia are able to manipulate plants into allocating more resources to trade even when it is not beneficial.

Finally, these allocation decisions and environmental conditions resulted in interesting trends in plant biomass. We detected significant interactions between nitrogen and rhizobia effects and between nitrogen and light effects on shoot biomass, but not between light and rhizobia (Table 3.5). Rhizobia are not beneficial to the plant at high soil nitrogen levels because of the high cost of nitrogen fixation (Silsbury, 1977; Voisin *et al.*, 2002; Andrews *et al.*, 2009) and the ability of the plant to obtain sufficient "cheap" nitrogen directly from the soil. The interaction of nitrogen and light can be explained by the plant being strongly nitrogen limited at low soil nitrogen levels, so that increasing light does not allow more growth because the limiting

resource is nitrogen. However, at high soil nitrogen and low light, carbon is limiting, so increasing light availability allows for a much larger nitrogen effect. At first it seems counterintuitive that investment in rhizobia is increased by increasing light, and that efficiency does not change depending on conditions, but the benefit from rhizobia does not change depending on light. This is likely due to the high cost of nitrogen fixation: since each unit of nitrogen fixed by rhizobia costs a relatively high amount of carbon, this change in allocation does not have statistically significant effects on shoot biomass. In addition, the carbon costs of rhizobia may be at least partially counteracted by the stimulation of photosynthesis by nodules, either by improved leaf nitrogen status (Kaschuk *et al.*, 2009) or through the extreme sink strength of nodules (Brown & Bethlenfalvay, 1987; Kaschuk *et al.*, 2009, 2010, 2012), meaning that these mechanisms may counteract the carbon dependency of benefit from nodules.

It is important to note that due to limitations of the facilities and equipment available, the light levels used in this experiment were a relatively small fraction of the maximum light intensity a plant might experience in full sun (200-400 μmol $m^{-2}s^{-1}$ compared to 2000 μmol $m^{-2}s^{-1}$; (Korczynski. *et al.*, 1991). However, these levels are within the range that legumes may face when being shaded by other plants (Burkey & Wells, 1991). Light level had a significant impact on plant biomass accumulation (Figure 3.5, Table 3.5), suggesting that light is limiting under these conditions. One important caveat of this work is the fact that manipulating light levels has broader effects beyond simply altering total carbon available to the plant. Changes in light availability, such as what plants in vegetation canopies experience, induces changes in traits such as shoot architecture (Cescatti & Niinemets, 2004; Poorter *et al.*, 2009) and chlorophyll content (Evans, 1993). The reduction in photosynthesis and thus growth due to low light reduces demand for soil nutrients (Cui & Caldwell, 1997). However, manipulating light levels offers a highly

feasible, ecologically relevant method of altering nutrient availability to examine its effects on mutualisms. Understanding the effect of shading on mutualisms is agriculturally important for the use of legumes in agroforestry (Houx *et al.*, 2009) and for determining optimum planting density in commercial soybean crops (Pons & Pearcy, 1994).

Because of the relative scarcity of literature regarding the impacts of both traded nutrients on plant-microbe nutritional symbioses, it is obvious that further research is required. It is particularly important to conduct rigorous testing of differences in optimal allocation patterns across different types of rhizobial nodules and different plant-microbe symbioses. This field offers a unique opportunity to use mathematical modeling and empirical testing to further our understanding of the stability of evolutionarily ancient symbioses (Clark *et al.*, 2017). In addition, this avenue of research will have important implications for understanding how anthropogenic nutrient deposition may affect plant-microbe symbioses both in agricultural and natural environments.

APPENDIX

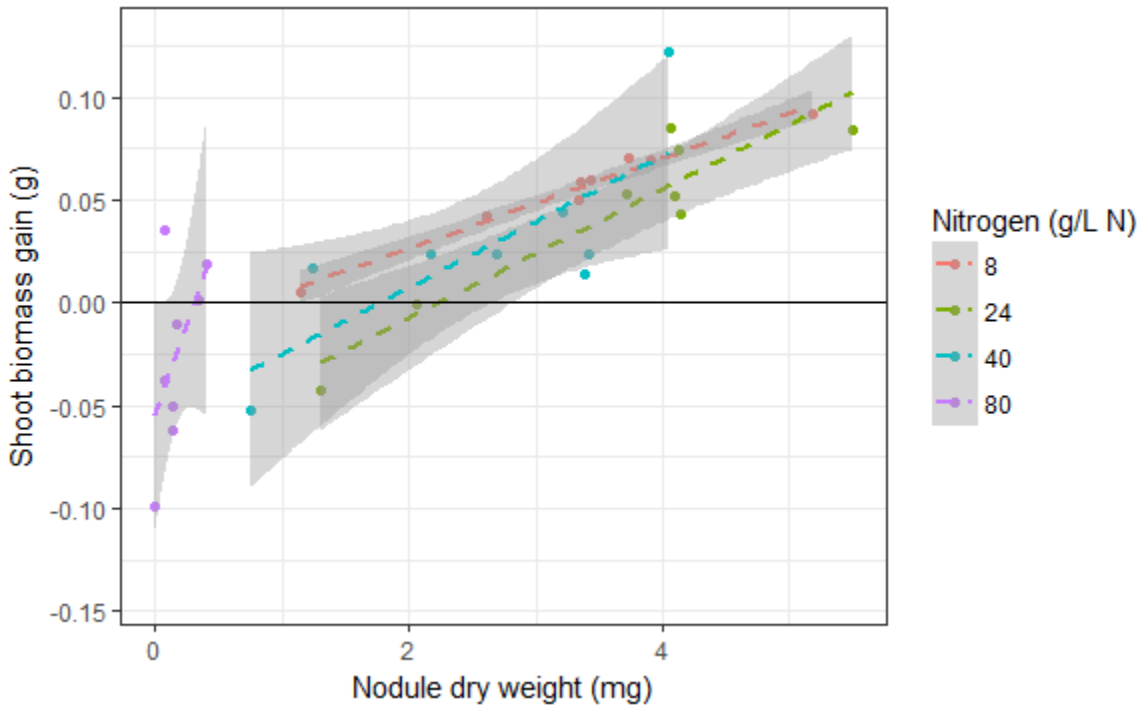

Figure 3.5 | Nodule efficiency in response to soil nitrogen. Shoot biomass gain (shoot biomass minus mean shoot biomass from uninoculated controls for each treatment) plotted against nodule biomass for four-week-old plants from Chapter 1. See Chapter 1 for full experimental details regarding these plants.

REFERENCES

REFERENCES

Andrews M, Lea PJ, Raven JA, Azevedo RA. **2009**. Nitrogen use efficiency. 3. Nitrogen fixation: genes and costs. *Annals of Applied Biology* **155**: 1–13.

Araujo KEC, Vergara C, Guimarães AP, Rouws JRC, Jantalia CP, Urquiaga S, Alves BJR, Boddey RM. **2018**. Changes in 15N natural abundance of biologically fixed N2 in soybean due to shading, rhizobium strain and plant growth stage. *Plant and Soil*: 1–16.

Bloom AJ, Chapin FS, Mooney HA. **1985**. Resource limitation in plants-an economic analogy. *Ann. Rev. Ecol. Sysl* **16**: 363–92.

Bronstein JL. **1994**. Conditional outcomes in mutualistic interactions. *Trends in Ecology & Evolution* **9**: 214–7.

Bronstein JL. **2001**. The exploitation of mutualisms. *Ecology Letters* **4**: 277–287.

Bronstein JL (Ed.). **2015**. *Mutualism*. Oxford: Oxford University Press.

Brown MS, Bethlenfalvay GJ. **1987**. Glycine-Glomus-Rhizobium Symbiosis : VI. Photosynthesis in Nodulated, Mycorrhizal, or N- and P-Fertilized Soybean Plants. *Plant physiology* **85**: 120–123.

Burkey KO, Wells R. **1991**. Response of soybean photosynthesis and chloroplast membrane function to canopy development and mutual shading. *Plant physiology* **97**: 245–52.

Cescatti A, Niinemets Ü. **2004**. Leaf to Landscape. In: Springer New York, 42–85.

Chu ACP, Robertson AG. **1974**. The effects of shading and defoliation on nodulation and nitrogen fixation by white clover. *Plant and Soil* **41**: 509–519.

Clark TJ, Friel CA, Grman E, Shachar-Hill Y, Friesen ML. **2017**. Modelling nutritional mutualisms: challenges and opportunities for data integration (M van Baalen, Ed.). *Ecology Letters* **20**: 1203–1215.

Cui M, Caldwell MM. **1997**. Shading reduces exploitation of soil nitrate and phosphate by Agropyron desertorum and Artemisia tridentata from soils with patchy and uniform nutrient distributions. *Oecologia* **109**: 177–183.

Denison RF. **2000**. Legume Sanctions and the Evolution of Symbiotic Cooperation by Rhizobia. *The American Naturalist* **156**: 567–576.

Erisman JW, Galloway JN, Seitzinger S, Bleeker A, Dise NB, Petrescu AMR, Leach AM, de Vries W. **2013**. Consequences of human modification of the global nitrogen cycle.

Philosophical transactions of the Royal Society of London. Series B, Biological sciences **368**: 20130116.

Evans J. **1993**. Photosynthetic Acclimation and Nitrogen Partitioning Within a Lucerne Canopy. II. Stability Through Time and Comparison With a Theoretical Optimum. *Australian Journal of Plant Physiology* **20**: 69.

Foley JA, Defries R, Asner GP, Barford C, Bonan G, Carpenter SR, Chapin FS, Coe MT, Daily GC, Gibbs HK, *et al.* 2005. Global consequences of land use. *Science (New York, N.Y.)* **309**: 570–4.

Glyan'ko AK, Vasil'eva GG, Mitanova NB, Ishchenko AA. **2009**. The influence of mineral nitrogen on legume-rhizobium symbiosis. *Biology Bulletin* **36**: 250–258.

Goh C-H, Nicotra AB, Mathesius U. **2016**. The presence of nodules on legume root systems can alter phenotypic plasticity in response to internal nitrogen independent of nitrogen fixation. *Plant, Cell & Environment* **39**: 883–896.

Hansen AP, Gresshoff PM, Pate JS, Day DA. **1990**. Interactions between Irradiance Levels, Nodulation and Nitrogenase Activity of Soybean cv. Bragg and a Supernodulating Mutant. *Journal of Plant Physiology* **136**: 172–179. *Journal of Plant Physiology* **136**: 172–179.

Heath KD, Stock AJ, Stinchcombe JR. **2010**. Mutualism variation in the nodulation response to nitrate. *Journal of Evolutionary Biology* **23**: 2494–2500.

van der Heijden MGA, Bardgett RD, van Straalen NM. **2008**. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* **11**: 296–310.

Houx JH, Mcgraw RL, Fritschi FB, Navarrete-Tindall NE. **2009**. Effects of shade on growth and nodulation of three native legumes with potential for use in agroforestry. *Native Plants Journal* **10**: 232–238.

Johnson NC, Graham JH, Smith FA. **1997**. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytologist* **135**: 575–585.

Kaschuk G, Kuyper TW, Leffelaar PA, Hungria M, Giller KE. **2009**. Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? *Soil Biology and Biochemistry* **41**: 1233–1244.

Kaschuk G, Leffelaar PA, Giller KE, Alberton O, Hungria M, Kuyper TW. **2010**. Responses of legumes to rhizobia and arbuscular mycorrhizal fungi: A meta-analysis of potential photosynthate limitation of symbioses. *Soil Biology and Biochemistry* **42**: 125–127.

Kaschuk G, Yin X, Hungria M, Leffelaar PA, Giller KE, Kuyper TW. **2012**. Photosynthetic adaptation of soybean due to varying effectiveness of N2 fixation by two distinct

Bradyrhizobium japonicum strains. *Environmental and Experimental Botany* **76**: 1–6.

Klinger CR, Lau JA, Heath KD. **2016**. Ecological genomics of mutualism decline in nitrogen-fixing bacteria. *Proceedings of the Royal Society of London B: Biological Sciences* **283**: 20152563.

Korczynski. PC, Logan J, Faust JE. **1991**. Mapping Monthly Distribution of Daily Light Integrals across the Contiguous United States. *HortTechnology* **12**: 12–16.

Lau JA, Bowling EJ, Gentry LE, Glasser PA, Monarch EA, Olesen WM, Waxmonsky J, Young RT. **2012**. Direct and interactive effects of light and nutrients on the legume-rhizobia mutualism. *Acta Oecologica* **39**: 80–86.

Lucinski R, Polcyn W, Ratajczak L. **2002**. Nitrate reduction and nitrogen fixation in symbiotic association Rhizobium - legumes. *ACTA BIOCHIMICA POLONICA* **49**: 537–546.

Neuhauser C, Fargione JE. **2004**. A mutualism-parasitism continuum model and its application to plant-mycorrhizae interactions. *Ecological Modelling* **177**: 337–352.

Oono R, Denison RF, Kiers ET. **2009**. Controlling the reproductive fate of rhizobia: how universal are legume sanctions? *The New phytologist* **183**: 967–79.

Pons TL, Pearcy RW. **1994**. Nitrogen reallocation and photosynthetic acclimation in response to partial shading in soybean plants. *Physiologia Plantarum* **92**: 636–644.

Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R. **2009**. Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New Phytologist* **182**: 565–588.

Rainbird RM, Hitz WD, Hardy RW. **1984**. Experimental determination of the respiration associated with soybean/rhizobium nitrogenase function, nodule maintenance, and total nodule nitrogen fixation. *Plant physiology* **75**: 49–53.

Ratcliff WC, Underbakke K, Denison RF. **2012**. Measuring the fitness of symbiotic rhizobia. *Symbiosis* **55**: 85–90.

Reeve W, Chain P, O'Hara G, Ardley J, Nandesena K, Bräu L, Tiwari R, Malfatti S, Kiss H, Lapidus A, *et al.* 2010. Complete genome sequence of the Medicago microsymbiont Ensifer (Sinorhizobium) medicae strain WSM419. *Standards in Genomic Sciences* **2**: 77–86.

Regus JU, Gano KA, Hollowell AC, Sofish V, Sachs JL. **2015**. Lotus hosts delimit the mutualism-parasitism continuum of Bradyrhizobium. *Journal of Evolutionary Biology* **28**: 447–456.

Santos VAF, Neves MCP, Rumjanek NG. **1997**. Differential symbiotic efficiency by shading of soybean nodulated by B. japonicum and B. elkanii strains. *Soil Biology and Biochemistry* **29**: 1015–1018.

Schwartz MW, Hoeksema JD. **1998**. Specialization and resource trade: Biological markets as a model of mutualisms. *Ecology* **79**: 1029–1038.

Silsbury JH. **1977**. Energy requirement for symbiotic nitrogen fixation. *Nature* **267**: 149–150.

Simonsen AK, Han S, Rekret P, Rentschler CS, Heath KD, Stinchcombe JR. **2015**. Short-term fertilizer application alters phenotypic traits of symbiotic nitrogen fixing bacteria. *PeerJ* **3**: e1291.

Streeter J, Wong PP. **1988**. Inhibition of legume nodule formation and N 2 fixation by nitrate. *Critical Reviews in Plant Sciences* **7**: 1–23.

Thornley JHM. **1972**. A Model to Describe the Partitioning of Photosynthate during Vegetative Plant Growth. *Annals of Botany* **36**: 419–430.

Udvardi M, Poole PS. **2013**. Transport and metabolism in legume-rhizobia symbioses. *Annual review of plant biology* **64**: 781–805.

Voisin A-S, Salon C, Munier-Jolain NG, Ney B. **2002**. Effect of mineral nitrogen on nitrogen nutrition and biomass partitioning between the shoot and roots of pea (Pisum sativum L.). *Plant and Soil* **242**: 251–262.

Weese DJ, Heath KD, Dentinger BTM, Lau JA. **2015**. Long-term nitrogen addition causes the evolution of less-cooperative mutualists. *Evolution; international journal of organic evolution* **69**: 631–42.

Young ND, Debellé F, Oldroyd GED, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KFX, Gouzy J, Schoof H, *et al.* 2011. The Medicago genome provides insight into the evolution of rhizobial symbioses. *Nature* **480**: 520–524.

CHAPTER 4

HOST SPECIFICITY IN THE TRIFOLIUM-RHIZOBIUM SYMBIOSIS

Abstract

Interactions with microbial mutualists may be able to promote plant coexistence if these interactions allow plants to occupy different niches. Plants may also be able to directly affect the composition of the soil microbial community, which then feeds back into plant fitness in a process known as plant-soil feedback. There is limited empirical evidence for the role of microbial mutualists in the promotion of plant coexistence, and it is focused mainly on plant-fungus interactions. Here, we assess the role rhizobia (soil bacteria that colonize plant roots and fix nitrogen in exchange for photosynthetic carbon) may play in the promotion of coexistence between 8 native species of *Trifolium* clover at our field site at Bodega Bay, California. We find that while both plants and rhizobia from this ecosystem are specialists, there is no clear pattern of increased benefit from or investment into conspecific rhizobia (those isolated from the same species as the test host), which we expected to perform better due to co-adaptation with their preferred hosts. We find that *Trifolium* species vary both in the amount they invest into rhizobia overall as well as the efficiency with which they extract benefit from rhizobia, leading to large differences in overall response to rhizobia. These results have important implications for our understanding of rhizobia's role in this ecosystem and the development of effective crop inoculants.

Introduction

Biodiversity in an ecosystem, both at the genetic and species level, is vital for ecosystem resilience to disturbance, response to environmental change, and productivity (Hughes & Stachowicz, 2004; Cardinale *et al.*, 2006, 2009; Fridley *et al.*, 2007; Hughes *et al.*, 2008; Cadotte *et al.*, 2009). Diversity at any level is maintained by a given species or allele increasing in relative abundance when it becomes rare in the system (Levene, 1953; Levine & HilleRisLambers, 2009). The ability to increase when rare depends on niche differentiation, or differences in the way organisms interact with or affect their environment (Chesson, 2000; Adler *et al.*, 2010; Turnbull *et al.*, 2013). Niche differentiation means that a given species will limit itself more than its competitors (Chesson, 2000). Plants may be able to engage in niche differentiation through interactions with beneficial soil microbes that allow them to specialize in various ways of interacting with the biotic and abiotic environment (Peay, 2016).

Ecological theory has presented two theories for how interactions with mutualists may promote coexistence. Parker, (1999) suggested that co-adaptation between host and symbiont can lead to a stable mosaic pattern. In this scenario, different physical locations are occupied by stable groups of co-adapted plants and microbes that resist invasion by outsiders, maintaining diversity through patchy spatial distribution (Parker, 1999). Bever, (1999) suggested that diversity can be maintained by microbial mutualists when the microbe that receives the most benefit from plant species A provides the most benefit to plant species B, resulting in negative feedback between plant species A and the microbe to which plant species A gives the most benefit (Bever, 1999). This negative feedback then maintains diversity of the plant species.

Niche differentiation on microbial partners requires the ability of plants to specialize in their interactions with microbes. If there is a disconnect between the amount of benefit a microbe

receives from its plant partner and the amount of resources it extracts from that plant, then these negative plant-soil feedbacks can maintain diversity between plant species as discussed above (Bever, 1999). In contrast, if a plant can select for its most beneficial microbes and preferentially amplify those microbes, then the plant may be able to engage in positive plant-soil feedbacks. In this case, the plant must condition the soil or alter the composition of the microbial community in such a way that makes that community more beneficial to that plant species than to other plant species (Bever, 2003; Hodge & Fitter, 2013). In these cases, plants are engaging in niche differentiation through their impacts on the environment, a process known as niche construction (van der Putten *et al.*, 2013). Plant-soil feedback can have major impacts on plant competition, altering whether conspecifics or heterospecifics are more limiting competitors (Pendergast *et al.*, 2013). Most examples of plant-soil feedback tend to be negative (Hodge & Fitter, 2013), resulting from the accumulation of pathogens (e.g. Klironomos, 2002). However, positive plant-soil feedbacks have been reported between neotropical trees and their mycorrhizal fungi (Mangan *et al.*, 2010).

There is relatively little empirical research on the effects of plant-microbe mutualisms on niche differentiation and host coexistence, and most of this research has focused on plant-fungus interactions (Peay, 2016). Fungal endophytes reduce the impact of drought stress on the grass *Bromus laevipes* and allow it to occupy habitats that are too dry for uninfected individuals (Afkhami *et al.*, 2014). In contrast, a fungal endophyte of the grass *Poa leptacoma* limits its host range at early life stages and promotes growth at later life stages (Kazenel *et al.*, 2015). Diverse communities of arbuscular mycorrhizal fungi can reduce interspecific competition between plants by reducing competition for soil resource (Wagg *et al.*, 2011). However, the role of other plant microbial symbionts in niche differentiation is unclear.

The legume-rhizobia symbiosis represents a thus-far untapped resource for exploring the effects of microbial symbionts on niche differentiation and plant-soil feedbacks. In this interaction, nitrogen-fixing rhizobia induce the formation of nodules on legume roots. Inside the nodules, the rhizobial bacteria fix atmospheric dinitrogen into ammonia for export to the plant in exchange for photosynthetically fixed carbon (Poole *et al.*, 2018). Legumes are a highly diverse group, and multiple legume species often co-occur (Sprent *et al.*, 2017). Co-occurring legumes can share symbionts that may vary in effectiveness between hosts (Ehinger *et al.*, 2014). There are examples of both highly specialized and promiscuous symbionts (Lira *et al.*, 2015). There is also evidence of legumes being able to differentiate between cooperative and uncooperative rhizobia (Kiers *et al.*, 2003; Yates *et al.*, 2005, 2008; Gubry-Rangin *et al.*, 2010). Thus, the legume-rhizobia symbiosis fulfills all the requirements for niche differentiation and/or plant-soil feedbacks as described above. The ability of legumes to differentiate between cooperative and uncooperative rhizobia suggest that Parker (1999)'s theory of co-adaptation is more likely than Bever (1999)'s to hold true in this system.

Our field site at Bodega Bay Marine Reserve in Bodega Bay, CA provides an excellent model to explore rhizobial axes of niche differentiation and test for plant-soil feedbacks. We have more than eight years of field data documenting the coexistence of eight native species of *Trifolium* in 330 2x2 m plots at this field site. All rhizobia that have been isolated from *Trifolium* nodules belong to *Rhizobium leguminosarum* bv. *trifolii* (Jordan, 1984). Members of this species exhibit geographic specificity--strains isolated from Africa, Europe, and North America generally cannot effectively colonize plants from different continents (Howieson *et al.*, 2005). In addition, strains from perennial species cannot effectively colonize annual species and vice versa (Howieson *et al.*, 2005). When strains from one *Trifolium* host can colonize a different host,

there is wide variation in the fitness benefits provided by a given strain across different hosts (Holland & Parker, 1966; Holland, 1970; Carlsson *et al.*, 2006). All of this suggests that interactions with rhizobia are a key aspect of niche differentiation in *Trifolium*, and that genetic diversity in the *Rhizobium* population at our field site may support plant diversity.

We explored the role that rhizobia may play in niche differentiation and plant-soil feedback at Bodega Bay by testing the benefits gained from and resources invested into 32 field-isolated rhizobial strains across all 8 *Trifolium* species. The rhizobia strains were isolated from the 7 *Trifolium* species with sufficient population size to destructively sample in the field. Thus, for these 7 species, we were able to contrast the effects of interacting with conspecific rhizobia (rhizobia for which the isolation host and test host are the same plant species) versus heterospecific rhizobia (rhizobia for which the isolation host and test host are different plant species). This setup allowed us to test the following hypotheses:

1. The plants and rhizobia in this experiment will tend to be specialists. Specialists will benefit more from their partners than generalists since they are co-adapted for higher levels of benefit.
2. If *Trifolium* specialize on rhizobia and are able to preferentially amplify their most beneficial strains, plant performance and investment in rhizobia should be higher when plants are inoculated with conspecific rhizobia than with heterospecific rhizobia.
3. *Trifolium* species that are more common in the field will have had a chancer to preferentially amplify their most beneficial strains in a larger proportion of the microbial community than rare species. Thus, common species are expected to extract more benefit from and invest more resources into rhizobia than rare species.

The results from this experiment will provide insight into the role of rhizobia in promoting plant diversity and shaping the community composition of ecosystems that include legumes (Keller, 2014). In addition, rhizobial specialization and specificity is an important phenomenon to understand for the development of effective rhizobial crop inoculants, which are frequently outcompeted by promiscuous and ineffective native rhizobia (e.g. Gerding *et al.*, 2014).

Materials and Methods

Pot preparation

We filled SC10 Cone-Tainers (Steuwe and Sons Inc., Corvallis, OR, USA) with a 1:1:1 mixture (by volume) of medium vermiculite, greenhouse sand, and Turface. We triple autoclaved the pots for 60 minutes with 24 hours between cycles to sterilize.

Seedling preparation

We scarified seeds of greenhouse-grown *Trifolium barbigerum, T. bifidum, T. fucatum, T. macraei, T. microdon, T. microcephalum,* and *T. willdenovii* by nicking the seed coat with a clean razor blade. We sterilized the seeds with chlorine gas by placing the seeds in a dessicator with a beaker of 100 mL of full strength commercial bleach (8.25% $NaHClPO_3$), to which we added 3 mL of HCl. We immediately sealed the dessicator and incubated the seeds in the gas for 4 hours. After 4 hours, we transferred seeds to sterile water agar plates sealed with Parafilm and incubated them in the dark at 4°C for 48 hours. We then germinated the seeds at room temperature in the dark for 24-48 hours. Seedlings with radicles at least 1 cm long were transplanted into prepared pots. After planting, the plants were fully randomized and grown in

the greenhouse at Michigan State University from [date] to [date]. Artificial lighting maintained a 16 hour day/8 hour night cycle.

Rhizobia isolation

We isolated rhizobia from nodules of field-collected *Trifolium* plants from our field site in Bodega Bay, California. We randomly sampled plants from across the field site and nodules were selected to represent the range of nodule size on a given plant. We washed the root systems with dH_2O on ice to remove soil and other debris. We sterilized each root system in 10% commercial bleach (0.825% $NaHClPO_3$) for 2 minutes, then rinsed 6 times with cold sterile dH_2O. We plucked individual nodules with flame sterilized forceps and crushed them with a plastic pestle in 150 uL of sterile crushing buffer (a 1:1 mixture of ½x phosphate buffered saline (PBS) and 80% glycerol). We streaked the resulting suspension onto tryptone yeast (TY) plates and incubated the plates at 30°C for 48-72 hours. Individual colonies were restreaked 2-3 times. A single colony was plucked and grown in liquid TY and frozen in 40% glycerol at -80°C for future use.

Rhizobia inoculation

We streaked 35 strains from the strain collection into a lawn onto replicate TY plates and grown at 30°C for 48 hours. These 35 strains represented 5 strains isolated from each of 7 plant species. We were unable to sample rhizobia from *T. microcephalum* plants because so few plants are found in the field that this destructive sampling would have major effects on their population numbers. Three strains did not grow sufficiently for inoculation, so the experiment was conducted with 5 strains each from *T. barbigerum, T. fucatum, T. gracilentum,* and *T. willdenovii* and 4 strains each from *T. bifidum, T. macraei ,* and *T. microdon.* These 32 strains were inoculated factorially across all 8 plant species with 4 replicates per condition. The bacterial

lawn was gently scraped from the plate and resuspended in 1 mL of ½x PBS. We determined cell density by measuring the OD600 of the culture using a NanoDrop. After one week of growth, plants received 1 mL of 10^7 CFU/mL rhizobial cells suspended in ½x phosphate buffered saline (PBS) and rhizobia-free control plants received 1 mL of sterile ½x PBS. We checked the sterility of the mock inoculum and viability of the rhizobial inoculum using spot plating on TY agar.

Plant growth and harvest

We planted the experiment February 13-16, 2017 and harvested all plants April 17-20, 2017. Each plant was given 2 pellets of Osmocote 14-14-14 slow release fertilizer on March 24, 2017 to provide a small amount of nutrients to maintain plant growth without disrupting nitrogen fixation. Plants were watered as needed by automatic misting from above. Misting was adjusted throughout the growth period to ensure adequate moisture in the pots without over-watering. At harvest, we separated the root, shoot, and flower tissue and counted and separated nodules from the root system using forceps. Tissue was dried at 60°C for one week and weighed. Only one uninoculated control developed nodules, and this plant was excluded from further analysis.

Statistical analysis

To assess the degree of specialization of the plants and rhizobia, we calculated the paired difference index (PDI; bipartite package, R 3.4.3). PDI is a highly robust and informative measure of specialization that ranges from 0-1, with larger values indicating increasing specialization (Poisot *et al.*, 2012). PDI is calculated use the formula

$$\mathrm{PDI} = \frac{\sum_{i=2}^{R} (\mathbf{P}_1 - \mathbf{P}_i)}{R - 1}$$

Where P_1 is the largest link strength (strength of the interaction with a given resource), P_i is the link strength with the ith resource, and *R* is the number of resources (Poisot *et al.*, 2012). From the plant perspective, each rhizobial strain is a resource, and each plant species is a resource for the rhizobia. We used scaled shoot biomass, specific nodulation, and nodule:root biomass ratio as link strengths to calculate three PDI values for each plant species and rhizobial strain. Scaled shoot biomass PDI (sPDI) represents specificity in the amount of benefit received or given, specific nodulation PDI (snPDI) represents specificity in the initiation of nodules, and nodule:root biomass PDI (nbPDI) represents specificity in the total amount of biomass allocated to/received by rhizobia. We used a linear model ANOVA to test whether isolation host affected sPDI, snPDI, and nbPDI. We also used linear model ANOVA to test whether any PDI measure affected mean and maximum scaled shoot biomass, specific nodulation, and nodule:root biomass. Maximum responses were calculated in a two-step process: first we calculated the mean response for each strain-plant species combination. We then selected the combination with the highest mean for each plant species or rhizobial strain. This allowed us to assess maximum possible investment while avoiding using a single plant as a data point, which could lead to excessive variation. The response variables in all of these models were log-transformed to improve normality and model fit. The p-values for the effects of various PDI measures on plant responses were Bonferroni-corrected to account for multiple testing.

We tested the effects of conspecific versus heterospecific rhizobia on plant performance using scaled shoot biomass (shoot biomass of an individual divided by the mean shoot biomass of the uninoculated controls of that species), flowering success (a binary yes/no measure of whether plants flowered or not) and flower number as proxies for plant fitness. In all cases, we included main effects of interaction type (conspecific versus heterospecific), test species, and

their interaction as predictors in the model. We used a linear model ANOVA for scaled shoot biomass and log transformed scaled shoot biomass to improve normality. We used a generalized linear model ANOVA with a binomial distribution for flowering success and a generalized linear model ANOVA with a Poisson distribution for flower number. Since flowering success was only dependent on plant species (Table 1), so we only assessed effects on flower number in plant species that had at least 40% of individuals flower (Table 4.9).

We also tested whether plants invest most in conspecific or heterospecific rhizobia using nodule number and nodule biomass. In both cases, we included main effects of interaction type, test species, and nodule counter (the identity of the researcher who plucked and counted nodules), and the interaction type x test species interaction term as predictors. We used a generalized linear model ANOVA with a Poisson distribution for nodule number and linear model ANOVA for nodule biomass. We log-transformed nodule biomass to improve normality.

We confirmed that patterns of investment between conspecific and heterospecific rhizobia were due to specificity in the interaction by testing the effects of isolation host (the plant species from which a rhizobial strain was isolated) on nodule number and biomass. In these models, we used the main effects of isolation host, test species, and nodule counter, and the interaction between isolation host and test species as predictors. We used a generalized linear model ANOVA with a Poisson distribution for nodule number and a linear model ANOVA for biomass. We log-transformed nodule biomass to improve normality.

To assess plant response to rhizobia, we measured scaled shoot biomass, specific nodulation (nodule number per g root biomass), and nodule:root biomass (mg nodule biomass per g root biomass) for each plant species averaged across all rhizobia treatments. For these models, we used a general linear model ANOVA with plant species as a predictor. We log-

transformed all of the response variables to improve normality. We tested whether response to rhizobia increases with commonness in the field with a linear model of each response as predicted by the fraction of plots in the field in which each species is present. We log-transformed the response variables to improve normality and model fit.

We assessed the efficiency of the rhizobia on each plant species in two different ways. To assess raw efficiency, we used the raw increase in shoot biomass (shoot biomass of the individual minus the mean shoot biomass of uninoculated controls of that plant species) as the response. We also assessed scaled efficiency by using scaled shoot biomass as the response. We used general linear model ANOVA with main effects of nodule biomass and test species and the nodule biomass x test species interaction as predictors. Since we were particularly interested in the linear relationship between nodule biomass and shoot biomass, we did not log-transform the response variables to improve normality. While coefficients of course varied between models with log-transformed and untransformed responses, there was no difference in whether each term in the model was significant. To visualize the relationship between nodule biomass and shoot biomass across species, we bootstrapped the nodule biomass coefficient for each plant species and displayed the mean and 95% CI (20,000 replicates; bias corrected and accelerated (Bca) output, boot package, R 3.4.3). We also assessed the linear relationship between nodule biomass and the raw increase in shoot biomass for each plant species by testing for correlation with Spearman's correlation coefficient (cor.test function, R 3.4.3). *P* values for the correlation tests were Bonferroni-corrected to account for multiple testing.

All linear models used the lm function (R 3.4.3), all generalized linear models used the glm function (R 3.4.3), and all ANOVAs were conducted with Type II sum of squares using the

Anova function (car package, R 3.4.3). Post-hoc testing was conducted with Tukey tests with the emmeans function (emmeans package, R 3.4.3).

Results

Plants and rhizobia in this ecosystem are mostly specialists

We found that all plant species were specialists (PDI > 0.5) when assessed by scaled shoot biomass PDI (sPDI) and specific nodulation PDI (snPDI) (Figure 4.1 a,c). When assessed by nbPDI, *T. fucatum* was found to be slightly generalist (nbPDI of 0.47), while all other plant species were specialists (nbPDI > 0.5; Figure 4.1e).

We found that most rhizobia were specialists, but the pattern depended on the type of PDI being calculated. For sPDI, 13/32 (40%) of strains were generalists (sPDI < 0.5), while 19/32 (60%) were specialists (sPDI > 0.5; Figure 4.1b). For snPDI, 5/32 (16%) of strains were generalists (snPDI < 0.5), while 27/32 (84%) of strains were specialists (snPDI > 0.5; Figure 4.1d). For nbPDI, all rhizobia were specialists (nbPDI > 0.5; Figure 4.1f). We did not detect a significant effect of isolation host on any measure of rhizobial PDI (Table 4.1). These results should be interpreted with caution because we were unable to calculate 95% confidence intervals for the PDI values and thus cannot determine how confident we are in the division of organisms between specialist and generalist. In addition, it is unlikely that there is a meaningful biological difference between an organism with a PDI of 0.49 and 0.51, though one would be classified as a weak generalist and one as a weak specialist. However, PDI is still useful for providing an overview of the patterns of generalization and specialization.

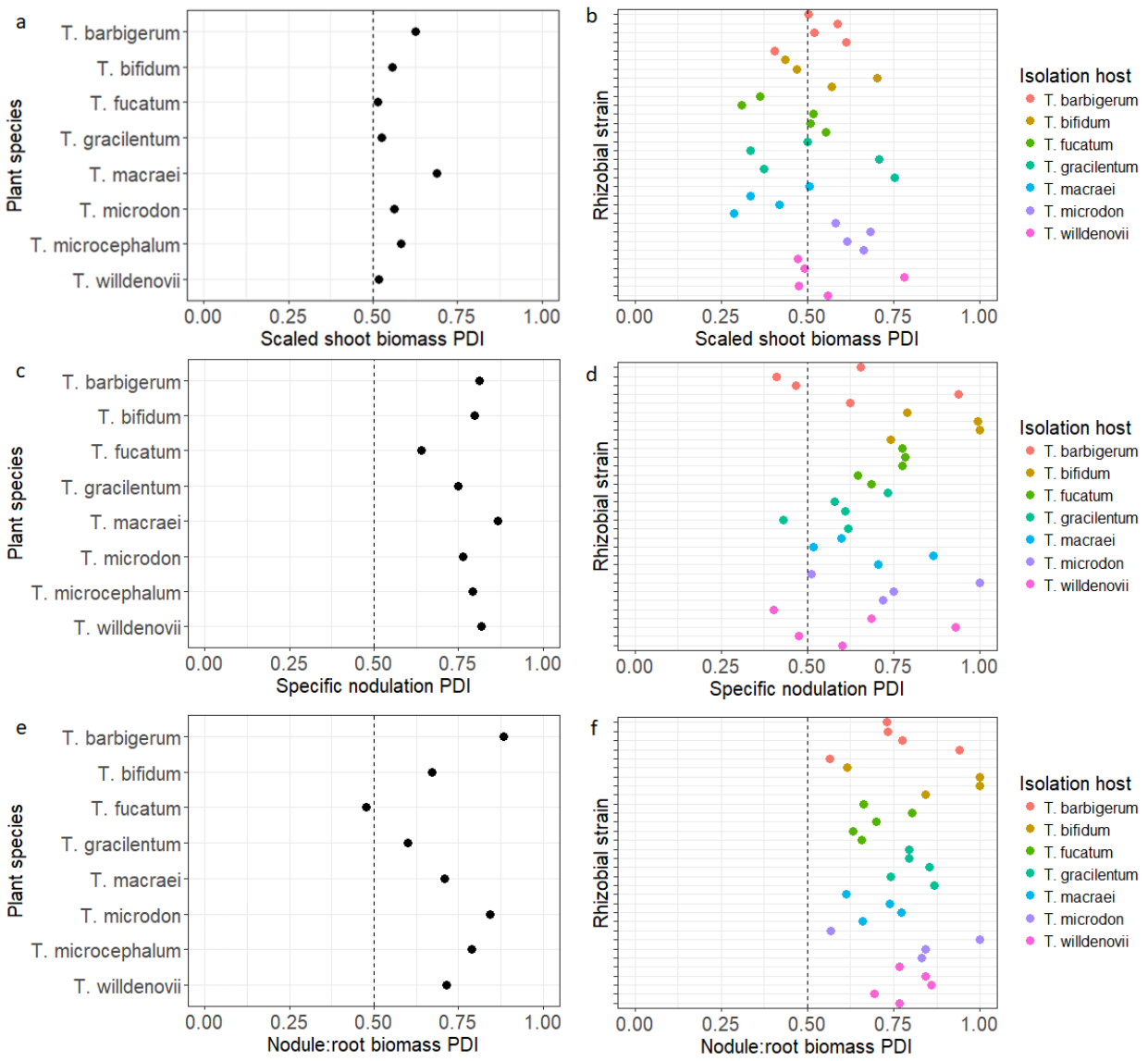

Figure 4.1 | Specialization of plants and rhizobia as measured by Paired Difference Index (PDI). PDI for each plant species calculated with a) scaled shoot biomass, c) specific nodulation, and e) nodule:root biomass. PDI for each rhizobia strain calculated with b) scaled shoot biomass, d) specific nodulation, and f) nodule:root biomass.

Table 4.1 | ANOVAs summarizing the effect of isolation host on rhizobial PDI. sPDI indicates scaled shoot biomass PDI, snPDI indicates specific nodulation PDI, and nbPDI indicates nodule biomass PDI.

	sPDI		snPDI		nbPDI	
	$F_{(6,25)}$	p	$F_{(6,25)}$	p	$F_{(6,25)}$	p
Isolation host	1.83	0.133	1.73	0.157	1.35	0.274

No effect of plant specialization on investment in or benefit from rhizobia

We did not detect a significant effect of any plant PDI measure on mean or maximum scaled shoot biomass, specific nodulation, or nodule:root biomass (Figure 4.2). See Table 4.2 for a summary of all *p*-values.

Effects of rhizobial specialization depend on the response being measured

We detected significant negative effects of rhizobial sPDI, snPDI, and nbPDI on nodule:root, but did not detect a significant effect of these PDIs on mean scaled shoot biomass, maximum specific nodulation, or nodule:root biomass (Figure 4.3, Table 4.3). Other effects of rhizobial specialization depend on the response being measured. For instance, we detected a significant positive effect of rhizobial sPDI but not snPDI or nbPDI on maximum scaled shoot biomass. Furthermore, we did not detect a significant effect of rhizobial sPDI on mean specific nodulation, but we did detect significant negative effects of rhizobial snPDI and nbPDI. See Table 4.3 for a summary of all *p*-values.

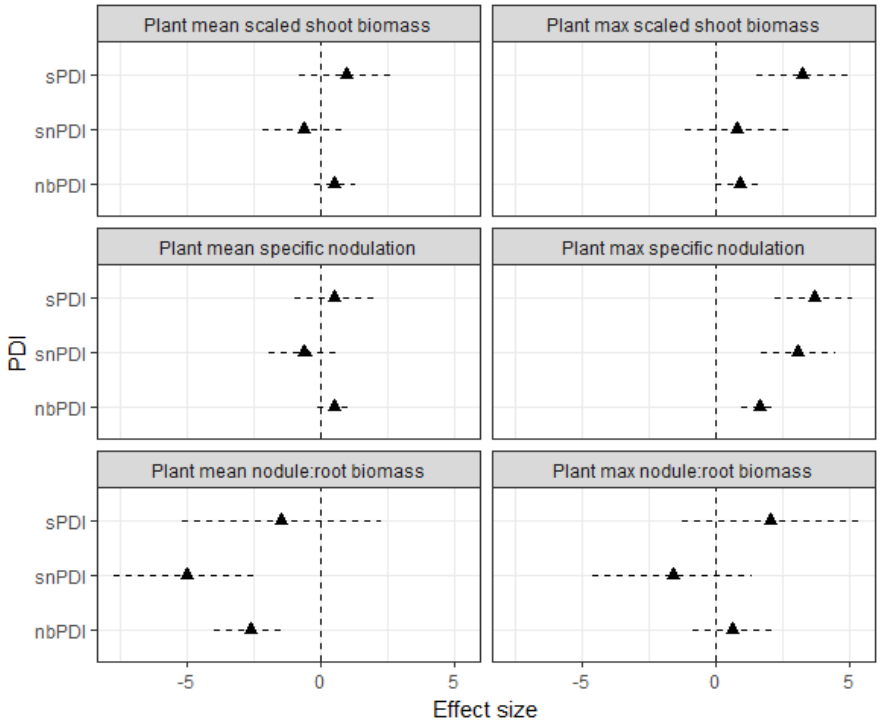

Figure 4.2 | Effect of plant specialization on response to rhizobia. Effect size of various plant PDI measures (y axis) on plant responses (facets)in a linear model of the form log(response) ~ PDI. A negative value in this figure indicates a negative effect of PDI on the response, and a positive value indicates a positive effect of PDI on the response. Since regressions were performed with log transformed responses, the mean of the response is multiplied by exp(effect size) when PDI increases by one unit. Triangles with dotted lines indicate a nonsignificant effect of the predictor on the response ($p > 0.05$ after Bonferroni correction) and circles with solid lines indicate a significant effects of the predictor on the response ($p < 0.05$ after Bonferroni correction). See Figure 4.9 and 4.10 for scatterplots.

Table 4.2 | ANOVAs summarizing the effects of plant PDI measures on plant responses to rhizobia. Note that predictors are the rows and responses the column; a separate ANOVA was conducted for each predictor-response combination, while in other tables each row contains a predictor that was included in a single model for each response. *P* values were Bonferroni corrected to account for multiple testing.

	Mean scaled shoot biomass		Mean specific nodulation		Mean nodule:root biomass		Max scaled shoot biomass		Max specific nodulation		Max nodule:root biomass	
	$F_{(1,6)}$	*p*	$F_{(1,6)}$	*p*	$F_{(1,6)}$	*p*	$F_{(1,6)}$	*p*	$F_{(1,6)}$	*p*	$F_{(1,6)}$	*p*
sPDI	0.315	<1	0.134	<1	0.152	0.977	3.59	0.961	6.52	0.390	0.394	>1
snPDI	0.139	<1	0.198	<1	3.34	<1	0.175	>1	4.97	0.606	0.283	>1
nbPDI	0.501	<1	0.446	<1	3.66	0.937	0.997	>1	6.37	0.405	0.193	>1

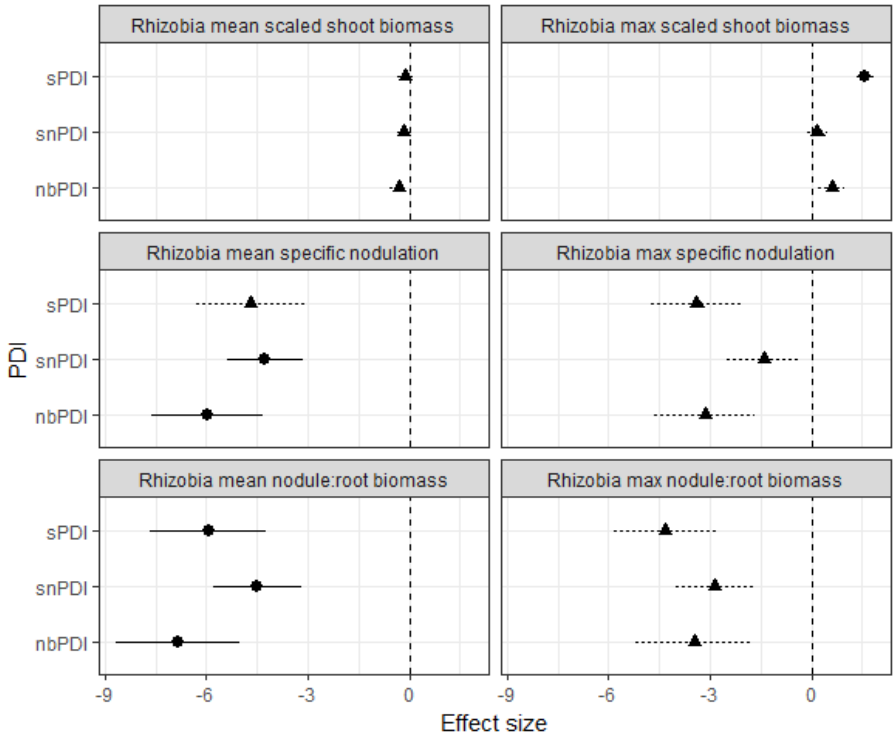

Figure 4.3 | Effect of rhizobial specialization on plant responses. Effect size of rhizobia PDI measures(y axis) on plant responses (facets) in a linear model of the form log(response) ~ PDI. A negative value in this figure indicates a negative effect of PDI on the response, and a positive value indicates a positive effect of PDI on the response. Since regressions were performed with log transformed responses, the mean of the response is multiplied by exp(effect size) when PDI increases by one unit. Triangles with dotted lines indicate a nonsignificant effect of the predictor on the response ($p > 0.05$ after Bonferroni correction) and circles with solid lines indicates a significant effect of the predictor on the response ($p < 0.05$ after Bonferroni correction). See Fig 4.11 and 4.12 for scatterplots.

Table 4.3 | ANOVAs summarizing the effects of rhizobial PDI measures on plant responses to rhizobia. Note that predictors are the rows and responses the column; a separate ANOVA was conducted for each predictor-response combination, while in other tables each row contains a predictor that was included in a single model for each response. *P* values were Bonferroni corrected to account for multiple testing.

	Mean scaled shoot biomass		Mean specific nodulation		Mean nodule:root biomass		Max scaled shoot biomass		Max specific nodulation		Max nodule:root biomass	
	$F_{(1,30)}$	*p*	$F_{(1,30)}$	*p*	$F_{(1,30)}$	*p*	$F_{(1,30)}$	*p*	$F_{(1,30)}$	*p*	$F_{(1,30)}$	*p*
sPDI	0.213	>1	8.70	0.055	**11.99**	**0.015**	**41.7**	**3.51e-06**	6.55	0.142	8.36	0.064
snPDI	0.831	>1	**14.50**	**0.006**	**12.0**	**0.015**	0.372	>1	1.73	>1	5.91	0.191
nbPDI	1.51	>1	**13.1**	**0.010**	**13.9**	**0.007**	2.22	>1	4.45	0.391	3.92	0.514

Positive effect of conspecific rhizobia on flower number but not shoot biomass

We detected a significant effect of test species on scaled shoot biomass ($p < 2e\text{-}16$), but we did not detect a significant main effect of interaction type ($p = 0.890$) or an interaction between interaction type and test species ($p = 0.731$; Table 1, Figure 4.4a, Figure 4.14a). We detected a significant effect of test species on flowering success ($p < 2e\text{-}16$), but we did not detect a significant effect of interaction type ($p = 0.989$) or a significant interaction between interaction type and test species ($p = 0.849$). Because the probability of flowering was only affected by plant species, and some species had such low flowering rates as to prevent meaningful analysis, we limited flower number analysis to the three plant species that had at least 40% of individuals flower (Table 4.9). Mean flower number in plants inoculated with heterospecific rhizobia was approximately 54% of mean flower number in plants inoculated with conspecific rhizobia ($p = 0.010$). We also detected a significant effect of plant species ($p < 2e\text{-}16$) on flower number in this reduced dataset, but we did not detect a significant interaction between interaction type and plant species ($p = 0.523$; Table 1, Figure 4.4b, Figure 4.14b).

Table 4.4 | ANOVAs summarizing the effects of interaction type and test species on plant performance. A linear model was used to assess scaled shoot biomass, while a generalized linear model with a binomial distribution was used to assess flowering probability and a generalized linear model with a Poisson distribution was used to assess flower number. Note that the full dataset was used for scaled shoot biomass and flowering probability models, while only *T. fucatum*, *T. macraei*, and *T. microdon* were included in the model for flower number since the other plant species exhibited very low flowering rates.

	Scaled shoot biomass		Flowering probability		Flower number	
	F	*p*	χ^2	*p*	χ^2	*p*
Interaction type (IT)	$F_{(1,1039)}$ = 0.019	0.890	$\chi^2_{(1,1039)}$ =0.989	0.320	$\chi^2_{(1,405)}$ =**6.63**	**0.010**
Test species (TS)	**$F_{(7,1039)}$ = 13.6**	**< 2e-16**	**$\chi^2_{(7,1039)}$= 250.1**	**<2e-16**	**$\chi^2_{(2,405)}$= 97.6**	**<2e-16**
IT*TS	$F_{(6,1039)}$ = 0.600	0.731	$\chi^2_{(6,1039)}$ =2.67	0.849	$\chi^2_{(2,405)}$ =1.30	0.523

Species-dependent differences in nodulation between conspecific and heterospecific rhizobia

Interaction type, test species, and their interaction significantly affected nodule number (p = 7.78e-08, p < 2.2e-16, and p < 2.2e-16, respectively; Table 4.5). We did not detect a significant main effect of interaction type on nodule biomass (p = 0.320), but we did detect a significant effect of test species (p < 2.2e-16) and a significant interaction between interaction type and test species (p = 2.41e-04; Table 4.5). After Tukey testing, we did not detect a significant difference in nodule number or nodule biomass in *T. barbigerum* plants inoculated with conspecific versus heterospecific rhizobia ((p = 0.482 and p = 0.839, respectively; Figure 4.4). *T. bidifum*, *T. microdon,* and *T. willdenovii* make significantly fewer nodules with conspecific rhizobia than with heterospecific rhizobia (p < 0.001, p = 0.014, and p = 0.024, respectively; Figure 4.4a). We did not detect a significant difference in nodules biomass between plants inoculated with conspecific versus heterospecific rhizobia for these three species (p = 0.288, p = 0.718, and p = 0.815, respectively; Figure 4.4b). *T. fucatum, T. gracilentum,* and *T. macraei* made significantly more nodules with conspecific rhizobia than with heterospecific rhizobia (p < 0.001, p = < 0.001, p = 0.003, respectively; Figure 4.4a). *T. fucatum* had significantly higher nodule biomass when inoculated with conspecific rhizobia than with heterospecific rhizobia (p < 0.001), but we did not detect a significant difference in nodule biomass between *T. gracilentum* and *T. macraei* inoculated with conspecific versus heterospecific rhizobia (p = 0.630 and p = 0.815, respectively; Fig 4.4b).

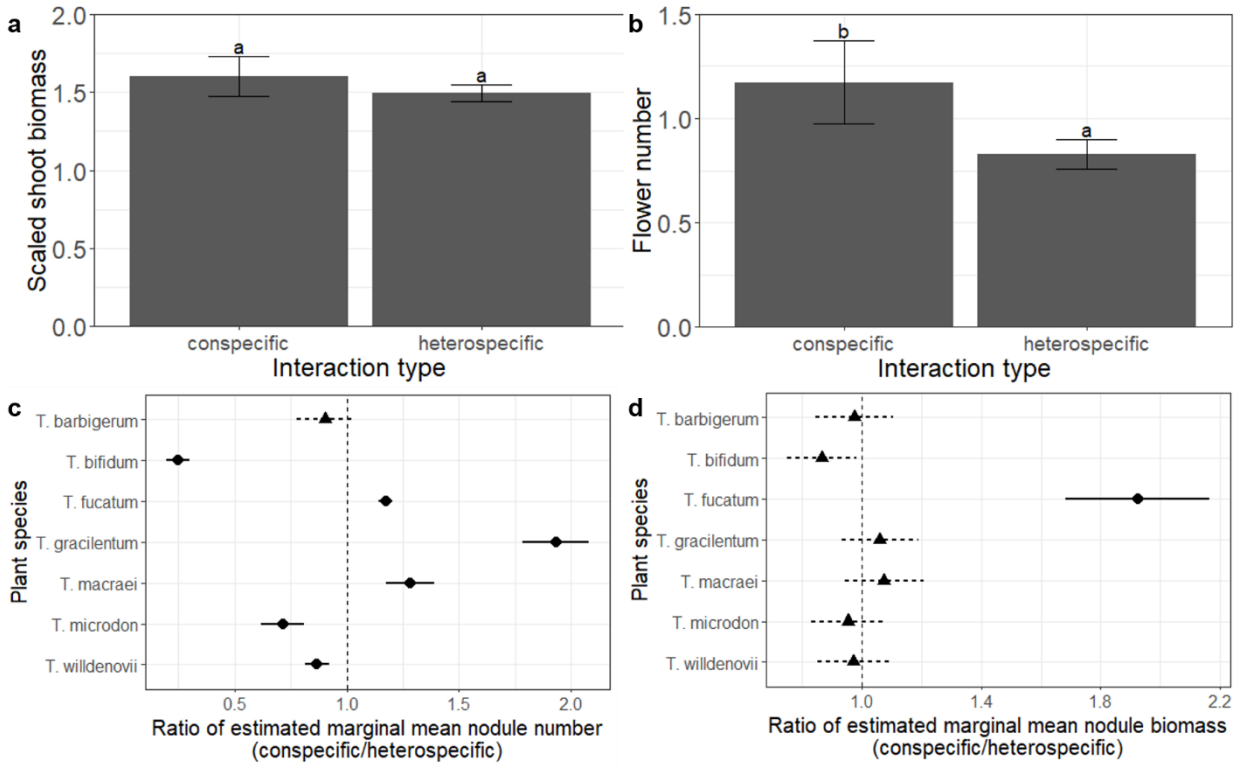

Figure 4.4 | Plant performance and nodulation with conspecific versus heterospecific rhizobia. a) Mean scaled shoot biomass across all test species. b) Mean flower number across the three test species analyzed for flowering. In a) and b) error bars represent +/- 1 SE. Bars with the same letter in a given panel are not statistically different ($p > 0.05$). c) Ratio of estimated marginal mean nodule number and d) Ratio of estimated marginal mean nodule biomass of plants inoculated with conspecific versus heterospecific rhizobia. The point range represents +/- 1 SE. Dotted lines with triangles indicate nonsignificant differences between mean responses of plants inoculated with conspecific and heterospecific rhizobia after Tukey testing ($p > 0.05$), while solid lines with circles indicate significant differences between mean responses of plants inoculated with conspecific and heterospecific rhizobia after Tukey testing ($p < 0.05$).

Table 4.5 ANOVAs summarizing the effects of interaction type and test species on nodulation. Results of generalized linear model ANOVA (nodule number) and linear model ANOVA (nodule biomass) summarizing the effects of interaction type and plant species on nodulation. Nodule counter was included to control for variation in nodule counting accuracy among researchers but is not of experimental interest.

	Nodule number		Nodule biomass	
	χ^2	*p*	F	*p*
Interaction type (IT)	**$\chi^2_{(1,1030)}$ =28.9**	**7.78e-08**	$F_{(1,1030)}$=3.01	0.083
Test species (TS)	**$\chi^2_{(7,1030)}$ =2.80e04**	**<2.2e-16**	**$F_{(7,1030)}$=210.5**	**<2.2e-16**
IT*TS	**$\chi^2_{(6,1030)}$ =171.9**	**<2.2e-16**	**$F_{(6,1030)}$=4.35**	**2.41e-04**
Nodule counter	**$\chi^2_{(3,1030)}$ =143.7**	**<2.2e-16**	$F_{(3,1030)}$=2.62	0.050

Specificity of interactions

To confirm that detected nodulation trends were due to specificity in the interactions and not because of uniformly poor colonization by any particular strain, we tested the effect of isolation host on nodulation measures. We detected a significant main effect of isolation host and test species ($p < 2.2e\text{-}16$ for both) on nodule number as well as a significant interaction between isolation host and test species ($p < 2.2e\text{-}16$; Table 4.6). We then determined the number of test species for which a given isolation host is the worst isolation host (i.e., that isolation host is statistically indistinguishable via Tukey testing from the isolation host with the smallest mean) and the best isolation host (i.e., that isolation host is statistically indistinguishable via Tukey testing from the isolation host with the largest mean). No isolation host was either the best nor the worst isolation host for all 8 test species, suggesting that their nodulation trends on conspecific hosts are not a universal characteristic (Table 4.7, Figure 4.5). In the most extreme example, *T. bifidum* was the worst isolation host for 7 out of 8 test species but was the best isolation host for the remaining test species (Table 4.7). Thus, even though these strains are generally poor colonizers, they are capable of relatively high colonization on at least one host.

We also assessed the effect of isolation host on nodule biomass. We detected significant main effects of isolation host and test species on nodule biomass and a significant interaction of the two ($p = 2.2e\text{-}07$, $p < 2.2e\text{-}16$, and $p = 6.30e\text{-}05$, respectively; Table 4.6). After Tukey testing, there were no significant differences in nodule biomass between isolation hosts for any other test species than *T. fucatum*, confirming that the high nodule biomass in *T. fucatum* individuals inoculated with conspecific strains is a specific interaction and not a universal feature of the rhizobia (Figure 4.6).

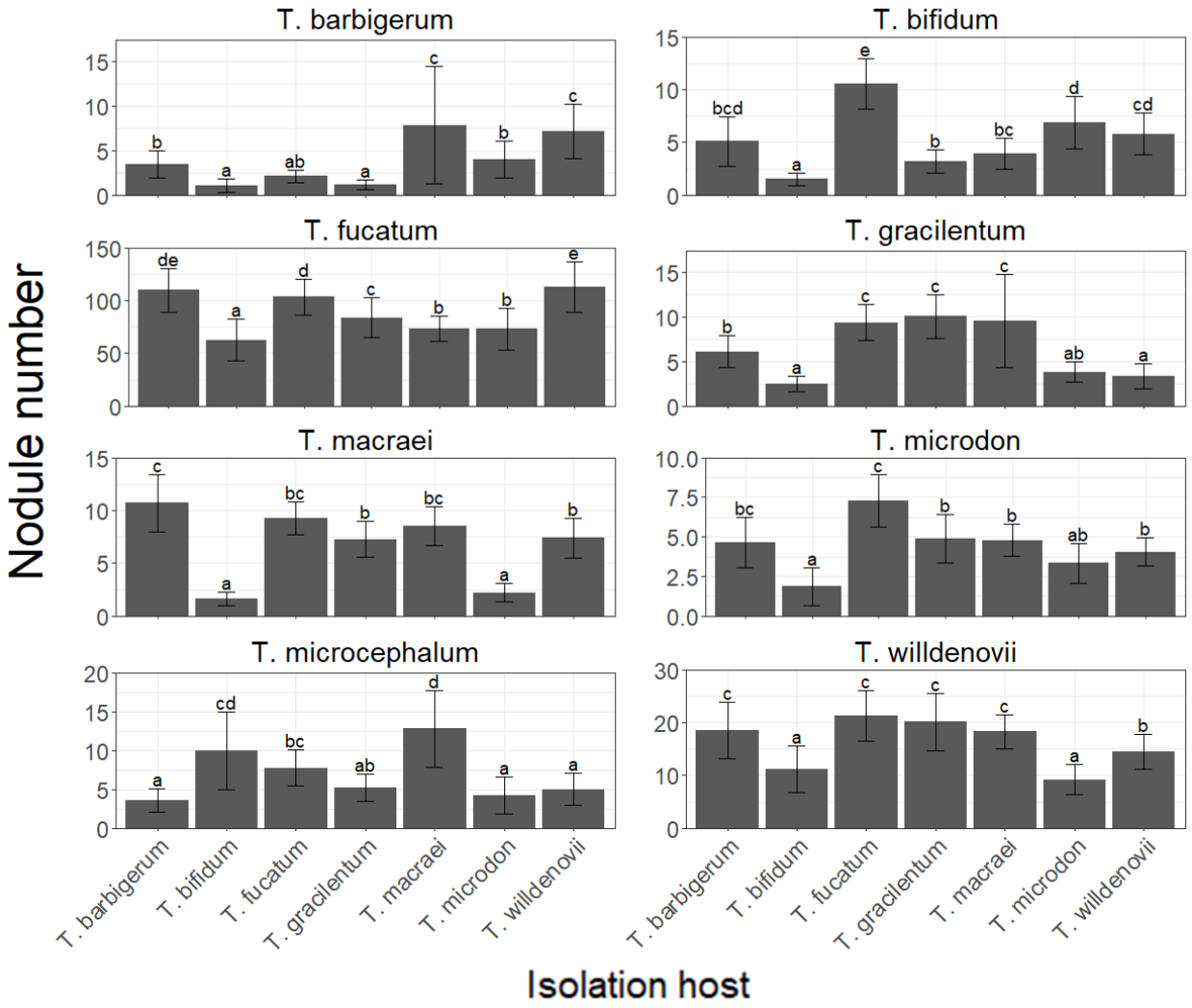

Figure 4.5 | Effects of isolation host on nodule number across all test species. Nodule number of each combination of isolation host (x axis; the plant species from which a given strain was isolated) and test host (facet; the plant species on which a strain was inoculated). Within a given facet, bars with the same letter do not significantly differ after Tukey testing ($p > 0.05$). Note that each facet has a different y axis range.

Table 4.6 ANOVAs summarizing the effects isolation host and test species on nodulation. Results of generalized linear model ANOVA (nodule number) and linear model ANOVA (nodule biomass) summarizing the effects of isolation host and test species on nodulation. Nodule counter was included to control for variation in nodule counting accuracy among researchers but is not of experimental interest.

	Nodule number		Nodule biomass	
	χ^2	p	F	p
Isolation host (IH)	**$\chi^2_{(6,989)}$ =727**	**<2.2e-16**	**$F_{(6,989)}$=7.07**	**2.2e-07**
Test species (TS)	**$\chi^2_{(7,989)}$ =2.81e04**	**<2.2e-16**	**$F_{(7,989)}$=224**	**<2.2e-16**
IH*TS	**$\chi^2_{(42,989)}$ =969**	**<2.2e-16**	**$F_{(42,989)}$=2.11**	**6.30e-05**
Nodule counter	**$\chi^2_{(3,989)}$ =135**	**<2.2e-16**	$F_{(3,989)}$=2.15	0.092

Table 4 .7 | Summary of number of test species for which each isolation host was the worst isolation host or best isolation host. Worst isolation host indicates mean nodule number was statistically indistinguishable from the lowest mean nodule number, $p > 0.05$ and best isolation host indicates mean nodule number was statistically indistinguishable from the highest mean nodule number, $p > 0.05$. See Figure 4.5 for full data.

	Worst isolation host	Best isolation host
T. barbigerum	4	1
T. bifidum	1	7
T. fucatum	5	1
T. gracilentum	2	2
T. macraei	5	0
T. microdon	0	5
T. willdenovii	2	2

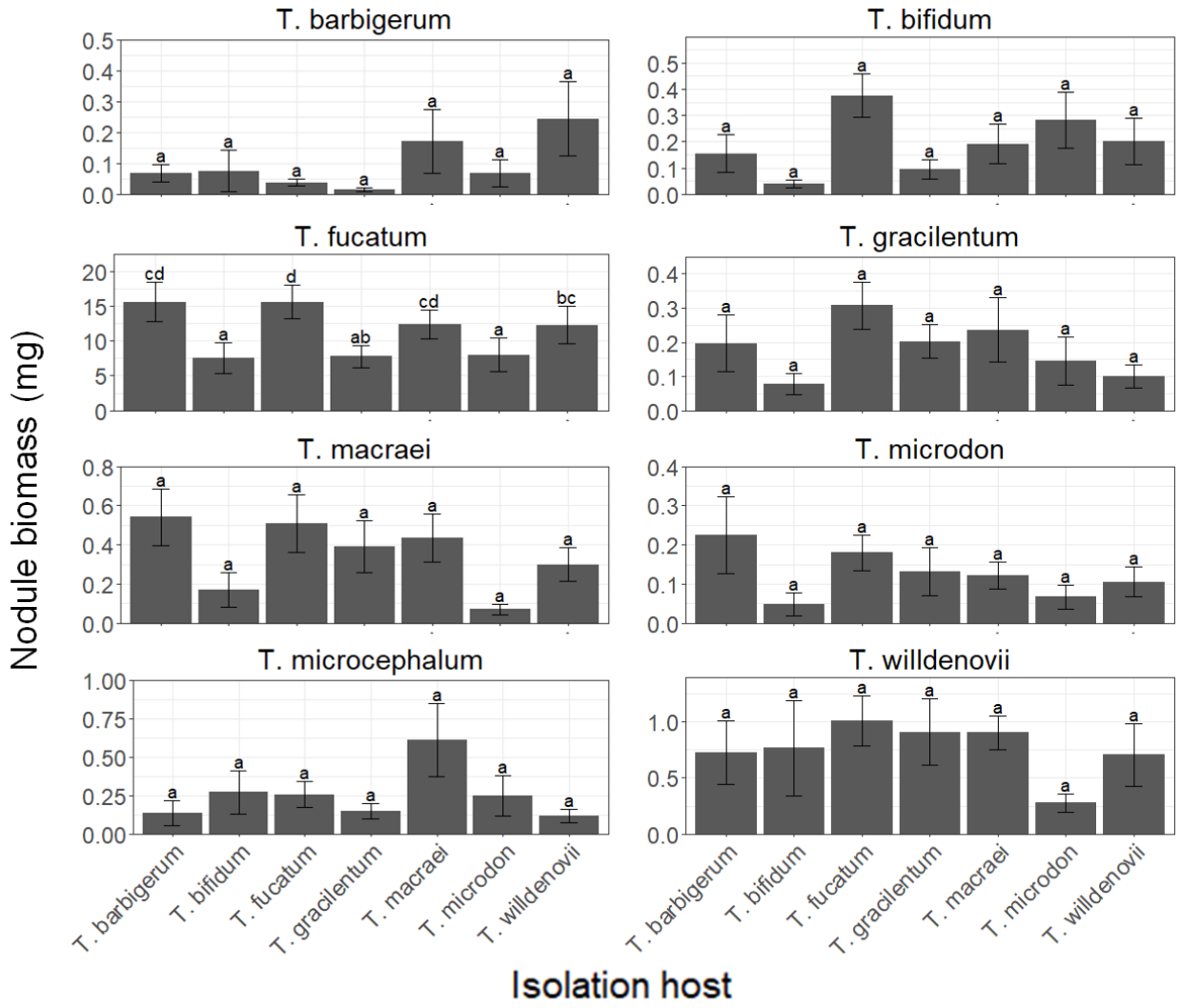

Figure 4.6 | Effects of isolation host on nodule biomass across all test species. Nodule biomass of each combination of isolation host (x axis; the plant species from which a given strain was isolated) and test host (facet; the plant species onto which a strain was inoculated). Within a given facet, bars with the same letter do not significantly differ after Tukey testing ($p > 0.05$). Note that each facet has a different y axis range.

Trifolium species vary in their responsiveness to rhizobia

We detected a significant effect of plant species on scaled shoot dry weight ($p < 2$ e-16; Table 4.4). To assess the net effect of rhizobia on scaled shoot biomass, we determined whether the 95% CI of each species scaled shoot biomass overlapped with 1. Most species performed better when inoculated than without rhizobia: inoculated *T. barbigerum, T. fucatum, T. gracilentum, T. macraei, T. microdon,* and *T. willdenovii* were on average 2.0 (95% CI 1.7-2.5), 1.6 (95% CI 1.5-1.8), 1.4 (95% CI 1.5-1.8) 1.5 (95% CI 1.3-1.8), 2.0 (95% CI 1.7-2.4), and 1.3 (95% CI 1.1-1.5), respectively, times larger than control plants (Figure 4.7a). The remaining two species did not perform better with rhizobia than without: inoculated *T. bifidum* and *T. microcephalum* were on average 1.1 (95% CI 0.9-1.3) and 1.0 (95% CI 0.8-1.3) times the size of control plants (Figure 4.7a).

We also assessed response to rhizobia in terms of investment through specific nodulation and nodule:root biomass. There was relatively little variation in specific nodulation (Figure 4.7b). *T. fucatum* displayed significantly higher nodule:root biomass than any other species (Figure 4.7c).

We detected a marginally significant positive effect of commonness on scaled shoot biomass ($p = 0.078$; Fig 4.13). We did not detect a significant effect of commonness on specific nodulation ($p = 0.338$), but there was a general positive trend (Fig 4.13). We did not detect a significant effect of commonness on nodule:root biomass ($p < 1$ after Bonferroni correction) and there was no clear relationship between the two (Fig 4.13). It is important to note that with only eight points in this regression, the lack of significant effects could be due to lack of power due to small sample number, but the overall trends are still informative.

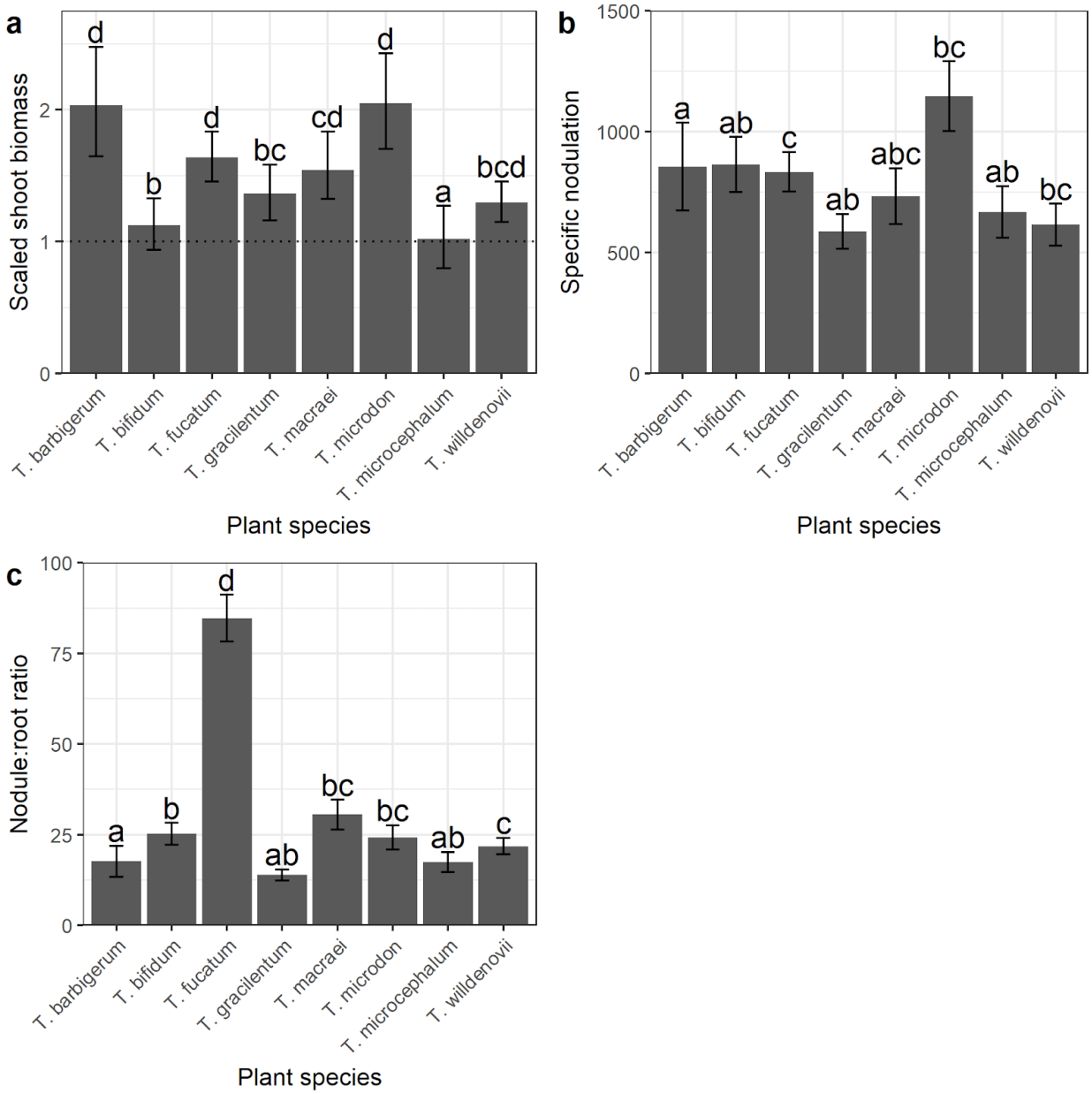

Figure 4.7 | Response to rhizobia for each plant species averaged across all rhizobia treatments. a) Mean scaled shoot dry weight, b) mean specific nodulation (nodule number per gram root biomass), and c) mean nodule:root biomass ratio (mg nodule per g root biomass) for each plant species across all inoculated treatments Error bars represent the 95% CI in a) and +/- 1 SEM in b) and c). Bars with the same letter in a given panel are not significantly different after Tukey testing ($p > 0.05$).

Trifolium species vary in return on investment in rhizobia

We also measured raw efficiency (g of shoot biomass minus mean control biomass for that species, per mg of nodule biomass) and scaled efficiency (g of shoot biomass divided by mean control biomass for that species, per mg of nodule biomass). We detected a significant correlations and positive correlation coefficients between nodule biomass and shoot biomass for all plant species (Table 4.10, Figure 4.8). The magnitude of the correlation coefficient varied by plant species. *T. fucatum* and *T. willdenovii* had the highest correlation coefficients (0.759 and 0.710, respectively), while the 95% confidence intervals of the remaining species all overlapped (Figure 4.8). Note that scaled shoot biomass and raw efficiency are interchangeable in a correlation for a single species because they have a linear relationship.

We also assessed efficiency through linear regression. We detected significant main effects of plant species ($p < 2.2e-16$) and nodule biomass ($p = 1.85$ e-11) and a significant plant species x nodule biomass interaction ($p = 0.01$) on shoot biomass minus mean control biomass (raw efficiency; Table 4.8). Similarly, we detected significant main effects of plant species ($p < 2.2e-16$) and nodule biomass ($p < 2.2e-16$) and a significant plant species x nodule biomass interaction ($p < 2.2e-16$) on shoot biomass minus mean control biomass (scaled efficiency; Table 4.8). Raw efficiency ranged from approximately 0.02 g shoot/mg nodule to 0.06 g shoot/mg nodule (Figure 4.8a). *T. gracilentum* had the highest raw efficiency, while *T. fucatum* had the lowest raw efficiency (Figure 4.8a). Scaled efficiency ranged from approximately 0.1x mean control biomass to 4x mean control biomass per mg nodule. *T. barbigerum* and *T. microdon* had the highest relative efficiency, followed by *T. microcephalum* (Figure 4.8b). *T. fucatum* had by far the lowest relative efficiency (Figure 4.8b).

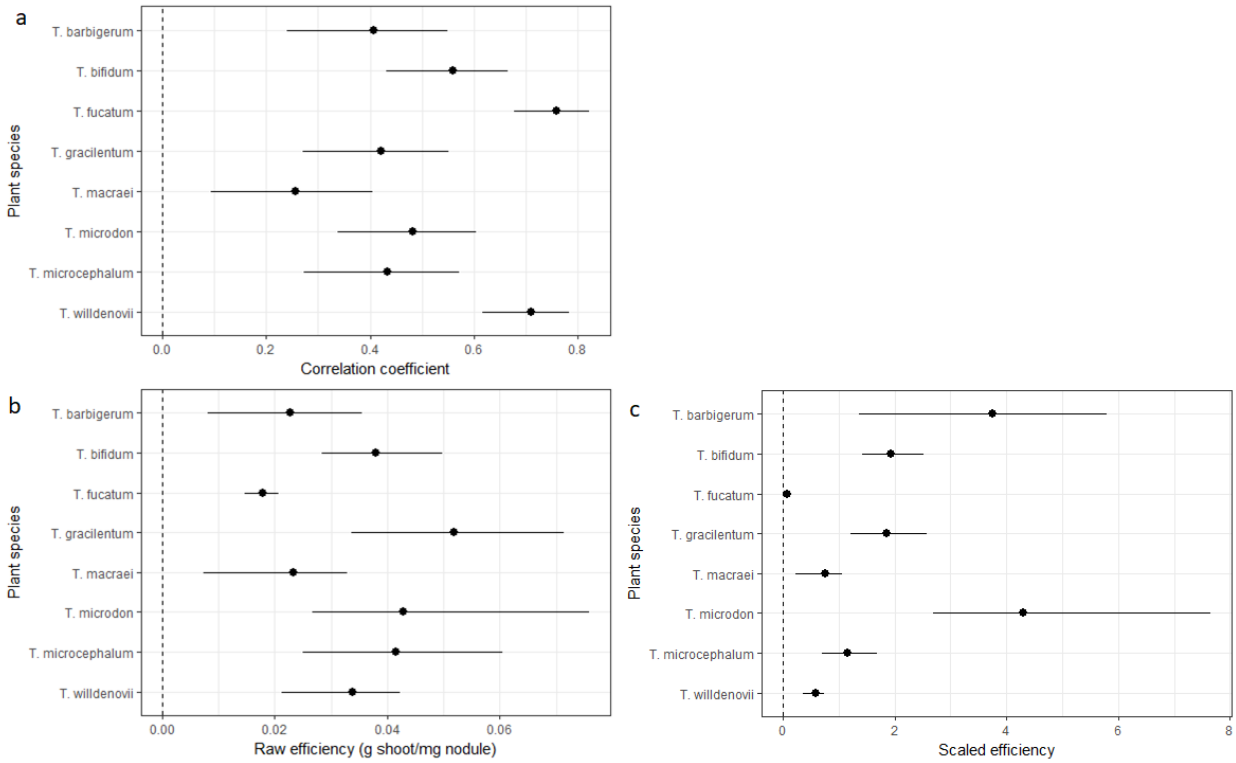

Figure 4.8 | Correlation coefficient and effect size for efficiency across all test species. Mean and 95% confidence interval for a) the correlation coefficient for nodule biomass and scaled shoot biomass, b) the slope of raw shoot biomass gain (shoot biomass minus mean control shoot biomass for each plant species) plotted against nodule biomass, i.e., g shoot gained per mg invested in nodule biomass, and c) the slope of scaled shoot biomass plotted against nodule biomass, i.e., proportion of control biomass gained per mg invested in nodule biomass.

Table 4.8 | ANCOVA summarizing the effects of nodule biomass and test species on shoot biomass gain. Raw biomass gain was calculated as shoot biomass minus the mean shoot biomass of uninoculated controls, while scaled biomass gain was calculated as shoot biomass divided by the mean shoot biomass of uninoculated controls.

	Raw biomass gain		Scaled biomass gain	
	F	*p*	F	*p*
Nodule biomass (NB)	**$F_{(1,1032)}$=1.17e03**	**<2.2e-16**	**$F_{(1,1032)}$=82.0**	**<2.2e-16**
Plant species (PS)	**$F_{(7,1032)}$=9.49**	**1.85e-11**	**$F_{(7,1037)}$=14.6**	**<2.2e-16**
NB*PS	**$F_{(7,1032)}$=2.53**	**0.014**	**$F_{(3,1037)}$=31.9**	**<2.2e-16**

Discussion

If it is assumed that interacting with any partner is better for a symbiont than interacting with no partners at all, then one would expect that generalist symbionts would have higher fitness because they have a wider range of potential partners available to them (Futuyma & Moreno, 1988; Wilson & Yoshimura, 1994). However, the jack of all trades, master of none hypothesis suggests that specialist symbionts exist because there is heterogeneity in hosts, and specialists have higher fitness on their preferred host than a generalist would have on that host (Wilson & Yoshimura, 1994; Burdon *et al.*, 1999). This theory assumes that there is a trade-off between traits that promote adaptation to various hosts (Futuyma & Moreno, 1988; Kassen, 2002), which has been demonstrated in the legume-rhizobia symbiosis (Ehinger *et al.*, 2014). In this experiment, plants were almost always classified as specialists based on PDI and most rhizobia were classified as specialists based on PDI (Figure 4.1). It is difficult to clarify the relationship between plant specialization and plant benefit based on our data. We did not detect a significant effect of any measure of PDI on plant benefit from rhizobia, though it is difficult to say whether this is simply due to the small number of points in the regression (Figure 4.2). Since an individual plant can interact with many rhizobial partners at once and are predicted to be able to preferentially associate with their most beneficial rhizobia, we expect that the maximum plant responses to rhizobia in these single strain inoculations are more representative of the benefit plants would get from their more beneficial partners in the field. The effects of all measures of PDI on plant scaled shoot biomass are positive, suggesting that plants have higher potential fitness in the field as they become more specialized (Figure 4.2). This agrees with Ehinger *et al.*, (2014), who found that the specialist native Californian legume *Acmispon strigosus* obtained more benefit from and invested more resources into rhizobia than the generalist native

Californian legume *Lupinus bicolor*. However, the results from this study should be approached cautiously due to the lack of power of these tests.

Our results for the correlation between specialization and benefit gained by rhizobia sharply contrast to the plant results. We detected significant negative effects of rhizobial specialization on mean investment in rhizobia, both in terms of nodule number and nodule biomass (Figure 4.3). However, we did not detect a significant effect of specialization on maximum investment in rhizobia, suggesting that maximum benefit from symbiosis does not increase with specialization for the rhizobia as it does for the plant. Unlike plants, a single rhizobial cell can only colonize one plant, so the relationship between mean responses and rhizobial PDI is probably more representative of what occurs in the field. In this case, there is fairly strong negative selection pressure against being a specialist, since mean allocation to rhizobia decreases with increasing rhizobial specialization (Figure 4.3). However, a large proportion of the rhizobia we tested are still specialists (Figure 4.1), suggesting that there must be something counteracting this negative selection. Perhaps existing in a more realistic field setting with multiple strains of rhizobia competing for nodule space would change the relative benefits of specialization.

We had hypothesized that if *Trifolium* species were specialized in their interactions with rhizobia, and were able to amplify their most beneficial rhizobia, they would perform better when inoculated with conspecific versus heterospecific rhizobia. Our results do not provide strong support for this hypothesis because interaction type did not significantly affect scaled shoot biomass (Table 4.4). However, in the small subset of plant species in which we could assess flower number, we detected a small positive effect of conspecific rhizobia (Table 4.4, Figure 4.14). Flower number is a more direct measure of plant fitness than shoot biomass, and it

is theoretically possible for a plant to produce more flowers without a significant increase in shoot biomass. Thus, the positive effect of conspecific rhizobia on flower number in three species may extend to the remaining species, if they had been given time to flower. However, we cannot be sure of this without further experimentation, and thus evidence of more benefit for plants from conspecific rhizobia must be taken with caution. A number of wild legumes have been shown to perform better with conspecific rhizobia than with heterospecific rhizobia (Wilkinson & Parker, 1996; Thrall *et al.*, 2000; Murray *et al.*, 2001; Ehinger *et al.*, 2014), making this result particularly surprising.

There are two possible explanations for the lack of difference between benefits received from conspecific and heterospecific rhizobia. The first is that there is no difference in the level of benefit provided by the 32 strains tested in this experiment. Indeed, we did not detect a significant effect of inoculum (a factor with 32 levels, one for each different rhizobial strain) or a significant inoculum x test species interaction on scaled shoot biomass (Table 4.11). We did, however, detect a significant main effect of inoculum and a significant inoculum x test species interaction on flower number in the reduced flower number dataset (Table 4.11). Thus, we once again have only weak evidence for variation in benefit gained from each individual strain that may or may not extend to all 8 *Trifolium* species. While we do not have data for this particular field site, field sites with *Trifolium* have been reported to contain 10^6 cells of *R. leguminosarum* bv. *trifolii* per gram (Hirsch *et al.*, 1993). In addition, 72 distinct strains of *R. leguminosarum* were isolated from a 1 m^2 plot in York, United Kingdom (Kumar *et al.*, 2015). This high level of diversity coupled with high levels of specificity in plant-rhizobia interactions in *Trifolium* (Andrews & Andrews, 2017) make it unlikely that there is simply no variation in benefit.

The second potential explanation is that if there is variation in the benefit gained from these rhizobial strains, it is not clearly divided by the conspecific and heterospecific groupings that we tested. Since we only sampled 4-5 strains per isolation host, the mean benefit from the conspecific strains may be skewed by the inclusion of just a few strains that were not preferred symbionts for that host but managed to form the single nodule we sampled. The benefit provided by rhizobia also does not appear to be partitioned by isolation host (Figure 4.15 & 4.16), though this assessment required a large number of pairwise tests, so the power to detect individual differences is fairly low. Finally, it is also possible that there is variation in benefit provided by rhizobial strains, and it is distributed throughout these 32 strains without regard to the interaction type or isolation host. There may be underlying genetic patterns in these strains that explain variation in benefit that we cannot detect without sequencing the rhizobia. This sequencing is in progress currently and will provide insight into the population structure of the rhizobia and the genetic factors that may shape their effectiveness. Such comparative genomic studies of other rhizobia strain collections have revealed novel insights about population structure and the myriad ways the rhizobia adapted to their native soil and host plants (Sugawara *et al.*, 2013; Kumar *et al.*, 2015; Porter *et al.*, 2016).

Our results were also unclear regarding the hypothesis that conspecific rhizobia would benefit more from their hosts. *T. barbigerum* showed no differences in nodule number or biomass between conspecific and heterospecific rhizobia, while three species made fewer nodules with conspecific rhizobia with no change in nodule biomass, two species made more nodules with conspecific rhizobia with no change in nodule biomass, and *T. fucatum* made more nodules and had higher nodule biomass with conspecific rhizobia than with heterospecific rhizobia (Figure 4.4). This trend suggests that there is significant variation in the way *Trifolium*

species adjust their allocation to rhizobia, and that there is a disconnect between nodule number and nodule biomass.

Legumes can engage in partner choice, or a pre-infection selection of partners that relies on some sort of signal communicating partner quality (Simms & Taylor, 2002; Sachs *et al.*, 2004). Plants can also engage in sanctions/preferential allocation, which occurs post-infection and involves the plant punishing poor quality partners and/or allocating more resources to more beneficial partners (Kiers *et al.*, 2002, 2003). Many plant species changed nodule number without changing average or total nodule biomass, suggesting that they tend to engage in partner choice rather than sanctions/preferential allocations. However, this experiment cannot be considered a direct test of partner choice since the plants did not have multiple strains of rhizobia to choose from. Further experimentation would be required to confirm this trend. Partner choice has been demonstrated in *Trifolium polymorhpum* and *Trifolium purpureum* (Yates *et al.*, 2005, 2008), though this was an extreme case with rhizobia from different continents and a choice between an effective fixer and a strain that did not fix any nitrogen(Yates *et al.*, 2005, 2008). The closely related model legume *Medicago truncatula* has been shown to engage in partner choice (Gubry-Rangin *et al.*, 2010). These trends suggest that the *Trifolium* species in this experiment may be able to engage in partner choice. However, since autoregulation of nodulation is tied to nitrogen status (Mortier *et al.*, 2012), an increase in nodule number may also be explained by the plant initiating more nodules in hopes of finding a high quality rhizobium if all of its current partners are poor nitrogen fixers. In contrast, *T. fucatum* makes more and larger nodules with its conspecific rhizobia, suggesting that it may engage in both partner choice and sanctions/preferential allocation. The congener *Trifolium pratense* preferentially allocates more resources to more cooperative arbuscular mycorrhizal fungi (Argüello *et al.*, 2016), and *M.*

trucatula has been shown to employ changes in nodule number and average nodule biomass simultaneously, though the changes in nodule biomass did not affect viable rhizobia cells per nodule (Gubry-Rangin *et al.*, 2010). However, without a clear connection to increased plant benefit, it is unclear how effective these forms of partner policing are. The overall trend of changes in nodulation patterns without resulting changes in plant performance have been detected in *Medicago lupulina*, and may indicate that the plant is increasings its allocation of resources to rhizobia to compensate for less beneficial partners (Harrison *et al.*, 2017).

Interpreting the effects of these trends on rhizobial fitness is complicated, since nodule number and nodule biomass are both fitness components for rhizobia (Ratcliff *et al.*, 2012). The *Trifolium-Rhizobium* symbiosis produces indeterminate nodules in which N-fixing bacteroids are terminally differentiated (Poole *et al.*, 2018). Thus, the only rhizobia that are able to reproduce once the nodule senesces at the end of the growing season are the undifferentiated cells that remain near the nodule meristem and do not fix nitrogen (Oono *et al.*, 2009). Crushing and plating experiments would be required to track the ratio of live cells to nodule number and nodule biomass to determine direct effects on rhizobial fitness. These ratios may also vary by rhizobial strain, making things even more complicated. In addition, nodule number in single strain inoculations can be correlated to nodulation competitiveness in multi-strain inoculations (Friesen, 2012). Thus, nodule number differences in these single strain inoculations may have important implications in more natural settings where multiple strains of rhizobia are competing to nodulate plant roots. However, a direct understanding of how our nodulation results translate into rhizobial fitness would require a large number of tests that assessed which rhizobia strains nodulated which plant species in a natural soil environment, and how many rhizobial offspring arose from each nodule.

While we did not detect a significant effect of commonness in the field on plant response to rhizobia, the general trend is positive as predicted, and the lack of significant relationship may be due to lack of power from only having 8 points (Figure 4.13). A species that is more common in the field is more likely to have co-adapted to a given rhizobial strain since they would have had more opportunities to interact over evolutionary time, all other factors equal. While specific nodulation also had a positive relationship with commonness, nodule:root biomass did not, highlighting the disconnect between nodule number and nodule biomass (Figure 4.13). Plant growth response to rhizobia is determined both by the amount of investment and the return on that investment, or the nodule efficiency. *T. barbigerum* and *T. microdon* are the most common species in the field that also show the largest growth response to rhizobia (Figure 4.7). These species have fairly average biomass investment in rhizobia, but have much higher scaled efficiency than any other species, explaining their large response (Figure 4.7). On the other hand, *T. fucatum* invests a huge proportion of biomass in nodules that have a relatively low raw efficiency (Figure 4.7). This relatively low raw efficiency translates into an exceptionally low scaled efficiency because *T. fucatum* is on average significantly larger than any other species. *T. fucatum* makes the largest average biomass per nodule by far, suggesting that perhaps there are inefficiencies in gas exchange or nutrient exchange in excessively large nodules.

It is important to consider these results in light of a few caveats about the setup of this experiment. First, we only sampled 4-5 rhizobial strains per plant species, which is likely to be a very small subset of the strains available in the field. We also artificially forced a single *Trifolium* to interact with a highly concentrated inoculum of a single strain of rhizobia. In the field, *Trifolium* would have to contend with inter- and intraspecific competition, abiotic stresses, and a full microbial community including other strains of rhizobia and pathogens. These factors

can affect the dynamics of nutrient trade between the plant and rhizobia. The presence of other rhizobial strains is particularly important, since this introduces competition for nodulation, which has serious impacts on colonization success, and it gives the plant the ability to exert partner choice (Gerding *et al.*, 2014). In addition, the colonization success and/or benefit provided by a given strain may depend on other microbes that were not included in this experiment (Martínez-Hidalgo & Hirsch, 2017). These trends could be explored through inoculating *Trifolium* with a mix of rhizobial strains and tracking which strains nodulate and fix nitrogen best on the different plant species in a more complex environment.

In conclusion, the results of this experiment do not fit with either of the theoretical explanations of how mutualisms may promote plant coexistence. If Parker (1999)'s theory of co-adaptation leading to coexistence through spatial patchiness held true, we would have expected to see positive plant-rhizobia feedbacks, with plants benefiting more from and investing more into conspecific rhizobia. Alternatively, if Bever (1999)'s theory of coexistence through negative feedbacks between rhizobia and plant were true, we would have expected that plants would perform worse with conspecific rhizobia and invest more into heterospecific rhizobia. We did not see either of these trends, suggesting that some other trend is driving coexistence between *Trifolium* species. Both the plants and rhizobia were usually specialists, suggesting that there is specialization, but maximum benefit is not clearly partitioned by interaction type or by isolation host. Future work will include sequencing the genomes of the rhizobial strains, which should help to clarify the genomic patterns that will hopefully explain the patterns of benefit. These and future results will have important implications for understanding the role of rhizobia in natural ecosystems and the development of suitably effective and specialized rhizobial crop inoculants.

APPENDIX

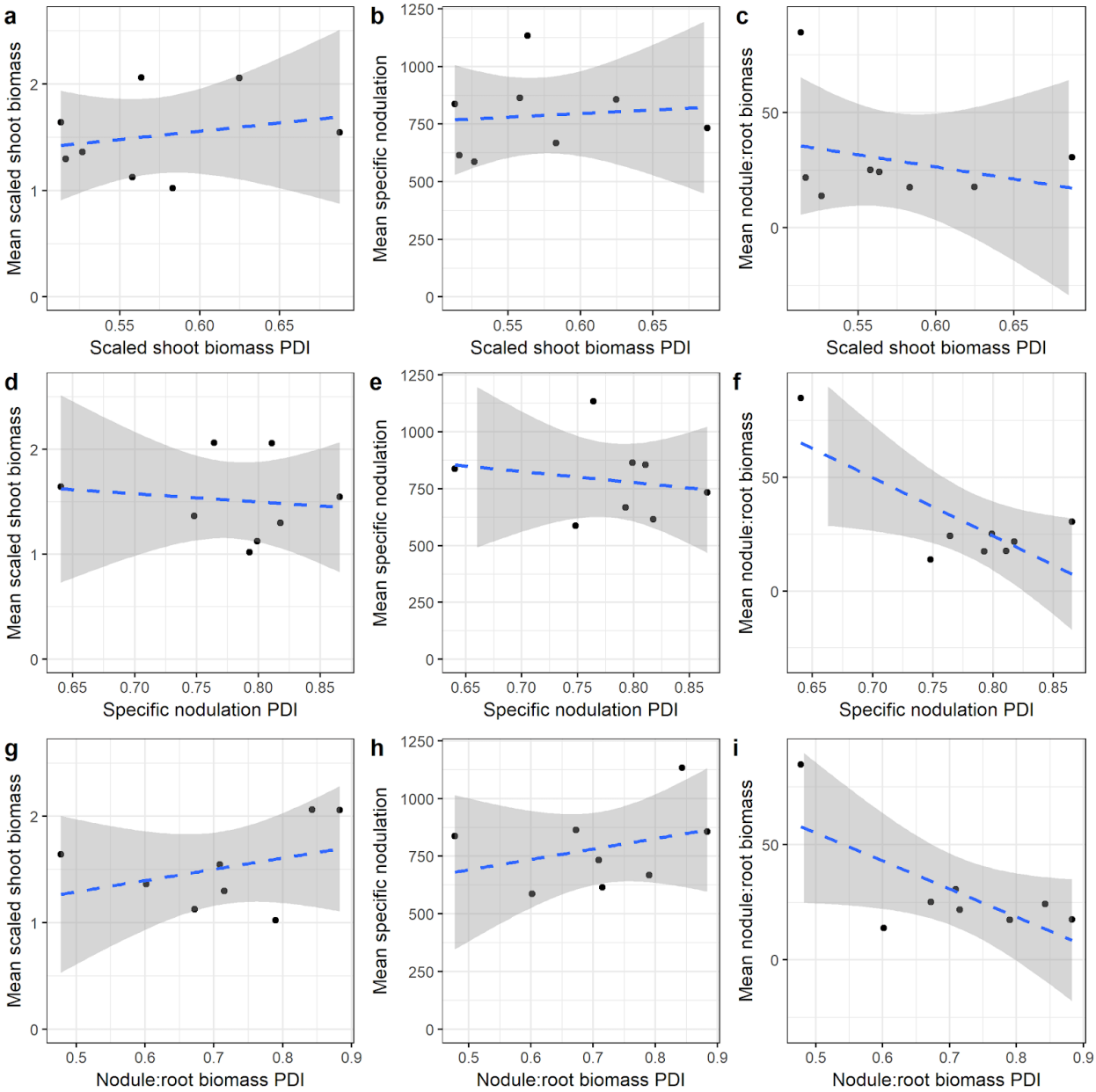

Figure 4.9 | Scatterplots of mean plant responses to rhizobia versus plant PDI measures. Mean scaled shoot biomass per plant species plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule:root biomass PDI. Mean plant specific nodulation per plant species plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule root biomass PDI. Mean plant nodule:root biomass plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule root biomass PDI. Dotted lines indicate a nonsignificant effect of x on y ($p > 0.05$ after Bonferroni correction) in a linear model of the form log(response) ~ x.

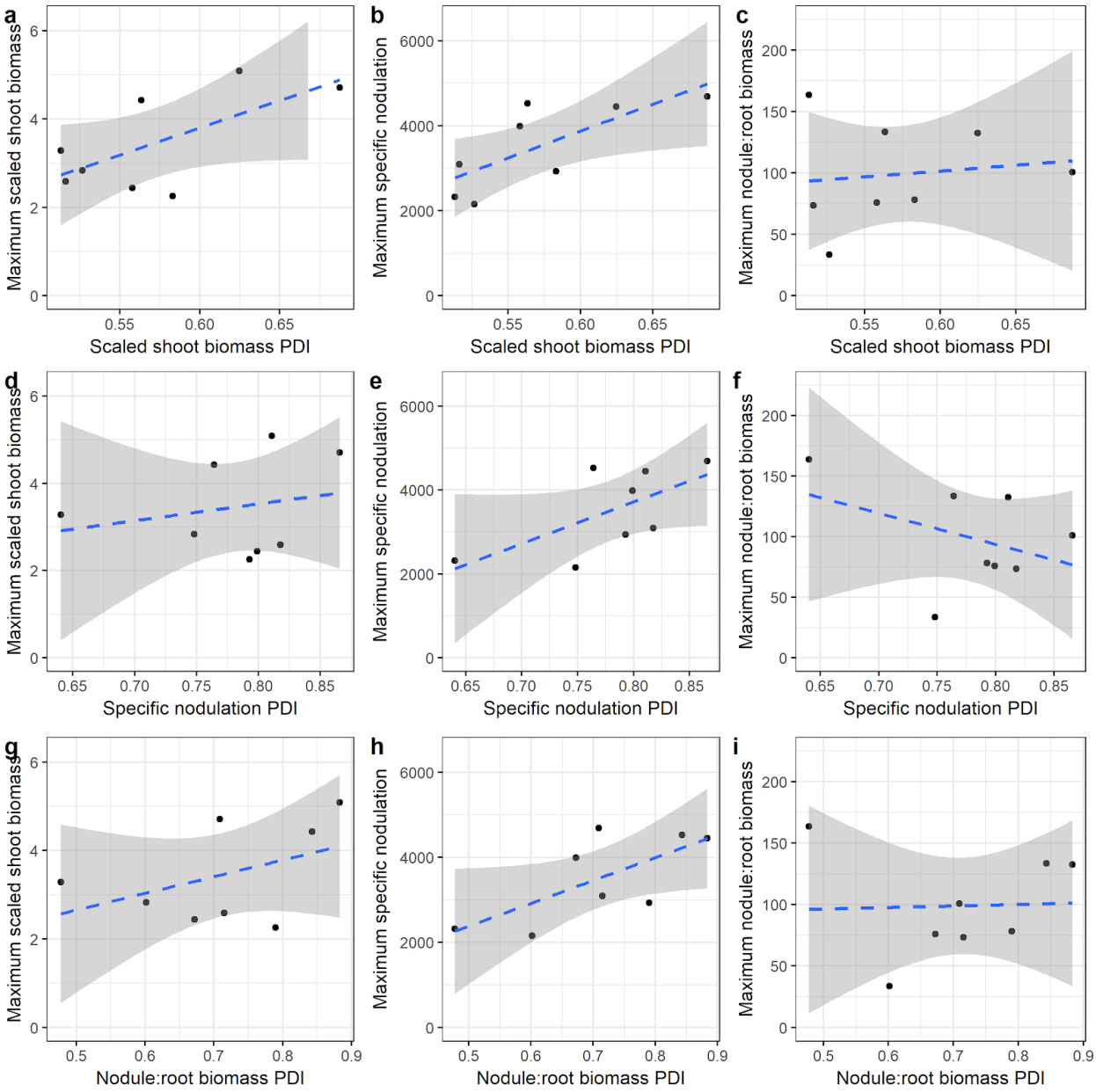

Figure 4.10 | Scatterplots of maximum plant responses to rhizobia versus plant PDI measures. Maximum scaled shoot biomass per plant species plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule:root biomass PDI. Maximum specific nodulation per plant species plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule root biomass PDI. Maximum nodule:root biomass per plant species plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule root biomass PDI. Dotted lines indicate a nonsignificant effect of x on y ($p > 0.05$ after Bonferroni correction) in a linear model of the form log(response) ~ PDI. Solid lines indicate a significant effect of x on y ($p < 0.05$ after Bonferroni correction). Maximum values of scaled shoot biomass, specific nodulation, and nodule:root ratio were calculated by finding the mean of each response for each combination of inoculum and plant species, and then selecting the maximum mean for each plant species.

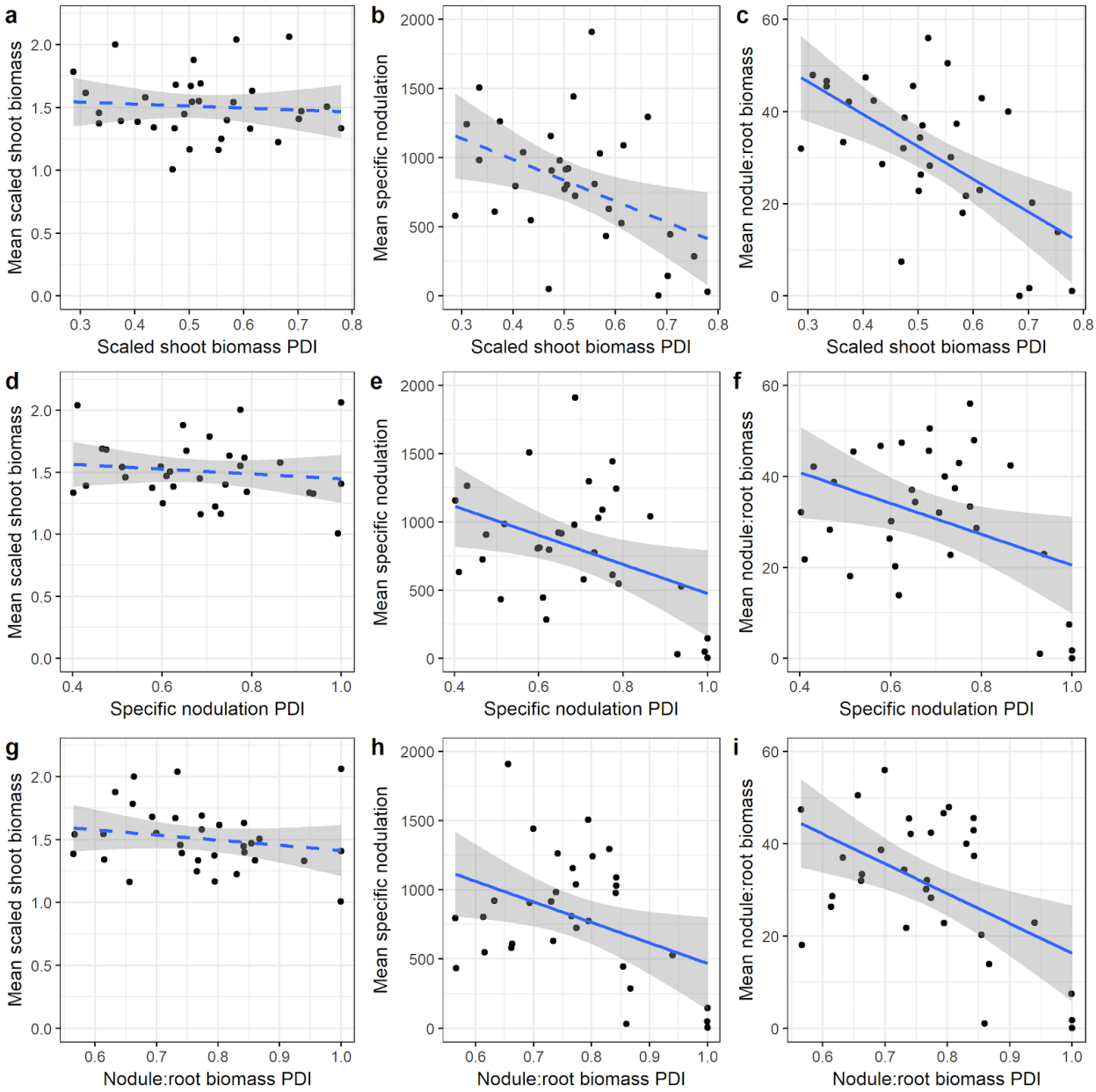

Figure 4.11 | Scatterplots of mean plant responses to rhizobia versus rhizobial PDI measures. Mean scaled shoot biomass per rhizobial strain plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule:root biomass PDI. Mean plant specific nodulation per plant species plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule root biomass PDI. Mean plant nodule:root biomass plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule root biomass PDI. Dotted lines indicate a nonsignificant effect of x on y ($p > 0.05$ after Bonferroni correction) in a linear model of the form log(response) ~ PDI. Solid lines indicate a significant effect of x on y ($p < 0.05$ after Bonferroni correction).

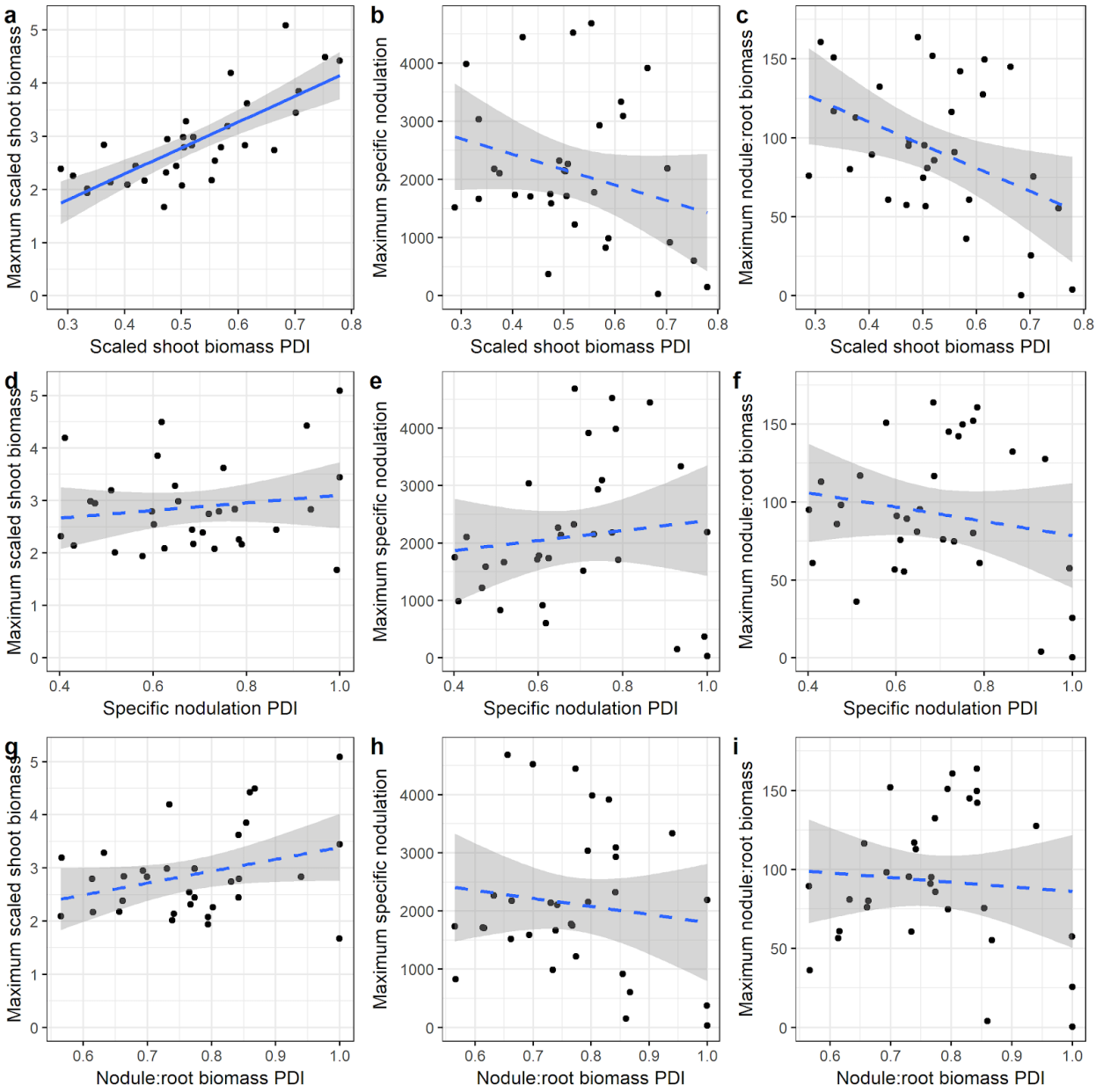

Figure 4.12 | Scatterplots of maximum plant responses to rhizobia versus rhizobial PDI measures. Maximum scaled shoot biomass per rhizobial strain plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule:root biomass PDI. Maximum specific nodulation per rhizobial strain plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule root biomass PDI. Maximum nodule:root biomass per rhizobial strain plotted against a) scaled shoot biomass PDI, d) specific nodulation PDI, and g) nodule root biomass PDI. Dotted lines indicate a nonsignificant effect of x on y ($p > 0.05$) in a linear model. Maximum values of scaled shoot biomass, specific nodulation, and nodule:root ratio were calculated by finding the mean of each response for each combination of inoculum and plant species, and then selecting the maximum mean for each plant species.

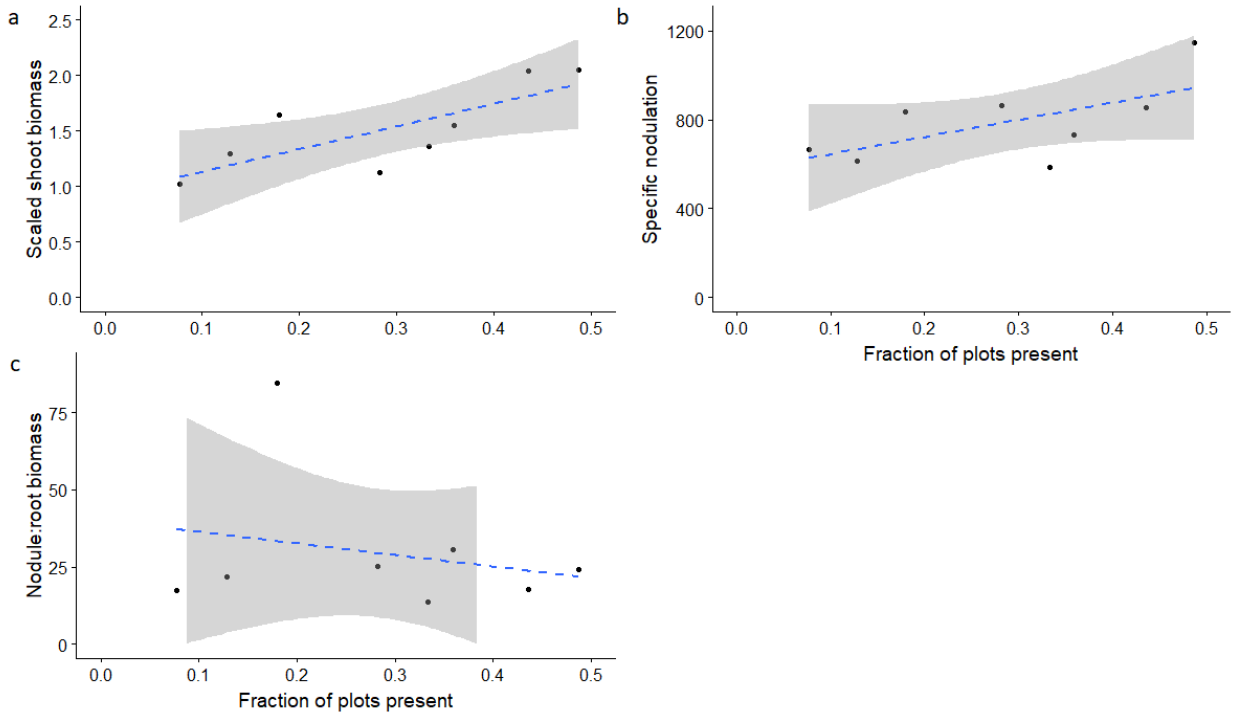

Figure 4.13 | Response to rhizobia versus commonness in the field. a) Scaled shoot biomass, b) specific nodulation, and c) nodule:root biomass plotted against the fraction of plots in the field in which a species is present. We detected a marginally significant effect of commonness on scaled shoot biomass ($F(1,6)=8.57$, $p = 0.078$ after Bonferroni correction). We did not detect a significant effect of commonness on specific nodulation $F(1,6)=3.45$, $p = 0.338$ after Bonferroni correction). We did not detect a significant effect of commonness on specific nodulation ($F(1,6)=0.213$, $p < 1$ after Bonferroni correction).

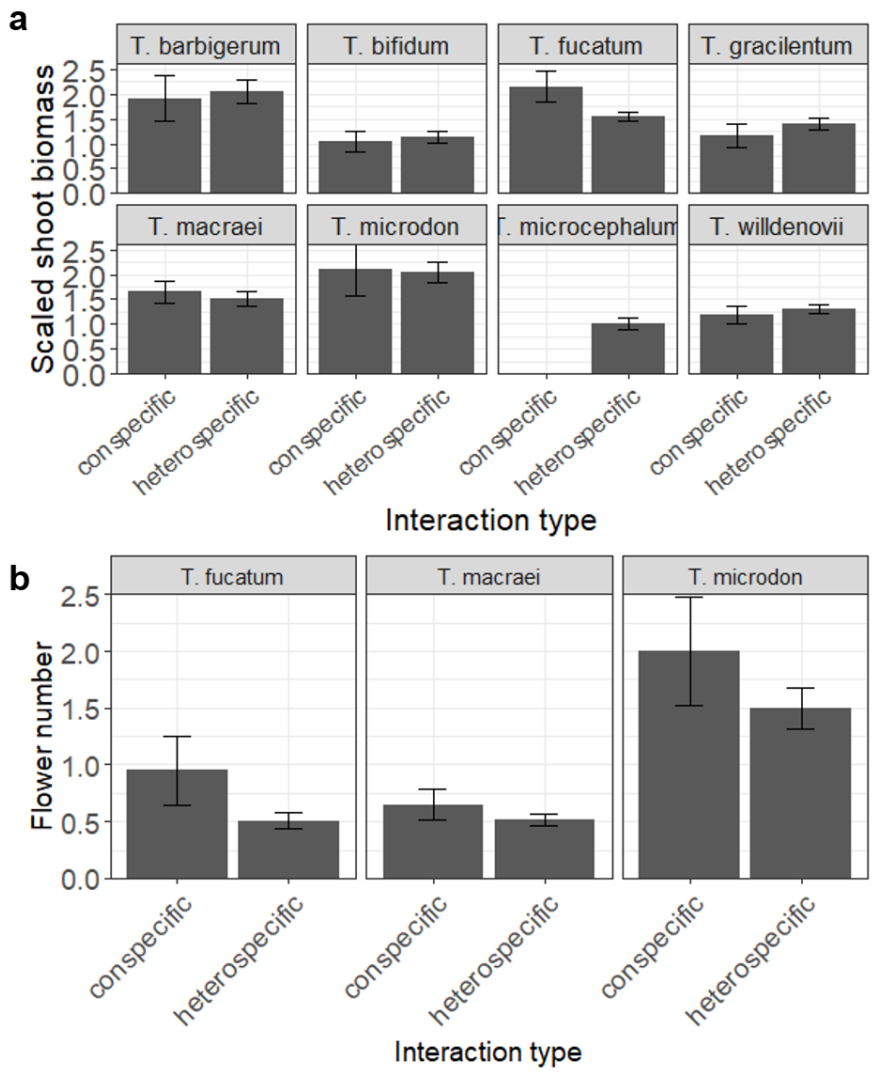

Figure 4.14 | Mean scaled shoot biomass and flower number for each test species inoculated with conspecific or heterospecific rhizobia. a) Mean scaled shoot biomass for each test species inoculated with conspecific or heterospecific rhizobia. We did not detect a significant effect of interaction type or an interaction between interaction type and test species on scaled shoot biomass (p = 0.890 and p = 0.731, respectively). b) Mean flower number for the reduced set of test species analyzed for flower number. Plants inoculated with conspecific rhizobia made significantly more flowers than plants inoculated with heterospecific rhizobia overall (p = 0.010), but we did not detect an interaction between flower number and plant species (p = 0.523).

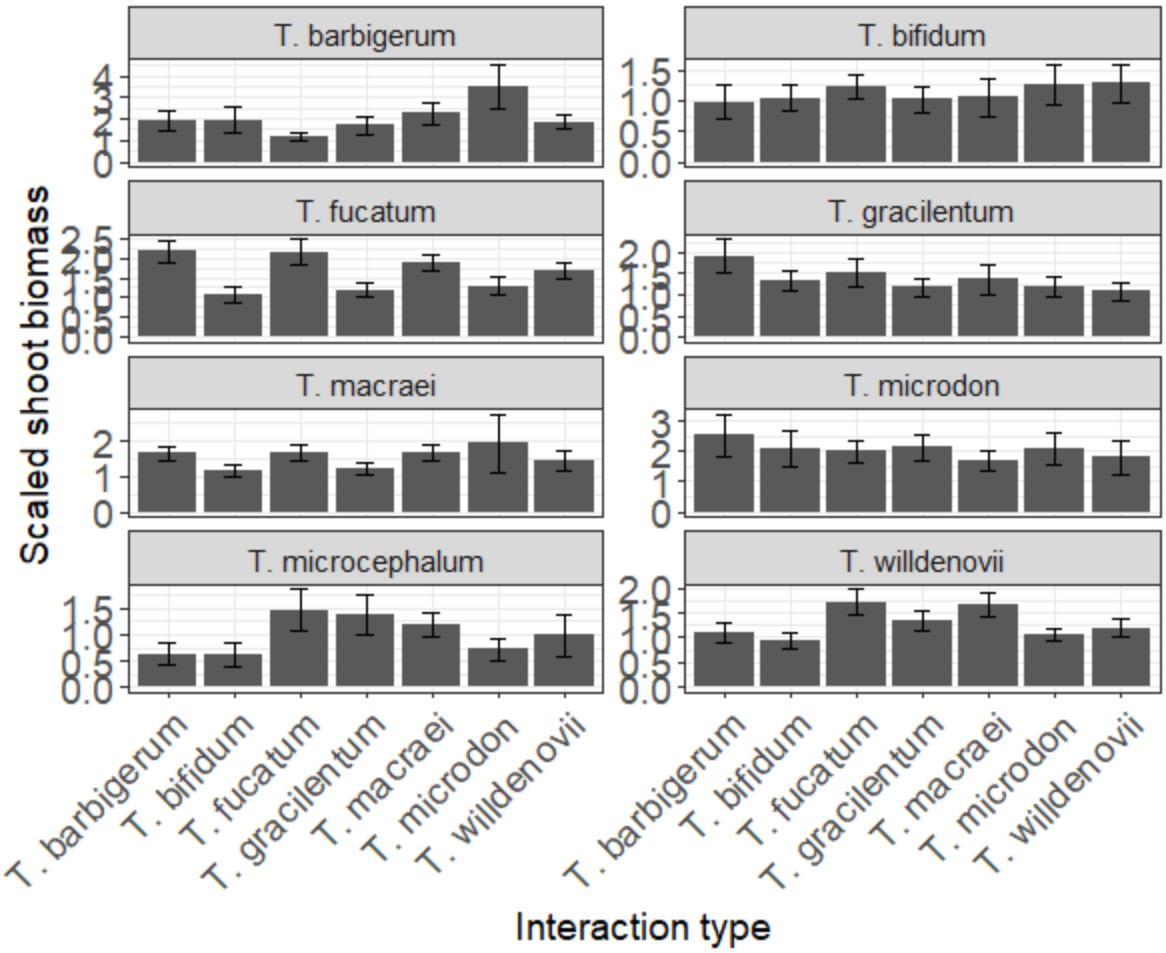

Figure 4.15 | Mean scaled shoot biomass for each plant species inoculated with rhizobia from each isolation host. We detected a significant effect of test host species ($F_{7,998}$=17.3, p < 2e-16), but we did not detect a significant effect of isolation host ($F_{6,998}$=1.54, p = 0.161) or a significant interaction between isolation host and test host species ($F_{42,998}$=1.33, p = 0.078).

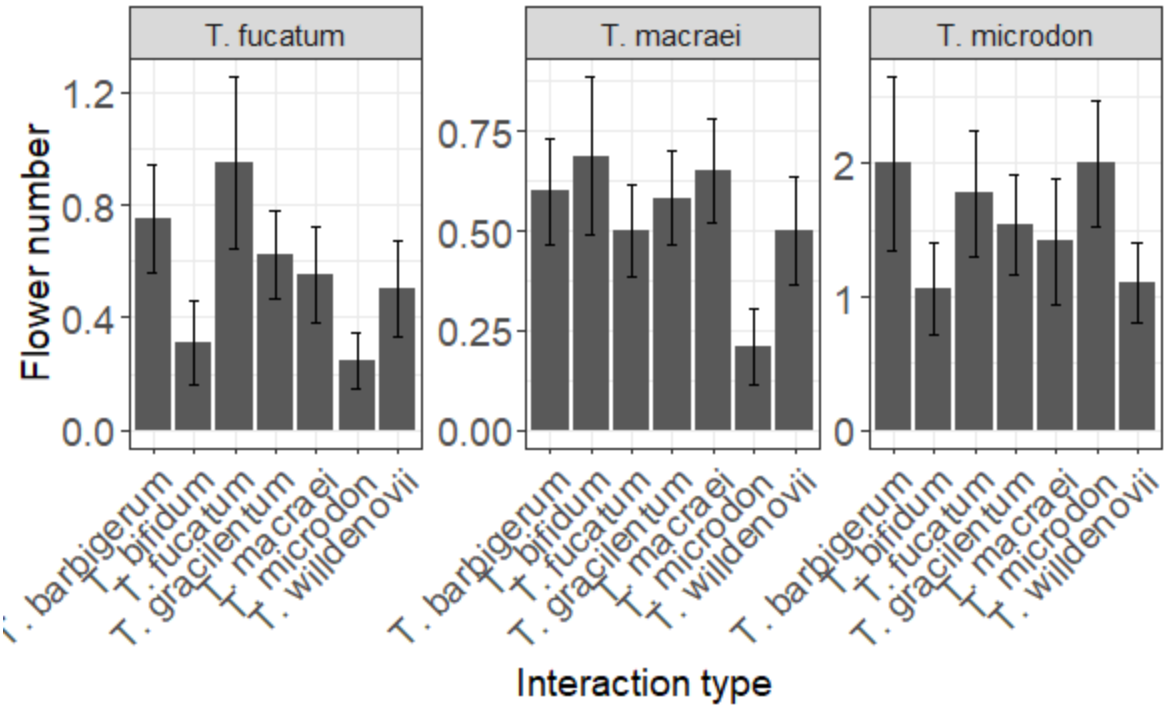

Figure 4.16 | Mean flower number for each plant species inoculated with rhizobia from each isolation host. We detected a significant effect of test host species ($\chi^2_{(2,390)}$=97.2, p < 2e-16), but we did not detect a significant effect of isolation host ($\chi^2_{(6,390)}$=10.4, p = 0.108) or a significant interaction between isolation host and test host species ($\chi^2_{(12,390)}$=18.7, p = 0.097).

Table 4.9 | Percentage of inoculated individuals of each plant species that flowered.

Plant species	Flowering percentage
T.barbigerum	2.54
T. bifidum	19.1
T. fucatum	40.7
T. gracilentum	23.1
T. macraei	48.9
T. microdon	50.8
T. microcephalum	2.59
T. willdenovii	1.44

Table 4.10 | Correlation between nodule biomass and scaled shoot biomass for each test species. Pearson's correlation coefficient, the upper and lower bound of the 95% confidence interval for the coefficient, and t and Bonferroni-corrected *p* values for the correlation test between nodule biomass and scaled shoot biomass for each plant species.

	Correlation coefficient	2.5%	97.5%	*t*	*p*
T. barbigerum	0.406	0.241	0.548	4.72	**6.03e-05**
T. bifidum	0.559	0.431	0.664	7.79	**1.44e-11**
T. fucatum	0.759	0.678	0.821	13.6	**< 1.76e-15**
T. gracilentum	0.420	0.270	0.551	5.32	**3.81e-06**
T. macraei	0.256	0.093	0.40	3.10	**0.021**
T. microdon	0.482	0.337	0.604	6.22	**5.25e-08**
T. microcephalum	0.433	0.273	0.571	5.13	**9.43e-06**
T. willdenovii	0.710	0.616	0.784	11.8	**< 1.76e-15**

Table 4.11 | ANOVA summarizing the effects of inoculum and test species on plant performance. Raw biomass gain was calculated as shoot biomass minus the mean shoot biomass of uninoculated controls, while scaled biomass gain was calculated as shoot biomass divided by the mean shoot biomass of uninoculated controls.

	Scaled shoot biomass		Flower number	
	F	*p*	χ^2	*p*
Inoculum (I)	$F_{(31,798)} = 0.921$	0.592	$\mathbf{\chi^2_{(31,315)} = 63.0}$	**5.87e-04**
Test species (TS)	$\mathbf{F_{(7,798)} = 17.5}$	**< 2e-16**	$\mathbf{\chi^2_{(2,315)} = 95.2}$	**<2e-16**
I*TS	$F_{(217,798)} = 1.11$	0.152	$\mathbf{\chi^2_{(62,315)} = 83.3}$	**0.037**

REFERENCES

REFERENCES

Adler PB, Ellner SP, Levine JM. **2010**. Coexistence of perennial plants: an embarrassment of niches. *Ecology Letters* **13**: 1019–1029.

Afkhami ME, McIntyre PJ, Strauss SY. **2014**. Mutualist-mediated effects on species' range limits across large geographic scales. *Ecology Letters* **17**: 1265–1273.

Andrews M, Andrews ME. **2017**. Specificity in Legume-Rhizobia Symbioses. *International Journal of Molecular Sciences* **18**: 705.

Argüello A, O'Brien MJ, van der Heijden MGA, Wiemken A, Schmid B, Niklaus PA. **2016**. Options of partners improve carbon for phosphorus trade in the arbuscular mycorrhizal mutualism (H Maherali, Ed.). *Ecology Letters* **19**: 648–656.

Bever JD. **1999**. Dynamics within mutualism and the maintenance of diversity: inference from a model of interguild frequency dependence. *Ecology Letters* **2**: 52–61.

Bever JD. **2003**. Soil community feedback and the coexistence of competitors: conceptual frameworks and empirical tests. *New Phytologist* **157**: 465–473.

Burdon JJ, Gibson AH, Searle SD, Woods MJ, Brockwell J. **1999**. Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian Acacia: within-species interactions. *Journal of Applied Ecology* **36**: 398–408.

Cadotte MW, Cavender-Bares J, Tilman D, Oakley TH. **2009**. Using Phylogenetic, Functional and Trait Diversity to Understand Patterns of Plant Community Productivity (RP Freckleton, Ed.). *PLoS ONE* **4**: e5695.

Cardinale BJ, Srivastava DS, Duffy JE, Wright JP, Downing AL, Sankaran M, Jouseau C, Cadotte MW, Carroll IT, Weis JJ, *et al.* 2009. Effects of biodiversity on the functioning of ecosystems: a summary of 164 experimental manipulations of species richness (WK Michener, Ed.). *Ecology* **90**: 854–854.

Cardinale BJ, Srivastava DS, Emmett Duffy J, Wright JP, Downing AL, Sankaran M, Jouseau C. **2006**. Effects of biodiversity on the functioning of trophic groups and ecosystems. *Nature* **443**: 989–992.

Carlsson G, Palmborg C, Huss-Danell K. **2006**. Discrimination against ^{15}N in three N_2-fixing *Trifolium* species as influenced by *Rhizobium* strain and plant age. *Acta Agriculturae Scandinavica, Section B - Plant Soil Science* **56**: 31–38.

Chesson P. **2000**. Mechanisms of Maintenance of Species Diversity. *Annual Review of Ecology and Systematics* **31**: 343–366.

Ehinger M, Mohr TJ, Starcevich JB, Sachs JL, Porter SS, Simms EL. **2014**. Specialization-generalization trade-off in a Bradyrhizobium symbiosis with wild legume hosts. *BMC Ecology* **14**: 8.

Fridley JD, Grime JP, Bilton M. **2007**. Genetic identity of interspecific neighbours mediates plant responses to competition and environmental variation in a species-rich grassland. *Journal of Ecology* **95**: 908–915.

Friesen ML. **2012**. Widespread fitness alignment in the legume-rhizobium symbiosis. *The New phytologist* **194**: 1096–111.

Futuyma DJ, Moreno G. **1988**. The evolution of ecological specialization. *Ann. Rev. Ecol. Syst* **19**: 207–240.

Gerding M, O'Hara GW, Howieson JG, Bräu L. **2014**. Overcoming non-selective nodulation of Lessertia by soil-borne rhizobium in the presence of inoculant mesorhizobium. *Plant and Soil* **380**: 117–132.

Gubry-Rangin C, Garcia M, Béna G. **2010**. Partner choice in Medicago truncatula-Sinorhizobium symbiosis. *Proceedings. Biological sciences / The Royal Society* **277**: 1947–51.

Harrison TL, Wood CW, Borges IL, Stinchcombe JR. **2017**. No evidence for adaptation to local rhizobial mutualists in the legume Medicago lupulina. *Ecology and Evolution* **00**: 1–10.

Hirsch PR, Jones MJ, McGrath SP, Giller KE. **1993**. Heavy metals from past applications of sewage sludge decrease the genetic diversity of rhizobium leguminosarum biovar trifolii populations. *Soil Biology and Biochemistry* **25**: 1485–1490.

Hodge A, Fitter AH. **2013**. Microbial mediation of plant competition and community structure (D Robinson, Ed.). *Functional Ecology* **27**: 865–875.

Holland AA. **1970**. Competition between soil- and seed-borneRhizobium trifolii in nodulation of introducedTrifolium subterraneum. *Plant and Soil* **32**: 293–302.

Holland AA, Parker CA. **1966**. Studies on microbial antagonism in the establishment of clover pasture. *Plant and Soil* **25**: 329–340.

Howieson JG, Yates RJ, O'Hara GW, Ryder M, Real D. **2005**. The interactions of *Rhizobium leguminosarum* biovar *trifolii* in nodulation of annual and perennial *Trifolium* spp. from diverse centres of origin. *Australian Journal of Experimental Agriculture* **45**: 199.

Hughes AR, Inouye BD, Johnson MTJ, Underwood N, Vellend M. **2008**. Ecological consequences of genetic diversity. *Ecology Letters* **11**: 609–623.

Hughes AR, Stachowicz JJ. **2004**. Genetic diversity enhances the resistance of a seagrass ecosystem to disturbance. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 8998–9002.

Jordan D. **1984**. Family 111. Rhizobiaceae. In: Krieg N, JG H, eds. Bergey's Manual of Systematic Bacteriology. Baltimore, MD: The Williams & Wilkins Co., 234–244.

Kassen R. **2002**. The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology* **15**: 173–190.

Kazenel MR, Debban CL, Ranelli L, Hendricks WQ, Chung YA, Pendergast TH, Charlton ND, Young CA, Rudgers JA. **2015**. A mutualistic endophyte alters the niche dimensions of its host plant. *AoB PLANTS* **7**: plv005-plv005.

Keller KR. **2014**. Mutualistic rhizobia reduce plant diversity and alter community composition. *Oecologia* **176**: 1101–1109.

Kiers ET, Rousseau RA, West SA, Denison RF. **2003**. Host sanctions and the legume–rhizobium mutualism. *Nature* **425**: 78–81.

Kiers ET, West SA, Denison RF. **2002**. Mediating mutualisms: farm management practices and evolutionary changes in symbiont co-operation. *Journal of Applied Ecology* **39**: 745–754.

Klironomos JN. **2002**. Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature* **417**: 67–70.

Kumar N, Lad G, Giuntini E, Kaye ME, Udomwong P, Shamsani NJ, Young JPW, Bailly X. **2015**. Bacterial genospecies that are not ecologically coherent: population genomics of Rhizobium leguminosarum. *Open Biology* **5**: 140133–140133.

Levene H. **1953**. Genetic Equilibrium When More Than One Ecological Niche is Available. *The American Naturalist* **87**: 331–333.

Levine JM, HilleRisLambers J. **2009**. The importance of niches for the maintenance of species diversity. *Nature* **461**: 254–257.

Lira MA, Nascimento LRS, Fracetto GGM. **2015**. Legume-rhizobia signal exchange: promiscuity and environmental effects. *Frontiers in Microbiology* **6**: 945.

Mangan SA, Herre EA, Bever JD. **2010**. Specificity between Neotropical tree seedlings and their fungal mutualists leads to plant–soil feedback. *Ecology* **91**: 2594–2603.

Martínez-Hidalgo P, Hirsch AM. **2017**. The Nodule Microbiome: N 2 -Fixing Rhizobia Do Not Live Alone. *Phytobiomes*: PBIOMES-12-16-0.

Mortier V, Holsters M, Goormachtig S. **2012**. Never too many? How legumes control nodule numbers. *Plant, Cell & Environment* **35**: 245–258.

Murray BBR, Thrall PH, Woods MJ. **2001**. Acacia species and rhizobial interactions: Implications for restoration of native vegetation. *Ecological Management and Restoration* **2**:

213–219.

Oono R, Denison RF, Kiers ET. **2009**. Controlling the reproductive fate of rhizobia: how universal are legume sanctions? *The New phytologist* **183**: 967–79.

Parker MA. **1999**. Mutualism in Metapopulations of Legumes and Rhizobia. *The American naturalist* **153**: S48–S60.

Peay KG. **2016**. The Mutualistic Niche: Mycorrhizal Symbiosis and Community Dynamics. *Annual Review of Ecology, Evolution, and Systematics* **47**: 143–164.

Pendergast TH, Burke DJ, Carson WP. **2013**. Belowground biotic complexity drives aboveground dynamics: a test of the soil community feedback model. *New Phytologist* **197**: 1300–1310.

Poisot T, Canard E, Mouquet N, Hochberg ME. **2012**. A comparative study of ecological specialization estimators. *Methods in Ecology and Evolution* **3**: 537–544.

Poole P, Ramachandran V, Terpolilli J. **2018**. Rhizobia: from saprophytes to endosymbionts. *Nature Reviews Microbiology* **16**: 291–303.

Porter SS, Chang PL, Conow CA, Dunham JP, Friesen ML. **2016**. Association mapping reveals novel serpentine adaptation gene clusters in a population of symbiotic Mesorhizobium. *The ISME Journal*.

van der Putten WH, Bardgett RD, Bever JD, Bezemer TM, Casper BB, Fukami T, Kardol P, Klironomos JN, Kulmatiski A, Schweitzer JA, *et al.* 2013. Plant-soil feedbacks: the past, the present and future challenges (M Hutchings, Ed.). *Journal of Ecology* **101**: 265–276.

Ratcliff WC, Underbakke K, Denison RF. **2012**. Measuring the fitness of symbiotic rhizobia. *Symbiosis* **55**: 85–90.

Sachs JL, Mueller UG, Wilcox TP, Bull JJ. **2004**. The Evolution of Cooperation. *The Quarterly Review of Biology* **79**: 135–160.

Simms EL, Taylor DL. **2002**. Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. *Integrative and comparative biology* **42**: 369–80.

Sugawara M, Epstein B, Badgley BD, Unno T, Xu L, Reese J, Gyaneshwar P, Denny R, Mudge J, Bharti AK, *et al.* 2013. Comparative genomics of the core and accessory genomes of 48 Sinorhizobium strains comprising five genospecies. *Genome Biology* **14**: R17.

Thrall PH, Burdon JJ, Woods MJ. **2000**. Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian legumes: interactions within and between genera. *Journal of Applied Ecology* **37**: 52–65.

Turnbull LA, Levine JM, Loreau M, Hector A. **2013**. Coexistence, niches and biodiversity effects on ecosystem functioning (JHR Lambers, Ed.). *Ecology Letters* **16**: 116–127.

Wagg C, Jansa J, Stadler M, Schmid B, van der Heijden MGA. **2011**. Mycorrhizal fungal identity and diversity relaxes plant–plant competition. *Ecology* **92**: 1303–1313.

Wilkinson HH, Parker MA. **1996**. Symbiotic specialization and the potential for genotypic coexistence in a plant-bacterial mutualism. *Oecologia* **108**: 361–367.

Wilson DS, Yoshimura J. **1994**. On the Coexistence of Specialists and Generalists. *The American Naturalist* **144**: 692–707.

Yates RJ, Howieson JG, Real D, Reeve WG, Vivas-Marfisi A, O'Hara GW. **2005**. Evidence of selection for effective nodulation in the Trifolium spp. saymbiosis with Rhizobium leguminosarum biovar trifolii. *Australian Journal of Experimental Agriculture* **45**: 189.

Yates RJ, Howieson JG, Reeve WG, Brau L, Speijers J, Nandasena K, Real D, Sezmis E, O'Hara GW. **2008**. Host–strain mediated selection for an effective nitrogen-fixing symbiosis between Trifolium spp. and Rhizobium leguminosarum biovar trifolii. *Soil Biology and Biochemistry* **40**: 822–833.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The interaction between legumes and rhizobia is a vital interaction that has shaped natural and agricultural ecosystems and has huge implications for ecosystem function and agricultural sustainability (Peoples *et al.*, 2009; Denison & Kiers, 2011). This interaction can be considered in the framework of a biological market (Noë & Hammerstein, 1995; Schwartz & Hoeksema, 1998). In this simplified market of carbon and nitrogen, the plant must decide how much of its resources to allocate to direct nitrogen uptake and how much to allocate to trade. This balance of resource allocation can be driven by the external abiotic environment, as discussed in Chapters 2 and 3. In Chapter 2, I collaborated with an interdisciplinary group of ecologists and metabolic modelers to explore how nitrogen affects a model of resource trade based on the Nash bargaining solution, a concept adopted from economics (Nash & Jr., 1950; Nash, 1953; Grman *et al.*, 2012). Our data did not support the assumption of symmetric bargaining power of the Nash bargaining solution. Instead, plants have more bargaining power than rhizobia, and, unexpectedly, this differential is more pronounced at low soil nitrogen. Importantly, while plants alter the amount of resources they invest in rhizobia, the "price" of carbon (the C:N trade ratio) does not change.

This result of changing allocation but not return on investment was carried over into Chapter 3. In this chapter, I altered market conditions in two ways by changing both light and nitrogen at the same time. These manipulations should change both the amount of carbon available to the plant to allocate as well as the availability and relative cost of nitrogen uptake

from the soil (Schwartz & Hoeksema, 1998; Kiers & van der Heijden, 2006; Sachs & Simms, 2006). We detected interactions between the effects of light and nitrogen on investment in rhizobia: light generally only altered total investment in rhizobia at low nitrogen when rhizobia were beneficial to the plant. At high nitrogen, plant allocation was very low and tended not to change with light. This chapter highlighted the complicated set of inputs that a plant in the field must integrate to determine optimal allocation to rhizobia and suggested that the effects of one input will depend on the levels of other nutrients. On a technical note, this chapter also illustrated the importance of carefully considering the method in which nodulation is measured. Increasing light had opposite effects on unscaled nodule number and specific nodulation, *i.e.*, nodule number per unit of root biomass. Many studies that report the effects of external nutrients on nodulation report only unscaled nodulation measures. Unscaled nodulation is important since it provides information about the fitness benefits a rhizobium receives from its host (Ratcliff *et al.*, 2012), but may lead to misleading conclusions about plant allocation patterns.

There are a number of future directions that would synthesize the results and approaches of Chapters 2 and 3. Our results in Chapter 3 suggest that nitrogen and light have interactive effects on certain aspects of the legume-rhizobia symbiosis. Parameterizing the model from Chapter 2 with factorial manipulation of nitrogen and light would allow us to explore these interactions in more detail. Increasing light has the opposite effect on investment in rhizobia that increasing soil nitrogen does. Thus, it would be interesting to explore whether light has a similar magnitude and direction of effect on bargaining power as nitrogen does.

It would also be interesting to extend Chapters 2 and 3 to other legume-rhizobia pairs. Research suggests that the response of the legume-rhizobia interaction to nitrogen depends on the specific pairing of the legume and plant even within the same plant species (Heath *et al.*,

2010). Thus, it is vital to test our results in a variety of systems to see how universal they are within the legume-rhizobia symbiosis, and perhaps even in other plant-microbe mutualisms. One option for extension is to compare model results when *M. truncatula* is inoculated with a set of strains that range from uncooperative (fixing very little nitrogen) to highly cooperative (fixing large amounts of nitrogen). If we consider a less effective strain that induces the formation of the same amount of nodule tissue but provides the plant with less growth benefit, that strain presumably has negotiated a higher C:N exchange ratio (i.e., they receive more carbon for every unit of nitrogen). This higher price for nitrogen suggests that the plant may have lower bargaining power in this situation.

There are many possible extensions to this question in other legume-rhizobia systems. Some rhizobia accumulate poly-β-hydroxybutyrate (PHB) (Trainer & Charles, 2006). PHB can be viewed as a way to "hoard" resources from the plant to fuel later rhizobial reproduction instead of being funneled into nitrogen fixation for plant benefit (Ratcliff *et al.*, 2008). This resource hoarding would likely affect the price a plant must pay for each unit of nitrogen, while also setting up a fitness conflict between the plant and rhizobium, which may alter the balance of bargaining power. It would also be interesting to explore C:N trade dynamics in legumes that form determinate nodules. *M. truncatula* forms indeterminate nodules, with persistent meristems and terminally differentiated swollen bacteroids (Masson-Boivin *et al.*, 2009). Other legumes form determinate nodules, with transient meristems and non-terminally differentiated bacteroids that can reproduce after nodule senescence (Masson-Boivin & Sachs, 2018). Indeterminate nodules may be more efficient than determinate nodules, but this is difficult to test since no plant forms both types of nodule (Oono & Denison, 2010). These differences have many important implications for C:N trade dynamics. The reproductive ability of nitrogen fixing bacteroids may

translate into a more direct correlation between plant and rhizobial fitness, while higher efficiency may reduce the C:N ratio.

There are a number of important technical considerations that would be required to carry out these extensions. First, it would be valuable to expand the range of nitrogen and light conditions tested so that they range from the minimum level that supports uninoculated plant survival through the experiment to a growth-saturating maximum. These minimum and maximum levels would vary for each plant species tested. This setup could also be used to test hypotheses about resource availability, trade dynamics, and plant control of their rhizobial partners. For example, you could test whether plants that can generally enforce more favorable C:N trade ratios and/or tend to have higher bargaining power with their rhizobia, and whether nutrient availability affects the strength of partner choice or sanctions, such as in (Grillo *et al.*, 2016).

Returning to the market framework for legume-rhizobia resource trade, in Chapter 4 I manipulated the partners available for trade on the market. Different partners come with different returns on investment and thus different optimal investment in those partners (Noë & Hammerstein, 1994; Werner *et al.*, 2014). This chapter also produced results that challenged our assumptions based in ecological theory. While both the plants and rhizobia were specialists, plants did not receive more benefit from or invest more resources into conspecific rhizobia than heterospecific rhizobia. Benefit from and investment in rhizobia was also not clearly partitioned by isolation host. This suggests that there is some other underlying population structure that we were not able to determine with our knowledge of the isolation host for each rhizobial strain. Sequencing the genomes of the rhizobia involved in this experiment, as well as the hundreds of other strains we have isolated from our field site, will go a long way to address this question.

These results will provide information about the genetic diversity of the rhizobial population at Bodega Bay and will help us assess whether there are patterns in the genome that explain the pattern of benefit and investment we detected in this experiment. We can also examine how rhizobia have co-adapted to their plant hosts by checking for genes in which the phylogeny of the rhizobia track the phylogeny of the plants. This analysis will reveal genes that may be important for the specificity of the interaction.

An important follow-up involves increasing the complexity of the biotic environment in which the interactions occur. The next logical step would be performing pairwise inoculations with a subset of the plant species to test the correlation between nodulation and benefit in single-strain inoculations and in multi-strain inoculations. This would require marking the strains in some way, such as *gusA* and *cellB*, which allows easy visualization of nodule occupancy after staining the root system (Sessitsch *et al.*, 1996). Assessment of nodulation success and rhizobial fitness in more complex inoculations would require DNA barcoding and sequencing to track population sizes. Alternatively, it would be both fascinating and challenging to track rhizobial populations in the soil at the field site and correlate their population levels in space with that of the plants.

A plant participating in the biological carbon:nitrogen market must integrate a large number of signals about resource availability and partner quality to make continuous adjustments to its nitrogen acquisition strategy. This dissertation has focused on exploring these questions at an organismal level, but I am particularly interested in understanding the molecular mechanisms that underlie these trends. This is of course a tremendous task, since the pathways that integrate such a large number of signals to regulate many plant processes are likely to be complex and intertwined. Research on autoregulation of nodulation has been ongoing for decades, but there

are still several pieces of the pathway that we still do not understand (Nishida & Suzaki, 2018). There are some experiments that may provide initial insight into these pathways. Two complementary approaches would include transcriptomics and mutant analysis. Transcriptomics could be used to compare in broad strokes which pathways plants use to downregulate allocation to nodules in response to increasing soil nitrogen, decreasing light, or poor rhizobial cooperation. The specifics of the experiment would be challenging: one would need to compare across many tissues, such as nodule to uninfected root, and effective nodule to ineffective nodule. However, since the shoot is involved in autoregulation of nodulation, it would be informative to assess shoot transcriptomic changes as well. Alternatively, one could test nodulation responses to one input in a mutant deficient in response to a different input, such as testing nodulation responses to light in a mutant deficient in autoregulation of nodulation (Bacanamwo & Harper, 1997). This would provide preliminary evidence about the involvement of known pathways in novel responses.

It is important to note the limitations of the work described in this dissertation. It would be ideal to repeat these experiments to determine the variability in the trends observed. In addition, this work employed a combination of growth chamber and greenhouse studies. Growth chambers and greenhouses are reductionist by nature, since they eliminate stresses from biotic and abiotic sources that plants would face in the field. Including relevant stresses from the field, such as nutrient limitation, pathogen stress, or plant-plant competition could help increase the relevance of these results to field conditions. However, increasing the complexity of the experimental setup will make interpretation of results more difficult.

In conclusion, this dissertation assessed the effects of the abiotic environment and genotype by genotype interactions on resource allocation and trade in the legume-rhizobia

interactions. These projects have helped me understand the value of combining ecological theory and modeling with rigorous empirical testing (Clark, *et al.*, 2017). It has also taught me the value of questioning assumptions and carefully considering the meaning of the data reported. These results provide an excellent first step for continuing to explore the regulation and ecology of the legume-rhizobia symbiosis.

REFERENCES

REFERENCES

Bacanamwo M, Harper JE. **1997**. Response of a hypernodulating soybean mutant to increased photosynthate supply. *Plant Science* **124**: 119–129.

Denison RF, Kiers ET. **2011**. Life histories of symbiotic rhizobia and mycorrhizal fungi. *Current biology : CB* **21**: R775-85.

Clark TJ, Friel CA, Grman E, Shachar-Hill Y, Friesen ML. **2017**. Modelling nutritional mutualisms: challenges and opportunities for data integration (M van Baalen, Ed.). *Ecology Letters* **20**: 1203–1215.

Grillo MA, Stinchcombe JR, Heath KD. **2016**. Nitrogen addition does not influence pre-infection partner choice in the legume-rhizobium symbiosis. *American journal of botany* **103**: 1763–1770.

Grman E, Robinson TMP, Klausmeier CA. **2012**. Ecological specialization and trade affect the outcome of negotiations in mutualism. *The American naturalist* **179**: 567–81.

Heath KD, Stock AJ, Stinchcombe JR. **2010**. Mutualism variation in the nodulation response to nitrate. *Journal of Evolutionary Biology* **23**: 2494–2500.

Kiers ET, van der Heijden MGA. **2006**. Mutualistic stability in the the arbuscular mycorrhizal symbiosis: exploring hypotheses of evolutionary cooperation. *Ecology* **87**: 1627–1636.

Masson-Boivin C, Giraud E, Perret X, Batut J. **2009**. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? *Trends in Microbiology* **17**: 458–466.

Masson-Boivin C, Sachs JL. **2018**. Symbiotic nitrogen fixation by rhizobia — the roots of a success story. *Current Opinion in Plant Biology* **44**.

Nash J. **1953**. Two-Person Cooperative Games. *Econometrica* **21**: 128.

Nash JF, Jr. 1950. The Bargaining Problem. *Econometrica* **18**: 155.

Nishida H, Suzaki T. **2018**. Nitrate-mediated control of root nodule symbiosis. *Current Opinion in Plant Biology* **44**: 129–136.

Noë R, Hammerstein P. **1994**. Biological markets: supply and demand determine the effect of partner choice in cooperation, mutualism and mating. *Behavioral Ecology and Sociobiology* **35**: 1–11.

Noë R, Hammerstein P. **1995**. Biological markets. *Trends in Ecology & Evolution* **10**: 336–339.

Oono R, Denison RF. **2010**. Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant physiology* **154**: 1541–8.

Peoples MB, Brockwell J, Herridge DF, Rochester IJ, Alves BJR, Urquiaga S, Boddey RM, Dakora FD, Bhattarai S, Maskey SL, *et al.* 2009. The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis* **48**: 1–17.

Ratcliff WC, Kadam S V., Denison RF. **2008**. Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. *FEMS Microbiology Ecology* **65**: 391–399.

Ratcliff WC, Underbakke K, Denison RF. **2012**. Measuring the fitness of symbiotic rhizobia. *Symbiosis* **55**: 85–90.

Sachs JL, Simms EL. **2006**. Pathways to mutualism breakdown. *Trends in Ecology & Evolution* **21**: 585–592.

Schwartz MW, Hoeksema JD. **1998**. Specialization and resource trade: Biological markets as a model of mutualisms. *Ecology* **79**: 1029–1038.

Sessitsch A, Wilson K, Akkermans A, de Vos W. **1996**. Simultaneous detection of different Rhizobium strains marked with either the Escherichia coli gusA gene or the Pyrococcus furiosus celB gene. *Appl. Envir. Microbiol.* **62**: 4191–4194.

Trainer MA, Charles TC. **2006**. The role of PHB metabolism in the symbiosis of rhizobia with legumes. *Applied Microbiology and Biotechnology* **71**: 377–386.

Werner GDA, Strassmann JE, Ivens ABF, Engelmoer DJP, Verbruggen E, Queller DC, Noë R, Johnson NC, Hammerstein P, Kiers ET. **2014**. Evolution of microbial markets. *Proceedings of the National Academy of Sciences of the United States of America* **111**: 1237–44.