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# Hosts, Symbionts, and Soils: Contextual Drivers of the Cowpea-Rhizobium Symbiosis

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by

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The co-author Joel Sachs listed in that publication directed and supervised the research which forms the basis for this dissertation.

For this publication, co-authors include Oscar G. Mercado, Raphiel X. Camantigue, Fizzah Khairi, Sierra Neal, Warisha F. Farsamin, and Matthew T. Lampe who performed the experiment, Oscar G. Mercado, Raphiel X. Camantigue, Teresa Nguyen, Warisha F. Farsamin, Matthew T. Lampe, Ivan A. Perez, Jacob Rothschild, Tram H. Le, and Gabriel S. Ortiz-Barbosa who collected the data, and Lorena Torres- Martínez, and Joel L. Sachs who analyzed the data and wrote the manuscript.

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# DEDICATION

I dedicate this dissertation to my wife, Brie

for late nights at the greenhouse,

for long drives to the field,

for growing and changing with me,

and for the infinite joy of these last 10 years.

Brie, you're the co-author of my dreams.

Next, to my wonderful mom and dad,

who always knew I would be a scientist,

who immersed me in science from a young age,

and who championed me every step of the way.

I can never thank you enough for your support.

Last, to the educators in my life

whose passion made science come alive:

Mr. Meese, Mr. Lynch, Mr. McFarland, Mr. Diaz,

Dr. Breakwell, Dr. Hope, Dr. Griffitts,

And, of course, Joel.

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ABSTRACT OF THE DISSERTATION

Hosts, Symbionts, and Soils: Contextual Drivers of the Cowpea-Rhizobium Symbiosis

by

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University of California, Riverside, June 2023
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Plant-associated microbes can provide substantial benefits to crops, improving yields and reducing necessary nutrient inputs. The benefits of plant-microbial symbioses are highly variable and rely on several contextual drivers, including host symbiosis traits, makeup of available soil microbes, and environmental conditions such as soil nutrient levels and field management practices. Legume species receive fixed nitrogen from symbiosis with rhizobia, soil bacteria which can infect roots. However, many legume species still require nitrogen fertilization, suggesting breakdowns in the forces directing this symbiosis.

To examine these forces, we turned to cowpeas (*Vigna unguiculata*)—a legume crop grown across Africa, Asia, and the Americas—and tested the effects of host genotype, microbial community, and soil conditions on plant growth benefits. In Chapter 1, we performed a full factorial soil inoculation experiment on twenty wild and domesticated

cowpea genotypes to test the effects of cowpea domestication, host genotype, and soil microbial community on cowpea growth benefits in a greenhouse setting. In Chapter 2, we tested whether cowpea-associating rhizobial communities are structured spatially or by host genotype in a field that had a long history of cowpea growth and was demonstrated to have a highly beneficial soil microbial community. In Chapter 3, we planted a different subset of nineteen cowpea genotypes in the same field and an adjacent field with no history of cowpea growth to test whether seed coat inoculants could improve growth in either field.

We found strong evidence that cowpea host benefits are primarily shaped by soils, which drove most of the observed variation in both nodulation and host growth. We found that cowpea host genotype has very little effect on structuring variation in host benefits from inoculation, with no significant variation in nodule rhizobial communities across host genotype. UCR Field 11 is enriched with *Bradyrhizobium* and dominated by a small handful of strains, with soil that offers high benefits to plants under greenhouse conditions. However, neither soil nor strains from this field were effective inoculants under field conditions, and the commercial inoculant tested also did not perform under field conditions.

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# INTRODUCTION

Plant-associated microbes are abundant, diverse, and can offer a wide range of benefits to plant hosts. Microbial mutualists can improve nutrient availability, expand the ecological niche of their hosts, protect hosts from pathogens, and enhance stress tolerance. In crops, this plant-microbial symbiosis can reduce reliance on fertilizers, expand the regions where a crop can be grown successfully, and improve crop yields. However, the benefits of soil microbes on crops are tenuous, context-dependent, and vary widely among strains and communities. Understanding the factors which shape this association and its benefits could allow growers to better leverage soil microbes and produce more crops in diverse locations with fewer costly nutrient inputs.

Cowpea (*Vigna unguiculata*) is a crop legume which associates with several species of *Bradyrhizobium* bacteria. Cowpea boast a high proportion of edible plant mass, require minimal nutrient inputs, are broadly drought and heat tolerant, and are grown throughout Africa, Asia, and the Americas. As with other legume-rhizobium symbioses, *Bradyrhizobium* can infect plant roots and multiply within nodules where they can provide fixed nitrogen to the host. Cowpea have some capacity to restrict infection and nutrient access to undesirable strains, referred to as host control. Rates of infection and nitrogen fixation vary widely among *Bradyrhizobium* strains, as do the growth benefits received by cowpea hosts. These growth benefits might also be contingent on host and environmental factors, including variation in host control among cowpea genotypes, as well as localized soil and environmental factors.

In testing the possible factors which might influence *Bradyrhizobium* infection and resulting cowpea host benefits, we found that soils were the predominant driver of variation, while host genotype played a lesser role. Soils from cowpea fields across southern and central California provided a striking range of growth benefits to cowpea; host genotype also played a significant role, though differences between individual genotypes were minimal. The growth differences attributed to soil type were due to differences in the soil microbiota and were also indirectly tied to soil nutrient profile. In another study, we found no evidence of spatial or host structuring of *Bradyrhizobium* communities in field cowpea nodules. Instead, we found that an epidemic lineage dominated the field, regardless of host genotype or field quadrat. In a third study, we inoculated cowpea seeds with several microbial formulations, and found that none of them provided growth benefits in either field where cowpeas were grown. Instead, we found that growth differences were strongly shaped by field effects.

# CHAPTER 1

**Title:** Live soil inocula, not host population or domestication status, is the predominant driver of growth benefits to cowpea

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**Abstract**

Crops rely on microbes for critical services, but host benefits can be influenced by local makeup of microbiota and the host's capacity to select optimal strains. We investigated host benefits that cowpeas receive from microbiota depending on plant genotype, their domestication status, and soil source.

We performed a full factorial soil inoculation experiment. Twenty diverse cowpea genotypes, selected from wild and domesticated populations, were exposed to soil rinsates from four agricultural sites across California, all having cowpea cultivation and varied physicochemical features. Cowpea investment in and benefit from microbiota was quantified by measuring host growth response to inoculation, nodulation, and segregating trait variation.

Variation in induction of root nodulation and strikingly heterogenous benefits to host growth were observed among soil sites. These effects were restricted to live soil inocula but were absent in autoclaved soil controls that lacked microbiota. Cowpeas expressed heritable variation in nodulation, but there was negligible effect of plant population or domestication status on the net benefit that hosts gained from microbiota.

Soils varied substantially and consistently among cultivation sites and were the most prominent driver shaping host growth effects on cowpeas. While growth benefits vary among host cultivars, soil microbiota (and the conditions that maintain them) predominantly shape plant performance in agricultural settings.

## Introduction

Plant-associated microbial mutualists are abundant, exceptionally diverse, and provide varied services to hosts (Friesen et al. 2011). However, the taxonomic makeup of microbial communities – and consequently the benefits they provide – can vary a great deal over space and time (Heath & Stinchcombe 2014). The drivers that shape soil microbiota can be broadly categorized as top down and bottom up forces. Top down forces are driven by symbiosis traits, host phenotypes that regulate the colonization and infection of associated microbes (Bulgarelli et al. 2013; Porter and Sachs 2020). Symbiosis traits are predicted to play a significant role in shaping symbiont communities (Foster et al. 2017). For instance, plants can release specific flavonoids and other compounds from roots to attract and regulate the growth of microbial partners (Sasse et al. 2018; van Dam and Bouwmeester 2016). Plant exudates can reshape the associated microbial community by enriching or reducing specific microbial taxa on plant roots and in the rhizosphere, and parallel processes occur on leaves (Balachandar et al. 2006; Micallef et al. 2009; Morella et al. 2020). Plants are also thought to impose selection by restricting infection to a subset of microbial strains, and by selectively rewarding or punishing strains post-infection depending on the benefits that they provide (Denison 2000; West et al. 2002). However, symbiosis traits can vary substantially among plant species and even among host genotypes or populations of the same species (Haney et al. 2015; Pahua et al. 2018; Torres-Martínez et al. 2021; Wendlandt et al. 2019), potentially mitigating microbial benefits on plant health, yield, and fitness (Lareen et al. 2016; Mueller and Sachs 2015).

Bottom up forces shape the community makeup of microbes during free-living phases in soil, including abiotic factors such as soil pH, particle size, water availability, nutrient composition, and biotic factors such as microbial predators, competitors, and facilitators (Agler et al. 2016; Bonkowski et al. 2004; Fitzpatrick et al. 2019; Hussain et al. 2018; Leite et al. 2017; Li et al. 2019). In natural settings, soil texture and nutrient availability are primary factors that alter the composition and abundance of bacterial communities (Xu et al. 2018). In managed settings, tillage and fertilization impact soil abiotic factors, affecting species richness and evenness in soil microbial communities (He et al. 2007; Legrand et al. 2018; Zhong et al. 2010). These environmental factors can interact with host selection to drive variation in plant-associated microbial communities (G x E interactions; Peiffer et al. 2013; Wagner et al. 2016). Additionally, the expression of genetic variation for symbiosis traits among related host genotypes can vary with environmental inputs (Batstone et al. 2020; Wood and Brodie III 2016). Moreover, symbiosis traits can be degraded in agricultural settings, as domesticated plants often gain less fitness benefits from microbiota than their wild relatives (Porter and Sachs 2020). Staple crops with evidence of reduced benefits from microbiota include soybean, maize, potatoes, wheat, and rice (Bouffaud et al. 2012; Engelhard et al. 2000; Hetrick et al. 2011; Kiers et al. 2007; Zhu et al. 2001). Degradation of symbiosis can be due to artificial selection of above-ground plant traits that tradeoff with belowground symbiosis functions, relaxed selection on belowground traits in rich agricultural settings, or demographic changes in crop plants such as inbreeding or founder effects (Denison 2015; Porter and Sachs 2020). A key aspect of domestication is the movement of plant genotypes to new

regions (Gaut et al. 2018), introducing plants to novel soil characteristics and belowground communities which can directly impact host benefits from symbiosis and the expression of symbiosis traits. Examining the relative effects and interplay between host-mediated and environmental forces on soil microbiota and the expression of host symbiosis traits is critical to predicting soil health and plant fitness in natural and agronomic settings.

Plants in the legume family (Fabaceae) associate with rhizobia, proteobacteria that trigger formation of symbiotic root nodules and fix nitrogen (Kakraliya et al. 2018; Sawada et al. 2003), and other rhizosphere associated bacteria that can provide metabolite solubilization, phytostimulation, and other services (Rascovan et al. 2016). Rhizobia can provide substantial amounts of fixed nitrogen, such that host plants can thrive with little or no added nitrogen in the soil (Regus et al. 2017). Individual rhizobia strains, both in natural and agricultural soils, vary tremendously in their effects on hosts, ranging from highly beneficial strains to ones that are ineffective for nitrogen fixation (Gano-Cohen et al. 2020; Moawad and Beck 1991; Thrall et al. 2000). Legumes exert host control by selecting genetically compatible rhizobia and by sanctioning less beneficial strains (Kiers et al. 2003; Heath and Tiffin 2009; Oono et al. 2011; Sachs et al. 2010). These symbiosis traits can vary among legume populations (Heath and Tiffin 2009; Wendlandt et al. 2019). Furthermore, variation in expression of symbiosis traits (such as nodulation) among legume genotypes can be influenced by environmental factors, such as planting location and light availability (Batstone et al. 2020; Heath et al. 2020).

Cowpea (*Vigna unguiculata*) is a genetically diverse legume with cultivars that require minimal nutrient inputs, offer a high proportion of edible plant mass, and are ideal

for regions with limited economic or agricultural resources (Herniter et al. 2020; Muñoz-Amatriaín et al. 2017). Wild cowpeas (subsp. *dekindtiana*) are distributed throughout Africa and are the progenitors of cultivated cowpea varieties (Ali et al. 2015; Coulibaly et al. 2002). Early domesticated cowpeas, known as landraces, are comprised of two distinct populations, Genepool-1 and Genepool-2 (FST = 0.18), distributed across separate regions in northern and southern Africa, respectively, and each of which is diverged from wild cowpeas (FST = 0.13; Ortiz-Barbosa et al. 2022). The patterns suggest that divergent subsets of wild cowpeas were transported and bred in northern and southern regions of Africa during waves of human migration, with only modest gene flow between them, indicating separate domestication events (Huynh et al. 2013; Muñoz-Amatriaín et al. 2017). Both populations of landraces share a suite of improved traits, including large seeds, shatter-resistant pods, and flexible flowering time (Lo et al. 2018; Xiong et al. 2016). Cowpea landraces are grown under simple agricultural conditions and have not been expanded or adapted to new regions, consistent with an early stage of crop domestication (i.e., stage two of four proposed stages; Gaut et al. 2018).

Here, we examined the roles of plant genotype and soil source in shaping the expression of cowpea host symbiosis traits in response to soil microbiota. We conducted a full factorial soil inoculation experiment where the effects of cowpea host population, genotype, and soil inoculum source were simultaneously analyzed. We used eight wild cowpea genotypes and twelve early-domesticated landraces to examine the role of host genotype – and effects of host domestication – on the expression of host performance and symbiosis traits. We selected cowpea from different populations to account for known

variation in symbiosis traits among legume populations and to examine the effects of separate domestication events among the two landrace populations (Ortiz-Barbosa et al. 2022). Plants were inoculated with soil rinsates generated from four agricultural field sites distributed across a 460 km transect in California, having current or recent cultivation with cowpea. We quantified aboveground plant biomass and root-nodulation patterns to estimate host growth response to inoculation. Additionally, we tested whether the differences in the soil sources could influence the expression of segregating variation in symbiosis traits by quantifying soil physicochemical properties and estimating additive genetic variances and heritability among cowpea genotypes. The goals were to i) evaluate the roles of cowpea host genotype and soil source in mediating the expression of plant symbiosis traits, ii) examine whether domestication has influenced plant investment into and benefits from symbiosis when exposed to diverse soil sources, and iii) quantify heritable variation in symbiosis traits and test whether association with diverse soil microbial sources can shape this expression.

## Materials and Methods

**Cowpea genotypes:** Eight wild cowpea accessions (i.e., genotypes) were sampled from natural populations in Botswana (PI 632890), Zimbabwe (PI 632891), Tanzania (PI 632876, PI 632892), and Niger (PI 632882, PI 632879, PI 632880, PI 632881). Twelve cowpea landraces were selected from populations in northern and southern Africa. For the northern population, genotypes were sampled from Egypt (TVu-9492), Senegal (TVu-14346), Benin (TVu-8834), Niger (TVu-15591, TVu-14971), and Nigeria (TVu-3804), and southern population genotypes were sampled from Mozambique (NamuesseD,

Nhacoongo-3, Muinana-Lawe), Tanzania (TVu-1280), Malawi (TVu-9848), and Zambia (TVu-13305) (Huynh et al. 2013). The African cowpea genotypes are photoperiod sensitive and do not flower or set seed under the summer conditions tested herein. Under shorter day lengths, these lines take about 40 days to flower and 70 days to form pods. Landraces were chosen to maximize genetic diversity and were only selected from germplasm collections made before 1975, after which African breeding programs began transferring cowpea germplasm, leading to admixture among genotypes (Huynh et al. 2013; Ortiz-Barbosa et al. 2022). Accessions were previously genotyped using an Ilumina iSelect Consortium array developed for cowpea, which targets more than 50,000 single-nucleotide polymorphisms. (Muñoz-Amatriaín et al. 2017; Ortiz-Barbosa et al. 2022). Seeds were obtained from the USDA germplasm collection (Griffin, GA).

**Soil inocula preparation:** Soil sampling locations were selected from fields based on history of agricultural management and sampling accessibility, including at the Coachella Valley Agricultural Research Station in Thermal, CA, the University of California Riverside Agricultural Experiment Station, the Kearney Agricultural Research and Extension Center in Parlier, and a commercial cowpea grower's field near Shafter, CA (Fig. 1.1, Table S1.1).

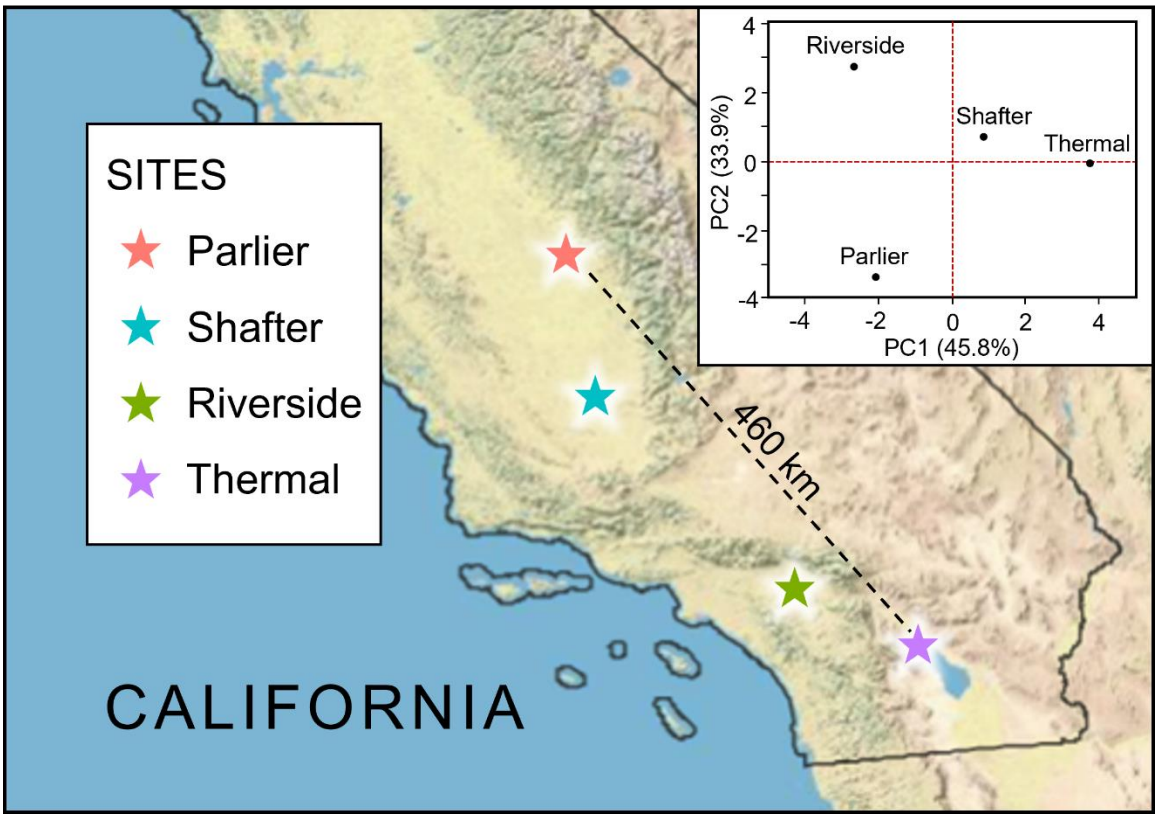


**Fig. 1.1** Sampling sites for soils, including a principal components analysis of soil nutrient composition and texture at each site. The x-axis indicates PC1, which explained 45.8% of the soil variation. The y-axis indicates PC2, which explained 33.9% of the variation. Site names, collection dates, sampling coordinates, and crop history from each of the four sites are listed in Table S1.1.

The Thermal, Riverside, and Parlier sites were fallow during sampling and had not been recently irrigated or fertilized, though they did receive low levels of fertilization during prior growth seasons. The grower's field in Shafter was unique in that it had growing cowpeas at the time of sampling, was recently fertilized, and cowpea had been inoculated via a peat-based seed-coat inoculant prior to planting (Exceed Peat for Cowpea/Lespedeza/Mung Bean, product #: 2013; Visjon Biologics). The conditions of fertilization and seed inoculation at the Shafter site are typical of the current cowpea

agricultural process in California (Long et al. 2010). Parlier & Shafter sites were sampled on 6/17/19, Thermal was sampled on 6/21/19, and Riverside was sampled on 6/29/19.

Approximately six liters of topsoil were sampled from four randomized sampling plots at each field site. Soil samples were pooled by field site, sieved, mixed with an equal portion of sterile water, filtered through cheesecloth, left to settle overnight, and the supernatant from each flask was removed (i.e., top ~50%) and divided into five portions. This protocol enables plants to be inoculated with dominant microbiota, while minimizing addition of nutrients that could change the soil makeup (Unkovich and Pate 1998). Three portions were reserved at room temperature to be used as a 'live' inoculum, while the rest were autoclaved and allowed to cool to serve as a dead control. The next day, seedlings were inoculated with 10 ml of the appropriate inoculum. Live and dead inocula from each site were separately spread inoculated (100 µl) onto plates with a modified arabinose gluconate medium (MAG; Sachs et al. 2009) and incubated at 29$^{o}$C for eight days to confirm the presence of soil microbiota in live inocula and likewise confirm the sterility of dead inocula. Live inocula from all four source soils formed dense lawns on the MAG plates, whereas control dead inocula did not generate any colonies. Soil inocula were prepared at two time points from the same sampled soils to account for variation in germination speed among the diverse cowpea genotypes (7/6/19, 8/3/19).

**Soil analysis:** Soil samples collected at each site in February 2021 were analyzed for organic matter, nitrogen, phosphorus (weak Bray and sodium bicarbonate-P), pH, extractable cations (potassium, magnesium, calcium, sodium), hydrogen, sulfate-S, cation exchange capacity, percent cation saturation, and soil texture (A&L Western Labs,

Modesto, CA). A portion of the original soils from 2019 were also analyzed for nitrate nitrogen as a comparison. Principal components analysis (PCA) of quantitative soil measures was performed to reduce dimensionality. Data on soil composition, available water storage, drainage, and proportion of hydric soils were extracted via geolocation from the UC Davis California Soil Research Lab.

**Pot and seed preparation:** One-gallon nursery pots were filled with wetted soil and autoclaved twice (50:50 silica sand mix of #12 and #30 size). Seeds were surface sterilized in a 6% sodium hypochlorite solution and vortexed intermittently for 3 minutes, then rinsed four to six times with sterile water, nick-scarified, and planted the same day. Wild cowpea genotypes were planted on 6/12/19 and landraces were planted on 6/19/19 to account for germination timing and growth. Seeds were planted in triplicate per pot and extra seedlings were later removed or redistributed to pots lacking visible growth. Each treatment by genotype combination had 5 live inoculation replicates and 3 controls that received the dead inoculum. These replicates were divided across 8 blocks in the greenhouse, each containing a random arrangement of all treatment combinations (20 plant genotypes x 4 soil sources = 80 plants per block). Controls for each treatment combination were randomly assigned among the eight blocks, with each block containing a mix of live and control-inoculated plants to reduce confounding block effects. Beginning the first week of July, plants with true leaves were fertilized twice weekly with 10mL of sterilized Jensen's solution, which contains micronutrients and was supplemented with a minimal concentration of nitrogen to allow for cowpea survival under symbiont free conditions (0.4 g/L of $KNO_3$; Somasegaran and Hoben 1994). Germination was unexpectedly slow for the

wild cowpeas, and five additional seeds were planted in pots without visible seedlings on 6/25/19. Prior to inoculation, pots with visible seedlings were rearranged with unsuccessful pots from blocks 1-3 to complete as many blocks as possible. Inoculation of germinated plants (including all landraces and roughly half of the wild plants) took place on 7/7/19. By 7/15/19, nearly all previously planted wild seeds had germinated. These late-germinated plants were then inoculated on 8/4/19. Plants with true leaves were treated with 10 ml of inoculum, directly onto the soil. The greenhouse received weekly pesticide treatments.

**Measurement of plant and symbiosis traits:** Harvest of plants occurred block by block starting on 8/19/19 and ending on 10/26/19 (Table S1.2) to account for time necessary to dissect and process plants. Plants which had germinated earlier and received the first round of inoculation were harvested first to minimize variation in growth period. Plants were de-potted, true leaves were counted, and roots were rinsed of soil. Nodules were dissected, counted, photographed, and dried in an oven at 60$^{o}$C to weigh biomass. If available, up to ten nodules per plant were set aside prior to drying, surface sterilized, and stored at -80$^{o}$C for a separate genotyping study. Roots and shoots were separated and dried in an oven at 60$^{o}$C to weigh biomass. To account for the weight of nodules set aside for culturing, fifty nodules of varied size (i.e., nodule radius) were photographed, dried, and weighed individually to generate an area-by-weight curve: estimated nodule mass = 0.00602 + (0.000135 x nodule volume). This curve was used to estimate nodule biomass for plants with 20 or fewer total nodules to reduce potential bias from extrapolation of biomass from low nodule counts. For plants with greater than 20 total nodules, total nodule

biomass was estimated by extrapolating from the initial biomass to account for nodules that were set aside for genotyping and were not weighed.

Traits were quantified, including the number of nodules formed, total nodule biomass, mean individual biomass of nodules, total plant biomass, and host growth response. Host growth response was calculated by dividing the total dry biomass of each inoculated plant by the mean dry biomass of the dead inoculum controls of the same genotype. The resulting ratio reflects the effects of inoculated microbiota on plant growth, separate from growth effects due to other soil features (i.e., nutrient variation). This calculation also controls for variation in plant size among cultivars, indicating that genotype or population-level effects in our models are due to variation in response to inoculation, rather than natural size differences (Sachs et al. 2010; Regus et al. 2015; Ortiz-Barbosa et al. 2022).

Linear mixed models were implemented to test whether the trait response varied among soil treatments, among wild and domesticated cowpea populations, and whether differences between wild and domesticated populations depended on the soil treatment while accounting for the cowpea genotypic effects. Soil treatment, cowpea population, and their interaction were treated as fixed factors, and cowpea genotype as a random factor. Days post inoculation was added as a covariate to account for the variation attributed to the different harvest time points. Models with block as a random factor indicated that block was not significant, so it was excluded. For all analyses, host growth and mean nodule biomass were log-transformed, and the number of nodules was square root transformed to meet the assumptions of normality and heteroscedasticity. Tukey's post-hoc tests were

conducted to test for differences among soil treatments and cowpea populations. A variance partitioning test, which assesses the proportionate variation explained by two or more variables, was performed to compare the relative influence of host genotype and soil treatment on host growth response using the publicly-available POV Engine JSL script for JMP, developed by Thomas A. Little Consulting (TLC), 2022. All analyses were performed in JMP® Pro, Version 15.0.0. SAS Institute Inc., Cary, NC, 1989–2022.

**Expression of trait genetic variation and heritability*:*** Genetic variation was assessed for symbiosis traits by examining the significance of the random factor with a log-likelihood ratio test between a null model that excluded the genotypic factor and the main model described above. We tested whether the genetic variance component varied significantly among soil inoculum treatments by comparing models with different variance-covariance structures (Shaw 1991; Saxton 2004; Torres-Martínez et al. 2019). A model where the genotype variance component was allowed to vary among soil treatments (heterogeneous variance model) was compared to a model where the genotype variance component was constrained to be identical across soil treatments (homogeneous variance model; Table S1.3). To evaluate whether a genotype-by-environment (G x E) interaction was observed, we also compared a model where no G x E is assumed with a model where G x E is present (Table S1.3).

Broad and narrow sense heritability were estimated for traits where a significant genotypic variation was observed. A soil treatment-specific heritability was estimated when the expression of trait genetic variation varied among soil treatments. To better visualize changes in genetic variance, we estimated breeding values of each cowpea

genotype under each soil inoculum with Best Linear Unbiased Predictions (BLUPS; Henderson 1975; Liu et al. 2008). BLUPs were calculated from the model that best fit the variation for each trait (Tables S1.3 and S1.4). Genetic variation estimates were calculated using the R package sommer (Covarrubias-Pazaran 2016).

**Results**

**Soil physicochemical features:** The four soil sources varied in physical and chemical features. Soil textural analysis revealed that all soils were predominantly sandy (i.e., particles 50-2000 µm in diameter), but that the Thermal soil had the highest sand proportion (78%, compared to an average of 54% for the remaining sites; Table S1.5). The first principal component (PC1) of the quantitative soil analysis explained 45.8% of the variation in physicochemical properties (Fig. 1.1, Table S1.5). PC1 was mainly driven by variation in the proportion of silt and sand particles, available phosphorous, and salinity (Table S1.5). Parlier and Riverside field sites were classified by the Hanford soil series, with Shafter and Thermal sites classified by the Lewkalb & Myoma series, respectively (Table S1.1). Both Hanford and Lewkalb soils are characterized as deep, well-drained, coarse-loamy, and mixed, while Lewkalb soils are also calcareous (Soil Survey Staff, USDA). Myoma soils are characterized by fine, moderately alkaline sands which are somewhat excessively drained (Soil Survey Staff, USDA).

**Total plant biomass:** Live and dead soil treatments resulted in significant differences in plant biomass, as did the soil source and the interaction effect (live/dead x soil source; Table S1.6). Cowpea host population and genotype also had significant effects on plant biomass, indicating natural size variation among genotypes not due to inoculation.

In all models for total plant biomass, days post inoculation was a significant factor and was therefore included as a covariate in analysis (Tables S1.6 & S1.7).

**Host growth response:** Live soil treatments had significant positive effects on host growth, with significant variation among soil sources that ranged 2x-3x in magnitude (Fig. 1.2a). In contrast, treatment with sterilized dead soils did not produce any significant differences in total plant biomass among soil treatments (Table S1.7). While total plant biomass did vary significantly among cowpea populations (Table S1.7), indicating natural differences in plant size, host growth response to inoculation did not vary significantly among cowpea populations, and mean host growth response values by population did not vary by soil treatment (no Population X Treatment interaction effect, Table 1.1). Host genotype had a significant effect on host growth response, but these differences were modest, and most genotypes (16/20) were not significantly different from one another (Table 1.1; Fig. 1.3). A partition of variance (POV) test indicated that differences among soil treatments explained almost twice the variance in host growth response (25.98%) compared to differences among host genotypes (14.94%; Table 1.2). While host genotype and soil source were both significant factors in our model of host growth response, these data indicate that variation in soil treatment (rather than host domestication or provenance) was the prominent factor mediating host benefits. Days post inoculation was also a significant factor and was included as a covariate during analysis (Table 1.1).

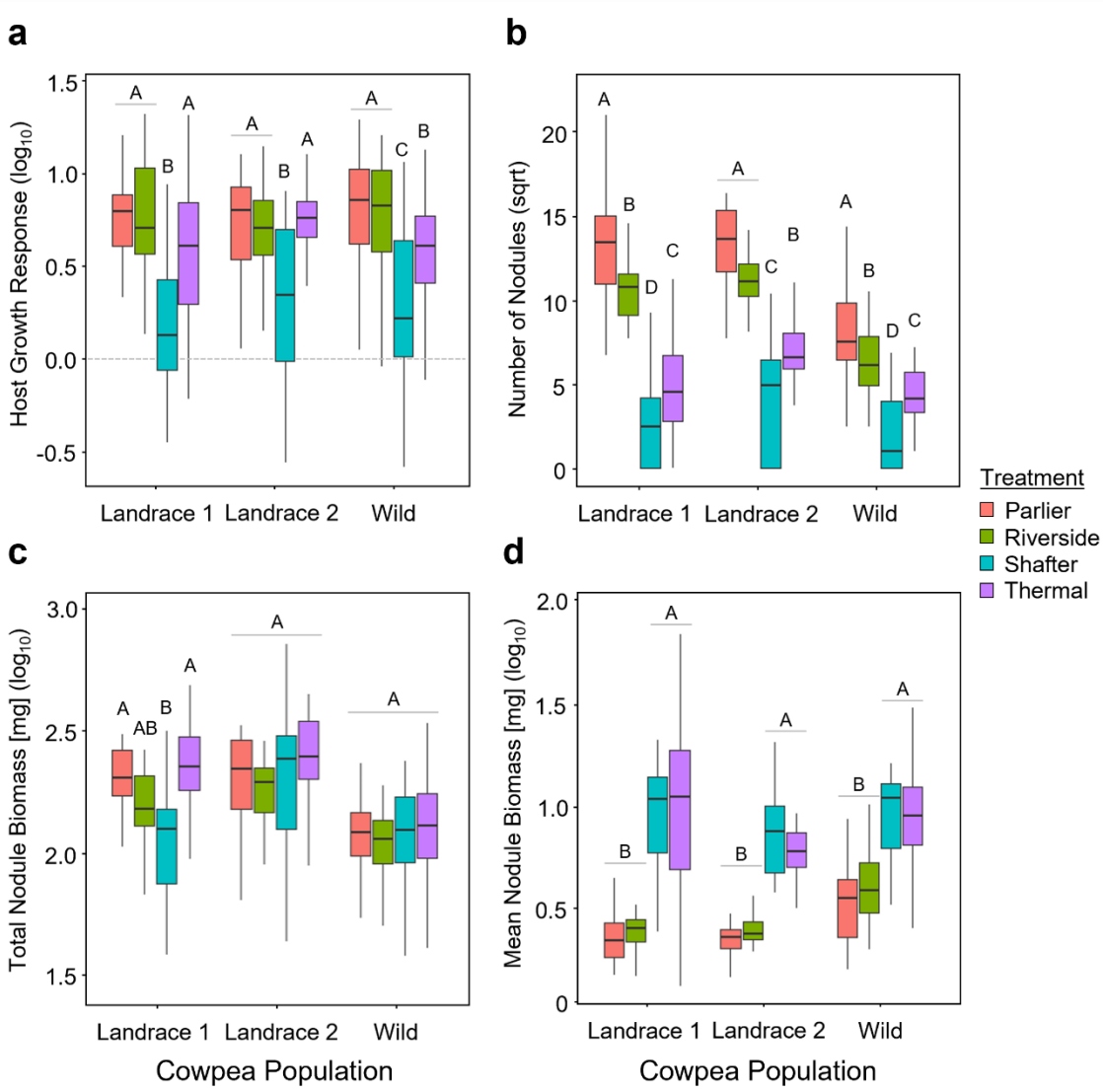


**Fig. 1.2** Variation in symbiosis traits among populations. Boxplots of (a) Host growth response, (b) Number of nodules, (c) Total nodule biomass, and (d) Mean nodule biomass in response to inoculation from 4 distinct sites and across three populations of African cowpea (two landrace populations and one wild population). Treatments are denoted by color (pink = Parlier, green = Riverside, blue = Shafter, purple = Thermal). Connected letters represent Tukey groupings from linear mixed models, calculated within each lineage. For both Total nodule biomass and Mean nodule biomass, plants without nodules were excluded. Host growth and mean nodule biomass were log-transformed, and the number of nodules was squared root transformed. Outliers are hidden for visual simplicity.

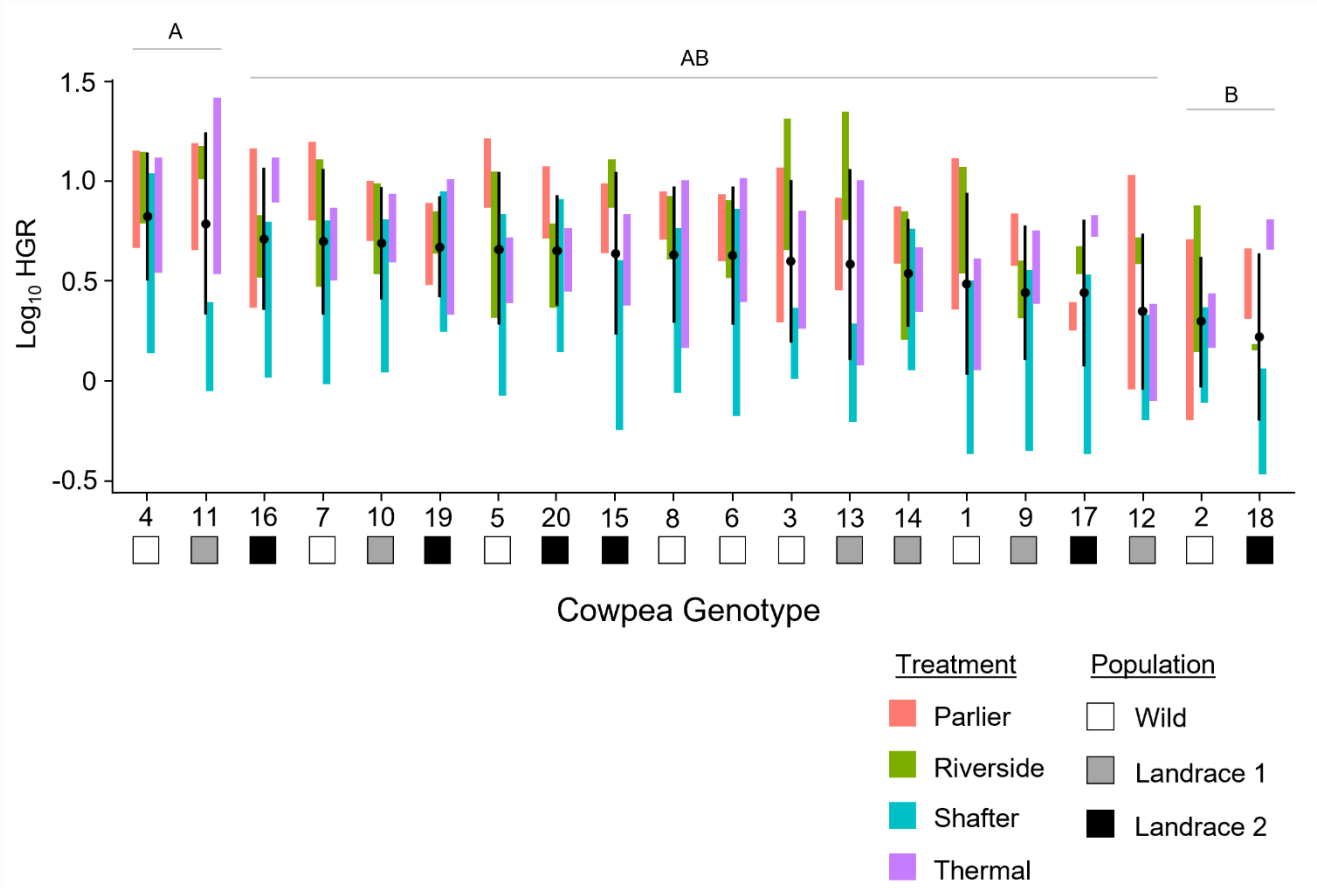


**Fig. 1.3** Variation in host growth response (HGR) among host genotypes. Grouped interval plot indicates standard deviation in response to inoculation from soil treatments, denoted by color (pink = Parlier, green = Riverside, blue = Shafter, purple = Thermal) across twenty cowpea lines, ordered from highest to lowest mean HGR. Connected letters show Tukey groupings. Global mean HGR and standard deviation of each host genotype is shown in black. Boxes below each host genotype denote the host population (white = wild, grey = landrace population 1, black = landrace population 2), showing that host populations are highly intermixed when genotypes are ranked by HGR

**Number of nodules:** Each soil treatment resulted in significantly different nodule counts (Table S1.9). Despite the similar appearance of treatment ranking among populations with regard to nodulation (Fig. 1.2b), there was a significant population X treatment effect (Table 1.1). The Shafter soil inoculation induced nodules in only 59 of 98 plants (~60%). In contrast, the Thermal soil induced nodulation in 98% of plants, and Parlier and Riverside soils had 100% nodulation. Within the Parlier and Riverside inoculum treatment groups, both landrace populations formed significantly more nodules than the wild population, while within the Thermal and Shafter treatment groups, landrace population 2 formed significantly more nodules than either of the other populations (Table S1.9).

Twelve of 239 control plants had nodules (~5%), indicating contamination, and were excluded from analysis (Table S1.2). Nine of the contaminated plants had 8 or fewer nodules, whereas the mean nodule count for an inoculated plant was 66. We were unable to detect potential cross-contamination by other microbiota. Additionally, 28 plants had lost over 50% of their leaves, indicating senescence likely due to stress from late-harvest pest control spray treatments, or had mature seed pods, indicating senescence due to shorter day lengths. These plants were also excluded from analysis. Among these senesced plants, 16 individuals (64%) belonged to two host genotypes, TVu-1280 and TVu-9848, both from landrace population 2. One individual was incorrectly harvested at 22 days post inoculation. For all remaining plants, days post inoculation ranged from 42 days to 105 days. The majority of plants were harvested within two weeks of the mean days post inoculation (66 days, 52% of plants).

**Nodule biomass**: Host population, soil source, and their interaction all had significant effects on total and mean individual nodule biomass (Table 1.1). Nodules from landrace population 1 were the largest, and population 2 were the smallest nodules (Tables 1.2 & 1.3). Shafter and Thermal soils induced significantly larger nodules on average than either of the other treatment groups, despite their association with lower levels of host growth (Table S1.9, Fig. 1.2d). Both cowpea landrace populations had a higher total nodule biomass than the wild population (49% and 77% higher, respectively), which was consistent across most treatments (Table S1.9). We found no significant differences in total nodule biomass among soil treatments within landrace population 2 or the wild population (Fig. 1.2c). With total plant biomass as a covariate, the wild population still had a lower total nodule biomass than either landrace 1 or 2 populations ($T_{325} = 3.07$, $p < 0.01$; $T_{325} = 5.06$, $p < 0.01$). These data suggest that wild cowpeas had a proportionally lower investment into nodule tissues. Soil from Thermal induced the highest total nodule biomass (mean = 202.72 mg, Table S1.8), which was significantly higher than both the Shafter and Riverside soils. Days post inoculation had a significant effect on total nodule biomass and was included as a covariate; however, days post inoculation was not a significant factor in our model for mean nodule biomass.

**Effects of soil characteristics on symbiosis traits:** In a linear mixed model with PC1 (from the quantitative soil analysis), host population, and their interaction as fixed effects and host genotype as a random effect, we found that PC1 had a significant effect on host growth response, number of nodules, and mean nodule biomass (Table 1.3). We also found a significant host population x PC1 interaction effect for both number of nodules

and mean nodule biomass (Table 1.3). Conversely, we found no significant differences among autoclaved inoculum treatments. These data suggest that growth differences among soil treatments are driven primarily by variation in microbial community, which is modulated by the soil physicochemical characteristics (Table S1.7). For all traits except for number of nodules, days post inoculation was a significant factor, and was included as a covariate (Table 1.3).

**Cowpea genetic variation and heritability**: The expression of genetic variation ($\sigma^2_G$) for the number of nodules and total nodule biomass varied with the soil inoculum imposed, respectively ($\chi^2_5 = 21.85$, $p < 0.01$; $\chi^2_5 = 14.20$, $p = 0.01$), but host growth response and mean nodule biomass did not ($\chi^2_5 = 2.3$, $p = 0.80$; $\chi^2_5 = 10.6$, $p = 0.06$; Fig. 1.4; Table S1.3), consistent with soil rather than plant genotype being the prominent driver shaping host growth effects on cowpeas, despite both host genotype and soil affecting nodulation patterns. The highest expression of $\sigma^2_G$ for the number of nodules was observed within the soil inoculum from Parlier, and the lowest $\sigma^2_G$ was observed within the soil inoculum from Thermal, further indicating the highest and lowest heritability for this trait, respectively (Table S1.4). These patterns were maintained for the additive genetic variation ($\sigma^2_A$) when considering the additive relationship among cowpea genotypes (Table S1.4). With total nodule biomass, the highest expression of $\sigma^2_G$ and heritability was observed within Shafter followed by Thermal, and no genetic variation was evident within Riverside and Parlier, which shared the Hanford soil composition (Table S1.4).

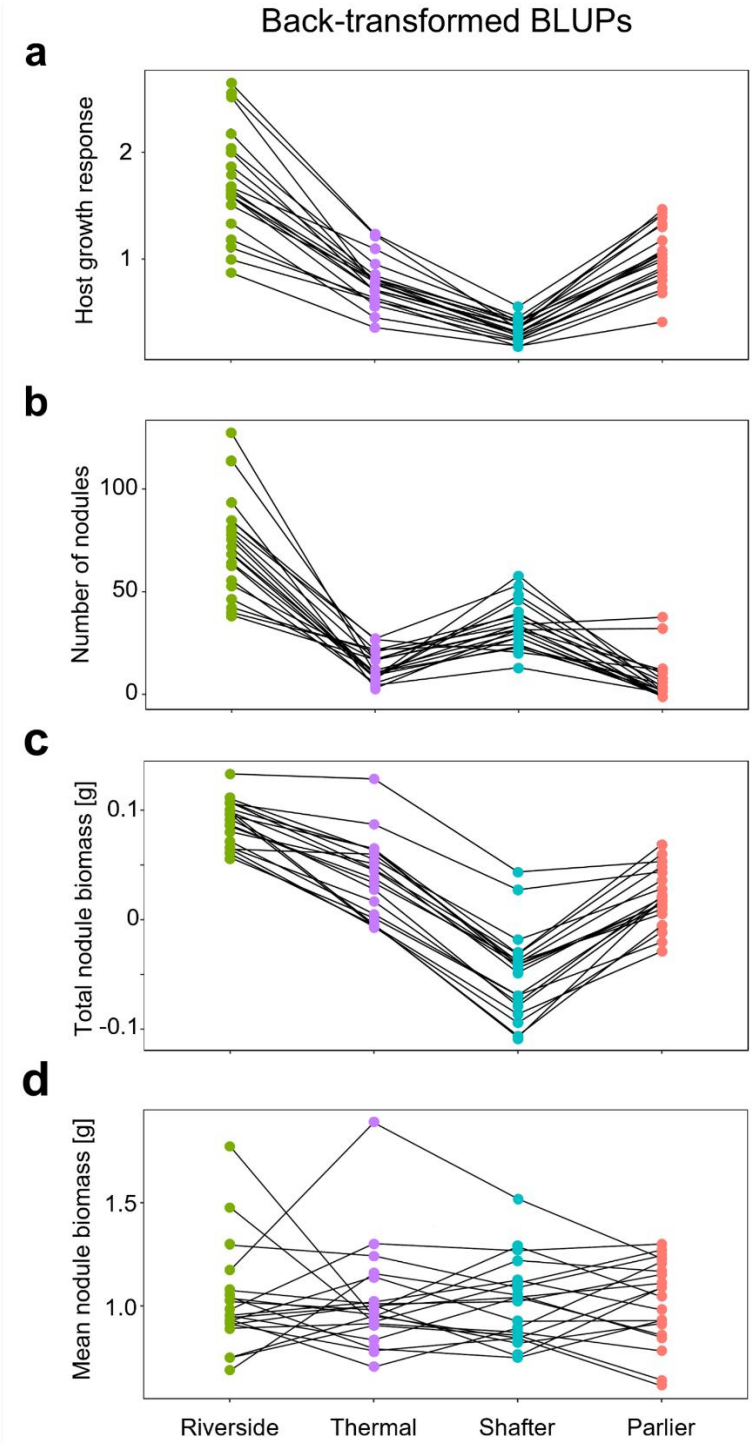


**Fig. 1.4** Reaction norms of symbiosis traits with a significant genotype effect. (a) Host growth response, (b) Number of nodules, (c) Total nodule biomass, (d) Mean nodule biomass. In the y-axis are the estimated breeding values for each genotype based on adjusted BLUP values from each variance–covariance model that best fit the data. These values were back transformed to their original scale. Each dot represents an individual cowpea genotype.

The addition of the relationship matrix caused an overfit of the model and estimates of $\sigma^2_A$ within each soil treatment were not obtained for total nodule biomass, so narrow sense heritability was excluded from total nodule biomass reports (Table S1.4). A G x E interaction was observed for both host growth and mean nodule biomass ($\chi^2_1 = 6.11$, $p = 0.01$; $\chi^2_1 = 7.97$, $p < 0.01$; Table S1.3) despite the homogeneity in genetic variances among soil treatments for these traits (Fig. 1.4). For the number of nodules and total nodule biomass, a significant G x E was evident when genetic variances and covariances were allowed to differ among treatments, indicating differences in the phenotypic plasticity of cowpea genotypes, as well as genetic variation in phenotypic plasticity (Table S1.3).

**Discussion**

We found that soil source strongly influenced both host benefits and expression of belowground plant traits in the cowpea-rhizobia symbiosis. Soil source was a significant factor contributing to host growth response and nodule counts (Fig. 1.2, Table 1.1), and soil composition appeared to play a prominent role in these effects. Both Riverside and Parlier sites – which induced the strongest host growth response – share the same soil series type (Hanford), and a similar among-genotype variation was observed in these soil sources for both nodule counts and total nodule biomass (Table S1.1, Table S1.4). Conversely, the Shafter and Thermal soils have distinct soil compositions (Lewkalb, Myoma) and different among-genotype variation for these same traits (Table S1.1, Table S1.4). We also found that soil physicochemical properties (PC1) had a significant effect on host growth response, number of nodules, and mean nodule biomass (Table 1.3). As there were no significant differences in plant biomass among the dead inoculum controls due to either soil treatment

or PC1 (Table S1.7), this suggests that soil physicochemical properties shape microbiota in each soil, thus indirectly driving plant benefits from inoculation. Our analysis of trait heritability suggests that different soil treatments can shift the expression of genetic variation in the number and size of nodules, but not for the host growth response of cowpea. For host growth, we found a significant G x E interaction, suggesting the presence of differences in phenotypic plasticity of host growth in cowpea genotypes in response to the soil rhizobia community.

Previous studies have also suggested soil-driven effects in the cowpea-microbial symbiosis. For instance, soil particle makeup and pH influenced the rhizobia populations in cowpeas sampled from agronomic fields in Kenya, as well as rhizobia cultured from nearby uncultivated soils (Ndungu et al. 2018). Similarly, soil type played a larger role than plant genotype in shaping non-rhizobia cowpea nodule microbial communities (Leite et al. 2017). However, neither of these studies examined the effect of soil conditions on plant growth or benefits from those microbes. Other studies that focus on legume inoculation benefits have shown that host genotype, inoculation, and soil type are all significant drivers of host growth and nodulation; however, in each case, plants were inoculated with a single strain of rhizobia (Amha and Fassil 2018; Keller and Lau 2018; Sánchez et al. 2014).

It was striking that no significant differences in host benefits from soil inoculation were observed among cowpea populations, given that the cowpea genotypes span the diversity of this species (Huynh et al. 2013). This also supports the hypothesis that domestication has not degraded cowpea symbiosis benefits, as wild and domesticated cowpea respond similarly when treated with the same soil communities (Ortiz-Barbosa et

al. 2022). Nonetheless, landrace population 2 formed significantly more nodules than population 1 with the soil treatments from Thermal and Shafter, and had a significantly higher host growth response than population 1 within the Thermal treatment (Table S1.9). This indicates that landrace population 2 might be more resilient under challenging soil conditions, as the Thermal and Shafter treatment groups were the least beneficial overall (Table S1.8, Fig. 1.2). However, there are also limitations in our approach that should be considered. Preparation of soil for inoculation can change qualitative and functional diversity of rhizobia present (Alberton et al. 2006). Additionally, some of the observed soil inoculation effects could be due to density, rather than community makeup, of compatible microbes that varied among sites. In particular, low nodulation effects from the Shafter inoculum could indicate either a reduced or significantly altered rhizobial population. Nonetheless, for growers considering different cowpea cultivars as well as different field plots, our data suggests that the field soil – and the microbial community it contains – is more important for determining yield. Additionally, analysis of genotypic variation & expression among specific genotypes suggests that cowpea genotypes respond to changes in soil microbial communities in different ways, and that a change in soil inoculum can alter the ranking of genotypes when examining host growth (Table S1.10). This is a factor which should be considered by plant breeders and those making planting decisions, when cultivar-specific consistency in growth response to inocula is desirable.

The lower nodulation and host growth associated with the Shafter soil inoculation was surprising, as this was the only soil that had been treated with a *Bradyrhizobium* biofertilizer, as well as the only inoculum from a field with live cowpea at the time of

sampling, both factors that we expected to enhance nodulation. However multiple factors can mediate the success of inoculation. When inocula were derived from field soils which had been recently fertilized, treated plants experienced significantly reduced biomass compared with non-fertilized soil inoculation, suggesting that fertilization impacts soil populations of nitrogen-fixing rhizobia (Simonsen et al. 2015). Long term field nitrogen fertilization has also been shown to stimulate the evolution of less-mutualistic rhizobia strains (Klinger et al. 2016; Weese et al. 2015). However, chemical analysis of Shafter soils showed that in 2019, the nitrogen levels (NO3-N) at this site were low relative to other sites (Table S1.5), suggesting that any negative impacts to local microbiota are likely not due to recent fertilization. The crop history at Shafter might also explain some of the variation seen, as each of the other soils originated from sites where a multi-parent intercross population of cowpea genotypes have been propagated for multiple seasons (Huynh et al. 2018), while Shafter had a mixed crop history. In the 3 years prior to sampling, the Shafter field had been used to grow carrots and cotton. Prior to that, it had been an alfalfa field for 4 years. Like cowpea, alfalfa is a legume; however, it generally associates with *Ensifer*, and does not form nodules with *Bradyrhizobium* (Stajković-Srbinović et al. 2012; Wang et al. 2018; Woliy et al. 2019). Bioinoculants such as the one used at Shafter are employed to prime soils without a history of successful prior production of a particular legume, making this site a perfect candidate for inoculation. Since Shafter was sampled during the first season of cowpea growth, it's possible that the soils had not yet been sufficiently enriched with *Bradyrhizobium*.

Later stages of plant domestication often involve the introduction of plant genotypes to new regions and thus to soils with novel characteristics and microbial communities (Gaut et al. 2018). Thus, while the African cowpea populations might not be adapted to microbes in Californian soils, introduction to novel soils and microbes is a fundamental aspect of agriculture (Gaut et al. 2018). We found that the expression of genetic variation in host growth response to soil treatments did not vary significantly among cowpea genotypes, and that variance in host growth response was more strongly associated with soil treatment than host genotype, suggesting that field soil locations (and their associated microbiota) are more important than host cultivar when predicting host benefits and expected yields. The yield gap – the difference between actual and maximum expected crop yield – is substantial for cowpeas grown in Africa (Foyer et al. 2016). Like other domesticated legumes, modern cowpeas are most often fertilized to maximize growth, suggesting that key below-ground traits have been lost or neglected in the process of domestication (Denison et al. 2004). However, we did not find significant differences in host growth response among wild and landrace populations, confirming results from Ortiz-Barbosa (2022) that early cowpea domestication has not degraded host benefits from symbiosis. Future studies could illuminate how rhizobia communities in nodules vary among wild and domesticated genotypes. With the increase in above-ground plant mass associated with domestication, cowpea could have adapted strategies to maintain fitness benefits. If so, identifying these traits would prove useful in breeding cowpea and other domesticated legumes to harness local rhizobia, improve crop yields, and reduce inorganic fertilization practices.

**Table 1.1:** Variation in plant symbiosis traits among cowpea populations exposed to different soil inocula (Linear mixed model)

| | Host growth response | | | Number of nodules | | | Total nodule biomass | | | Mean nodule biomass | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fixed effects** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** |
| Host population | 0.140 | 2 | 0.880 | 20.871 | 2 | <.0001* | 16.341 | 2 | <.0001* | 5.702 | 2 | 0.0122* |
| Soil treatment | 63.129 | 3 | <.0001* | 212.718 | 3 | <.0001* | 10.389 | 3 | <.0001* | 137.290 | 3 | <.0001* |
| Population x Treatment | 1.262 | 6 | 0.2968 | 9.797 | 6 | <.0001* | 1.150 | 6 | 0.3335 | 3.528 | 6 | 0.0053* |
| Days post inoculation [a] | 78.249 | 1 | <.0001* | 6.144 | 1 | 0.0137* | 42.415 | 1 | <.0001* | 2.868 | 1 | 0.0913 |
| **Random effects** | | | | | | | | | | | | |
| Host genotype [b] | | 1 | 0.0171* | | 1 | 0.0260* | | 1 | 0.0287* | | 1 | 0.0886 |

(*****) indicates significant effect, where $p < 0.05$

[a] Days post inoculation has 1 degree of freedom (df) as it is a continuous variable with a single regression coefficient (i.e. slope).

[b] Host genotype has 1 degree of freedom, as calculated by JMP' s Wald Test, because it is a random effect.

**Table 1.2:** Partitioned effects of host genotype and soil inoculation treatment (Partition of variance analysis)

| Component | Population variance | % of Total | Sqrt (Variance components) | F Ratio | *p* |
|---|---|---|---|---|---|
| *Between total* | *0.0604* | *40.91* | *0.2457* | *10.9522* | *0.000** |
| Between genotype | 0.0220 | 14.94 | 0.1485 | 4.6296 | 0.000* |
| Between treatment | 0.0383 | 25.98 | 0.1958 | 50.9950 | 0.000* |
| *Within total* | *0.0872* | *59.09* | *0.2953* | | |
| Within genotype | 0.0153 | 10.36 | 0.1236 | | |
| Within treatment | 0.0277 | 18.80 | 0.1666 | | |
| Common | 0.0001 | 0.08 | 0.0111 | | |
| Within error | 0.000 | 0.00 | 0.0000 | | |
| *Total* | *0.1476* | *100.00* | *0.3842* | | |

**Table 1.3:** Effects of soil characteristics on symbiosis traits (Linear mixed model)

| | Host growth response | | | Number of nodules | | | Total nodule biomass | | | Mean nodule biomass | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fixed effects** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** |
| PC1[a] | 23.988 | 1 | <.0001* | 183.388 | 1 | <.0001* | 3.476 | 1 | 0.0632 | 292.302 | 2 | <.0001* |
| Population | 0.063 | 2 | 0.9394 | 17.219 | 2 | <.0001* | 16.273 | 2 | 0.0001* | 5.677 | 2 | 0.0130* |
| Population x PC1 | 2.038 | 2 | 0.1319 | 10.383 | 2 | <.0001* | 0.640 | 2 | 0.5279 | 8.271 | 2 | 0.0002* |
| Days post inoculation | 47.138 | 1 | <.0001* | 1.877 | 1 | 0.1716 | 34.110 | 1 | <.0001* | 5.175 | 1 | 0.0236* |
| **Random effects** | | | | | | | | | | | | |
| Host genotype | | | 0.0288* | | | 0.0890 | | | 0.0529 | | | 0.1291 |

[a] See Table S1.5 for soil physicochemical properties

## References


Agler MT, Ruhe J, Kroll S, et al (2016) Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. PLOS Biology 14:e1002352. https://doi.org/10.1371/journal.pbio.1002352

Alberton O, Kaschuk G, Hungria M (2006) Sampling effects on the assessment of genetic diversity of rhizobia associated with soybean and common bean. Soil Biology and Biochemistry 38:1298–1307. https://doi.org/10.1016/j.soilbio.2005.08.018

Ali ZB, Yao KN, Odeny DA, et al (2015) Assessing the genetic diversity of cowpea [Vigna unguiculata (L.) Walp.] accessions from Sudan using simple sequence repeat (SSR) markers. Afr J Plant Sci 9:293–304. https://doi.org/10.5897/AJPS2015.1313

Amha G, Fassil A (2018) The effect of inter cross-inoculation host group rhizobia on the growth and nitrogen fixation of Faba Bean (Vicia faba L.) varieties in North Showa, Amhara Regional State, Ethiopia. J Agric Biotech Sustain Dev 10:25–33. https://doi.org/10.5897/JABSD2018.0307

Balachandar D, Sandhiya GS, Sugitha TCK, Kumar K (2006) Flavonoids and Growth Hormones Influence Endophytic Colonization and in Planta Nitrogen Fixation by a Diazotrophic Serratia sp. in Rice. World J Microbiol Biotechnol 22:707–712. https://doi.org/10.1007/s11274-005-9094-0

Batstone RT, Peters MAE, Simonsen AK, et al (2020) Environmental variation impacts trait expression and selection in the legume–rhizobium symbiosis. American Journal of Botany 107:195–208. https://doi.org/10.1002/ajb2.1432

Bonkowski M (2004) Protozoa and plant growth: the microbial loop in soil revisited. New Phytologist 162:617–631. https://doi.org/10.1111/j.1469-8137.2004.01066.x

Bouffaud M-L, Kyselková M, Gouesnard B, et al (2012) Is diversification history of maize influencing selection of soil bacteria by roots? Mol Ecol 21:195–206. https://doi.org/10.1111/j.1365-294X.2011.05359.x

Bulgarelli D, Schlaeppi K, Spaepen S, et al (2013) Structure and Functions of the Bacterial Microbiota of Plants. Annual Review of Plant Biology 64:807–838. https://doi.org/10.1146/annurev-arplant-050312-120106

Coulibaly S, Pasquet RS, Papa R, Gepts P (2002) AFLP analysis of the phenetic organization and genetic diversity of Vigna unguiculata L. Walp. reveals extensive gene flow between wild and domesticated types. Theor Appl Genet 104:358–366. https://doi.org/10.1007/s001220100740

Covarrubias-Pazaran G (2016) Genome-Assisted Prediction of Quantitative Traits Using the R Package sommer. PLOS ONE 11:e0156744. https://doi.org/10.1371/journal.pone.0156744

Denison RF (2000) Legume Sanctions and the Evolution of Symbiotic Cooperation by Rhizobia. The American Naturalist 156:567–576. https://doi.org/10.1086/316994

Denison RF (2015) Chapter 9 - A Darwinian perspective on improving nitrogen-fixation efficiency of legume crops and forages. In: Sadras VO, Calderini DF (eds) Crop Physiology (Second Edition). Academic Press, San Diego, pp 207–222

Denison RF, Kiers ET (2004) Lifestyle alternatives for rhizobia: mutualism, parasitism, and forgoing symbiosis. FEMS Microbiol Lett 237:187–193. https://doi.org/10.1111/j.1574-6968.2004.tb09695.x

Engelhard M, Hurek T, Reinhold-Hurek B (2000) Preferential occurrence of diazotrophic endophytes, Azoarcus spp., in wild rice species and land races of Oryza sativa in comparison with modern races. Environ Microbiol 2:131–141. https://doi.org/10.1046/j.1462-2920.2000.00078.x

Fitzpatrick CR, Mustafa Z, Viliunas J (2019) Soil microbes alter plant fitness under competition and drought. Journal of Evolutionary Biology 32:438–450. https://doi.org/10.1111/jeb.13426

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

Foyer CH, Lam H-M, Nguyen HT, et al (2016) Neglecting legumes has compromised human health and sustainable food production. Nat Plants 2:16112. https://doi.org/10.1038/nplants.2016.112

Friesen ML, Porter SS, Stark SC, et al (2011) Microbially Mediated Plant Functional Traits. Annual Review of Ecology, Evolution, and Systematics 42:23–46. https://doi.org/10.1146/annurev-ecolsys-102710-145039

Gano-Cohen K, Wendlandt C, Moussawi K, et al (2020) Recurrent mutualism breakdown events in a legume rhizobia metapopulation. Proceedings of the Royal Society B: Biological Sciences 287:20192549. https://doi.org/10.1098/rspb.2019.2549

Gaut BS, Seymour DK, Liu Q, Zhou Y (2018) Demography and its effects on genomic variation in crop domestication. Nature Plants 4:512–520. https://doi.org/10.1038/S41477-018-0210-1

Gupta PK, K.J Y, B.O SV, et al (2019) Impact of Plant-Microbe Interactions on Plant Metabolism Under Saline Environment. In: Akhtar MS (ed) Salt Stress, Microbes, and Plant Interactions: Causes and Solution: Volume 1. Springer, Singapore, pp 113–127

Haney CH, Samuel BS, Bush J, Ausubel FM (2015) Associations with rhizosphere bacteria can confer an adaptive advantage to plants. Nature Plants 1:1–9. https://doi.org/10.1038/nplants.2015.51

He J-Z, Shen J-P, Zhang L, et al (2007) Quantitative Analyses of the Abundance and Composition of Ammonia-Oxidizing Bacteria and Ammonia-Oxidizing Archaea of a Chinese Upland Red Soil under Long-Term Fertilization Practices. Environmental microbiology 9:2364–74. https://doi.org/10.1111/j.1462-2920.2007.01358.x

Heath KD, Podowski JC, Heniff S, et al (2020) Light availability and rhizobium variation interactively mediate the outcomes of legume–rhizobium symbiosis. American Journal of Botany 107:229–238. https://doi.org/10.1002/ajb2.1435

Heath KD, Stinchcombe JR (2014) Explaining Mutualism Variation: A New Evolutionary Paradox? Evolution 68:309–317. https://doi.org/10.1111/evo.12292

Heath KD, Tiffin P (2009) Stabilizing Mechanisms in a Legume-Rhizobium Mutualism. evol 63:652–662. https://doi.org/10.1111/j.1558-5646.2008.00582.x

Henderson CR (1975) Best Linear Unbiased Estimation and Prediction under a Selection Model. Biometrics 31:423–447. https://doi.org/10.2307/2529430

Herniter IA, Muñoz-Amatriaín M, Close TJ (2020) Genetic, textual, and archeological evidence of the historical global spread of cowpea (Vigna unguiculata [L.] Walp.). Legume Science 2:e57. https://doi.org/10.1002/leg3.57

Hetrick B, Wilson G, Cox T (2011) Mycorrhizal dependence of modern wheat varieties, landraces, and ancestors. Canadian Journal of Botany 70:2032–2040. https://doi.org/10.1139/b92-253

Hussain M, Hamid MI, Tian J, et al (2018) Bacterial community assemblages in the rhizosphere soil, root endosphere and cyst of soybean cyst nematode-suppressive soil challenged with nematodes. FEMS Microbiology Ecology 94:fiy142. https://doi.org/10.1093/femsec/fiy142

Huynh B-L, Close TJ, Roberts PA, et al (2013) Gene Pools and the Genetic Architecture of Domesticated Cowpea. The Plant Genome 6:plantgenome2013.03.0005. https://doi.org/10.3835/plantgenome2013.03.0005

Huynh B-L, Ehlers JD, Huang BE, et al (2018) A multi-parent advanced generation inter-cross (MAGIC) population for genetic analysis and improvement of cowpea (Vigna unguiculata L. Walp.). Plant J 93:1129–1142. https://doi.org/10.1111/tpj.13827

Kakraliya SK, Singh U, Bohra A, et al (2018) Nitrogen and Legumes: A Meta-analysis. In: Meena RS, Das A, Yadav GS, Lal R (eds) Legumes for Soil Health and Sustainable Management. Springer, Singapore, pp 277–314

Keller KR, Lau JA (2018) When mutualisms matter: Rhizobia effects on plant communities depend on host plant population and soil nitrogen availability. Journal of Ecology 106:1046–1056. https://doi.org/10.1111/1365-2745.12938

Kiers ET, Hutton MG, Denison RF (2007) Human selection and the relaxation of legume defences against ineffective rhizobia. Proceedings of the Royal Society B: Biological Sciences 274:3119–3126. https://doi.org/10.1098/rspb.2007.1187

Kiers ET, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. Nature 425:78–81. https://doi.org/10.1038/nature01931

Klinger CR, Lau JA, Heath KD (2016) Ecological genomics of mutualism decline in nitrogen-fixing bacteria. Proceedings of the Royal Society B: Biological Sciences 283:20152563. https://doi.org/10.1098/rspb.2015.2563

Lareen A, Burton F, Schäfer P (2016) Plant root-microbe communication in shaping root microbiomes. Plant Mol Biol 90:575–587. https://doi.org/10.1007/s11103-015-0417-8

Legrand F, Picot A, Cobo-Díaz JF, et al (2018) Effect of tillage and static abiotic soil properties on microbial diversity. Applied Soil Ecology 132:135–145. https://doi.org/10.1016/j.apsoil.2018.08.016

Leite J, Fischer D, Rouws LFM, et al (2017) Cowpea Nodules Harbor Non-rhizobial Bacterial Communities that Are Shaped by Soil Type Rather than Plant Genotype. Front Plant Sci 7:. https://doi.org/10.3389/fpls.2016.02064

Li M, Wei Z, Wang J, et al (2019) Facilitation promotes invasions in plant-associated microbial communities. Ecology Letters 22:149–158. https://doi.org/10.1111/ele.13177

Liu XQ, Rong JY, Liu XY (2008) Best linear unbiased prediction for linear combinations in general mixed linear models. Journal of Multivariate Analysis 99:1503–1517. https://doi.org/10.1016/j.jmva.2008.01.004

Lo S, Muñoz-Amatriaín M, Boukar O, et al (2018) Identification of QTL controlling domestication-related traits in cowpea ( Vigna unguiculata L. Walp). Scientific Reports 8:6261. https://doi.org/10.1038/S41598-018-24349-4

Long R, Temple S, Schmierer J, et al (2010) Common Dry Bean Production in California, Second Edition. University of California, Agriculture and Natural Resources

Micallef SA, Shiaris MP, Colón-Carmona A (2009) Influence of Arabidopsis thaliana accessions on rhizobacterial communities and natural variation in root exudates. J Exp Bot 60:1729–1742. https://doi.org/10.1093/jxb/erp053

Moawad H, Beck DP (1991) Some characteristics of Rhizobium leguminosarum isolates from uninoculated field-grown lentil. Soil Biology and Biochemistry 23:933–937. https://doi.org/10.1016/0038-0717(91)90173-H

Morella NM, Weng FC-H, Joubert PM, et al (2020) Successive passaging of a plant-associated microbiome reveals robust habitat and host genotype-dependent selection. PNAS 117:1148–1159. https://doi.org/10.1073/pnas.1908600116

Mueller UG, Sachs JL (2015) Engineering Microbiomes to Improve Plant and Animal Health. Trends in Microbiology 23:606–617. https://doi.org/10.1016/j.tim.2015.07.009

Muñoz-Amatriaín M, Mirebrahim H, Xu P, et al (2017) Genome resources for climate-resilient cowpea, an essential crop for food security. The Plant Journal 89:1042–1054. https://doi.org/10.1111/tpj.13404

Ndungu SM, Messmer MM, Ziegler D, et al (2018) Cowpea (Vigna unguiculata L. Walp) hosts several widespread bradyrhizobial root nodule symbionts across contrasting agro-ecological production areas in Kenya. Agriculture, Ecosystems & Environment 261:161–171. https://doi.org/10.1016/j.agee.2017.12.014

Oono R, Anderson CG, Denison RF (2011) Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates. Proceedings of the Royal Society B: Biological Sciences 278:2698–2703. https://doi.org/10.1098/rspb.2010.2193

Ortiz-Barbosa GS, Torres-Martínez L, Manci A, et al (2022) No disruption of rhizobial symbiosis during early stages of cowpea domestication. Evolution evo.14424. https://doi.org/10.1111/evo.14424

Pahua VJ, Stokes PJN, Hollowell AC, et al (2018) Fitness variation among host species and the paradox of ineffective rhizobia. Journal of Evolutionary Biology 31:599–610. https://doi.org/10.1111/jeb.13249

Peiffer JA, Spor A, Koren O, et al (2013) Diversity and heritability of the maize rhizosphere microbiome under field conditions. PNAS 110:6548–6553. https://doi.org/10.1073/pnas.1302837110

Porter SS, Sachs JL (2020) Agriculture and the Disruption of Plant–Microbial Symbiosis. Trends in Ecology & Evolution 35:426–439. https://doi.org/10.1016/j.tree.2020.01.006

Rascovan N, Carbonetto B, Perrig D, et al (2016) Integrated analysis of root microbiomes of soybean and wheat from agricultural fields. Sci Rep 6:28084. https://doi.org/10.1038/srep28084

Regus JU, Gano KA, Hollowell AC, et al (2015) Lotus hosts delimit the mutualism–parasitism continuum of Bradyrhizobium. Journal of Evolutionary Biology 28:447–456. https://doi.org/10.1111/jeb.12579

Regus JU, Wendlandt CE, Bantay RM, et al (2017) Nitrogen deposition decreases the benefits of symbiosis in a native legume. Plant Soil 414:159–170. https://doi.org/10.1007/s11104-016-3114-8

Sachs JL, Kembel SW, Lau AH, Simms EL (2009) In Situ Phylogenetic Structure and Diversity of Wild Bradyrhizobium Communities. Applied and Environmental Microbiology 75:4727–4735. https://doi.org/10.1128/AEM.00667-09

Sachs JL, Russell JE, Lii YE, et al (2010) Host control over infection and proliferation of a cheater symbiont. Journal of Evolutionary Biology 23:1919–1927. https://doi.org/10.1111/j.1420-9101.2010.02056.x

Sachs JL, Ehinger MO, Simms EL (2010) Origins of cheating and loss of symbiosis in wild Bradyrhizobium. Journal of Evolutionary Biology 23:1075–1089. https://doi.org/10.1111/j.1420-9101.2010.01980.x

Sánchez AC, Gutiérrez RT, Santana RC, et al (2014) Effects of co-inoculation of native Rhizobium and Pseudomonas strains on growth parameters and yield of two contrasting Phaseolus vulgaris L. genotypes under Cuban soil conditions. European Journal of Soil Biology 62:105–112. https://doi.org/10.1016/j.ejsobi.2014.03.004

Sasse J, Martinoia E, Northen T (2018) Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? Trends in Plant Science 23:25–41. https://doi.org/10.1016/j.tplants.2017.09.003

Sawada H, Kuykendall LD, Young JM (2003) Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. J Gen Appl Microbiol 49:155–179. https://doi.org/10.2323/jgam.49.155

Saxton AM, SAS Institute. Genetic Analysis of Complex Traits Using SAS. SAS Institute; 2004.

Shaw RG (1991) The Comparison of Quantitative Genetic Parameters Between Populations. Evolution 45:143–151. https://doi.org/10.1111/j.1558-5646.1991.tb05273.x

Simonsen AK, Han S, Rekret P, et al (2015) Short-term fertilizer application alters phenotypic traits of symbiotic nitrogen fixing bacteria. PeerJ 3:e1291. https://doi.org/10.7717/peerj.1291

Somasegaran P, Hoben HJ (1994) Handbook for Rhizobia: Methods in legume-Rhizobium technology. xvi, 450. https://doi.org/10.1007/978-1-4613-8375-8

Stajković-Srbinović O, De Meyer SE, Miličić B, et al (2012) Genetic diversity of rhizobia associated with alfalfa in Serbian soils. Biol Fertil Soils 48:531–545. https://doi.org/10.1007/s00374-011-0646-1

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. https://doi.org/10.1046/j.1365-2664.2000.00470.x

Torres-Martínez L, McCarten N, Emery NC (2019) The adaptive potential of plant populations in response to extreme climate events. Ecology Letters 22:866–874. https://doi.org/10.1111/ele.13244

Torres-Martínez L, Porter SS, Wendlandt C, et al (2021) Evolution of specialization in a plant-microbial mutualism is explained by the oscillation theory of speciation. Evolution 75:1070–1086. https://doi.org/10.1111/evo.14222

Unkovich MJ, Pate JS (1998) Symbiotic effectiveness and tolerance to early season nitrate in indigenous populations of subterranean clover rhizobia from S.W. Australian pastures. Soil Biology and Biochemistry 30:1435–1443. https://doi.org/10.1016/S0038-0717(97)00258-7

van Dam NM, Bouwmeester HJ (2016) Metabolomics in the Rhizosphere: Tapping into Belowground Chemical Communication. Trends in Plant Science 21:256–265. https://doi.org/10.1016/j.tplants.2016.01.008

Wagner MR, Lundberg DS, del Rio TG, et al (2016) Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nat Commun 7:12151. https://doi.org/10.1038/ncomms12151

Wang XL, Cui WJ, Feng XY, et al (2018) Rhizobia inhabiting nodules and rhizosphere soils of alfalfa: A strong selection of facultative microsymbionts. Soil Biology and Biochemistry 116:340–350. https://doi.org/10.1016/j.soilbio.2017.10.033

Weese DJ, Heath KD, Dentinger BTM, Lau JA (2015) Long-term nitrogen addition causes the evolution of less-cooperative mutualists. Evolution 69:631–642. https://doi.org/10.1111/evo.12594

Wendlandt CE, Regus JU, Gano-Cohen KA, et al (2019) Host investment into symbiosis varies among genotypes of the legume Acmispon strigosus, but host sanctions are uniform. New Phytologist 221:446–458. https://doi.org/10.1111/nph.15378

West SA, Kiers ET, Simms EL, Denison RF (2002) Sanctions and mutualism stability: why do rhizobia fix nitrogen? Proc Biol Sci 269:685–694. https://doi.org/10.1098/rspb.2001.1878

Woliy K, Degefu T, Frostegård Å (2019) Host Range and Symbiotic Effectiveness of N2O Reducing Bradyrhizobium Strains. Frontiers in Microbiology 10:2746. https://doi.org/10.3389/fmicb.2019.02746

Wood CW, Brodie III ED (2016) Evolutionary response when selection and genetic variation covary across environments. Ecology Letters 19:1189–1200. https://doi.org/10.1111/ele.12662

Xiong H, Shi A, Mou B, et al (2016) Genetic Diversity and Population Structure of Cowpea (Vigna unguiculata L. Walp). PLOS ONE 11:e0160941. https://doi.org/10.1371/journal.pone.0160941

Xu Z, Yu G, Zhang X, et al (2018) Biogeographical patterns of soil microbial community as influenced by soil characteristics and climate across Chinese forest biomes. Applied Soil Ecology 124:298–305. https://doi.org/10.1016/j.apsoil.2017.11.019

Zhong W, Gu T, Wang W, et al (2010) The effects of mineral fertilizer and organic manure on soil microbial community and diversity. Plant Soil 326:511–522. https://doi.org/10.1007/s11104-009-9988-y

Zhu Y-G, Smith SE, Barritt AR, Smith FA (2001) Phosphorus (P) efficiencies and mycorrhizal responsiveness of old and modern wheat cultivars. Plant and Soil 237:249–255. https://doi.org/10.1023/A:1013343811110

## CHAPTER 2

**Title:** Epidemic *Bradyrhizobium* strains dominate cowpea roots irrespective of host genotype or field location

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**Abstract**

Plant-associating soil microbes can improve crop growth, but these communities are highly variable and little is known about the forces that structure them. We investigated cowpea-associating rhizobia in an agricultural field, and tested for the role of host genotype, field location, and selection versus drift processes in structuring the microbial community.

Nineteen genotypes of cowpea, with a diverse set of agronomic traits, were planted in three replicated quadrats across an experimental field with a decade-long history of cowpea cultivation. Rhizobial isolates from nodules were cultured and sequenced. Whole genomes were analyzed and rhizobial genotypes were delineated using Bayesian analysis of population structure.

The rhizobia community was dominated by a small handful of highly related *Bradyrhizobium yuanmingense* genotypes. There was no effect of plant genotype or soil location on the microbial community structure. The experimental field community had no evidence of gene flow with nearby natural populations of *Bradyrhizobium.*

The epidemic lineage of *Bradyrhizobium* likely has a local fitness advantage and may have arisen due to host-driven selection from the recurrent cultivation of cowpea in this field. The lack of host-driven structuring of this community suggests that partner selection may be a fixed trait in cowpeas, as it is in other legumes.

## Introduction

Microbial communities in field soils can have significant impacts on the growth, yield, and productivity of crops (Vacheron et al. 2013; Zakira et al. 2007). Microbes can improve nutrient availability to plants, protect hosts from pathogens, and enhance stress tolerance to drought and salinity, reducing reliance on fertilizers and expanding geographical areas where crops can be grown (Friesen et al. 2011; Rolli et al. 2015; Kuypers et al. 2018; Fitzpatrick et al. 2019; Khan et al. 2019; Ali and Xie 2019). The services provided by soil microbial communities are context-dependent, highly varied, and range significantly in their magnitude of effects on host performance (Heath and Stinchcombe 2014; Kaminsky et al. 2019). This variation in both colonization and microbial services can arise due to genetic variation among microbial strains and communities, as well as external forces shaping this variation including host plant genotype and environmental factors. Understanding the complex genetic and environmental drivers that shape microbial benefits to plants is critical to developing more sustainable agricultural practices.

Plants have a remarkable capacity to structure their associated microbial communities, favoring microbes that provide beneficial services to the plant host (Mueller & Sachs 2015). Plants can select among microbial partners by restricting which strains colonize or infect the host, and by punishing or rewarding specific taxa depending on their degree of benefit (Kiers et al. 2003; Wendlandt et al. 2019; Denison 2000; West et al. 2002; Sachs et al. 2004). Plant hosts can selectively enrich beneficial microbes and can have significant impacts on microbial diversity. For example, in prairie communities, plant

community richness is negatively correlated with both bacterial diversity and the proportion of antagonistic microbes in soils, while plant diversity and culturable soil bacterial diversity were positively correlated in experimental grassland communities (Stephan et al. 2001; Schlatter et al. 2015). While these patterns appear to diverge, both examples illustrate that plant communities can shape their associated soil microbial communities. In agricultural settings, seasonal planting strategies can alter these host-driven effects, as intercropping of different plant species can alter soil microbial composition over time (Tian et al., 2019) while continuous cropping can magnify host selection, reducing rhizosphere diversity (Xiong et al. 2015; Wu et al. 2015). Plant genotypes often vary in the populations of microbes they support (Haney et al. 2015; Pahua et al. 2018; Wendlandt et al. 2019). This variation can be linked with differences in agronomic traits which impact symbiosis. For example, the Verticillium wilt disease resistance trait in olives has been associated with an altered microbial community in the rhizosphere (Fernández-González et al. 2020). Root exudates, composed of sugars, amino acids, and other compounds, are thought to be an important driver of belowground microbial communities by host plants. These biochemicals can vary within and among plant species (Micallef et al. 2009), driving among-species variation in rhizosphere community structure (Haichar et al. 2008). Because host selection can favor beneficial microbial partners, breeding for these traits in crops, combined with the application of beneficial strains, offers a compelling strategy to more sustainably increase crop yields.

Soil conditions, both abiotic and biotic, also affect crop-associated microbial communities. Abiotic factors such as pH, particle size, drainage, and nutrient availability

influence microbial community diversity and structure (Agler et al. 2016; Leite et al. 2017). Common grower practices such as tillage and fertilization can alter these abiotic soil factors and are asscociated with restructured microbial communities (Simonsen et al. 2015; Weese et al. 2015; Legrand et al. 2018). Plant responses to both mycorrhizal and rhizobial inoculation can be negatively impacted under fertilization, especially with nitrogen (Hoeksema et al. 2010; Regus et al. 2017; Gano-Cohen et al. 2020; Moawad and Beck 1991; Thrall et al. 2000; Weese et al., 2015). Inoculants can be sensitive to variation in soil conditions across fields (Thilakarathna and Raizada 2017). Variation in microbial community structure and function can also be driven by biotic interactions within the soil, such as competition, facilitation, and predation among microbes and other soil organisms (Hussain et al. 2018; Li et al. 2019). Inoculated strains must compete with local soil microbial communities for resources and host infection (Triplett and Sadowsky 1992; Weert et al. 2007; Lugtenberg and Kamilova 2009) which can mitigate which strains colonize the target hosts (De Roy et al. 2013; Mallon et al. 2018). Microbial communities are not uniform in crop fields, so biotic effects might vary, even within individual fields. Multiple scales of nested soil microbial community structure have been found in agricultural fields, with sub-communities distributed in distinct patterns across plots, likely due to differing population responses to spatially variable soil properties (Nunan et al., 2002; Franklin & Mills, 2003). Agricultural soils can be influenced by dispersal from nearby natural microbial communities, as well as by selection during the process of land conversion. Soils in natural landscapes are often dominated by high-abundance microbial strains, consistent with fitness superiority of these microbial genotypes, but it is not clear

how often these strains cross over into managed systems (Smith et al. 2000; Hollowell et al. 2016a; Hollowell et al. 2016b; McInnes et al. 2004; Sachs et al. 2009; VanInsberghe et al. 2015).

Rhizobia are a group of diazotrophic proteobacteria which induce specialized root structures called nodules, where they fix atmospheric nitrogen for the plant host in exchange for carbon (Kakraliya et al. 2018; Sawada et al. 2003). Cooperating rhizobia can provide substantial levels of fixed nitrogen to legumes and can reduce or eliminate the need for additional nitrogen fertilization (Regus et al. 2017). Because of their positive effects on plant growth and their potential to reduce reliance on costly nutrient inputs, rhizobia are often used as bioinoculants (O'Callaghan et al. 2022). However, as with other microbes, these benefits are context-dependent and vary widely among rhizobia strains, with strains ranging from highly beneficial to ineffective at fixing nitrogen (Gano-Cohen et al. 2020; Moawad and Beck 1991; Thrall et al. 2000).

Cowpea (*Vigna unguiculata*) is a crop legume which benefits from association with several species of *Bradyrhizobium*. Originally domesticated in Africa, they are now also grown across Asia and the Americas, including California where this study took place. Cowpeas are valued for their nutrient-dense seed pods, minimal nutrient input requirements, and high proportion of edible plant mass, as well as their broad resistance to heat and drought (Huynh et al. 2013; Muñoz-Amatriaín et al. 2017; Herniter et al. 2020). Modern cowpea genotypes are often bred for additional agronomic traits, including resistance to pests and disease. Despite these desirable traits, modern cowpeas are frequently fertilized to improve growth, and the yield gap (i.e. the difference between

expected and actual crop yield) for cowpeas grown in Africa is substantial (Denison et al. 2004; Foyer et al. 2016). Rhizobia inoculants have been developed to address these issues and improve cowpea growth, but inoculant strains are often outcompeted by native rhizobia in field soils (Mbah et al. 2022; Law et al. 2007). Understanding the forces driving variation in the cowpea-*Bradyrhizobium* symbiosis could inform better field practices, improving crop yields.

Here, we characterized the community structure of cowpea associated rhizobia present in a focal cultivated field. This field has a multidecade history of cowpea cultivation and intercropping with other legume species and was previously demonstrated to have a highly beneficial soil microbial community with superior benefits compared to other cowpea field soils (Huynh et al. 2019; Manci et al. 2022). Additionally, soil microbiota from this field was found to be 52 times more beneficial to cowpea host growth than USDA110, a rhizobial inoculant which provides widespread benefits to cowpea and other legumes, suggesting this field is enriched with highly beneficial cowpea-associating rhizobia (Ortiz-Barbosa et al. 2022). We investigated the community genetic structure of cowpea-associating rhizobia in this field. Nineteen cowpea genotypes were selected that exhibit a diversity of agronomic traits to test for the role of plant genotype in structuring the rhizobia community. The cowpea genotypes were planted in three experimental quadrats spread across the field to dissect plant genotype-driven versus soil-driven spatial variation. Rhizobia were isolated from root nodules from 79 field plants, and their genomes were sequenced to quantify the roles of host and planting location in structuring the rhizobial communities. To examine possible source populations and gene flow, sequences

were compared to rhizobia isolated from nearby natural sites, including legume and soil-associated isolates. Our goals were to test whether this community of crop associated rhizobia are primarily structured by host genotype, by abiotic conditions that vary across the field, or by selection and drift processes within the microbial community.

**Materials & Methods**

**History of experimental Field 11:** This field contains several subsections, including Field 11H where this experiment took place in 2015, and Field 11G where soils were used in a greenhouse experiment 2019 (Manci et al., 2022). Field subsections 11G and 11H are adjacent to one another. Cowpea was planted in Field 11H during the summer seasons of even-numbered years, beginning in 2004, and included both breeding lines and germplasm collections (Huynh et al., 2019). Field 11G had a parallel history, with cowpea planting during odd-numbered years. Both field subsections were occasionally used for other crops, including soybean and pigeonpea and was intercropped with barley. Prior to this experiment, the field had been treated regularly with pre-emergence herbicides and occasionally with irrigated liquid fertilizer.

Soil microbiota in Field 11 are highly beneficial to cowpea, providing the highest growth benefits compared three other cowpea fields sampled across California (Manci et al. 2022), and 52 times the raw host growth benefits received from a commonly applied beneficial strain, *Bradyrhizobium* strain USDA110 (Ortiz-Barbosa et al. 2022). USDA110 provides significant benefits to a wide diversity of cowpea, as well as other legumes including soybean (Keyser et al. 1982; Yelton et al. 1983; Chamber and Iruthay-athas

1988). These significant growth benefits suggest that Field 11 is enriched with beneficial cowpea-associating rhizobia.

**Cowpea Cultivars:** Nineteen cowpea genotypes were selected for planting into Field 11. These genotypes were chosen from a multi-parent intercross population used for a genome-informed cowpea breeding program at the University of California, Riverside (UCR) (Boukar et al., 2016). These genotypes exhibit a variety of agriculturally relevant traits, including resistance to important pests such as aphids, thrips, and nematodes, disease resistance to *Fusarium* wilt and bacterial blight, and tolerance of abiotic stressors such as drought and heat (Table 2.1).

**Experimental design**: Cowpeas were planted on 6/16/15 in three randomly located quadrats across Field 11H, a 3-acre field at the UCR Agricultural Experiment Station (N 33.967, W −117.339) (see Fig. S2.1). The minimum distance between quadrats was 28 m, and the maximum was 53 m. Within each 16.5 $m^2$ quadrat, 19 cowpea genotypes were planted in randomly arrayed groups of 20. Plants were watered via flood irrigation every seven days. Fields were not fertilized.

Two harvests occurred at three and six weeks post planting (7/7/15 – 7/8/15 & 7/28/15 – 8/1/15), and involved collection of roots, shoots, and nodules from one plant per host genotype per quadrat. Plants were extracted from soil to a depth of ~50cm to retrieve as much of the intact root system as possible. Root systems were rinsed and photographed. Nodules were dissected and counted. From each harvested plant, ten nodules were randomly selected, surface-sterilized, cultured on a solid medium of modified arabinose gluconate (i.e., MAG; Sachs et al. 2009), incubated at 29$^{o}$C for ten days to generate

colonies, and then colonies were picked and grown in liquid MAG media for archiving at -80°F. Shoots, roots, and remaining nodules were separated, dried at 60°C, and weighed for biomass. Two additional harvests occurred at four and eight weeks post planting to measure aboveground plant traits. As many as ten plant replicates per plant genotype were detopped to measure stem length, number of leaves, and length of the longest leaf.

**Sequencing & analysis:** From the Field 11 experiment, up to five nodule isolates from each host genotype and planting location treatment combination were selected for whole-genome sequencing. In all cases, cultures were isolated from an individual colony plated from a single nodule, based on the assumption that most nodules harbor an individual strain of rhizobia (Simms et al. 2006). In total, 288 nodule isolates were sequenced from Field 11.

Root nodule bacteria from two adjacent fields were also sequenced. From the adjacent Field subsection 11G, used in alternate years with parallel crop history, 5 isolates were cultured and processed from a 2019 greenhouse study for comparison with those from field 11H (Manci et al. 2022). From Field 10E, neighboring to Field 11H, but with no history of cowpea planting, 14 nodule isolates were cultured and processed for comparison.

DNA was extracted from 2 µl of archived nodule cultures using DNeasy Blood & Tissue Kit (Qiagen) and DNA quality was checked via Nanodrop and Qubit Bioanalyzer. Whole genomes were sequenced via Illumina HiSeq 3000 (Center for Genome Research & Biocomputing, Oregon State University) using whole-genome library prep (seqWell). Raw sequencing reads were trimmed with fastp to remove adapter sequences and low-

quality regions (Chen et al. 2018). Trimmed reads were assembled *de novo* using SPAdes, and assemblies were annotated with prokka (Bankevich et al. 2012; Seemann 2014).

Genome sequences of isolates were categorized into clonal groups and species, and phylogenetic relationships were reconstructed among them. Among-sequence pairwise average nucleotide identity (ANI) was calculated, and species groups were defined using the 95% ANI threshold. Percentage of conserved genes (POCP) were measured using Panaroo (Tonkin-Kill et al. 2020). SNP differences were analyzed among strains using the GATK pipeline (McKenna et al. 2010). Genotypes were hierarchically delineated from SNP calls using Bayesian Analysis of Population Structure (BAPS; Corander and Tang 2007). Minimum spanning networks were generated using Poppr using the cutoff of >800 SNPs to cluster genotypes, as this measure represents roughly 0.01% divergence for 8Mb *Bradyrhizobium* genomes (Kamvar et al. 2014). A k-mer based method was used to generate species designations within *Bradyrhizobium*, using bbsketch of the BBTools Suite (Bushnell 2014).

A Chi-Square test of independence was used to test for structuring of rhizobial lineages by by quadrat (i.e. spatial structuring) while Fisher's Exact test was used to test for structuring by cowpea genotype (i.e., host structuring) to account for low expected counts. Sequence data from the Field 11H trial were compared to 19 isolates from the two neighboring fields to identify shared clones among sites. Isolates from all three fields were also compared to a published sequence database of *Bradyrhizobium* isolates from a nearby natural site, where *Bradyrhizobium* was cultured from nodules, soil, and the root interface of *Acmispon strigosus*, a native annual legume, and sequenced for two loci, *recA* and *glnII*

(Hollowell et al., 2016). Genomes from the Field 11 trial cowpea dataset were analyzed using NCBI nucleotide BLAST and sequences for *recA* and *glnII* were extracted. Sequences from the Field 11H trial and Riverside Hills *Acmispon* isolate dataset were aligned using mafft (Katoh et al. 2002) and concatenated with catfasta2phyml (Nylander 2011), then re-aligned. Pairwise identities were calculated using ClustalOmega (Sievers et al. 2011) and phylogenetic trees were constructed in IQ-TREE (Nguyen et al. 2015) to check for overlapping or closely related genotypes, as well as to compare the rhizobial community structures between natural and agricultural sites.

## Results

Our goals for this study were to examine the cowpea-associating rhizobial strains in Field 11 and understand how they are structured, as previous work has shown the soil microbial community in this field is highly beneficial to cowpea (Manci et al. 2022). Our results revealed two core conclusions: 1) the field is dominated by a small handful of very closely related *Bradyrhizobium* strains, and 2) the community is unstructured by either plant genotype or soil location.

***Bradyrhizobium* community structure:** BAPS hierarchically delineated the Field 11H isolates into 2 species groups, 9 lineages, and 22 genotypes (Fig. S2.2). Most of the 287 nodule isolates (>97%) belong to species group 1, categorized as *Bradyrhizobium yuanmingense* (n = 278, Fig. S2.2). This low-diversity species group is primarily comprised of two abundant clonal-like lineages (n = 231, 41) with within-group pairwise ANI of >99% and differing by ~78,000 SNPs (Tables S2.1 & S2.2). Isolates within species group

1 share nearly identical gene content, with the percentage of conserved proteins (POCP) between isolates >99% in all cases (Table S2.3). Species group 1 can be further subdivided into 5 lineages, comprised of 14 distinct genotypes (Table S2.1; Fig. S2.2). The remaining isolates belong to a second clonal-like species group of *Bradyrhizobium liaoningense* (species group 2, n = 9), which shares ~90% ANI with species group 1 (Table S2.2; Fig. S2.2). This single-lineage species group can be divided into 8 distinct genotypes (Table S2.1). While the 287 isolates from both species groups comprised 22 genotypes total, 79% of isolates belong to a single genotype within species group 1 (genotype 1 of lineage 1; n = 228; Fig. S2.2).

The majority of isolates sequenced from Field 10 and Field 11G also belong to the epidemic lineage 1 of species group 1, suggesting that these strains dispersed beyond where cowpeas were grown, persisted in the soil for years after cowpea planting stopped, and diverged very little in that time. Ten of the fourteen isolates from Field 10E belong to *Bradyrhizobium yuanmingense* species group 1, with eight of these isolates belonging to lineage 1 and two belonging to lineage 5, and two of the remaining isolates closely related to species group 1. All five of the sequenced isolates from Field 11H also belong to the same epidemic *Bradyrhizobium* lineage (species group 1, lineage 1, Table S2.1; Manci et al., 2022). This suggests that the epidemic lineage 1 of species group 1 may be particularly well-adapted to these fields, as it not only dominated the 2015 Field 11H study, but was also recovered at proportionately high rates in nearby fields years later.

The rhizobial community exhibited no significant structuring by cowpea genotype or field quadrat. When comparing the distribution of lineage 1 (species group 1, n = 231)

against the combined distribution of remaining lineages (n = 56) among quadrats, we found no significant association between rhizobial lineage and field quadrat, $X^2$ (2, $N$ = 287) = 4.72, $p$ = 0.095. Lineage 1 and lineage 2 of species group 1 (i.e. the most abundant clades) were recovered across each of the host genotypes and field transects. The most abundant haplotype (n = 217) was retrieved from each of the 19 host genotypes, as well as across each of the three field sites (Fig. 2.1).

**Comparison with native *Acmispon* community:** We found no shared cowpea-associating rhizobia clones or species between the nearby natural *A. strigosus* populations and any of the neighboring fields (10E, 11G, 11H). Both the agricultural and natural soil communities exhibited epidemic population structure, defined by high relative frequency (>10%) of one or several genotypes at one or multiple sites (Hollowell et al., 2016, Fig. 2.2). Extracting the *recA* and *glnII* genes within the cowpea-associating Field 11 dataset led to a reduction in diversity among *Bradyrhizobium* isolates, with only 6 unique concatenated haplotypes detected, and 95% (n = 273) of isolates belonging to a single clonal haplotype (Fig. 2.2). In contrast, isolates from the natural *Acmispon* site were much more diverse and comprised 110 unique concatenated haplotypes. This lack of overlap might suggest that these communities are differentially adapted to their distinct plant hosts or soil conditions, and that the Field 11 strains did not originally disperse from this nearby natural population.

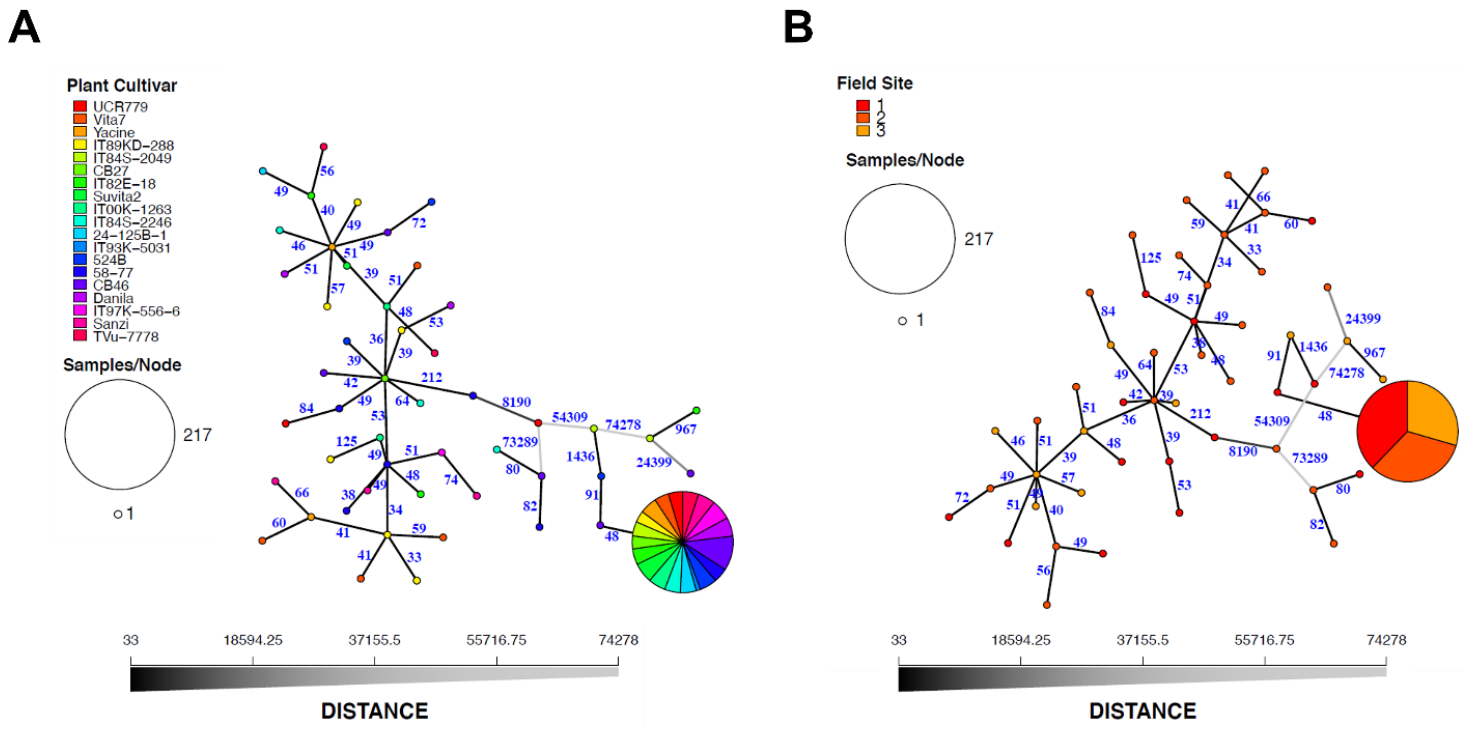


**Figure 2.1.** Minimum Spanning Networks showing the genetic relationships among isolates within the 268-isolate species group, where nodes (or circles) and their size represent genotypes and their abundance, and the density of lines connecting nodes represents the distance (in SNPs) between genotypes. SNP differences of ≤ 33 relative to references for each species group were used to cluster genotypes.

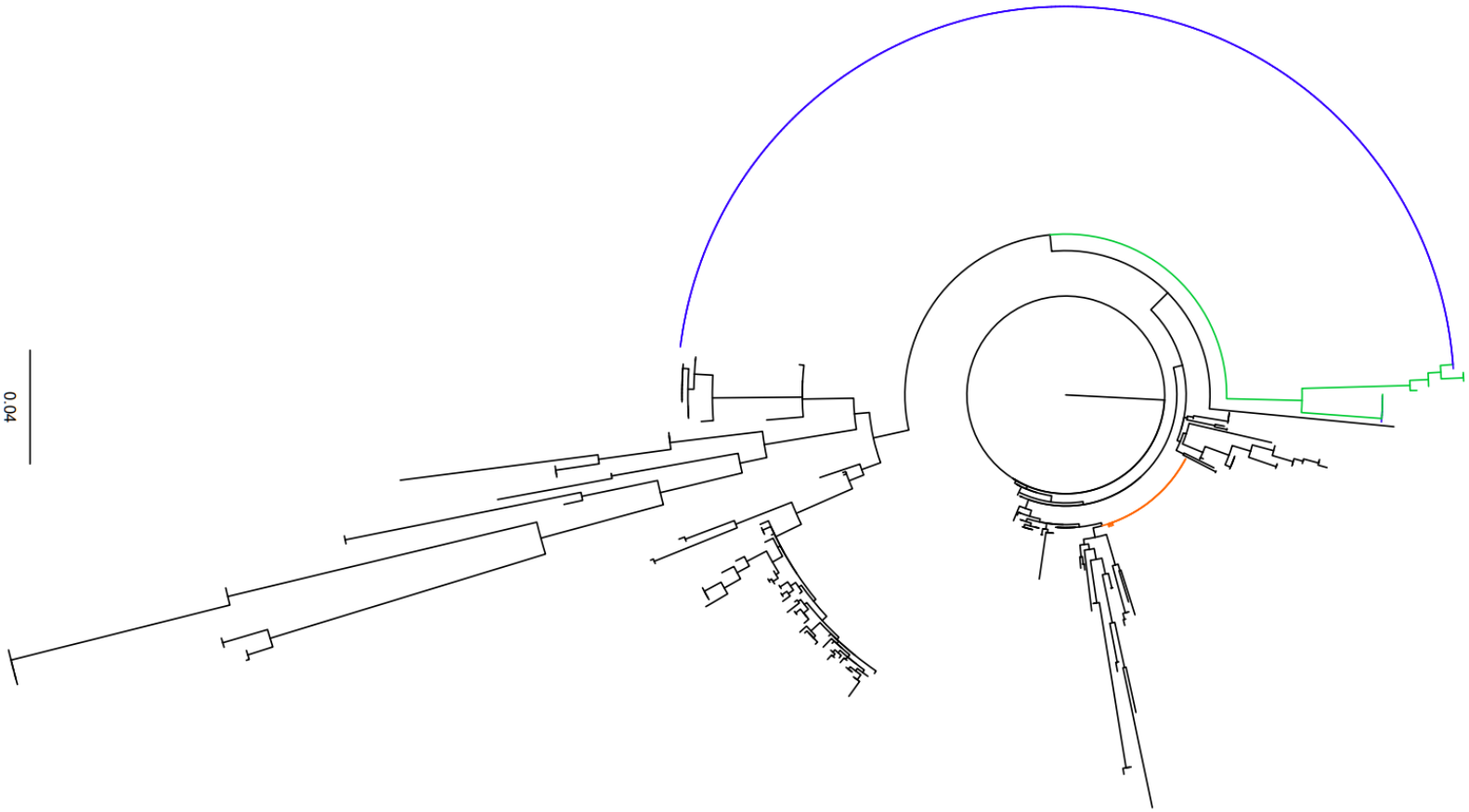


**Figure 2.2.** Phylogenetic tree of concatenated *glnII* and *recA* loci comparing *Bradyrhizobium* isolates from field cowpea and natural *Acmispon* populations. Clades isolated from cowpea are marked in green, with members of the most epidemic haplotype (n = 273) marked in blue. Clades isolated from *Acmispon* are black, with members of the most epidemic haplotype (n = 71) marked in orange.

## Discussion

Our findings revealed two core conclusions regarding the cowpea-associating *Bradyrhizobium* in Field 11H: (1) This low-diversity community exhibits extreme epidemic structure, and (2) This community does not exhibit any spatial or host-driven structuring.

Regarding the epidemic community structure, 93% of the Field 11H isolates belong to 3 highly related genotypes of *Bradyrhizobium yuanmingense* (genotypes 1, 5, and 8; n = 257; Table S2.1; Figure S2.2). Of the six total lineages recovered, five of these lineages (i.e. species group 1, n = 278) share >98% ANI. While we did not expect this low level of diversity, other studies corroborate that field soil conditions can drive epidemic bacterial populations. Modern agronomic practices aim to maximize plant yield, but these practices can also have unexpected effects on bacterial taxonomic and functional diversity in soils. For example, conventional tillage practices can decrease soil microbial biomass and diversity (Ibekwe et al., 2002; Loureiro et al. 2007) as well as decrease species evenness (Legrand et al., 2018) when compared to no or reduced tillage. Tilling increases soil uniformity across a given field, and uniform environments are more likely to lead to microbial competitive exclusion (Cardinale 2011). Fertilization can also reduce diversity in soils (Simonsen et al., 2015; Zhong et al., 2010) in ways that can reduce plant benefits (Weese et al., 2015). The research field used in this study was regularly tilled and occasionally fertilized, both of which could have reduced the belowground rhizobial diversity over time. Field cultivation and management practices can likewise structure belowground microbial communities to favor a small handful of strains. Fields with recent

and distant (eight years) cultivation with clover both had epidemic rhizobial populations, with one or two strains dominating each site in addition to many others at lower frequency, and one strain found in high abundance at both sites (Duodu et al., 2006).

These results somewhat mirror our findings of a core epidemic lineage dominating both Field 11H and 10E, regardless of field planting history. There was significant overlap between the *Bradyrhizobium* recovered from Field 11H in 2015 and the associated isolates from Field 10E and 11G in 2019, with most isolates from each site belonging to the same epidemic lineage (lineage 1, Table S2.2). Field 10E had no prior history of growing legumes, suggesting that the epidemic strains recovered there were dispersed from Field 11 and adapted to similar field conditions, as host-driven selection for these strains would have been absent in this field. This also suggests that these strains have persisted over the years in the same location, including several years without cowpea growth, and that Field 11 soils might be driven by a small handful of highly beneficial strains, rather than a complex community. Similarly, a comparison of rhizobia at arable fields and roadside verge sites found dominant strains in both an arable field where legumes had not been grown for 20 years, as well as an uncultivated verge where wild vetches grew annually, suggesting that epidemic structure can be found regardless of land cultivation or recent/recurring presence of legumes, and that heightened fitness in soils (compared to fitness in planta) can allow rhizobia to persist in diverse environments (Handley et al., 1998). Cowpea-associated rhizobial communities in cultivated and uncultivated regions of Africa did not significantly differ in terms of overall abundance or the abundance of bacterial clades, with differences in community structure among sites more correlated with

environmental factors (Ndungu et al., 2018). In a coal field recovery study, the same few rhizobial genotypes dominated all fields, regardless of trap host species or recent cultivation of alfalfa, again pointing to soil fitness as a driving factor of rhizobial dominance (Zhang et al., 2001). These datasets suggest that epidemic rhizobia populations are not uncommon, and that rhizobia fitness and community structure are primarily driven by soils, not necessarily by variation in host plants or land use.

In contrast to the adjacent fields, the epidemic genotypes recovered in 11H were absent in deeply sampled neighboring natural *A. strigosus* communities, suggesting differential adaptation either to plant hosts (i.e. different legume species) or soil conditions (cultivated vs. uncultivated). While these strains were isolated from a different host species, the most highly-recovered haplotype has the capacity to broadly nodulate a diversity of other legume species (La Pierre et al. 2017). Since natural populations can serve as a source for managed soil microbial populations, and horizontal gene transfer of chromosomal elements is common among rhizobia, we expected some overlap with the Field 11 isolates. However, we found no overlap when comparing alleles at two chromosomal loci (*recA, glnII*). This suggests that the source of these epidemic strains is not from nearby natural soil populations but is perhaps due to long-term host selection and adaptation, selection by field cultivation due to tillage and soil conditions, or genetic drift. In this case, genetic drift seems unlikely, as the microbial community is highly beneficial to cowpea.

There was no evidence that the *Bradyrhizobium* community was structured by cowpea host genotype or by field quadrat, as the most abundant genotype dominates each of the 19 host genotypes and 3 field regions. The lack of community structuring by host

genotype was surprising, as rhizobial strain occupancy and host compatibility can significantly differ among legume cultivars within the same host species (George et al., 1997; Rigg et al., 2021). However, the power of host control is limited; belowground microbial communities can also exhibit a lack structuring by plants, especially among plants within the same species, where host genotype can have little to no effect on the structure or diversity of associated bacterial communities (Corneo et al. 2016; Leite et al. 2017). Additionally, soil microbial community structure can persist long-term, even when plant functional groups are selectively removed, suggesting insignificant plant-driven structuring over time (Marshall et al. 2011). We had also expected some structuring by field region, as rhizobial communities are typically structured by soil conditions, including soil type, pH, and nutrient availability, which can vary across a field (Chaudri et al. 2000; Leite et al. 2017; de Castro Pires et al. 2018; Rascovan et al. 2016). Soil nutrients and biotic factors, such as nematodes, can also influence rhizobial access to roots and drive significant differences in nodulation (Horiuchi et al. 2005; Wang et al. 2022). However, some rhizobia dominate landscapes irrespective of host or soil variation (McInnes et al. 2004; Vinuesa et al. 2005). These genotypes are hypothesized to be favored by fitness superiority relative to other strains (Hollowell et al. 2015, 2016). Additionally, Fields 11H, 11G, and 10E are each made up of the same soil type (Chapter 3) and all have a history of tillage. While we did not uncover evidence of spatial structuring, we cannot conclude whether variation in soil conditions might have influenced the recovered epidemic community structure.

Selection by plant hosts can, however, drive a reduction in microbial diversity long-term via repeated selection of the most desirable partners, suggesting that cowpea hosts

might be driving the unstructured community of rhizobia in this field over time. In managed settings where a crop species is recurrently grown, soils tend to have a lower diversity of associated microbial partners (Rodrigues et al. 2013; French et al. 2017; Legrand et al. 2018; Stephan et al. 2000; Wang et al. 2021). While several species of legume had been intercropped at the field site used in this experiment, cowpeas were grown every other year for 10 years, suggesting recurrent host selection for competitive rhizobial strains. Additionally, there is some evidence that post-infection sanctioning of undesirable microbial partners may be a fixed trait for certain species of legumes, suggesting limited within-species variation in host control and subsequent effects on soil microbial structure in field settings (Wendlandt et al. 2019; Ortiz-Barbosa et al. 2022). This could serve as a potential explanation of the lack of plant-driven structuring of rhizobial communities in Field 11, as each of the genotypes tested belong to the same plant species. Without variation in host-driven selection or spatial selection due to conventional cropping practices, the expectation would be consistent, low-diversity microbial communities across field locales. When nodules were collected from fifty soybean fields across China, geographic distance did not significantly influence nodule rhizobial communities, and 14 of the 16 rhizobial OTUs occupied at least 50% of the sites sampled, suggesting both that these isolates are generalists and that soybean selection of rhizobia is relatively consistent among sites and cultivars (Zhang et al., 2018).

In conclusion, these results suggest that the high benefits of certain field soils might be explained by the presence of a small handful of strains due to increased fitness in soils or plant roots. In conjunction with previous studies, these data suggest that neither host

genotype nor within field spatial structure are predominant drivers of legume-associated rhizobia communities (Ortiz-Barbosa et al., 2022; Manci et al., 2022). Selection of dominant strains might be driven by cowpea hosts over time, though this selection appears to not be differentially shaped by host genotype. These data support the hypothesis that partner selection is uniform across cowpea cultivars, suggesting that it may be a fixed trait as it is in other legumes (Wendlandt et al. 2019; Ortiz-Barbosa et al. 2022). The dominance of these genotypes among cowpea genotypes also suggests that these genotypes of *Bradyrhizobium* are generalists in their association with cowpea.

## Tables

**Table 2.1.** Cowpea genotypes used in this study, including their origin of development and traits. Origin abbreviations refer to: the University of California, Riverside (UCR); the International Institute of Tropical Agriculture in Nigeria (IITA), the Institute of Agricultural Research & Development in Cameroon (IRAD), the Institute of Agricultural Research in Senegal (ISRA), and the Institute of Environmental & Agricultural Research in Burkina Faso (INERA). Citations: 1. Muñoz-Amatriaín et al. 2017 (Table S3), 2. Agbicodo et al. 2009, 3. Hall et al. 2003, 4. Huynh et al. 2018, 5. Pottorff et al. 2012, 6. Huynh et al. 2022, 7. Roberts et al. 1996, 8. Silva et al. 2019

| Genotype | Origin | Biotic Stress | Abiotic Stress | Agronomic Traits | Citations |
|---|---|---|---|---|---|
| 524-B | UCR | Nematode, *Fusarium* wilt resistant | | Large seed, blackeye type | 1 |
| Danila | IITA | Nematode, Fusarium, Foliar thrips resistant | Drought tolerant | Rough white seeds | 1 |
| TVu-7778 | IITA | N/A | Drought susceptible | | 1, 2 |
| UCR 779 | Botswana | Aphid resistant | | | 1, 3 |
| IT84S-2049 | IITA | Aphid, bacterial blight, nematode resistant | | Small seeds | 1 |
| CB27 | UCR | Fusarium and nematode resistant | Heat tolerant | Black-eye type | 1, 4 |
| 24-125B-1 | IRAD | Fusarium resistant**,** Fot race 4 susceptible | | Sweet taste | 1, 5 |
| 58-77 | ISRA | Aphid, & thrips resistant | | | |
| CB46 | California | Fusarium & nematode resistant, aphid susceptible | | Black-eye type | 1, 6 |
| IT97K-556-6 | IITA | Aphid resistant | | | 1 |
| Sanzi | Ghana | Flower thrips resistant | | Sub-globose leaves | 1 |
| Vita 7 | IITA | Macrophomina resistant, aphid susceptible | | Smooth tan seeds, hastate leaf shape | 1, 8 |
| Yacine | ISRA | Aphid, bacterial blight, cowpea aphid-borne mosaic virus resistant | | Early maturity, brown seeds | 1 |
| IT89KD-288 | IITA | Nematode resistant | | High yield, white rough seeds | 1, 4 |
| IT82E-18 | IITA | Nematode resistant | | High yield, light brown seed | 1, 4 |
| Suvita 2 | INERA | Striga, macrophomina resistant | Drought tolerant | High yield | 1 |
| IT00K-1263 | IITA | Striga, aphid, fusarium, and nematode resistant | | | 1, 4 |
| IT84S-2246 | IITA | Aphid, bacterial blight, viruses, nematode resistant | | | 1, 4 |
| IT93K-503-1 | IITA | Nematode, macrophomina, striga, and fusarium resistant | Drought tolerant | Stay-green | 1, 4 |

## References


Agbicodo EM, Fatokun CA, Muranaka S, et al (2009) Breeding drought tolerant cowpea: constraints, accomplishments, and future prospects. Euphytica 167:353–370. https://doi.org/10.1007/s10681-009-9893-8

Ali S, Xie L (2019) Plant Growth Promoting and Stress mitigating abilities of Soil Born Microorganisms. Recent Pat Food Nutr Agric. https://doi.org/10.2174/2212798410666190515115548

Amaresan N, Jayakumar V, Kumar K, Thajuddin N (2019) Biocontrol and plant growth-promoting ability of plant-associated bacteria from tomato (Lycopersicum esculentum) under field condition. Microbial Pathogenesis 136:103713. https://doi.org/10.1016/j.micpath.2019.103713

Balatti PA, Pueppke SG (1990) Cultivar-Specific Interactions of Soybean with Rhizobium fredii Are Regulated by the Genotype of the Root 1. Plant Physiol 94:1907–1909

Bankevich A, Nurk S, Antipov D, et al (2012) SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. Journal of Computational Biology 19:455–477. https://doi.org/10.1089/cmb.2012.0021

Berg G (2009) Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl Microbiol Biotechnol 84:11–18. https://doi.org/10.1007/s00253-009-2092-7

Bhardwaj D, Ansari MW, Sahoo RK, Tuteja N (2014) Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. Microb Cell Fact 13:66. https://doi.org/10.1186/1475-2859-13-66

Bissett A, Richardson AE, Baker G, Thrall PH (2011) Long-term land use effects on soil microbial community structure and function. Applied Soil Ecology 51:66–78. https://doi.org/10.1016/j.apsoil.2011.08.010

Boukar O, Fatokun CA, Huynh B-L, et al (2016) Genomic Tools in Cowpea Breeding Programs: Status and Perspectives. Front Plant Sci 7:. https://doi.org/10.3389/fpls.2016.00757

Buckley DH, Schmidt TM (2001) The structure of microbial communities in soil and the lasting impact of cultivation. Microb Ecol 42:11–21. https://doi.org/10.1007/s002480000108

Cardinale BJ (2011) Biodiversity improves water quality through niche partitioning. Nature 472:86–89. https://doi.org/10.1038/nature09904

Chamber MA, Iruthayathas EE (1988) Nodulation and nitrogen fixation by fast- and slow-growing rhizobia strains of soybean on several temperate and tropical legumes. Plant Soil 112:239–245. https://doi.org/10.1007/BF02140001

Chaudhry V, Rehman A, Mishra A, et al (2012) Changes in Bacterial Community Structure of Agricultural Land Due to Long-Term Organic and Chemical Amendments. Microb Ecol 64:450–460. https://doi.org/10.1007/s00248-012-0025-y

Chaudri AM, Allain CMG, Barbosa-Jefferson VL, et al (2000) A study of the impacts of Zn and Cu on two rhizobial species in soils of a long-term field experiment. Plant and Soil 221:167–179. https://doi.org/10.1023/A:1004735705492

Chen S, Zhou Y, Chen Y, Gu J (2018) fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34:i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Corander J, Tang J (2007) Bayesian analysis of population structure based on linked molecular information. Mathematical Biosciences 205:19–31. https://doi.org/10.1016/j.mbs.2006.09.015

Corneo PE, Suenaga H, Kertesz MA, Dijkstra FA (2016) Effect of twenty four wheat genotypes on soil biochemical and microbial properties. Plant Soil 404:141–155. https://doi.org/10.1007/s11104-016-2833-1

de Castro Pires R, dos Reis Junior FB, Zilli JE, et al (2018) Soil characteristics determine the rhizobia in association with different species of Mimosa in central Brazil. Plant Soil 423:411–428. https://doi.org/10.1007/s11104-017-3521-5

De Roy K, Marzorati M, Negroni A, et al (2013) Environmental conditions and community evenness determine the outcome of biological invasion. Nat Commun 4:1383. https://doi.org/10.1038/ncomms2392

Denison RF, Kiers ET (2004) Lifestyle alternatives for rhizobia: mutualism, parasitism, and forgoing symbiosis. FEMS Microbiol Lett 237:187–193. https://doi.org/10.1111/j.1574-6968.2004.tb09695.x

Duodu S, Carlsson G, Huss-Danell K, Svenning M m. (2007) Large genotypic variation but small variation in N2 fixation among rhizobia nodulating red clover in soils of northern Scandinavia. Journal of Applied Microbiology 102:1625–1635. https://doi.org/10.1111/j.1365-2672.2006.03196.x

Fernández-González AJ, Cardoni M, Gómez-Lama Cabanás C, et al (2020) Linking belowground microbial network changes to different tolerance level towards Verticillium wilt of olive. Microbiome 8:11. https://doi.org/10.1186/s40168-020-0787-2

Fitzpatrick CR, Mustafa Z, Viliunas J (2019) Soil microbes alter plant fitness under competition and drought. Journal of Evolutionary Biology 32:438–450. https://doi.org/10.1111/jeb.13426

Foyer CH, Lam H-M, Nguyen HT, et al (2016) Neglecting legumes has compromised human health and sustainable food production. Nat Plants 2:16112. https://doi.org/10.1038/nplants.2016.112

Franklin RB, Mills AL (2003) Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. FEMS Microbiology Ecology 44:335–346. https://doi.org/10.1016/S0168-6496(03)00074-6

French KE, Tkacz A, Turnbull LA (2017) Conversion of grassland to arable decreases microbial diversity and alters community composition. Applied Soil Ecology 110:43–52. https://doi.org/10.1016/j.apsoil.2016.10.015

Friesen ML, Porter SS, Stark SC, et al (2011) Microbially Mediated Plant Functional Traits. Annual Review of Ecology, Evolution, and Systematics 42:23–46. https://doi.org/10.1146/annurev-ecolsys-102710-145039

Gano-Cohen K, Wendlandt C, Moussawi K, et al (2020) Recurrent mutualism breakdown events in a legume rhizobia metapopulation. Proceedings of the Royal Society B: Biological Sciences 287:20192549. https://doi.org/10.1098/rspb.2019.2549

George T, Bohlool BB, Singleton PW (1987) Bradyrhizobium japonicum-Environment Interactions: Nodulation and Interstrain Competition in Soils along an Elevational Transect. Appl Environ Microbiol 53:1113–1117

Grossman JM, Schipanski ME, Sooksanguan T, et al (2011) Diversity of rhizobia in soybean [Glycine max (Vinton)] nodules varies under organic and conventional management. Applied Soil Ecology 50:14–20. https://doi.org/10.1016/j.apsoil.2011.08.003

Haichar F el Z, Marol C, Berge O, et al (2008) Plant host habitat and root exudates shape soil bacterial community structure. The ISME Journal 2:1221–1230. https://doi.org/10.1038/ismej.2008.80

Hall AE, Cisse N, Thiaw S, et al (2003) Development of cowpea cultivars and germplasm by the Bean/Cowpea CRSP. Field Crops Research 82:103–134. https://doi.org/10.1016/S0378-4290(03)00033-9

Handley BA, Hedges AJ, Beringer JE (1998) Importance of host plants for detecting the population diversity of Rhizobium leguminosarum biovar viciae in soil. Soil Biology and Biochemistry 30:241–249. https://doi.org/10.1016/S0038-0717(97)00103-X

Haney CH, Samuel BS, Bush J, Ausubel FM (2015) Associations with rhizosphere bacteria can confer an adaptive advantage to plants. Nature Plants 1:1–9. https://doi.org/10.1038/nplants.2015.51

Hargreaves SK, Williams RJ, Hofmockel KS (2015) Environmental Filtering of Microbial Communities in Agricultural Soil Shifts with Crop Growth. PLOS ONE 10:e0134345. https://doi.org/10.1371/journal.pone.0134345

Hayat R, Ali S, Amara U, et al (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598. https://doi.org/10.1007/s13213-010-0117-1

Heath KD, Stinchcombe JR (2014) Explaining Mutualism Variation: A New Evolutionary Paradox? Evolution 68:309–317. https://doi.org/10.1111/evo.12292

Herniter IA, Muñoz-Amatriaín M, Close TJ (2020) Genetic, textual, and archeological evidence of the historical global spread of cowpea (Vigna unguiculata [L.] Walp.). Legume Science 2:e57. https://doi.org/10.1002/leg3.57

Hoeksema JD, Chaudhary VB, Gehring CA, et al (2010) A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. Ecology Letters 13:394–407. https://doi.org/10.1111/j.1461-0248.2009.01430.x

Hollowell AC, Gano KA, Lopez G, et al (2015) Native California soils are selective reservoirs for multidrug-resistant bacteria. Environ Microbiol Rep 7:442–449. https://doi.org/10.1111/1758-2229.12269

Hollowell AC, Regus JU, Gano KA, et al (2016a) Epidemic Spread of Symbiotic and Non-Symbiotic Bradyrhizobium Genotypes Across California. Microb Ecol 71:700–710. https://doi.org/10.1007/s00248-015-0685-5

Hollowell AC, Regus JU, Turissini D, et al (2016b) Metapopulation dominance and genomic-island acquisition of Bradyrhizobium with superior catabolic capabilities. Proceedings of the Royal Society B: Biological Sciences 283:20160496. https://doi.org/10.1098/rspb.2016.0496

Horiuchi J, Prithiviraj B, Bais HP, et al (2005) Soil nematodes mediate positive interactions between legume plants and rhizobium bacteria. Planta 222:848–857. https://doi.org/10.1007/s00425-005-0025-y

Hosseinalizadeh Nobarinezhad M, Wallace LE (2020) Fine-Scale Patterns of Genetic Structure in the Host Plant Chamaecrista fasciculata (Fabaceae) and Its Nodulating Rhizobia Symbionts. Plants 9:1719. https://doi.org/10.3390/plants9121719

Huynh B-L, Close TJ, Roberts PA, et al (2013) Gene Pools and the Genetic Architecture of Domesticated Cowpea. The Plant Genome 6:plantgenome2013.03.0005. https://doi.org/10.3835/plantgenome2013.03.0005

Huynh B-L, Duong T, Clark NE, et al (2022) Registration of aphid-resistant 'California Blackeye 77' cowpea. Journal of Plant Registrations 16:13–20. https://doi.org/10.1002/plr2.20176

Huynh B-L, Ehlers JD, Close TJ, Roberts PA (2019) Registration of a Cowpea [Vigna unguiculata (L.) Walp.] Multiparent Advanced Generation Intercross (MAGIC) Population. Journal of Plant Registrations 13:281–286. https://doi.org/10.3198/jpr2018.04.0020crmp

Huynh B-L, Ehlers JD, Huang BE, et al (2018) A multi-parent advanced generation inter-cross (MAGIC) population for genetic analysis and improvement of cowpea (Vigna unguiculata L. Walp.). The Plant Journal 93:1129–1142. https://doi.org/10.1111/tpj.13827

Ibekwe AM, Kennedy AC, Frohne PS, et al (2002) Microbial diversity along a transect of agronomic zones. FEMS Microbiology Ecology 39:183–191. https://doi.org/10.1111/j.1574-6941.2002.tb00921.x

Kakraliya SK, Singh U, Bohra A, et al (2018) Nitrogen and Legumes: A Meta-analysis. In: Meena RS, Das A, Yadav GS, Lal R (eds) Legumes for Soil Health and Sustainable Management. Springer, Singapore, pp 277–314

Kaminsky LM, Trexler RV, Malik RJ, et al (2019) The Inherent Conflicts in Developing Soil Microbial Inoculants. Trends in Biotechnology 37:140–151. https://doi.org/10.1016/j.tibtech.2018.11.011

Kamvar ZN, Tabima JF, Grünwald NJ (2014) Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2:e281. https://doi.org/10.7717/peerj.281

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30:3059–3066. https://doi.org/10.1093/nar/gkf436

Keyser HH, van Berkum P, Weber DF (1982) A Comparative Study of the Physiology of Symbioses Formed by Rhizobium japonicum with Glycine max, Vigna unguiculata, and Macroptilium atropurpurem 1. Plant Physiology 70:1626–1630. https://doi.org/10.1104/pp.70.6.1626

Khan MN, Ijaz M, Ali Q, et al (2019) Biological Nitrogen Fixation in Nutrient Management. In: Hasanuzzaman M (ed) Agronomic Crops: Volume 2: Management Practices. Springer, Singapore, pp 127–147

Kiers ET, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume–rhizobium mutualism. Nature 425:78–81. https://doi.org/10.1038/nature01931

Kuypers MMM, Marchant HK, Kartal B (2018) The microbial nitrogen-cycling network. Nat Rev Microbiol 16:263–276. https://doi.org/10.1038/nrmicro.2018.9

La Pierre KJ, Simms EL, Tariq M, et al (2017) Invasive legumes can associate with many mutualists of native legumes, but usually do not. Ecology and Evolution 7:8599–8611. https://doi.org/10.1002/ece3.3310

Lau JA, Lennon JT (2012) Rapid responses of soil microorganisms improve plant fitness in novel environments. Proceedings of the National Academy of Sciences 109:14058–14062. https://doi.org/10.1073/pnas.1202319109

Legrand F, Picot A, Cobo-Díaz JF, et al (2018) Effect of tillage and static abiotic soil properties on microbial diversity. Applied Soil Ecology 132:135–145. https://doi.org/10.1016/j.apsoil.2018.08.016

Leite J, Fischer D, Rouws LFM, et al (2017) Cowpea Nodules Harbor Non-rhizobial Bacterial Communities that Are Shaped by Soil Type Rather than Plant Genotype. Front Plant Sci 7:. https://doi.org/10.3389/fpls.2016.02064

Leite J, Seido SL, Passos SR, et al (2009) Biodiversity of rhizobia associated with cowpea cultivars in soils of the lower half of the São Francisco River Valley. Rev Bras Ciênc Solo 33:1215–1226. https://doi.org/10.1590/S0100-06832009000500015

Lopez-Velasco G, Welbaum GE, Falkinham III JO, Ponder MA (2011) Phyllopshere Bacterial Community Structure of Spinach (Spinacia oleracea) as Affected by Cultivar and Environmental Conditions at Time of Harvest. Diversity 3:721–738. https://doi.org/10.3390/d3040721

Loureiro M de F, Kaschuk G, Alberton O, Hungria M (2007) Soybean [Glycine max (L.) Merrill] rhizobial diversity in Brazilian oxisols under various soil, cropping, and inoculation managements. Biol Fertil Soils 43:665–674. https://doi.org/10.1007/s00374-006-0146-x

Lugtenberg B, Kamilova F (2009) Plant-Growth-Promoting Rhizobacteria. Annual Review of Microbiology 63:541–556. https://doi.org/10.1146/annurev.micro.62.081307.162918

Mallon CA, Le Roux X, van Doorn GS, et al (2018) The impact of failure: unsuccessful bacterial invasions steer the soil microbial community away from the invader's niche. ISME J 12:728–741. https://doi.org/10.1038/s41396-017-0003-y

Manci M, Mercado OG, Camantigue RX, et al (2022) Live soil inocula, not host population or domestication status, is the predominant driver of growth benefits to cowpea. Plant Soil. https://doi.org/10.1007/s11104-022-05709-6

Marshall CB, McLaren JR, Turkington R (2011) Soil microbial communities resistant to changes in plant functional group composition. Soil Biology and Biochemistry 43:78–85. https://doi.org/10.1016/j.soilbio.2010.09.016

McInnes A, Thies JE, Abbott LK, Howieson JG (2004) Structure and diversity among rhizobial strains, populations and communities–a review. Soil Biology and Biochemistry 36:1295–1308. https://doi.org/10.1016/j.soilbio.2004.04.011

McKenna A, Hanna M, Banks E, et al (2010) The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20:1297–1303. https://doi.org/10.1101/gr.107524.110

Micallef SA, Shiaris MP, Colón-Carmona A (2009) Influence of Arabidopsis thaliana accessions on rhizobacterial communities and natural variation in root exudates. J Exp Bot 60:1729–1742. https://doi.org/10.1093/jxb/erp053

Moawad H, Beck DP (1991) Some characteristics of Rhizobium leguminosarum isolates from uninoculated field-grown lentil. Soil Biology and Biochemistry 23:933–937. https://doi.org/10.1016/0038-0717(91)90173-H

Mueller UG, Sachs JL (2015) Engineering Microbiomes to Improve Plant and Animal Health. Trends in Microbiology 23:606–617. https://doi.org/10.1016/j.tim.2015.07.009

Muñoz-Amatriaín M, Mirebrahim H, Xu P, et al (2017) Genome resources for climate-resilient cowpea, an essential crop for food security. The Plant Journal 89:1042–1054. https://doi.org/10.1111/tpj.13404

Mutch LA, Young JPW (2004) Diversity and specificity of Rhizobium leguminosarum biovar viciae on wild and cultivated legumes. Molecular Ecology 13:2435–2444. https://doi.org/10.1111/j.1365-294X.2004.02259.x

Ndungu SM, Messmer MM, Ziegler D, et al (2018) Cowpea (Vigna unguiculata L. Walp) hosts several widespread bradyrhizobial root nodule symbionts across contrasting agro-ecological production areas in Kenya. Agriculture, Ecosystems & Environment 261:161–171. https://doi.org/10.1016/j.agee.2017.12.014

Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. Molecular Biology and Evolution 32:268–274. https://doi.org/10.1093/molbev/msu300

Nunan N, Wu K, Young IM, et al (2002) In Situ Spatial Patterns of Soil Bacterial Populations, Mapped at Multiple Scales, in an Arable Soil. Microb Ecol 44:296–305. https://doi.org/10.1007/s00248-002-2021-0

O'Callaghan M, Ballard RA, Wright D (2022) Soil microbial inoculants for sustainable agriculture: Limitations and opportunities. Soil Use and Management 38:1340–1369. https://doi.org/10.1111/sum.12811

Ortiz-Barbosa GS, Torres-Martínez L, Manci A, et al (2022) No disruption of rhizobial symbiosis during early stages of cowpea domestication. Evolution 76:496–511. https://doi.org/10.1111/evo.14424

Owen D, Williams AP, Griffith GW, Withers PJA (2015) Use of commercial bio-inoculants to increase agricultural production through improved phosphrous acquisition. Applied Soil Ecology 86:41–54. https://doi.org/10.1016/j.apsoil.2014.09.012

Pahua VJ, Stokes PJN, Hollowell AC, et al (2018) Fitness variation among host species and the paradox of ineffective rhizobia. Journal of Evolutionary Biology 31:599–610. https://doi.org/10.1111/jeb.13249

Palansooriya KN, Wong JTF, Hashimoto Y, et al (2019) Response of microbial communities to biochar-amended soils: a critical review. Biochar 1:3–22. https://doi.org/10.1007/s42773-019-00009-2

Palmer KM, Young JPW (2000) Higher Diversity of Rhizobium leguminosarum Biovar viciae Populations in Arable Soils than in Grass Soils. Applied and Environmental Microbiology 66:2445–2450. https://doi.org/10.1128/AEM.66.6.2445-2450.2000

Pottorff M, Wanamaker S, Ma YQ, et al (2012) Genetic and Physical Mapping of Candidate Genes for Resistance to Fusarium oxysporum f.sp. tracheiphilum Race 3 in Cowpea [Vigna unguiculata (L.) Walp]. PLoS One 7:e41600. https://doi.org/10.1371/journal.pone.0041600

Rascovan N, Carbonetto B, Perrig D, et al (2016) Integrated analysis of root microbiomes of soybean and wheat from agricultural fields. Sci Rep 6:28084. https://doi.org/10.1038/srep28084

Regus JU, Wendlandt CE, Bantay RM, et al (2017) Nitrogen deposition decreases the benefits of symbiosis in a native legume. Plant Soil 414:159–170. https://doi.org/10.1007/s11104-016-3114-8

Rigg JL, Webster AT, Harvey DM, et al (2021) Cross-host compatibility of commercial rhizobial strains for new and existing pasture legume cultivars in south-eastern Australia. Crop Pasture Sci. https://doi.org/10.1071/CP20234

Roberts PA, Matthews WC, Ehlers JD (1996) New Resistance to Virulent Root-Knot Nematodes Linked to the Rk Locus of Cowpea. Crop Science 36:cropsci1996.0011183X0036000400012x. https://doi.org/10.2135/cropsci1996.0011183X0036000400012x

Rodrigues JLM, Pellizari VH, Mueller R, et al (2013) Conversion of the Amazon rainforest to agriculture results in biotic homogenization of soil bacterial communities. Proceedings of the National Academy of Sciences 110:988–993. https://doi.org/10.1073/pnas.1220608110

Rolli E, Marasco R, Vigani G, et al (2015) Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. Environmental Microbiology 17:316–331. https://doi.org/10.1111/1462-2920.12439

Sachs JL, Kembel SW, Lau AH, Simms EL (2009) In Situ Phylogenetic Structure and Diversity of Wild Bradyrhizobium Communities. Applied and Environmental Microbiology 75:4727–4735. https://doi.org/10.1128/AEM.00667-09

Sawada H, Kuykendall LD, Young JM (2003) Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. J Gen Appl Microbiol 49:155–179. https://doi.org/10.2323/jgam.49.155

Schlatter DC, Bakker MG, Bradeen JM, Kinkel LL (2015) Plant community richness and microbial interactions structure bacterial communities in soil. Ecology 96:134–142. https://doi.org/10.1890/13-1648.1

Seemann T (2014) Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153

Sharaf H, Rodrigues RR, Moon J, et al (2019) Unprecedented bacterial community richness in soybean nodules vary with cultivar and water status. Microbiome 7:63. https://doi.org/10.1186/s40168-019-0676-8

Sievers F, Wilm A, Dineen D, et al (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular Systems Biology 7:539. https://doi.org/10.1038/msb.2011.75

Silva C, Eguiarte LE, Souza V (1999) Reticulated and epidemic population genetic structure of Rhizobium etli biovar phaseoli in a traditionally managed locality in Mexico. Molecular Ecology 8:277–287. https://doi.org/10.1046/j.1365-294X.1999.00564.x

Silva LC da, Nere DR, Bleicher E, et al (2019) Preferences and demographic parameters of cowpea aphid on advanced lines of semiprostrate cowpea. Pesq agropec bras 54:. https://doi.org/10.1590/S1678-3921.pab2019.v54.00230

Simms EL, Taylor DL, Povich J, et al (2006) An empirical test of partner choice mechanisms in a wild legume–rhizobium interaction. Proceedings of the Royal Society B: Biological Sciences 273:77–81. https://doi.org/10.1098/rspb.2005.3292

Simonsen AK, Han S, Rekret P, et al (2015) Short-term fertilizer application alters phenotypic traits of symbiotic nitrogen fixing bacteria. PeerJ 3:e1291. https://doi.org/10.7717/peerj.1291

Smith JM, Feil EJ, Smith NH (2000) Population structure and evolutionary dynamics of pathogenic bacteria. BioEssays 22:1115–1122. https://doi.org/10.1002/1521-1878(200012)22:12<1115::AID-BIES9>3.0.CO;2-R

Stephan A, Meyer AH, Schmid B (2000) Plant diversity affects culturable soil bacteria in experimental grassland communities. Journal of Ecology 88:988–998. https://doi.org/10.1046/j.1365-2745.2000.00510.x

Tan S, Jiang Y, Song S, et al (2013) Two Bacillus amyloliquefaciens strains isolated using the competitive tomato root enrichment method and their effects on suppressing Ralstonia solanacearum and promoting tomato plant growth. Crop Protection 43:134–140. https://doi.org/10.1016/j.cropro.2012.08.003

Thilakarathna MS, Raizada MN (2017) A meta-analysis of the effectiveness of diverse rhizobia inoculants on soybean traits under field conditions. Soil Biology and Biochemistry 105:177–196. https://doi.org/10.1016/j.soilbio.2016.11.022

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. https://doi.org/10.1046/j.1365-2664.2000.00470.x

Tian X, Wang C, Bao X, et al (2019) Crop diversity facilitates soil aggregation in relation to soil microbial community composition driven by intercropping. Plant Soil 436:173–192. https://doi.org/10.1007/s11104-018-03924-8

Tiemann LK, Grandy AS, Atkinson EE, et al (2015) Crop rotational diversity enhances belowground communities and functions in an agroecosystem. Ecology Letters 18:761–771. https://doi.org/10.1111/ele.12453

Tonkin-Hill G, MacAlasdair N, Ruis C, et al (2020) Producing polished prokaryotic pangenomes with the Panaroo pipeline. Genome Biol 21:180. https://doi.org/10.1186/s13059-020-02090-4

Triplett EW, Sadowsky MJ (1992) Genetics of Competition for Nodulation of Legumes. Annual Review of Microbiology 46:399–422. https://doi.org/10.1146/annurev.mi.46.100192.002151

Vacheron J, Desbrosses G, Bouffaud M-L, et al (2013) Plant growth-promoting rhizobacteria and root system functioning. Front Plant Sci 4:. https://doi.org/10.3389/fpls.2013.00356

van Loon LC (2007) Plant responses to plant growth-promoting rhizobacteria. Eur J Plant Pathol 119:243–254. https://doi.org/10.1007/s10658-007-9165-1

VanInsberghe D, Maas KR, Cardenas E, et al (2015) Non-symbiotic Bradyrhizobium ecotypes dominate North American forest soils. ISME J 9:2435–2441. https://doi.org/10.1038/ismej.2015.54

Vinuesa P, Silva C, Werner D, Martínez-Romero E (2005) Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in Bradyrhizobium species cohesion and delineation. Mol Phylogenet Evol 34:29–54. https://doi.org/10.1016/j.ympev.2004.08.020

Wang G, Bei S, Li J, et al (2021) Soil microbial legacy drives crop diversity advantage: Linking ecological plant–soil feedback with agricultural intercropping. Journal of Applied Ecology 58:496–506. https://doi.org/10.1111/1365-2664.13802

Wang Q, Sheng J, Pan L, et al (2022) Soil property determines the ability of rhizobial inoculation to enhance nitrogen fixation and phosphorus acquisition in soybean. Applied Soil Ecology 171:104346. https://doi.org/10.1016/j.apsoil.2021.104346

Weert S de, Kuiper I, Kamilova FD, et al (2007) The role of competitive root tip colonization in the biological control of tomato foot and root rot. Biological control of plant diseases 103–122

Weese DJ, Heath KD, Dentinger BTM, Lau JA (2015) Long-term nitrogen addition causes the evolution of less-cooperative mutualists. Evolution 69:631–642. https://doi.org/10.1111/evo.12594

Wendlandt CE, Regus JU, Gano-Cohen KA, et al (2019a) Host investment into symbiosis varies among genotypes of the legume Acmispon strigosus, but host sanctions are uniform. New Phytologist 221:446–458. https://doi.org/10.1111/nph.15378

Wendlandt CE, Regus JU, Gano-Cohen KA, et al (2019b) Host investment into symbiosis varies among genotypes of the legume Acmispon strigosus, but host sanctions are uniform. New Phytologist 221:446–458. https://doi.org/10.1111/nph.15378

Wu L, Wang J, Huang W, et al (2015) Plant-microbe rhizosphere interactions mediated by Rehmannia glutinosa root exudates under consecutive monoculture. Sci Rep 5:15871. https://doi.org/10.1038/srep15871

Xiong W, Li Z, Liu H, et al (2015) The Effect of Long-Term Continuous Cropping of Black Pepper on Soil Bacterial Communities as Determined by 454 Pyrosequencing. PLOS ONE 10:e0136946. https://doi.org/10.1371/journal.pone.0136946

Xue D, Christenson R, Genger R, et al (2018) Soil Microbial Communities Reflect both Inherent Soil Properties and Management Practices in Wisconsin Potato Fields. Am J Potato Res 95:696–708. https://doi.org/10.1007/s12230-018-9677-6

Yelton MM, Yang SS, Edie SA, Lim STY 1983 Characterization of an Effective Salt-tolerant, Fast-growing Strain of Rhizobium japonicum. Microbiology 129:1537–1547. https://doi.org/10.1099/00221287-129-5-1537

Zakria M, Njoloma J, Saeki Y, Akao S (2007) Colonization and Nitrogen-Fixing Ability of Herbaspirillum sp. Strain B501 gfp1 and Assessment of Its Growth-Promoting Ability in Cultivated Rice. Microbes and Environments - MICROBES ENVIRONMENTS 22:197–206. https://doi.org/10.1264/jsme2.22.197

Zhang B, Du N, Li Y, et al (2018) Distinct biogeographic patterns of rhizobia and non-rhizobial endophytes associated with soybean nodules across China. Science of The Total Environment 643:569–578. https://doi.org/10.1016/j.scitotenv.2018.06.240

Zhang X-X, Kosier B, Priefer UB (2001) Genetic diversity of indigenous Rhizobium leguminosarum bv. viciae isolates nodulating two different host plants during soil restoration with alfalfa. Molecular Ecology 10:2297–2305. https://doi.org/10.1046/j.0962-1083.2001.01364.x

Zhong W, Gu T, Wang W, et al (2010) The effects of mineral fertilizer and organic manure on soil microbial community and diversity. Plant Soil 326:511–522. https://doi.org/10.1007/s11104-009-9988-y

## CHAPTER 3

**Title:** Seed-coat inoculation promotes nodulation but provides no benefits to cultivated cowpeas

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**Abstract**

Plant-associated microbes can have strong positive effects on crop growth and yields, particularly in legumes. Efforts to harness the symbiosis between legumes and rhizobia have largely focused on the development of field inoculants. However, inoculated strains are often outcompeted by native rhizobia and fail to infect targeted plants. We investigated the host benefits that cowpea receive from seed-coat inoculants depending on plant genotype, field history, and soil properties.

Three seed-coat inoculants were tested on four genotypes of cowpea across two adjacent fields. Seed treatments included a synthetic community of cowpea-associating strains, soil from the reciprocal field, and a commercial inoculant. One field had a long history of cowpea growth, while the other had no history of legume cultivation. Cowpea growth and nodulation were measured to assess inoculant effects.

None of the inoculant treatments resulted in significant growth benefits to cowpea in either field. The synthetic community was the only treatment to significantly induce nodulation, yet it resulted in a net negative effect on host growth. The commercial inoculant had no significant effect on nodulation or benefits in either field. We also found a significant field effect on host biomass, though proportional host growth benefits were consistent in both fields.

Field conditions add complexity which can hinder the performance of inoculants which can nodulate or provide benefits under simplified conditions. Growers should be aware that soil nutrient levels may impact symbiosis benefits from inoculation, and that nodulation may not be an indicator of inoculant benefit.

## Introduction

Plant-associated microbes can provide major benefits to crop productivity, improving plant growth and protecting against disease (van Loon 2007; Berg 2009; Hayat et al. 2010; Tan et al. 2013; Amaresan et al. 2019). Multiple factors can impact the success of plant-microbial symbioses, including the plant host genotypes, the soil conditions, and the microbial community, but efforts to harness benefits from microbes to improve crop yields have largely focused on altering their microbial communities via inoculation, with less attention towards the other factors (Sasse et al. 2018; Pahua et al. 2018; Agler et al. 2016; Fitzpatrick et al. 2019; Sinclair and Nogueira 2018). Inoculant strains often underperform in field settings due to their failure to establish under local soil conditions and because of competition with native strains for host infection (Chai et al. 2022; Thilakarathna and Raizada 2017; Ulzen et al. 2018; Kaminsky et al. 2019; Triplett and Sadowsky 1992; Yates et al. 2011; Sinclair and Nogueira 2018; Nazir et al. 2013; Zilli et al. 2013). The complex interactions between host plants, soils, and soil microbes are difficult to predict, and the relative importance of these factors on the success of inoculants are poorly understood in the field. To better guide growers in their decisions, we must gain a better understanding of the role of field planting site, belowground microbial communities, and crop genotype, and how each factor might contribute to benefits from both native and inoculated plant-associated microbes.

Developing and selecting hosts with a greater capacity to harness benefits from soil microbes could also improve the performance of inoculants and crops. Plant hosts can modulate infection and benefits from soil microbiota by attracting certain microbial

partners through the release of root exudates, restricting infection by undesirable partners through the detection of molecular signals, and finally by 'sanctioning' strains which do not provide sufficient benefits (Denison 2000; West et al. 2002; Sasse et al. 2018; van Dam and Bouwmeester 2016). These host control traits can vary among plant genotypes, meaning growers can select for these traits through breeding and choose plant genotypes that will gain more benefits from interactions with soil microbial communities (Haney et al. 2015; Pahua et al. 2018; Torres-Martínez et al. 2021; Wendlandt et al. 2019). In addition to selecting desirable crop genotypes, growers must consider the cropping history of a field, as it can have significant effects on the local microbial community and plant-soil-microbial feedbacks (Wu et al. 2015). Soil microbial communities can be substantially altered by the cultivated crops in a field, especially when there is a long history of growing a particular crop (Xiong et al. 2015; Tian et al. 2019; Wang et al. 2021).

Field soil conditions can shape microbial associations with crops and might mediate growth benefits from inoculants. Abiotic characteristics of soils can shape microbial communities, including pH, climate, drought, nutrients, and soil drainage (Agler et al. 2016; Fitzpatrick et al. 2019; Leite et al. 2017). Field microbial communities are also shaped by agricultural practices such as fertilization, watering regime, tillage, as well as cropping history (Simonsen et al. 2015; Weese et al. 2015; Legrand et al. 2018). Long-term land management can also impact belowground microbial community structure and function; when compared against non-agricultural grassland sites, agricultural soils show lower microbial biomass and potential nitrification rates, both of which can impact plant growth (Bissett et al. 2011). Soil characteristics can also alter interactions between soil

microbes and hosts, driving variation in the benefits that host plants receive. Across potato varieties (*Solanum spp.)*, variation in plant benefits from live soil microbial inocula is context-dependent based on nutrient levels, with landraces responding more positively to inocula under low nutrient conditions, and modern potatoes responding positively under high nutrient conditions (Miao & Lankau, 2022). In maize (*Zea mays* L. var Colisee), plant growth benefits from phosphorus-solubilizing bacterial inocula were strongly dependent on the form of nitrogen fertilization used (Mpanga et al. 2019). Calcareous soil environments improve the efficacy of rhizobial inoculants on soybean (*Glycine* max) by increasing root nodulation and, subsequently, plant nitrogen levels and growth (Wang et al. 2022). Long-term nitrogen addition can impede the symbiosis between legumes and rhizobia, as fields exposed to nitrogen fertilization are associated with rhizobia that provide significantly less growth benefits to *Trifolium* spp (Weese et al. 2015). Thus, characteristics of field soils and the crop genotypes planted must both be considered when inoculating crops with beneficial soil microbes.

Cowpea (*Vigna unguiculata*) is an important crop across regions of Africa, Asia, and the Americas, as it contains a high proportion of edible plant mass, requires minimal nutrient inputs, and is broadly adapted to resist heat and drought (Huynh et al. 2013; Muñoz-Amatriaín et al. 2017; Herniter et al. 2020). Cowpeas associate with nitrogen fixing bacteria (i.e., rhizobia), primarily in the genus *Bradyrhizobium*, that infect cowpea roots and instigate the formation of symbiotic root nodules. The benefits that cowpeas gain from this association appear to be shaped by multiple factors. Soil rhizobial communities strongly shaped host growth benefits and nodulation in cowpea, both of which vary broadly

(Manci et al. 2022). Soil communities of *Bradyrhizobium* are sensitive to pH and vary with nutrients including phosphorus, zinc, sodium, and potassium (Puozaa et al. 2019). These data suggest that successfully altering the soil rhizobial community profile could have significant effects on plant performance across diverse cowpea genotypes. The most popular inoculant application method for commercial-scale is seed-coat inoculation, wherein seeds are pre-mixed and coated with a powder or peat-based formulation prior to planting. Peat-based seed-coat inoculation is especially common for cowpea and related legumes, such as soybean, as *Bradyrhizobium* exhibit high survivability in peat mixtures (Casteriano et al. 2013). The same factors which shape field soil microbial communities (fertilization, cropping history, etc.) can also impact the viability or efficacy of legume inoculants. However, relatively few studies have examined the conditional effects of commercial inoculants in field settings to determine how inoculant features and field conditions can shape plant benefits. Inoculants have been used to improve cowpea growth, however, they are often outcompeted by native rhizobia in field settings, with nodules dominated by local strains (Mbah et al. 2022; Law et al. 2007). In some cases, field application of *Bradyrhizobium* can successfully improve grain yields. In Cuba, locally isolated *Bradyrhizobium* strains were able to survive high salt conditions, outcompete native rhizobia, and provide significant growth benefits to cowpea (Gómez Padilla et al. 2016). Heat-tolerant strains isolated and tested in Brazil were also found to be beneficial to cowpea in Ghana in fields previously sown with corn, without prior inoculation history (Ulzen et al. 2016). One of these strains of *Bradyrhizobium* more than doubled grain yields

of field cowpea in a later experiment (BR 3267, Boddey et al. 2017), suggesting that some strains can survive diverse soil conditions, even across separate continents.

Here, we grew four genotypes of cowpeas in two adjacent fields: one field with a decade long history of growing cowpeas and other legumes, with previous work demonstrating a high level of benefit to cowpeas from this soil community (Manci et al. 2022), and another field where legumes had never been grown. We tested three seed-coat treatments: 1) a commercial *Bradyrhizobium* peat inoculant with widespread usage in California, 2) a synthetic mixture of three strains of *Bradyrhizobium* isolated from the field with cowpea history and demonstrated cowpea benefits, and 3) soil from each respective field. Inoculants were assessed based on their ability to stimulate nodulation, infect roots, fix nitrogen, and improve plant growth. We hypothesized that soil inoculation confers greater fitness benefits than synthetic inoculation of nodulating strains, that microbial treatments derived from local soils are better adapted to survive and provide benefits than commercial inoculants, and that field cropping history shapes plant benefits from inoculation. Despite conditionally stimulating nodulation (and even fixing nitrogen), none of the microbial treatments resulted in a beneficial host growth response, and some were costly to host growth. While host growth benefits did not vary by field, we did find striking differences in nodulation and shoot biomass between fields. These findings support other studies which suggest that under some conditions, inoculated strains might not survive field conditions or effectively compete with native microbiota. Moreover, these results serve as a warning that bioinoculants can actually be harmful under some conditions.

## Materials and Methods

**Cowpea genotypes:** Plant genotypes were selected from two divergent landrace populations of cowpea in northern and southern Africa ($F_{ST}$ = 0.18, Ortiz et al. 2022), which appear to have arisen from independent domestication events (Muñoz-Amatriaín et al. 2017; Huynh et al. 2013). These plant genotypes represent a broad diversity of cowpea genotypes and have already demonstrated to respond well to Californian soils (Manci et al., 2022; Ortiz-Barbosa et al., 2022). Two cowpea genotypes were selected from each of these populations, including TVu-14346 (Senegal) and TVu-3804 (Nigeria) from northern Africa and Muinana-Lawe (Mozambique) and TVu-13305 (Zambia) from southern Africa (Muñoz-Amatriaín et al. 2017). These genotypes were selected based on their relatively high host growth response to soil inoculation treatments in previous studies (Manci et al., 2022; Ortiz-Barbosa et al., 2022).

**Field sites:** Field inoculation experiments took place at the University of California Riverside Agricultural Experiment Station, in two nearby field plots: one with more than ten years of cowpea cultivation alternated with other crops, and the other with no recent history of cowpea cultivation. Both fields were previously fertilized with nitrogen, phosphorous, and potassium via irrigation lines and were treated with pre-emergence herbicides during planting seasons. Field 10 (Lat. 33.965637, Long. -117.340989), the field where cowpeas had not been planted previously, has a negligible density of cowpea compatible rhizobia. A pilot experiment was conducted in June 2022 in Field 10 to test for root nodulating rhizobia that are compatible with cowpea. For this pilot, 18 plants for each of four isogenic cowpea genotypes (RK, RK2, CB3, and NULL) were planted in Field 10,

for a total of 72 cowpeas. Of those, 65 plants (> 90%) failed to form even a single nodule. Field 10 is divided into 10 subsections (A-J); the pilot experiment took place in subsection 10E, while the inoculation experiment took place in 10F, which is directly adjacent. Field 10F had been fallow for 2 years, and before that period had been used to grow melons for 10 years.

Field 11 (Lat. 33.967568, Long. -117.339573) had been used to grow cowpea during alternating years from 2004 through 2017, intercropping with barley (*Hordeum vulgare*) and other legumes including soybean (*Glycine max*) and pigeonpea (*Cajanus cajan*). Unlike Field 10, where we recovered very low levels of rhizobia that are compatible with cowpea, soil rinsates from Field 11 resulted in high nodule counts on cowpeas (mean = 92.91 ± 5.82; 100% nodulation rate; Ortiz-Barbosa et al. 2022) and were associated with a high level of host growth response across twenty diverse cowpea genotypes (mean = 6.65 ± 0.44; Manci et al. 2022). Field 11 is divided into 7 subsections (B-H); this experiment took place in subsection 11H.

**Microbial inoculant treatments:** Four microbial treatments were applied to seeds prior to planting in each field, including three microbial seed coat applications and a sterile water control. Microbial treatments included a synthetic community of rhizobia isolated from Field 11, a commercial cowpea inoculant, and sieved soil from the reciprocal field. The synthetic community was composed of three *Bradyrhizobium yuanmingense* strains isolated in 2015 from cowpea nodules in Field 11 (H1_1_1.3, H1_2_2.1, and H1_1_41.1; Chapter 2). These strains were part of a clonal-like group of *Bradyrhizobium* that dominated the site (268/286 sequenced isolates, Chapter 2). Soil from this site provided

substantial growth benefits to cowpea in a greenhouse experiment (Manci et al. 2022). The commercial inoculant was Exceed Superior Legume Inoculant for Cowpea (Visjon Biologics), a peat-based inoculant distributed by Cal-Bean & Grain Co-Op Inc (Pixley, CA) and reported to be widely used by cowpea growers in California. The reciprocal soil treatments were sampled from four randomized sites each at Field 10 and Field 11, which were pooled, sieved, and mixed separately for each field. Soil samples from each field were quantified for soil organic matter, nitrogen, phosphorus (weak Bray and sodium bicarbonate-P), pH, extractable cations (potassium, magnesium, calcium, sodium), hydrogen, sulfate-S, cation exchange capacity, percent cation saturation, and soil texture (A&L Western Labs).

Prior to inoculation, seeds were surface-sterilized in bleach (5% sodium hypochlorite) and rinsed multiple times in autoclave-sterilized water. Microbial treatments were added to wetted seeds such that they completely covered the seed coats, except in the case of the control plants, wherein the seeds were pre-wetted only with sterile water. For the synthetic community, the three strains were spread onto modified arabinose gluconate medium plates (MAG; Sachs et al. 2009) and incubated at 29$^{o}$ C for eight days. Cultures were scraped from the plates, pelleted in liquid MAG, combined, and vortexed briefly to form a slurry, and 1 ml of this slurry was added to each genotype of seeds. Individual strain pellets and the final slurry mix were each serially diluted and plated on MAG to quantify the concentration of cells in the inoculant, and proportions of each strain. For the commercial inoculant, seed coating followed manufacturer instructions. The inoculum was added to surface-sterilized, wetted seeds, which were vigorously mixed with the inoculum

to evenly coat seeds. For the soil treatments, 1 g of sieved soil was applied to each genotype of seeds to be planted in the reciprocal field. After the seed coating treatments, each of the cowpea genotypes were planted the same day.

**Quantifying rhizobia concentrations in microbial treatments:** The concentration of rhizobia compatible with cowpea was quantified for each of the Field 11 and commercial inoculant treatments, to establish a baseline for infectivity, using the Most Probable Number approach (i.e. MPN; Somasegaran & Hoben, 1994). Ten, fourfold serial dilutions of Field 11 soil were created and inoculated on cowpea accession TVu-13305 with fourfold replication, and nodules were counted 5 weeks later. This commercial inoculant was tested with tenfold dilutions as recommended for peat inocula (Somasegaran & Hoben, 1994). Soil for the MPN was collected on 2/28/21, and seeds were planted on 4/29/21. The Field 10 soil previously demonstrated to have negligible rhizobia, with only 7 of 72 field cowpeas (10% of plants) forming nodules, with 32 total nodules formed (mean number of nodules = 0.44; Ortiz-Barbosa et al. 2022).

**Planting:** Each field was planted with five replicate blocks per microbial treatment and plant genotype combination (including the water control), with a total of 20 blocks in each field. Placement of blocks across each field was randomized across the field, as was the location of each cowpea genotype cluster within the blocks (i.e., sub-blocks). Each genotype sub-block contained 4 replicate plants, for a total of 16 plants per block and 320 plants in each field. Blocks were placed 2 meters apart, with roughly 30 cm between sub-blocks. Seeds were planted 15-20 cm apart. Control & reciprocal soil treatment blocks were planted on 8/11/21, and the remaining microbial treatments were planted on 8/12/21. After

planting, fields were treated with pre-emergence herbicides (Dual Magnum and Prowl H2O) and watered by sprinkler twice over the course of 1 week. Thereafter, plants were watered as needed via drip irrigation (usually twice weekly).

**Harvest:** Two different harvests took place. The early harvest took place four weeks after planting, when all plants had visible true leaves, as this is the stage where nodules can be most reliably harvested and cultured prior to senescing. Three plants (each from separate blocks) were randomly selected from each treatment and genotype combination, and were removed from each field. A trenching spade was used to extract roots to at least 30cm depth including roughly four liters of surrounding soil. This spade was wiped down and sprayed with ethanol between treatments, as well as between fields. For each plant selected, the next adjacent plant was also harvested by de-topping to assess shoot biomass and host growth response to inoculation. In all other sub-blocks, when possible, two adjacent plants were randomly selected and de-topped. In sub-blocks with only two or three viable plants, one was harvested. In sub-blocks with a single viable plant, none were harvested. Harvested plants were rinsed and photographed. Nodules were dissected from roots, counted, and photographed. In Field 10, one plant from each microbial treatment x host genotype combination was randomly selected, and a portion of roots was dug up and rinsed to capture nodules for culturing. This process was repeated in Field 11 for both the water and commercial inoculant treatments. Synthetic and reciprocal soil treatments were excluded from Field 11 sequencing as it is unlikely the recovered strains could be reliably distinguished from the dominant Field 11 community. When available, up to ten nodules were randomly selected per plant and frozen at -80$^{o}$ C. These

nodules were later surface sterilized with bleach and cultured on rhizobium defined medium (RDM) agar plates (Vincent 1970). DNA was extracted using the Qiagen DNeasy Blood & Tissue kit and submitted with isolates from early harvest for whole-genome sequencing at the SeqCenter (Pittsburgh, PA). Roots and shoots (including those de-topped in the field were separated and dried in an oven at 60$^{o}$C to weigh biomass. After the early harvest, fields were treated for aphids, and were spot treated for weeds prior to the late harvest.

The late harvest occurred 16 weeks after planting to estimate agriculturally relevant host growth, as most plants at this stage had mature seed pods. Since root nodule senescence has been previously associated with pod formation, nodulation was not quantified during this harvest. Plants were detopped at the soil surface, and shoots were dried and weighed. A representative portion of dried leaf tissue from plants in the synthetic community, commercial inoculant, and water control treatment groups were encapsulated and submitted for $^{15}$N isotopic analysis (University of California Davis, Stable Isotope Facility). Plants in the reciprocal soil treatment were excluded as they represent two distinct sub treatments (i.e. ,Field 11 soil applied to Field 10, and Field 10 soil applied to Field 11) which cannot be statistically compared between fields.

**Statistical analyses:** Linear mixed models were employed to test the effects of microbial treatment, cowpea genotype, and their interaction on nodulation, shoot biomass, host growth response, and the proportion of nitrogen derived from the air (%Ndfa) in each field. Proportional host growth response (HGR) to inoculation was calculated by dividing individual plant biomass by the average biomass of water-treated control plants within the

same field, genotype, and harvest (Manci et al. 2022, Ortiz-Barbosa et al. 2022). A separate linear mixed model was used to assess the effects of field, microbial treatment, genotype, and their interactions on the above response variables for all plants combined, excluding soil-treated plants (as this inoculum treatment varied by field). For all analyses, host growth response was log-transformed and number of nodules was square root transformed to meet assumptions of normality and heteroscedasticity. Tukey's post-hoc tests were conducted to test for differences among microbial treatments in each field, and Student's T tests were used to test for differences between fields. All analyses were performed in JMP® Pro, Version 15.0.0. SAS Institute Inc., Cary, NC, 1989–2022.

**Results**

**Field soil composition:** Field 10 and 11 differed primarily in pH and in content of nitrogen, phosphorus, and sulfur. Field 11 had 67% more nitrate nitrogen than soil sampled from Field 10 (Table S3.2), likely due to Field 11's long history of legume cultivation. Phosphorus and sulfur were both higher in Field 10 (57% and 55%, respectively). Potassium and sodium levels were also higher in soil in Field 10 compared to 11 (30% and 20%, respectively). Soil organic matter and magnesium levels in each field were similar. The pH of soil from Field 11 was slightly more basic (7.8 compared to 7.4 in Field 10), so the Weak Bray phosphorous level in Field 11 was noted as unreliable due to high pH (A&L Western Labs). Soils from both fields were characterized as sandy loam, with similar particle size distributions (% Sand/Silt/Clay, Table S3.2.)

**Inoculant concentrations:** The MPN experiment indicated that the commercial inoculant had more than an order of magnitude higher concentration of cowpea compatible

rhizobia compared to Field 11 soil (~5.0 x $10^4$ cells/g vs. 4.4 x $10^3$ cells/g, respectively, Table S3.4). Serial dilutions of the mixed synthetic community estimate a cell density of 3.4 x $10^{10}$ CFU/ml (CFU: colony-forming units), which was made up equally of the three individual strains (2.3 x $10^{10}$ CFU/ml, 2.4 x $10^{10}$ CFU/ml, and 1.8 x $10^{10}$ CFU/ml from H1_1_1.3, H1_2_2.1, and H1_1_41.1 respectively, Table S3.4). The Field 10 soil was previously demonstrated to have negligible rhizobia, with only 32 total nodules formed across 72 field cowpeas (mean number of nodules per plant = 0.44; Ortiz-Barbosa et al. 2022). This level of nodulation most closely corresponds to the $4^{-8}$ dilution of Field 11 soil when tested in the greenhouse (mean number of nodules = 0.25) and is far surpassed by the nodules observed from the $4^{-7}$ dilution of Field 11 soil (mean number of nodules = 4.25). This suggests that the concentration of cowpea associating rhizobia in Field 11 is at least 6.6 x $10^4$ times the concentration of rhizobia found in Field 10, as greenhouse inoculations only introduced a portion of soil material, rather than the full soil environment found in the field.

**Nodulation:** Both the microbial treatment and the planted field had significant effects on the number of nodules formed, but there was no significant plant genotype effect (Table 3.1, Table 3.2). Field 11 had significantly more nodules than Field 10 (Field 10, 18.02 ± 3.01; Field 11, 37.56 ± 3.17, Table S3.5; $p < 0.01$, Table S3.6). Plants treated with the synthetic community had the highest number of nodules in both fields (Field 10, 40.75 ± 6.53; Field 11, 51.33 ± 7.72; Fig. 3.1, Table S3.5), significantly more than plants treated with water or the commercial inoculant (Field 10: $p < 0.01$ for both treatments; Field 11: $p < 0.05$ for both treatments; Table S3.6). Plants treated with the commercial inoculant

consistently formed the lowest number of nodules in both fields and could not be significantly differentiated from the water controls (Fig. 3.1, Table S3.6).

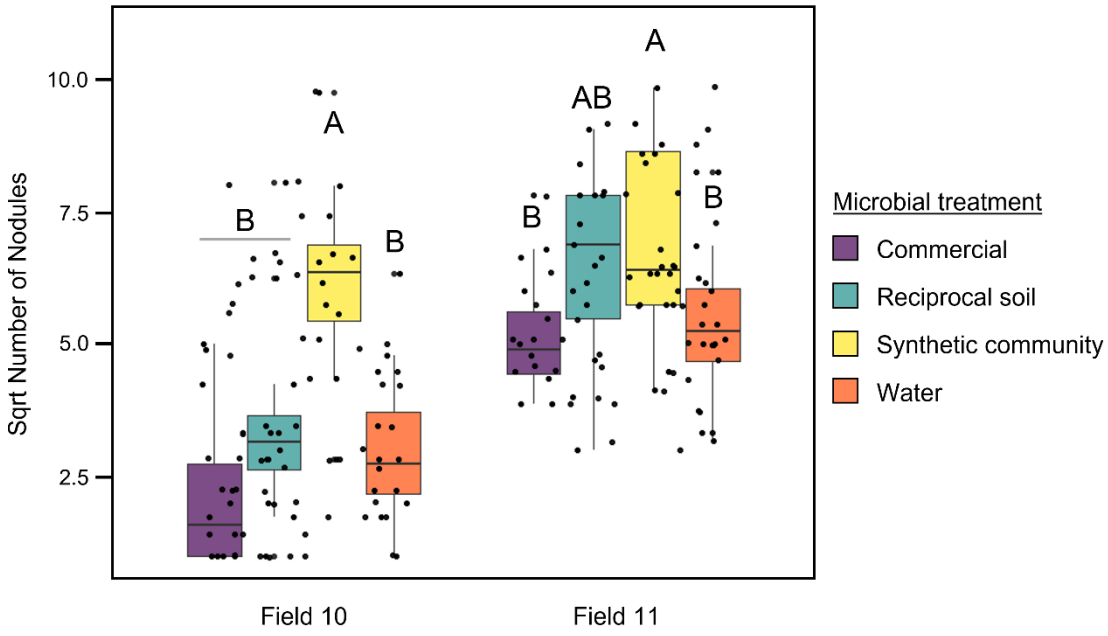


**Fig. 3.1** Number of nodules recovered from a randomized subset of cowpeas during early harvest from Fields 10 and 11 in response to microbial treatment. Dots represent individual data points. Connected letter reports are from Tukey groupings. Number of nodules is square root transformed for normality.

**Plant growth:** Planting field had a strong effect on late harvest shoot biomass ($p <$ 0.0001, T1), with plants growing over six times larger on average in Field 10 than in Field 11 (Field 10 = 338.23 ± 208.43 g, Field 11 = 50.74 ± 35.98, Table S3.5). Microbial treatment had a significant effect on shoot biomass in both fields during the early harvest, with reciprocal soil and synthetic community treatments resulting in lower shoot biomass than water controls and the commercial treatment, though this effect was not significant in Field 10 during the later harvest (Table 3.1, Table S3.6). Cowpea genotype had a

significant effect on shoot biomass at both harvest timepoints in Field 11, but not in Field 10 (Table 3.1).

None of the microbial treatments were associated with a significant, positive host growth response relative to uninoculated controls, though there were significant growth differences among treatments at both harvest timepoints (Table 3.1 & Table 3.2). The commercial treatment group had a significantly higher host growth response compared to the reciprocal soil and synthetic community microbial treatments in both fields, as the reciprocal soil and synthetic community both imposed negative growth responses; however, the growth effect from the commercial treatment was not significantly different from the water controls (Table S3.6, Fig. 3.2). Cowpea genotype had a significant effect on host growth response, but only in Field 10.

Late harvest host growth response was significantly different among cowpea genotypes and among microbial treatments in Field 11 (Table 3.1); in this field, the synthetic community treatment was associated with significantly lower host growth response than the commercial inoculant and the water controls, with an overall negative growth effect ($p$ <0.01 for each, Table S3.6, Fig. 3.2). In Field 10, each of the inoculation treatments had a negative growth effect, and there were no significant differences among the treatments (Table 3.1, Table S3.6). There was no significant difference in host growth response between fields for either harvest (Table S3.6).

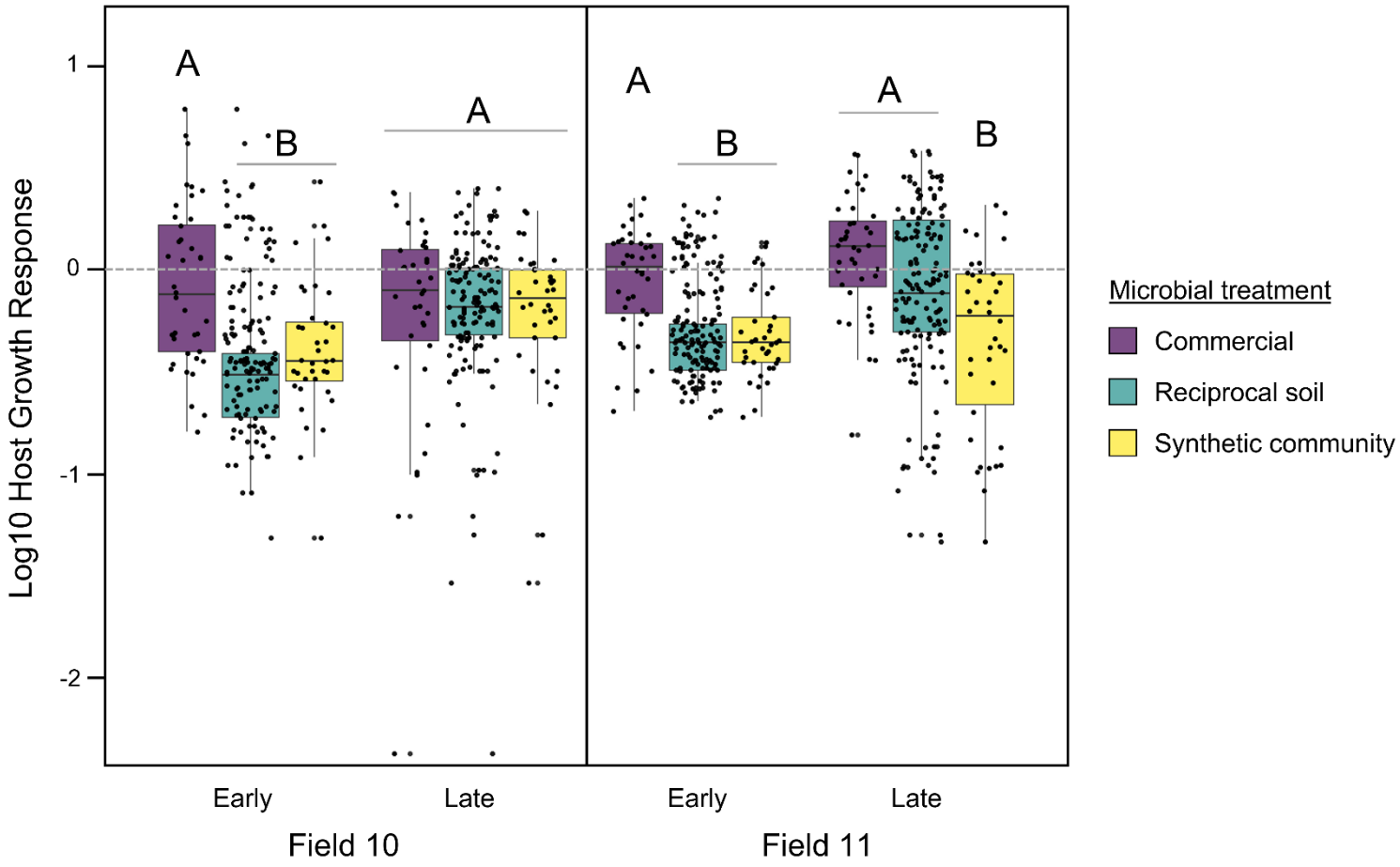


**Figure 3.2.** Early and late host growth response of cowpea grown in Fields 10 and 11 in response to microbial treatments (see color legend). Connected letter reports are from Tukey groupings. The dashed zero line represents the value where the plant biomass of an individual equals that of its associated water control (i.e. no additional growth). Host growth response was log-transformed for normality.

**Nitrogen fixation:** Plants in Field 11 had significantly higher levels of symbiotic nitrogen fixation, calculated as %Ndfa (nitrogen derived from the atmosphere; Field 10 = 33.12 ± 1.38, Field 11 = 51.48 ± 1.22, Table S3.5; $p < 0.01$, Table S3.6). Within Field 11, there was a significant microbial treatment effect on %Ndfa (Table 3.1); shoots from plants treated with the synthetic community had significantly lower %Ndfa when compared to both water controls and the commercial inoculant, indicating a lower level of nitrogen fixation within the synthetic treatment ($p < 0.05$ for both, Table S3.6). We found no significant microbial treatment effect on %Ndfa within Field 10.

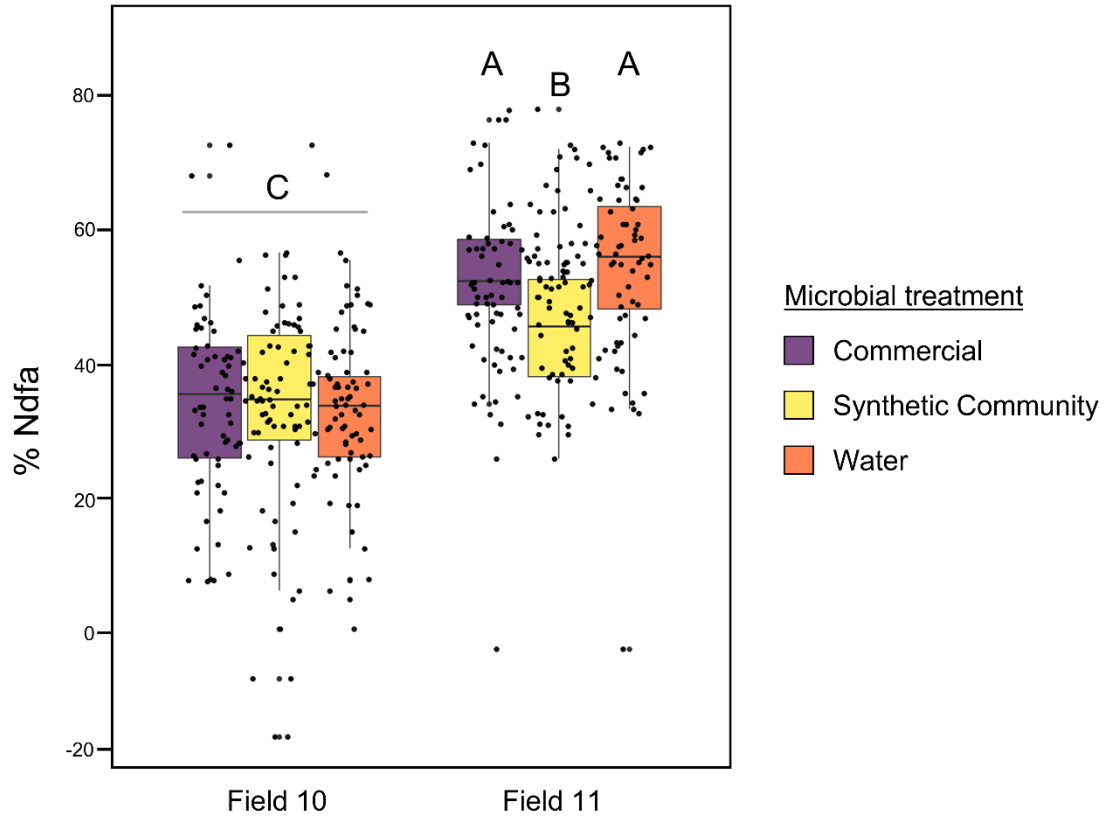


**Fig. 3.3** Nitrogen derived from atmosphere (%Ndfa) from late-harvested cowpeas in Fields 10 and 11. Connected letter reports are from Tukey groupings. Reciprocal soil treatments were excluded.

## Discussion

We tested several inoculants that would be expected to produce growth benefits to cowpea hosts, including a commercial inoculant designed and advertised to stimulate cowpea growth, as well as a soil and synthetic community shown to be enriched with beneficial cowpea microbiota, including *Bradyrhizobium spp.* (Chapter 2; Manci et al. 2022). We found that none of the inoculation treatments stimulated a positive response in cowpea growth (Fig. 3.2, Table S3.6). Plants treated with the synthetic community formed significantly more nodules in both fields compared to controls, but the plants had reduced host growth response in both fields (Fig. 3.1, Table S3.6). This inverse relationship between nodulation and host benefits may be due to the levels of nitrogen in both fields (i.e., NO3-N; 45 ppm in Field 11, 27 ppm in Field 10), as nodulation itself can be costly to legumes (Ryle et al. 1979; Gutschick 1981; Markham 2005) and fixed nitrogen provides no benefit when soil nitrogen levels are moderate or high. Extension recommendations suggest that cowpeas do not require nitrogen fertilization at soil NO3-N levels of 30 ppm and above (Davis and Brick, 2009), so nitrogen levels in both fields might have been sufficient for cowpea growth without additional biological fixed nitrogen. Interestingly, the reciprocal soil treatment had no significant effect on nodulation in either field, but plants from this treatment still had a reduced host growth response in both fields, suggesting hidden negative growth effects of inoculation, unrelated to nodulation (Fig. 3.1, Fig. 3.2, Table S3.6). Inoculating seed coats with soil could lead to early infection by harmful strains or reduce infection by beneficial strains due to antagonism between strains from each field. These results are particularly relevant to growers who intend to apply

inoculants to their legume fields, as nodules are easy to detect, but are an unreliable indicator of desired benefits from inoculation. Additionally, field inoculants are often added to an existing fertilization scheme (i.e. without reducing N fertilization), which could further reduce the efficacy of inoculation (Simonsen et al. 2015; Otieno et al. 2009; Zhong et al. 2010; Rawsthorne et al. 1985; Walley et al. 2005; Otieno et al. 2009).

Strikingly, the commercial inoculant had no effect on either nodulation or host growth response in the field, despite stimulating nodulation in the axenic greenhouse experiment, suggesting that this inoculant has poor viability in the field (Fig. 2, Tables S3.3 & S3.6). Rhizobial strains delivered by this inoculant might be unable to survive the abiotic conditions found in these fields or might be unable to sufficiently infect plant roots due to antagonism or competition with local rhizobial strains—application issues which have been repeatedly observed for other inoculants (Chai et al. 2022; Thilakarathna and Raizada 2017; Kaminsky et al. 2019; Triplett and Sadowsky 1992; Yates et al. 2011; Sinclair and Nogueira 2018; Nazir et al. 2013; Zilli et al. 2013). In a previous experiment, a soil slurry derived from an active cowpea field which had been recently treated with this same commercial inoculant induced minimal growth effects and nodulation when applied to cowpeas in a greenhouse setting, with many individuals resembling controls (Shafter treatment, Manci et al. 2022). These results could support the hypothesis that these commercial strains are unable to survive or persist in soils under certain field conditions. Furthermore, under greenhouse conditions, the commercial inoculant had no discernable effect on cowpea growth (Table S3.7). Host growth response, calculated by dividing the total dry biomass of each plant by the mean dry biomass of plants which did not form

nodules within the same inoculation treatment (Ortiz-Barbosa et al. 2022; Manci et al. 2022), did not significantly vary between commercial dilutions in which all plants formed nodules, and dilutions where no plants formed nodules (mixed model $p = 0.5162$; Table S3.7). In fact, the commercially treated plant with the highest calculated host growth response had zero nodules (mean commercial HGR = 1.02, individual HGR = 1.57; Table S3.7). Together, these results call into question the efficacy of this commercial inoculant, as it was unable to stimulate cowpea growth benefits under sterile greenhouse conditions (Table S3.8, $p = 0.2061$) or in the field and did not promote nodulation in either field.

We observed a strong and unexpectedly large field effect on host biomass, with plants in Field 10 growing significantly larger than those in Field 11, despite no significant difference in host growth response between fields (i.e., from inoculation), and significantly higher nodule counts across all treatments in Field 11. This field effect is perhaps due to soil nutrient differences directly impacting plant growth, as Field 10 had higher levels of phosphorous, sulfur, potassium, and sodium prior to planting (Table S3.2). Additionally, Field 10 had a more neutral pH, whereas Field 11 was more basic (Table S3.2). This difference is important as both cowpeas and rhizobia prefer neutral or slightly acidic soils (Duke 1981; McLeod 1982; Miguel and Moreira 2001; Indrasumunar et al. 2012; Missbah El Idrissi et al. 2021; Mbah et al. 2022; Puozaa et al. 2019). Soil nitrogen content and cowpea symbiotic nitrogen fixation levels were both significantly higher in Field 11 (Table S3.6), suggesting that a portion of the field effect on cowpea biomass could be due to the energetic and nutritional costs of nodulation in plants when available nitrogen is high (Ryle et al. 1979; Gutschick 1981; Markham 2005), as the fixed nitrogen from nodules provides

no added benefit. The stark growth difference observed in plants between these adjacent fields re-iterates the critical effect that soil history and nutrients can have on plants, both directly and on their symbiosis with soil microbes.

Under controlled axenic conditions, microbes can offer significant benefits when applied to plants. However, translating and scaling this effect to real life settings is extraordinarily difficult (Kaminsky et al. 2019). Elite inoculate strains are often tested in growth chambers or greenhouses, which lack many of the variable and complex biotic and abiotic factors present in fields. Isolated benefits from native or inoculated microbes on plants are more challenging to detect under field conditions; however, these types of experiments are critical to understand the context-dependent effects of microbial inoculation in agricultural settings. Efforts to develop effective broad-spectrum microbial inoculants face several longstanding challenges, including that elite strains often have trouble establishing in diverse soils and competing for infection with local strains (Kaminsky et al. 2019; Triplett and Sadowsky 1992; Thilakarathna and Raizada 2017). We hoped to address this issue by testing inoculants in a field without a strongly established rhizobial community, with no history of legume cultivation or inoculation, where both the synthetic community and reciprocal soil rhizobial strains would be locally adapted to survive. We chose to test inoculants via seed-coating as this method is currently prevalent among legume growers and has some evidence of benefit under field conditions (Thilakarathna and Raizada 2017; Chai et al. 2022). Despite these considerations, this experiment failed to produce a beneficial inoculant.

For decades, work to harness the legume-rhizobium symbiosis has largely focused on adjusting microbial communities, examining rhizobial symbiosis traits and developing inoculants from strains which confer higher benefits in limited experimental settings (Sinclair and Nogueira 2018). While there have been some advancements in inoculant formulation, challenges to this microbe-first approach have persisted, as there are many factors which can reduce or eliminate inoculation benefits in the field (Triplett and Sadowsky 1992; Thilakarathna and Raizada 2017; Kaminsky et al. 2019). Many inoculant strains are unable to survive diverse soil conditions, and those that do are unpredictable in their ability to persist in field soils over time. In some cases, inoculants must be re-applied seasonally (Chowdhury et al. 1983; Albareda et al. 2009). Even with formulations which reliably support rhizobial viability in soils, scientists and growers have failed to solve the rhizobial competition problem, with local ineffective strains regularly out-competing inoculate strains for access to infection (Triplett and Sadowsky 1992; Thilakarathna and Raizada 2017). Additionally, growers who use inoculants often apply nitrogen fertilizers within the same season; under these high nitrogen conditions, nodule formation can be costly to the plant, and benefits from inoculation are lost (Ryle et al. 1979; Gutschick 1981; Markham 2005).

Alternatives to this microbe-first approach include shifting focus to either plant hosts or soil conditions to improve the legume-rhizobium symbiosis (Sinclair and Nogueira 2018). There is some genotypic variation in host control and relative growth benefits from inoculation among cowpea genotypes (Manci et al. 2022), and breeding programs could focus on improving host control traits in select genotypes to better direct symbiosis from

the top down, allowing plants to select and reward beneficial partners even from limited communities. However, the results from this study support the hypothesis that soil conditions are the primary drivers of variation in host benefits (Manci et al. 2022); future studies could further disentangle the confounding effects of soil nutrients and native rhizobial communities on the legume-rhizobium symbiosis and the efficacy of proposed inoculants. Understanding the interplay between soils, hosts, and symbionts in driving host benefits could allow growers to utilize improved plant genotypes and tailor soil conditions to specifically benefit plant-associated microbes. If harnessed carefully, crop-associated microbes have the potential to improve crop yields, reduce reliance on nutrient inputs, and improve the economical use of field land.

## Tables

**Table 3.1:** Linear mixed model effects on plant inoculation response within Fields 10 and 11. Number of nodules is square-root transformed for normality, while HGR and shoot biomass are log-transformed. For HGR, water-treated controls are excluded, as they were used to calculate HGR. Soil treatments were excluded from the nitrogen analyses. * Indicates significant effects ($p < 0.05$).

| **Field 10** | **Number of Nodules** | | | **Early Shoot Biomass** | | | **Late Shoot Biomass** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Fixed effects** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** |
| Microbial treatment | 17.483 | 3 | **<.0001*** | 10.896 | 3 | **<.0001*** | 1.666 | 3 | 0.1777 |
| Cowpea genotype | 1.636 | 3 | 0.2004 | 2.094 | 3 | 0.1041 | 0.207 | 3 | 0.8917 |
| Treatment x genotype | 2.77 | 9 | **0.0161*** | 0.471 | 9 | 0.8918 | 0.867 | 9 | 0.5561 |

| **Field 10** | **Early HGR** | | | **Late HGR** | | | **%Ndfa** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Fixed effects** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** |
| Microbial treatment | 13.897 | 3 | **<.0001*** | 0.268 | 3 | 0.7654 | 0.349 | 2 | 0.7063 |
| Cowpea genotype | 5.231 | 3 | **0.0020*** | 2.891 | 3 | 0.0922 | 2.55 | 3 | 0.0599 |
| Treatment x genotype | 0.24 | 9 | 0.9621 | 0.79 | 9 | 0.4565 | 0.357 | 6 | 0.904 |

| **Field 11** | **Number of Nodules** | | | **Early Shoot Biomass** | | | **Late Shoot Biomass** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Fixed effects** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** |
| Microbial treatment | 4.486 | 3 | **0.0097*** | 15.926 | 3 | **<.0001*** | 9.038 | 3 | **<.0001*** |
| Cowpea genotype | 1.855 | 3 | 0.1571 | 7.22 | 3 | **0.0002*** | 10.075 | 3 | **<.0001*** |
| Treatment x genotype | 1.236 | 9 | 0.3086 | 0.489 | 9 | 0.8798 | 0.986 | 9 | 0.4543 |

| **Field 11** | **Early HGR** | | | **Late HGR** | | | **%Ndfa** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Fixed effects** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** |
| Microbial treatment | 15.972 | 3 | **<.0001*** | 13.697 | 3 | **<.0001*** | 5.382 | 2 | **0.0061*** |
| Cowpea genotype | 0.402 | 3 | 0.7517 | 4.567 | 3 | **0.0048*** | 0.878 | 3 | 0.4554 |
| Treatment x genotype | 0.595 | 9 | 0.7338 | 1.426 | 9 | 0.2118 | 1.153 | 6 | 0.338 |

**Table 3.2:** Linear mixed model effects on plant inoculation response in both fields combined. Number of nodules is square-root transformed for normality, while HGR and shoot biomass are log-transformed. For HGR, water-treated control plants were excluded, as they were used to calculate HGR. Soil treatments were excluded as they represented different treatments in each field. Soil treatments were also excluded from the nitrogen analyses. * Indicates significant effects ($p < 0.05$).

| **Combined Fields** | **Number of Nodules** | | | **Early Shoot Biomass** | | | **Late Shoot Biomass** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Fixed effects** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** |
| Field | 40.196 | 1 | **<.0001*** | 2.244 | 1 | 0.1356 | 308.627 | 1 | **<.0001*** |
| Microbial treatment | 34.884 | 2 | **<.0001*** | 19.884 | 2 | **<.0001*** | 6.898 | 2 | **0.0013*** |
| Cowpea genotype | 1.892 | 3 | 0.1419 | 4.45 | 3 | **0.0047*** | 4.019 | 3 | **0.0083*** |
| Field x treatment | 3.993 | 2 | **0.0241*** | 0.102 | 2 | 0.9034 | 7.596 | 2 | **0.0007*** |
| Field x genotype | 1.21 | 3 | 0.3151 | 0.669 | 3 | 0.5719 | 2.21 | 3 | 0.088 |
| Treatment x genotype | 4.941 | 6 | **0.0004*** | 0.501 | 6 | 0.8069 | 1.651 | 6 | 0.1346 |

| **Combined Fields** | **Early HGR** | | | **Late HGR** | | | **%Ndfa** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Fixed effects** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** | **F ratio** | **df** | ***p*** |
| Field | 0.846 | 1 | 0.3593 | 1.241 | 1 | 0.2672 | 100.002 | 1 | **<.0001*** |
| Microbial treatment | 33.22 | 1 | **<.0001*** | 9.04 | 1 | **0.0031*** | 2.83 | 2 | 0.0614 |
| Cowpea genotype | 2.881 | 3 | **0.0382*** | 2.067 | 3 | 0.1076 | 1.193 | 3 | 0.3137 |
| Field x treatment | 0.144 | 1 | 0.7051 | 11.464 | 1 | **0.0009*** | 2.139 | 2 | 0.1205 |
| Field x genotype | 1.992 | 3 | 0.118 | 1.139 | 3 | 0.3359 | 2.47 | 3 | 0.0631 |
| Treatment x genotype | 0.121 | 3 | 0.9474 | 2.741 | 3 | **0.0457*** | 0.445 | 6 | 0.8478 |

## References


Afkhami ME, Almeida BK, Hernandez DJ, et al (2020) Tripartite mutualisms as models for understanding plant–microbial interactions. Current Opinion in Plant Biology 56:28–36. https://doi.org/10.1016/j.pbi.2020.02.003

Agler MT, Ruhe J, Kroll S, et al (2016) Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. PLOS Biology 14:e1002352. https://doi.org/10.1371/journal.pbio.1002352

Albareda M, Rodríguez-Navarro DN, Temprano FJ (2009) Soybean inoculation: Dose, N fertilizer supplementation and rhizobia persistence in soil. Field Crops Research 113:352–356. https://doi.org/10.1016/j.fcr.2009.05.013

Amaresan N, Jayakumar V, Kumar K, Thajuddin N (2019) Biocontrol and plant growth-promoting ability of plant-associated bacteria from tomato (Lycopersicum esculentum) under field condition. Microbial Pathogenesis 136:103713. https://doi.org/10.1016/j.micpath.2019.103713

Bashan Y, de-Bashan LE, Prabhu SR, Hernandez J-P (2014) Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). Plant Soil 378:1–33. https://doi.org/10.1007/s11104-013-1956-x

Berg G (2009) Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl Microbiol Biotechnol 84:11–18. https://doi.org/10.1007/s00253-009-2092-7

Bissett A, Richardson AE, Baker G, Thrall PH (2011) Long-term land use effects on soil microbial community structure and function. Applied Soil Ecology 51:66–78. https://doi.org/10.1016/j.apsoil.2011.08.010

Boddey RM, Fosu M, Atakora WK, et al (2017) COWPEA (VIGNA UNGUICULATA) CROPS IN AFRICA CAN RESPOND TO INOCULATION WITH RHIZOBIUM. Experimental Agriculture 53:578–587. https://doi.org/10.1017/S0014479716000594

Casteriano A, Wilkes MA, Deaker R (2013) Physiological Changes in Rhizobia after Growth in Peat Extract May Be Related to Improved Desiccation Tolerance. Applied and Environmental Microbiology 79:3998–4007. https://doi.org/10.1128/AEM.00082-13

Chai YN, Futrell S, Schachtman DP (2022) Assessment of Bacterial Inoculant Delivery Methods for Cereal Crops. Frontiers in Microbiology 13:

Chowdhury MS, Msumali GP, Malekela GP (1983) Need for Seasonal Inoculation of Soybeans with Rhizobia at Morogoro, Tanzania. Biological Agriculture & Horticulture 1:219–228. https://doi.org/10.1080/01448765.1983.9754397

Davis J, Brick M Fertilizing Dry Beans

Denison RF (2000) Legume Sanctions and the Evolution of Symbiotic Cooperation by Rhizobia. The American Naturalist 156:567–576. https://doi.org/10.1086/316994

Duke JA (1981) Handbook of LEGUMES of World Economic Importance. Springer US, Boston, MA

Fitzpatrick CR, Mustafa Z, Viliunas J (2019) Soil microbes alter plant fitness under competition and drought. Journal of Evolutionary Biology 32:438–450. https://doi.org/10.1111/jeb.13426

Gómez Padilla E, Ruiz-Díez B, Fernández-Pascual M, et al (2016) Inoculation with Native Bradyrhizobia Strains Improved Growth of Cowpea Plants Cultivated on a Saline Soil. Communications in Soil Science and Plant Analysis 47:2218–2224. https://doi.org/10.1080/00103624.2016.1228950

Gutschick VP (1981) Evolved Strategies in Nitrogen Acquisition by Plants. The American Naturalist 118:607–637. https://doi.org/10.1086/283858

Haney CH, Samuel BS, Bush J, Ausubel FM (2015) Associations with rhizosphere bacteria can confer an adaptive advantage to plants. Nature Plants 1:1–9. https://doi.org/10.1038/nplants.2015.51

Hayat R, Ali S, Amara U, et al (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598. https://doi.org/10.1007/s13213-010-0117-1

Herniter IA, Muñoz-Amatriaín M, Close TJ (2020) Genetic, textual, and archeological evidence of the historical global spread of cowpea (Vigna unguiculata [L.] Walp.). Legume Science 2:e57. https://doi.org/10.1002/leg3.57

Huynh B-L, Close TJ, Roberts PA, et al (2013) Gene Pools and the Genetic Architecture of Domesticated Cowpea. The Plant Genome 6:plantgenome2013.03.0005. https://doi.org/10.3835/plantgenome2013.03.0005

Indrasumunar A, Menzies NW, Dart PJ (2012) Laboratory prescreening of Bradyrhizobium japonicum for low pH, Al and Mn tolerance can be used to predict their survival in acid soils. Soil Biology and Biochemistry 48:135–141. https://doi.org/10.1016/j.soilbio.2012.01.019

Johnson NC, Wilson GWT, Bowker MA, et al (2010) Resource limitation is a driver of local adaptation in mycorrhizal symbioses. Proceedings of the National Academy of Sciences 107:2093–2098. https://doi.org/10.1073/pnas.0906710107

Kaminsky LM, Trexler RV, Malik RJ, et al (2019) The Inherent Conflicts in Developing Soil Microbial Inoculants. Trends in Biotechnology 37:140–151. https://doi.org/10.1016/j.tibtech.2018.11.011

Law IJ, Botha WF, Majaule UC, Phalane FL (2007) Symbiotic and genomic diversity of 'cowpea' bradyrhizobia from soils in Botswana and South Africa. Biol Fertil Soils 43:653–663. https://doi.org/10.1007/s00374-006-0145-y

Legrand F, Picot A, Cobo-Díaz JF, et al (2018) Effect of tillage and static abiotic soil properties on microbial diversity. Applied Soil Ecology 132:135–145. https://doi.org/10.1016/j.apsoil.2018.08.016

Leite J, Fischer D, Rouws LFM, et al (2017) Cowpea Nodules Harbor Non-rhizobial Bacterial Communities that Are Shaped by Soil Type Rather than Plant Genotype. Front Plant Sci 7:. https://doi.org/10.3389/fpls.2016.02064

Manci M, Mercado OG, Camantigue RX, et al (2022) Live soil inocula, not host population or domestication status, is the predominant driver of growth benefits to cowpea. Plant Soil. https://doi.org/10.1007/s11104-022-05709-6

Markham JH (2005) The effect of Frankia and Paxillus involutus on the performance of Alnus incana subsp. rugosa in mine tailings. Can J Bot 83:1384–1390. https://doi.org/10.1139/b05-108

Mbah GC, Mohammed M, Jaiswal SK, Dakora FD (2022) Phylogenetic Relationship, Symbiotic Effectiveness, and Biochemical Traits of Native Rhizobial Symbionts of Cowpea (Vigna unguiculata L. Walp) in South African Soil. J Soil Sci Plant Nutr 22:2235–2254. https://doi.org/10.1007/s42729-022-00805-z

McLeod E (1982) Feed the soil. Organic Agriculture Research Institute, Graton, Calif.

Miao M, Lankau R (2022) Plant host domestication and soil nutrient availability determine positive plant microbial response across the Solanum genus. Journal of Experimental Botany erac453. https://doi.org/10.1093/jxb/erac453

Miguel DL, Moreira FMS (2001) Influence of medium and peat pH on the behaviour of Bradyrhizobium strains. Rev Bras Ciênc Solo 25:873–883. https://doi.org/10.1590/S0100-06832001000400010

Missbah El Idrissi M, Bouhnik O, ElFaik S, et al (2021) Characterization of Bradyrhizobium spp. Nodulating Lupinus cosentinii and L. luteus Microsymbionts in Morocco. Frontiers in Agronomy 3:

Mpanga IK, Nkebiwe PM, Kuhlmann M, et al (2019) The Form of N Supply Determines Plant Growth Promotion by P-Solubilizing Microorganisms in Maize. Microorganisms 7:38. https://doi.org/10.3390/microorganisms7020038

Muñoz-Amatriaín M, Mirebrahim H, Xu P, et al (2017) Genome resources for climate-resilient cowpea, an essential crop for food security. The Plant Journal 89:1042–1054. https://doi.org/10.1111/tpj.13404

Nazir R, Semenov AV, Sarigul N, Elsas JD van (2013) Bacterial community establishment in native and non-native soils and the effect of fungal colonization. Microbiol Discov 1:8. https://doi.org/10.7243/2052-6180-1-8

Ortiz-Barbosa GS, Torres-Martínez L, Manci A, et al (2022) No disruption of rhizobial symbiosis during early stages of cowpea domestication. Evolution evo.14424. https://doi.org/10.1111/evo.14424

Otieno PE, Muthomi JW, Chemining'wa GN, Nderitu JH (2009) Effect of rhizobia inoculation, farm yard manure and nitrogen fertilizer on nodulation and yield of food grain legumes. Journal of Biological Sciences 9:326–332

Pahua VJ, Stokes PJN, Hollowell AC, et al (2018) Fitness variation among host species and the paradox of ineffective rhizobia. Journal of Evolutionary Biology 31:599–610. https://doi.org/10.1111/jeb.13249

Puozaa DK, Jaiswal SK, Dakora FD (2019) Phylogeny and distribution of Bradyrhizobium symbionts nodulating cowpea (Vigna unguiculata L. Walp) and their association with the physicochemical properties of acidic African soils. Syst Appl Microbiol 42:403–414. https://doi.org/10.1016/j.syapm.2019.02.004

Rawsthorne S, Hadley P, Summerfield RJ, Roberts EH (1985) Effects of supplemental nitrate and thermal regime on the nitrogen nutrition of chickpea (Cicer arietinum L.). Plant Soil 83:279–293. https://doi.org/10.1007/BF02184299

Rigg JL, Webster AT, Harvey DM, et al (2021) Cross-host compatibility of commercial rhizobial strains for new and existing pasture legume cultivars in south-eastern Australia. cpsc 72:652–665. https://doi.org/10.1071/CP20234

Rúa MA, Antoninka A, Antunes PM, et al (2016) Home-field advantage? evidence of local adaptation among plants, soil, and arbuscular mycorrhizal fungi through meta-analysis. BMC Evol Biol 16:122. https://doi.org/10.1186/s12862-016-0698-9

RYLE GJA, POWELL CE, GORDON AJ (1979) The Respiratory Costs of Nitrogen Fixation in Soyabean, Cowpea, and White Clover: II. COMPARISONS OF THE COST OF NITROGEN FIXATION AND THE UTILIZATION OF COMBINED NITROGEN. Journal of Experimental Botany 30:145–153. https://doi.org/10.1093/jxb/30.1.145

Sasse J, Martinoia E, Northen T (2018) Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? Trends in Plant Science 23:25–41. https://doi.org/10.1016/j.tplants.2017.09.003

Simonsen AK, Han S, Rekret P, et al (2015) Short-term fertilizer application alters phenotypic traits of symbiotic nitrogen fixing bacteria. PeerJ 3:e1291. https://doi.org/10.7717/peerj.1291

Sinclair TR, Nogueira MA (2018) Selection of host-plant genotype: the next step to increase grain legume N2 fixation activity. Journal of Experimental Botany 69:3523–3530. https://doi.org/10.1093/jxb/ery115

Thilakarathna MS, Raizada MN (2017) A meta-analysis of the effectiveness of diverse rhizobia inoculants on soybean traits under field conditions. Soil Biology and Biochemistry 105:177–196. https://doi.org/10.1016/j.soilbio.2016.11.022

Tian X, Wang C, Bao X, et al (2019) Crop diversity facilitates soil aggregation in relation to soil microbial community composition driven by intercropping. Plant Soil 436:173–192. https://doi.org/10.1007/s11104-018-03924-8

Torres-Martínez L, Porter SS, Wendlandt C, et al (2021) Evolution of specialization in a plant-microbial mutualism is explained by the oscillation theory of speciation. Evolution 75:1070–1086. https://doi.org/10.1111/evo.14222

Triplett EW, Sadowsky MJ (1992) Genetics of competition for nodulation of legumes. Annual review of microbiology 46:. https://doi.org/10.1146/annurev.mi.46.100192.002151

Ulzen J, Abaidoo RC, Masso C, et al (2018) Is there a need for Bradyrhizobium yuanmingense and B. japonicum reinoculation in subsequent cropping seasons under smallholder farmers' conditions? Applied Soil Ecology 128:54–60. https://doi.org/10.1016/j.apsoil.2018.04.003

Ulzen J, Abaidoo RC, Mensah NE, et al (2016) Bradyrhizobium Inoculants Enhance Grain Yields of Soybean and Cowpea in Northern Ghana. Frontiers in Plant Science 7:

van Dam NM, Bouwmeester HJ (2016) Metabolomics in the Rhizosphere: Tapping into Belowground Chemical Communication. Trends in Plant Science 21:256–265. https://doi.org/10.1016/j.tplants.2016.01.008

van Loon LC (2007) Plant responses to plant growth-promoting rhizobacteria. Eur J Plant Pathol 119:243–254. https://doi.org/10.1007/s10658-007-9165-1

Vincent JM (1970) A manual for the practical study of the root-nodule bacteria. A manual for the practical study of the root-nodule bacteria

Walley FL, Kyei-Boahen S, Hnatowich G, Stevenson C (2005) Nitrogen and phosphorus fertility management for desi and kabuli chickpea. Can J Plant Sci 85:73–79. https://doi.org/10.4141/P04-039

Wang G, Bei S, Li J, et al (2021) Soil microbial legacy drives crop diversity advantage: Linking ecological plant–soil feedback with agricultural intercropping. Journal of Applied Ecology 58:496–506. https://doi.org/10.1111/1365-2664.13802

Weese DJ, Heath KD, Dentinger BTM, Lau JA (2015) Long-term nitrogen addition causes the evolution of less-cooperative mutualists. Evolution 69:631–642. https://doi.org/10.1111/evo.12594

Wendlandt CE, Regus JU, Gano-Cohen KA, et al (2019) Host investment into symbiosis varies among genotypes of the legume Acmispon strigosus, but host sanctions are uniform. New Phytologist 221:446–458. https://doi.org/10.1111/nph.15378

West SA, Kiers ET, Simms EL, Denison RF (2002) Sanctions and mutualism stability: why do rhizobia fix nitrogen? Proc Biol Sci 269:685–694. https://doi.org/10.1098/rspb.2001.1878

Woliy K, Degefu T, Frostegård Å (2019) Host Range and Symbiotic Effectiveness of N2O Reducing Bradyrhizobium Strains. Frontiers in Microbiology 10:2746. https://doi.org/10.3389/fmicb.2019.02746

Wu L, Wang J, Huang W, et al (2015) Plant-microbe rhizosphere interactions mediated by Rehmannia glutinosa root exudates under consecutive monoculture. Sci Rep 5:15871. https://doi.org/10.1038/srep15871

Xiong W, Li Z, Liu H, et al (2015) The Effect of Long-Term Continuous Cropping of Black Pepper on Soil Bacterial Communities as Determined by 454 Pyrosequencing. PLOS ONE 10:e0136946. https://doi.org/10.1371/journal.pone.0136946

Yates RJ, Howieson JG, Reeve WG, O'Hara GW (2011) A re-appraisal of the biology and terminology describing rhizobial strain success in nodule occupancy of

legumes in agriculture. Plant Soil 348:255–267. https://doi.org/10.1007/s11104-011-0971-z

Zhong W, Gu T, Wang W, et al (2010) The effects of mineral fertilizer and organic manure on soil microbial community and diversity. Plant Soil 326:511–522. https://doi.org/10.1007/s11104-009-9988-y

Zilli JÉ, Pereira GMD, França Júnior I, et al (2013) Dinâmica de rizóbios em solo do cerrado de Roraima durante o período de estiagem. Acta Amaz 43:153–160. https://doi.org/10.1590/S0044-59672013000200004