EXPLORING PRIMITIVE LEGUME SYMBIOSIS USING *Chamaecrista fasciculata* AS A MODEL

by

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ABSTRACT

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The University of Wisconsin-Milwaukee, 2019
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Legumes form symbiotic associations with diazotrophs collectively termed “rhizobia” leading to the formation of nodules and N_2-fixation and contribute significant amounts of fixed N to agricultural and natural environments. The mechanisms of nodulation and N_2-fixation are extensively studied using model legumes that belong to the Papilionoideae, in which nodulation is widespread and advanced. In contrast, very little is known about the mechanisms of nodulation and N_2-fixation in in basal Caesalpinioideae subfamily in which nodulation is rare. The nodulating caesalpiniod legume *Chamaecrista fasciculata* is widespread in North America and is suggested as a complementary model to study legume nodulation because of its divergence from the papilionoids nearly 60 million years ago. As a first step in understanding nodulation in basal legumes, we have isolated rhizobial strains from soils of Mid-Western USA that form N2-fixing nodules on *C. fasciculata* roots. Molecular phylogenetic analysis based on 16S rDNA was performed on 13 unique isolates and revealed *C. fasciculata* is exclusively nodulated by *Bradyrhizobium spp,* a group of rhizobia considered more ancient than other papilionoid nodulating rhizobial strains. Multi-locus phylogenetic analysis using ITS, *nodA*, *nifH*,

recA, and *dnaK* was also used to further classify these unique isolates from one another. Using a GFP marker introduced in one unique isolate, as well as light microscopy and TEM, we were able to obtain a better understanding of how *Bradyrhizobium spp* is able to colonize and effectively nodulate in *C. fasciculata.* With the basic understanding of what these isolates are and how they compare to one another on a phylogenetic level, we looked at the growth promoting abilities of these isolates to determine nodulation and N_2-fixing abilities of each and how they relate to one another.

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Introduction:

Nitrogen is one of the most essential nutrients in plants, responsible for many vital functions. Without nitrogen present, plants simply cannot function. Although nitrogen is one of the most abundant elements found in our atmosphere, it is found in an unusable form by plants, N_2 gas. This creates a challenge for plants and makes nitrogen one of the limiting nutrients in terms of plant production (Fageria and Baligar, 2005). To get around this, we have found a way to create usable nitrogen sources for plants in the form of fertilizers using the Haber-Bosch process. This solves the issue of getting nitrogen to our plants but causes new issues in pollution and potentials for global warming (Razon, 2013) (Vitousek et al, 1997). The Haber-Bosch process uses extensive amounts of fossil fuels and releases tons of unnecessary greenhouse gases back into the atmosphere, approximately 3100 kg of CO_2 per 1000 kg of liquid ammonia produced (Razon, 2013). And while the outcome of this process is essential to helping produce the large number of crops made around the world each year, the use of fertilizers is still less than ideal. When analyzing the nitrogen recovery in these plants, it has found that these plants retain less than half of the nitrogen that is placed down (Fageria and Baligar, 2005). A vast majority of the nitrogen used from these fertilizers is lost to the environment leading to contamination of ground and surface water sources as well as back to the atmosphere resulting in an increase in air pollution (Fageria and Baligar, 2005). This manipulation of available nitrogen has also had effects on the natural nitrogen cycle as well as approximately doubling the rate nitrogen entering the cycle. Increased nitrogen entering into the nitrogen cycle helps play a role in decreased soil fertility, air quality, and pollution as well as the decrease in biological diversity of

plants, animals, and microorganisms while also contributing to the long-term decline of coastal fisheries (Vitousek et al, 1997). It is therefore imperative to find more sustainable system to supply nitrogen to plants. An alternative to the chemical nitrogen in using biological nitrogen fixation (BNF). BNF is widespread in bacteria and archea and is postulated to fix significant amount of nitrogen in the globe. Of the various forms of BNF, the symbiosis between legumes and rhizobia is one of the most important contributors of fixed nitrogen in agricultural and aquatic systems.

Legumes make up one of the largest flowering plant families, with over 700 genera and just under 20,000 species split between three distinct subfamilies, the *Caesalpinioideae*, the *Mimosoideae*, and the *Papilionoideae* (Singer et al. 2009). Legumes as a whole are one of the most studied plant groups in the world because of their unique ability to form a symbiotic relationship with a specific bacterial group, rhizobia (dos Santos et al. 2016). This symbiotic relationship leads to the formation of nodules on the roots of the plants which allows the bacteria inside the nodules to fix nitrogen into a usable state and supply it not only to the host plant, but also back into the environment. In exchange for fixing nitrogen, the bacteria is supplied with carbon which can be used as an energy source (Desbrosses and Stougaard, 2011) (dos Santos et al. 2016).

Mechanisms of rhizobial-legume interactions

Due to the importance of legumes in agriculture, their symbiosis with rhizobia has been extensively studied. The initiation of this symbiosis requires signal exchanges between the host plant and its microbial partner in which legume roots secrete flavonoids and isoflavonoids that activate the expression of bacterial *nod* genes (Venkateshwaran et al., 2013; Oldroyd, 2013).

The Nod proteins are involved in the production of signal molecules, called Nod factors (NFs), which harbor various substitutions on a lipochitooloigosaccharide (LCO) backbone. The NFs are then recognized by the legume host through specific receptors and trigger plasma membrane ion fluxes, root hair deformations, nuclear calcium spiking and "nodulin" gene expression (Dénarié et al., 1992, 1996; Madsen et al., 2003; Venkateshwaran et al. 2013; Oldroyd, 2013). Interestingly, a similar molecular dialog has been identified during the early steps of the arbuscular mycorrhizal symbiosis (Harrison, 2005; Maillet et al., 2011). The mechanisms of nodulation have been studied extensively in the Papilionoideae family due to the presence of a majority of the common and model legumes (Singer et al. 2009) in this family. In contrast, not much is known about nodulation mechanisms in subfamilies of more primitive legumes, where nodulation is rarer.

***Chamaecrista* as a model for determining rhizobial interactions with primitive legumes.**

To fully understand symbiotic nitrogen fixation, it is important to extend research beyond the subfamily Papilionoideae (Sprent and James, 2007; Singer *et al*., 2009, Sprent *et al*., 2017). The paraphyletic subfamily Caesalpinioideae in the mimosoid clade diverged from the Papilionoideae nearly 60 million years ago (Lavin *et al*., 2005). The mimosoid clade comprises 23% of legumes but has no model established model system. In contrast to Papilionoideae in which nodulation is widespread, nodulation in Caesalpinioideae is rare (LWG2017) but does occur in the genus *Chamaecrista.* It has been suggested that in *Chamaecrista* nodulation may have evolved independently from Papilionoideae but also from Mimosoideae (Doyle, 2011). A few studies have shown that *Chamaecrista* forms indeterminate nodules but also show presence of fixation thread, a trait considered primitive (Naisbitt *et al*., 1992). In addition,

Chamaecrista contain a putative ancestral hemoglobin that is an intermediate between non-symbiotic and symbiotic hemoglobins. Owing to its nodulation ability, *C. fasciculata* was suggested to be a model for determining rhizobial interactions with Caesalpinioideae (Singer *et al*., 2009). Rhizobia from *C. fasciculata* nodules have been isolated and belong to the USDA collection. Apart from the potential to enhance fundamental knowledge, *Chamaecrista* is an ideal legume for establishing mixed prairie and can be an alternative crop for biofuels.

C. fasciculata is a species of *Chamaecrista* that is native to North America and is commonly used as one of the model plants from the caesalpiniods (Cronk et al, 2006). Previous studies have revealed that *Bradyrhizobium* appears to be the primary nodulator in not only *C. fasciculata*, but in other legumes belonging to the *Caesalpinioideae* sub family as well (Parker, 2014, Sprent et al, 2017). Studies on *Chamaecrista* native to Brazil and India have also revealed similar results, suggesting that *Bradyrhizobium* is the primary nodulator of *Chamaecrista* not only in the US but all over the world (dos Santos et al, 2016) (Rathi et al, 2018). Phylogenetic analysis of key genomic and symbiotic genes is necessary to not only confirm what other studies have shown but to also narrow down the evolutionary changes that have occurred in these strains over time and if these changes coincide with the evolutionary changes observed in the legume family. Unlike other strains of Rhizobia, *Bradyrhizobium* does not contain a symbiotic plasmid on which genes are able to spread. Instead they contain more of a symbiotic island of genes found clustered together on a portion of their chromosome (Parker, 2014). Analysis of genes found on this cluster could potential provide some insight into what makes this genus of bacteria the primary nodulator of the rarely nodulating *Caesalpinioideae* subfamily and how this relates evolutionarily to the rise of the *Papilionoidea*e.

A few studies have isolated bradyrhizobial symbiont from *C. fasciculata* growing in USA. However, it is not known if *Bradyrhizobia* predominant in extended area or if there are other rhizobia that can nodulate *C. fasciculata*. In addition, the mechanisms important for colonization and nodulation of *C. fasciculata* are yet to be studied. This study was aimed at determining the rhizobial symbiont of *C. fasciculata* in the native soils of broader Mid-Western USA soils and to study the colonization and nodulation of *C. fasciculata.*

Materials and Methods:

Collection of nodules from field grown *C. fasciculata* and form *C. fasciculata* used as host-trap.

C. fasciculata plants were collected from fields grown on University of Wisconsin-Milwaukee's campus and brought back to the lab. Plants were washed with dH_2O to remove all excess soil and nodules were then isolated from the plant. Host trap *C. fasciculata* seeds were sterilized, germinated, and grown in pots with 3 plants per pot. For these plants, collected soils from various locations in mid-west states were used as inoculum (Table 2, Figure 1). Plants were allowed to grow for 21 days before being removed from their pot and checked for nodulation. Plants that had nodules were washed of excess soil and then the nodules were isolated from the plant. Typically, 2-5 nodules per plant were used to isolate bacteria from.

Isolation of bacterial symbionts from nodules of *C. fasciculata*

Nodules from *C. fasciculata* were removed from plant roots using sterile forceps and placed in an Eppendorf tube containing dH_2O. After all of the nodules had been removed and placed in the Eppendorf tube, the dH_2O was removed and the nodules were surfaced sterilized using 96%

ethanol for 5 minutes followed by 100% Clorox Bleach for 30 seconds. The nodules were then washed 6 times using sterile dH_2O. The final wash was plated onto one TY and one YM plate as a control. Surface-sterilized nodules were placed onto one TY and one YM plate containing 100ng/µL of nystatin (to prevent fungal contamination). On average, 2-5 nodules from each plant were plated onto each plate type. Nodules were then squeezed with sterile forceps to release the contents of the nodules onto the plate. The contents of the nodules were streaked for isolation and allowed to incubate for 4-7 days, or until colonies began to grow. Colonies with distinct morphology were selected and plated out onto a fresh plate of the same media type. This process was replicated 4-6 times, until all of the bacteria on the plate had the same colony morphology. Each unique colony type was selected for further testing. Table 2 shows the name and location of all isolates used in this study.

Isolation of genomic DNA.

Cultures for genomic DNA isolation were grown by inoculating strains into yeast-Mannitol (Vincent, 1970) **(**YM) or Tryptone yeast-extract (TY) media. Cultures were grown aerobically at 30°C. 2mL of culture was pelleted by centrifugation at 13,200 rpm for 1 minute and used for genomic DNA isolation using Wizard Genomic DNA Isolation Kit (Promega, USA) and following the manufacturer's protocol.

DNA quality and quantity estimations.

The concentration and quality of genomic DNA isolations, PCR purifications, and gel purifications was determined using a NanoDrop ND-100 Spectrophotometer. The absorption of the samples were recorded and the 260/280 and the 260/280 wavelengths were recorded for

each sample to determine the concentration and quality of the samples. Samples were then stored at -20°C for further analysis.

Primer design:

Primers were designed to amplify specific regions/genes from various strains of *Bradyrhizobium*. Primers were typically designed to include between 18 and 22 base pairs of identity to the template genomic DNA with a GC content of around 50%. The T_m of the primers were designed to be within a few (2-5°C) of one another for proper annealing during PCR. Primer fragments that were to be used in restriction cloning had proper restriction sites added to the 5' end of each primer. A web cutter tool was used to ensure that the restriction sites used in the primer didn't cut inside of the template DNA. An oligo analysis program was used to calculate GC content as well as T_m for each primer.

Primer Name	Sequences 5' - 3'	PCR Program	Reference
16S F	CAGGCCTAACACATGCAAGTC		
16S R	ACGGGCGGTGTGTACAAG	2' 95° C, 30 x (30'' 95° C, 30'' 57° C, 2' 72° C), 5' 72° C	
ITS F	TCGGGCTGGATCACCTCCTT		(dos Santos et al, 2017)
ITS R	CCGGGTTTCCCCATTCGG	5' 94° C, 35 x (30'' 94° C, 30'' 55° C, 30'' 72° C), 7' 72° C	(dos Santos et al, 2017)
NifH F	AARGGNGGNATYGGHAARTC	5' 94° C, 20 x (30'' 94° C, 30'' 60° C -0.5° C/cycle, 1' 72° C), 25 x	(dos Santos et al, 2017)
NifH R	GCRTAVAKNGCCATCATYTC	(30'' 94° C, 30'' 50° C, 1' 72° C), 5' 72° C	(dos Santos et al, 2017)
RecA F	CAACTGCMYTGCGTATCGAAGG		(dos Santos et al, 2017)
RecA R	CGGATGTGGTTGATGAAGATCACCATG	2' 95° C, 35 x (45'' 95° C, 30'' 58° C, 1'30'' 72° C), 7' 72° C	(dos Santos et al, 2017)
NodA 1	TGCRGTGGAARNTRNNCTGGGAAA		
NodA 2	GGNCCGTCRTCRAAWGTCARGTA	2' 95° C, (30'' 95° C, 1' 49° C, 1' 72° C), 5' 72° C	
DnaK F	TTCGACATCGACGCSAACGG		(dos Santos et al, 2017)
DnaK R	GCCTGCTGCKTGTACATGGC	2' 95° C, 35 x (45'' 95° C, 30'' 56.8° C, 1'30'' 72° C), 7' 72° C	(dos Santos et al, 2017)
p318GFP F	GAGAGAACTAGTGGAGGAAGAAAAA		
p319GFP R	CTCTCGAGCTGATTTGTATAGTTCAT	2' 95° C, 30 x (30'' 95° C, 30'' 55° C, 1' 72° C), 5' 72° C	
NodAMut F	CAGCGAATTCATGAACATTGCCGTCTCC		
NodAMut R	GTATGCGGCCGCTTCACAACTCAGGCCCGTTAC	2' 95° C, 30 x (30'' 95° C, 30'' 66° C, 1' 72° C), 5' 72° C	
NodA(Mut)Bjap F	GATTCTAGATCAAGTGCAGTGGAGCCTTCG		
NodA(Mut)Bjap R1	TACAGATCTACCAGTGCAGAGCCGTGAG	2' 95° C, 30 x (30'' 95° C, 1' 63° C, 1'30'' 72° C), 5' 72° C	

Table 1. Table showing the primers and PCR program conditions used throughout this study for amplification of genes for phylogenetic analysis, cloning, and screening of GFP strains.

Amplification, purification and sequencing of gene fragments.

Genomic DNA isolated from each strain was used as a template to amplify genes using polymerase chain reaction (PCR) and gene specific primers (Table 1). PCRs were set up according to the instructions for the GoTaq Green Master Mix (Promega). Master mix was diluted to 1x final concentration while the primers were diluted to .5 μM concentration. The final concentration of the template used was no more than 250ng of DNA, or 5μL of genomic DNA. The amplified products were separated by gel electrophoresis, stained with ethidium bromide and visualized under UV light. The PCR products were purified using PureLink PCR Micro Kit (Invitrogen). In case of more than one PCR products, the band of expected size was excised from agarose gels and purified using Wizard SV Gel and PCR Clean-Up System kit (Promega). The purified products were sequenced at the University of Chicago DNA sequencing facility. Sequences were analyzed using the FinchTV program for completeness and accuracy by comparing peaks to the registered nucleotide. Sequences were analyzed through the NCBI Blast database to determine the closest match and accuracy of the amplification. Sequences were submitted to NCBIs GenBank.

Construction of Phylogenetic Trees:

Sequences of all strains for phylogenetic analysis were uploaded to NCBI GenBank. Sequences were then obtained from online and aligned using the Clustal W program. Aligned sequences were then used to construct phylogenetic trees utilizing the MEGA7 program. Neighbor-Joining trees were built in MEGA 7 using 1000 bootstrap replications for each phylogenetic tree. Table 1 shows the primers and PCR settings for all sequences analyzed.

Plant colonization, nodulation and growth promotion.

C. fasciculata and soybean seeds were surfaced sterilized by treatment with 96% ethanol for 5 minutes followed by (1:1 v/v) of Clorox Bleach and sterile dH_2O for 30 seconds. The seeds were then washed 6 times using sterile dH_2O. After the final wash, seeds were treated with sulfuric acid for 10 minutes. Sulfuric acid was removed from the tube and the seeds were washed 6 times using sterile dH_20. After the final wash, the tube was filled with sterile dH_2O to allow the seeds to imbibe water and seeds were incubated at 30˚C for 48 hours. After incubation, germinated seeds were then aseptically transferred onto the petri dishes lined with sterile paper towels, and further incubated at 30˚C for 48 hours. The seedlings were either planted into sterile pots or into sterile test tubes containing paper towels. Freshly planted seedlings were watered with sterile nitrogen free plant growth media (PGM) and placed into a growth chamber to begin growing for approximately 2-3 days before inoculation by the selected rhizobial isolates. *Bradyrhizobium spp.* were grown in either YM broth or TY broth at 30˚C for approximately 4-7 days, depending on the media used. Cells were pelleted by centrifugation at 13,200 rpm for 1 minute, washed with 1mL of nitrogen free PGM and centrifuged at 13,200 rpm for 1 minute. The cell pellet was resuspended in fresh nitrogen free PGM. Each pot or test tube containing germinated seedlings were removed from the growth chamber and received 1mL of 1.0 OD_{600nm} culture. Plants grown in pots were watered with fresh nitrogen free PGM to allow the bacteria to spread throughout the entirety of the pot. Plants grown in test tubes were rewrapped with parafilm to secure seedlings so that the roots were still contacting the paper towel and to prevent contamination. Plants were allowed to grow in the growth chamber and watered daily. The plants were removed from the growth media, washed with tap water and

the roots were observed for nodule formation. The effectiveness of the symbiosis was determined by counting nodule numbers and nodule coloration (red, pink, white) and measuring plant dry weights.

Plant growth promotion

Plant growth promotion by the *Bradyrhizobium* spp. isolates was determined by analyzing the fresh and dry weight of the roots and shoots. Plants were grown in pots with 2-3 plants per pot, using two separate pots for each inoculated strain. Plants were removed from their pots at 40 days after inoculation and their roots were washed with dH_2O of all excess soil material. The roots and shoots for each plant were separated from one another and fresh weight was measured. After the initial fresh weight measurement, the individual roots and shoots for each plant were wrapped in foil and placed in an incubator at 65°C to dry. The weights for each plant were taken twice daily until there was no change in the weight between measurements. This weight was recorded for the final dry weight for each plant.

Marking of *Bradyrhizobium* sp Nodule1 with β-glucuronidase (GUS) and green fluorescent protein (GFP)

Bradyrhizobium sp. Nodule1 (isolated from nodule of *C. fasciculata* growing at UWM) was marked with a transposon containing a constitutively expressed GUS (Wilson et al., 1995) by conjugation. *E. coli* containing pCAM121 and Bradyrhizobium sp. Nodule1 were grown in LB or TY, respectively and then mixed 1:1, 1:3, and 1:5 ratios of *E. coli* to *Bradyrhizobium.* Mixed cultures were centrifuged for 1 minute at 8000 rpm to loosely pellet the cells. The supernatant was removed, and cells were concentrated into 30μL of TY media. Cells were placed onto sterile

filter discs on TY agar plates and incubated at 30°C for 2-3 days. After incubation, cells from the filter were resuspended into a test tube containing 1mL of fresh TY and plated out on TY containing spectinomycin and incubated at 30°C for 4-6 days. The transconjugants were purified 2 times and GUS activity was visualized by blue color in presence of X-gluc. One transconjugant showing GUS activity and no apparent growth defect as compared to the wild type strain was selected for further studies.

The GUS expressing strain was further marked with GFP. A plasmid constitutively expressing GFP (pHC60) (Cheng and Walker 1998) was transferred from *E. coli* into the GUS marked strain by biparental conjugation. Transconjugants were selected on YM medium, containing 50 µg/ml tetracycline. The presence of plasmid in strains was confirmed by direct fluorescence microscopy.

Construction of *nod* mutant of *Bradyrhizobium* sp. Nodule1

To construct the nod mutant, the *nodA* gene was PCR-amplified using primers with restriction digest sites for XbaI, digested and ligated into a suicide vector pSUP202 pol4 digested with the same enzymes. *E. coli* DH5α was transformed with the ligation mixture and the recombinants were selected on LB media containing 10 µg/ml tetracycline. The construct was then confirmed by PCR and recombinant plasmid was isolated and purified using ChargeSwitch Pro Plasmid MiniPrep Kit (Invitrogen). The recombinant plasmid was then transferred to *Bradyrhizobium* sp. Nodule1 by tri-parental conjugation using pRK2013 as helper plasmid or electroporation. The conjugated or the electroporated cells were plated onto selective media to isolate single recombinant cells that are disrupted by the suicide plasmid.

Histochemical and microscopic studies of plant root colonization by *Bradyrhizobium* sp. Nodule1.

Seeds of *C. fasciculata* and soybean were surface-sterilized and germinated as described above. The contamination-free seedlings were transferred to small paper cups containing sterile vermiculite soaked with growth media or in glass tubes containing liquid growth media. The seedlings were inoculated with the GUS and GFP marked *Bradyrhizobium* sp. Nodule1 and incubated in the growth chamber. Plants were removed from the pots/tubes at 3, 5, 7, and 10 days post inoculation (DPI) and stained for GUS activity as described (Mitra et al., 2016). Briefly, the plant roots were aseptically transferred to petri dishes containing sterile 0.1M phosphate buffer (pH 7.0) with 50μg per mL X-Gluc and placed in a vacuum chamber for 10 minutes. After vacuuming, the samples were incubated at 37˚C in dark and monitored for the formation of blue color indicating GUS activity. The GUS stained root portions were imaged, excised and fixed with 25% glutaraldehyde (Fisher Scientific) for further examination using light and electron microscopy.

The colonization was also analyzed by visualizing GFP fluorescence using both epifluorescence and confocal microscopy. For epifluorescence microscopy, samples were prepared by placing a small portion of the plant material of interest (root section or nodule) onto a microscope slide containing a drop of dH_2O. The slides were observed using a Zeiss Axio Imager M2 microscope using a Zeiss 424931 GFP cube. For confocal microscopy, slides were prepared by placing a small rubber spacer onto a glass slide. The spacer was filled with sterile dH_2O and the sample was placed inside of the spacer. A No. 1½ 22mm^2 glass cover slip was placed on top of the

spacer to create a seal. The prepared slides were then analyzed using a Leica DM R Confocal microscope to detect the GFP.

Results:

Isolation of nodulating strains from field-grown and "host trap" *C. fasciculata*.

The symbionts of *C. fasciculata* growing in natural environments or in soils collected from various parts of Midwestern USA were isolated from surface-sterilized nodules using YM agar. *C. fasciculata* nodulated with all soils tested, except soils collected from Georgia red clay, Madison WI cornfield soil, Purdue IN cornfield soil, and Morris MN cornfield soil (Fig 1). The isolates from all the nodules showed a similar morphology and slow growth phenotype. Six isolates representing all the sites were selected for further analysis. To these we added four C. fasciculata nodulating strains obtained from USDA rhizobial germplasm collection. The isolates and their isolation source are listed in Table 2.

Isolate	Isolation Source
Nodule 1	UWM Chamaecrista Isloate
Nodule 4	UWM Chamaecrista Isloate
ZZ#1	UWM Chamaecrista Isloate
USDA794	USDA Strain 3010
USDA797	USDA Strain 3014
USDA798	USDA Strain 3574
USDA800	USDA Strain 3572
ASDSOY	Aurora South Dakota Soil
SDOY	South Dakota Soybean Soil
MSOY	Madison Soybean Soil
RMN	Rosemont MN Soil
HUMN	Hamline University MN Soil
HCOKY	Henderson County Kentucky Soil

Table 2. Table showing the isolates used in this study as well as their isolation source. All strains used were found to nodulate in *C. fasciculata*. Soil from Georgia red clay, Madison Cornfield, Purdue cornfield, and Morris MN cornfield did not nodulate in the host-trap experiments and no isolates were able to be obtained from these soils.

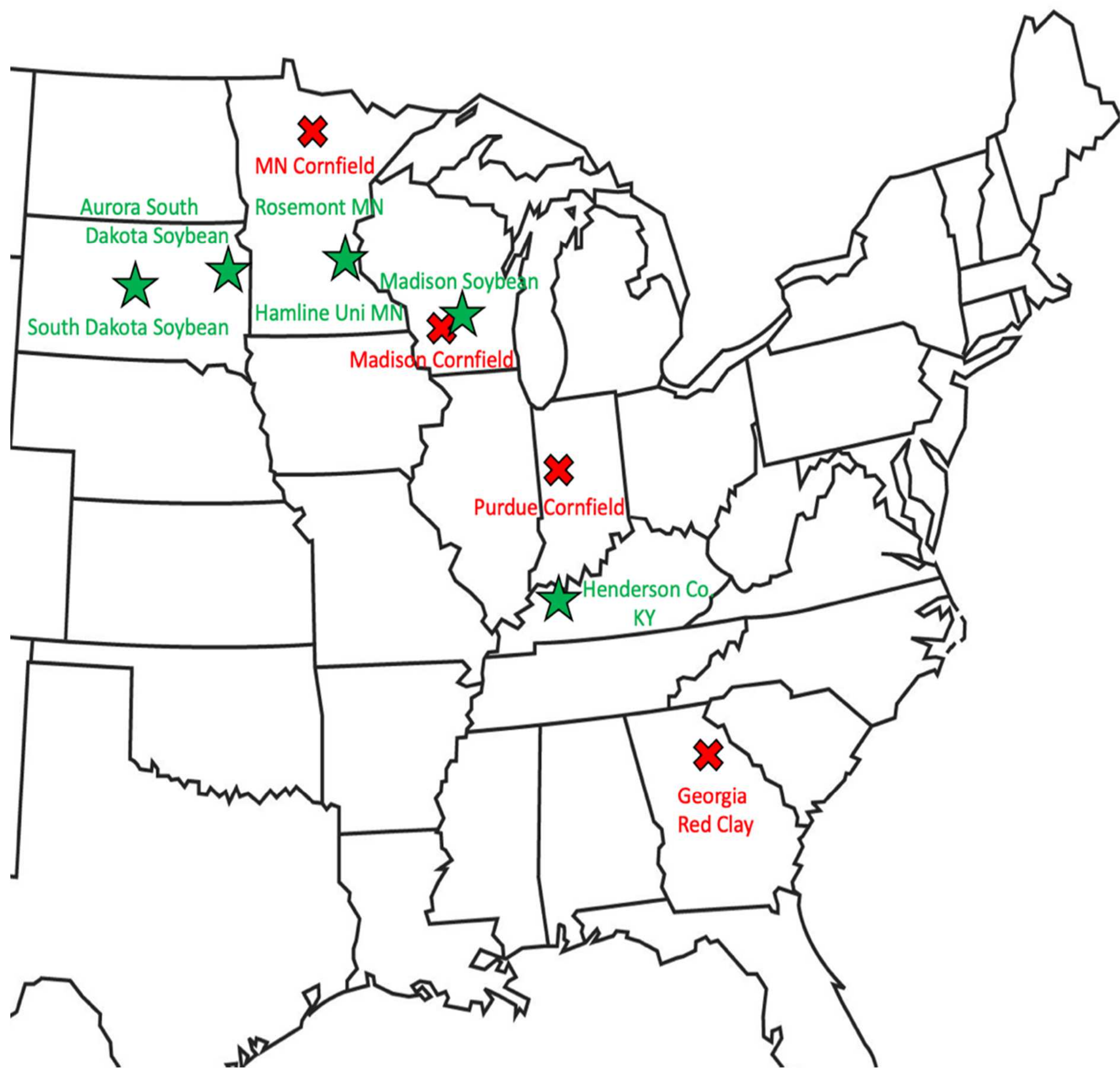

Figure 1. Map of the US mid-west showing where the soils used in the host trap inoculation of *C. fasciculata* came from. Red X's indicate that the soil from that location was used as inoculum but was unable to nodulate in *C. fasciculata* while the green stars indicate that soil from the location was used and was able to nodulate in *C. fasciculata*.

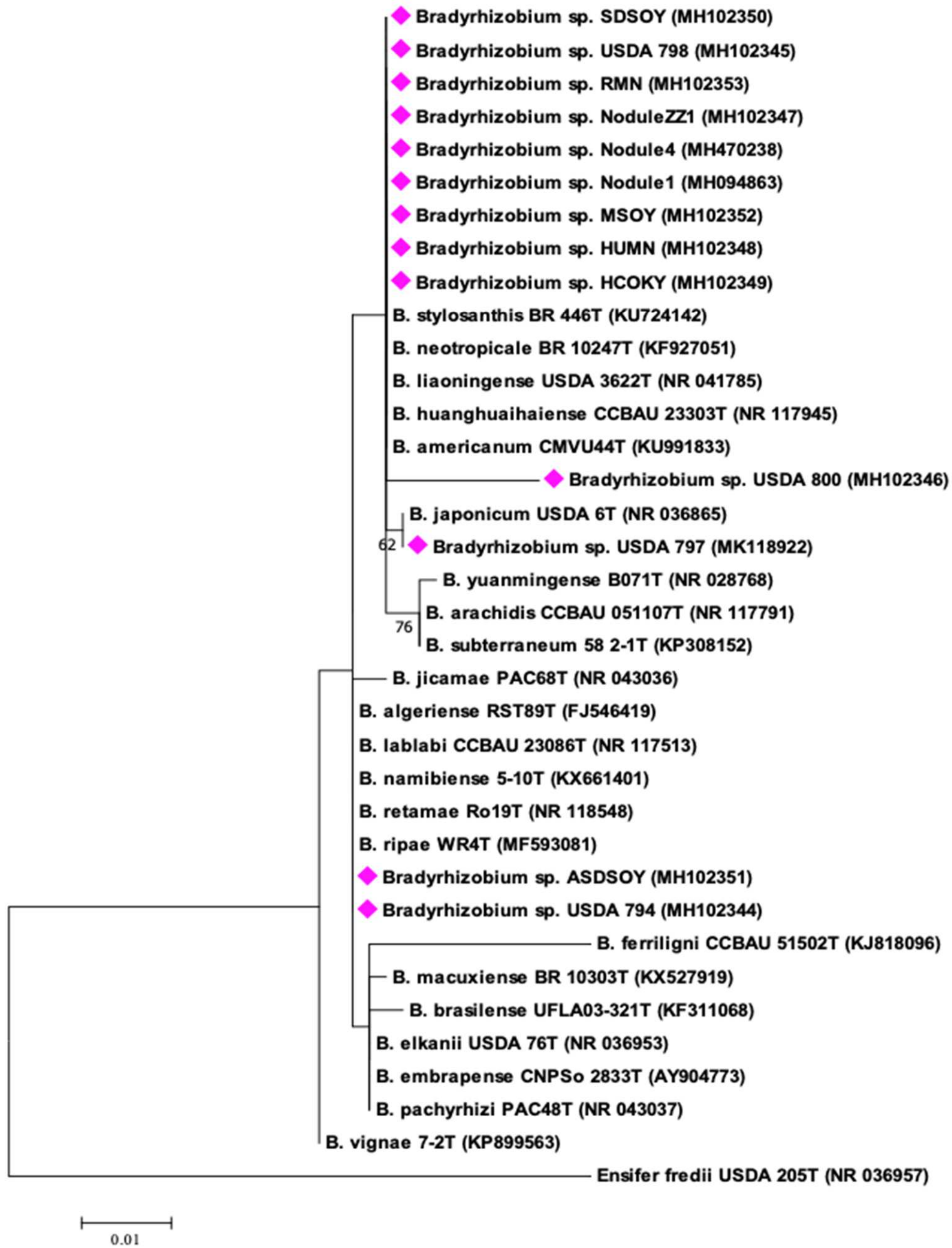

Figure 2. Phylogenetic tree based on the 16S rRNA sequences of bacteria isolated from *Chamaecrista spp*. Purple diamonds indicate strains used in this study; all other strains were taken from GenBank to use for comparison.

Phylogenetic analysis of 16S rRNA and ITS

To identify the selected strains, approximately 1kb fragments of 16S rRNA gene were amplified by PCR and sequenced. Sequences were then aligned with database using NCBI's BLASTn program. The 16S rRNA sequences form all isolates showed similarity with *Bradyrhizobium* spp. The phylogenetic analysis further showed the strains to be widely distributed within the *Bradyrhizobium* genera (Fig 2). Most of the strains clustered together and formed a separate clade. However, four of the thirteen strains showed more heterogeneous distribution indicating that the 16S rDNA sequences were not diverse enough for determining the phylogeny of the isolates. To further determine the phylogeny, the 16S-23S ribosomal RNA intergenic spacer (ITS) fragments of about 900-1000 base pairs were PCR-amplified, sequenced and analyzed. *Bradyrhizobium* strains that were isolated from the Brazilian *Chamaecrista* species; *C. bahiae, C. desvauxii, C. ensiformis, C. flexuosa, C. rotundifolia, C. serpens, C. supplex,* and *Chamaecrista spp*, were also included in this phylogenetic tree. The phylogeny based on ITS resolved the strain better than just the 16S rRNA sequences (Fig 3). Similar to the 16S rRNA phylogeny, nine strains formed a tight cluster and the remaining four formed two separate clusters (Fig 3).

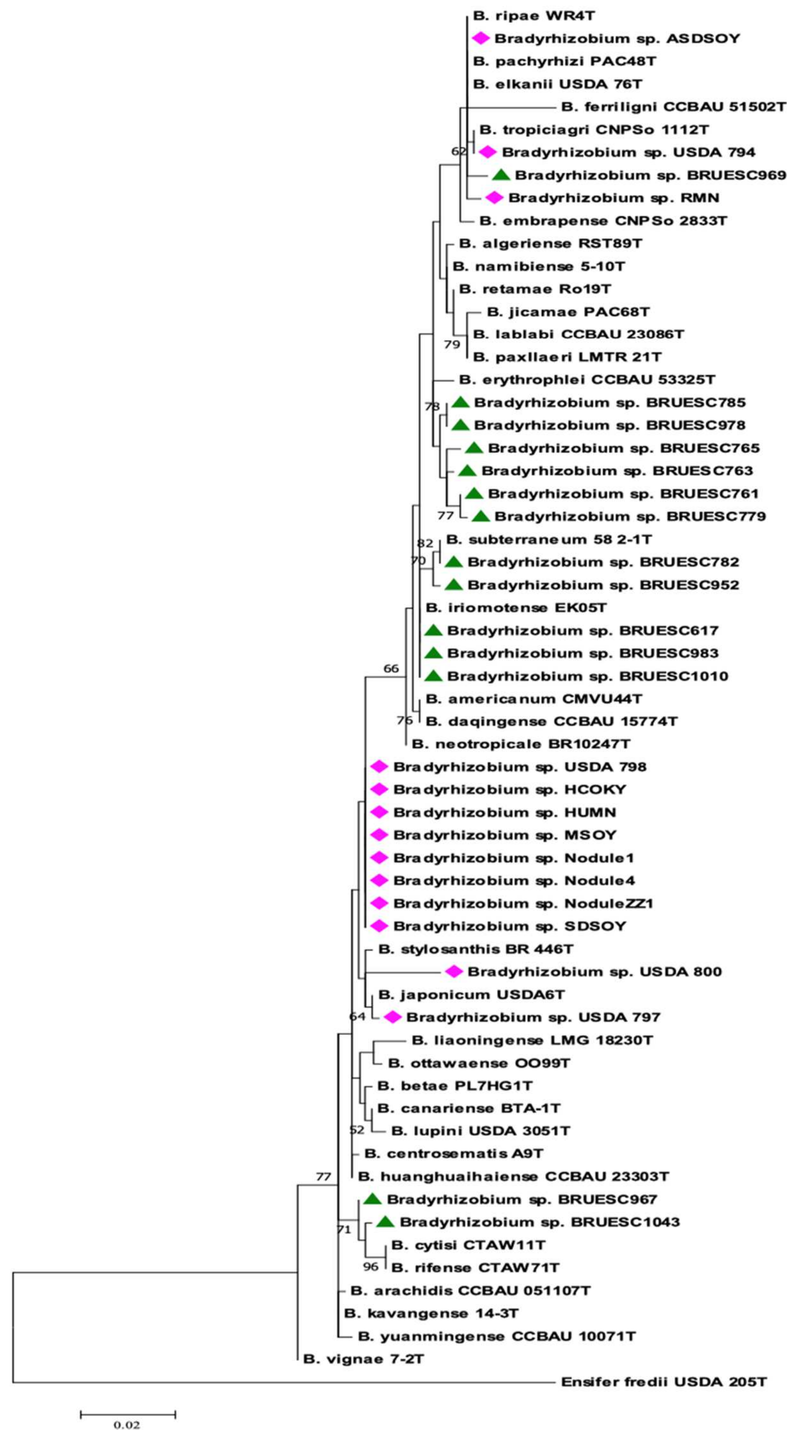

Figure 3. Phylogenetic tree based on both 16S and ITS region sequences of bacteria isolated from *Chamaecrista spp*. Purple diamonds indicate strains used in this study while green triangles indicate strains isolated from Brazilian *Chamaecrista*; all other strains were taken from GenBank to use for comparison *spp*.

Phylogenetic analysis of housekeeping genes *recA and dnaK*

Apart from 16S rRNA gene, bacterial phylogeny can be further resolved using other conserved housekeeping genes such as *recA* and *dnaK*. To further explore the phylogeny of the C. fasciculata symbionts, approximately 600 base pair fragments of *recA* and 300 base pair fragments of *dnaK* were PCR-amplified, sequenced and analyzed. The phylogeny based on these housekeeping genes correlated with the 16S-rRNA based phylogeny (Fig 4, 5). The eight strains grouped together. *Bradyrhizobium spp* USDA 800 and *Bradyrhizobium spp* USDA 797 showed more divergence but were still clustered. *Bradyrhizobium spp* ASDSOY and *Bradyrhizobium spp* RMN appear to be closely related when looking at the housekeeping genes with *Bradyrhizobium spp* USDA 794 being further apart. This is different compared to the 16S rRNA + ITS tree where *Bradyrhizobium spp* ASDSOY was further apart and *Bradyrhizobium spp* RMN and *Bradyrhizobium spp* USDA 794 appeared to be closely related.

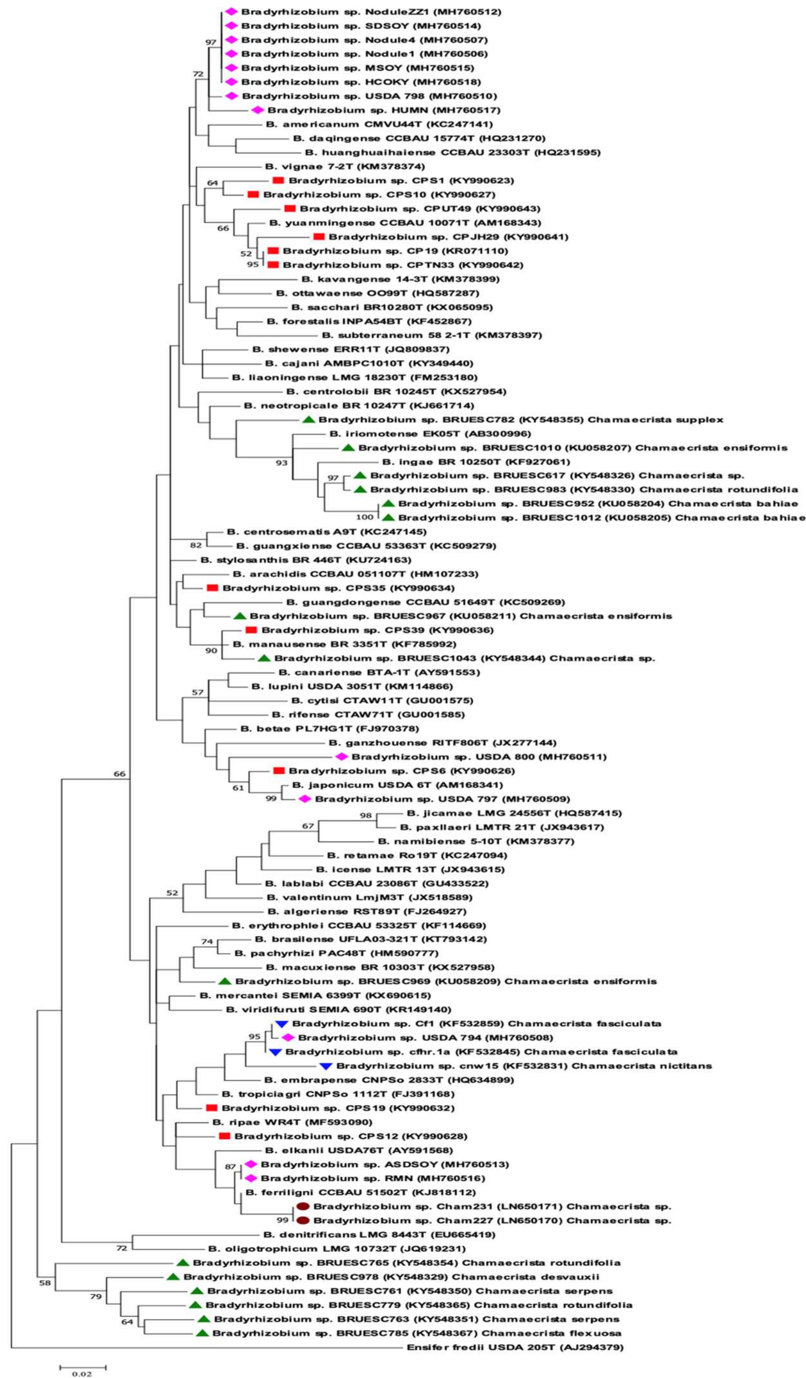

Figure 4. Phylogenetic tree based on the house keeping *recA* gene sequences of bacteria isolated from *Chamaecrista spp*. Purple diamonds indicate strains used in this study while green triangles indicate strains isolated from Brazilian *Chamaecrista*. The red squares indicate bacterial strains isolated from Indian *Chamaecrista spp.* while the maroon circles indicate strains isolated from African *Chamaecrista spp*. The blue triangles are strains that have also been isolated from *Chamaecrista* spp from US soils. All other strains were taken from GenBank to use for comparison *spp*.

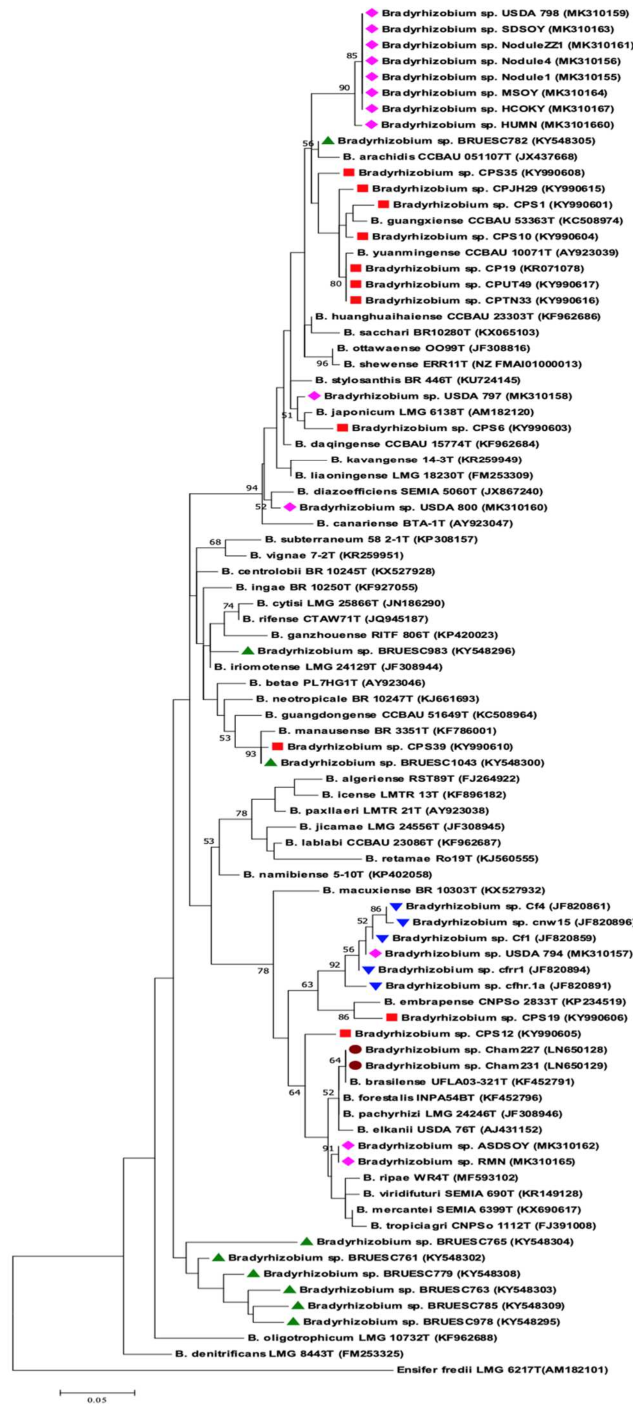

Figure 5. Phylogenetic tree based on the house keeping *dnaK* gene sequences of bacteria isolated from *Chamaecrista spp*. Purple diamonds indicate strains used in this study while green triangles indicate strains isolated from Brazilian *Chamaecrista*. The red squares indicate bacterial strains isolated from Indian *Chamaecrista spp.* while the maroon circles indicate strains isolated from African *Chamaecrista spp*. The blue triangles are strains that have also been isolated from *Chamaecrista* spp from US soils. All other strains were taken from GenBank to use for comparison *spp*.

Phylogenetic analysis of symbiotic genes *nodA* and *nifH*

To determine the genetics of symbiosis for the *C. fasciculata* nodulating strains two genes essential for symbiosis (*nodA* and *nifH*) were amplified by PCR, sequenced and analyzed. In the *nodA* gene phylogeny four distinct types were observed (Fig 6). Seven strains formed a distinct clade close to *B. forestalis* that was isolated from nodules of forest legumes in the Amazon (da Costa et al., 2018). The *nodA* sequences of three strains were identical to *B. elkani*, a strain that nodulate soybean. In contrast to these nod genes, *nodA* of one strain was divergent from *Bradyrhizobium* and shared similarities with *nodA* of *Ensifer meliloti*, an alfalfa nodulating rhizobia. These results indicate that *C. fasciculata* is likely able to recognize diverse Nod factors secreted by the nodulating strains. The *nifH* phylogeny was similar to the *nodA* gene phylogeny as the eight strains clustered with *B. forestalis*. Other strains showed similarities to soybean nodulating *B. elkani* and *B. japonicum* (Fig 7). These results indicate tight linkage between the two symbiotic genes.

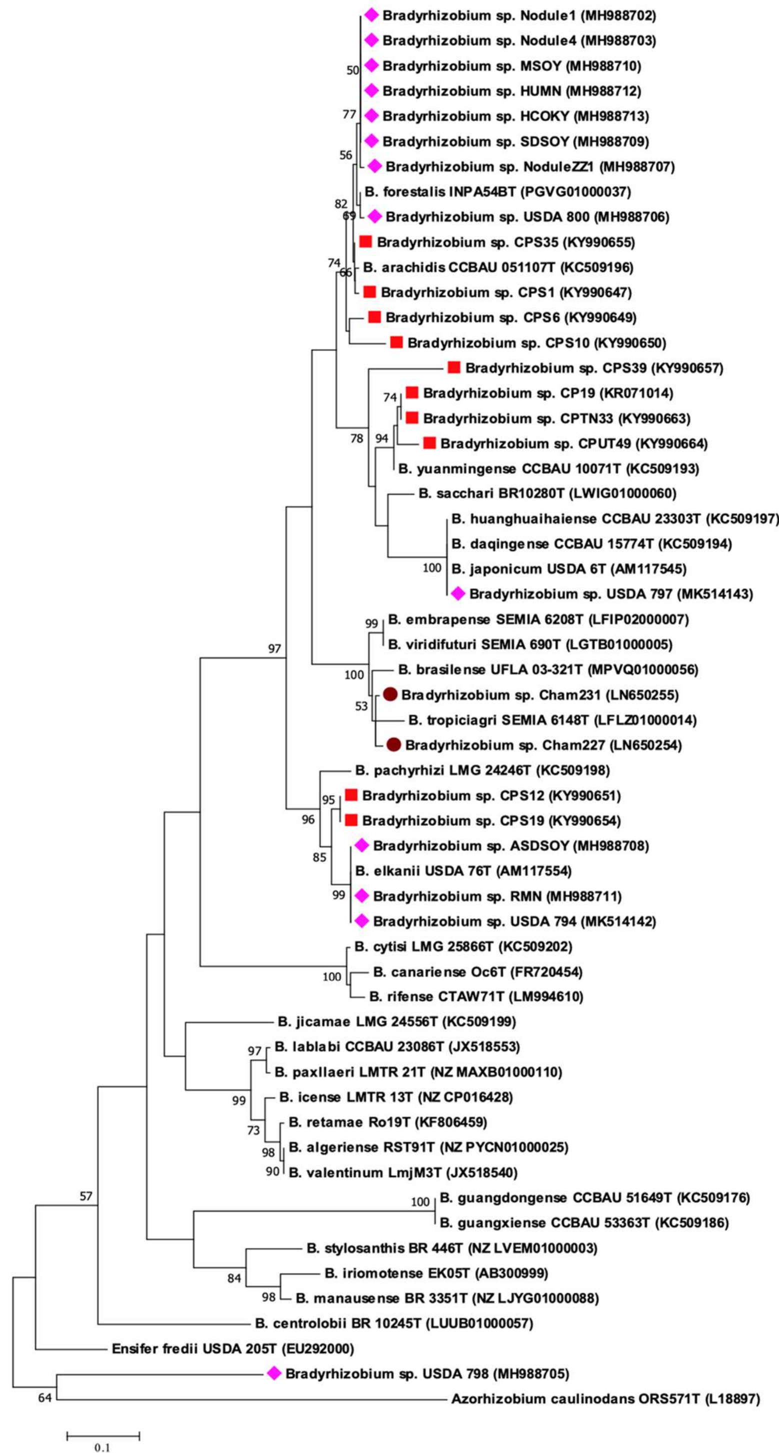

Figure 6. Phylogenetic tree based on the *nodA* nodulation gene sequences of bacteria isolated from *Chamaecrista spp*. Purple diamonds indicate strains used in this study while the red squares indicate bacterial strains isolated from Indian *Chamaecrista spp.* and the maroon circles indicate strains isolated from African *Chamaecrista spp*. All other strains were taken from GenBank to use for comparison *spp*.

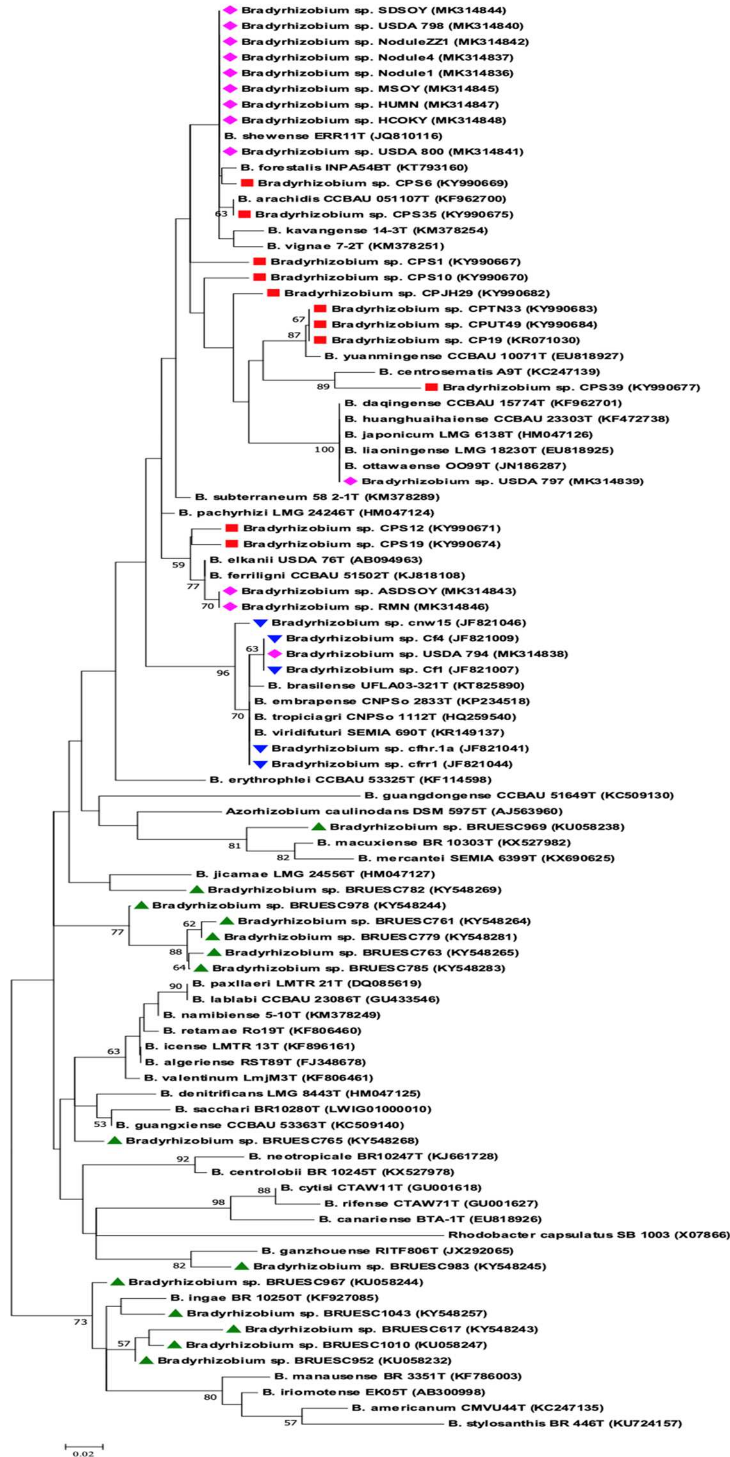

Figure 7. Phylogenetic tree based on the *nifH* nitrogenase gene sequences of bacteria isolated from *Chamaecrista spp*. Purple diamonds indicate strains used in this study while blue triangles are strains that have also been isolated from *Chamaecrista* spp from US soils. The green triangles indicate strains isolated from Brazilian *Chamaecrista* and the red squares indicate bacterial strains isolated from Indian *Chamaecrista spp.* All other strains were taken from GenBank to use for comparison *spp*.

Nodulation and growth promotion of *C. fasciculata* and soybean by *Bradyrhizobium spp* isolates.

The molecular phylogeny of *C. fasciculata* nodule isolates, especially the divergence of symbiotic genes indicates that these rhizobia could differ in their ability to nodulate and fix nitrogen. Additionally, the relatedness of these isolates to soybean symbionts coupled with the use of soybean farm soils as a source of these isolates suggests that these could also form symbiosis with soybean. To determine the symbiotic interactions, the thirteen isolates and *B. japonicum* USDA110 (a model soybean nodulating strain) were inoculated onto *C. fasciculata* and soybean seedlings under axenic conditions. The symbiosis was evaluated by determining the number of nodules and total plant dry weights. Non-inoculated seedlings served as a negative control. All strains formed nodules on *C. fasciculata* but there were significant differences in the ability of these strain to enhance plant-growth (Fig 8). These results indicate that there are phenotypic differences in the symbiotic interactions. In accordance with the molecular phylogeny of the symbiotic genes, all of these strains could also nodulate soybean, a legume that is phylogenetically distinct from *C. fasciculata*. Similar to the results with *C. fasciculata*, there were significant differences in the growth promotion of soybean by these isolates (Fig 9). In contrast, the soybean symbiont *B. japonicum* USDA110 formed only a few nodules on *C. fasciculata* and did not enhance plant growth (Fig 8).

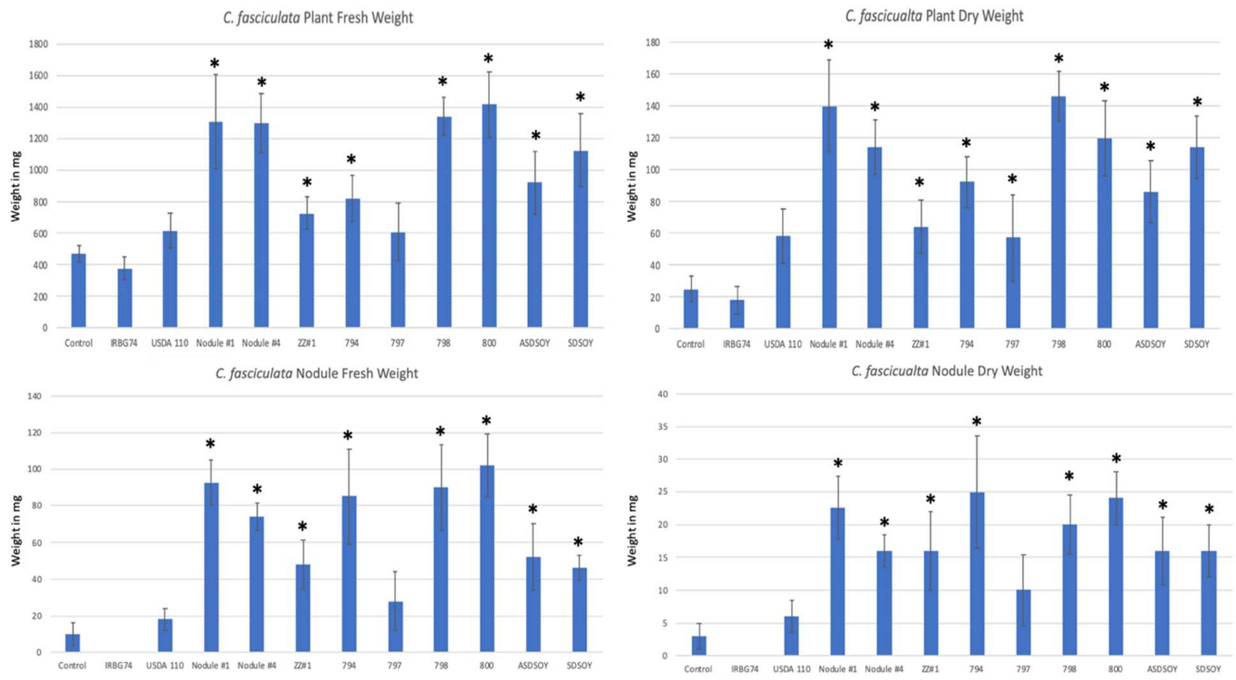

Figure 8. *Chamaecrista fasciculata* plant growth promotion comparing fresh and dry weights of the whole plant as well as the nodules present, of all isolates from this study. Y-axis represents weight in mg. * denotes significance as determined by t-test assuming unequal variance ($p < 0.05$).

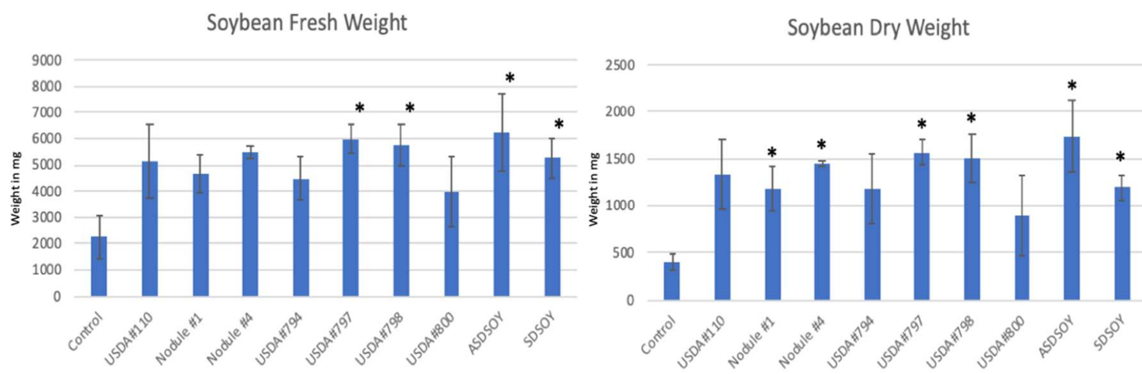

Figure 9. Soybean plant growth promotion comparing fresh and dry weights of the whole plant, of isolates used in this study. Y-axis represents weight in mg. * denotes significance as determined by t-test assuming unequal variance ($p < 0.05$).

Colonization and infection mechanisms of host-plant by *Bradyrhizobium* sp. Nodule1.

In most of the well-studied legumes, rhizobia colonize and infect through root hair in which the bacteria invade plant root intracellularly via a root hair infection thread. However, in certain other legumes, rhizobia can infect intercellularly, either at fissures in the epidermal layer or through the cracks formed during lateral root formation (crack entry) (Ibáñez et al, 2016). These infection mechanisms have been studied in the more advanced papilionoid legumes. In contrast, very little is known about the infection mechanisms in the more primitive and ancestral caesalpiniod legumes such as *C. fasciculata*. To determine the infection mechanisms, *Bradyrhizobium* sp. Nodule1, isolated from the nodules of field-grown *C. fasciculata* was marked with GUS and GFP. The marked strain was inoculated onto *C. fasciculata* seedlings in axenic conditions and colonization and infection was studied using histochemical GUS staining, GFP fluorescence and microscopy of the fixed samples. Roots of the inoculated plants were stained with X-gluc at various time points to localize the infecting bacteria. GUS staining (blue coloration) was observed both on the surface of the roots and in the root hairs (Fig 10). However, the staining was most intense on the root surface and no infection thread in the root hairs was observed. The GUS staining pattern indicates that *Bradyrhizobium* sp. Nodule1 likely infects C. fasciculata through epidermal layer although the root hair infection is also possible.

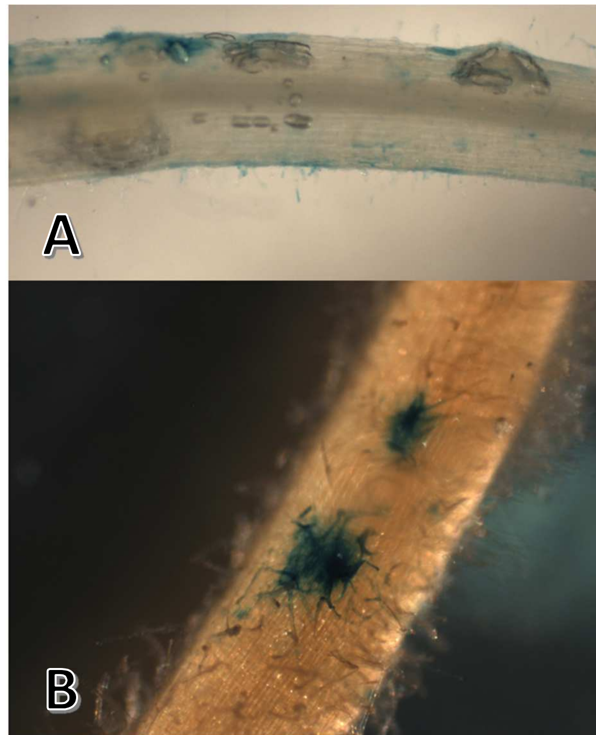

Figure 10. *Chamaecrista fasciculata* root samples that have been inoculated with *Bradyrhizobium spp.* strain containing the β-Glucuronidase reporter system and stained with the X-Gluc substrate. Presence of blue color on the roots indicates that bacteria is present to some extent. **A.** Shows highlights presence of bacteria associating with the root hairs. **B.** Shows spot on root surface as well as root hairs.

To further determine the root infection, the GUS stained root portions were examined for GFP fluorescence using microscopy. Epifluorescence microscopy showed that GFP expressing bacteria were present inside root hair (Fig 11). However, formation of an infection thread was not clearly seen. In addition, the root hair infecting bacteria did not extend into the cortical cells to form nodule primordia. To study the infection in more detail, confocal microscopy was utilized to get a better understanding. Confocal microscopy at 20x magnification reveals what does appear to be an infection thread running the entire length of a root hair (Figure 12). However, the infection thread did not propagate into internal cells of the root. In contrast to the cells within the root hair, GFP expressing cells were observed inside the

GUS stained nodule primordia that seem to have been infected by epidermal infection (Fig 14 A, B). These results suggest that *Bradyrhizobium* sp. Nodule1 infects *C. fasciculata* by both root hair and intercellularly through epidermal layer, but the epidermal infection is likely to be involved in nodule formation.

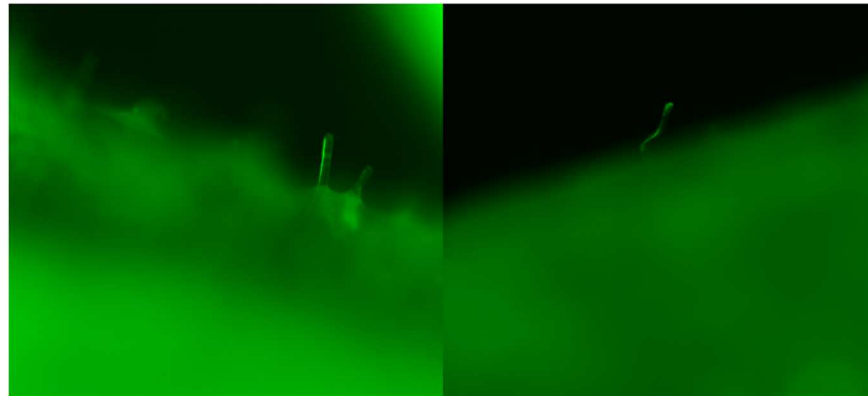

Figure 11. Fluorescent imaging of *Chamaecrista fasciculata* root samples inoculated with *Bradyrhizobium spp.* strain containing a GFP plasmid. Samples were imaged using epifluorescence. Imaging reveals potential infection thread inside of the root hair of plant.

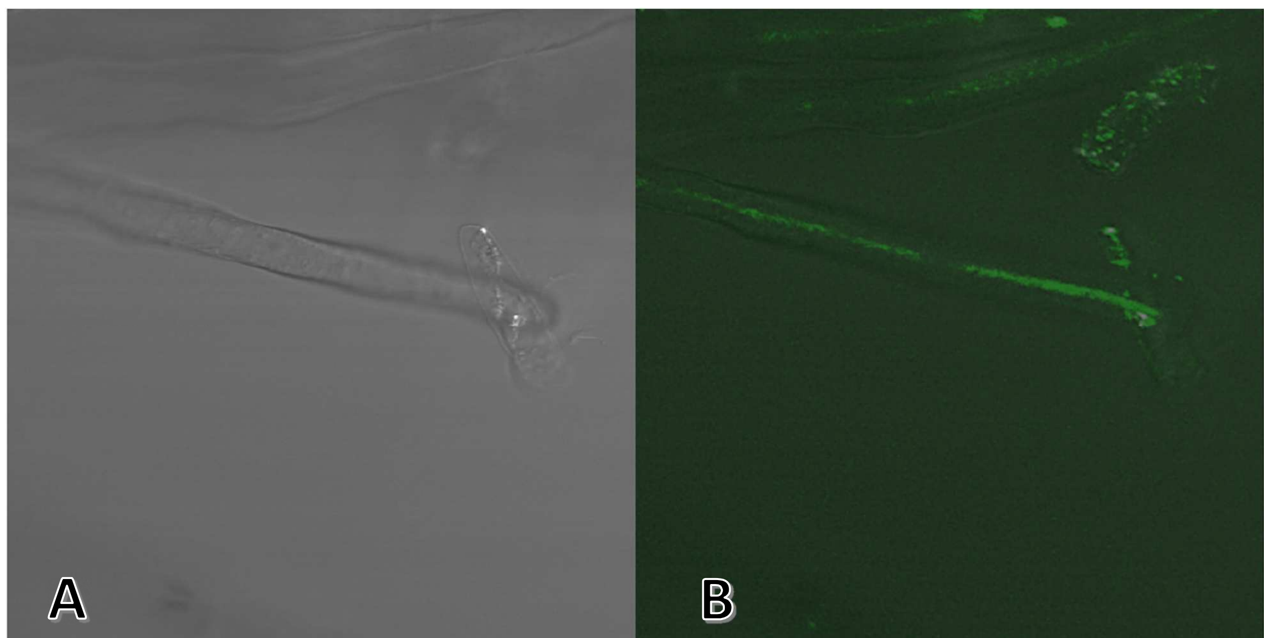

Figure 12. Fluorescent imaging of *Chamaecrista fasciculata* root samples grown aerobically inoculated with *Bradyrhizobium spp.* strain containing a GFP plasmid. Samples were imaged using confocal microscopy. **A.** Transmitted light image showing the outline of the root hair being observed. **B.** GFP overlay onto the transmitted light image, showing what appears to be an infection thread traveling the length of the root hair being shown.

In addition to the root hair and epidermal infection, some legumes (especially those growing in submerged conditions) form nodules through “crack entry”. To determine if *Bradyrhizobium sp* Nodule1 is able utilize “crack entry” for nodule formation the lateral root junctions were observed using confocal microscopy. As shown in Fig 13B GFP fluorescence was observed at the junction between the main root and the lateral root. To further confirm the “crack entry” *C. fasciculata* seedlings were inoculated and grown in test tubes containing liquid media such that the roots were submerged. It is known that root growth in submerged conditions inhibit formation of root hair and thus allow bacteria to enter through lateral root cracks. Microscopic analysis of these roots showed that GFP fluorescent cells were Figure 13D shows a localization in between the cells of the main and lateral root. These results indicate that *C. fasciculata* could also be nodulated via “crack entry”.

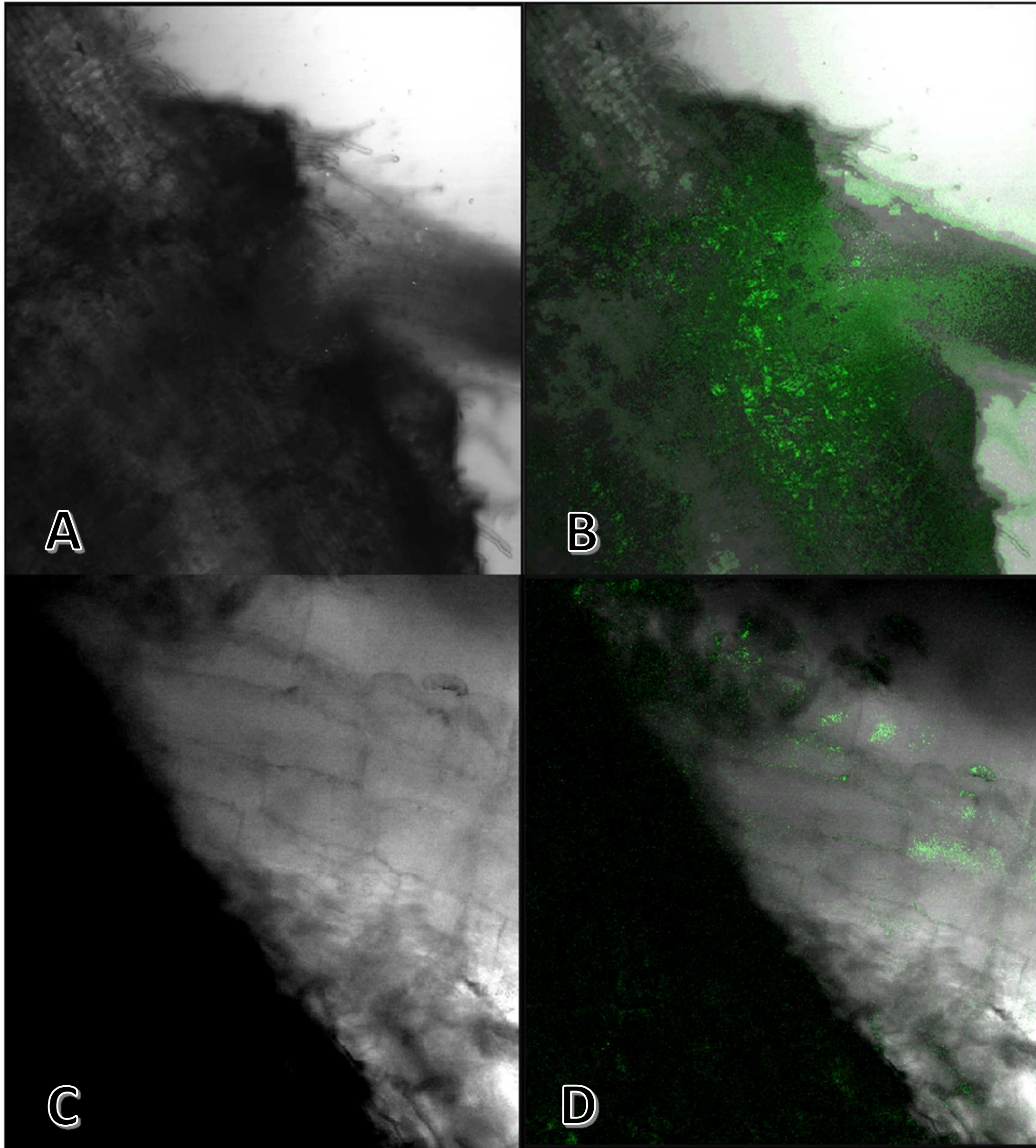

Figure 13. Fluorescent imaging of *Chamaecrista fasciculata* root samples inoculated with *Bradyrhizobium spp.* strain containing a GFP plasmid. Samples were imaged using confocal microscopy. **A.** Transmitted light image showing the junction of the main root and a lateral root from *Chamaecrista fasciculata* grown aerobically. **B.** GFP overlay onto the transmitted light image shown in 10A. GFP signal is found mostly localized to the junction between the main root and the lateral root. **C.** Transmitted light image zoomed in on a lateral root from *Chamaecrista fasciculata* grown in submerged conditions. **D.** GFP overlay onto the transmitted light image shown in 10C. Faint GFP signal can be seen mostly localized to the space in between neighboring cells.

The results of fluorescent microscopy were further confirmed using light microscopy of the GUS stained root portions. The roots were fixed, and semi-thin sections were observed under microscope. The toluidine-blue staining of the root sections revealed that bacteria were located around the surface of the nodule primordia-like structure (Figure 14B). Observations under higher magnification (100x) showed bacteria likely entering the nodule primordia of *C. fasciculata* through spaces between the epidermal cells and progressing inside some internal cells that could presumably lead to nodule formation (Figure 14C and Figure 14D).

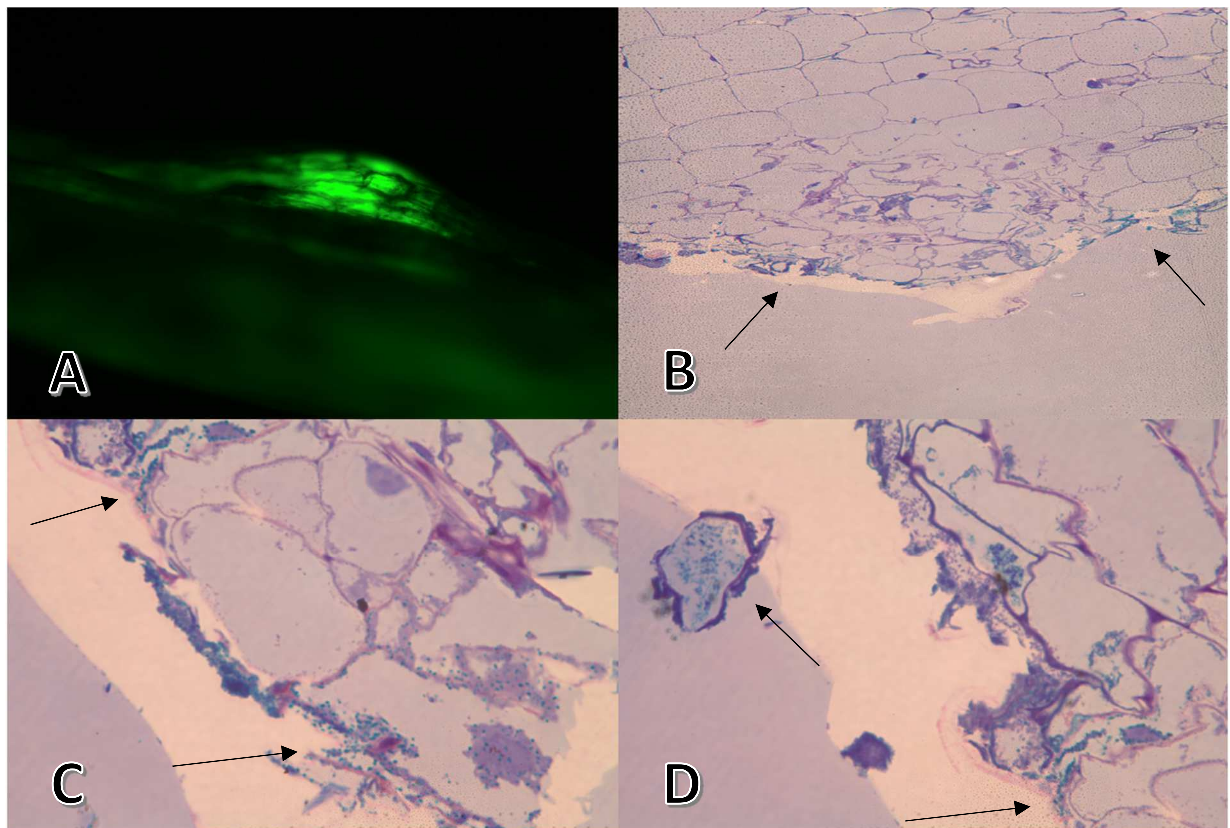

Figure 14. Microscopy images analyzing the nodule primordial formed on *Chamaecrista fasciculata* roots. **A.** Epifluorescence imaging with GFP reveals that bacteria is present inside of the nodule primordial after entering into the root. **B.** Low magnification (40x) light microscopy image of semi-thin sections of *Chamaecrista fasciculata* root tissues 5 days post inoculation. Arrows indicate areas that were zoomed in and focused on in panels C and D. **C and D.** High magnification (100x) light microscopy images of 14B. Arrows in these figures point out small portions of stained bacteria (in blue) that appear to be entering the nodule primordial through epidermal means rather than coming from traditional crack or root hair entry methods.

Microscopic analysis of *C. fasciculata* nodules

The above results show that *Bradyrhizobium s*p. Nodule1 can infect *C. fasciculata* through epidermal fissures or through "crack entry" at the later root junctions. Irrespective of the entry mechanisms, the symbiotic interaction finally leads to the formation of nodules. To confirm that the observed nodules were formed by the GFP marked *Bradyrhizobium* sp. Nodule1, the nodules were observed by confocal microscopy. As shown in Fig 15B, GFP fluorescing bacteria could be seen inside intact nodule indicating that the GFP plasmid in stable and this marked strain can be used in further studies to identify the infection and nodulation mechanisms in more details.

To further analyze the symbiotic interaction ultrastructure of nodules were determined using transmission electron microscopy (TEM). The nodules were fixed into resin and ultra-thin sections were observed under TEM. TEM micrographs showed elongated bacteroids present inside the plant cells and were surrounded by the peri-bacteroid membrane (Fig 15C, D). However, the bacteroids seem to lack polyhydroxy butyrate granules that are the hallmark of soybean nodules formed by *Bradyrhizobium* spp. In addition, few cells were only partially filled with bacteroids (Fig 15D). These results indicate that symbiotic *C. fasciculata* nodules are different than the soybean nodules and this is likely reflecting the primitive symbiotic interactions.

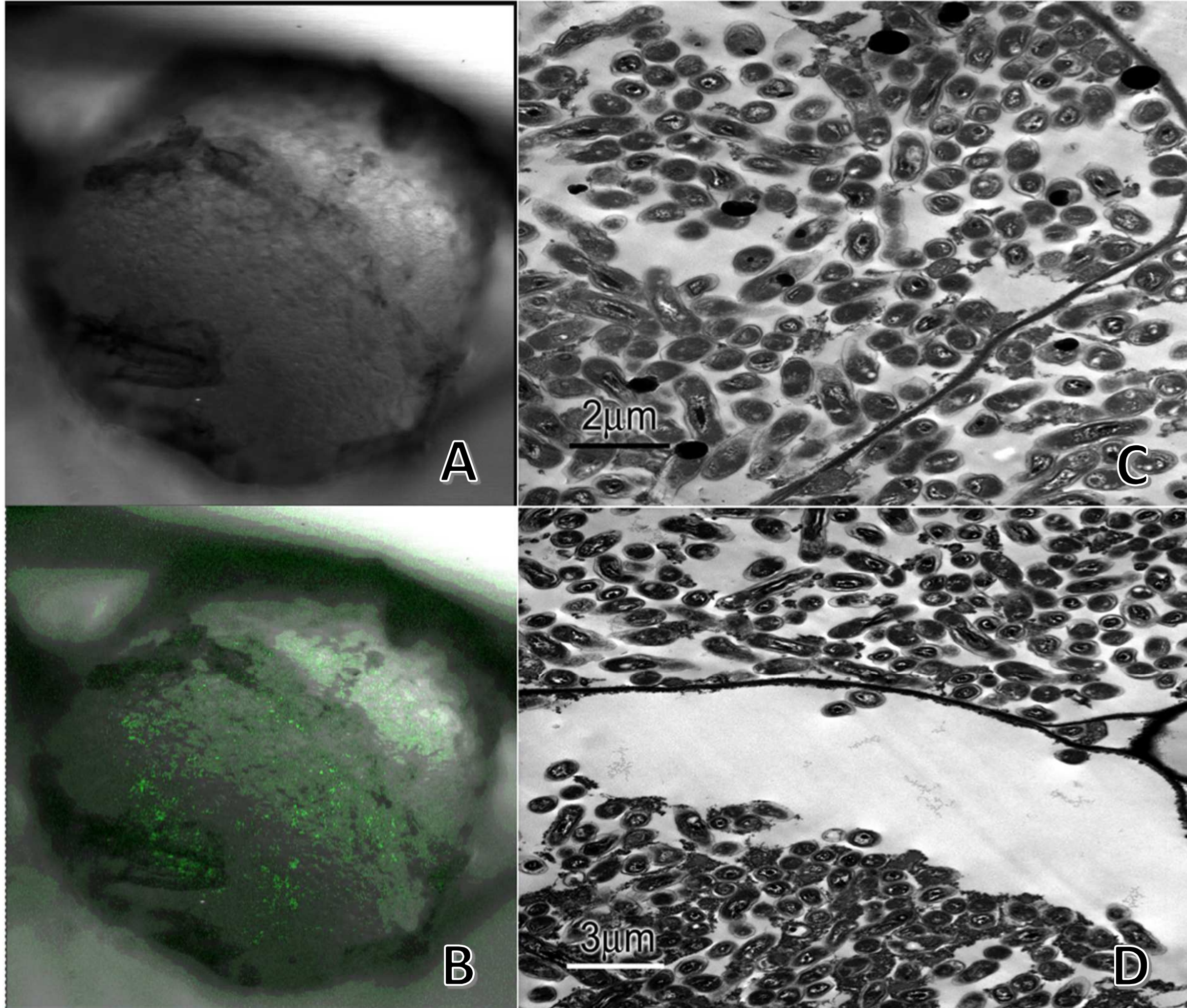

Figure 15. Microscopy images analyzing the nodules of *Chamaecrista fasciculata* using both confocal and transmission electron microscopy imaging. **A.** Transmitted light image showing a nodule formed on the root of *Chamaecrista fasciculata*. **B.** GFP overlay onto the transmitted light image shown in 15A. GFP signal shows that the *Bradyrhizobium spp.* used in inoculation does in fact differentiate and form nodules in *Chamaecrista* fasciculata. **C and D.** TEM micrographs showing the inoculated *Bradyrhizobium spp*. inside the nodule from *Chamaecrista fasciculata*. Scale bars are included and labeled for each image for reference for 15C and 15D.

Discussion:

Bradyrhizobium spp* are the preferred symbionts of *Chamaecrista fasciculata

Chamaecrista is the only nodulating genus in the ancestral caesalpiniod subfamily of legumes and is of interest because of its primitive nodule morphology and its significance to understand evolution of nodulation and nitrogen fixation in legumes (dos Santos et al., 2017, Rathi et al., 2018). Diverse species of *Bradyrhizobium* have been reported to nodulate various Chamaecrista spp. found globally (dos Santos et al., 2017, Rathi et al., 2018). In accordance with these observations, our studies show that *C. fasciculata* is exclusively nodulated by *Bradyrhizobium* spp. in the Mid-Western USA. The strains were phylogenetically analyzed using sequences of multiple genes such as *rrs*, *ITS*, *recA* and *dnaK*. These strains are phylogenetically diverse and form novel clades and lineage. Further analysis using genomics is needed to determine if these are new species of *Bradyrhizobium*. Similarly, Rathi et al. (2018) reported the diversity of root nodule bacteria associated with *C. pumila* growing in alkaline-neutral-acidic soils of India based on multi locus sequence analysis. On comparative phylogenetic analysis of *Bradyrhizobium* strains reported to be associated with *Chamaecrista* species growing in alkaline and acidic soils of different continents it was revealed that except for the strain USDA 3010 all *Bradyrhizobium* strains from this study were genetically distinct from the *Chamaecrista-Bradyrhizobium* strains isolated from India, Africa, Brazil (South America) and USA (North America).

Diversity of symbiotic genes in *Bradyrhizobium* strain nodulating species of *Chamaecrista* in different continents

Symbiotic gene phylogenies (based on *nodA* and *nifH* genes) were in congruence with analysis done using conserved protein coding core genes (*recA* and *dnaK*) and 16S-23S ribosomal RNA intergenic spacer region. Such congruence is characteristic of genus *Bradyrhizobium* that possess symbiotic island on the chromosome. However there were differences in terms of the legume hosts for the strains that showed maximum similarities to the *C. fasciculata* isolates. Only one of the ten *C*. *fasciculata* strains of Clade-I had *nod* and *nif* genes identical to *B*. *japonicum* and the strains clustering in this clade such as *B*. *daqingense*, *B*. *huanghuaihaiense*, *B*. *liaoningense* and *B*. *ottawaense*. All three strains of the different clade had *nodA* genes identical to *B*. *elkanii* USDA 76^T a microsymbiont of soybean. There are several reports of genetically diverse *Bradyrhizobium* strains possessing common *nod* sequences (Andrews et al., 2018).

Based on *nifH* phylogeny strains ASDSOY and RMN shared close similarities with *B*. *ferriligni* CCBAU 51502^T (isolated from root nodule of tree *Erythrophloeum fordii* and to *B*. *elkanii* whereas strain USDA 3010 shared high similarity with sequences of *B*. *embrapense* CNPSo 2833^T, *B*. *tropiciagri* CNPSo 1112^T, *B*. *viridifuruti* SEMIA 690^T and microsymbionts of *C*. *fasciculata* from USA (Dorman and Wallace 2019) as observed for its analysis based on housekeeping genes. Except strain USDA 3010 all other *Bradyrhizobium* strains isolated from *C. fasciculata* in USA from this study were symbiotically and genetically distinct from previously reported strains of USA. Rathi et al. (2018) in their study reported about diversity in *sym* genes of *Bradyrhizobium* strains isolated from species of *Chamaecrista* growing in acidic soils in India, Africa and Brazil. In the present study, the *C*. *fasciculata-Bradyrhizobium* strains were also closely related to few (CPS1, CPS6 and CPS35) strains of *Bradyrhizobium* isolated from *C*. *pumila* (from acidic soils of Shillong,

India; Rathi et al., 2018) indicating that these (*B. arachidis*, *B. forestalis* and *B. shewense*) symbiotic genes have wide distribution both in the Old and the New World. This suggests that the *Bradyrhizobium* strains associated with species of *Chamaecrista* have genetically diverged in terms of their core genes but still harbouring closely related symbiotic genes in USA and India. In contrast to the strains from India, the *C. fasciculata-Bradyrhizobium* strains from this present study were symbiotically distinct from microsymbionts of *Chamaecrista* sp. reported from Africa and Brazil.

Bradyrhizobium symbionts can enhance growth of C. fasciculata and soybean

Earlier studies have isolated and phylogenetically characterized the *C. fasciculata* symbionts. However, the ability of these isolates to nodulate and enhance plant growth in axenic conditions is not known. It is possible that strains isolated from nodules may not reflect the most effective symbiotic strain and thus it is important to determine if the isolates can nodulate the host-legume in pure conditions. Our results show that all of the isolates formed pink nodules on *C. fasciculata* and significantly enhanced the growth of *C. fasciculata* in nitrogen-free conditions demonstrating their nitrogen-fixing symbiosis. Interestingly, all isolates also formed effective symbiosis with soybean, a distantly related and more advanced legume. The ability of these isolates to nodulate soybean could be due to extensive soybean cultivation in Mid-Western USA soils, an environment native to *C. fasciculata.*

***Bradyrhizobium spp* infects *Chamaecrista fasciculata* through both intercellular and intracellular mechanisms.**

Rhizobia infect legumes by either inter and intra-cellular mechanisms (Wang et al, 2018) (Ibáñez et al, 2016). However, these infection mechanisms have been studied in the model legumes such as *Medicago, Lotus* and soybean that belong to the phylogenetically recent Papilionoideae subfamily. Our results using a GUS and GFP marked bradyrhizobial strain and microscopy demonstrate that *C. fasciculata* is nodulated most likely through bacterial entry intercellularly via gaps in epidermal cells. However, bacteria were also observed in the root hairs, but these did not lead to infection thread mediated invasion of cortical cells. In contrast to the epidermal infection in plants grown aerobically, the *Bradyrhizobia* was mostly localized at the lateral root junctions when plants were grown in submerged conditions. This indicates that *C. fasciculata* can also be infected via “crack entry” under aquatic environments. More detailed imaging is needed to further confirm the infection mechanism that leads to nodule formation.

Conclusion:

The results of this study show that an ancestral legume *C. fasciculata* is exclusively nodulated by *Bradyrhizobium* spp. in soils of Mid-Western USA. These isolates form separate phylogenetic clusters than the other known *Bradyrhizobia* but are similar to some of the soybean symbionts. These isolates are able to form nitrogen-fixing symbiosis with both *C. fasciculata* and soybean. One of these isolates marked with GUS and GFP showed that *C. fasciculata* is infected through epidermal layers as well as by “crack entry”. The occurrence of diverse and region-specific *Bradyrhizobium* strains as microsymbionts of species of *Chamaecrista* growing in different continents indicates the promiscuous nature of this genus, which has helped it to co-evolve with local rhizobia and effectively fix nitrogen in widespread habitats.

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