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Domestication, Fertilization, and Nodulation: Assessing the Role of Anthropogenic Impositions on the Symbiosis between *Phaseolus vulgaris* L. and Nitrogen-fixing Bacteria

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ABSTRACT

Phaseolus vulgaris L. (Latin American common bean) is a leguminous crop that is significant in the life histories of both human civilization and nitrogen-fixing bacteria called rhizobia. These associations are evident from the impacts they have left on the genome of the common bean. As a legume, the common bean has several genes that code for the initiation and maintenance of mutualistic endosymbiosis with rhizobia. Over the course of its domestication and cultivation, the common bean has also undergone simultaneous artificial selection and multiple bottlenecks. As it exists in commercial agriculture, crops of common bean lack within-population genetic diversity and are widely considered poor at facilitating nitrogen fixation. Recent studies suggest nitrogen fertilization to be key in the decline of nodulation in cultivars of common bean. In addition, preliminary research suggests that wild lineages of bean have greater numbers of nodules. However, these data do not directly compare proxies of nitrogen fixation, like nodulation, with genotype and the presence of nitrogen fertilizer. I hypothesized that bean accession would be a more significant factor than nitrogen presence in determining the ability to facilitate nitrogen fixation. Through a randomized block greenhouse experiment that observed the variables of accession and nitrogen treatment, I found that both nitrogen fertilization and genotype were significant contributors to root:shoot ratio, nodule number, and chlorophyll content index (CCI) in common bean. However, bean genotype was more significant in determining ability to facilitate nitrogen fixation.

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Chapter 1

Introduction & Background

The Latin American common bean (Phaseolus vulgaris L.) is rich in cultural and biological significance. Its anthropological role as an ancient, domesticated crop and its botanical classification as a legume make the common bean an interesting focal point for understanding how human involvement impacts both genetic and environmental components of crop phenotypes. While genotypes, or the specific combinations of genes that make up an individual, are heritable and encode for traits, phenotypes are the culmination of what genes are present to be expressed and how those genes are expressed. As gene expression can be regulated by both genetic and environmental cues, phenotypes are influenced to various degrees by a combination of these factors. A better understanding of the genetic and environmental changes that are brought on by human intervention may potentially lend insight into how if at all, these actions impose upon plant-microbiome relationships. By studying how a combination of reduced genetic variation and excessive usage of anthropogenic nitrogen fertilizers may be related to a reduced ability to facilitate nitrogen fixation, I aim to better understand the degree to which bean genotype versus nitrogen fertilization impacts traits associated with nitrogen fixation. An improved comprehension of how these human impositions on the genetics and environment of common bean impact its capacity to facilitate nitrogen fixation is critical. The implications of this knowledge could aid in lessening global agricultural nitrogen fertilizer needs, increasing productivity to meet global nutritional needs, and determining how to increase the efficacy of nitrogen fixation in common bean.

A Natural History: Legumes and Nitrogen Fixation

Legumes, or plants in the family *Fabaceae*, are categorized primarily by their endosymbiotic relationship with a wide range of bacteria categorized as rhizobia. The nitrogen fixation process facilitated by this endosymbiosis is called symbiotic nitrogen fixation, or SNF. The mutualistic partnership is dated at approximately 58 million years ago and the ability to form nitrogen-fixing associations persists in over 80% of extant legume species (Ferguson et al., 2019). Though legumes can vary greatly in their size and appearance, from small crops that feed livestock, like alfalfa, to larger cash crops that feed humans, like soybean, they are generally linked by their ability to exchange carbon for fixed nitrogen via this mutualism.

The continued prevalence of SNF over evolutionary time is likely due to the competitive benefits it provides legumes over non-leguminous plants living in the same environment. Empirical support for this hypothesis comes from a global study published in 2021. In examining data collected globally in grassland legumes over six years, researchers found that the regular addition of nitrogen compounds to otherwise nitrogen-poor soil caused a significant decline in legume biomass and population richness compared to non-legumes living in the same environments, even when other necessary nutrients such as phosphorus were added simultaneously with the nitrogen (Tognetti et al., 2021). Further support of SNF acting as an adaptation in legumes is found in a specialized regulatory system that responds to environmental nitrogen. The presence of nitrogen compounds in surrounding soil has the potential to reduce or inhibit nodulation. Nitrate and ammonia-containing compounds are seen to generally inhibit the number of nodules formed on a plant and some species of legumes have adapted to systematically disable nodulation in high nitrogen environments to conserve energy (Ferguson et

al., 2019). The ability of legumes to source their nitrogen from the atmosphere confers an advantage over non-legumes in nitrogen-poor environments, and thus may also confer a fitness advantage to plants that can facilitate nitrogen fixation more effectively.

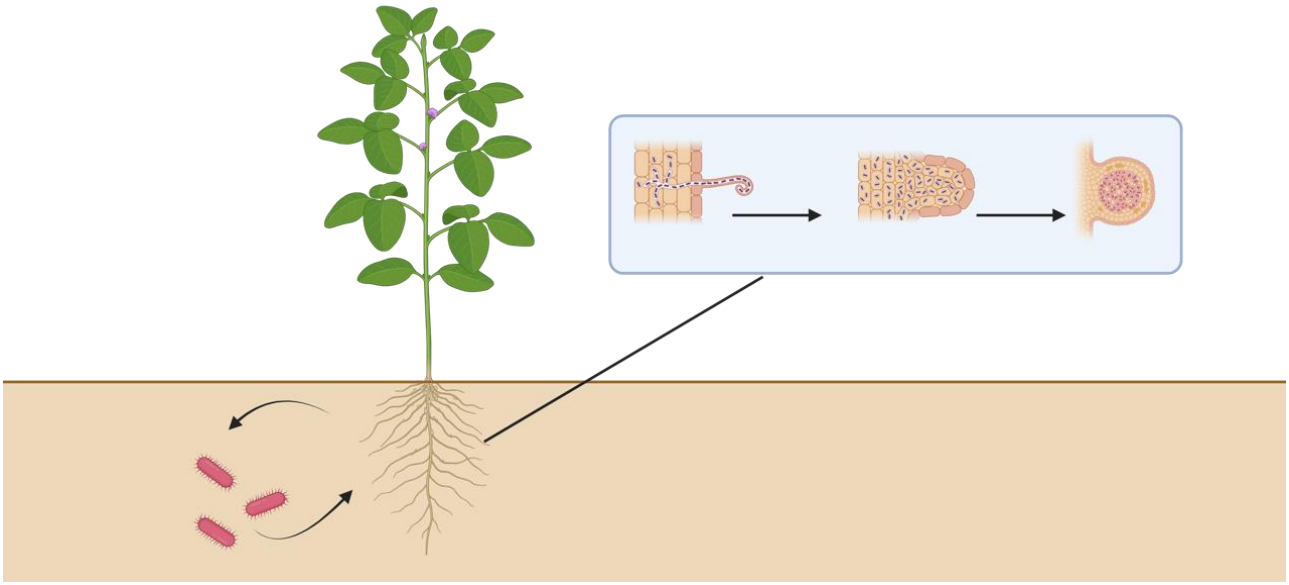

Figure 1 . A simplified diagram of the initiation of nodules in legumes [made using BioRender]

Though the specific genes involved in initiating nodulation differ by species, the general pathways are similar across species of legumes. First, the legume host produces compounds that signal to potential bacterial symbionts in the surrounding rhizosphere. Flavonoids and isoflavonoids are released via secretions from the roots called exudates and attract rhizobial endosymbionts compatible with the host legume (Ferguson et al., 2019). Chemical communication between rhizobia and the legume continues and eventually leads to rhizobial infection of the roots. While the exact means of rhizobia entering the plant can vary, the rhizobia enter the cortex cells of the root and undergo multiple rounds of cell division (Lindemann & Glover, 2015). Subsequently, the molecular signaling pathway Autoregulation of Nodulation

(AON) mediates nodule organogenesis; this is distinct from the pathway that regulates nodulation in response to nitrogen presence (Ferguson et al., 2019). Overall, the ability to initiate, form, and maintain nodules is exceedingly complex, with a minimum of 196 genes currently implicated as necessary for successful symbiosis (Roy et al., 2020).

Phaseolus vulgaris L. is considered a poor nitrogen fixer compared to other legumes. While other leguminous crops can fix over a hundred pounds of nitrogen per acre per year in a cropping system, common bean can only fix 50 pounds of nitrogen per acre during its growing season as an annual plant and is typically unable to meet its nitrogen needs solely via fixation (Lindemann & Glover, 2015). This is not because the common bean requires a specific species of rhizobia to fix nitrogen. While there are specialist legumes in which root exudates recruit specific species of rhizobia, common bean is understood to be promiscuous, meaning that it is capable of forming symbioses with a wide range of rhizobia (Moura et al., 2022). Other researchers studying SNF have determined that varying levels of nitrogen addition impact the ability and efficiency of nitrogen fixation, concluding that selective breeding allows nodulation at higher external nitrogen levels (Reinprecht, Schram, Marsolais, et al., 2020). However, these data consistently see genotype in cultivars, or true-to-seed breeding lines, as significant to the quality of nitrogen fixation, but do not present consistent results on the relationships between other variables, such as nitrogen addition (Reinprecht, Schram, Marsolais, et al., 2020). Ultimately, the inefficiency with which common bean fixes nitrogen is not well understood but may be related to rhizobial partners, host genetics, environmental nitrogen addition, and/or a combination of two or more of these factors.

Pre-Domestication: Origins in Mexico and Distribution Across Latin America

Though they now exist in many distinct global populations, the origins of common bean can be traced to wild progenitors in Latin America. Interactions initiating the domestication of *P. vulgaris* L. have been estimated to date back as far as 5 million years, with the sequencing of the Mesoamerican gene pool strongly suggesting origins in central Mexico for all Latin American gene pools (Bitocchi et al., 2011). The archeological record for common bean reveals the beginnings of domestication as early as 7,000 years ago in Mexico and Central and South America, with archeological sites attributed to Infiernillo Phase hunter-gatherers revealing the remains of domesticated bean pods (Kaplan & MacNeish, 1960). Later archeological sites in the Americas also demonstrate the prevalence of common bean as a domesticate and indicate a timeline of its spread. For instance, recoveries of *P. vulgaris* L. seed fragments and cotyledons dating as far back as the sixteenth century have been found in the ancestral territory of the Iroquois, on land now incorporated into modern New York, Quebec, and Ontario (Hart, 2022). While this time point falls significantly after the domestication of the common bean, it provides useful information when considering the difficulty of obtaining a more complete timeline of the common bean's range expansion.

Gaps in the macro-botanical record for *P. vulgaris* L. are common. Previously, archeologists suggested an Andean origin of the common bean due to a lack of wild bean remains found in Mexico (Kaplan, 1981). Current theories have changed in light of a general understanding that common bean, particularly its seeds or germplasm, does not preserve well in

the context of the methods indigenous peoples used to process them for consumption (Hart, 2022). The advent of genomic comparisons has allowed for the relative dating of different common bean populations as they differentiated. The genetic distance between beans from different parts of Mexico and Central and South America can be used to assess the time since allopatry, allowing geneticists to determine the relative ages of common bean populations in order of dispersal time from central Mexico to the Andes via human migration; human movement and separation of different common bean individuals from the larger wild population left genetic signatures in the genomes of different populations of common bean (Bitocchi et al., 2011). These separations and migrations defined allopatric communities of *Phaseolus vulgaris* L. in which daughter populations became distinct from the parent populations in terms of available genetic variation. These genetic data complement anthropological data in constructing the origin story of the domesticated common bean.

Domestication Syndrome in Common Bean

Trends in traits that tend to be acquired together in domesticated organisms are described by the phrase ‘domestication syndrome.’ The phenotypes associated with domestication tend to come from pre-existing genotypic variation within progenitor gene pools. In the common bean, domestication syndrome appears as an inability to disperse seeds, a wider range of temperature tolerance, preference for longer day length, changes in growth modality, and differences in plant chemical composition.

In the process of domestication, genetic variation is reduced within the domesticated population. Part of this reduction is artificial selection for traits desired by humans.

Simultaneously, genetic bottlenecks occur. As individuals with a desired trait are taken from the wild parent population, the genetic variation in the new daughter population is reduced. Frequencies of alleles, or different versions of a gene, associated with desired or undesired phenotypes are directly altered by artificial selection while all other alleles of related or unrelated genes are also subject to rise or decline within a daughter population. Populations of Western domesticates, referred to as cultivars, of common bean, have experienced the most severe reduction of genetic variation of *P. vulgaris* L. (Gepts, 1998). Because cultivars are derived from accessions, or breeding lines, of landraces, Indigenous American domesticates, this means that cultivar populations are the least genetically diverse bean lineage, or type of population, compared to wild *P. vulgaris* L. populations, demonstrating a decline in genetic diversity with continued selective breeding (Gepts, 1998).

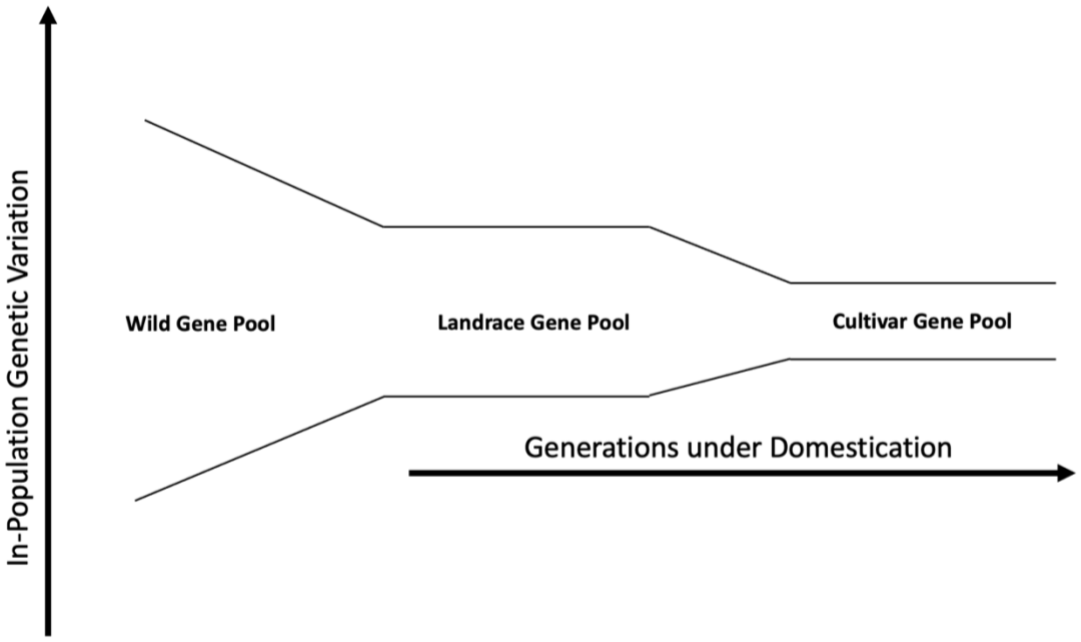

Figure 2. As removal from the larger population and selection has occurred over time, genetic diversity has declined in subsequent populations of common bean

Many current samples of wild *Phaseolus vulgaris* L. do not necessarily represent populations present at the beginning of the species domestication process. Populations are subject to changes in allele frequency over time and wild bean allele frequencies today may not reflect those in pre-domestication populations. In addition, stored germplasm has been shown to accumulate mutations in the form of single nucleotide polymorphisms, or SNPs, over time when stored in seed banks (Fu et al., 2023). This suggests that even preserved bean seeds from times closer to domestication may not provide entirely accurate pictures of pre-domestication bean genomes. While modern wild bean germplasm reflects more current allelic frequency shifts, they can still be used to understand the greater genetic variety that exists outside of domesticated lineages of common bean.

Several traits distinguish wild *P. vulgaris* L. from its landrace and cultivar counterparts. Some of these traits relate to the mode and condition of growth. The ancestral state's mode of growth is thought to be the climbing phenotype, in which the shoot of the bean elongates vertically, necessitating structural support during its growth; cultivars and landraces have greater variation in phenotype, with bush phenotypes, in which shoot growth is lateral, ancestral climbing phenotypes, and intermediate half-climb phenotypes, which exhibit a mixture of climbing and bush phenotypes (Gepts, 1998). In addition, wild common bean has a lower range of tolerance to day length and to warm temperatures, with pre-landrace migration and Western cultivation broadening these ranges of day length and temperature tolerance at different points in time (Gepts, 1998). More drastic changes in environment in which common bean was sowed, such as in the Andes Mountains of South America and in the higher latitudes of Europe, also

necessitated human intervention via selective breeding to extend these tolerances to desired growth conditions (Koinange et al., 1996).

Further and arguably more distinguishing differences between domesticated and wild common bean can be found when investigating its means of reproduction. The general trend of acquired traits that cause a population to become obligately dependent on humans for its survival is referred to as domestication syndrome. In many crops, including common bean, domestication syndrome renders the plant unable to disperse seeds without human intervention, increases the dormancy time of seeds, enlarges fruit and seed products, and decreases the time interval from germination to reproductive maturity (Koinange et al., 1996). In wild lineages of *Phaseolus vulgaris* L., seeds can be dispersed via seed shattering, wherein seeds are released upon ripening. Seed shattering is a trait that is both genetically and environmentally influenced, with strong natural selection for the ability to seed shatter re-emerging in feral domestic crop populations previously bred to be negative for this trait (Maity et al., 2021). In cultivated common bean, seed shattering is disabled by selecting for mutations that inhibit the formation of fibers in the sutures and walls of the bean pods (Koinange et al., 1996). This is favorable for farmers as it allows for control of crop harvest. Fundamental changes in phenotypes acquired over the course of domestication do not necessarily correspond with what would confer fitness outside of a cropping system.

A Cultural Symbol: Post-Colonial and Post-Industrial Perspectives

Likewise, multiple cultures have adopted *Phaseolus vulgaris* L. to some degree across history. One of the most emblematic ties between human cultural practices and the persistence of

common bean is the milpa agricultural system. In some of its simplest definitions, milpa is a Mayan food system that is always, but not necessarily exclusively, comprised of maize, common bean, and squash. Previous literature and research in this area have found more extensive definitions of milpa, the most common expanding the definition to encompass sociocultural, economic, and or environmental effects attributed to the crop system itself (Rodríguez-Robayo et al., 2020). Milpa is not merely viewed superficially as an agricultural method, but something embedded at a deeper level within the lives of those who practice it. For instance, during the Guatemalan revolutions of the latter half of the twentieth century, milpa became an identifying symbol of Mayan-Guatemalans, for better or for worse. Given little land for agriculture, Mayan-Guatemalans utilized milpa, allowing them to achieve high crop yields relative to the land occupied; alternatively, milpa was a visible symbol of Mayan heritage and was often destroyed by agents of the Guatemalan government as a demonstration of the oppressive power held over the Mayan-Guatemalans (Handy, 2022). Variations on the milpa system of agriculture, such as Three Sister Gardens, have been found in North American indigenous groups, such as in the Iroquois, for whom common bean comprised an estimated 13% of daily dietary calories based on historic and ethnographic records from the early 1600s (Hart, 2022). Generations of cultivation, whether by American indigenous peoples using milpa or farmers utilizing Western agricultural techniques, ultimately contribute to the genetic make-up of current germlines of common bean.

In its cultivated form, *Phaseolus vulgaris* L. is a global crop of great significance. The harvest of both the beans and their edible pods feeds over 300 million people annually (Nasar et al., 2023), with common bean contributing up to 30% of the world's caloric consumption (Nadeem et al., 2021). Furthermore, common bean is economically important on multiple levels.

Rural farmers in less industrialized countries stand to profit from common bean farming for local distribution, as in the communities of the Kashmir Himalayas (Nasar et al., 2023). In other countries, such as South Africa, poorer farmers are encouraged by the federal government to increase their common bean yields through freely provided seeds and anthropogenic nitrogen fertilizers, such as NPK (nitrogen-phosphorus-potassium), to promote food security (Habinshuti, et al., 2021). For these reasons, the common bean exemplifies the importance of nutrient-dense and economical crops in countries with developing and often post-colonial economies.

Worldwide current trends suggest a continued if not greater reliance on the common bean in years to come; however, it is questionable whether the common bean as it exists today can meet these demands. Common bean production has been predicted to play a significant role in increasing global food production by 70% by 2050 to feed a growing global population (Nadeem et al., 2021) and has been tied to attempts to combat global hunger alongside new farming technologies in 20th-century movements referred to as the Green Revolutions (Reinprecht, Schram, Smith, et al., 2020). Originating as a means to increase food productivity after World War II, the Green Revolutions involved bringing industrial agricultural techniques, such as nitrogen fertilizers and seed cultivars, to non-industrialized nations, where crop populations were selectively bred to be more adapted to local environments (Wu & Butz, 2004). In the present day, data from countries supplied with tools to industrialize their agriculture reveal a general and significant decline in genetic diversity within their crop populations, which is likely due to the popularization of mega-variety cultivars, or exceedingly popular cultivars of plants expected to produce near-identical crops (Khoury et al., 2022). This lack of diversity creates a risk for entire populations of crop loss in the event of certain stresses, such as disease, drought, or pest

infestation. The reliance of common bean on anthropogenic nitrogen fertilizers for yields large enough to meet demands is also an issue. As of 2016, over 28 billion USD is spent globally on the application of more than 108 million metric tonnes of nitrogen fertilizers, of which only half is able to be taken up by the plants to which it is applied (Ferguson et al., 2019). The nitrogen fertilizers that are not taken up can run into bodies of water, causing accelerated eutrophication, population imbalances in aquatic ecosystems, and eventual local loss of organisms that require oxygenated water for life; humans can also feel these repercussions firsthand through runoff contaminating sources of drinking water (Chislock et al., 2013). While the common bean has been a viable response to the call for cost-effective and nutritionally dense food thus far, its severe decline in genetic diversity and the environmental costs of growing the crop in industrialized agricultural systems cast a grim outlook on its ability to continue serving its current function.

Domestication, Nitrogen Addition, and Nodulation

Despite the concerns that arise from a lack of genetic variation within cultivar populations of *Phaseolus vulgaris* L. and the amount of fertilizer needed to create crop yields that meet demand, the relationships between domestication, excessive nitrogen fertilization, and the common bean's reputation as a poor nitrogen fixer are not well understood. Preliminary research from the Haus Lab at Michigan State University indicates better nodulation in experimentally grown wild beans from the Mexican gene pool compared to similarly grown domesticated beans, which may be indicative of better facilitation of nitrogen fixation. While both individual plant genotypes and environment are understood to impact the nodulation

phenotype of common bean, the reduction of this trait in domesticated lineages may be associated with reductions in genetic variation; cultivar accessions of common bean have greatly reduced allelic diversity when compared to both landrace and wild accessions (Gepts, 1998). Conversely, nitrogen presence in soil can also lead to a reduction in nodulation in legumes, causing reduced associations with nitrogen-fixing rhizobia (Ferguson et al., 2019). Due to these factors that influence nodulation phenotype, and potentially the ability to facilitate nitrogen fixation, it would not be unreasonable to suggest a connection between the human impositions on genetic diversity, via domestication, and nitrogen addition, via fertilizer application, and poor capacity to facilitate nitrogen fixation. My thesis experiments evaluate why nitrogen fixation may appear more efficient in wild beans than less genetically diverse accessions, especially cultivars. By growing a total of ten accessions of genotyped beans from wild, landrace, and cultivar-type lineages in the presence or absence of nitrogen fertilizer, I intend to address the following questions:

1. Are traits commonly associated with measuring nitrogen fixation, especially nodule number, root:shoot ratio, and chlorophyll content index (CCI) consistently higher in wild beans than in accessions of domesticated lineages, regardless of nitrogen addition?
2. Is nodule number positively related to other proxies of nitrogen fixation, such as root:shoot ratio and CCI, in common bean?

I anticipate that the availability of genetic diversity will be more significant in determining nodulation phenotype. Because of this, I hypothesize that my wild accessions, which have more in-population genetic variation than the landrace and cultivar accessions, will have

higher nodule numbers, higher root:shoot ratios, and higher CCI values. These traits are all positively related to nitrogen fixation in distinct ways. A higher nodule count is indicative of more associations with rhizobia, which live and fix nitrogen inside nodules. A larger root:shoot ratio, or the dry mass of the belowground biomass divided by the dry mass of the aboveground biomass, can be indicative of greater investment in the plant's roots and thus, more investment in nodulation. A high CCI value indicates that there are higher concentrations of the photosynthetic pigment chlorophyll, which can be made using nitrogen accessed from nitrogen fixation. Because increased nodule number is not directly correlated with increased nitrogen fixation (Ferguson et al., 2019), it is meaningful to assess these data in light of how it corresponds to nitrogen fixation. I predict that higher nodule number will be positively associated with higher CCI and higher root:shoot ratios.

Chapter 2

Methods

To test my hypotheses, I conducted two experiments: a pilot experiment to capture soil bacteria, and a final experiment in which nitrogen was applied. The pilot provided me with a way to test the germination of my germplasm and to see if nodules would form on the roots of my beans. Using materials and knowledge from the pilot, I was then able to conduct my final experiment and collect data to answer my research questions.

Germplasm Utilized

The bean seeds, or germplasm, that I used were sourced from the lab of Dr. Miranda Haus, an assistant professor in the Department of Horticulture at the Michigan State University College of Agriculture and Natural Resources. These germplasms came from 10 different accessions that had been genotyped by the Haus Lab and had been categorized by lineage type: wild, landrace, and cultivar (**Table 1**). The germplasms I used are all from or derived from the Mesoamerican gene pool of common bean. Individual germplasms from each accession were assumed to have negligible genetic differences from other individual germplasms in the same accession. The landrace accessions included in my experiment are representative of the populations from which the included cultivar accessions were derived. This means that landraces with the same market class as cultivars in **Table 1** have non-negligible genetic similarity to each other. I sowed the same 10 accessions in both my first and second experiments. Germplasm from different accessions varied in seed coat color, patterns, and size, highlighting both the selective

pressures of domestication in the landrace and cultivars as well as the degrees of variation that emerge within the Mesoamerican gene pool of common bean (**Figure 3)**.

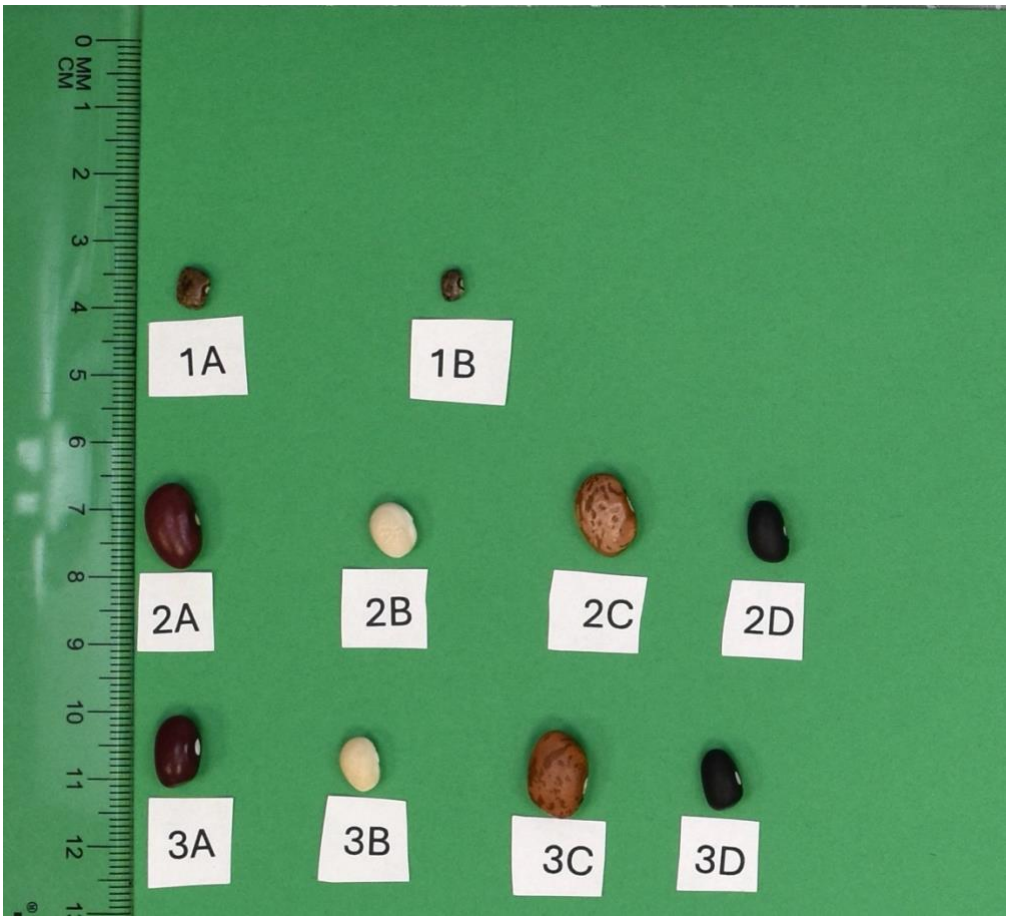

Figure 3. Germplasm of accessions used in my experiments arranged by lineage type (1A: WBO; 1B: WBT; 2A: CRM; 2B: ROB; 2C: COP; 2D: BTS; 3A: CAY; 3B: ALP; 3C: ELD; 3D: ZOR)

Table 1. Classification of Experimental Accessions of Common Bean

Accession Name	Lineage	Market Class	3 Letter Code	Earliest Recorded Year of Accession
Wild Bean 1 (One)	Wild	-	WBO	1989
Wild Bean 2 (Two)	Wild	-	WBT	2002
Black Turtle Soup	Landrace	Black	BTS	1900
Common Pinto	Landrace	Pinto	COP	1900
Common Red Mexican	Landrace	Red	CRM	1900
Robust	Landrace	Navy	ROB	1900
Zorro	Cultivar	Black	ZOR	2009
Eldorado	Cultivar	Pinto	ELD	2012
Cayenne	Cultivar	Red	CAY	2018
Alpena	Cultivar	Navy	ALP	2014

Randomized Block Design

I used a randomized block design in both experiments to mitigate any discrepancies in environmental conditions across the greenhouse. Each block was made up of two trays of plants

that were kept together. Each tray held eight plants for a total of 16 per block. In an Excel sheet, I assigned each tag a random number using the function =RAND() and sorted either by ascending or descending to assign order. I limited randomization to within blocks to prevent the likelihood of one block having a majority of representatives of an accession and/or a treatment.

Experiment 1: An Assessment of Germination and Nodulation in My Experimental Accessions and Capture of Rhizobia Compatible with Common Bean

I conducted my preliminary pilot greenhouse experiment over approximately seven weeks. My primary goal was to capture rhizobia that were compatible with my accessions, as this was vital to conducting my second experiment. To adequately study my research questions, I needed to grow common beans that would develop nodules, or nodulate. By capturing rhizobia from nodules that formed on the roots of the same bean accessions that I would grow in my final experiment, I could make an inoculum that I knew could form nodules with my plants. Having an inoculum for my second experiment meant that I could control for what bacteria lived in the rhizosphere, or media surrounding the roots, of my beans and use cleaner pot media that would be less likely than field soil to carry harmful plant pathogens.

Selection of Field Soils and Nodule Harvest for Inoculum

I sowed my seeds in a combination of non-sterile field soil, calcined clay, and silica sand. Approximately 20% of the field soil utilized originated from the 2023 green bean plots at the Keiko Miwa Ross Student Farm at the Pennsylvania State University and the remaining 80% of field soil originated from cover crop mixture plots managed by the Russel E. Larson Agricultural

Research Center at Rock Springs, PA. The 2023 Student Farm green bean plots specifically had grown *Phaseolus vulgaris* L. and had been in use for green bean production since the spring of 2020. The soil from the cover crop mixture plots had hosted various legume species over the past 11 years, including cash crop *Glycine max* (L.) Merr (soybean) and cover crops *Trifolium pratense* L. (common medium red clover) and *Pisum sativum L.* (Austrian winter pea), but not *P. vulgaris* L (Murrell et al., 2017). I purposefully used field soil from areas that had grown legumes before to increase the likelihood that my beans would find compatible bacteria to form nodules with. I used combinations of different field soils so that I could give my beans as many options of rhizobial endosymbionts to choose from as possible. I also added calcined clay and silica sand to this mixture to make cleaning the roots and subsequent nodule harvesting easier.

At harvest, I collected a maximum of 20 nodules from each accession that survived to harvest. If one representative of an accession had less than 20 nodules, another representative of that accession had nodules harvested until nodules from representatives of that accession totaled 20. If there were not enough plants to do this, then the largest possible number of nodules were picked. I combined all the picked nodules before bleaching them and storing them in 0.85% NaCl, which would prevent the rupture of cells from osmotic pressure.

Experiment 2: An Assessment of How Different Accessions of Common Bean and Application of Nitrogen Fertilizer Impacts Legume-Rhizobia Symbiosis

In my second experiment, I evaluated the significance of accession versus nitrogen application in the ability of common bean to facilitate nitrogen fixation of symbiosis with

rhizobia. This experiment yielded the data that I would use to make my final analyses for supporting or rejecting my hypotheses.

Potting Media and Pot Preparation

For my potting media, I combined a 1:1 mixture of calcined clay and silica sand using a cement mixer. Before mixing, the cement mixer was sterilized with dilute GreenShield II Disinfectant and Algicide - containing active ingredients benzyl-C12-18-alkyldimethyl, chlorides; C12-14-alkyl[(ethylphenyl)methyl]dimethyl, chlorides; ethanol) (BSAF, 2022) - and rinsed with water to ensure the removal of any residues. The potting media was then mixed in the cement mixer, with added tap water to help my media mix evenly. Non-perlite vermiculite was sterilized via autoclave and was used to cover beans at planting. I omitted field soil because I would later apply inoculum as the source of rhizobia for my beans.

I filled disinfected 2 by 10-inch comical cell planters to approximately 2/3 full with potting media. To prevent loss of media, I blocked the bottoms of my pots with disinfected mesh synthetic fabric and secured them with UV-resistant rubber bands. Before planting, I watered the potting media until the water ran through the bottoms of the pots. This ensured that the media would be wet enough to encourage my beans to germinate, as they require a lot of water to do so. I also treated the media treated with 10 mL 2x Fahräeus medium as a measure for improved germination.

Seed Preparation, Planting, and Germination

For each accession, I set aside a maximum of 32 seeds. If less than 32 seeds were remaining from my experimental accessions, I set aside the highest even number of seeds as my supplies allowed. Seeds were scarified, or had small scratches made in their seed coats, using a small pair of scissors. Scarification was only necessary for germination in my wild accession beans, but all seeds were scarified to encourage more uniform germination. After scarification, I disinfected my seeds in a dilution of 10% bleach. This was done to remove any microbes that may have existed on my seeds before sowing and further control which microbes were present in the rhizosphere of my beans. After bleaching, I rinsed my beans until they no longer smelled of bleached, then divided them randomly by accession into two sterile 50 mL conical centrifuge tubes and covered the beans in sterile 0.85% NaCl solution and autoclaved deionized water. The submerged beans were left to imbibe in the dark for 2 to 3 hours, which allowed them to take in water and encouraged faster germination.

I planted my prepared seeds two to a pot, or as my supplies allowed. Seeds were planted hilum-down, covered with vermiculite for moisture retention, and misted with water. Immediately after sowing, I covered my pots with clean clear plastic painter's tarps to retain moisture for germination and watered daily. 10 days after they were sowed, I removed the tarp and moved the pots to their randomized blocks.

Growth Conditions

Beginning 7 days after planting, all plants were fertilized with 10 mL 2x Fahräeus medium. Fahräeus is a liquid nutrient medium that lacks nitrogen and is used to grow legumes in nutrient-poor potting media, like calcined clay silica sand. I recipe I used was made using a modified recipe from Appendix 2 in *The* Medicago truncatula *Handbook* (David G. Barker et al., 2006). 2x Fahräeus medium application continued once per for six weeks. For the remaining two weeks, 4x Fahräeus medium was applied to meet an increase in the needs of the plants. After the tarp was removed, I watered the beans as needed until harvest. Pots in which two germination events occurred were culled to one plant via cutting of the shoot approximately three weeks after sowing. Removing additional germinants from the same pots prevented competition for resources that may have skewed my results.

Homogenizing Nodules from Experiment 1 and Inoculation

I drained nodules harvested during the pilot of their 0.85% NaCl suspension and rebleached them in 10% bleach dilution for 2 minutes. I then added my nodules to a sterile 15 mL conical centrifuge tube, suspended them in sterile 0.85% NaCl solution, and homogenized them for 90 seconds utilizing an automated homogenizer.

I then centrifuged my homogenized nodules at 400 x g at 10° C for 7.5 minutes, forming a solid pellet at the bottom of the tube. This forms a layer of supernatant that contains less the less dense bacterial cells and the pellet, which is made up of denser plant matter. In a sterile environment, I poured off the supernatant into a sterile 15 mL conical centrifuge tube and

discarded the pellet. I combined 4 mL of rhizobia-rich supernatant with 900 mL of autoclaved 0.85% NaCl solution to make my inoculum. I poured 5 mL of this mixture into every pot 10 days after sowing to inoculate my beans.

Nitrogen Treatment

Initial nitrogen treatments began 14 days after sowing, or 4 days after inoculation. Once they began, nitrogen treatments took place once per week on the same day that all pots received Fahräeus medium. Based on the recipes for Fahräeus medium and hydroponic nutrient solution HY found in *The* Medicago truncatula *Handbook* (Baker et al., 2006) as well as annotations from Jennifer E. Harris, Dr. Regina B. Bledsoe, and Ellen M. C. Bingham, I decided to start adding diluted KNO_3 stock on my N_+ treatment group. I initially added a 0.02 M concentration of KNO_3 via a diluted stock solution. Each plant received 5 mL of 0.02 M KNO_3 stock. Each week, I increased the concentration of nitrogen applied to the N_+ treatment group by a factor of *[moles KNO_3 in week 1 treatment]*4*n* where *n* is equal to the number of weeks since the first nitrogen treatment (**Table 2)**. This was always proportioned to a volume of 5mL for application to prevent too much liquid from being added to the pots at once. Adding too much liquid could cause rhizobia in the rhizosphere or nutrients from the Fahräeus medium to flow out through the bottoms of the pots. To make sure both treatment groups received the same amount of liquid similarly, the N_0 group also received an 5mL of tap water on days when nitrogen fertilizer was added to the N_+ treatment group.

Table 2. Nitrogen Application Timeline

Week of Nitrogen Fertilization	Week of Experiment	Mol KNO_3 in Nitrogen Application Per Pot	Dilution Concentration (in 5mL)	1M KNO_3 volume per pot	DI H_2O volume per pot	Total Volume per Pot
1	3	0.0001	0.02M	0.1 mL	4.9 mL	5 mL
2	4	0.0004	0.08M	0.4 mL	4.6 mL	5 mL
3	5	0.0008	0.16M	0.8 mL	4.2 mL	5 mL
4	6	0.0012	0.24M	1.2 mL	3.8 mL	5 mL
5	7	0.0016	0.32M	1.6 mL	3.4 mL	5 mL

*after week 1, increases by (0.001*4n), where n is the number of weeks since week 1

Harvest Procedures and Data Collection

Near-daily observations on plant health, group, and germination were kept on a shared digital log. I noted when the plants germinated because this would let me determine the effective age of my plants from their date of germination to the day of harvest. I collected chlorophyll content index (CCI) using a chlorophyll meter from live leaves while the plants were growing. I would go to the youngest completely unfurled leaves of the plant and then go down one leaflet to determine which leaves to take CCI values from. I would three take values each from two leaves in the chosen leaflet and would average them together to get an average CCI value for the plant at that time point. Other data could only be collected at harvest.

I harvested my bean plants approximately eight weeks after sowing. Before harvest, I took six chlorophyll meter readings per plant to create an average CCI value for each remaining bean plant. I harvested the aboveground biomass, or the shoot, using sterilized scissors to cut the shoot where it emerged at the soil line in the pot. I then dried it for three days in a drying oven and took the dry masses of each. I harvested the belowground biomass, or the root system, by cleaning it from the pot media. I then floated the roots in water and imaged each using a flatbed scanner. I used ImageJ to count the nodules on each plant. After scanning, the belowground biomass was then oven-dried for two days and dry massed.

Statistical Analysis

RStudio was utilized to analyze data collected from germination time points and final harvests. I used packages dplyr and ggplot2 to create analytical pipelines to understand any

relationships between variables and differences between groups in my data. The significance of these relationships and differences were then assessed via ANOVA and Tukey tests. ANOVA tests were conducted to control for interference from confounding variables when applicable and Tukey tests were run with a 95% family-wise confidence interval. I interpreted the p-values output by these tests to be significant if they were less than 0.05 and further investigated these relationships between variables using Tukey tests.

Chapter 3

Results

Table 3. Germinations by Accession and Nitrogen Treatment in Experiment 2

Accession	Lineage	Germinating Plants in N_+ Group	Germinating Plants in N_0 Group
WBO	Wild	8	8
WBT	Wild	6	7
BTS	Landrace	7	4
COP	Landrace	0	0
CRM	Landrace	1	0
ROB	Landrace	0	0
ZOR	Cultivar	0	0
ELD	Cultivar	1	2
CAY	Cultivar	2	3
ALP	Cultivar	0	0

In both of my experiments, I saw lower rates of germination than I had anticipated. Failure to germinate, especially in the final experiment, limited the data I could collect on certain accessions and direct comparisons of nitrogen treatment groups (**Table 3**). Of the germinated plants, only 40 survived until the harvest date.

Accession Consistently Influences Traits Associated with Nodulation More Significantly than Nitrogen Treatment

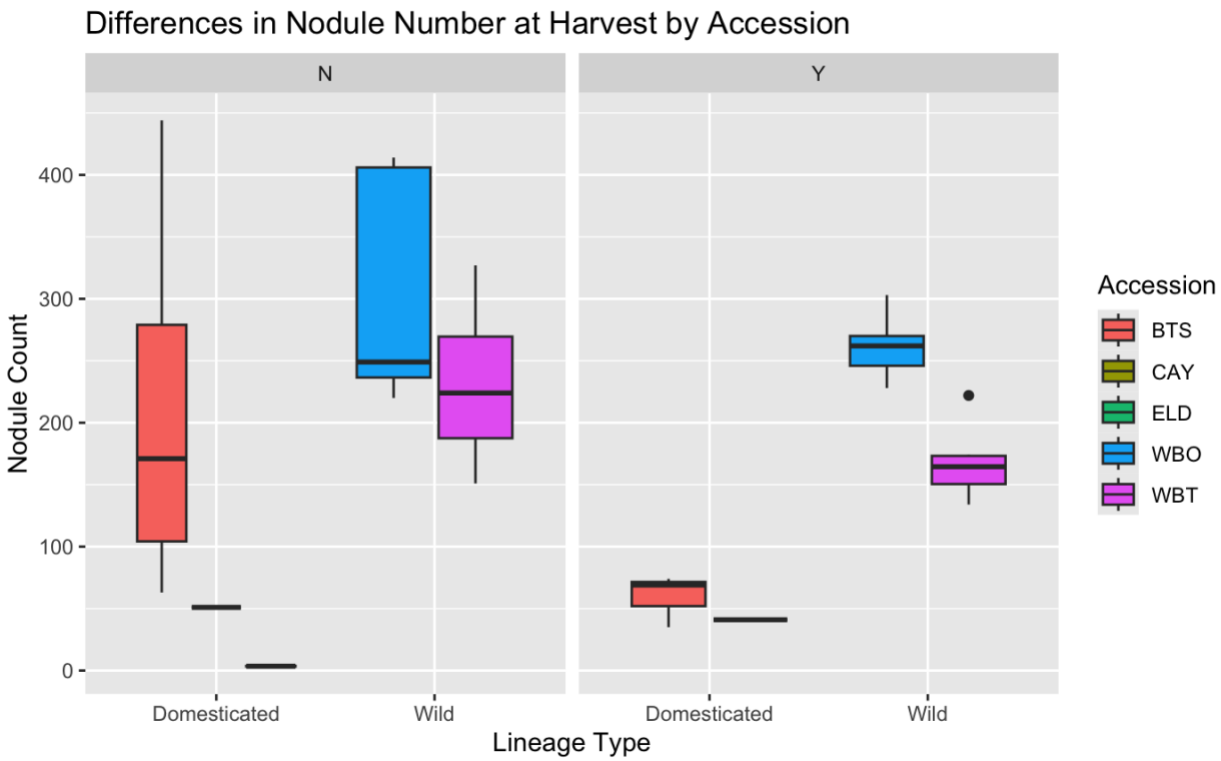

Figure 4. Nodules picked from wild lineage types, especially WBO, seem to form more nodules than domesticated lineage types.

I looked at the relationships between nodule number, accession, and nitrogen treatment to understand if the number of nodules formed was more dependent upon nitrogen treatment or accession (**Figure 4)**. Because I had a lot of variation in the number of representatives that had survived to harvest, I also visualized these data by lineage type ('wild' or 'domesticated'). When running the ANOVA to understand if these differences were significant, I found that accession had the most significant effect on nodule number (nodule number ~ accession*nitrogen treatment, p~0), with data collected from WBO being significantly different from CAY, ELD, and BTS and data from WBT being significantly different from ELD (**Appendix A: Table 4**).

The presence (Y) or absence (N) of the nitrogen treatment was also significant, but less so than accession was (nodule number ~ accession*nitrogen treatment, p=0.01653) (**Appendix A: Table 6**). These values do not show significant differences between WBT and WBO, which implies similar levels of nodulation across accessions that are wild lineage type. Though WBT does not differ significantly in nodule number compared to three of the other four accessions represented, that WBO differs significantly from every accession except WBT may suggest that wild lineage types are better able to nodulate than domesticated lineage types.

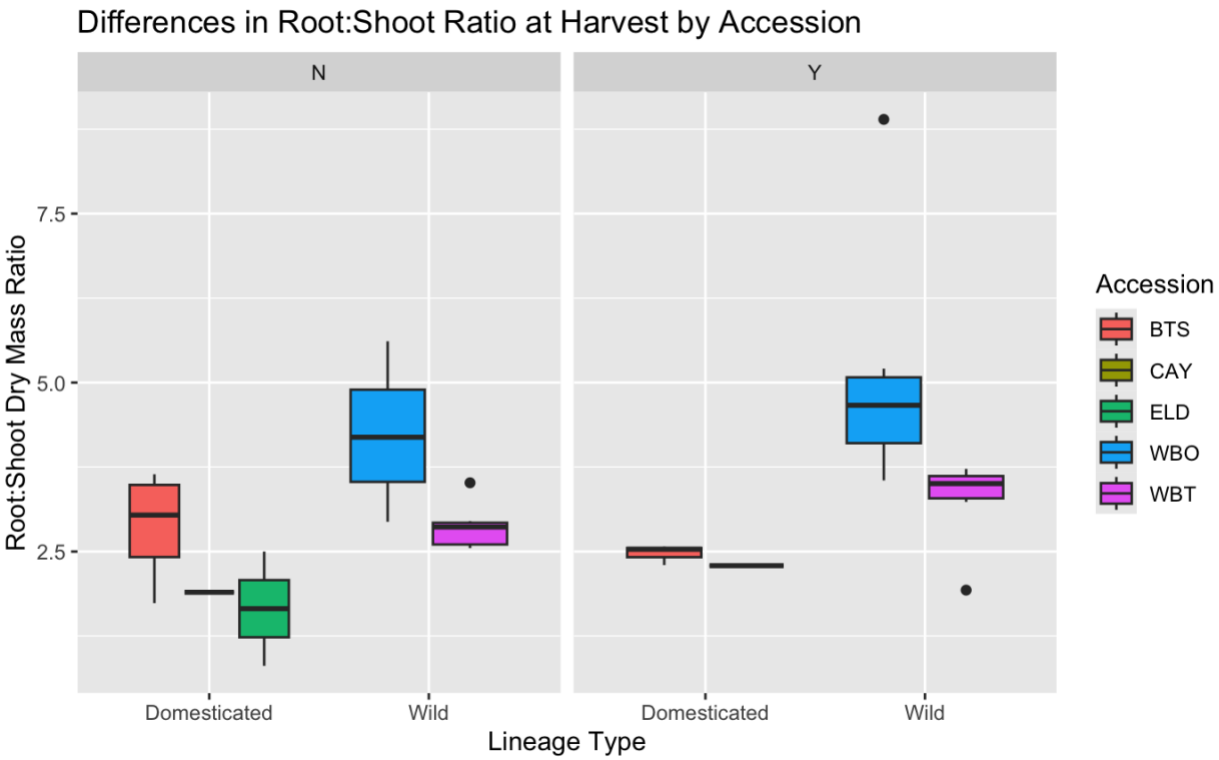

Figure 5. While accession significantly affected root:shoot ratio overall, there were no accessions with root:shoot ratios that were significantly different compared to all other accessions.

Similarly, root:shoot ratio differs most significantly by accession compared to nitrogen treatment group (root:shoot ratio ~ accession*nitrogen treatment, p~0) (**Figure 5**). Unlike the data I collected and analyzed for nodule number, only accession was seen to be significant, with

WBO having significantly different root:shoot ratios than BTS and ELD and WBT having significantly different root:shoot ratios than WBO (**Appendix A: Table 8**). Surprisingly, nitrogen treatment did not have a significant influence on root:shoot ratio, even though it provided the positive treatment group (Y) with more of a limiting resource, while the null treatment group (N) had only nitrogen fixation as a nitrogen source and would have presumably needed to invest more resources into its roots.

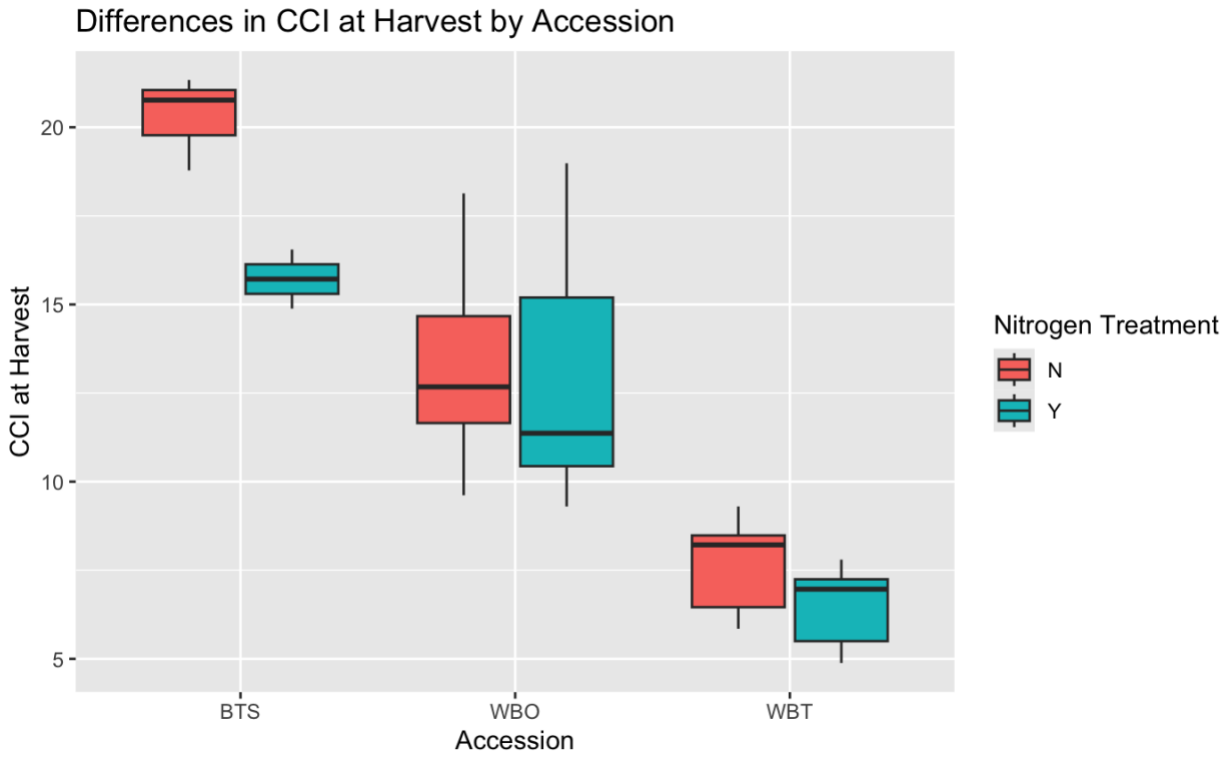

Figure 6. CCI differs significantly by accession, but not by nitrogen treatment.

CCI also saw significant differences in data across accessions (harvest CCI ~ accession*nitrogen treatment, p~0) but did not see any significance by nitrogen treatment group (**Figure 6**). CCI was significantly different between WBO and BTS as well as for WBT and WBO and WBT and BTS. CCI differences by accession may indicate that the differences

between the ability to facilitate nitrogen fixation among different accessions of common bean may have a larger genetic component tied to the genetic histories of their accessions than the nitrogen present in their environments.

Overall, accession consistently appeared as one of the most significant factors that influenced differences between metrics of traits associated with nitrogen fixation.

Nodule Number as a Measure of Nitrogen Fixation in Common Bean is Inconsistent with Other Related Measures

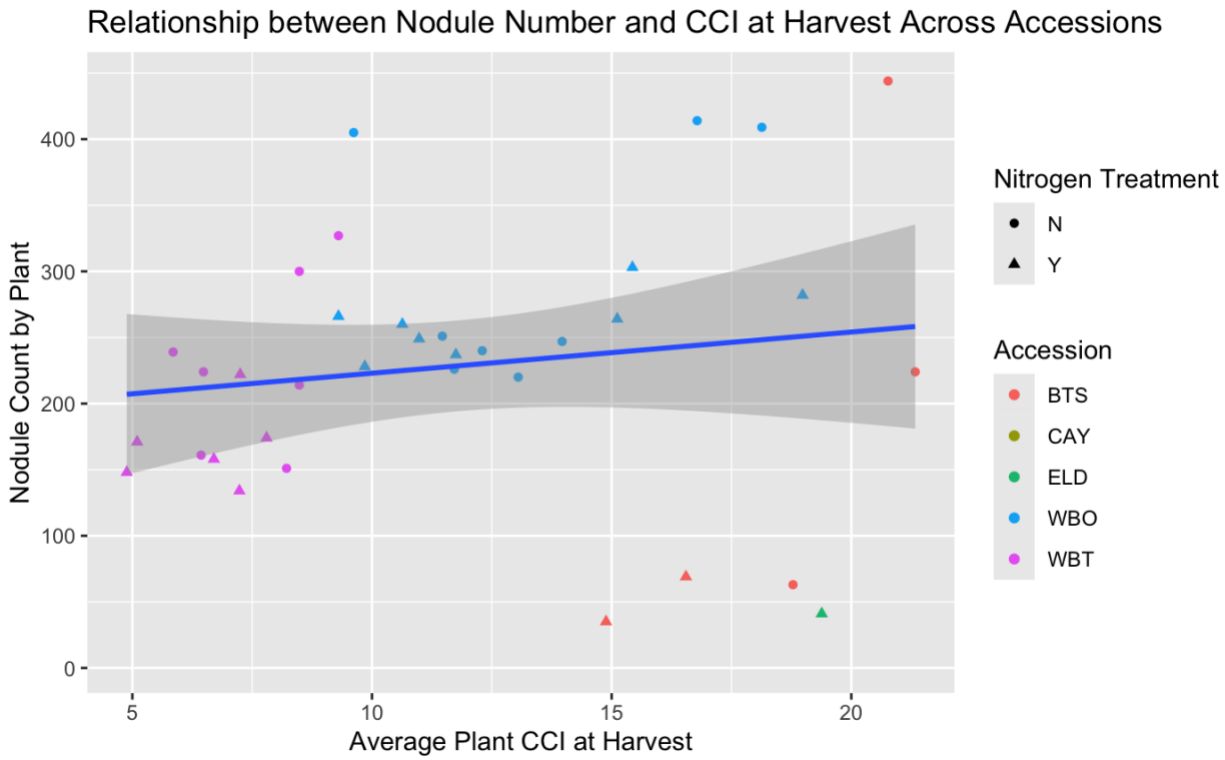

Figure 7. Greater nodule number does not positively correlate with higher CCI values.

When comparing nodule number across individuals across all accessions with CCI values recorded at harvest, there is no significant relationship between CCI and nodule number (**Figure**

7). I also considered accession and nitrogen treatment in this comparison but only found accession to be significant of the two additional variables analyzed (nodule number ~ harvest CCI value*accession*nitrogen treatment, p~0). This agrees with the results seen in **Figure 4** that indicate nodule number is significantly dependent on accession.

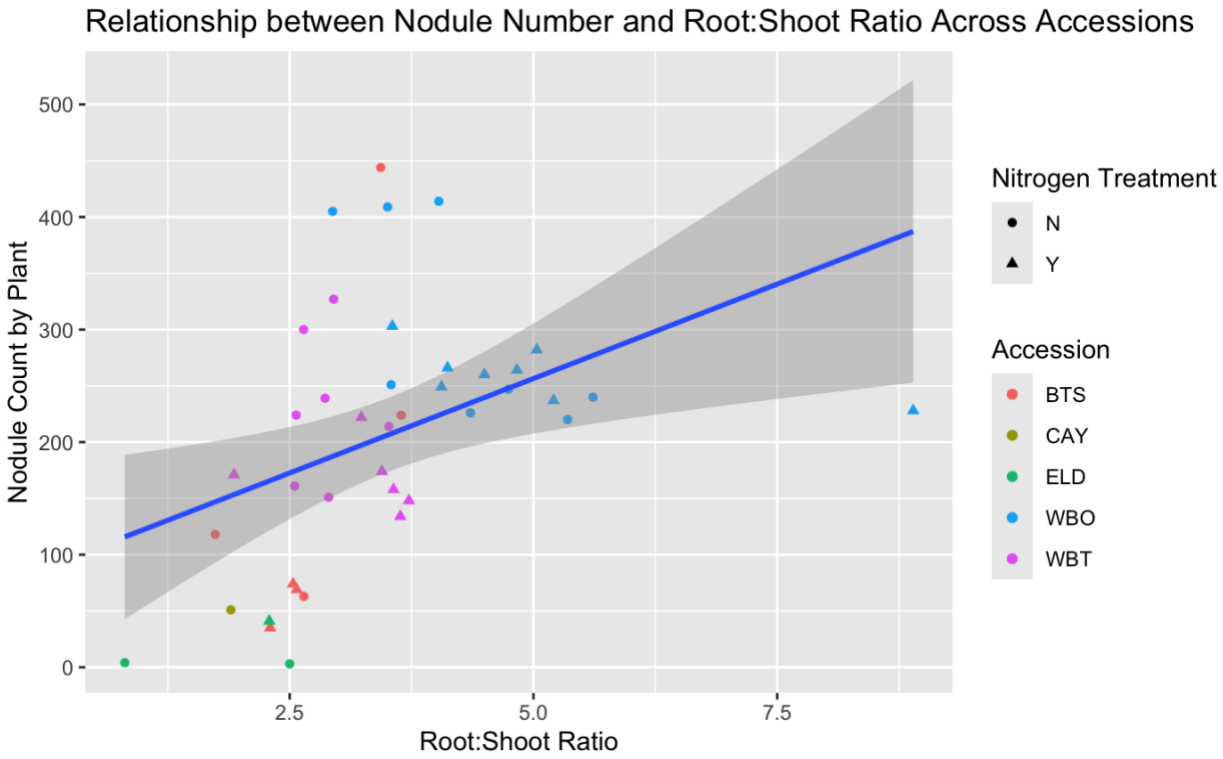

Figure 8. Root:shoot ratio is equally as related to nodule number as it is to accession.

Upon examination of the relationship between root:shoot ratio and nodule number, I found that root:shoot ratio is significantly related to nodule number (nodule number ~root:shoot ratio*accession*nitrogen treatment, p~0), but also equally significant is accession, as is demonstrated in **Figure 5**.

While nodule number is a trait that differs by accession, it does not appear to be a telling indicator for the efficacy of nitrogen fixation in common bean due to its lack of association with CCI value and root:shoot ratios.

Chapter 4

Discussion

Analysis and Future Experiments

Indications that the facilitation of nitrogen fixation in the nodules of legumes generally agree with the idea that initiation and maintenance of nodules fall under the domain of the host plant, particularly its accession. It is established in the literature that nitrogen-fixing capabilities in *P. vulgaris* L. are heavily reliant on the host-legume's genotype, with experimental data demonstrating advantages by cultivar accessions regardless of nitrogen application, fertilization, or rhizobial inoculation (Reinprecht, Schram, Marsolais, et al., 2020). In addition to recruiting rhizobia and nodule organogenesis, legumes also transcribe genes that ultimately serve to regulate nodule number (Reinprecht, Schram, Marsolais, et al., 2020). Thus, a host phenotype of low nodule number could be suggestive of low variation in genes responsible for regulating nodule number. Mutations leading to a large number of nodules forming on host legume roots, known as hyper-nodulation, are the result of mutated versions of genes used in the AON pathways of legumes and have been found in genes otherwise associated with shoot genotype, like *nts382* in soybeans (Roy et al., 2020). Examples of mutations in traits that are seemingly unrelated to nodulation, such as *nts382*, may suggest that heavily selecting for traits historically favored in common bean bears influence on nodulation. A larger sample size of beans from different accessions and lineages would support that the facilitation of nitrogen fixation is reduced in domesticated beans and may potentially provide insight into the pleiotropic nature of genes underlying traits under artificial selection.

Nitrogen application seemed to have less significance on nodulation within the context of my experiment than within experiments I referenced from the literature. In Habinshuti et al., 2021 which studied the relationship between nitrogen fertilizer application and inhibition of nitrogen fixation in South African fields of common bean, the plant average nodule number for each field involved in the experiment differed significantly with nitrogen fertilization type and number of applications for the duration of the experiment (Habinshuti et al., 2021). It should also be noted that while differences between the results from my experiment and those of Habinshuti et al., 2021 cannot be directly compared due to difference in design and types of data measured, the trends I observed merit further investigation given results from papers like Habinshuti et al., 2021.

This experiment was limited in size and this was further exacerbated by unanticipated issues with germination. Seed viability by genotype did not appear uniform, as in both the pilot and final experiments, seeds from the same genotype repeatedly failed to germinate. Because of this, representatives of beans by genotype were biased toward seeds that had higher germination rates in both my pilot and final experiments. It remains to be seen if attempting to germinate seeds of the same genotype from different sources would lead to greater germination rates. Ideally, a larger number of genotypes and number of representatives per genotype would yield a higher number of germinants and would increase the statistical power of downstream data analysis. Differences in germination time also led to a lack of uniformity across and within representatives of the same genotype. Future attempts at a similar experiment would likely benefit from sowing pre-germinated seeds to mitigate the usage of inviable germplasm and lessen discrepancies in germination times.

Data collection in this experiment also has the potential to be expanded upon. While data regarding nitrogen fixation were collected from nodule count and average plant CCI readings, examples of similar metrics for measuring rates of nitrogen fixation in common bean often utilize chemical assays. For instance, the percentage of nitrogen derived from the atmosphere, or the Ndfa % calculates the ratio of specific nitrogen isotopes in a dried and powdered sample of the plant and compares it to the ratio present in atmospheric nitrogen (Habinshuti et al., 2021). Another more direct usage of biochemistry to analyze plant nitrogen obtained via nitrogen fixation is the ureide assay. A ureide assay involves extracting ureide compounds, or compounds derived from nitrogen-containing urea, from the xylem, or vascular tissue, of a bean and uses these data to measure N2 fixation occurring in nodules (Herridge et al., 1988). In soybean models, ureide concentration in plants was seen to increase linearly with plant maturity, indicating that mature plants have a greater reliance on nitrogen fixed by their endosymbionts than on soil nitrogen (Herridge, 1982). The changing relationship between legume hosts and their nitrogen-fixing rhizobia thus can be more directly observed by measuring how fixed nitrogen exists in vivo before its breakdown to further derived nitrogen compounds. The association of ureide assays with different time points in common bean development could eventually underly strategies for nitrogen fertilization and prevent excess nitrogen application to monocrops of common bean that would not be as receptive to non-fixed nitrogen. Including ureide assays in future experiments could also further establish genetic linkages between domestication bottlenecks and molecular biological processes, such as transcription, translation, and differential gene expression, as they relate to SNF. Early chemical assays predating metabolomics and proteomics have determined that the proteins, amino acids, and other

nitrogen-containing biological compounds differed significantly between wild and domesticated genotypes of *P. vulgaris* L., essentially giving different lineage types unique fingerprints of nitrogen compounds (Sotelo et al., 1995). Given that accession is the driving factor in differences in nodule phenotypes, I hypothesize that further investigation of nitrogen-chemical composition across specific genotypes using ureide assays and other biochemical methods would allow for more precise descriptions of why facilitation of nitrogen fixation differs significantly between wild and domesticated populations of common bean.

Conclusions and Future Implications

Ultimately, domesticated *Phaseolus vulgaris* L. is a poor nitrogen fixer compared to other legumes and has greatly reduced genetic diversity compared to the wild populations it is understood to have descended from. While nitrogen addition to crops of common bean can be associated with differences in nodulation, it seems that heritable genetic variation is largely responsible for a poor nitrogen-fixing phenotype. To understand this further, biochemical methods can be used to collect additional data relating to nitrogen fixation and how this may differ between wild and domesticated accessions. For instance, performing assays of metabolites and mRNA from different accessions and lineage types could provide viable insight into differences in common bean gene expression relating to initiation, maintenance, and rates of nitrogen fixation. If alleles that enhance the ability to facilitate nitrogen fixation are more common in wild populations, then crossing wild and cultivar individuals may expand genetic diversity. Expanding genetic diversity for other traits, like disease resistance, within domesticated populations of common bean can potentially be achieved through this method but

are ultimately understudied (Gepts, 1998). Controlled mixing of cultivars has occurred by planting multiple cultivars in the same field in a study published in a 2020 study yielded success in creating hybrid vigor, but does not record much data on how these crosses impact host facilitation of nitrogen fixation (Reinprecht, Schram, Smith, et al., 2020). More data must be collected regarding the expansion of in-population genetic diversity in common bean.

Expanding our knowledge of the larger genetic variation in the common bean is of biocultural exigence as the chance to introduce more genetic diversity into cultivars from landraces and wild bean may not exist forever. As the climate changes and precipitation increases due to global warming, the landrace domesticates that are planted by indigenous farmers decline in favor of later planting types for maize in Milpa cropping systems (Ebel et al., 2018). Rising temperatures present a potential decline in the traditional growing practices of common bean, which is shown to have presented landraces with a significantly smaller genetic bottleneck than cultivars (Gepts, 1998). Furthermore, wild bean populations are less adapted to temperatures warmer than 21 C and would likely experience reduction in the event of significant increases in mean growing season temperatures and rainfall (Gepts, 1998). Time constraints to characterize genetic diversity lost through domestication bottlenecks are also enforced by worldwide population increase.

Introducing more genetic variation to domesticated common bean from wild sources could strengthen future global food systems. Environmental and financial constraints also push this exigence, as anthropogenic nitrogen fertilizer is both harmful to the environment and expensive (Chislock et al., 2013); (Ferguson et al., 2019). Global usage of nitrogen fertilizers in agriculture systems can potentially be lessened via a better understanding of nitrogen fixation in

the nodules of common bean. Through its development as a partner of both humans and rhizobia, it remains critical to understand how human influence has and continues to impact the relationship between rhizobia and common bean. However, human intervention is also needed to preserve the invaluable cultural and biological relationships that make *Phaseolus vulgaris* L. key to the persistence of both its human and bacterial partners.

Chapter 5 Appendix A

Statistical Models and Additional Tables

Statistical Models for Figure 4

Table 4. Figure 4: ANOVA (Nodule Number ~ Accession*Nitrogen Treatment)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Accession	4	247910	61978	10.866	1.27e-05***
Nitrogen Treatment	1	37737	37737	6.616	0.0515*
Accession:Nitrogen Treatment	3	22640	7547	1.323	0.2846
Residuals	31	176820	5704		

Signif. Codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

Table 5. Figure 4: Tukey Multiple Comparisons of Means: 95% Family-Wise Confidence Interval - Significant Relationships by Accession

	Diff	Lwr	Upr	p Adj
WBO-BTS	134.59821	35.523935	233.672494	0.0037516
WBO-CAY	230.31250	4.955795	455.669205	0.0432351
WBO-ELD	265.31250	127.761993	402.863007	0.0000377
WBT-ELD	185.76923	45.735164	325.803298	0.0047977

Table 6. Figure 4: Tukey Multiple Comparisons of Means: 95% Family-Wise Confidence Interval - Significant Relationships by Nitrogen Treatment

	Diff	Lwr	Upr	p Adj
Y - N	-60.83417	-109.7889	-11.87946	0.01653

Statistical Models for Figure 5

Table 7. Figure 5: ANOVA (Root:Shoot Ratio ~ Accession*Nitrogen Treatment)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Accession	4	38.71	9.678	9.364	4.41e-05 ***
Nitrogen Treatment	1	1.74	1.744	1.688	0.203
Accession:Nitrogen Treatment	3	1.65	0.550	0.532	0.663
Residuals	31	32.04	1.034		

Signif. Codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

Table 8. Figure 5: Tukey Multiple Comparisons of Means: 95% Family-Wise Confidence Interval - Significant Relationships by Accession

	Diff	Lwr	Upr	p Adj
WBO-BTS	1.94674540	0.6130998	3.2803910	0.0016999
WBO-ELD	2.77507884	0.9235022	4.6266555	0.0012468
WBT-WBO	-1.60132121	-2.7002077	- 0.5024347	0.0017338

Statistical Models for Figure 6

Table 9. Figure 6: ANOVA (Harvest CCI ~ Accession*Nitrogen Treatment)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Accession	2	534.0	266.99	46.771	1.19e-09 ***
Nitrogen Treatment	1	15.9	15.91	2.786	0.106
Accession:Nitrogen Treatment	2	14.8	7.40	1.296	0.290
Residuals	28	159.8	5.71		

Signif. Codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

Table 10. Figure 6: Tukey Multiple Comparisons of Means: 95% Family-Wise Confidence Interval - Significant Relationships by Accession

	Diff	Lwr	Upr	p Adj
WBO-BTS	-5.395624	-8.424511	-2.366737	0.0003992
WBT-BTS	-11.369742	-14.480728	-8.258757	0.0000000
WBT-WBO	-5.974118	-8.181542	-3.766694	0.0000008

Statistical Models for Figure 7

Table 11. Figure 7: ANOVA (Nodule Number ~ Harvest CCI Value*Accession*Nitrogen Treatment)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Harvest CCI Value	1	7600	7600	1.492	0.234881
Accession	3	154285	51428	10.094	0.000222***
Nitrogen Treatment	1	20743	20743	4.071	0.055981 .
Harvest CCI Value: Accession	1	130	130	0.026	0.874571
Accession: Nitrogen Treatment	2	12543	6272	1.231	0.311362
Harvest CCI Value: Accession: Nitrogen Treatment	2	4455	2227	0.437	0.651358
Residuals	22	112089	5095		

Signif. Codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

5 observations deleted due to missingness

Table 12. Figure 7: Tukey Multiple Comparisons of Means: 95% Family-Wise Confidence Interval - Significant Relationships by Accession

	Diff	**Lwr**	**Upr**	**p Adj**
WBO-BTS	131.11504	29.563716	232.66636	0.0083091
WBO-ELD	259.98001	55.671996	464.28802	0.0093653

Statistical Models for Figure 8

Table 13. Figure 8: ANOVA(Nodule Number ~Root:Shoot Ratio*Accession*Nitrogen Treatment)

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Root:Shoot Ratio	1	83564	83564	18.039	0.000282***
Accession	4	169453	42363	9.145	0.000123***
Nitrogen Treatment	1	33476	33476	7.226	0.012849*
Root:Shoot Ratio: Accession	3	60304	20101	4.339	0.014062*
Root:Shoot Ratio: Nitrogen Treatment	1	13323	13323	2.876	0.102845

Accession: Nitrogen Treatment	3	11135	3712	0.801	0.505388
Root:Shoot Ratio: Accession: Nitrogen Treatment	2	2675	1338	0.289	0.751764
Residuals	24	111177	4632		

Signif. Codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

Table 14. Figure 8: Tukey Multiple Comparisons of Means: 95% Family-Wise Confidence Interval - Significant Relationships by Accession

	Diff	**Lwr**	**Upr**	**p Adj**
WBO-BTS	1.25610329	0.1049770	2.4072296	0.0276883
WBT-WBO	-1.19317367	-2.1416695	- 0.2446779	0.0088501

Table 15. Figure 8: Tukey Multiple Comparisons of Means: 95% Family-Wise Confidence Interval - Significant Relationships by Nitrogen Treatment

	Diff	**Lwr**	**Upr**	**p Adj**
Y - N	0.3045131	-0.261077	0.8701032	0.2774905

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