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Characterizing Nodule Endophyte Communities in Glycine Max and Lablab Purpureus Using Next-Generation Sequencing

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CHARACTERIZING NODULE ENDOPHYTE COMMUNITIES IN *GLYCINE MAX* AND *LABLAB PURPUREUS* USING NEXT-GENERATION SEQUENCING

A Master's Thesis

Presented to

The Graduate College of

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In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Agriculture

By

Scott David McElveen

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ABSTRACT

Biological nitrogen fixation by rhizobia in the root nodules of legumes is a significant source of agricultural nitrogen in global crop production systems. The influence of and interaction of factors involved in nodule endophyte selection remain poorly understood. In the present study, the influences of crop rotation (soybean-legume vs. cotton-legume) and recalcitrant soil organic matter (compost amendment) on the relative distribution of endophytic bacteria in the root nodules of greenhouse-grown soybean and lablab were investigated by extracting, amplifying, and sequencing 16S ribosomal RNA (rRNA) and *nifH* genes. Neither preceding crop nor compost amendment were found to have an influence on microsymbiont selection at the level of genus. In both crops and in all treatments, *Bradyrhizobium* spp. were the dominant rhizobial symbionts, accounting for 95.9% of all recovered 16S rRNA sequences from root nodules, suggesting strong selection exhibited by both soybean and lablab. Likewise, the genera *Nitrobacter* and *Tardiphaga*, close relatives of *Bradyrhizobium*, were present in all root nodules, accounting for an average of 2.9% and 1.0% of nodule sequences, respectively. Previously reported non-rhizobial endophytes were present only inconsistently and at low abundances if at all, suggesting that they may not play a significant role in plant growth as nodule endophytes. These findings indicate that the isolation, characterization, and subsequent inoculation of seeds with non-rhizobial species may not be sufficient to establish their role as endophytes. Their relative abundance in the root nodules should be regarded an important means of certifying a suspected endophyte.

KEYWORDS: soybean microsymbionts, lablab microsymbionts, non-rhizobial endophytes, 16S ribosomal RNA (rRNA) and *nifH* gene high-throughput sequencing, *Bradyrhizobium*, *Nitrobacter*, *Tardiphaga*

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INTRODUCTION

There is much to be gained in enhancing nitrogen fixation in cultivated legumes, but massive improvements still exist only as potentialities. While, the identification and development of rhizobial strains highly efficient in nitrogen fixation is a well-advanced project, such strains all too often fail to successfully compete for nodule occupancy. The interaction between host plant and microsymbiont rhizobia is complex and can be influenced by several factors such as host plant genetics and behavior, rhizobium species genetics and behavior, abiotic environmental factors, and soil and root microbes [1–8]. Moreover, the endophytic microbial community, rhizobial and non-rhizobial alike, has yet to be examined at the level of detail afforded by the latest advancements represented by Next-Generation DNA sequencing. The present study investigated recent crop history and organic matter amendment with highly weathered compost as two potential environmental influences on the nodulation/partner-selection process. Either of these factors, if effective at influencing endosymbiotic partner choice, could represent low cost methods of achieving greater nodule occupancy of more efficient or otherwise desirable rhizobial and/or non-rhizobial endophytes [9–12]. Next-Generation DNA sequencing techniques were employed in the analysis of the soil and nodule microbial communities, providing greater detail, specificity, and reliability than traditional culture techniques employed by similar studies in the past [13–18]. Soybean and lablab were cultivated in a greenhouse setting in soil from the Missouri Bootheel taken either from a field previously under soybean or previously under cotton, and with or without a highly weathered compost amendment.

LITERATURE REVIEW

Nitrogen is an essential nutrient for all living organisms. It is also an important limiting nutrient in plant species. Primary productivity in terrestrial ecosystems around the world is limited by available nitrogen [19, 20]. The vast majority of plants depend on soil nitrogen (N) sources to fulfill their need, but members of the family Fabaceae (formerly Leguminosae), or legumes, are equipped to form direct symbiotic mutualisms with nitrogen-fixing species belonging to the order Rhizobiales [21–23]. In this symbiotic interaction, the plant provides the rhizobia with carbon (C), energy resources, and a protected shelter in the form of root nodules (Figure 1) in exchange for ammoniacal biologically fixed N [24, 25].

Figure 1. Rhizobia nodules on the roots of *Vigna unguiculata*

There are many species of rhizobia in the soil, yet typically only a small subset of that community successfully establish themselves in root nodules of the host plant. The means and

mechanisms of this establishing this symbiosis, referred to as "partner selection", is of key interest to researchers and agriculturalists alike. Many factors that influence partner selection have been identified, though many more are likely to be added to the equation in time. In their 2018 review, diCenzo et al. highlight the fact that improved rhizobial inocula often fail to enhance legume crop yields not due to deficient nitrogen-fixing ability, but rather due to a failure to outcompete indigenous rhizobial strains for nodule occupancy [1]. They enumerate the many factors identified as influencing this competitiveness and categorize them into four groups to conceptualize partner choice as the product of interaction between "G (plant genotype) × E (environment) × M (root and soil microbiota) × R (rhizobium)" [1]. This process remains poorly understood. A more complete understanding of this process may offer legume producers the ability to manage partner selection to favor some desired endosymbionts, rhizobial or otherwise, over others. Currently, relatively inefficient native rhizobia routinely outcompete more efficient non-native inocula for nodule occupancy, resulting in suboptimal plant growth promotion, not to mention wasted effort and resources [2–8]. The ability to manage this process, therefore, holds promise for enhancing legume crop yield, quality, and robustness to environmental stresses, gains that could extend to non-legume crops if rhizobial symbiosis is successfully engineered into non-legume crop species in the future.

The present study undertakes to evaluate the influence of two factors, preceding crop and recalcitrant soil organic matter, on legume-rhizobia partner choice in *Glycine max* L. Merr (soybean) and *Lablab purpureus*, as well as their influence on the selection of non-rhizobial endophytes (NREs).

Until relatively recently, the best methods employed in studying the rhizobial species in root nodules were cultural isolation methods, which suffer from numerous, inherent selective

biases [13]. The development of means of species identification based on gene sequence isolation has massively enhanced the completeness and resolution of studies of microbial communities [13]. In the present study, nucleic-acid-based species identified the abundance and distribution of rhizobia and other endophyte species in soil and root nodules of soybean and lablab, offering insight into which species in soil are available for selection, and can this selection be altered by varying soil amendments.

Biological Nitrogen Fixation in Agriculture

Estimates of the total amount of nitrogen that biological nitrogen fixation (BNF) fixes from the atmosphere to terrestrial ecosystems vary widely from 58 Tg yr^{-1} to 128 Tg yr^{-1}, whether through cultivated legumes or wild legumes and other nitrogen fixers [26–29]. The other most significant flux of nitrogen into terrestrial ecosystems comes by way of the Haber-Bosch process, in which the transformation of N_2 gas and hydrogen derived from methane into ammonia is catalyzed under conditions of extreme heat and high pressure [30–32]. While only 20 years ago, industrial nitrogen fixation was estimated to account for 25% of annually fixed nitrogen compared to 60% for biologically fixed nitrogen, that proportion has steadily increased and the two values are estimated more recently to be roughly even [26, 33]. The development of the Haber-Bosch process tremendously raised the theoretical carrying capacity of human population globally, and in many agricultural contexts turned nitrogen from a limiting nutrient to one in excess to the point of pollution [31, 34, 35]. Typically carried out at a temperature of 400°-500° C and pressure of 15-25 MPa, this process is energetically costly, depending heavily on hydrocarbon fuels and releasing 9.7-13.5 Mg of carbon dioxide equivalents emitted, not to

mention greenhouse gas emissions from fuel use during transport and application of fertilizer [36].

Greater efficiency and utilization of biological nitrogen fixation could serve to offset emissions by decreasing the need for chemical fertilizer. Improved understanding of management effects on the symbiotic process in legumes may provide benefits to production of legume forages, decrease the need for inorganic fertilizer in the subsequent crop, and enhance soil fertility and microbial species diversity [37–43]. Low-tech means of managing BNF may be of special economic benefit to small-holder farmers in developing countries who often lack the necessary capital to access, purchase, and transport inorganic nitrogen fertilizer [44–49]. Consider that in many cases, a non-native rhizobial inoculum may be significantly more efficient at nitrogen fixation than the native strain, but the native strain is a much more effective competitor for nodule occupancy, such that the host plant experiences no benefit of improved nitrogen fixation, despite the time and expense of applying the inoculum [2, 3, 50–52]. Conversely, indigenous rhizobia are often better suited to a range of local environmental stresses than a foreign inoculant and may outperform the introduced strains under suboptimal conditions; under such conditions, it would in fact be ideal to select against an introduced rhizobial strain [53].

Two major research questions that needs to be addressed are; (i) Does crop rotation influence the selection of microsymbionts and (ii) can this selection of microsymbionts be altered by organic matter incorporation. Lastly, if these factors influence the selection of partner choice then is it consistent across multiple host plant species.

Crops of Interest: Soybean and Lablab

Two legume species were selected for the present study: soybean, *Glycine max* L. Merr., and lablab, *Lablab purpureus*. Soybean (Figure 2) is the most important legume in terms of economic value and total biologically fixed nitrogen domestically and globally. The USA was the largest national producer of soybean in 2018 (Brazil has lead recent years since about 2013), harvesting 125 million Mg, 73,000 Mg of which was produced in Missouri [54] Soybean is a commercially important crop in Missouri, constituting an average annual value upwards of $2.3 billion between 2012 and 2016 [54]. Most soybean production occurs north of the Missouri River and in the "Bootheel" region in the Southeast of the state. The Missouri Bootheel is part of the Mississippi River Delta, which provides fertile soils and plentiful quality water for irrigation, making the few counties in the Bootheel some of the most productive producers of rice, cotton, and soybean in the state. The soils employed in the present study were collected from this region.

Figure 2. *Glycine max* pods and foliage

Soybean are nodulated by a diverse set of *Bradyrhizobium* and *Ensifer* species, though, on the basis of traditional isolation-based methods, one species or another will be the dominant endophyte in any given specific set of circumstances [44, 46-56]. Dinesh et al. (2010) observed that *B. japonicum* dominates in temperate, Nepali soils, but "in subtropical locations, *B. elkanii*, *B. yuanmingense*, and *B. liaoningense* dominated at acidic, moderately acidic, and slightly alkaline soils, respectively" [55]. Other evidence also suggests that *Bradyrhizobium* species other than *B. japonicum* dominate under the right circumstances, and that fast-growing rhizobial species of other genera, such as *Ensifer fredii*, *E. xinjiangensis*, and *Mesorhizobium thianshanense*, fix nitrogen in association with soybean with an efficiency comparable to *B. japonicum* [56–65]. The predominant identification of *B. japonicum* as the dominant or sole symbiotic partner of soybean may have been overstated because of the prevalence of research into the most commercially employed soybean varieties [61, 66]. Consequently, the relative distribution of rhizobial endosymbionts of soybean, and factors influencing all operative selective influences in the process of partner choice remain open questions. A recent meta-analysis of soybean rhizobial inoculants catalogued the effective nodulation of soybean by a diverse set of *Bradyrhizobium* and *Ensifer* species, citing, "soil organic matter, nutrients, pH, salinity, agricultural practices (e.g. organic, no till, rotations, application of pesticides) as well as temperature and drought" as influences on inoculant survival and competitiveness [53].

Analysis of 16S rRNA and *nifH* sequences retrieved from nodules of mung bean (*Vigna radiata* [L.] R. Wilczek) revealed a codominance of *Bradyrhizobium* and *Ensifer* species that was not identified by traditional cultural methods [15]. Sequence analysis in soybean may very

likely also reveal greater complexity and rhizobial species diversity than did the results of traditional species isolation.

Lablab (Figure 3), known by many other names, most notably dolichos bean and hyacinth bean, is a vigorous, trailing, perennial native to most of Africa [67]. Today it is cultivated globally in tropical climates [67]. It is not a commercially significant crop in most contexts, but is a valuable, drought tolerant legume for food (seeds, pods, and foliage), forage, cover, green manure, and herbal medicine in many humid and semiarid, tropical and subtropical agroecosystems [68–71]. Lablab is one of the most diverse domesticated legume species and it exhibits greater drought tolerance than cowpea (*Vigna unguiculata* L. Walp.) and common bean (*Phaseolus vulgaris* L.), as well as notable tolerance of salinity [68, 72, 73]. A rather neglected crop in recent history, lablab production is seeing a resurgence as a reliable, high protein source of supplemental forage and hay for livestock in areas of unreliable rainfall and as an advantageous intercropping option alongside a main crop [74–78]. In Missouri, lablab is employed principally in food plot polycultures for wildlife conservation and hunting purposes [79].

On the basis of studies employing isolation and gene sequence analysis, lablab is considered to be promiscuous in its rhizobial associations, associating with fast- and slow-growing species, mainly *Bradyrhizobium* species [80, 81]. Rhizobial symbiosis in lablab enhances tolerance of drought and salinity [72]. Cobalt and copper are essential to Lablab nodulation, and phosphorus fertilization beyond what is needed for maximal growth may increase nodulation and nitrogen concentration [82–84]. Studies on factors which influence the selection and distribution of rhizobial species in lablab are scarce relative to available information for soybean.

Figure 3. *Lablab purpureus* pods and foliage

Cultural and Nucleic-acid-based Means of Bacterial Study

Until recently, understanding of soil and root microbial communities has been limited by the selective effects of cultural isolation of rhizobial species. While species of all four rhizobial genera have been successfully cultured, the conditions of the culture (incubation time and temperature, moisture, nutrient medium, light, oxygen, etc.) are selective as to which genera or species present in the sample can survive, grow, and reproduce. Soil is highly heterogeneous, containing microhabitats and niches of unique combinations of pH, pore size, moisture, oxygen concentration, light availability, nutrient ion concentration, proximity to roots of different plant

species, presence of competing or symbiotic microbes, etc. [13]. Carefully isolated soil microaggregates, possessing unique combinations of these conditions tend to be the sites of greatest microbial diversity relative to bulk soil (i.e. sampled whole soil) [85]. A petri dish of agar in an incubator is a homogeneous environment, offering only one specific value for each of those abiotic and biotic conditions in each petri dish, resulting in the successful survival and isolation of only a small portion of the total microbial community present *in situ*. Culturing also requires that cells be viable, but some microbial species would not survive the trauma of the sample extraction process [13]. The time, necessary space, and cost of traditional culturing methods also presented challenges to producing a comprehensive picture of soil microbiological communities. Rhizobia extracted from wild legume nodules appear to be more commonly nonculturable than culturable [86].

Recent advances in DNA sequencing techniques has enabled researchers to increase capture and resolution of bacterial community composition by orders of magnitude [13]. Because species are identified on the basis of DNA nucleotide sequences, cells do not need to survive the extraction process or the distinct environmental conditions in the lab in order to be identified by species [13]. Most prominent among these technique is the polymerase chain reaction (PCR) [14]. PCR employs a thermostable polymerase enzyme original isolate from *Thermus aquaticus* to produce multiple copies of an isolated gene of interest. The gene of interest is delineated by means of specifically selected and designed forward and reverse primers [14]. The isolated, amplified DNA sequences (PCR product) may then be sequenced (read) and used to identify millions of species that were present in the environmental sample from which the sequences were extracted [13]. While PCR eliminates the selective biases of conditions employed in cultural isolation studies, the technique does have inherent biases, mainly in the form of primer

bias, in which the primer designed or selected for use in isolation may be a closer match (and therefore bind at higher rates) to the target sequence of some taxa than others [13, 87, 88]. One safeguard for avoiding primer bias is to target two or more genes for amplification in the taxa of interest, which provides redundancy by which potential primer bias in one target gene may be identified and corrected by another [15].

One means of sequencing PCR product is known as sequencing-by-synthesis, best known under the trade name Illumina®. In Illumina® sequencing, the primers employed include an adapter sequence that is designed to hybridize with an oligonucleotide repeated across a glass flow cell [16, 89]. Sample DNA binds to the oligonucleotides on the flow cell and is replicated via bridge amplification with nearby reverse oligos to form clusters of identical sequences. Finally, nucleotides tagged with a fluorescent molecule are added stepwise to the flow cell. When a nucleotide is added to the chain, near-UV irradiation cleaves the fluorophore from the nucleotide and a characteristic fluorescent signal (wavelength and intensity) from that sequence cluster is detected by a computer and translated into the letter representing that nucleotide base in sequence [16, 17]. Reverse reads are completed the same way as a measure to enhance accuracy. Millions of sequences can be produced in this process. Sequences from numerous samples may be simultaneously sequenced and later distinguished by including a unique index sequence (MiSeq™) in the forward and reverse primers utilized in PCR [18].

In this study, in order to avoid primer bias in species gene isolation, fragments of two genes were isolated by PCR for sequencing: 16S rRNA and *nifH*. The *nifH* gene is unique to nitrogen fixers, coding the Fe subunits on either end of the nitrogenase enzyme [90]. It contains well-conserved segments and distinctly variable segments, making it an ideal candidate for isolation of nitrogen fixers and differentiation between species on the basis of characteristic base

pair variations [91–94]. 16s rRNA is present in all prokaryotes [95]. With alternating conserved and variable regions, it represents the "gold standard" of microbial identification through PCR [13, 95, 96]. Isolating *nifH* sequences enables us to analyze the community of rhizobia and other nitrogen fixers specifically, while isolating 16S rRNA sequences enables us to analyze other endophytes and bacterial populations in general, while also confirming or challenging the rhizobial findings provided by *nifH* [15, 97].

Influence of Antecedent Crop and Compost Amendment on Rhizobial Symbiosis

Thus far, a number of environmental and ecological factors have been identified as having some effect on nodulation, partner choice, and/or nitrogen fixation rate in legumes. Many legumes have a preferred partner, a dominant microsymbiont often referred to as the favorable or highly competitive symbiont [98–102]. However, which species is most preferred by the host may shift across different geography or soil conditions, and the extent of its dominance may vary as well [101, 103–106]. When the host's preferred rhizobial partner is not present in the rhizosphere at the time of first infection, other rhizobia have the opportunity to establish symbiosis [107]. The rhizobial species with which *Vicia cracca* associates depends more on the rhizobial species abundance in the soil than on partner choice by the host [108]. Previous studies by Lopez-Garcia et al. (2002) and McDermott and Graham (1989) suggested that the position of rhizobia in the soil is of greater importance to nodulation than competitiveness, as a significantly less competitive strain of *Bradyrhizobium* previously established in the soil occupied more than 72% of nodules over a more competitive strain used to inoculate seeds at planting; while the inoculum species nodulated the tap root in the area around and near the seed, its nodule occupancy rates decrease significantly with distance from the planting site, especially in lateral

roots, despite the greater competitive ability of the inoculum species over indigenous species [99, 109]. Similarly, Zhang et al. (2014) observed that, while two non-native *Mesorhizobium* species were more competitive than the predominant native strain at nodulating chickpea in sterilized vermiculite, the native, less competitive strain remained the dominant nodule occupier over the non-native strains in non-sterilized soil [110]. *Fabaceae* may not exercise partner choice between otherwise identical strains of rhizobia capable and incapable of fixing nitrogen, as in the case examined by Westhoek et al. (2017) in which peas exhibited no discrimination between strains of *Rhizobium leguminosarum* bv. viciae possessing or lacking a functional *nifH* gene; after nodulation however, non-fixing nodules were sanctioned with restricted supplies of carbohydrates, oxygen, and other nutrients [111]. Much work remains to be done to elucidate the distribution and selection of many rhizobial species, particularly in lablab, as well as how factors like preceding crop and soil organic matter influence them.

Kumar et al. (2017) investigated the influence of crop rotations of cereal grains (maize, rice, wheat) with and without legumes (soybean or chickpea) and observed that soil rhizobial populations were 22-fold larger in rotations that included soybean compared to those that did not [9]. They also observed that continuous soybean "led to a greater proliferation of fast-growing rhizobia", despite the finding that slow-growing rhizobial symbionts produced greater dry plant matter, nodule mass, and fixed nitrogen than fast-growing species [9]. They concluded that because rotating soybean with other crops increased the proportions of symbiotically superior slow-growers, rotation was preferable to continuous soybean [9]. That would suggest that in the present study we may observe more slow-growing species in the nodules of soybean or lablab grown in the soil previously sown to cotton compared to that under continuous soybean. Yan et al. (2014) documented distinct compositions of three species of *Bradyrhizobium* in soybean

nodules under different crop rotations (bare land; grassland; monocultures of soybean, maize, or wheat; and a maize-soybean-wheat rotation) and under different soil parameters (organic carbon, available phosphorus, and pH) [10]. In terms of nodulation in soybean, *Bradyrhizobium japonicum* outperformed *Ensifer fredii* in an acid environment, and vice-versa in an alkaline environment [105, 112–115]. Other management decisions, such as the use of various pesticides may also influence soil and endophyte species composition [116–119]. Pre-planting application of glyphosate may alter endophytic bacterial communities [120]. The use of modern sequencing technologies to identify rhizobial species at greater resolution within and among nodules is very likely to enhance our understanding of the influence crop rotation and management may have on rhizobial species selection.

Studies investigating the specific relationships between compost, rhizobial species, and legume production are relatively few. Of particular note, however, is Kostov and Lynch (1998), in which they determined that composted sawdust was an effective carrier and inoculum of *Bradyrhizobium*, *Rhizobium*, and *Azospirillum* species, enhancing yields in "soybean (34–62%), groundnuts (4–39%), lucerne (24–82%) and a grass mixture of bird's foot trefoil and ryegrass (20–21%)" [12]. Additionally, Iqbal et al. (2012) observed improved nodulation, nitrogen content, and yield in *Lens culinaris* Medik. (lentils) with the integrated use of *R. leguminosarum*, plant growth promoting *Pseudomonas* spp., and phosphorus-enriched compost [11]. While such studies observed certain beneficial effects of composts on rhizobial associations, the effect of compost amendments on microbial symbiont species selection is poorly understood.

Non-rhizobial Endophytes

While nitrogen-fixing rhizobia are accepted as the dominant inhabitants of legume root nodules under most conditions, several studies identified the presence of many other bacterial species, classifying them as non-rhizobial endophytes (NREs) or nodule-associated bacteria [121, 122]. Due to the biases and risks inherent in isolation work, great care and multiple safeguards are necessary to prevent the false positive identification of endophytes, such as rolling nodules in nutrient agar following surface sterilization to verify whether viable CFUs remain [123, 124]. Nonetheless, a large diversity of microbes have been identified as NREs of legume nodules, even several which possess the capability to induce nodulation in some species in the absence of nitrogen-fixing symbionts [125]. Kuklinsky-Sobral et al. (2004) identified numerous endophytic species associated with soybean with plant-growth promoting traits (e.g. indole acetic acid production [IAA], phosphate solubilization, etc) belonging to the genera *Pseudomonas*, *Ralstonia*, *Enterobacter*, *Pantoea* and *Acinetobacter*, noting differences in bacterial population densities depending on soybean growth stage, plant tissue, and season of isolation [120]. The diversity and abundance of NREs within root nodules may be so high that they outnumber even the nitrogen-fixing endosymbionts [86]. Bai et al. (2002) found *Bacillus thuringiensis* and *B. subtilis* co-inhabiting soybean root nodules with *Bradyrhizobium japonicum* and confirmed a plant growth promoting effect of both strains in a coinoculation experiment [126]. De Almeida Lopes et al.'s (2016) most abundant soybean root endophytes isolated by 16S rRNA sequence analysis were identified as *Enterobacter ludwigii* and *Variovorax paradoxus*; 44.4% of their endophytic isolates were capable of promoting plant growth by either producing IAA or solubilizing phosphates [127]. Hung et al. (2007) also identified IAA producers as a large portion of the soybean endophytic community isolated from stems, roots, and nodules; most of their

isolates were motile species, with 70% excreting cellulase, and 33% excreting pectinase [128]. *Agrobacterium*, *Klebsiella*, *Gluconacetobacter*, *Burkholderia*, *Bacillus*, *Psuedomonas*, *Pantoea*, *Serratia*, and *Acinetobacter* have all been identified as nodule endophytes in soybean [125, 129–133].

Despite all of this information about NREs, little is yet understood about whether or how the host plant entices or excludes potential endophytes, how their distribution consequently differs between nodules and surrounding soil, and what factors may influence this process. Given the plant-growth promoting potential of many NREs, the prospect of managing their infection of host root nodules through soil management and crop rotation could be of significant benefit to legume production.

Hypotheses

The present study was undertaken to investigate two potential factors affecting nodulation, species selection, and NRE nodule occupancy in the Missouri bootheel: the preceding crop (whether a legume [soybean] or non-legume [cotton]), and the recalcitrant organic matter present (a highly weathered compost amendment in this case). We hypothesize (i) that *Bradyrhizobium* spp. are the dominant endosymbionts of soybean and lablab; (ii) that fast-growing species of *Bradyrhizobium* are in high abundance in soil previously planted to soybean, leading the a higher portion of fast-growing species in nodules; (iii) that nodule microsymbiont diversity is greater in lablab than in soybean, though less so when both follow soybean in rotation; and (iv) that increased recalcitrant organic matter in the form of a compost amendment does not influence endophyte selection in either legume species.

METHODS

Soil, Compost, and Seed Collection and Preparation

Soils were collected from the Rhodes Farm in Clarkton Missouri January of 2018 and stored for two weeks in large plastic boxes in the greenhouse head house at a temperature of 21°C. The farm belongs to University of Missouri and is used for evaluating practices for managing pathogenic nematodes and wind erosion. Our goal was to select comparable soils that differed in terms of the preceding crop, whether soybean, which we would expect to have a viable rhizobial population, or cotton, which we would expect to have far lower numbers of rhizobia in the microbial community. The first soil was a ridge-tilled Malden fine sand (MFS) that has been under continuous cotton production for the past six years (Figure 4). The second was a Bosket fine sandy loam (BFSL) that has produced soybean (single crop) for the past two seasons, following five years of cotton production (Figure 5). Previous soybean crops had not been treated with fungicide, nematicide, or rhizobial inoculum. Hydrometer tests for soil texture confirm these soil classifications. Compost was collected from the Springfield Yardwaste Recycling Facility (Figure 6). The compost was produced largely through passive composting, being watered by rain, checked for temperature weekly, and turned weekly if needed. In order to control for rhizobia that may be introduced in the compost amendment, commercially packaged, coarse “patio sand” (source: Lowes Home Improvement) was autoclaved at 121°C and 15 psi for one hour in 30 cm^2 bagged batches in order to sterilize the soil of bacteria; this autoclaved sand was amended with compost such that any rhizobial DNA sequences recovered from nodules or soil samples may be presumed to have come from the compost.

Figure 4. Malden fine sand on the Rhodes Farm, Clarkton, MO

Figure 5. Bosket fine sandy loam on the Rhodes Farm, Clarkton, MO


Figure 6. Compost windrows at the Springfield Yardwaste Recycling Facility

Both soils, the autoclaved sand, and the compost were analyzed at the Missouri University Soil and Plant Testing Laboratory. The soils and sand were tested for pH, soluble macronutrient concentrations, nitrate concentration, ammonium concentration, total Kjeldahl nitrogen, and organic and inorganic nitrogen [134]. The compost was tested for nutrient concentrations.

The *G. max* seed was untreated Asgrow 38x7s. The *L. purpureus* seed was of the variety Highworth from the Hancock Seed Co. in Dade City, FL and was also not treated with any inoculum or pesticide.

Experimental Setting and Design

Experimental units were potted in hypochlorite-sterilized pots on hypochlorite-sterilized greenhouse benches (Figure 7). An experimental unit consisted of one 1.9 L, plastic pot with one

of the soil types and three plants of the same species. Experimental units were arranged in a randomized block design; pots were blocked by replicate and their placement randomized within blocks. The position of the blocks and of the experimental units within each block was re-randomized twice over the course of the experiment to mitigate the effects of any minor spatial differences in light level, ventilation, or water interception along the greenhouse bench. Each treatment group included at least three and as many as five experimental units, depending on resource availability and adequate plant germination and survival, with the exception of the autoclaved sand and compost control treatments of each species which each included two experimental units. For compost treatments, compost was mixed with each soil at 10% total volume. The treatments are defined in Table 1.

Figure 7. Experimental units at planting

Table 1. Experimental Treatments

Treatment ID	Species	Preceding Crop	Compost (y/n)
1	Soybean	Soybean	n
2	Soybean	Cotton	n
3	Lablab	Soybean	n
4	Lablab	Cotton	n
5	Soybean	Soybean	y
6	Soybean	Cotton	y
7	Lablab	Soybean	y
8	Lablab	Cotton	y
9	Soybean	None (autoclaved sand)	y
10	Lablab	None (autoclaved sand)	y

Seed was surface sterilized before planting as follows: lablab seed was soaked for 2 minutes in 70% ethanol, then 10 minutes in 2.6% hypochorlite, and then being rinsed with deionized water. Soybean seed was sterilized in like manner, but in 40% ethanol and 1.6% hypochlorite in the interest of reducing the chance of injury to the seed due to the difference in size and seed coat thickness between soybean and lablab. Plants were sown initially on February 9, 2018. Nine seeds were sown in each pot with separation between each seed. These were later

thinned to three plants per pot at two weeks from planting. After the initial set of soybeans exhibited very poor germination, any seedlings were removed and the pots re-sown with soybean seed from a different source (untreated Asgrow 38x7s) on February 17, eight days after the initial planting (Figure 8).

Figure 8. Germination test with original soybean seeds on the left and replacement soybean seeds on the right.

Irrigation and Fertilization

Plants were watered as needed with only deionized water. A nutrient solution was incorporated into the water at every other watering. The nutrient solution consisted of 2.5 ml/L 800 mM $CaCl_2 \cdot 2H_2O$, 2 ml/L 160 mM KH_2PO_4, 0.5 ml/L K_2HPO_4, 2.5 ml/L 200 mM $MgSO_4 \cdot 7H_2O$, 4 ml/L 12.5 mM Fe Sequestrene, 1 ml/L 800 mM KCl, and 1 ml/L micronutrient solution.

The presence of thrips and whiteflies was observed on the plants. Evenly spaced yellow, adhesive traps were placed on March 5 and replaced on March 23 and April 12. Safer Brand

Concentrate II Insect Killing Soap, mixed according to the manufacturer's instructions, was applied to the leaves of all plants on March 16, March 22, April 12, and April 19.

On April 25, a leak was discovered in the greenhouse roof above the new bench area to which the experiment had been moved on April 6. Rain storms on April 22 and April 25 led to flooded conditions in at least four experimental units, and caused water and surface sediment to be transported from those four experimental units to numerous experimental units in proximity when water droplets fell from the roof into the inundated pots. While it is possible that the bacteria present in some bulk and rhizosphere soil samples may be influenced by this cross-contamination, we expect such effects to be minimal, as samples were harvested eight days after the first rain event. We anticipate no influence on the species present in nodules, as the active nodulation stage of the crop life cycle had almost certainly already passed (Figure 9).

Figure 9. Experimental units in the greenhouse on April 16

Harvest and Sampling

Soil samples and root nodules were harvested on April 30, at 81 days after planting for lablab and 72 days for soybean. The decision to harvest was precipitated by the earlier-than-anticipated flowering of several soybean plants, potentially due to photoperiod. A SPAD chlorophyll meter (Minolta) reading was taken from one leaflet of a penultimate trifoliate leaf from each plant at harvest and the average recorded from each experimental unit. Three sample cores of bulk soil were taken from each experimental unit. Loose soil was massaged to fall away from the roots with minimum disturbance to roots and nodules (Figure 10). Soil that remained attached to the roots after this massaging was shaken loose and collected as rhizosphere soil. The shoots were removed, and the root systems placed in Falcon tubes with ethanol. Presence and number of nodules and uniformity of leaf color was also noted. All samples of soil and roots were immediately stored in a freezer.

Sample Processing and DNA Extraction

Nodules were collected from root systems on a hypochlorite-steriled surface, and carefully and individually surface-cleaned by scraping in ethanol to remove most sediment and bacteria adhered to the nodule surface. The cleaned nodules were ground with mortar and pestle (Figure 11), and suspended in sterile deionized water. The mortar and pestle were cleaned, sterilized, and wiped dry with a Kimwipe in between each sample. DNA from soil and from ground nodule material was extracted using the Qiagen DNeasy PowerLyzer PowerSoil Kit according to the manufacturer's instructions (Figure 12); this process included both physical (bead beating) and chemical means of cell lysis. Early DNA extractions were assessed by gel electrophoresis to confirm efficacy.

Figure 10: Nodules on a soybean plant grown in sand with compost at harvest

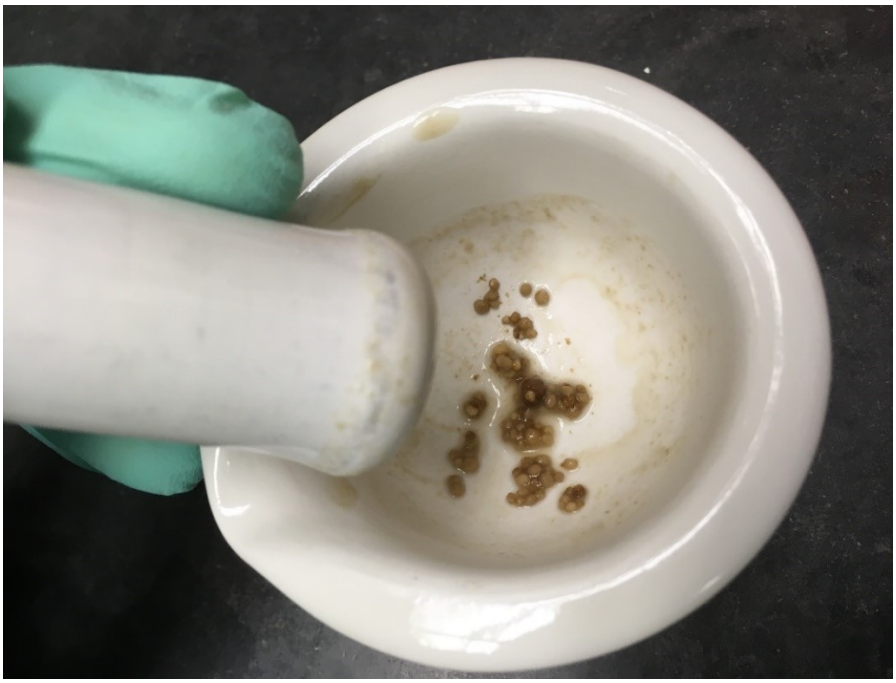

Figure 11. Nodules were ground with mortar and pestle

Figure 12. DNA extraction using the Qiagen PowerSoil DNA kit

Target Gene Isolation and Amplification

Target segments of 16S rRNA and the *nifH* gene were amplified by thermostable enzyme *Taq* polymerase [135]. Amplification via polymerase chain reaction (PCR) was carried out in two stages. In the first round, "hot start" PCR was employed in order to improve amplification in samples of potentially low microbial abundance, particularly of rhizobia in bulk soil and in

treatments with very few and/or very small nodules. In this technique, DNA was first denatured in the thermocycler for 5 mins at 94°C for 16S, 95°C for *nifH*, and then dNTPs and Invitrogen AccuPrime™ Taq DNA polymerase are added to enhance initial binding of Taq polymerase to single-stranded DNA. The primers employed in the first stage of PCR for 16S and *nifH* genes were manufactured by Eurofins and targeted conserved sequences of 392 bps and 360 bps respectively (16S forward primer: ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGC CAGCMGCCGCGG, 16S reverse primer: GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CTCCGTCAATT CMTTTRAGTTT, *nifH* forward primer: ACACTCTTTCCCTACACGACGC TCTTCCGATCTTGCGAYCCSAARGCBGACTC, and *nifH* reverse primer: GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATSGCCATCATYTCRCCGGA). The 16S targeted sequence enclosed the region of the gene from 515 bp to 907 bp. These primers also contained adapter sequences that would complement the Miseq primers employed in the second stage of PCR. This first PCR program ran through 35 cycles of amplification--94°C for 30s, 56°C for 30s, and 72°C for 30s for 16S; 95°C for 30s, 59°C for 30s, and 72°C for 30s for *nifH*--followed by an additional seven minutes at 72°C. The reaction volume was 25 microliters.

Amplification was verified by 1% agarose gel electrophoresis; 7µl of PCR product mixed with 2µl 1X loading dye was loaded into 1% agarose gel and electrophoresed in 1X TAE buffer (40mM Tris free base, 20mM glacial acetatic acid, 1mM disodium EDTA; pH ~ 8.6) for 30 minutes. The gel was then stained with ethidium bromide. The stained gel was observed and photographed on a UV transilluminator (Figure 13). PCR results were categorized on the basis of the brightness of the amplicon band in the gel and on the presence and brightness of excess primer dimers. One microliter of stage 1 PCR product was cleaned up using ExoSAP-IT™ in preparation for stage two.

The second stage of PCR was carried out normally (i.e. not hot start) using Invitrogen - Platinum™ Taq DNA polymerase and PCR product from round one as template DNA. Unique Miseq primer pairs were employed for each sample; these primers bound to complementary adapter sequences in the amplicon from the first round of PCR. This enabled differentiation of each sample from all other samples following sequencing. Following 5mins at 90°, stage two ran through ten amplification cycles--90°C for 30s, 60°C for 30s, and 72°C for 30s--followed by an additional seven minutes at 72°C. Reaction volume was again 25µl. Stage 2 PCR product was verified via gel electrophoresis as described above.

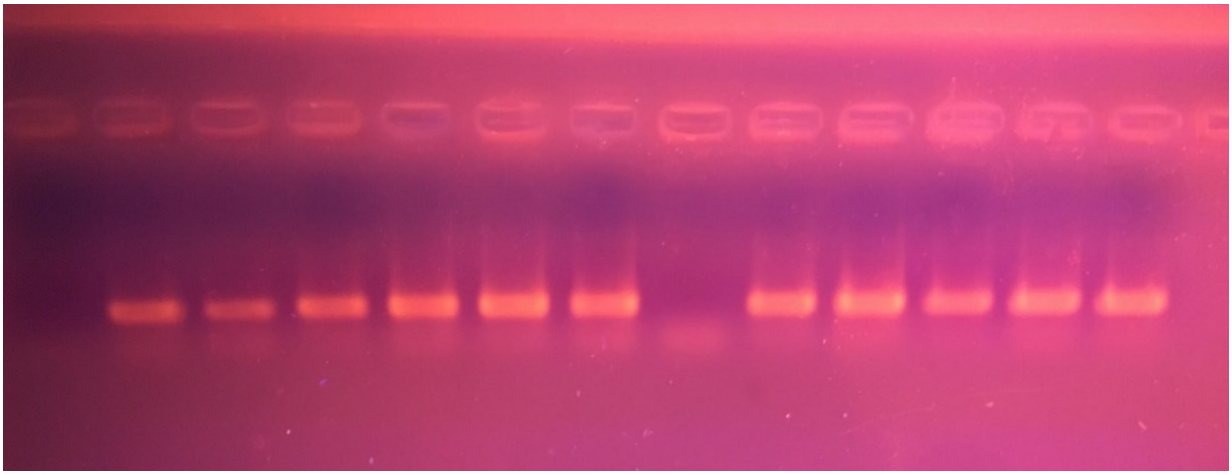

Figure 13. Gel electrophoresis showing bands of amplified 16S rRNA

The PCR products from various samples were pooled into six groups on the basis of the prevalence of primer dimers visible in stained gels and the target gene, i.e. three groups (minimal primers, moderate primers, and excessive primers) for each target gene. The quantity of product added to each pooled sample varied based on observed brightness (3µl from very bright samples, 5µl from moderately bright samples, and 8µl from comparatively dim samples). These pooled samples were cleaned up using AMPure XP magnetic beads. Product from the cleanup process

was verified by gel electrophoresis as described above. These six groups of pooled samples were ultimately pooled together and cleaned once more with AMPure XP magnetic beads.

Sequencing and Identification

Following cleanup, samples were sequenced via Illumina high-throughput sequencing. Sequence phyla and genera were summarized through the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) Classifier tool and aggregated in Microsoft Excel [136]. On the basis of the classifications made by RDP, Cyanobacterial sequences were removed and Rhizobiales sequences isolated using mothur [137]. Primers sequences were then excised and unidentified bases (N) replaced using Sequencher DNA Sequence Analysis software. Sequences were then aligned using the RDP Aligner tool and then clustered into operational taxonomic units (OTUs) at 97% DNA identity using the RDP Cluster tool [136]. For each OTU containing more than five sequences, a representative sequence was phylogenetically analyzed using MEGA software version # [138]. Species corresponding to each sequence were identified by means of BLAST searches of the NCBI 16S rRNA sequence database.

Statistical Analysis

Statistical analyses were carried out in SAS® 14.2 (Cary, NC, USA). The effects of preceding crop or compost amendment responses of interest were determined by analysis of variance (ANOVA), and then delineated by Tukey's Honest Significant Difference Test. Results were considered significant at the level of $p = 0.05$.

RESULTS

Soil Analysis

The two soils employed in this study were substantially similar, with some notable differences. The Malden fine sand had 17.1% more sand, making it a noticeably lighter-textured soil, which may account for its higher germination rates of soybean. Malden fine sand also had nearly four-fold more available phosphorus than the Bosket fine sandy loam, as well as pH a full point more acidic. The compost was 1.86% nitrogen by weight, not sufficient to supply the nitrogen needs of the plants, and therefore not sufficient to deter nodulation. See Table 2 for all soil and compost chemical and textural analyses.

The soils also differed in their microbial communities (Figure 14). Presumably, the sequences identified in the autoclaved sand belonged to bacteria that did not survive autoclaving, though fragments of their DNA persisted. Sequences belonging to *Bradyrhizobium*, *Rhizobium*, and *Mesorhizobium* were all identified in both soil types. Only *Rhizobium* sequences were identified in the compost. Importantly, and contrary to expectations, a greater proportion of the sequences recovered from the Malden fine sand, which had previously grown five years of cotton crops, belonged to *Bradyrhizobium* spp. (1.4%) than that of the Bosket fine sandy loam (0.8%), which had previously grown two years of soybean. The Malden fine sand was also home to greater proportions of *Rhizobium* and *Mesorhizobium* sequences. A far greater proportion of rhizobial species had been anticipated in the Bosket fine sandy loam compared to the Malden fine sand. This result calls into question whether these measurements, and therefore the treatment outcomes due to soil type, are atypical of soil differences observed under different rotations in experiments such as Kumar et al. (2017) [9].

Table 2: Soil and Compost Chemical and Textural Analyses

	Soils			Compost	
	Bosket FSL	**Malden FS**	**Autoclaved Sand**		
pH_s	5.8	4.8	7.2	N (%)	1.86
P (lbs/A) (Bray-I)	40	153	16	N (lbs/ton)	37.2
K (lbs/A)	270	274	79	P (%)	0.180
Ca (lbs/A)	1244	840	2013	P2O5 (lbs/ton)	8.25
Mg (lbs/A)	145	104	89	K (%)	0.594
Organic Matter (%)	0.8	0.7	0.0	K2O (lbs/ton)	13.3
Neutralizable Acidity (meq/100g)	1.0	2.5	0.0	Ca (%)	3.75
CAC (meq/100g)	5.1	5.4	5.5	Mg (%)	0.261
NO3 ppm	14.6	14.4	2.3	Zn (ppm)	102
NH4 ppm	2.607	2.028	2.425	Fe (ppm)	3455
TKN (%)	0.102	0.073	0.020	Mn (ppm)	1173
Inorganic N ppm	17	16	5	Cu (ppm)	21
Organic N (%)	0	0	0		
Sand (%)	68.9	86	>99		
Silt (%)	22	7.45	<1		
Clay (%)	9.1	6.55	<1		
Textural Class	Sandy Loam	Loamy Sand	Sand		

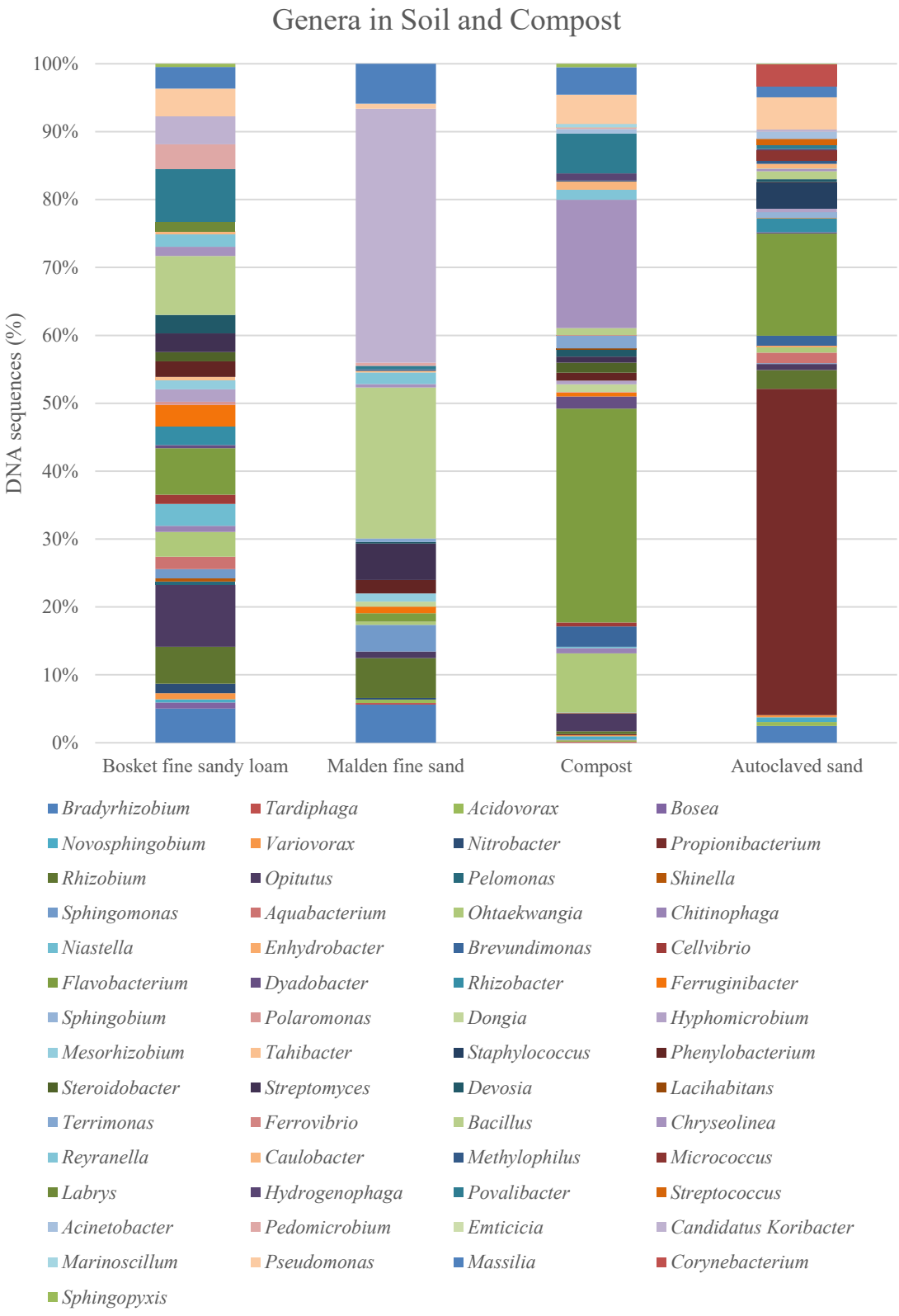

Figure 14. Genera of DNA sequences recovered from pre-experimental soils and compost

Germination

Soybean sown in Malden fine sand exhibited significantly higher rates of germination than either Bosket fine sandy loam or autoclaved sand ($p < 0.0001$) (Table 3).

Table 3. Germination across soil types

	Bosket FSL	Malden FS	Autoclaved Sand
Rep	*seeds germinated*	*seeds germinated*	*seeds germinated*
1	3	8	4
2	3	7	5
3	1	6	4
4	3	8	
5	2	5	
6	3	8	
7	1	7	
8	3	7	
9	2	7	
Mean	2.3	7.0	4.3

Nodulation

Neither compost amendment, nor preceding crop (soil type), nor the interaction thereof had a significant effect on SPAD value of penultimate trifoliate leaves or the number of nodules recovered from the root systems of each experimental unit. Successful nodulation was entirely

absent for lablab plants grown in autoclaved sand, but all other treatments exhibited successful nodulation.

Isolation and Amplification

Extraction and amplification of 16S rRNA from soil and nodule samples was universally successful. The resulting number of successful, high-quality sequences reads from all samples, and from nodule samples in particular, was highly variable (Table 4).

nifH gene sequences were amplified and sequenced, but due to time constraints, these data have yet to be fully processed, a task which is to be completed by a successive researcher in order to complement the results of 16S rRNA analysis.

Table 4: High-quality Sequences Obtained from Samples

	Total	Min	Max	Average	Standard Deviation
All soybean samples	404,917	904	24,462	9,666	5,477
Soybean nodule samples	85,841	1,089	11,606	4,088	2,476
All lablab samples	661,725	886	25,904	10,180	5,864
Lablab nodule samples	41,810	196	10,682	3,484	3,878

Soybean Endophyte Phyla and Genera

Soybean strongly selected for *Bradyrhizobium*, which amounted to less than 1% of the total bulk soil sequences and nearly 96% percent of all nodule sequences. As with soybean, the most abundant bacteria in bulk soil—*Gp1*, *Gp4*, and *Gp6* of the Acidobacteria, *Gaiella*,

Nitrososphaera, *Nitrospira*, *Bacillus*, *Chryseolinea*, *Candidatus Koribacter*, *Chryseobacter*, *Povalibacter*—were almost completely excluded from the nodules. The balance of nodule sequences was composed almost entirely of *Nitrobacter* and *Tardiphaga* sequences. On average across all soil samples, there were 23 *Bradyrhizobium* sequences, 4 *Nitrobacter* sequences, and 3 *Tardiphaga* sequences per sample. Across all nodule samples, *Bradyrhizobium*, *Nitrobacter*, and *Tardiphaga* were represented by 1,683; 50; and 17 sequences per sample, respectively. Table 5 summarizes the predominant endophyte sequences recovered.

Table 5: Nodule Endophyte Abundances Summarized

Bacterial Genera	Nodule Sequences	Proportion
Bradyrhizobium	55,542	95.9%
Nitrobacter	1,651	2.9%
Tardiphaga	571	1.0%
Other NREs	135	0.2%

In both soybean and lablab, comparisons between sequences recovered from bulk soil and rhizosphere soil are so similar as to suggest strongly that the methodology employed for collecting soil from the rhizosphere was insufficient to successfully isolate rhizosphere soil. Nonetheless, strong selection is still evinced by the significant differences between nodule samples and both bulk and “rhizosphere” soil (Figure 15).

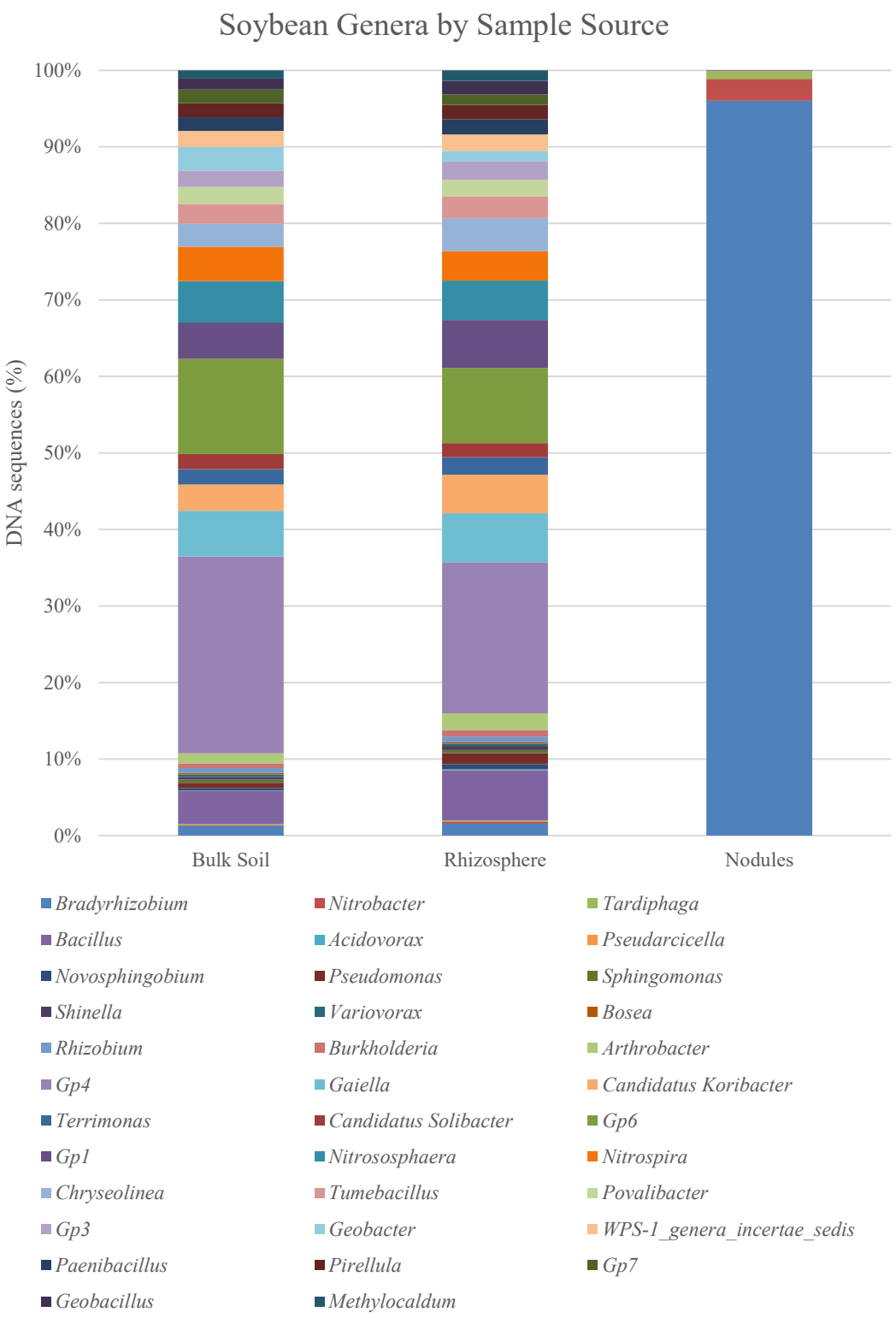

Figure 15. Genera of DNA sequences recovered from the bulk soil, rhizosphere, and nodules of experimental units planted to soybean

Despite the textural, chemical, and microbiological differences in the two soil types and compost, no difference was found in the phyla and genera whose sequences were identified from the nodules of soybean plants grown in any of the treatments (Figures 16-19). Note that the *y*-axes below are truncated to facilitate displaying great detail.

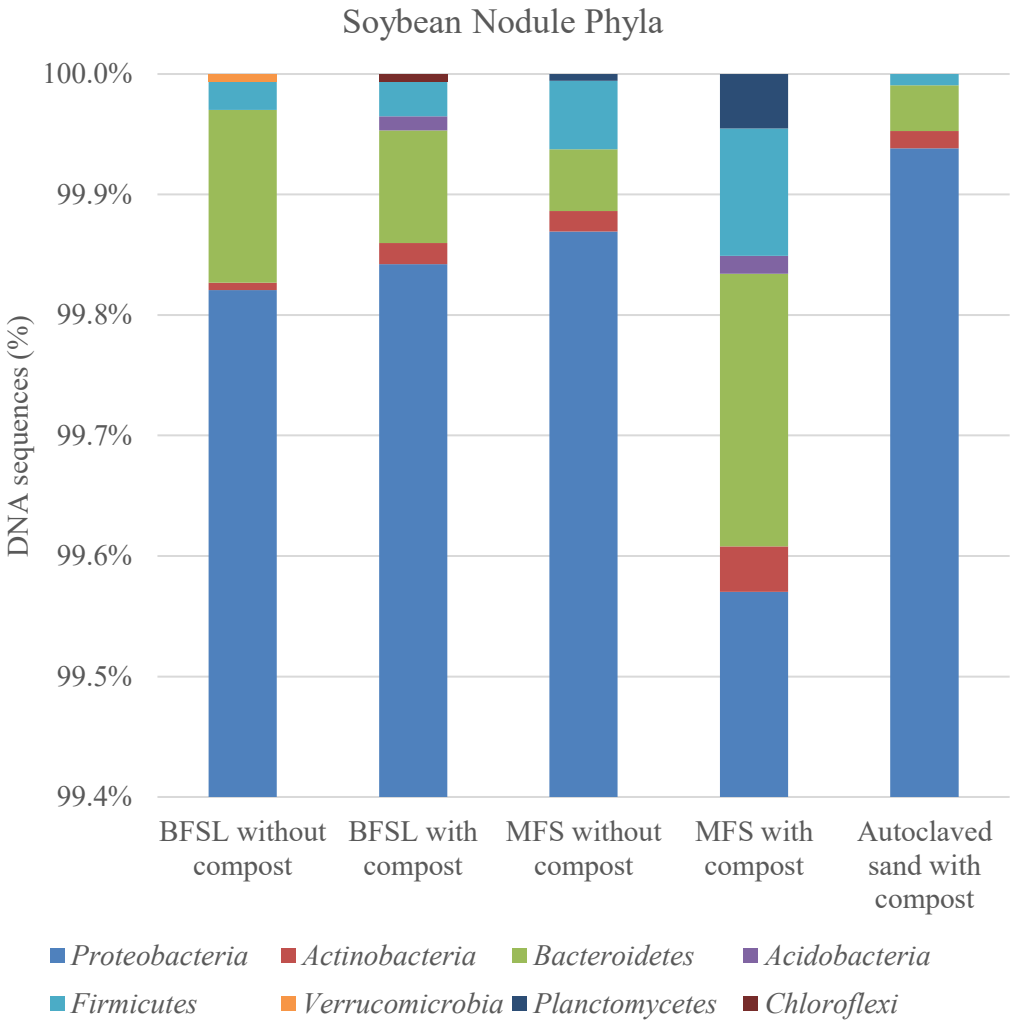

Figure 16. Phyla of DNA sequences recovered from soybean nodules from each treatment group

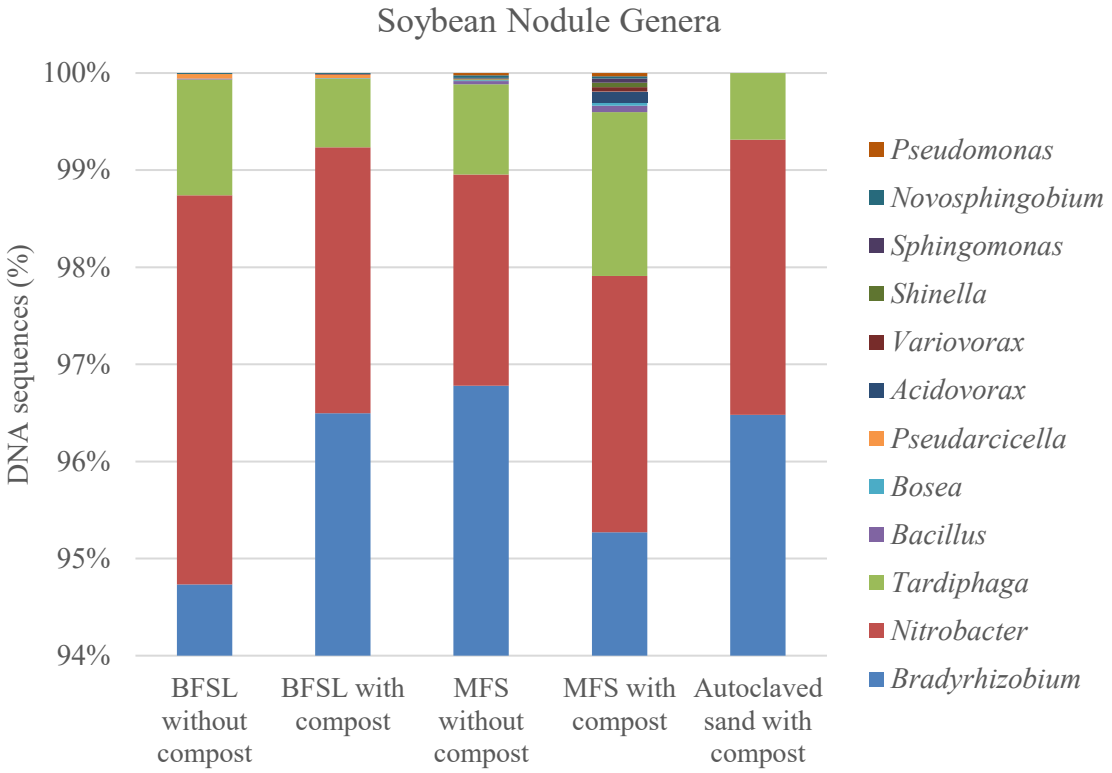

Figure 17. Genera of DNA sequences recovered from soybean nodules from each treatment group

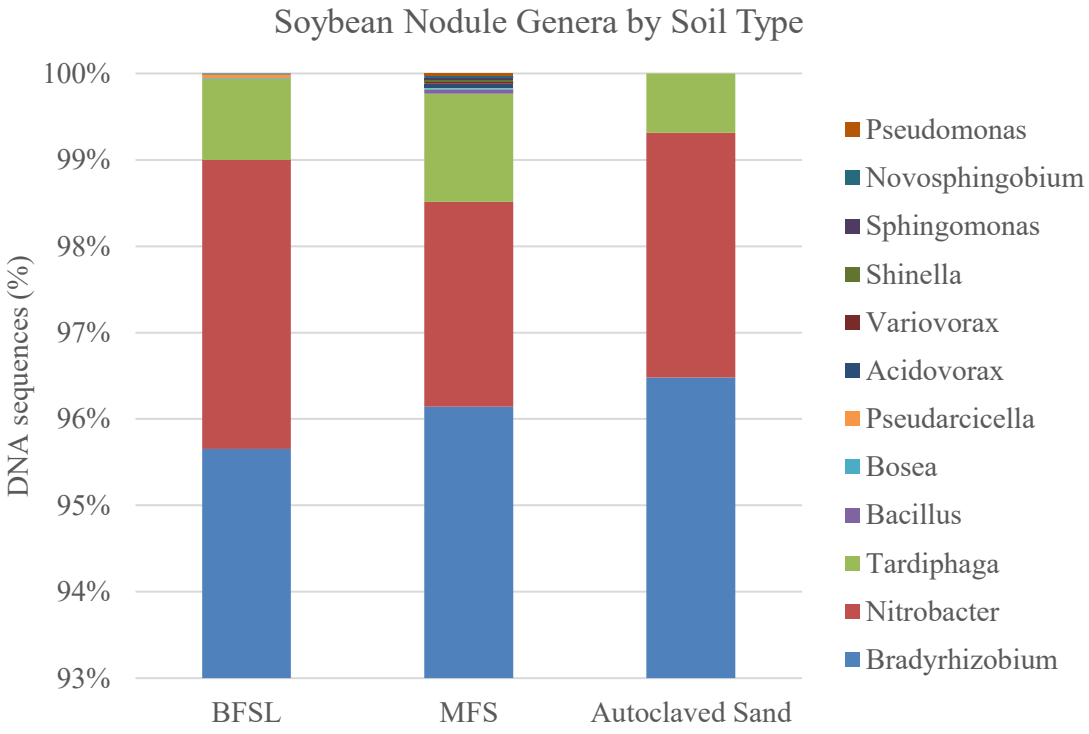

Figure 18. Genera of DNA sequences recovered from soybean nodules from each soil

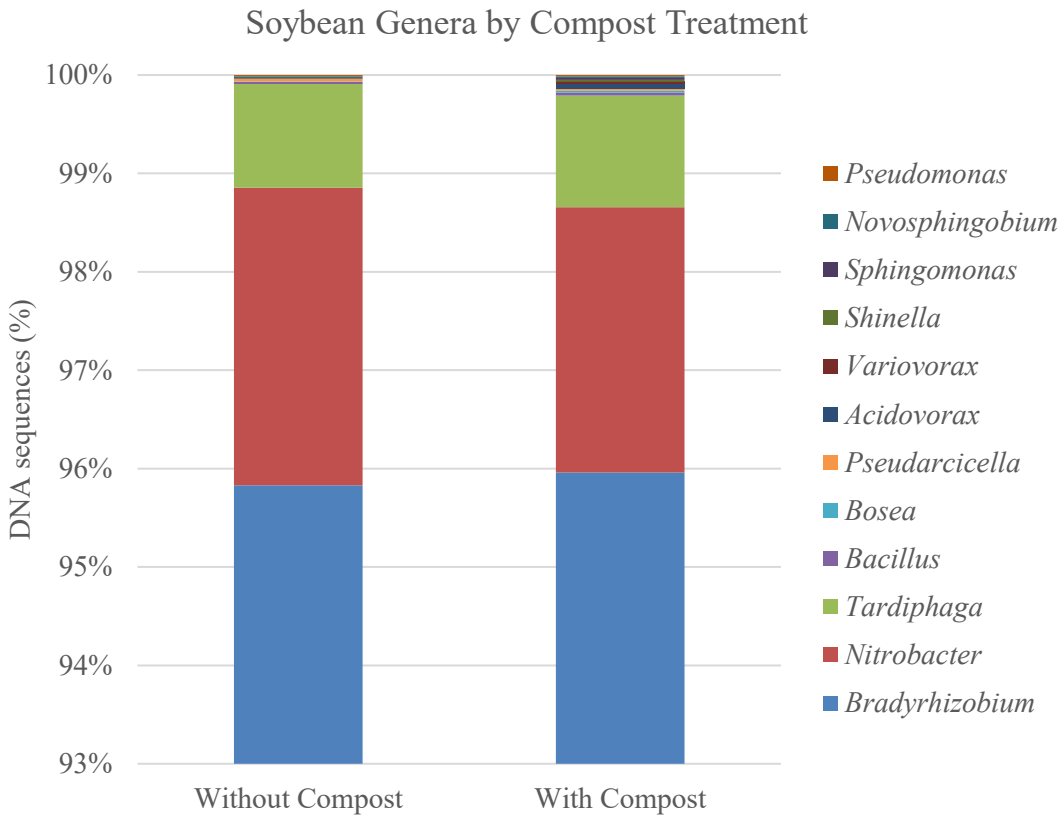

Figure 19. Genera of DNA sequences recovered from soybean nodules from each compost treatment

Lablab Endophyte Phyla and Genera

Strong selection was also evident in lablab. *Bradyrhizobium* sequences again represented 95% of the sequences identified within nodules. As with soybean, the most abundant bacteria in bulk soil were almost completely excluded from the nodules (e.g. *Gp4*, *Gp6*, *Gaiella*, *Nitrosospheara*, *Bacillus*) (Figure 20).

Despite the textural, chemical, and microbiological differences in the two soil types and compost, no difference was found in the phyla and genera whose sequences were identified from the nodules of lablab plants grown in any of the treatments (Figures 21-24). Data is not available for autoclaved sand with compost, because, as noted above, none of the lablab plants successfully nodulated in that soil. The apparent differences visible among the data displayed in

Figures 21-24 is the result of poor or inconsistent nodulation rather than that of any real treatment effect. To be more specific, only one experimental unit in the group grown in BFSL without compost produced any recoverable nodules. That sample, along with two samples grown in BFSL with compost, had very few and small nodules, small enough that it was very difficult to clean off all of the plant material from around the outside of the nodule. These samples were not discarded because each had at least 70% of its sequences belonging to rhizobial species, confirming that nodule material was recovered, but they should be regarded as contaminated by plant material, as the non-rhizobial sequences they contained were not repeated in other samples even with the same treatment. Again, note that the *y*-axes below are truncated to facilitate greater detail.

As with soybean, there were only three genera consistently found in all nodule samples: *Bradyrhizobium*, *Tardiphaga*, and *Nitrobacter*, with the notable distinctive that, contrary to soybean, the abundance of *Tardiphaga* sequences in lablab nodules were more than twice as abundant as *Nitrobacter* sequences. While a higher total number of *Acidovorax* and *Variovorax* sequences was measured, those abundances are the artifact of a single sample of small nodules from which it was impossible to effectively remove the epidermis without destroying the nodule itself; those genera were not found in any other nodule sample, save for a single *Acidovorax* sequence. One or two genus *Rhizobium* sequences were identified in half of the lablab nodule samples. When compared to the average number of *Bradyrhizobium* sequences, which was 2600/sample, it is evident that *Rhizobium* species are not the target endophyte.

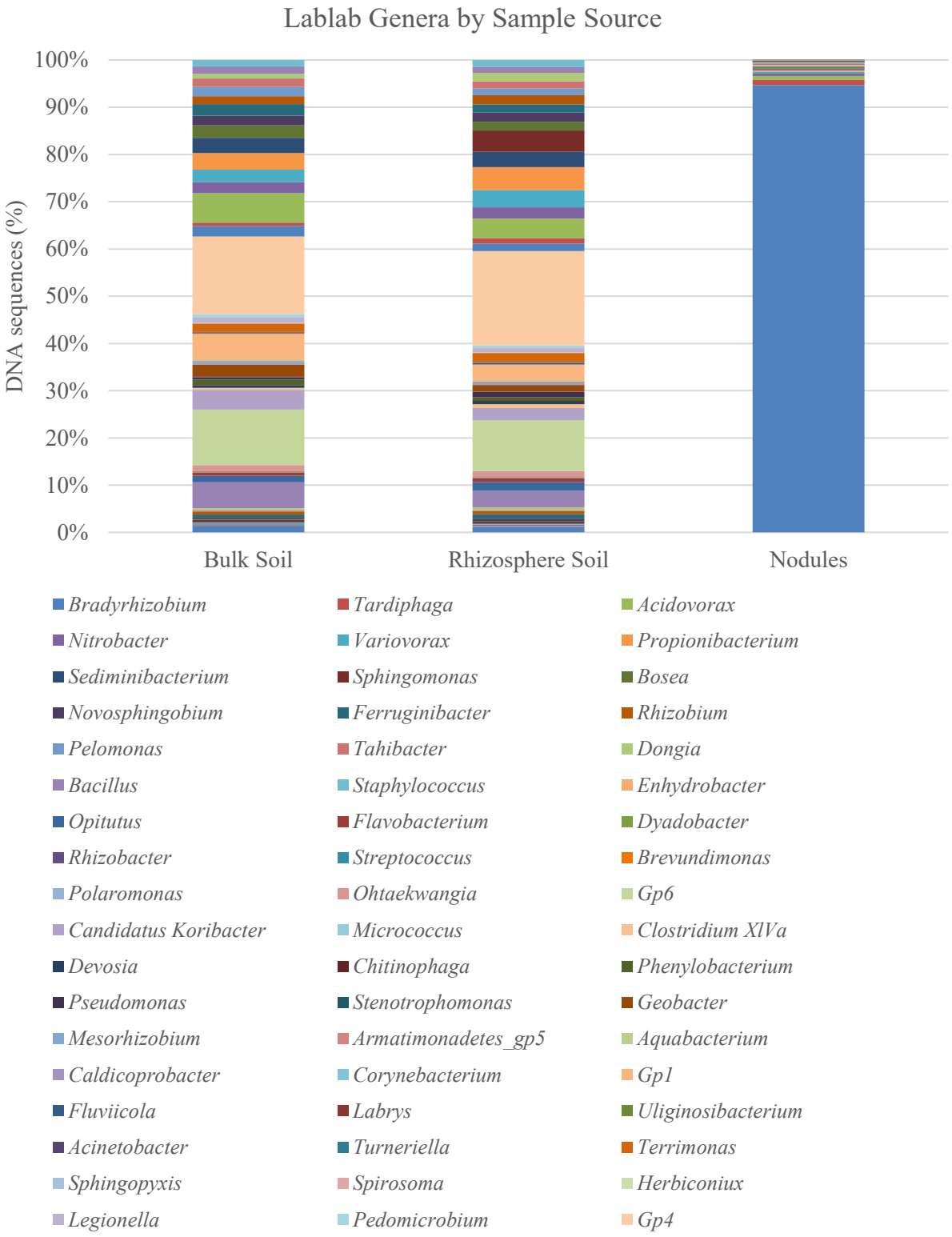

Figure 20. Genera of DNA sequences recovered from the bulk soil, rhizosphere, and nodules of experimental units planted to lablab

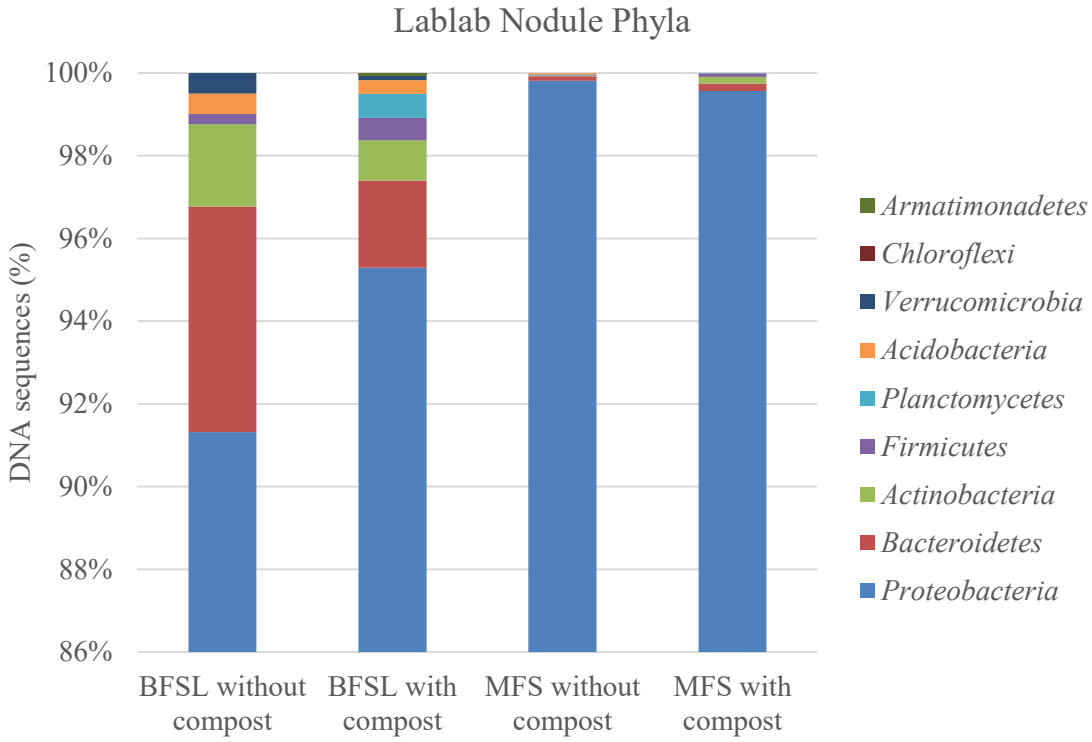

Figure 21. Phyla of DNA sequences recovered from lablab nodules from each treatment group

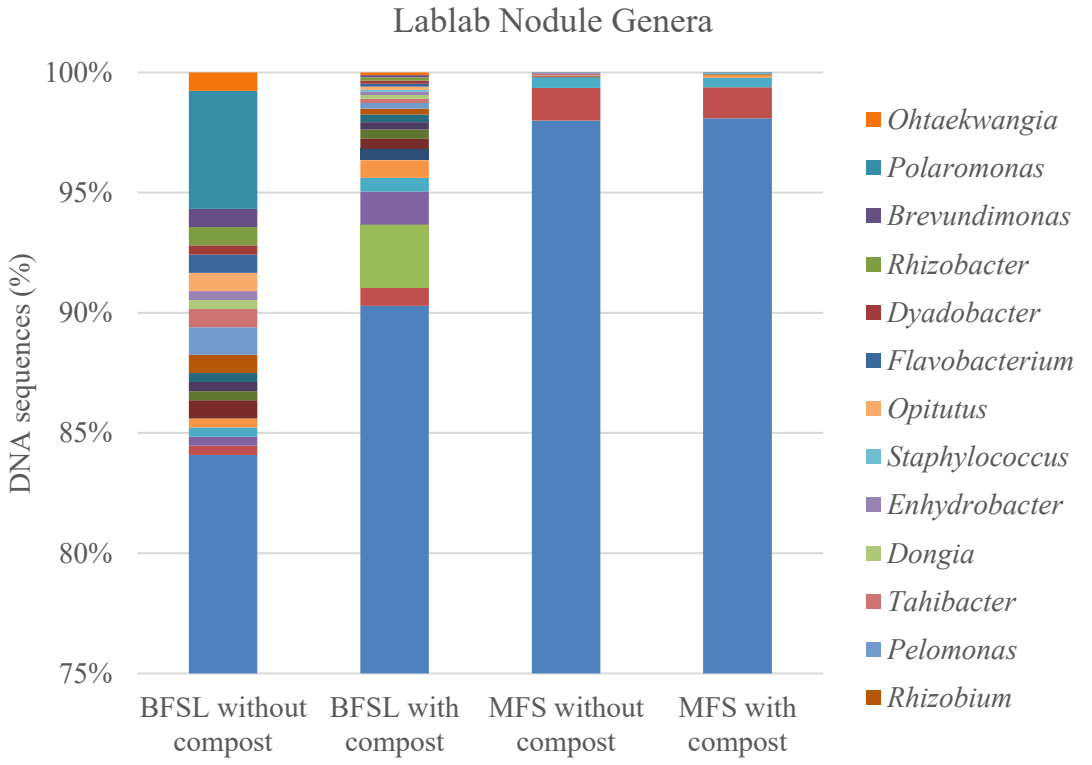

Figure 22. Genera of DNA sequences recovered from lablab nodules from each treatment group

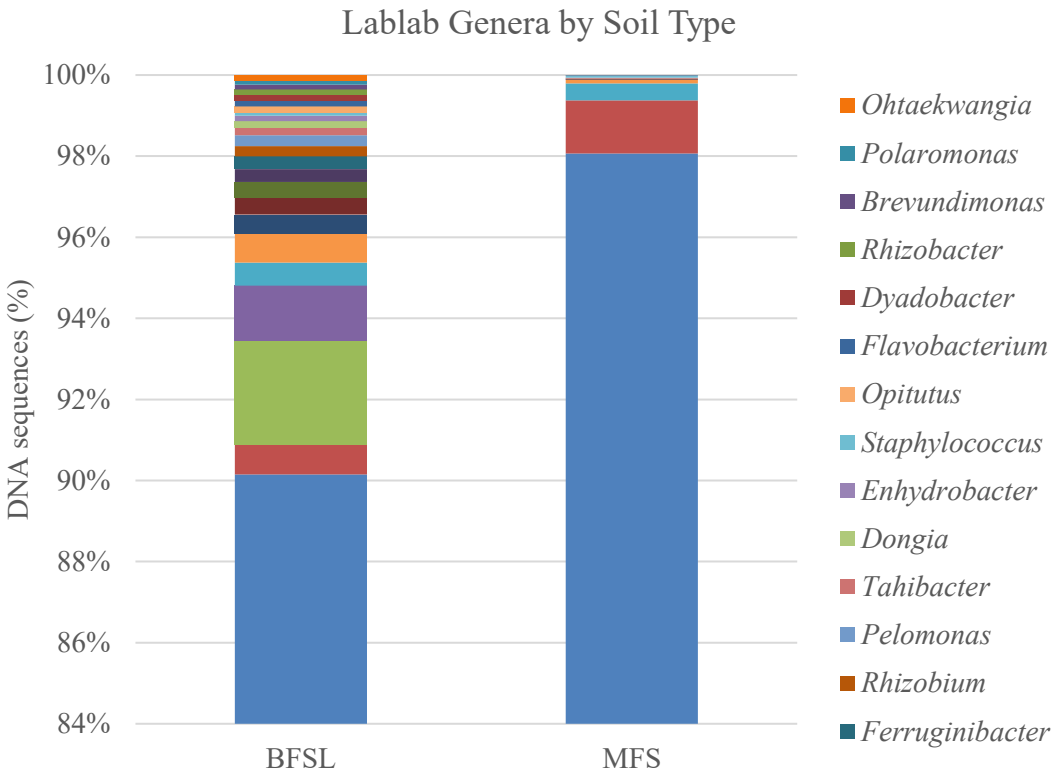

Figure 23. Genera of DNA sequences recovered from soybean nodules from each soil

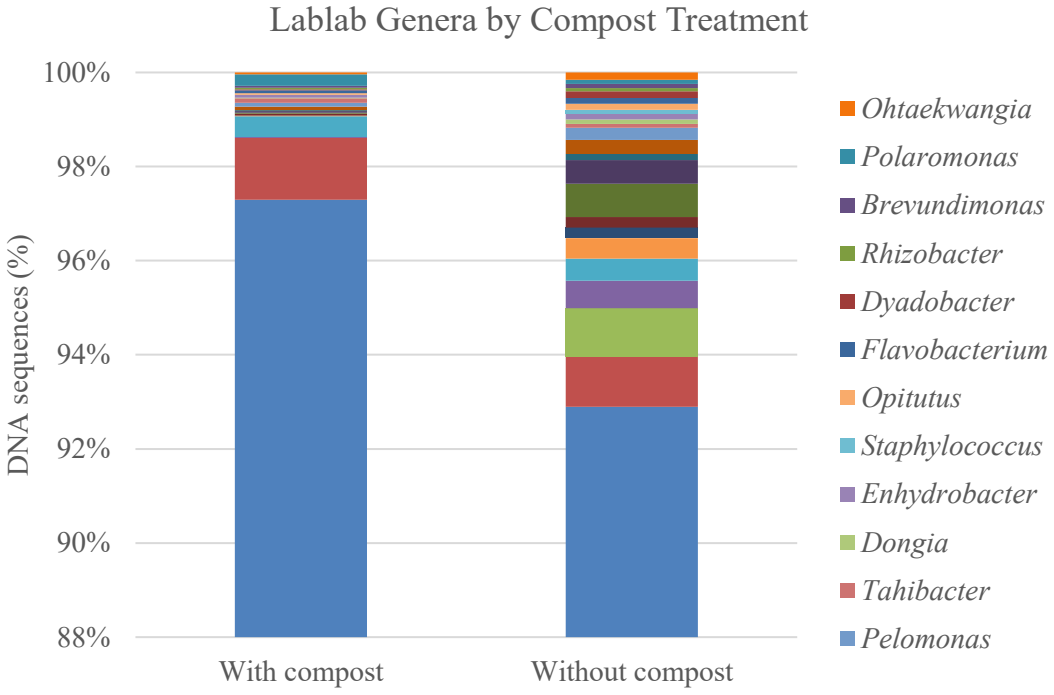

Figure 24. Genera of DNA sequences recovered from soybean nodules from each compost treatment

DISCUSSION

Soil Differences and Preceding Crop

Contrary to our hypothesis, the two different soils did not produce significant differences in the nodule endophytes selected by either lablab or soybean plants, meaning that neither the distinct preceding crops nor any other distinctives of the two soils exerted notable selective discrimination. Nonetheless, there were a number of notable and interesting differences observed about the soil microbial communities. Bosket Fine Sandy Loam (pH_s 5.8) was dominated (~30-50% of sequences) by Acidobacteria from the genera *Gp4*, and *Gp6*. While these genera also made up around 25% of sequences in Malden Fine Sand (pH_s 4.8), other genera were in greater relative abundance, namely, *Gp1* and *Candidatus Koribacter* of Acidobacteria, *Gaiella* of Actinobacteria, and *Bacillus* of the Firmicutes. The preponderance of Acidobacteria in the soils is notable because members of the phylum are underrepresented in cultural analyses [139, 140]. Acidobacteria were far less abundant, relative to other phyla, in the autoclaved sand with compost (coarse, pH_s 7.2), which exhibited greater population proportions of Proteobacteria and Firmicutes.

The compost was dominated (~60% of sequences) by *Chryseolinea*, a genus whose members are capable of degrading lignocellulose, and *Methalocaldum*, a genus of thermotolerant and thermophilic methanotrophs [141, 142]. Nonetheless, its amendment to soil produced no change in endophyte species selection.

Rhizobial Endophytes

Despite the presence of *Rhizobium* and *Mesorhizobium* species in the soils, *Bradyrhizobium* spp. were overwhelmingly selected for nodule occupancy. *Bradyrhizobium* spp. were abundant in all nodules analyzed, despite differences in compost, amendment, preceding crop, or any of the differences in the growth media, suggesting strong selection by the host plant specifically for *Bradyrhizobium*. This result is entirely consistent with previous findings that *Bradyrhizobium* is the dominant endophyte of soybean under acidic conditions [105, 112–115]. As the soils used in the present study had pH values of 5.8 (Bosket Fine Sandy Loam), 4.8 (Malden Fine Sand), and 7.2 (autoclaved sand), our findings support the findings of such previous studies with the greater clarity afforded by Next-Gen DNA sequencing. It is significant that none of the different treatments employed produced any change in rhizobial endophyte selection.

Non-rhizobial Endophytes

While *Bradyrhizobum* spp. amounted to 92-98% of the total sequences isolated from each sample's root nodules in both species (with the exception of those lablab samples expected to include some amount of plant material), two other genera are notable for their consistency and number of sequences: *Nitrobacter* and *Tardiphaga*. Both genera belong to the family *Bradyrhizobaceae*, sharing a number of important genetic similarities (16S rRNA, *atpD*, *dnaK*, *gyrB*, *recA*, *rpoB*) [143, 144].

Nitrobacter is *Bradyrhizobium*'s closest genetic relative; *Nitrobacter* shares extensive similarity with *Bradyrhizobium* in 1300 of its 3143 total genes [145]. *Nitrobacter* is not a commonly reported endophyte, possibly due to its very slow growth as a chemoorganotroph

[146, 147]. *Nitrobacter* may likely overcome soybean host defenses and survive in the nodules as a chemolithotroph by making use of NO_2 in the nodule as an electron source [147–149]. It was by including nitrite into the growth substrate employed in their experiment that Ibiene et al. (2012) were able to isolate and identify *Nitrobacter* as an endophyte of *Lycopersicum esculentus* [148]. Most studies do not include nitrite in isolation media [123, 124, 150].

In the conventional understanding based on laboratory studies, nitrogen-fixing bacteria fix N_2 into NH_4, which is then oxidized by *Nitrosomonas* or *Nitrosopira* to produce NO_2, which in turn is oxidized by *Nitrobacter* to produce NO_3 [149, 151]. Until recently, it was thought that ammonia oxidation and nitrite oxidation were always carried out by distinct species in cooperative consortia, a situation puzzling to scientist, since it would be energetically advantageous to carry out the complete oxidation of ammonia [152]. Daims et al. (2015) then discovered a completely-nitrifying *Nitrospira* strain, "fundamentally chang[ing] our picture of nitrification" [152]. Under current understanding, nitrite would be necessary for *Nitrobacter* is surviving as a chemolithotroph in the nodule rather than as a chemoorganotroph, but no sequences belonging to ammonia-oxidizing bacteria were identified, so there is no clear nitrite source. A follow up study that attempts to amplify ammonia-oxidizing genes from *Nitrobacter* isolated from legume nodules would be able to confirm whether endophytic *Nitrobacter* is capable of complete ammonia oxidation as *Nitrospira* was found to be. Complicating this picture is the understanding that Alanine, not ammonia, is the nitrogen-carrying molecule excreted by nodule bacteroids for transfer to the host plant, not ammonia [153]. In fact, Streeter (1989) estimated the ammonium concentration in the cytosol of soybean nodules at "essentially nil" [154]. Alternatively, *Nitrobacter* may survive by making use of glucose from the host plant as a

chemoorganotroph [145, 146]. In short, the metabolic means *Nitrobacter*'s persistence of within root nodules is not at all clear and bears further investigation.

Ibiene et al. (2012) identified *Nitrobacter* spp. as plant growth promoting rhizobacteria due to their ability to solubilize phosphate [148]. It is unclear, however, whether *Nitrobacter* serves as a plant-growth promoting endophyte in legume nodules, or whether it is simply able to overcome the host's defenses and then living in a state of commensalism or parasitism in the nodule without conferring benefits to the host.

The other endophyte consistently identified from soybean root nodules was *Tardiphaga*. *Tardiphaga* is also rarely cited as an endophyte due to its extremely slow-growth rate; Safronova et al. (2015) measured its doubling time at 10 days, well beyond the incubation times used in most isolation studies [155]. Based on isolates from the root nodules of *Robinina pseudoacacia*, *Tardiphaga* is also genetically quite similar to *Bradyrhizobium* [144]. *Tardiphaga* has also been isolated from the root nodules of *Vavilovia Formosa* in a study which additionally amplified *nodM* and *nodT* genes from the bacteria [155].

The presence of these two genera in the nodules might be explained by their high degree of genetic similarity to the apparent target symbiote, *Bradyrhizobium*. This genetic similarity must include precisely those factors that enable *Bradyrhizobium* to bypass the host plant's defense against infection. Once established in the root nodule, *Nitrobacter* and *Tardiphaga* are able to persist. It is unclear whether their far lower numbers in the nodules are due to poor competitiveness with *Bradyrhizbium* due to poorer adaptation to the nodule environment, or to significantly slower growth. It is possible that both factors may contribute. Any potential roles of *Nitrobacter* and *Tardiphaga* in plant growth have yet to be established.

It is important to note than no sample found nodules dominated by either *Nitrobacter* or *Tardiphaga*; *Bradyrhizobium* was both present and dominant in every nodule sample, suggesting that *Nitrobacter* and *Tardiphaga* are incapable of unilaterally nodulating soybean, but are able to enter the host plant when nodulation with *Bradyrhizobium* occurs.

Regarding other, commonly reported non-rhizobial endophytes such as *Variovorax*, *Enterobacter*, *Ralstonia*, *Agrobacterium*, *Klebsiella*, *Gluconacetobacter*, *Burkholderia*, *Bacillus*, *Psuedomonas*, *Pantoea*, *Serratia*, *Acinetobacter*, their inconsistent presence within root nodules suggests that they are not important nodule endophytes in soybean or lablab [86, 120, 126, 127, 129–133, 156]. Many of these were identified sporadically in the present study, which may be the result of random selection or passive penetration into the root nodule, or their extracted DNA may simply have originated from the outer surface of the root nodules and actually belong properly to the rhizosphere rather than the nodule interior. Consider, for example, *Bacillus* representing the fourth most common genera in soybean nodules after *Tardiphaga*; between one and four 16S sequences belonging to *Bacillus* were recovered (out of thousands of total sequences per sample) from five out of 21 total soybean nodule samples. Co-inoculation-based studies suggest that genera from some or all of these genera may play the role of free-living PGPR [126, 132, 157]. These genera are much more frequently cited in the literature than *Nitrobacter* or *Tardiphaga*, potentially because of their faster growth. Their inconsistent presence and low abundances in the present study strongly suggest that these are not species selected for by soybean to be nodule endophytes, and that their importance in the literature is overstated in the literature due to their ease of isolation.

While these genera have been commonly identified as potential endophytes, the present study highlights the importance of measuring relative abundance with the nodules over against

traditional isolation, characterization, and subsequent inoculation. The low abundance and random selection of non-rhizobial species suggest that they may not play a significant role in plant growth as endophytes. They may nonetheless be important to plant growth as free-living rhizobacteria.

For some non-rhizobial species in lablab, such *Enhydrobacter*, *Propionibacterium*, *Staphylococcus* and the aforementioned *Acidovorax* and *Variovorax*, 86-100% of their sequences derived from nodule samples, though neither was their presence consistent across samples nor their total proportions great relative to the three main genera discussed above. Due to the great difficulty in cleaning all of the plant material away from the lablab nodules, it is possible that these species are soybean root endophytes whose sequences derived from the epidermis, or at the very least closely associated with the root surface, though they are not nodule endophytes.

Conclusion

Both Soybean and Lablab exhibited strong selection, effectively excluding all but three genera from their root nodules—*Bradyrhizobium* and its close relatives *Nitrobacter* and *Tardiphaga*. The potential role of the latter two taxa in plant growth has yet to be established. The low abundance and random selection of non-rhizobial endophytes previously identified in the literature suggests that these organisms may not play a significant role in plant growth as endophytes, though they may very well still be plant-growth-promoting symbionts as free-living residents of the rhizosphere. These findings indicate that the isolation, characterization, and subsequent inoculation of non-rhizobial species may not be sufficient to establish their role as endophytes. Their relative abundance in the root nodules should be regarded an important means of certifying a suspected endophyte.

Neither soil type (preceding crop) nor compost amendment were found to have an influence on endophyte selection at the level of genus.

The successor to this study will be able to identify nodule endophyte sequences at the species level as well as analyze the recovered *nifH* sequences. Possibilities for future research include isolation and co-inoculation studies of *Nitrobacter* spp. and *Tardiphaga* spp. and even whole genome analysis of *Bradyrhizobium*, *Nitrobacter*, and *Tardiphaga* in order better to understand what genetic elements may be important to overcoming host plant defenses in the process of nodulation.

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