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Investigation of the Individual Soybean Root Nodule Microbiome

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INVESTIGATION OF THE INDIVIDUAL SOYBEAN ROOT NODULE MICROBIOME

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Parris Mica Mayhood

May 2020

INVESTIGATION OF THE INDIVIDUAL SOYBEAN ROOT NODULE MICROBIOME

Biology

Missouri State University, May 2020

Master of Science

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ABSTRACT

Soybean is a valuable crop that establishes a symbiotic association with nitrogen-fixing microorganisms. Previous research suggests members of the genus *Bradyrhizobium* are the major nitrogen-fixing inhabitants of soybean root nodules. However, results from recent studies identified the presence of other bacteria, so called non-rhizobial endophytes (NREs), inside root nodules. All previous research has pooled nodules of different plants for bacterial community analysis, but this study investigated the presence and relative abundance of rhizobia and NREs within multiple root nodules of a single plant. Three sites were selected on Kendrick Farm in Springfield, MO, where three plants and rhizosphere soil were collected at each (nine total plants). I have assessed the microbiomes of 193 individual soybean root nodules from nine different plants using Illumina MiSeq paired-end DNA sequencing. *Bradyrhizobium japonicum* strains were preferentially selected in high abundance within all root nodules despite the presence of other soybean nodulating rhizobia. *Nitrobacter* and *Tardiphaga* were the two non-rhizobial genera that were consistently detected in low abundance within almost all root nodules. DNA sequences related to other frequently reported soybean NREs were detected within a few nodules only. The overall, very low abundance and inconsistent presence of most commonly reported NREs within different root nodules of a single plant suggest that these NREs are not being preferentially selected as endophytes by host plants and most likely have a very limited role in plant growth as endophytes. However, these NREs may have a significant role in plant growth as free-living plant growth-promoting rhizobacteria, which was observed in several co-inoculation-based studies.

KEYWORDS: Soybean, *Glycine max*, root nodules, non-rhizobial endophytes, *Bradyrhizobium*, *Nitrobacter*, *Tardiphaga*

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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LITERATURE REVIEW

Soybean and Nitrogen Fixation

Soybean, *Glycine max*, is the most important source of protein in agriculture, an important oil crop and key component of human nutrition worldwide. Soybean is grown widely around the globe, fueling many economies (Figure 1)(1). Soybean is the most cultivated legume globally, as the largest legume cropland acreage is attributed to its production (2). Soybean is processed into oils and high-protein content soy flour that is commonly used in animal feed, tofu, cake and bread products (3). Like other crops, soybean also requires nitrogen to produce high yield.



Figure 1. Soybean (*Glycine max*) pods.

Nitrogen (N) availability is one of the major plant growth limiting nutrients in the terrestrial ecosystem (4). Although plenty of nitrogen is present in the atmosphere (78% of the air is N_2), it is the major limiting nutrient for the growth of valuable agricultural crops. The atmospheric N (N_2) is highly stable and cannot be directly used by plants for their growth and development. Plants can fulfill their N requirements either by taking up inorganic nitrogen

(NH_4^+ , NO_3^-) from soil or develop a symbiotic association with the N-fixing microorganisms.

The inorganic N in soil can come from the decomposition of organic matter or added into the soil in the form of chemical fertilizers.

Chemical Fertilizers

To achieve maximum crop yield by improving soil fertility, the application of chemical fertilizers and other nutrients became common practice (5). Although the application of chemical fertilizers increases agricultural production, it also has a detrimental effect on terrestrial and aquatic ecosystems (6). Only a small amount of applied chemical fertilizer is taken up by the plants and the remaining N can be lost due to agricultural runoff or leaches into groundwater, causing harmful effects on the freshwater or groundwater quality (7, 8). The long-term use of chemical fertilizer has been shown to deteriorate soil quality and fertility, ultimately lowering agricultural efficiency over time (6).

In addition, the production of chemical fertilizers also contributes significantly toward global climate change (9). A significant amount of fuel resources have been used to synthesize nitrogenous fertilizers. For example, the Haber-Bosch process of making nitrogenous fertilizers consumes a high amount of energy to create high temperatures ($400^\circ\text{--}500^\circ\text{C}$) and pressures (150-250 bar) that are required to combine nitrogen gas with hydrogen to make ammonia (10). Secondly, a high concentration of greenhouse gas emissions such as N_2O (1.5%) has also been associated with the extensive use of chemical fertilizers, as microbial transformations within the soil can generate this greenhouse gas (11-13). According to an estimate by Skiba and Rees (2013), about 27% of the global N_2O gas comes from the transformation of chemical fertilizer (14). Considering the deleterious effects of chemical nitrogenous fertilizers on the environment

as well as limited fuel resources for their production, the development of alternate resources is needed to fulfill plant N requirements through a sustainable basis.

Biological Nitrogen Fixation

The atmospheric N (N_2) is highly stable and cannot be directly used by plants. However, some prokaryotes in the soil can convert this N_2 into plant useable form with the help of the nitrogenase enzyme (15). These N-fixing prokaryotes either develop a symbiotic association with legume crops or can fix N_2 as asymbiotically (free-living N fixing bacteria). The biologically fixed N can be directly available to the host plant or can be added into a pool of soil as inorganic N that is available for plant uptake (4, 16). Symbiotic N fixation is extremely important because the amount of N fixed through symbiotic association is much higher (>100 kg N/ha/year) than the amount of N fixed by free-living N-fixing microorganisms (1-20 kg N/ha/year)(4). This amount of N fixed through legume-rhizobia association is approximately 16.4 Tg N per year, which accounts for approximately 77% of total biologically fixed N (2).

Soybean (*Glycine max*) is an important legume crop that belongs to the *Fabaceae* family. Like other legumes, soybean develops a symbiotic association with rhizobia. Rhizobia is a broad term that collectively includes several bacterial genera such as *Sinorhizobium* (*Ensifer*), *Rhizobium*, *Neorhizobium*, *Mesorhizobium*, and *Bradyrhizobium*. These rhizobia taxonomically fall into the *Proteobacteria* phylum, into class *alpha-proteobacteria* (16). In general, soybean can be nodulated by all rhizobial genera including *Bradyrhizobium* (17, 18), *Ensifer* (19, 20), *Mesorhizobium* (21) and *Rhizobium* (22). The microsymbiont resides within root nodules (Figure 2) and provides the host plant with fixed N. Whereas, in return the host plant provides shelter and carbohydrates for rhizobial growth within root nodules (23).



Figure 2. Root nodules along soybean root system.

The establishment of the symbiotic relationship between plants and rhizobia is a highly regulated process. During early plant growth, specific signals from the host plant root system are released into the rhizosphere soil to attract specific N-fixing rhizobia (24). The host compatible rhizobia then release signals that initiate the formation of the root hair infection thread. The plant tissue responds and eventually develops into a root nodule, which is a complex organ of specialized tissues. Within root nodules, rhizobial cells transform into bacteroids which perform biological N fixation (16). Some legumes are promiscuous hosts i.e., can be nodulated by diverse rhizobial species, whereas others opt for specific strains of rhizobia as a microsymbiont.

The specific selection of rhizobia within soybean plant is relatively well studied and suggested to be influenced by different soil factors such as pH, salinity, nutrient availability, water availability, geographic locations, and the diversity of the rhizobial population within the

soil (25-28). Depending on the soil pH, soybean nodules are frequently dominated by *Bradyrhizobium* or *Ensifer* spp. (29-32). Under acidic soil conditions, the most dominant endophytes reported within soybean root nodules are *Bradyrhizobium* sp., whereas, under alkaline soil conditions *Ensifer* were detected as major endophytes (18, 33). Similarly, several other environmental or genetic factors have also been reported to influence the selection of specific rhizobial genera or strains within root nodules. In the United States, the inoculation of soybean plants with *Bradyrhizobium japonicum* has started in the early 20th century (34), however many indigenous *Bradyrhizobium* strains, which can outcompete the inoculated strain for root nodules occupancy, have also been reported (35). Understanding the factors that drive the selection of the soybean microsymbiont is crucial to the future development of N-fixing bioinoculants.

Factors that Drive Selection of Rhizobia in Soybean Root Nodules

As mentioned above, many genetic and environmental factors may affect the selection of specific rhizobia within soybean root nodules. The specific selection of rhizobia within soybean nodules is influenced by several factors such as soil pH, salinity, nutrient availability, geographic locations, and diversity of the rhizobial population within soil (25-28). Large scale biogeographical differences have accounted for much variation between rhizobial populations in soybean (26, 36, 37), with pH and/or latitude as the major selection-driving environmental parameters (33, 37). Host plant genetics have also been shown to contribute to the selection of the microsymbiont, as the R_j genotypes of soybean have also been shown to influence the selection of specific *Bradyrhizobium* strains (37, 38).

Despite all these studies, the information on the role of a small-scale spatial heterogeneity of soil on the selection of rhizobial endophytes within different root nodules of a single plant is unknown. Soil is a highly heterogeneous environment, and its biochemical conditions can vary greatly at short distances of 1 cm or less (39). It is unclear if some rhizobial species get selected within all root nodules of a single plant or if it can vary within individual root nodules depending upon location of the root nodules on a root system or nearby soil conditions. One of the major research questions of the current study was to assess the role of small-scale spatial heterogeneity of soil on the selection of rhizobial endophytes within different root nodules of a single plant.

Plant Growth Promoting Rhizobacteria (PGPR)

In contrast to the root nodule, which is an enriched locale for specific rhizobial endophytes, soil contains high bacterial diversity (up to 10^8 to 10^9 cells per gram soil)(40). The rhizosphere soil is rich in plant root exudates (such as sugars, amino acids, phenolic compounds and organic acids), which enrich specific bacterial communities that can play an important role in plant growth (41). These bacterial genera are known as plant growth promoting rhizobacteria (PGPR). The suggested mechanisms through which these PGPR enhance plant growth include phytohormone production, increased nutrient availability in soil, or by acting as a biocontrol agent by limiting the growth of plant pathogens (41, 42). These bacteria can inhabit the rhizosphere, colonize the root system, or more recently have been reported to inhabit nodule tissue in legumes (43).

So far, several bacterial species have been identified as PGPR. For example, bacterial genera such as *Pseudomonas*, *Bacillus*, *Flavobacterium*, etc. have been reported to produce organic acids that can lower soil pH. The decrease in soil pH enhances phosphate (P)

solubilization in the soil which increases the P availability to the plant (44). Similarly, many rhizobacteria isolates have shown to have the capacity of phytohormone production (auxins, gibberellic acid and cytokines) which enhance root growth and development (45). Like PGPR, phytohormone production by rhizobial strains such as *Bradyrhizobium elkanii* has also been reported (46). Phytohormones also allow the plant to modulate their stress responses under stressful environmental conditions, such as salinity, drought, flooding, pollution, and wounding (47). Bacteria that produce phytohormones found in plants would be candidates for potential selection by the host plant.

Indirectly, PGPR can suppress or inhibit the infection of a plant by phytopathogens. Production of antibiotics and degradative enzymes by PGPR has been reported to inhibit the infection of many phytopathogens (48). The hydrolytic enzyme chitinase may be produced, which targets the cell wall of fungi, a major group of plant pathogens (49). The continuous presence of PGPR in the rhizosphere can induce systemic resistance by triggering the plant's immune system, allowing defenses to be primed to respond to a pathogenic infection (50). The roles of PGPR in plant growth have been well documented within the rhizosphere, however, more recently several studies have reported some common PGPR species as root nodule endophytes (Table 1). The information on the selection of these PGPR as root nodule endophytes has been limited. Hence one of the research questions of the current study is the selection of PGPR within soybean root nodules. If these PGPR endophytes are truly beneficial for the host plant, then they will be selected with all root nodules of a single plant. Alternatively, if these non-rhizobial endophytes enter passively within root nodules, they will not be consistently present in all nodules of a plant and may not have a role in plant growth within the nodule.

Table 1. Commonly implicated PGPR more recently detected and/or isolated from legume root nodules.

PGPR/NRE Genus	Host Plant	Plant Growth	Citations
		Promotion Assay	
<i>Bacillus</i>	Soybean	+	51
<i>Pseudomonas</i>	Soybean	+	52
<i>Enterobacter</i>	Soybean	+	53
<i>Paenibacillus</i>	Soybean	+	54
<i>Stenotrophomonas</i>	Soybean	-	55
<i>Rhodococcus</i>	Soybean	+	56
<i>Ochrobacterum</i>	Soybean	+	52
<i>Serratia</i>	Soybean	-	57
<i>Chryseobacterium</i>	Soybean	-	55
<i>Agrobacterium</i>	Soybean	+	53
<i>Pantoea</i>	Soybean	+	53
<i>Acinetobacter</i>	Soybean	+	53
<i>Sphingobacterium</i>	Soybean	-	58
<i>Novosphingobium</i>	Indigenous	-	59
<i>Variovorax</i>	Indigenous	-	59

Non-Rhizobial Endophytes

In addition to rhizobia, several studies have observed the presence of various non-rhizobial endophytes (NREs) within soybean root nodules (Table 1). Many culture-based studies have isolated and identified several NREs from the root nodules and reported their potential beneficial effects on the growth of host plant (51-54, 57). The suggested mechanisms through which these NREs may enhance plant growth includes synthesis of plant growth regulators, phosphate solubilization, suppressing the growth of plant pathogens, and by helping the host plant through enhanced stress regulation (60). Inoculation of isolated NREs onto the host plant

can often improve plant growth, however, it remains unclear if these benefits occur in the root nodule or within the rhizosphere in nature.

Unlike the rhizobia-soybean relationship, much less is known about when and how NREs enter the nodule, however, it has been shown they can enter with rhizobia during nodule formation in *Vigna radiata* (61). The role of host plant species or genotype, soil conditions, and salinity stress on the presence of these NREs have been investigated, yet it remains unclear if there is selection operating on the curation of the nodule microbiome and if so, what environmental factors drive the selection (59, 62). These culture-based studies have provided useful information about the potential beneficial effects of NREs on the host plant. However, these studies were not able to provide any information on the relative abundance of NREs within root nodules or their preferential selection by host plant as commonly observed in the case of rhizobial endophytes.

Bias in Current Methodology Studying Nodule Endophytes

So far, most of the studies on NREs are culture-dependent. The procedure adopted in these studies was surface sterilization followed by culturing on nutrient-rich growth media. Isolated bacterial colonies were characterized by different physiological methods and subsequently identified using 16S rRNA gene sequencing (61, 63, 64). While these studies provide useful insight about the NREs within soybean root nodules, they fail to provide information on their relative abundance within root nodules (65).

In contrast to culture-based studies, culture-independent (sequencing) studies suggested a high diversity of NREs (55, 59, 66). Next-generation sequencing has allowed the study of total communities using metagenomic approaches that target the total genetic product of a sample and

subsequently identify the members of the community. This method also allows insight to abundance ratios between members of the nodule community, also known as relative abundance. However, these culture-independent studies are unable to provide any information about the preferential selection of NREs within soybean root nodules. In this research, studies that employ next generation sequencing techniques typically pool many nodules from the sample plant together and is treated as one sample, increasing amount of nodule surface area (55, 59, 62, 66, 67). All these studies have used multiple root nodules that were collected from different plants to assess endophytic diversity. It remains unknown whether the high diversity of NREs is uniformly distributed across all root nodules or is randomly present within a few root nodules. Hence, these culture independent studies were unable to provide information on the selection of NREs. Therefore, the focus of the current study was to assess the NRE diversity within different root nodules of a single plant.

Other problems exist with sequencing-based studies. While these nodules are surface sterilized, DNA may be left behind from nodule surface bacteria, amplified, and detected. Another problem, the layer of plant tissue surrounds these nodules, termed the epidermis, that is in direct contact with microbe-concentrated rhizospheric soils (Figure 3). It is likely that these adjacent microbes can attach and/or penetrate this tissue, but not the inner portion of the nodule. While these microbes are important for study, their classification as nodule endophytes may be inaccurate. They may be opportunistic endophytes that can enter less specified tissue such as roots, but not the more selective tissue like the root nodule, which has been observed previously (67). The surface-attached microbes may not survive sterilization, but their DNA may still be detected and confound the results of microbiome analysis. Slight alterations in procedure, such as individual nodule sampling and epidermis removal, may limit the biases addressed above and

provide new insight to the community composition and the selection processes that are known to operate on rhizobia and potentially operate on NREs.

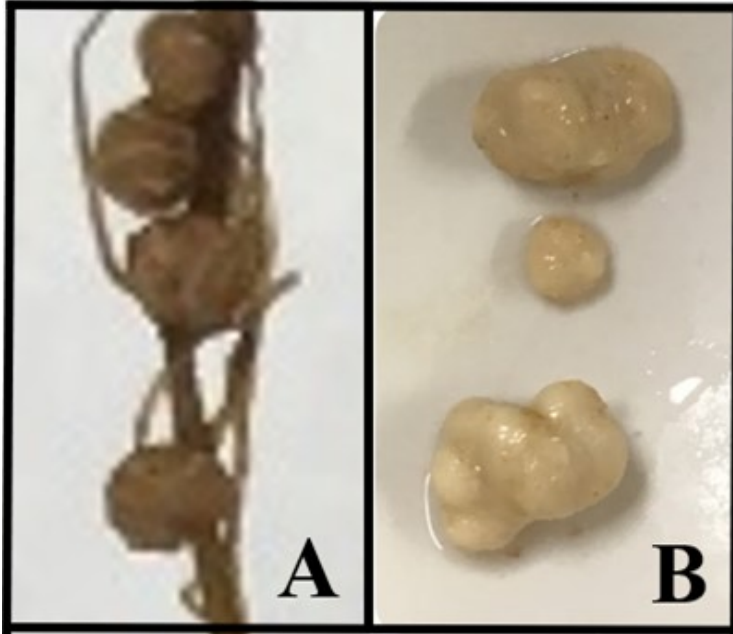


Figure 3. A: Soybean root nodules. B: Root nodules after removal of epidermis.

Present Study and Hypotheses

The primary objectives of the current study were to assess the (i) preferential selection and relative distribution of bacterial endophytes within different root nodules of a single plant, (ii) determine the influence of small-scale biochemical heterogeneity of soil on the selection of both rhizobial and NREs within nodules and (iii) determine the influence of large-scale heterogeneity of soil on the selection of endophytes. I have analyzed the microbiome of 193 individual root nodules of soybean, with the epidermis removed, from nine different plants that were grown under field conditions originating from three locations. I randomly selected ~25 root nodules per plant across the entire root system and the bacterial endophytes were analyzed using Illumina MiSeq paired-end DNA sequencing. This methodology was created to limit the biases listed above and may reveal effects of timing of nodule formation, placement upon the root

system or other small-scale differences within the soil on the communities of endophytes within nodules.

I hypothesized *Bradyrhizobium* and/or *Ensifer* would dominate in the nodules, as observed previously. I hypothesized that if NREs do provide benefits to host plants within the nodule, the host plant would select for their presence. If operating under selection, I should see them at considerable abundance within a majority of sampled root nodules, as observed with rhizobial endophytes. If these NREs are opportunistic endophytes, I expect to see them at low relative abundance and randomly between nodules of the same root system. I expect to see differences between the nodule microbiomes of different plants and locations, as soil parameters have been reported to modulate host plant selection.

METHODS

Research Site, Plant, Nodule, and Soil Collection

The research site for this study was the Missouri State University Kendrick Family Farm, located in Greene County, Missouri, approximately two miles southwest of the Springfield Branson Regional Airport. The farm has a corn, soybean, and wheat crop history. Three locations at this site were sampled. Location 1 (L1) was within a toe slope within the field (Figure 4). Location 2 (L2) was north of L1 and located east of a sinkhole on the property. Location 3 (L3) was located between the sinkhole and L1. Each sampled location was approximately 200 m away from the other. Three plants from each location were uprooted with their surrounding rhizosphere soil and bagged. Bags were kept on ice until arriving at the Missouri State University Department of Biology, then stored at -20°C until further processing.

Roots of selected plants were detached of rhizosphere soil and then washed with DI water to remove excess soil and organic matter. For rhizosphere soil samples, a subsequent 1-mL wash of each plant's root system was collected to get soil bound to roots and nodules. Samples were centrifuged at 10,000 rpm for 10 min and the supernatant discarded. For assessment of soil geochemical parameters such as nitrate, ammonium, and phosphate concentrations, 10 g of rhizosphere soils were sent to the University of Missouri for further analysis. To determine significant difference between locations a one-way ANOVA post hoc Tukey test was employed.

The root systems were then trimmed of thin roots with no nodules for better imaging (Figure 5). After imaging the nodules, 25 were selected at random and labeled for selection and storage. They were removed from the roots with sterile forceps and rinsed with DI water if excess soil remained. Detached nodules were suspended in 99% ethanol and stored at -20°C until



Figure 4. Ariel view of sampled locations L1, L2, and L3 at Kindrick Farm in Springfield, MO. Supplemental photos from sampling.

further processing. Nodules were then thoroughly washed with distilled water and surface sterilized with 75% ethanol. The root nodule epidermis was aseptically removed using sterile forceps while submerged in 75% ethanol. Surface sterilized nodules were crushed individually in 1-mL of sterile water using sterilized mortar and pestle (Figure 6). Crushed nodule material was centrifuged into a pellet at 10,000 rpm for 10 min.

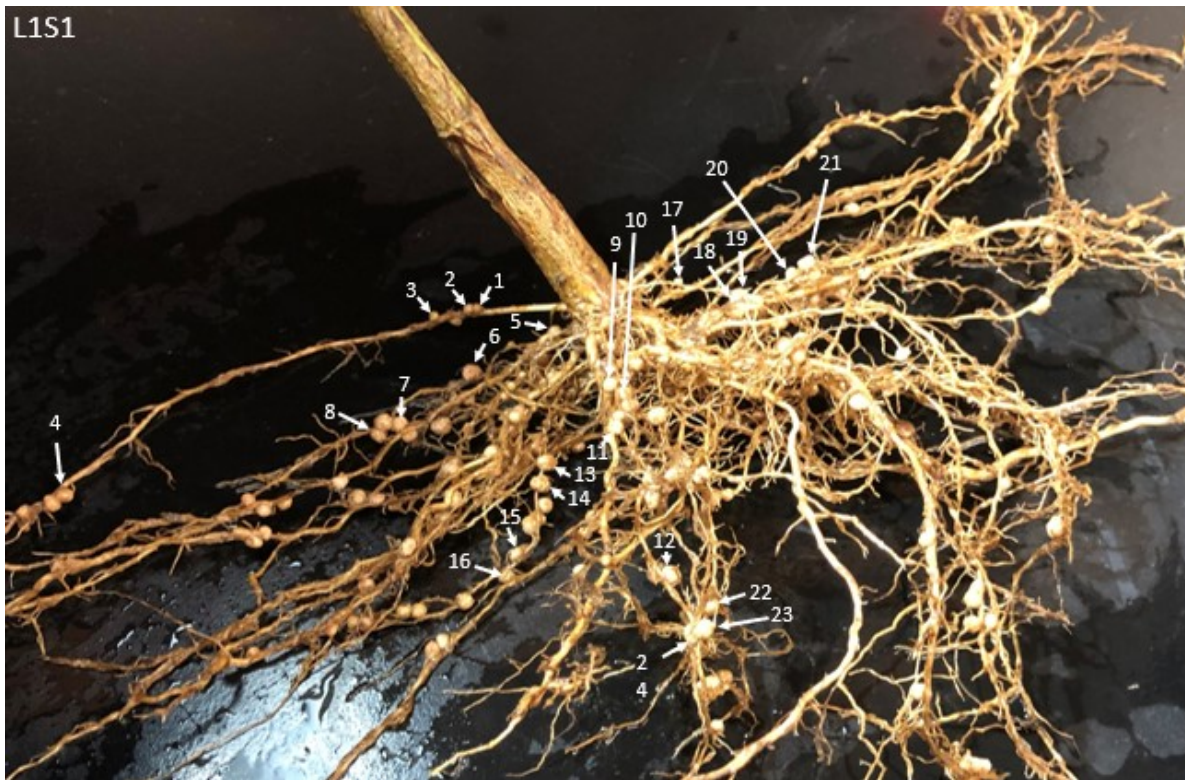


Figure 5. Selected nodules for sequencing from sample plant 1 (L1, plant 1).



Figure 6. Crushed nodule in sterile mortar and pestle.

DNA Extraction and Sequencing Preparation

DNA was extracted from each individual root nodule as well as from rhizosphere soil samples using DNeasy PowerLyzer PowerSoil DNA Extraction Kit (Qiagen, MoBio, USA). The pellets containing bacterial cells were resuspended with 'PowerBead Solution' provided in the PowerSoil kit. DNA was then extracted according to manufacturer's instructions. Extracted DNA was eluted in 25 µL nuclease-free PCR-grade sterile water and stored at -20°C until further use.

To access the bacterial communities within root nodules and rhizosphere soil, Illumina MiSeq paired-end DNA sequencing approach was used. Briefly, I have used a two-PCR approach. During the first PCR, partial bacterial 16S rRNA gene fragment of the V3-V5 region was amplified using universal primers, F515 (5'-GTGCCAGCMGCCGCGG-3') and R907 (5'-CCGTCAATTCMTTTRAGTTT-3'). These primers were previously ligated with Illumina DNA sequencing primers. PCR was performed in 25 µL reaction, each containing 1X buffer, 0.2 µM of each primer, 2.0 mM MgSO₄, 0.2 µM of each deoxynucleoside triphosphate (dNTPs), 1.0 µL of template DNA, and 0.1 µL High Fidelity Platinum Taq Polymerase High-Fidelity PCR System enzyme (Invitrogen, USA). The PCR conditions are as follows: 4 min at 96°C, followed by 30 cycles of denaturation at 94°C for 45 sec, primer annealing at 56°C for 45 sec, extension at 72°C for 45 sec, and a final extension for 7 minutes. Amplification was confirmed by running the products on 1% agarose gel and visualized with ethidium bromide (10 mg/mL). Amplified PCR products were cleaned with ExoSAP-IT PCR Cleanup System (Invitrogen, USA), according to manufacturer's instructions.

Cleaned PCR products were diluted 10-fold and used as a template in a 2nd PCR. All the reagents used in the second PCR were the same as described above with the exception of PCR

primers. The primers used in the second PCR contained the unique index sequences for each sample and Illumina adaptor sequences. The PCR conditions for the second PCR were: initial denaturation for 3 min at 90°C, followed by 10 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, with a final extension for seven minutes. Amplified, uniquely indexed PCR products were pooled in equimolar concentrations. Pooled, amplified DNA products were purified with Agencourt AMPure Magnetic Bead System (Beckman Coulter, Brea, CA). Purified PCR products were sequenced using MiSeq paired-end platform. DNA sequencing was performed at the Center for Integrated Biosystems (CIB), Utah State University, USA.

Data Analysis

Paired-end Illumina reads were assembled to generate consensus sequence and initial quality filtration was done using Mothur software (68). Initial quality filtrations (minlength=370, maxambig=3, maxhomp=8) were performed as previously described (69). Filtered sequences were screened for chimeras using the Ribosomal Database Project (RDP) platform. High-quality DNA sequences (10,962,675) were classified using RDPipeline function of the Ribosomal Database Project Naïve Bayesian Classifier 2.5 (70). RDP output provides classified genera and their sequence counts within every unique sample, providing community composition data for each individual nodule sample. Sequences corresponding to chloroplast and mitochondrial DNA were removed accordingly. Totals for bacterial genera were calculated and genera totaling more than 10 sequences within the 193 nodule samples were used for following analysis. To simplify visualization, the top 15 nodule genera were then used for community composition figures.

With Mothur, *Bradyrhizobium* sequences and remaining unclassified *Rhizobiales* sequences (4,085,797) were extracted. These sequences were cleaned at 98% similarity using Sequencher 5.4.6 Software (GeneCodes), correcting for errors in sequencing. An abundance table was generated using Galaxy (71), adding back unique names to get the true sequence count number for individual nodule and rhizosphere samples. Cleaned *Bradyrhizobium* sequences were aligned using RDPipeline Aligner tool and clustered into operational taxonomic units (OTUs) at 99% similarity with the clustering tool. Representative sequences were selected for each OTU (99% DNA similarity level) with more than 50 sequences using Python. A maximum likelihood tree was generated using MEGAX (72), using the maximum likelihood method with 1000 bootstraps. Branches were collapsed into subgroups based on OTUs (99% DNA similarity level) to assess the variation within the *Bradyrhizobium* spp. that were selected for nodulation and were present in the rhizosphere soil. Reference sequences were added to determine closely related species to OTU groups.

Representative sequences of all rhizobial genera (*Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, *Neorhizobium*, and *Rhizobium*) were used to generate a tree displaying all rhizobial members within nodules and soil. Other abundant, relevant genera were extracted and processed as described above. OTUs were assembled at 99% similarity for all genera, and representative sequences selected for OTUs with more than 50 sequences.

RESULTS

Soil Analysis

Geochemical conditions such as soil pH, organic matter, N, P, K, Ca^{++} , and Mg^{++} were analyzed for each location (Table 2). Analysis of variance (one-way ANOVA) suggested significant differences in soil content of NH_4 ($p<0.01$; $F=12.9$), NO_3 ($p<0.001$; $F=188.7$), and P ($p<0.001$; $F=71.9$) between the three locations. The location L3 displayed a significantly higher concentration of $\text{NO}_3\text{-N}$ as compared to L1 or L2 locations. The concentration of $\text{NH}_4\text{-N}$ was significantly higher at L1 as compared to the other two locations (Table 2). Phosphate concentrations were significantly lower at L2 compared to the other locations. In contrast, other geochemical characteristics such as soil pH, OM%, K, Ca^{++} , Mg^{++} were remarkably similar at all three locations. Overall, the soil pH was slightly acidic (6.5-6.8).

Table 2. Geochemical soil conditions taken from each sampled location. * = $p<0.01$, ** = $p<0.001$.

Parameter	L1	L2	L3
pH	6.6 ± 0.2	6.5 ± 0.1	6.8 ± 0.1
OM (%)	3.5 ± 0.1	3.1 ± 0.2	3.5 ± 0.2
$\text{NO}_3\text{-N}$ (ppm)	9.7 ± 0.8	8.7 ± 1	$26.7 \pm 1.8^{**}$
$\text{NH}_4\text{-N}$ (ppm)	$22.4 \pm 4.5^*$	11.4 ± 3.5	8.2 ± 2.5
P (kg/ha)	99 ± 4.5	$50 \pm 5.6^{**}$	105 ± 7.9
K (kg/ha)	318 ± 9	316 ± 16	410 ± 22
Ca (kg/ha)	3730 ± 560	3395 ± 348	4141 ± 36
Mg (kg/ha)	95 ± 24	73 ± 17	85 ± 13

Illumina MiSeq Sequencing and RDP Classification

Total Sequences. Overall, the DNA extraction and PCR amplification were successful from all root nodules and soil samples. Illumina MiSeq DNA sequencing resulted in 9,036,146 good quality sequences from 193 root nodules (Table 3). A relatively lower number of DNA sequences were retrieved from the nodules of location 3 (L3). Approximately, one million sequences were retrieved per plant with an exception of plants 1 and 2 at location L3 (L3P1, L3P2). After the initial quality control and removal of plant-associated sequences (chloroplast and mitochondrial sequences), the remaining endophytic bacterial sequences (3,667,312) were classified using RDP. On average, I have retrieved about 20,000 classified high-quality DNA sequences per root nodule (n=193).

DNA extraction and PCR amplifications were also successful for rhizosphere soil samples with the exception of one replicate sample from the location 2. This resulted in overall fewer rhizospheric sequences for L2, approximately 100,000 sequences at L2 versus 160,000+ sequences from L1 and L3 (Table 3). From eight soil samples, I retrieved approximately 455,333 DNA sequences. After initial quality control, remaining sequences (276,388) were classified using RDP.

Classification of Nodule Endophytes. Overall, 96.5% of total bacterial sequences retrieved from all root nodules were related to the genus *Bradyrhizobium* (Table 3). Likewise, a high abundance of *Bradyrhizobium* sequences (~94%) were detected within each individual root nodule sample (Figure 7). Remaining DNA sequences obtained from root nodules were classified as *Nitrobacter*, *Tardiphaga*, and a small fraction of them related to many other bacterial genera (Table 3).

Table 3. Total sequence count breakdown for all sampled plants and rhizospheres.

Sample	Raw Total	Bacterial Sequences	<i>Bradyrhizobium</i>	<i>Nitrobacter</i>	<i>Tardiphaga</i>	Others
L1P1	1,879,974	849,249	825,469 (97.2%)	18,909 (2.2%)	4,734 (0.56%)	137 (0.02%)
L1P2	1,165,885	440,484	422,869 (96.0%)	13,508 (3.1%)	3,978 (0.90%)	129 (0.03%)
L1P3	912,814	274,974	261,462 (95.1%)	11,032 (4.0%)	2,407 (0.88%)	73 (0.03%)
L2P1	1,097,772	399,498	376,742 (94.3%)	20,414 (5.1%)	2,239 (0.56%)	103 (0.03%)
L2P2	1,328,705	462,560	449,399 (97.2%)	9,671 (2.1%)	3,331 (0.72%)	159 (0.03%)
L2P3	1,159,579	555,593	546,057 (98.3%)	6,819 (1.2%)	2,517 (0.45%)	200 (0.04%)
L3P1	373,561	162,035	154,086 (95.1%)	7,544 (4.6%)	317 (0.23%)	34 (0.02%)
L3P2	223,862	82,347	80,004 (97.2%)	1,960 (2.3%)	315 (0.38%)	27 (0.03%)
L3P3	894,044	440,572	424,757 (96.4%)	14,670 (3.3%)	1,100 (0.25%)	45 (0.01%)
Nodule Total	9,036,146	3,667,312	3,540,868 (96.5%)	104,527 (2.9%)	20,992 (0.57%)	907 (0.02%)
L1 Soil	165,890	102,186	4,393 (4.3%)	113 (0.1%)	9 (<0.001%)	96,671 (94.6%)
L2 Soil	104,780	61,733	2,414 (3.9%)	73 (0.1%)	3 (<0.001%)	59,243 (96.0%)
L3 Soil	184,663	112,469	14,940 (13.3%)	915 (0.8%)	37 (<0.001%)	96,577 (86.0%)
Soil Total	455,333	276,388	21,747 (7.9%)	1,101 (0.4%)	49 (<0.001%)	199,172 (72.1%)

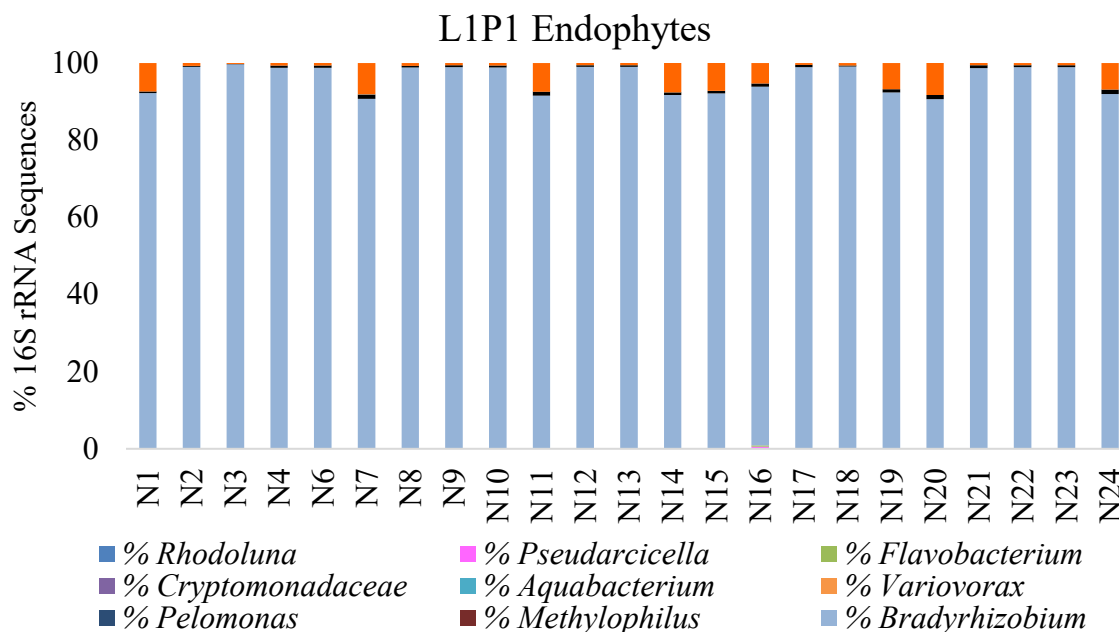


Figure 7. Relative abundance of all endophytes within individual nodules (1-24) from location 1, plant 1 (L1P1).

Among the NREs, *Nitrobacter* and *Tardiphaga* were two bacterial genera that were consistently detected within almost all individual root nodules (193 nodules) (Figure 7). The relative abundance of *Nitrobacter* sequences ranged from 1-5% within individual root nodules, representing 2.9% of nodule sequences on average (Figure 7; Table 3). *Tardiphaga*-classified sequences represented 0.2-0.9% of returned sequences for each plant, approximately 0.6% of total nodule sequences on average (Figure 7; Table 3).

The remaining DNA sequences from root nodules were classified as other NREs, and their abundance was relatively low in all plants, approximately 0.02% of all DNA sequences retrieved from root nodules (Table 3). The other NRE sequences were classified into several bacterial genera for each nodule. For the detailed evaluation of other NREs, the dominant bacterial genera (*Bradyrhizobium*, *Nitrobacter*, and *Tardiphaga*) were removed from Figure 7 to see the relative abundances of the remaining NREs within individual nodules of single plant

(Figure 8). The relative abundance of all other NREs collectively was very low (<0.1%) of total DNA sequences obtained from each root nodule.

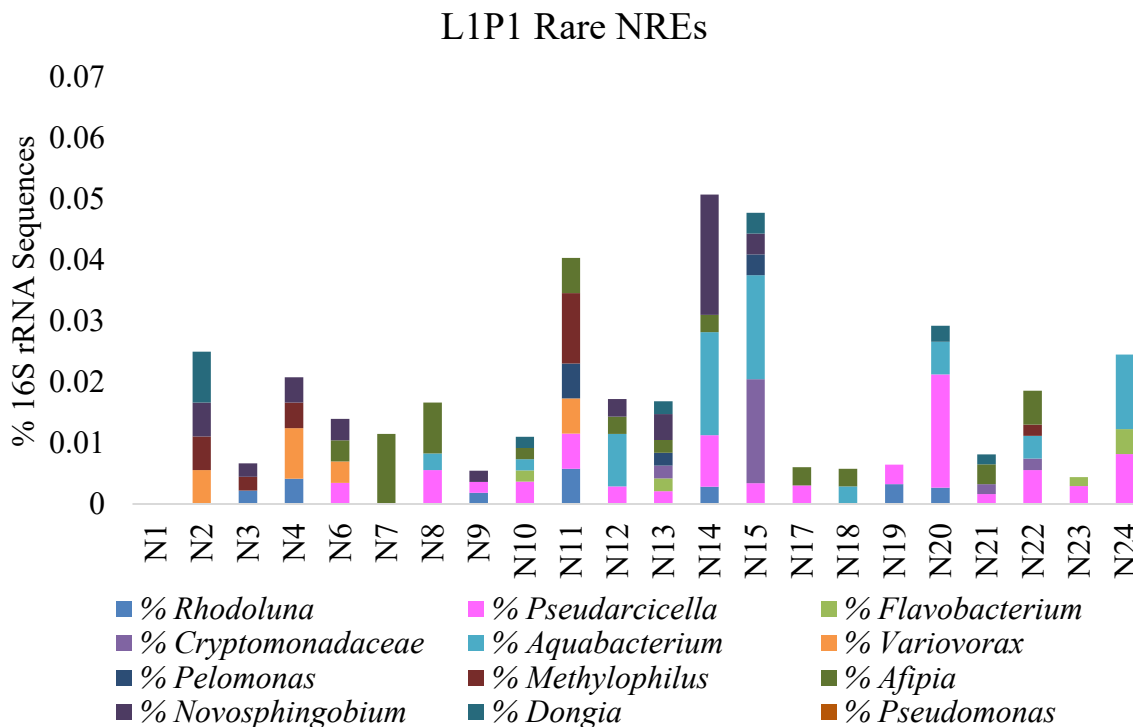


Figure 8. Nodule endophytes from location 1, plant 1 (L1P1) remaining after removal of *Bradyrhizobium*, *Nitrobacter*, and *Tardiphaga*.

The relative distribution of endophytes among all nine plants at three locations followed the trend elucidated above (Figure 9). *Bradyrhizobium* represented approximately 97% abundance DNA sequences retrieved from different nodules from all locations, followed by *Nitrobacter* (3%) and *Tardiphaga* (<1%). *Bradyrhizobium* was detected within all 193 root nodules from all three locations, while *Nitrobacter* and *Tardiphaga* sequences were detected within 98% and 96% nodules, respectively (Table 4). Rare NREs were detected at low abundances among all locations (Figure 10). These rare NREs were composed approximately 0.25% of total classified sequences at two locations (L1 and L2), whereas at L3 their abundance was <0.05%. The occupancy of these genera within individual nodules was much lower than

observed for our dominant genera, as they were detected in 12-49% of sampled nodules (Table 4).

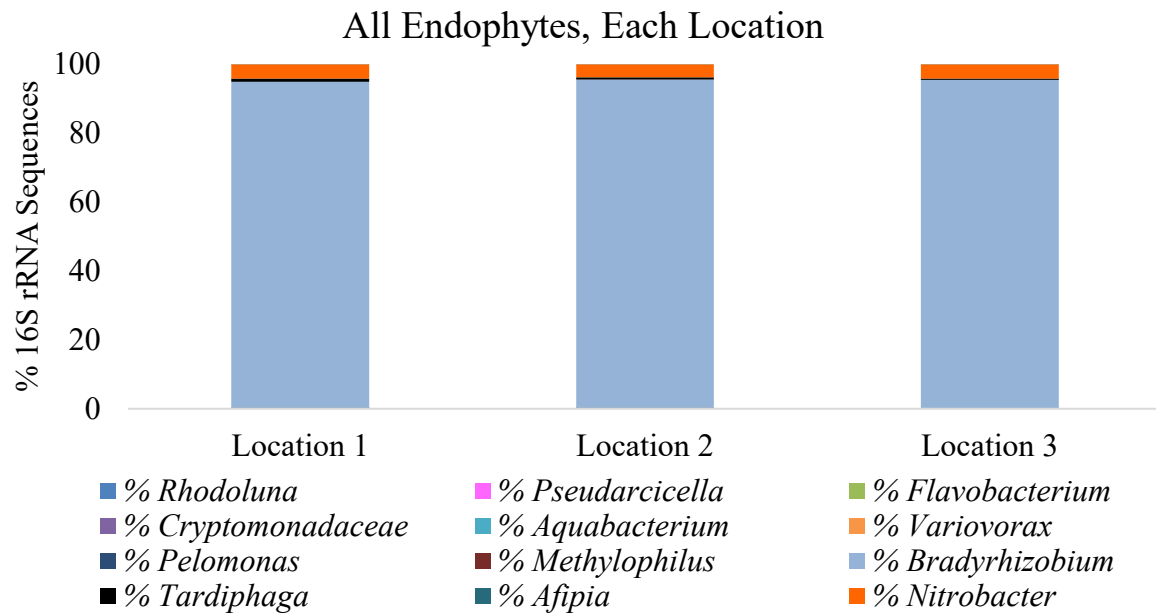


Figure 9. All nodule endophytes averaged between each sampled location.

Classification of Rhizospheric Communities. The DNA sequences retrieved from the rhizosphere soil (276,388) were classified into >300 bacterial genera. The dominant bacterial genera that were detected related to *Sporosarcina*, *Pseudomonas*, *Bradyrhizobium*, *Pedobacter*, *Planococcaceae*, *Flavobacterium*, *Sphingobacterium*, *Rhodococcus*, *Paenibacillus*, etc.

Rhizobia sequences were also detected within rhizosphere soil. Within rhizobia, DNA sequences related to the genus *Bradyrhizobium* were approximately 8% of total sequences (Table 5). Other rhizobial genera such as *Rhizobium*, *Mesorhizobium*, *Neorhizobium* and *Sinorhizobium* (*Ensifer*) were also detected at low abundance in the rhizosphere, corresponding to their low abundance within root nodules. The overall abundance of rhizobial sequences except *Bradyrhizobium* were extremely low (<2% of total soil sequences).

Table 4. Occupancy rate of nodules for each major detected genus.

Genus	Percent of Nodules Detected	Number of Seqs
<i>Bradyrhizobium</i>	100%	3,540,886
<i>Nitrobacter</i>	98%	104,527
<i>Tardiphaga</i>	96%	20,992
<i>Pseudarcicella</i>	49%	225
<i>Afipia</i>	29%	94
<i>Novosphingobium</i>	28%	105
<i>Dongia</i>	26%	98
<i>Rhodoluna</i>	20%	48
<i>Cryptomonadaceae</i>	15%	58
<i>Pseudomonas</i>	15%	56
<i>Pelomonas</i>	14%	37
<i>Flavobacterium</i>	13%	31
<i>Methylophilus</i>	12%	34

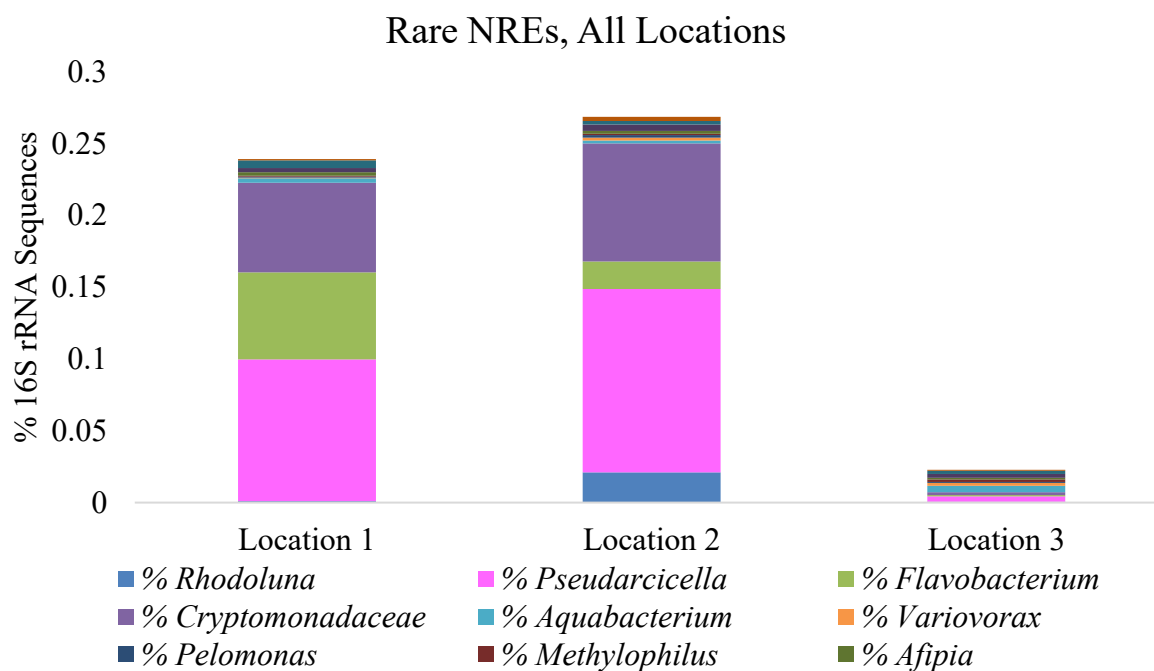


Figure 10. Rare NREs averaged between all sampled locations. *Bradyrhizobium*, *Nitrobacter*, and *Tardiphaga* were removed to aid visualization.

Table 5. Sequence counts of rhizobial genera between nodule and rhizosphere samples.

Genus	Sequences within Nodule	Sequences within Rhizosphere
<i>Bradyrhizobium</i>	3,540,868 (96.5%)	21,747 (7.9%)
<i>Rhizobium</i>	84 (<0.01%)	2,130 (0.77%)
<i>Mesorhizobium</i>	39 (<0.01%)	1,367 (0.49%)
<i>Neorhizobium</i>	29 (<0.01)	553 (0.2%)
<i>Sinorhizobium/Ensifer</i>	2 (<0.01%)	70 (<0.01%)

The dominant nodule bacteria, *Bradyrhizobium*, *Nitrobacter*, and *Tardiphaga* were also detected within the rhizosphere, however, their relative abundance was low as compared to their abundance within root nodules (Table 3). *Nitrobacter* sequences were also detected at low abundance (0.4%) in the soil as compared to their abundance within root nodules (2.9%) (Table 3). Similarly, *Tardiphaga* related sequences were less abundant within the soil (only 49 sequences, <0.001% of all soil sequences (Table 3). Conversely, the rare, inconsistently present NREs detected within the nodule were more abundant within the rhizosphere as compared to their abundance within root nodules (Table 6). For example, *Pseudomonas* was detected at <0.01% abundance within the nodule, however within the rhizosphere, *Pseudomonas* dominated in abundance (11.8%). *Pedobacter*, with 6 detected nodule sequences, was present in the rhizospheres at almost 8%.

Phylogenetic Analysis

All rhizobial sequences from nodule and rhizosphere (related to *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Neorhizobium*, and *Ensifer*) were extracted and analyzed

Table 6. Abundant nodule and rhizospheric genera comparative sequence numbers between nodule and rhizosphere samples.

Genus	Sequences within Nodule	Sequences within Rhizosphere
<i>Sporosarcina</i>	8 (<0.01%)	69,778 (25.3%)
<i>Pseudomonas</i>	56 (<0.01%)	32,614 (11.8%)
<i>Pedobacter</i>	7 (<0.01%)	21,654 (7.8%)
<i>Achromobacter</i>	2 (<0.01%)	15,487 (5.6%)
<i>Bradyrhizobium</i>	3,540,886 (96.5%)	21,747 (7.9%)
<i>Planococcaceae</i>	4 (<0.01%)	10,114 (3.7%)
<i>Flavobacterium</i>	33 (<0.01%)	9,815 (3.5%)
<i>Sphingobacterium</i>	8 (<0.01%)	8,121 (2.9%)
<i>Rhodococcus</i>	1 (<0.01%)	7,820 (2.8%)
<i>Paenibacillus</i>	1 (<0.01%)	6,554 (2.4%)
<i>Stenotrophomonas</i>	21 (<0.01%)	5,616 (2.0%)
<i>Variovorax</i>	90 (<0.01%)	2,835 (1.0%)
<i>Nitrobacter</i>	104,527 (2.9%)	1,101 (0.4%)
<i>Bacillus</i>	6 (<0.01%)	467 (0.1%)
<i>Novosphingobium</i>	189 (<0.01%)	285 (0.1%)
<i>Methylophilus</i>	105 (<0.01%)	140 (<0.01%)
<i>Tardiphaga</i>	20,992 (0.57%)	49 (<0.01%)

phylogenetically at 99% similarity. Four major groups were generated corresponding to *Bradyrhizobium*, *Neorhizobium*, *Rhizobium*, and *Mesorhizobium* (Figure 11). Greater than 99% of all rhizobial nodule sequences fell into the *Bradyrhizobium* group. Within the rhizosphere, *Bradyrhizobium* also dominated, but at much lower abundance (50-75% rhizobial sequences).

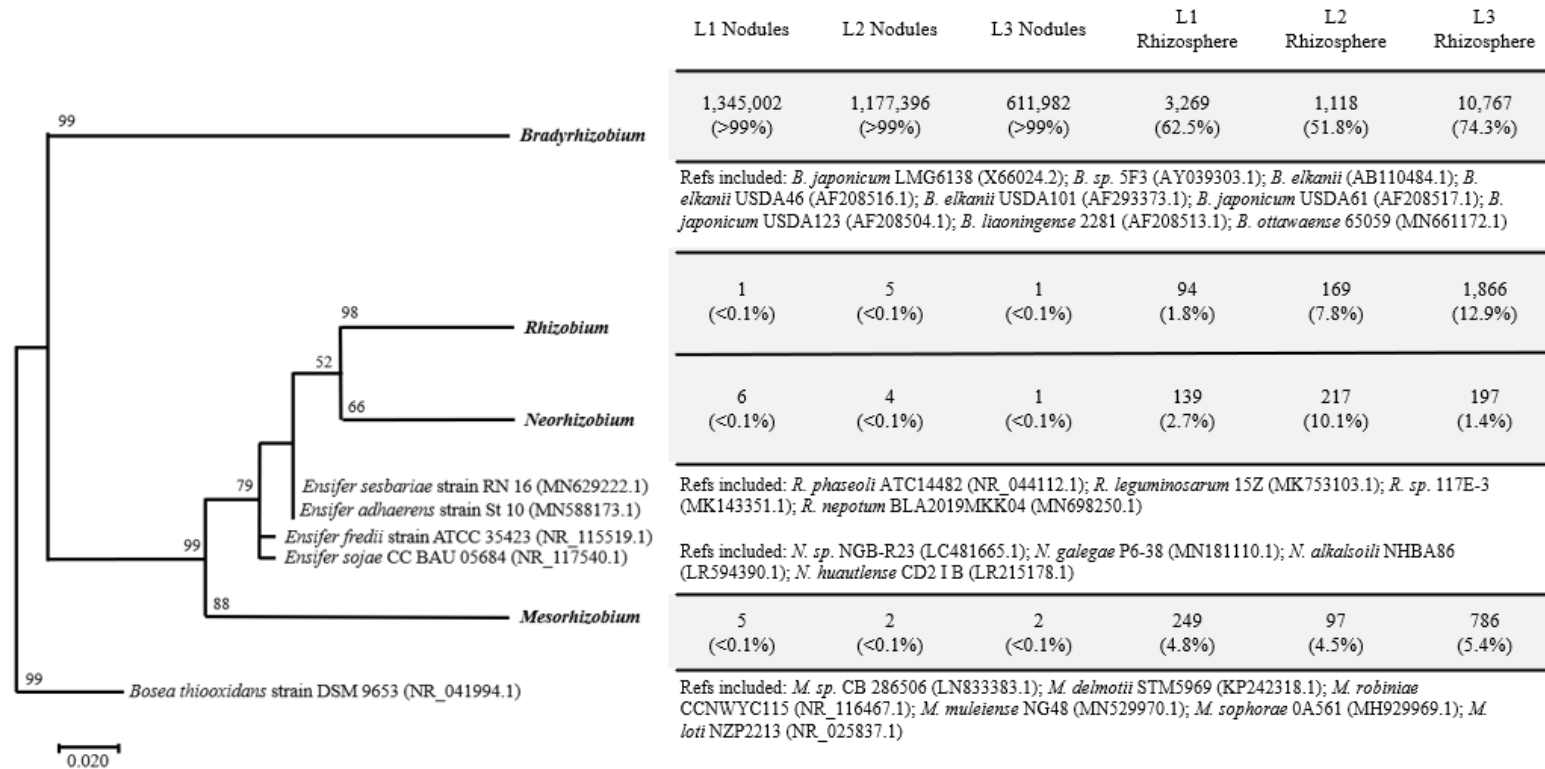


Figure 11. Maximum likelihood phylogenetic tree using partial 16S rRNA sequences. Bootstrap values >50 shown. Abundance counts for nodule and rhizosphere samples associated with detected rhizobial genera *Bradyrhizobium*, *Rhizobium*, *Neorhizobium*, and *Mesorhizobium* are to the right. Associated reference sequences are below each associated genus.

Genera *Neorhizobium*, *Rhizobium*, and *Mesorhizobium* were lowly abundant within the nodule (<0.1%), but more abundant within the rhizosphere. Sequences corresponding to *Sinorhizobium/Ensifer* were of too low abundance to be included for further analysis.

Bradyrhizobium-classified and unclassified *Rhizobiales* sequences were extracted and phylogenetically analyzed at 99% similarity. *Bradyrhizobium* sequences fell into 16 major OTUs, however only OTUs with >100 sequences are shown (Table 7). The most abundant OTU accounted for approximately 99% of all *Bradyrhizobium* sequences (4,049,669; Table 7) was detected within root nodules and soil at the all three locations. The most abundant OTU was closely related to *Bradyrhizobium japonicum* sequences from GenBank. The relative abundance of other OTUs was low and the number of DNA sequences related to other species ranged 15 to 3,414 sequences across all samples. These rare OTUs were closely related to other *Bradyrhizobium* spp. that have yet to be cultured and described (Table 7).

Table 7. OTU-based abundance table of the top 5 OTUs clustered from nodule-associated *Bradyrhizobium* sequences.

OTU	Sequences within Nodule	Related Reference
OTU1	4,049,669 (>99%)	<i>B. japonicum</i> USDA122 (AF208503.1)
OTU2	3,414 (<1%)	Uncultured <i>Bradyrhizobium</i> spp.
OTU3	1,061 (<1%)	Uncultured <i>Bradyrhizobium</i> spp.
OTU4	276 (<1%)	Uncultured <i>Bradyrhizobium</i> spp.
OTU5	131 (<1%)	Uncultured <i>Bradyrhizobium</i> spp.

Because of time constraints, the analysis of *Nitrobacter* sequences could not be completed. Further work is required to elucidate whether one major phylotype is present or many. The role of location and plant should also be investigated if more than one phylotype is present. *Tardiphaga* sequences from the nodule formed four major groups, containing a few

OTUs in each group (Figure 12). All groups are represented similarly at each location. The dominant phylotype is Group 2 as it includes 61% of all *Tardiphaga* sequences. Group 3 includes 21% of *Tardiphaga* sequences and is closely related to group 2. Both groups appear to be a relative of *Tardiphaga robiniae*. Group 1, which is more closely related to uncultured bacteria, only includes 18% of *Tardiphaga* sequences. Group 4 was the least abundant, representing 0.5% of related sequences.

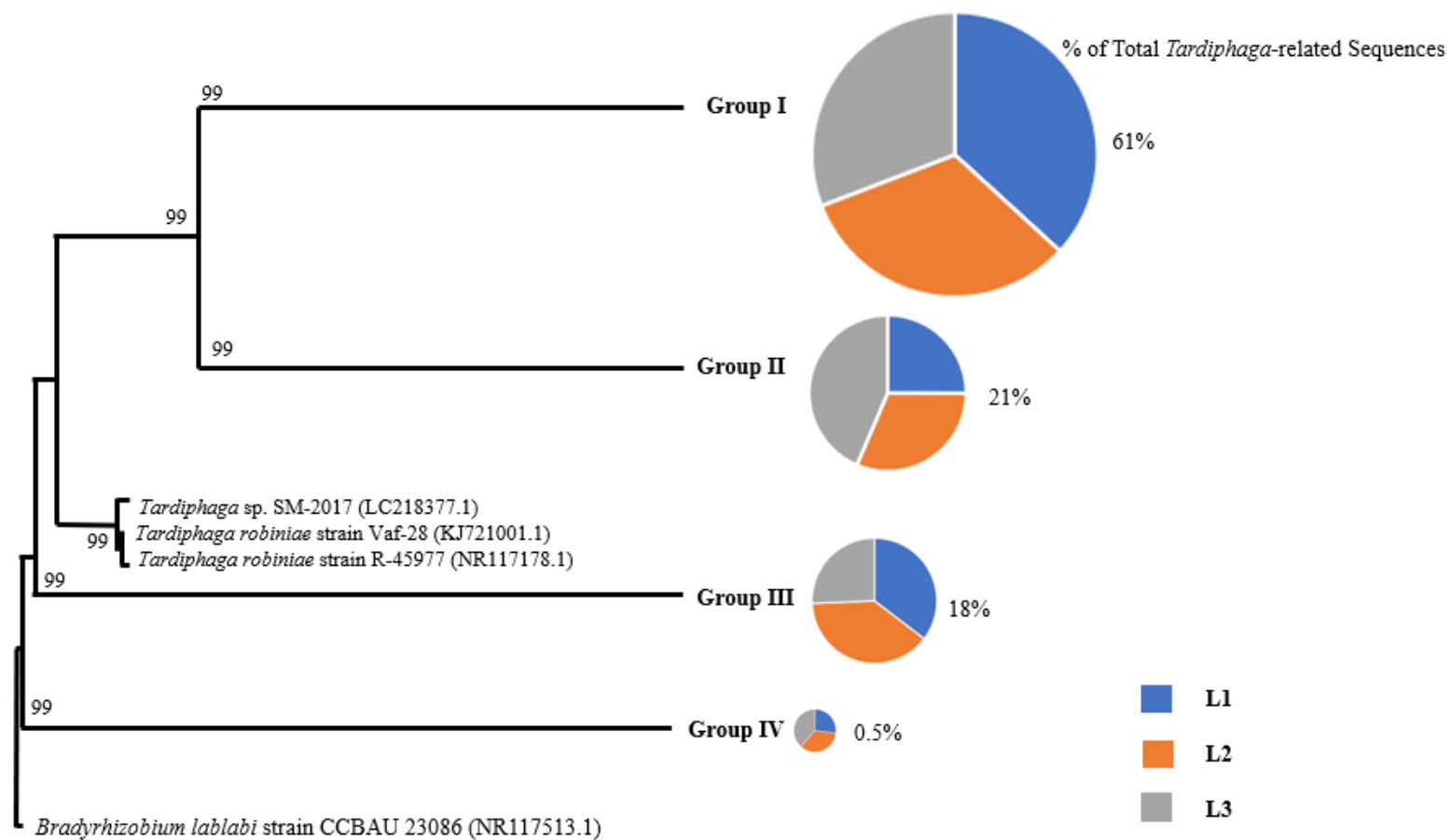


Figure 12. Maximum likelihood phylogenetic tree based on partial sequences of the 16S rRNA gene. Bootstrap values >50 shown. Groups I-IV represent the four major phylotypes of *Tardiphaga* sequences from the nodule.

DISCUSSION

Rhizobial Endophytes

Selection of Genus *Bradyrhizobium*. The dominant nitrogen-fixing rhizobial endophyte was *Bradyrhizobium*, as expected in our hypotheses. *Bradyrhizobium* spp. are thought to be the major microsymbiont within soybean root nodules (17), but other rhizobial genera such as *Ensifer* (19, 20), *Mesorhizobium* (21) and *Rhizobium* (22) have been reported to nodulate the host plant soybean (37). These alternate rhizobia were present in the rhizosphere samples and some sequences were also detected within nodules, but at considerably lower abundances. This suggests a very strong selection by the soybean host plant towards genus *Bradyrhizobium*.

A recent study displayed *B. japonicum* dominance at higher pH, although *E. fredii* still formed minority of nodules (20). However, it has been more commonly reported that soybean plants prefer *Bradyrhizobium* spp. as their microsymbiont in acidic or neutral soil conditions (33, 73), whereas *Ensifer* spp. are preferred in alkaline soils (18). A similar trend was reported in another host plant (*Chamaecrista pumila*), where only *Bradyrhizobium* was observed in nodules from acidic soils (74). Similarly, Temprano-Vera et al. (2018) also reported the dominance of *Bradyrhizobium* within nodules under acidic soil conditions (75). This phenomenon could explain why *Ensifer* sequences were less abundant within both the root nodules and rhizosphere samples in this study, as our soils were all slightly acidic.

Selection of *Bradyrhizobium japonicum*. I also determined whether it is a single species within genus *Bradyrhizobium* that dominates within all root nodules or several species were selected within different root nodules. Most of *Bradyrhizobium* sequences (>99%) closely related to *B. japonicum* spp., which suggests preferred selection operating within each individual

sampled nodule. Previously, Minami et al., (2010) suggested that soybean host plant's genotypes influence the selection of *Bradyrhizobium* within root nodules, potentially explaining the dominance of this species, as all our sample plants were of same genotype (76). I observed the presence of other *Bradyrhizobium* species that are capable to nodulate soybean but their relative abundance was very low. This suggests the small-scale heterogeneity in soil characteristics such as differences in soil pH, available nutrients, or the rhizosphere microbial community, did not influence the selection of rhizobial endophytes within soybean root nodules. The high abundance of *B. japonicum* with all root nodules suggests their preferred selection that could be due to various factors such as their high abundance in the rhizosphere, better ability to compete with other rhizobia, and/or preferred selection by the host plant genotype. Another possibility is that other species of *Bradyrhizobium* present in the rhizosphere were able to enter the nodule but were outcompeted once inside.

Dominant Non-Rhizobial Endophytes, *Nitrobacter* and *Tardiphaga*

Nitrobacter was the second most dominant genus within all nodules at all locations and the most dominant non-rhizobial genus. *Nitrobacter* has recently been detected as an endophyte of both soybean and alfalfa nodules, although at low abundance via combined nodule sequencing studies (77, 78). This genus belongs to the family *Bradyrhizobiaceae* and is closely related to the microsymbiont *Bradyrhizobium* based on 16S rRNA and housekeeping genes (63, 79). The consistent presence of *Nitrobacter* within the sampled soybean root nodules poses a few possible implications. First, the presence of *Nitrobacter* within the nodule may be due to selection by the host plant, potentially due to a role in host plant growth promotion. Previously, *Nitrobacter* demonstrated phosphate solubilization abilities and subsequently yielded additional growth when

the isolate was applied to its host plant, tomato (80). However, its low abundance within the rhizosphere samples (0.4%) suggests this genus was not attracted to the sample plant's roots through signaling molecules or plant exudates. The rare isolation of *Nitrobacter* as an endophyte in isolation-based studies could be due to its slow growth as a chemoorganotroph or the lack of NO_2^- in the growth medium that is required for its quick growth as a chemolithotroph.

Genome sequencing suggests a high genetic similarity between *Nitrobacter* and *Bradyrhizobium*, reporting 41% of genes (1300) in *Nitrobacter* strains are identical to those in *Bradyrhizobium* (79). The second possibility is that these shared genes may help *Nitrobacter* to overcome the host plant defense and reside within the nodule as a chemoorganotroph (79). Thirdly, the presence of *Nitrobacter* could be due to their potential role in nitrification, as *Nitrobacter* is known for converting nitrite to nitrate, the second and final step in the process of converting ammonia to nitrate. Conversely, one would expect *Nitrosomonas* or another capable bacterium to be present within the root nodules to complete the first step. However, recent studies have shown that genus *Nitrospira* can perform one-step nitrification by acquiring five times more energy than from converting nitrite to nitrate alone (81). It is possible that the anaerobic, enclosed root nodule environment provides the appropriate growth conditions for *Nitrobacter* to convert ammonia into nitrate and acquire significantly more energy (-349 kJ mol^{-1}) than just converting nitrite to nitrate in the soil (-74 kJ mol^{-1}) (81). Future research will be focused on culturing *Nitrobacter* strains from soybean root nodules and subsequent testing their influence on the plant growth.

Tardiphaga was the second most dominant non-rhizobial genus detected within most root nodules (97% of nodules). Their average relative abundance was approximately 0.6% of all bacterial sequences retrieved from root nodules. *Tardiphaga* sequences were phylogenetically

analyzed and placed within four major phylotypes. Overall, very few reference sequences related to *Tardiphaga* are available within GenBank. In fact, only one major species (*T. robiniae*) has been described so far for this genus. Most *Tardiphaga* sequences (61%) were closely related species *T. robiniae*. The relative distribution of *Tardiphaga* sequences was consistent at all three locations. This suggests that small-scale heterogeneity did not influence the entry and/or selection of *Tardiphaga*. This genus was established following its isolation from nodules of *Robinia pseudoacacia* (63) and *Vavilovia formosa* (82). Previous studies (78, 83) have detected this bacterial genus as a soybean endophyte, although at very low abundance. Like *Nitrobacter*, *Tardiphaga* isolates have shown slow growth rates (up to 10 days to grow) (82) and most of the culture-based methods incubate samples up to seven days. Hence, most of the culture-based studies have not been able to isolate this genus.

Tardiphaga's presence within nodules could be attributed to the some of the same possibilities as *Nitrobacter*. This genus could be selected for by the host plant, potentially due to additional benefits provided by this bacterium, however, none have been described previously. It is also possible *Tardiphaga* could be present due to genetic similarities to *Bradyrhizobium* allowing undetected entry into the nodule, as this genus displays high genetic similarity to *Bradyrhizobium* based on 16S rRNA and housekeeping genes *atpD*, *dnaK*, *gyrB*, *recA*, *ropB* (63). Additionally, the *Tardiphaga* genome includes *nodM* and *nodT* genes (82), which are involved in signaling to the host plant during nodule formation (84). However, their low abundance within the rhizosphere suggests they may not be actively attracted to the rhizosphere during the early stages of nodule formation. It is possible that nearby members participate in regulated entry but cannot respond to the initial signals released by the host plant to attract potential symbionts. *Tardiphaga* isolates have also been reported to have a role in N-cycle by

completing dissimilatory nitrate reduction under anaerobic conditions (85). This process works in the opposite direction of the nitrification reaction, but the possibility of a complex nitrogen metabolism within the nodule poses further investigation. *Nitrobacter* and *Tardiphaga*'s exact roles within the nodules remain unknown, but many possibilities exist to provide new avenues for nodule endophyte research.

Other Commonly Reported NREs

Low Abundance and Inconsistency within Nodules. One of the major research questions was to assess the influence of small-scale soil heterogeneity on the preferential selection, diversity, and relative abundance of NREs within different root nodules of a single plant. In the last few years, several studies have identified many of the NREs such as *Pseudomonas*, *Bacillus*, *Enterobacter*, *Paenibacillus*, *Stenotrophomonas*, *Rhodococcus*, *Agrobacterium*, *Achromobacter*, *Sphingobacterium*, etc. within soybean root nodules (See Table 1 for details). These studies have focused on the isolation and characterization of these NREs for several plant growth promoting attributes such as N fixation, phytohormone production, and potential as biocontrol agents (36, 51-54, 56). Most of these studies have also demonstrated the beneficial role of the isolated NREs on plant growth through subsequent co-inoculation-based studies (51-54, 86).

In the current study, I have also detected sequences related to some of these commonly reported NREs within soybean root nodules as well as in the rhizosphere soil. I have detected low abundance of DNA sequences related to these NREs within root nodules (<1% of total sequences) and their presence was also inconsistent within different root nodules of a single plant. For example, *Pseudomonas* related sequences were retrieved from only 15% of the 193

nodule samples and *Variovorax* related sequences within only 6% root nodules. This was true for almost all other commonly reported NRE genera. The overall very low abundance and inconsistent presence of these genera within different root nodules of a single plant suggest that these NREs are not being preferentially selected as endophytes by host plants and most likely have a very limited role in plant growth as endophytes. However, these NREs likely have a role in plant growth as free-living plant growth promoting rhizobacteria, as was observed in co-inoculation-based studies (51-54, 57).

Other NREs More Likely Function as PGPR. In support of this, I have observed a relatively higher abundance of some of the commonly reported NREs in the rhizosphere soil samples. Commonly reported NRE of soybean, *Pseudomonas*, corresponded to 12% of rhizospheric sequences. *Rhodococcus*, also reported as an NRE of soybean, provided one nodule sequence. However, in the rhizosphere, this genus represented almost 3% of sequences. Less reported NRE, *Achromobacter*, which has been reported to nodulate soybean (87), provided 2 nodules sequences but corresponded to almost 6% of rhizospheric sequences. Like *Pseudomonas*, this genus has also been reported to host a number of plant growth benefits such as P solubilization, phytohormone production, and siderophore production (88). Almost all the frequently reported bacterial genera detected within root nodules at low abundance were detected in the rhizosphere soils at higher abundance, further suggesting these commonly detected NREs may operate within the rhizosphere rather than the nodule.

Potential Entry Mechanisms of Other NREs. The presence of these NREs (lowly abundant, inconsistently present) may be attributed to several possibilities. Their attraction to the rhizosphere provides the opportunity for proximity to the host plant roots. Some may enter opportunistically during root nodule formation (61, 89) or via plant injuries. Some may enter less

specific tissue, such as the root (90), or potentially the nodule epidermis, and passively enter the root nodule. Whichever route is taken, it appears their presence is not selected for by the host plant. The frequent reports of *Pseudomonas*, *Bacillus*, and other NREs in soybean root nodule isolation studies may be due to culture bias. Other, more abundant, NREs observed in this study such as *Nitrobacter* and *Tardiphaga* are not commonly isolated from nodules, suggesting the methods currently employed are not getting the full scope of the nodule community. The media used for isolation may only culture a small subset of nodule bacteria, as observed for agricultural soils (91).

Comparative Abundance Values from Other Sources. Sequencing studies that determine relative abundance in root nodule microbiomes have reported different proportions of NREs in nodules. In salt-stressed soybean, NREs represented 14% of total returned sequences, higher than the 3.5% of returned classified sequences in this study (55). In a study in China, NREs were reported as 2.9% of returned sequences, much closer to our value (92). While reported at low abundances in some studies, others have reported up to 35% NRE sequences (66). It is possible that the soil conditions from these studies mitigated the proportion of NRE sequences. Some discrepancies in reports may be attributed to nodule sampling practices, as most studies combine many root nodules for one sequencing sample. No study has reported removing the outer surface of the root nodule, which may contain endophytes that can invade less specific tissue such as this layer, potentially mis-representing nodule community dynamics. In the future, methods surrounding nodule sampling should take further steps to prevent misrepresenting the nodule microbiome, in both isolation and sequencing studies.

CONCLUSIONS

I have assessed the microbiome of 193 individual root nodules from nine plants and associated rhizosphere soils. *Bradyrhizobium japonicum* was the most dominant nitrogen-fixing genus within all root nodules. *Nitrobacter* and *Tardiphaga* sequences were consistently detected within all root nodules. Their presence can be due to selection by the host plant because of their plant growth promoting benefits or their genetic similarity with *Bradyrhizobium*. Low abundance and inconsistent presence of other NREs suggest they may be entering accidentally and may not play a direct role in plant growth as endophytes.

While more was elucidated about the selection of *Bradyrhizobium* in soybean grown in acidic soils, many questions are left unanswered about NREs. It is still unknown if NREs operate under selection, but these results suggest they may. If selected for, what is the mechanism for regulated entry? What plant growth promoting benefits may be provided by *Nitrobacter* and *Tardiphaga*? If frequently reported nodule endophyte *Pseudomonas* is aiding plant growth as an NRE, why is it so scarce within nodules? Additional research is required to answer these important questions. Future answers may concentrate efforts for the future of sustainable agriculture.

A successor in this study will investigate the different phylotypes of *Nitrobacter* sequences detected within the nodule to further elucidate whether one species or many was present. Future research is required to isolate the detected species of *Bradyrhizobium*, *Nitrobacter*, and *Tardiphaga* from Kindrick Farm for plant growth promotion assays and co-inoculation studies.

LITERATURE CITED

1. Grau R, Gasparri NI, Aide TM. 2005. Globalization and Soybean Expansion into Semiarid Ecosystems of Argentina. *Ambio* 34(3):265-266.
2. Herridge DF, Peoples MB, Boddey RM. 2009. Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil* 311:1-18.
3. Barnes S. 2010. The Biochemistry, Chemistry, and Physiology of the Isoflavones in Soybeans and their Food Products. *Lymphat Res Biol* 8(1):89-98.
4. Reed SC, Cleveland CC, Townsend AR. 2011. Functional Ecology of Free-Living Nitrogen Fixation: A Contemporary Perspective. *Annu Rev of Ecol Evol and Syst* 42:489-512.
5. Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S. 2002. Agricultural sustainability and intensive production practices. *Nature* 418(6898):671-7.
6. Savci S. 2012. An Agricultural Pollutant: Chemical Fertilizers. *IJESD* 3(1):77-80.
7. Peoples MB, Herridge DF, Ladha JK. 1995. Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? *Plant Soil* 174:3-28.
8. Smith VH, Tilman GD, Nekola JC. 1999. Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environ Pollut* 100:179-196.
9. Stuart D, Schei RL, McDermott M. 2014. Reducing nitrogen fertilizer application as a climate change mitigation strategy: Understanding farmer decision-making and potential barriers to change in the US. *Land Use Policy* 36:210-8.
10. Razon LF. 2014. Life cycle analysis of an alternative to the Haber-Bosch process: Non-renewable energy usage and global warming potential of liquid ammonia from cyanobacteria. *Environ Prog Sustain Ener* 33(2):618-24.
11. Jiao F and Xu B. 2019. Electrochemical Ammonia Synthesis and Ammonia Fuel Cells. *Adv Mater* 31:1805173.
12. Martín AJ, Shinagawa T, Pérez-Ramírez J. 2019. Electrocatalytic reduction of nitrogen: From Haber-Bosch to ammonia artificial leaf. *Chem* 5(2):263-83.
13. Wang L, Xia M, Wang H, Huang K, Qian C, Maravelias CT, Ozin GA. 2018. Greening ammonia toward the solar ammonia refinery. *Joule* 2(6):1055-74.
14. Skiba U, Rees B. Nitrous oxide, climate change and agriculture. 2014. *CAB Rev* 9(010):1-7.

15. Burgess BK, LoI DJ. Mechanism of molybdenum nitrogenase. 1996. Chem Rev 96(7):2983-3012.
16. diCenzo GC, Zamani M, Ceccucci C, Fondi M, Griffiths JS, Finan TM, Mengoni A. 2019. Multidisciplinary approaches for studying rhizobium-legume symbioses. Can J Microbiol 65:1-33.
17. Masciarelli O, Llanes A, Luna V. 2014. A new PGPR co-inoculated with *Bradyrhizobium japonicum* enhances soybean nodulation. Microbiol Res 196:605-615.
18. Saeki Y, Kaneko A, Hara T, Suzuki K, Yamakawa T, Nguyen MT, Nagatomo Y, Akao S. 2005. Phylogenetic Analysis of Soybean-Nodulating Rhizobia Isolated from Alkaline Soils in Vietnam. J Soil Sci Plant Nutr 51(7):1043-1052.
19. Yan J, Han XZ, Ji ZH, Li Y, Wang ET, Xie ZH, Chen WF. 2014. Abundance and Diversity of Soybean-Nodulating Rhizobia in Black Soil Are Impacted by Land Use and Crop Management. Appl Environ Microbiol 80(17):5394-5402.
20. Pastorino GN, Alcantara VM, Malbran I, Videira L, Sarinelli J, Balatti PA. 2015. *Ensifer (Sinorhizobium) fredii* Interacted More Efficiently than *Bradyrhizobium japonicum* with Soybean. J Agric Ecol Res Int 2(1):10-19.
21. Adhikari D, Kaneto M, Itoh K, Suyama K, Pokharel BB, Gaihre YK. 2011. Genetic diversity of soybean-nodulating rhizobia in Nepal in relation to climate and soil properties. Plant Soil 357(1-2):131-145.
22. Kapembwa R, Mweetwa AM, Ngulube M, Yengi J. 2016. Morphological and Biochemical Characterization of Soybean Nodulating Rhizobia Indigenous to Zambia. Sustain Agric Res 5(3):84-92.
23. Dudeja SS, Giri R. 2014. Beneficial properties, colonization, establishment and molecular diversity of endophytic bacteria in legume and non-legume. Afr J Microbiol Res 8(15):1562-72.
24. Frayse N, Couderc F, Poinot V. 2003. Surface polysaccharide involvement in establishing the rhizobium–legume symbiosis. Eur J Biochem 270(7):1365-80.
25. Lira Jr MA. 2015. Legume-rhizobia signal exchange: promiscuity and environmental effects. Front Microbiol 6:945.
26. Han LL, Wang ET, Han TX, Liu J, Sui XH, Chen WF, Chen WX. 2009. Unique community structure and biogeography of soybean rhizobia in the saline-alkaline soils of Xinjiang, China. Plant Soil 324:291-305.
27. Mullen MD, Israel DW, Wollum AG. 1988. Effects of *Bradyrhizobium japonicum* and soybean (*Glycine max* (L.) Merr.) phosphorus nutrition on nodulation and dinitrogen fixation. Appl. Environ. Microbiol. 54(10):2387-92.

28. Koppell JH, Parker MA. 2012. Phylogenetic clustering of *Bradyrhizobium* symbionts on legumes indigenous to North America. *Microbiology* 158(8):2050-9.
29. Appunu C, Sasirekha N, Prabavathy VR, Nair S. 2009. A significant proportion of indigenous rhizobia from India associated with soybean (*Glycine max* L.) distinctly belong to *Bradyrhizobium* and *Ensifer* genera. *Biol Fert Soils* 46(1):57.
30. Youseif SH, El-Megeed FH, Khalifa MA, Saleh SA. 2014. Symbiotic effectiveness of *Rhizobium* (*Agrobacterium*) compared to *Ensifer* (*Sinorhizobium*) and *Bradyrhizobium* genera for soybean inoculation under field conditions. *Res J Microbiol* 9:151-62.
31. Jiménez-Guerrero I, Pérez-Montaña F, Monreal JA, Preston GM, Fones H, Vioque B, Ollero FJ, López-Baena FJ. 2015. The *Sinorhizobium* (*Ensifer*) *fredii* HH103 type 3 secretion system suppresses early defense responses to effectively nodulate soybean. *Mol Plant Microbe Interact* 28(7):790-9.
32. Brechenmacher L, Kim MY, Benitez M, Li M, Joshi T, Calla B, Lee MP, Libault M, Vodkin LO, Xu D, Lee SH. 2008. Transcription profiling of soybean nodulation by *Bradyrhizobium japonicum*. *Mol Plant Microbe Interact* 21(5):631-45.
33. Zhang YM, Li Jr. Y, Chen WF, Wang ET, Tian CF, Li QQ, Zhang YZ, Sui XH, Chen WX. 2011a. Biodiversity and Biogeography of Rhizobia Associated with Soybean Plants Grown in the North China Plain. *Appl Environ Microbiol* 77(18):6331-6342.
34. Pueppke, S.G. 2005. Nitrogen fixation by soybean in North America. 2005. In: D. Werner and W.E. Newton, editors, Nitrogen fixation in agriculture, forestry, ecology, and the environment. Vol. 4. Springer, Dordrecht, the Netherlands. p. 15–23.
35. Leggett M, Diaz-Zorita M, Koivunen M, Bowman R, Pesek R, Stevenson C, Leister T. 2017. Soybean Response to Inoculation with *Bradyrhizobium japonicum* in the United States and Argentina. *Soil Fert Crop Nutr* 109:3.
36. Li QQ, Wang ET, Zhang YZ, Zhang YM, Tian CF, Sui XH, Chen WF, Chen WX. 2011. Diversity and Biogeography of Rhizobia Isolated from Root Nodules of *Glycine max* Grown in Hebei Province, China. *Microb Ecol* 61:917-931.
37. Shiro S, Matsuura S, Saiki R, Sigua GC, Yamamoto A, Umehara Y, Hayashi M, Saeki Y. 2013. Genetic Diversity and Geographic Distribution of Indigenous Soybean-Nodulating *Bradyrhizobia* in the United States. *Appl Environ Microbiol* 79(12):3610-3618.
38. Ishizuka J, Suemasu Y, Mizogami K. 1991. Preference of Rj-soybean cultivars for *Bradyrhizobium japonicum* for nodulation. *Soil Sci Plant Nutr* 37(1):15-21.
39. Becker JM, Parkin T, Nakatsu CH, Wilbur JD, Konopka A. 2006. Bacterial activity, community structure, and centimeter-scale spatial heterogeneity in contaminated soil. *Microb Ecol* 51(2):220-31.

40. Schoenborn L, Yates PS, Grinton BE, Hugenholtz P, Janssen PH. 2004. Liquid serial dilution is inferior to solid media for isolation of cultures representative of the phylum-level diversity of soil bacteria. *Appl Environ Microbiol.* 70(7):4363-4366.
41. Glick BR. 2012. *Plant Growth-Promoting Bacteria: Mechanisms and Applications.* Scientifica 2012:963401.
42. Olanrewaju OS, Glick BR, Babalola OO. 2017. Mechanisms of action of plant growth promoting bacteria. *World J Microb Biot* 33(11):197.
43. Saharan BS, Nehra V. 2011. Plant growth promoting rhizobacteria: a critical review. *Life Sciences and Medicine Research* 21(1):30.
44. Rodriguez H, Fraga R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances* 17(4-5):319-39.
45. Bhattacharyya PN, Jha DK. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. 2012. *World J Microb Biot* 28(4):1327-50.
46. Fukuhara H, Minakawa Y, Akao S, Minamisawa K. 1994. The involvement of indole-3-acetic acid produced by *Bradyrhizobium elkanii* in nodule formation. *Plant Cell Physiol* 35(8):1261-1265.
47. Grobkinsky DK, van der Graaff E, Roitsch T. 2016. Regulation of abiotic and biotic stress responses by plant hormones. *Plant Path Resist Biotech* 131-154.
48. Etesami H. 2019. Plant Growth Promotion and Suppression of Fungal Pathogens in Rice (*Oryza Sativa* L.) by Plant Growth-Promoting Bacteria. In *Field Crops: Sustainable Management by PGPR* p. 351-383. Springer, Cham.
49. Negi YK, Prabha D, Garg SK, Kumar J. 2017. Biological control of ragi blast disease by chitinase producing fluorescent *Pseudomonas* isolates. *Org Agri* 7(1):63-71.
50. Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Samiyappan R. 2011. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. *Crop Protect* 20(1):1-1.
51. Bai Y, D'Aoust F, Smith DL, Driscoll BT. 2002. Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. *Can J Microbiol* 48:230-238.
52. Zhao L, Xu Y, Lai X. 2018. Antagonistic endophytic bacteria associated with nodules of soybean (*Glycine max* L.) and plant growth-promoting properties. *Braz J Microbiol* 49(2):269-78.
53. Tokgoz S. 2018. *Exploration of Soybean Nodule Microbiome for Plant Health Management [Thesis].* [UNL, USA]: University of Nebraska, Lincoln.

54. Senthilkumar M, Swarnalakshmi K, Govindasamy V, Lee YK, Annapurna K. 2008. Biocontrol potential of soybean bacteria against charcoal rot fungus, *Rhizoctonia bataticola*. *Curr Opin Microbiol* 58:288-293.
55. Zheng Y, Liang J, Zhao D-L, Meng C, Xu Z-C, Xie Z-H, Zhang C-S. 2020. The Root Nodule Microbiome of Cultivated and Wild Halophytic Legumes Showed Similar Diversity but Distinct Community Structure in Yellow River Delta Saline Soils. *Microorganisms* 8:207.
56. Aserse AA, Rasanen LA, Aseffa F, Hailemariam A, Lindström K. 2013. Diversity of sporadic symbionts and nonsymbiotic endophytic bacteria isolated from nodules of woody, shrub, and food legumes in Ethiopia. *Appl Microbiol Biotech* 97(23):10117-34.
57. Vargas-Díaz AA, Ferrera-Cerrato R, Silva-Rojas HV, Alarcón A. 2019. Isolation and evaluation of endophytic bacteria from root nodules of *Glycine max* L.(Merr.) and their potential use as biofertilizers. *Span J Agric Res* 17(3):1103.
58. Peng S, Hong DD, Xin YB, Jun LM, Hong WG. 2014. *Sphingobacterium yanglingense* spp.nov., isolated from the nodule surface of soybean. *IJSEM* 64:3862-3866.
59. De Meyer SE, De Beuf K, Vekeman B, Willems A. 2015. A large diversity of non-rhizobial endophytes found in legume root nodules in Flanders (Belgium). *Soil Biol Biochem* 83:1-11.
60. Martinez-Hidalgo P. 2017. The Nodule Microbiome: N₂ -Fixing Rhizobia Do Not Live Alone. *Phytobiomes* 1:70-82.
61. Pandya M, Kumar GN, Rajkumar S. 2013. Invasion of rhizobial infection thread by non rhizobia for colonization of *Vigna radiata* root nodules. *FEMS Microbiol Lett* 348:58–65.
62. Leite J, Fischer D, Rouws LFM, Fernandes-Junior PI, Hofmann A, Kublik S, Schlöter M, Xavier GR, Radi V. 2017. Cowpea Nodules Harbor Non-rhizobial Bacterial Communities that Are Shaped by Soil Type Rather than Plant Genotype. *Front Plant Sci* 7:2064.
63. De Meyer SE, Coorevits A, Willems A. 2012. *Tardiphaga robiniae* gen. nov., spp.nov., a new genus in the family *Bradyrhizobiaceae* isolated from *Robinia psuedoacacia* in Flanders (Belgium). *Syst Appl Microbiol* 35(4):205-214.
64. Stajkovic O, De Meyer S, Milicic B, Willems A. 2009. Isolation and characterization of endophytic non-rhizobial bacteria from root nodules of alfalfa (*Medicago sativa* L.). *Bot Serb* 33(1):107-14.
65. Tabacchioni S, Chiarini L, Bevivino A, Cantale C, Dalmastri C. 2000. Bias caused by using different isolation media for assessing the genetic diversity of a natural microbial population. *Microb Ecol* 40(3):169-76.

66. Sharaf H, Rodrigues RR, Moon J, Zhang B, Mills K, Williams MA. 2019. Unprecedented bacterial community richness in soybean nodules vary with cultivar and water status. *Microbiome* 7:63.
67. Xiao X, Chen W, Zong L, Yang J, Jiao S, Lin Y, Wang E, Wei G. 2017a. Two cultivated legume plants reveal the enrichment process of the microbiome in the rhizocompartments. *Mol Ecol* 26(6):1641-51.
68. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl Environ Microbiol* 75(23):7537-7541.
69. Mirza BS, Muruganandam S, Meng X, Sorensen DL, Dupont RR, McLean JE. 2014. Arsenic (V) Reduction in Relation to Iron (III) Transformation and Molecular Characterization of the Structural and Functional Microbial Community in Sediments of a Basin-Fill Aquifer in Northern Utah. *Appl Environ Microbiol* 80(10): 3198-3208.
70. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naïve Bayesian Classifier for the Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Appl Environ Microbiol* 73(16):5231-5237.
71. Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Grüning B, Guerler G, Hillman-Jackson J, Jalili V, Rasche H, Soranzo N, Goecks J, Taylor J, Nekrutenko A, Blankenberg D. 2018. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update, *Nucleic Acids Res* 46(1):537-544.
72. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 35(6):1547-9.
73. Qin Q, Wang ET, Zhang YZ, Zhang YM, Tian CF, Zui XH, Chen WF, Chen WX. 2011. Diversity and Biogeography of Rhizobia Isolated from Root Nodules of *Glycine max* Grown in Hebei Province, China. *Soil Microbiol* 61:917-931.
74. Rathi S, Tak N, Bissa G, Chouhan B, Ojha A, Adhikari D, Barik SK, Satyawada RR, Sprent JI, James EK, Gehlot HS. 2018. Selection of *Bradyrhizobium* or *Ensifer* symbionts by the native Indian caesalpinoid legume *Chamaecrista pumila* depends on soil pH and other edaphic and climatic factors. *FEMS Microbiol Ecol* 94(11):180.
75. Temprano-Vera F, Rodriguez-Navarro DN, Acosta-Jurado S, Perret X, Fossou RK, Navarro-Gomez P, Zhen T, Yu D, An Q, Buendia-Claveria AM, Moreno J, Lopez-Baena FJ, Ruiz-Sainz JE, Vinardell JM. 2018. *Sinorhizobium fredii* Strains HH103 and NGR234 Form Nitrogen Fixing Nodules with Diverse Wild Soybeans (*Glycine soja*) From Central China but are Ineffective on Northern China Accessions. *Front Microbiol* 9:2843.

76. Minami M, Yamakawa T, Yamamoto A, Akao S, Saeki Y. 2010. Estimation of nodulation tendency among Rj-genotype soybeans using the bradyrhizobial community isolated from an Andosol. *Soil Sci Plant Nutr* 55:65-72.
77. Chizhevskaya EP, Naidenova EA, Onishchuk OP, Andronov EE, Simarov BV. 2018. The Melanin Biosynthesis Gene from the CA15-1 Strain of Alfalfa Nodule Bacteria: Molecular Analysis and Phylogeny. *Russ J Genet* 54(8):925-932.
78. Xiao X, Fan M, Wang E, Chen W, Wei G. 2017b. Interactions of plant growth-promoting rhizobacteria and soil factors in two leguminous plants. *Appl Microbiol Biotech* 101(23-24):8485-97.
79. Starkenburg SR. 2007. An Investigation of Carbon and Nitrogen Metabolism through a Genomic Analysis of the Genus *Nitrobacter* [dissertation]. [Corvallis (OR)]: Oregon State University.
80. Ibiene AA, Agogbua JU, Okonko IO, Nwachi GN. 2012. Plant growth promoting rhizobacteria (PGPR) as biofertilizer: Effect on growth of *Lycopersicum esculentus*. *Am J Sci* 8(2):318-324.
81. Daims H, Lebedeva EV, Pjevac P, Han P, Herbold C, Albertsen M, Jehmlich N, Palatinszky M, Vierheilig J, Bulaev A, Kirkegaard RH, von Bergen M, Rattei T, Bendinger B, Nielsen PH, Wagner M. 2016. Complete nitrification by *Nitrospira* bacteria. *Nature* 528(7583):504-509.
82. Safronova VI, Kuznetzova IG, Sazanova AL, Kimeklis AK, Belimov AA, Andronov EE, Pinaev AG, Pukhaev AR, Popov KP, Akopian JA, Willems A, Tikhonovich IA. 2015. Extra-slow-growing *Tardiphaga* strains isolated from nodules of *Vavilovia formosa* (Stev.) *Arch Microbiol* 197(7):889-898.
83. Ramoneda J, Le Roux J, Frossard E, Frey B, Gamper HA. 2020. Ecological patterns of root nodule diversity in cultivated and wild rooibos populations: a community prediction approach. *bioRxiv* [preprint].
84. Streng A, Camp R, Bisseling T, Geurts R. 2011. Evolutionary origin of rhizobium Nod factor signaling. *Plant Signal Behav* 6(10):1510-1514.
85. Marcondes de Souza JA, Alves LMC, Varani AM, Lemos EGM. 2014. The Family *Bradyrhizobiaceae*. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, and Thompson F. *The Prokaryotes: Prokaryotic Biology and Symbiotic Associations*. 4th ed. New York (NY): Springer Reference p. 135-154.
86. Dashti, N., Zhang, F., Hynes, R., and Smith, D.L. 1997. Application of plant growth-promoting rhizobacteria to soybean [*Glycine max* (L.) Merrill] increases protein and dry matter yield under short-season conditions. *Plant Soil* 188: 33–41.

87. Wedhastri S, Fardhani DM, Kabirun S, Widada J, Widiyanto D, Evizal R, Prijambada ID. 2013. Legume nodulating bacterium, *Achromobacter xylosoxidans* found in tropical shrub agroecosystem, Sumatera, Indonesia. *Indones J Biotechnol* 18(2):161-7.
88. Egamberdieva D, Wirth SJ, Shurigin VV, Hashem A, Abd Allah EF. 2017. Endophytic bacteria improve plant growth, symbiotic performance of chickpea (*Cicer arietinum* L.) and induce suppression of root rot caused by *Fusarium solani* under salt stress. *Front Microbiol* 8:1887.
89. Ibanez F, Angelini J, Taurian T, Tonelli ML, Fabra A. 2009. Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. *Syst Appl Microbiol* 32:49-55.
90. Zhang YZ, Wang ET, Mao L, Li QQ, Zhang YM, Zhao SJ, Jia XL, Zhang LH, Chen WF, Chen WX. 2011b. Effects of rhizobial inoculation, cropping systems, and growth stages on endophytic bacterial community of soybean roots. *Plant Soil* 347:147-161.
91. Hill GT, Mitkowski NA, Aldrich-Wolfe L, Emele LR, Jurkonie DD, Ficke A, Maldonado-Ramirez S, Lynch ST, Nelson EB. 2000. Methods for assessing the composition and diversity of soil microbial communities. *Appl Soil Ecol* 15(1):25-36.
92. Zhang B, Du N, Li Y, Shi P, Wei G. 2018. Distinct biogeographic patterns of rhizobia and non-rhizobial endophytes associated with soybean nodules across China. *Sci Total Environ* 643:569-78.