

AN ABSTRACT OF THE THESIS OF

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Title: Utilization of rhizobia by the invasive legume, sericea lespedeza (*Lespedeza*
cuneata)

Abstract approved: _____

In the late 1800s, *Lespedeza cuneata* (sericea lespedeza) was introduced in North America. It became a noxious weed in Kansas due to its ability to invade and degrade native tallgrass prairie, and is currently one of the largest threats to existing tallgrass prairie. Because *L. cuneata* is a legume and is not native to North America, it must utilize existing rhizobia available in its environment because when a legume is transported to new locations as seed, the rhizobia that it normally utilizes are not transported with it. Previous studies have suggested that some legumes are highly specific, allowing a limited number of rhizobia species to form symbioses while other legumes are highly promiscuous. It was not clear if this was the case with *L. cuneata* in Kansas. In this study, the bacteria in the root nodules of *L. cuneata* were identified. Three species of rhizobia were found occupying the root nodules, *Bradyrhizobium liaoningense*, *Bradyrhizobium betae* and *Mesorhizobium caraganae*. The effects of *B. liaoningense* and *B. betae* on *L. cuneata* growth were tested in greenhouse grown plants. The results suggested that *L. cuneata* was relatively specific to the rhizobia species it allowed to infect its roots and

Bradyrhizobium could improve *L. cuneata* growth. The relationship between *M. caraganae* with *L. cuneata* is uncertain. Future control of this invasive plant might be developed by creating a mechanism to block the infection pathway between *L. cuneata* and *Bradyrhizobium* specifically.

UTILIZATION OF RHIZOBIA BY THE INVASIVE LEGUME, SERICEA

LESPEDEZA (*LESPEDEZA CUNEATA*)

A Thesis

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PREFACE

This thesis was prepared following the publication style of the *Biological Invasions*.

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CHAPTER 1

BACKGROUND

Nitrogen is a very important element for living organisms to produce DNA, amino acids and proteins. Like other organisms, plants require nitrogen for growth. Although nitrogen is abundant in the earth's atmosphere as dinitrogen gas (N_2), most organisms including plants cannot use it in this particular form directly. Some plants, particularly legumes, have evolved a way to increase the availability of nitrogen through a positive relationship with bacteria that can perform a process called biological nitrogen fixation. The critical players in this process are certain bacteria that reside in some plant roots and soil which can change atmospheric nitrogen into a form that plants can use. The most well-known kind of these bacteria is rhizobia. Rhizobia reside inside of a root nodule and enhance legume host growth by providing a usable form of nitrogen. This symbiotic relationship between legumes and rhizobia is advantageous for plant growth, especially in nitrogen-poor environments.

Rhizobia live independently in soils, but their ability to fix nitrogen is only realized when isolated in the unique environment provided by legume root nodules and supplemented with carbohydrates from the legume (Perret et al. 2000). Legumes solicit rhizobia from the soil environment by releasing an attractant (flavonoids) into the soil that turns on symbiotic recognition genes in each rhizobium (Ferguson et al. 2010; Desbrosses and Stougaard 2011). When the recognition genes are switched on, they initiate the synthesis of a series of compounds that act as “keys” to infect a root hair,

eliciting the formation of a root nodule to hold the rhizobia. Some rhizobia are able to infect a root hair, but lack the genes to create the “key” for root nodule formation in the legume while other rhizobia may initiate root nodule formation, but are unable to persist in the nodule (Perret et al. 2000).

Previous studies have shown that some legumes are promiscuous, allowing different strains of rhizobia to infect their roots and form nodules while other legumes, on the contrary, are highly selective of the strains of rhizobia they allow to infect their roots (Perret et al. 2000; Bala and Giller 2001; Tlusty et al. 2005). When a legume is removed from its native environment, the rhizobia it utilized are usually not transported with the seeds since rhizobia reside in the soil. Even closely related legumes within the same genus may not share similar rhizobia (Bala and Giller 2001). The few native legumes examined from North American grasslands have shown high variability in the number of rhizobial strains they utilize (Tlusty et al. 2005).

Non-native plants can either naturalize where they become a non-dominant member of the existing plant community, or they can become invasive where they crowd out and exclude existing plants. Accordingly, the invasive capacity of introduced legumes may be associated with the abundance and composition of rhizobial strains infecting their roots, and different microorganisms may play different roles in the invasion process of the plants (Yannarell et al. 2011). Invasive legumes may either be highly promiscuous in its acceptance of new rhizobia so that it can dominate in a new environment, or it may utilize existing strains to increase their nitrogen content and productivity.

In the late 1800s, *Lespedeza cuneata* (sericea lespedeza), a legume, was introduced in North America for wildlife cover and erosion control. Gradually, it has expanded its range and increased its population size to become a noxious weed in Kansas. *L. cuneata* can invade and degrade native tallgrass prairie and is currently one of the largest threats to existing tallgrass prairie because it reduces grass productivity and native species diversity (Eddy et al. 2003). Reduced productivity can have severe negative economic impacts on the ranching industry, while reduced diversity can hamper ecosystem function and make prairie ecosystems less resilient. Since *L. cuneata* is not native to North America, it must utilize existing rhizobia available in its new environments. Therefore, the key factor contributing to *L. cuneata*'s invasive capacity may be its symbiosis with rhizobia, which is still unknown.

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CHAPTER 2

UTILIZATION OF RHIZOBIA BY THE INVASIVE LEGUME, SERICEA LESPEDAZA (LESPEDAZA CUNEATA)

Abstract

In the late 1800s, *Lespedeza cuneata* (sericea lespedeza) was introduced in North America. It was listed as noxious weed in Kansas because of its invasive and detrimental impacts on native tallgrass prairie. As a legume, *L. cuneata* forms mutualistic relationships with rhizobia which are nitrogen fixing bacteria in addition to mycorrhizae. Since *L. cuneata* is introduced, it must utilize rhizobia available in its new environment. Previous studies suggested that some legumes are highly specific, allowing a limited number of rhizobia species to form symbioses while others are highly promiscuous. Therefore, the invasive capacity of *L. cuneata* might partly due to its symbiotic relationship with rhizobia available in its new habitats. The objective of my study was to evaluate rhizobia species that *L. cuneata* utilized and test the influence of these rhizobia on *L. cuneata* growth. *L. cuneata* individuals were collected from native and restored prairie sites. Bacteria in the nodules were cultured on YAM plates. Total DNA from each bacterium was extracted, amplified and sequenced. Two species of *Bradyrhizobium* were found frequently in the root nodules of field-collected *L. cuneata*. *B. liaoningense* matched *B. liaoningense* strain 2281, which is commonly associated with soybeans. *B. betae* matched *B. betae* strain PL7HG1, which has only been isolated from tumor-like

root formations on sugar beets. The influence of these two rhizobia on *L. cuneata* growth was tested in greenhouse experiments. Data showed that *B. betae* had the capacity to improve *L. cuneata* growth. Another rhizobium isolated was *Mesorhizobium caraganae*, matching *M. caraganae* strain CCBAU 11299, which is a novel rhizobial species nodulating *Caragana* spp. in China that was identified in 2008. The relationship between *M. caraganae* with *L. cuneata* is uncertain. Most of the rest bacteria were *Bacillus* bacteria, but their function is unknown. These results suggest that *L. cuneata* is relatively specific to the rhizobia species it allows to infect its roots. Agricultural activities, especially soybean production, may have facilitated its invasive success. The availability of the compatible rhizobia may be part of the reason *L. cuneata* has been invasive. The critical tipping point may be the amount of suitable rhizobia present in the soil. A targeted future control method for this invasive plant could be developed by creating a mechanism to block the infection pathway between *L. cuneata* and *Bradyrhizobium* spp.

Introduction

Nitrogen (N) is a necessary component for life because it is an integral component of nucleic acids, amino acids and proteins. N is abundant in the earth's atmosphere in the form of dinitrogen gas (N₂), but most organisms including plants cannot access it in this form directly. Rhizobia are one group of prokaryotic organisms that are able to access N₂ and convert it to biologically available N through biological N fixation. Some plants, particularly legumes, have evolved a mutualistic relationship with rhizobia to increase their N uptake.

Rhizobia live independently in soils and their N fixation ability is only realized when they are living in the unique environment provided by nodules, resulting in enhanced host growth (Perret et al. 2000). The nodule formation requires several signal exchanges between legumes and rhizobia. Legumes solicit rhizobia from the soil environment by releasing flavonoids as an attractant into the soil that may partially explain the specificity between legume hosts and rhizobia (Ferguson et al. 2010; Desbrosses and Stougaard 2011). The *nod* (nodulation) genes of rhizobia will be turned on to form a bacterial lipochitooligosaccharide with different side groups called Nod factors (Stougaard 2000). Rhizobial species with a broad specificity produce many different Nod factors. These factors' structure can determine whether a certain rhizobium will be recognized by a legume as a symbiont or a pathogen (Stougaard 2000; Ferguson et al. 2010). When these factors are received by the legume hosts, rhizobia are attached to the root hairs, forming

infection thread in the curl root hairs induced by Nod factors (Ferguson et al. 2010). The cells in the root cortex divide, forming the nodule to hold numerous rhizobia bacterioids of a single strain (Ferguson et al. 2010). Any signals missing in this procedure will lead to the failure of nodule formation (Ferguson et al. 2010; Reid et al. 2011). Other plant factors like auxin, cytokinin, ethylene, shoot-derived inhibitors and soil N levels also regulate nodulation process (Desbrosses and Stougaard 2011; Reid et al. 2011). Perret et al. (2000) suggested that some rhizobia are able to infect a root hair, but lack the genes to form root nodules while some rhizobia could initiate nodulation process, but are unable to persist within these nodules.

Legumes often fail at colonization in new environment where they lack their mutualistic partners (Parker 2001). Previous studies have suggested that some legumes are promiscuous, allowing different rhizobia strains to infect their roots and form nodules while other legumes are highly selective of rhizobia strains that they allow to infect their roots (Perret et al. 2000; Stougaard 2000; Bala and Giller 2001; Tlustý et al. 2005). Even closely related legumes within the same genus may do not share similar rhizobia (Bala and Giller 2001). Some promiscuous legumes find it easier to locate compatible symbionts in their new habitats, and some legumes can nodulate at lower rhizobial density to help them become well-established (Rodríguez-Echeverría et al. 2009). The few native legumes examined from North American grasslands have shown high

variability in the number of rhizobial strains they utilize (Tlustý et al. 2005). Little is known about how introduced legumes utilize rhizobia in their invasive range.

Introduced legumes can either naturalize where they become a non-dominant member of the existing plant community, or they can become invasive where they crowd out and exclude native plants. Invasive plants often experience more negative net soil biota effects in their native ranges than nonnative ranges because invasive species can escape from soil natural enemies and other negative effects of the whole soil community in their new habitats (Mitchell et al. 2006; Callaway et al. 2011). Invasive plants can modify their new soil environment, like elevating soil N, to facilitate their further invasion (Van Riper et al. 2010) and impede the growth of native plants by disrupting belowground mutualisms (Reinhart and Callaway 2006; Jordan et al. 2008; Rodriguez-Echeverria et al. 2012). Disturbances such as fire can also affect the plant-soil biota interactions where introduced plants can benefit more than native species (Carvalho et al. 2010). Microorganisms also play critical roles in the invasion process of the plants by their interaction with plants (Yannarell et al. 2011). Invasive plants may be able to benefit from the mycorrhizae network which has been established by native plants without the cost of maintaining this network (Reinhart and Callaway 2006). Most invasive plants can form mycorrhizal relationships and utilize them to facilitate the establishment of seedlings to compete with native species that also use the same mycorrhizae (Richardson et al. 2000). The relationship between rhizobia and legumes is

assumed to be mutualistic, but rhizobial strains vary in effectiveness of N fixation (Bender et al. 1989; Simms et al. 2006). Invasive plants may modulate resource allocation on individual nodules to save cost (Simms and Taylor 2002). When a legume is removed from its native environment, the rhizobia it utilized are usually not transported with the seeds since rhizobia typically reside in the soil. A nonnative plant could possibly find novel mutualist partners in its new habitat, allowing for its establishment (Reinhart and Callaway 2006). Accordingly, the invasive capacity of introduced legumes may be associated with the abundance and composition of rhizobial strains infecting their roots (Parker 2001; Rodriguez-Echeverria et al. 2012). Invasive plants may use existing rhizobia in local soils (Reinhart and Callaway 2006), or bring their native symbionts with them if transported as nursery plants (Weir et al. 2004). Therefore, invasive legumes may either be highly promiscuous in their acceptance of new rhizobia so that they can be successful in a new environment, or they may utilize specific compatible strains available in their new habitats to increase their N content and productivity.

In the late 1800s, a legume, *Lespedeza cuneata* (sericea lespedeza), was introduced into North America for wildlife cover and erosion control. Gradually, it has expanded its range and currently has become one of the largest threats to existing native tallgrass prairies in Kansas (Kansas Department of Agriculture, 2006). *L. cuneata* has detrimental impacts both environmentally and economically because it reduces native species diversity and grass productivity in addition to being unpalatable to livestock (Eddy et al.

2003). Since *L. cuneata* is an introduced legume thought to be originally introduced to the U.S. as seed, it must utilize existing rhizobia present in its new environment.

Therefore, the invasive capacity of *L. cuneata* may be associated with the abundance and composition of rhizobial strains present in soils and infecting its roots.

Even though studies of the symbioses between legumes and rhizobia have been ongoing for over 400 years, most of the current research has focused on crop species (i.e. alfalfa, soybeans, peas and beans) and the effectiveness of native rhizobia in inoculating these crops (Vasquez-Arroyo et al. 1998; Burdon et al. 1999; Sarr et al. 2005; Taurian et al. 2006). Less is known about how *L. cuneata*, an invasive legume, utilizes rhizobia in its invasive range. Previous studies have shown that introduced legumes sometimes use different rhizobia than native plants, and various *Lespedeza* spp., including *L. cuneata*, are promiscuous hosts for rhizobia in China (Weir et al. 2004; Gu et al. 2007; Yao et al. 2002). It is unclear if *L. cuneata* in North America also utilizes diverse rhizobia.

Given the unclear role that rhizobia play in their relationship with *L. cuneata* in its invasive ranges, I hypothesized that this invasive legume was highly promiscuous in its acceptance with new rhizobia because it should require a robust symbiosis with rhizobia to be highly invasive. In addition, the rhizobia present in *L. cuneata* root nodules should improve its growth. The objective of this study is to evaluate rhizobia strains infecting *L. cuneata* roots nodules in its invasive range and test the influence of these rhizobia on *L. cuneata* growth.

Materials and methods

Identification of rhizobia in field collected *L. cuneata* root nodules

Plant collection

All *L. cuneata* plants were collected at tallgrass prairie sites in Lyon and Greenwood Counties located in east-central Kansas. All sites were dominated by the warm-season grasses big bluestem (*Andropogon gerardii* Vitman), little bluestem (*Schizachyrium scoparium* [Michx.] Nash), indiagrass (*Sorghastrum nutans* [L.] Nash) and switchgrass (*Panicum virgatum* L.). Site types were either native or restored tallgrass prairies located in the Flint Hills region of Kansas. Restored sites included National Wildlife Refuge (FHNWR) in Hartford, KS, Ross Natural History Reservation (RNHR) near Americus, KS and four private landowner properties located in Lyon County (KD, SB RF, HB). Native tallgrass prairie sites included additional locations at FHNWR and HB and two distinct locations (MC1, MC2) in Greenwood County. Each site contained several dense patches of *L. cuneata*, some of which were treated with herbicide. Even with herbicide treatments, *L. cuneata* was still abundant at each site. Plants were collected between June 2012 and March 2013. At each site, 7 to 10 individuals of *L. cuneata* were sampled. For each plant collected, as much root mass as possible was excavated, approximately 30 cm from the edge of the plants to a 30 cm depth, to retain the majority of root system. Individual plants with the associated root ball were placed in 55 gallon plastic bags for transport back to the laboratory at Emporia State University.

Nodule collection and bacteria growth

Once back at the lab, each root ball was soaked in 48 L plastic container with water for 2 - 3 days to loosen soil around roots and root nodules. The soil was then washed from each plant. As many root nodules as possible were excised from the roots. Each nodule was soaked in sterilized distilled water for 1 hr (Tlusty et al. 2005) and submerged in 70% alcohol for 30 s (Vasquez-Arroyo et al. 1998). Then the nodules were sterilized for 15 min in bleach (6% sodium hypochlorite) followed by 5 rinses with distilled water (Weir et al. 2004). I cultured each nodule because each nodule only contains one strain of rhizobia, but one plant may have many different strains of rhizobia in all of its root nodules (Bala and Giller 2001). The nodules were determinate with spherical shape. Rhizobia were cultured on yeast mannitol agar (YAM) plates (Table 1). The plates were incubated at 28°C for 5 - 10 days. Each colony was amplified using YAM liquid medium. Pure isolates of bacteria were stored at -80°C in YAM liquid medium with 80% glycerol (4:1) until polymerase chain reaction (PCR) could be performed.

PCR amplification of 16s ribosomal DNA (rDNA) gene

Total DNA from each amplified bacteria isolate was extracted and amplified by PCR using rDNA primers from the 16S region of the rhizobia genome (Khbaya et al. 1998). One gene target region (16S) is required to determine bacteria species. 16S rRNA genes are similar throughout the bacterial world and contain highly conserved regions and regions vary in accordance with species and family. 16S rDNA sequence analyses

support the well-established subdivision of rhizobia into species and genera (Young and Haukka 1996). Primers used to target a conserved region of 16S rRNA genes were 8F (5' - AGAGTTTGATCCTGGCTCAG - 3') and 1492R (L) (5' - GGTTACCTTGTTACGACTT - 3') (Integrated DNA Technologies; San Diego, CA).

The reaction mixtures included 2.5 µl of each primer, 10 µl of template DNA, 10 µl of distilled water and 25 µl of Bullseye R-Taq DNA Polymerase Master Mix (Midsci™; St. Louis, MO). PCR was conducted under following conditions: Lid temperature was 94°C for 60 s with 29 cycles composed of denaturation at 94°C for 60 s, annealing at 48°C for 60 s and extension at 72°C for 90 s, followed by a final extension step for 5 min in an Eppendorf Thermal Cycler (Eppendorf®; Hauppauge, NY). Subsequently, each PCR product was run on an agarose gel (0.7%) to capture a pure DNA band from each isolate using a EC 105 model power supply (Apparatus Corporation; St. Petersburg, FL), at 110 volts for approximately 40 min (Fig 1). PCR products were visualized after electrophoresis using a UV Intensity Transilluminator (Fisher Scientific™; St. Louis, MO) and recovered using a Zymoclean™ Gel DNA Recovery Kit (ZYMO Research; Irvine, CA). Concentration of each PCR product was determined using a NANODROP 2000c Spectrophotometer (Thermo Scientific™; Pittsburgh, PA). The PCR products were sequenced by the University of Arkansas DNA Core Sequencing Facility (Little Rock, AR).

Sequence analyses

The identity of each isolated bacterium was determined by the Basic Local Alignment Search Tool (BLAST database, NCBI; Bethesda, MD), which contains known sequences of the organisms whose genomes have been identified. If multiple matches occurred for a single sequence, the bacteria species with highest score from the comparison results was considered. Only matches greater than 97% were considered as a positive identification.

Growth responses of *L. cuneata* with symbionts

Rhizobia inoculant preparation

Once the identity of the bacteria isolated was determined to be rhizobia, their symbiotic ability with *L. cuneata* was tested in greenhouse. Two species of rhizobia, *Bradyrhizobium liaoningense* and *Bradyrhizobium betae* were selected from the cultures stored from field-collected *L. cuneata* root nodules at the restored sites. Rhizobia were cultured on YAM plates for 5 - 7 days at 28°C and were amplified in YAM liquid medium at 28°C for 3 - 4 days to inoculate greenhouse grown *L. cuneata*. The concentration of each rhizobia species was determined using Bausch and Lomb Spectronic 20 Spectrophotometer at OD₄₆₀ (Bausch& Lomb®; Rochester, NY). The absorbance of *B. liaoningense* was 0.61 (~ 2.1 x 10⁹ colonies per milliliter) and of *B. betae* was 0.6 (~ 2.9 x 10⁹ colonies per milliliter).

L. cuneata seed collection and treatment

L. cuneata seeds were collected from the Bressner Range Research Unit, Yates Center, KS in late October 2008. Because *L. cuneata* seeds were difficult to germinate, the following treatments were used to enhance germination. All seeds were soaked for 1 hr in water brought to a boil and subsequently removed from heat source. Then the seeds were scarified using sand paper (500 grit).

Experimental design

The ability of isolated rhizobia to nodulate *L. cuneata* seedlings and their influences on *L. cuneata* growth was tested on plants grown in the Emporia State University greenhouse. *L. cuneata* plants were grown at temperatures ranging between 20 - 30°C and a 12 hr photoperiod. Fifty pots (0.95 liter) were planted with treated *L. cuneata* seeds for each rhizobia treatment. All the plants were grown in a planting mix of 50% coarse perlite (SUNSHINE®; Agawam, MA) and 50% medium vermiculite (SUNSHINE®). Initially, 5 - 7 seeds were sown in each pot to ensure each pot could contain one plant. A modified complete Hoagland's nutrient solution (Table 2) was applied according to the plants' moisture condition, approximately every 4 days (Hoagland and Arnon 1950). After 8 weeks, plants were culled to 2 seedlings per pot. Treatments including *B. liaoningense*, *B. betae* and the control group (no rhizobia) were applied to 50 randomly selected pots. Each pot was inoculated with 2 ml of corresponding rhizobia inoculum (Rodriguez-Echeverria et al. 2012). The non-inoculated control group received 2 ml of

YAM liquid medium devoid of any bacteria. Half of the pots within a single rhizobia treatment received 50 ml of a mycorrhiza spore solution prepared by combining 5 ml mycorrhizae spore powder (Hydro-Oranics™; Chico, CA) with 4 L of nutrient solution. Plants were harvested 32 weeks after germination and roots were carefully washed to remove the planting mix. Nodules were collected and counted. The identity of the rhizobia in the nodules was determined using the method previously described for identification of bacteria in field collected *L. cuneata* root nodules.

Plant measurements

The number of seeds, leaves and nodules per plant were counted. The root was separated from the shoot at the base of the crown. The length of the root and shoots were measured, and dry mass was also measured after drying at 55°C for 72 hr. Shoot mass ratio was calculated by the shoot mass divided by total plant mass. Root mass ratio was calculated by root mass divided by total plant mass. Shoot to root mass ratio was calculated by dividing shoot mass by root mass.

Mycorrhizal roots staining testing

Root tip sections from *L. cuneata* grown in greenhouse were placed in plastic cassettes and soaked in 10% hot KOH for 20 min. The root sections were then acidified using 2% HCl for 20 min. The root sections were stained using hot acid fuchsin in a 1:1:1 ratio of water, glycerin and lactic acid for 5 min. The cassettes containing these roots

were washed with sterile water several times to remove excess acid fuchsin and stored in water at 4°C for up to 2 weeks before observation. Roots were scanned using a microscope (Olympus America Inc.; Center Valley, PA) at 400x magnification for the presence of mycorrhizae. This method was also used for several randomly selected field collected *L. cuneata* plants to confirm that *L. cuneata* is mycorrhizal in the field.

Statistical analysis

Normality assessments and statistical tests were performed with SAS 9.2 software (SAS Institute Inc.; Cary, NC). A non-parametric two-sample t test ($\alpha = 0.05$) was used to determine site type differences in the proportion of successfully cultured nodules and the proportion of nodules containing rhizobia. The normality of data was tested using Shapiro-Wilk test ($\alpha = 0.05$) after outliers were removed. Total biomass, shoot to root mass ratio, leaf number, shoot mass, shoot length and root mass were transformed using a \log_{10} function while the shoot mass ratio was transformed using a quadratic polynomial function. A Two-way ANOVA ($\alpha = 0.05$) was used to evaluate the influence of rhizobia and mycorrhizae including an interaction on *L. cuneata* growth measures, like total biomass. No mycorrhizae were found in roots and the tests showed that no interactions between rhizobia and mycorrhizae on *L. cuneata* growth. Therefore, after confirmation that mycorrhizae application did not significantly affect any measured variables, non-mycorrhizal and mycorrhizae groups with the same rhizobia treatment were pooled. A one-way ANOVA ($\alpha = 0.05$) was used to test rhizobia treatments effects on *L. cuneata*

growth parameters. Once differences were established, I used a Tukey adjustment to distinguish between rhizobia treatments. A significance level of $\alpha = 0.05$ was used for all ANOVAs and mean separation tests. All ANOVA tests were performed using PROC ANOVA in SAS 9.2 (SAS Institute Inc.).

Results

Identification of rhizobia in field collected *L. cuneata* root nodules

The mean percentage of nodules from *L. cuneata* roots containing culturable bacteria collected from 6 restored sites and 4 native sites was $19.30 \pm 0.14\%$ and $20.22 \pm 0.12\%$, respectively (Fig. 2). No significant difference in the number of successfully cultured nodules was found between site types ($P = 1.000$). The mean percentage of nodules containing rhizobia from restored and native sites was $6.43 \pm 0.02\%$ and $0.72 \pm 0.11\%$, respectively, indicating that the percentage of nodules occupied by rhizobia from restored sites was significantly higher than native sites ($P = 0.009$) (Fig. 3).

The rhizobia found in nodules from restored sites matched *Bradyrhizobium liaoningense* strain 2281 (97 - 100% homology to GenBank NR 041785.1) and *Bradyrhizobium betae* strain PL7HG1 (99 - 100% homology to GenBank NR029104.1) in the BLAST database (Table 3, Appendix 1). Most of the rest of the nodules contained bacteria belonged to the genus *Bacillus*, which are common soil bacteria. At the native sites, only 7 nodules from 3 native sites contained rhizobia. Of those, 6 nodules came from 2 sites and contained *B. liaoningense*, the same rhizobia isolated from restored sites (Table 4). The other rhizobium isolated from a single nodule at one native site matched *Mesorhizobium caraganae* strain CCBAU 11299 (99% homology to GenBank NR044118.1) in the BLAST database (Appendix 1). Most of the rest of the nodules from the native sites contained bacteria in the genus *Bacillus*.

Growth responses of *L. cuneata* with symbionts

Mycorrhizae were present in the field collected roots of *L. cuneata*, but no mycorrhizae were found from any greenhouse grown *L. cuneata* roots. According to the two-way ANOVA, no interaction between rhizobia and mycorrhizae existed on any *L. cuneata* growth parameter measured so I pooled mycorrhizae treated and non-treated plants within the same rhizobia treatment together.

The plants were washed carefully to remove the soil mix and all the nodules were collected and counted. The mean nodule number of *B. betae* and *B. liaoningense* was 4.47 ± 0.57 and 2.21 ± 0.76 , respectively, and no nodules were found in the control group suggesting no cross contamination occurred between treatments (Table 5). A subset of plants from each rhizobia treatment was analyzed to determine the identity of the rhizobia in the nodules and for all plants tested, the nodules contained only the rhizobia with which they were treated. The analysis of the bacteria inside these nodules suggested no cross-contamination occurred between treatments. Plants receiving *B. betae* inoculant had significantly more nodules than either the *B. liaoningense* treatment or the control group ($F = 24.97, P < 0.001$).

No significant differences between the seed number and leaf number of *L. cuneata* occurred among the three treatments (Table 5). The plants treated with *B. betae* had a significantly longer shoot than the control group but not the *B. liaoningense* group ($F = 4.27, P = 0.019$). Root length was not significantly different between any of the treatments.

The rhizobia treatments did not significantly affect the total biomass of *L. cuneata* (Table 6). Root biomass also was not significantly different between rhizobia treatments. The plants treated with *B. betae* had significantly more shoot mass than the control group but not the plants inoculated with *B. liaoningense* ($F = 4.89, P = 0.012$).

Only the *B. betae* increased the mass ratios of *L. cuneata* compared to the control (Table 7). Shoot mass ratio and shoot to root mass ratio of *B. betae* was significantly higher than the control group ($F = 5.41, P = 0.008$; $F = 5.1, P = 0.007$) while root mass ratio was significantly lower than the control group ($F = 3.49, P = 0.038$), indicating that the plants treated with *B. betae* had greater allocation to shoot biomass.

Discussion

The invasive capacity of *L. cuneata* might partly due to its symbiotic relationship with rhizobia available in its new environment. Two *Bradyrhizobium* species were found in the root nodules of field-collected *L. cuneata*, and of the two, *B. betae* has the ability to significantly improve *L. cuneata* growth patterns, potentially contributing to its invasive capacity. At least one of the identified rhizobia, *B. liaoningense*, is a common inoculant of soybean roots, suggesting that previous or nearby agriculture practices could have also contributed to *L. cuneata*'s invasive success. In addition to rhizobia, *Bacillus* bacteria were found in root nodules, and may also contribute to *L. cuneata* growth.

Identification of rhizobia in field collected *L. cuneata* root nodules

According to the average percentage nodule number containing rhizobia isolated from restored and native grassland sites, I found nodules contained rhizobia more frequently at restored sites, even though the number of successfully cultured nodules was not different between site types (Fig. 2, 3). This suggested that *L. cuneata* had nearly the same proportion of bacteria-occupied nodules regardless of its location. On the contrary, the number of nodules containing rhizobia was nearly 10x higher in restored tallgrass prairie sites than native prairie, suggesting that there are more compatible rhizobia at restored sites for *L. cuneata*, and this may explain, at least in part, why *L. cuneata* plants at restored sites appeared more robust than plants at native sites. The nodule number occupied by rhizobia at both site types is not very high, suggesting that there are few

compatible rhizobia in the soil for *L. cuneata*. Therefore, *L. cuneata* may have the ability to nodulate at a lower compatible rhizobial density which may contribute to its invasive capacity.

Population density at each site was not measured because management and control strategies differed between sites regardless of site type. Some restorations of tallgrass prairie occurred up to 80 years ago, therefore *L. cuneata* may have been planted during early restoration activities and became well-established. Simms and Taylor (2002) suggested that legumes might be infected by rhizobial strains that do not fix N effectively, but signal reciprocity exists between the legume and rhizobia where the legume reduces allocation of resources to these non-beneficial rhizobia. Therefore, some legumes might detect N flux from a nodule as a cue to evaluate rhizobia quality which will help plants limit the costs of associating with ineffective rhizobia (Simms and Taylor 2002). One mechanism some legumes use to control ineffective rhizobia development is restricting nodule size, suggesting that these legumes allocate more resources to higher quality rhizobia (Simms and Taylor 2002; Sprent 2003). Nodule size control mechanism might also include partial alteration of oxygen supply (Kiers et al. 2003). I did not measure nodule size, but the nodules that contained rhizobia appeared to be larger. Regardless of the site type, many nodules were empty or unculturable, indicating that there might be other rhizobia present in the soil could induce nodule formation on *L. cuneata* roots but could not persist within the nodules due to incompatible genes or signals (Sprent and

James 2007). By restricting nodule size of ineffective rhizobia and rejecting other incompatible rhizobia, *L. cuneata* could utilize rhizobia more effectively and save the costs of mutualisms.

The rhizobia presented in *L. cuneata* root nodules at restored sites were *Bradyrhizobium liaoningense* and *Bradyrhizobium betae* (Table 3), both of which are slow-growing rhizobia (Sprent and James 2007). The *B. liaoningense* likely originated in China (Yao et al. 2002), and is commonly associated with *Glycine max* (L.) Merrill (soybean) root nodules (Yang and Zhou 2008), but may also form symbiotic relationships with other legumes.

The *B. betae* was first found to initiate tumor-like root formations on some varieties of *Beta vulgaris* (sugar beet) in northern Spain and its N-fixation capacity is unknown (Rivas et al. 2004). The origin of *B. betae* is unknown. It may have been transported by other legumes, or it may be a native species enhanced by nearby agricultural practices at restored sites.

Simms et al. (2006) found that larger root nodules on *Lupinus arboreus* contained more cells infected by *Bradyrhizobium*, suggesting that *Bradyrhizobium* were probably effective rhizobia that could benefit *L. cuneata* (Bender et al. 1985).

At the native locations, only seven root nodules contained rhizobia. Among the 7 rhizobia, 6 were *B. liaoningense*, the same rhizobia most commonly found at restored

sites. The remaining rhizobium isolated was *Mesorhizobium caraganae*, which is a novel rhizobial species nodulating *Caragana* spp. in China that was identified in 2008 (Guan et al. 2008). Its appearance may be due to contamination since only a single nodule contained it, or it may have been transported by other legumes. The relationship between *M. caraganae* and *L. cuneata* is uncertain.

Most of the rest bacteria isolated from root nodules of *L. cuneata* at both site types were various *Bacillus* species that are common soil bacteria. Their appearance may be due to contamination during the culturing process, or perhaps other rhizobia present in the soil were capable of initiating the nodulation process, but did not infect the nodules because of their incompatibility. Contamination seems unlikely because of the large difference in rhizobia present in nodules between site types. In addition, regardless of the site type, seven *Bacillus* species were found multiple times at different sites. Other *Bacillus* bacteria, such as *Bacillus radicumicola*, have been shown to cause nodulation (Sprent and James 2007). Therefore, the *Bacillus* bacteria found in this study may have the same ability to form nodules or infect the empty nodules formed by incompatible rhizobia. The functional relationship between the *Bacillus* bacteria identified in this study and *L. cuneata* is still unknown. Additional studies are needed to confirm if the *Bacillus* bacteria identified in this study are nodulating, as well as their potential function is within the nodule.

These results do not support my hypothesis that *L. cuneata* is a promiscuous legume

because only 3 rhizobia species were identified in the nodules, suggesting that *L. cuneata* is relatively conservative with the rhizobia species it allows to infect its roots. Specificity was found to be an important mechanism by which legumes could regulate rhizobia nodulation to save resources and be more effective (Simms and Taylor 2002). Since most nodules contained were *B. liaoningense* which is commonly associated with soybeans, the agricultural production of soybeans may have introduced the necessary rhizobia for *L. cuneata*. Agricultural activities may have introduced or enhanced *B. betae* for *L. cuneata*. The availability of these compatible rhizobia may be part of the reason that why *L. cuneata* has been able to shift from being a non-native, naturalized member of plant communities to a highly invasive component of grassland communities. The critical tipping point may be the amount of suitable rhizobia present in the soil. This specificity between *L. cuneata* and rhizobia suggests that a targeted future control method for this invasive plant could potentially be developed by creating a mechanism to block the infection pathway between *L. cuneata* and *Bradyrhizobium* spp.

Growth responses of *L. cuneata* with symbionts

The greenhouse experiment showed the influence of *B. liaoningense* and *B. betae* on *L. cuneata* growth and supports my hypothesis that the rhizobia in *L. cuneata* root nodules can improve its growth. Based on microscopic observation of *L. cuneata* roots, no mycorrhizae were found in plants that received a treatment of mycorrhizal spores indicating the amount of mycorrhizae applied was insufficient for infection, or the

mycorrhizae species in the commercial mix was incompatible with *L. cuneata*. *L. cuneata* does associate with mycorrhizae because mycorrhizae were found in the roots of field-collected *L. cuneata*. In addition, Sprent and James (2007) showed that most legumes, including *L. cuneata*, have the potential to associate with arbuscular mycorrhizae. Both *B. liaoningense* and *B. betae* could induce nodule formation and infect into the nodules, but *B. betae* was twice as effective at inducing nodulation than *B. liaoningense*, suggesting that *B. betae* may have greater capacity for nodule formation on *L. cuneata* than *B. liaoningense*. Only *B. betae* significantly influenced *L. cuneata* growth patterns. *B. betae* treatment caused significantly higher shoot length, shoot mass, shoot mass ratio, shoot to root mass ratio, and a lower root mass ratio than the non-rhizobial control group, indicating that *L. cuneata* treated with *B. betae* allocates more resources to shoot growth than root growth because *B. betae* may provide N for *L. cuneata* growth to save the cost of developing root system. On the contrary, *L. cuneata* without rhizobia allocates a greater proportion of mass to root likely to acquire sufficient N from the soil. Higher shoot allocation with rhizobia helps *L. cuneata* gain more light and shade its neighbors which also contributes to its invasive capacity. *B. liaoningense* did not improve *L. cuneata* growth significantly, but it had similar trend ($P < 0.1$) for shoot length growth and shoot mass growth, suggesting that it may help *L. cuneata* allocate resources to shoot growth but is not as effective as *B. betae*. Given the frequency that field collected nodules contain *B. liaoningense* and its similar trend on shoot growth, it is likely that these two rhizobia species work together to improve *L. cuneata* growth.

Many possible factors likely contribute to the invasive capacity of *L. cuneata*. First, *L. cuneata* is relatively conservative in the rhizobia species it utilizes. Only 3 rhizobia species were found to form mutualisms with it. These compatible rhizobia may improve the N status of *L. cuneata*. Other rhizobia in the soil may be able to initiate the nodulation process, but are unable to persist within the nodules. *Bacillus* bacteria occupied a large proportion of the root nodules, but their function is unknown. Previous studies have found that some *Bacillus* bacteria, like *Bacillus megaterium* S49, can enhance root nodule formation and increase plant root weight and total biomass (Chanway et al. 1990; Srinivasan et al. 1997). Enzymes produced by some soil bacteria and fungi might also improve nodule formation (Ghazal and Azzazy 1994). Additionally, *L. cuneata* appears to be most successful at restoration sites, possibly as a result of a modified soil microbial community and reduced plant competition. This study helps improve the understanding of why some introduced legumes either naturalize or become highly invasive. By increasing our understanding of why *L. cuneata* is able to succeed in new environments, and the role of symbiotic rhizobia play in the success of it, new control measures may be found.

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Table 1 Ingredients for Yeast Mannitol Agar medium (1 L) used to culture rhizobia from root nodules of *L. cuneata*

Ingredients	Amount (g)
Mannitol	10.0
Dispotassium Phosphate	0.4
Magnesium Sulphate	0.2
Sodium Chloride	0.2
Iron Chloride	0.1
Yeast Extract	0.5
Agar	15.0

Table 2 Composition of the modified complete Hoagland's nutrient solution (1L) applied to *L. cuneata* grown in the Emporia State University greenhouse

Ingredients	Volume(ml)
1M KNO ₃	2
1M KH ₂ PO ₄	2
1M Ca(NO ₃) ₂	3
1M MgSO ₄	2
Microelements	1
Iron	1

Table 3 Identity of bacteria found from each nodule on *L. cuneata* roots collected from restored sites

Bacteria Isolated from Nodules	Sites ^a	Nodule number	Range ^b
<i>Bradyrhizobium liaoningense</i>	SB	9	99-100
	KD	3	99-100
	FHNWR	6	100
	RF	5	99-100
	RNHR	4	99-100
	HB	7	97-100
<i>Bradyrhizobium betae</i>	RNHR	5	98-100
	SB	1	100
<i>Bacillus sp. LMG 20238</i>	SB	5	100
	FHNWR	1	
<i>Bacillus megaterium</i>	KD	1	100
	SB	3	99-100
<i>Bacillus luciferensis</i>	KD	2	100

Table 3 continued

	RNHR	1	100
<i>Bacillus acidiceler</i>	RF	1	100
	RNHR	1	
<i>Rubidibacter lacunae</i>	SB	2	99-100
<i>Brevibacillus brevis</i>	SB	2	100
<i>Bacillus subtilis subsp. subtilis</i>	SB	2	100
<i>Bacillus flexus</i>	RNHR	1	100
<i>Bacillus funiculus</i>	SB	1	100
<i>Brevibacillus panacihumi</i>	KD	1	100
<i>Arthrobacter phenanthrenivorans</i>	KD	1	100

^a Site stands for the location of each bacterium that was isolated. ^b Range is the match range found in BLAST database

Table 4 Identity of bacteria found from each nodule on *L. cuneata* roots collected from native sites

Bacteria Isolated from Nodules	Sites ^a	Nodule number	Range ^b
<i>Bradyrhizobium liaoningense</i>	MC1	1	99
	MC2	2	99
	HB	3	98-99
<i>Mesorhizobium caraganae</i>	HB	1	99
<i>Bacillus megaterium</i>	MC1	1	97
	MC2	8	99-100
<i>Bacillus nealsonii</i>	FHNWR	6	100
<i>Bacillus drentensis</i>	MC1	4	99
	MC2	2	99
<i>Bacillus sp. LMG 20238</i>	FHNWR	1	100
	MC1	1	100
	MC2	4	99-100
<i>Bacillus luciferensis</i>	FHNWR	3	100

Table 4 continued

<i>Bacillus acidiceler</i>	FHNWR	2	100
<i>Bacillus subtilis subsp. subtilis</i>	MC1	2	98-99
<i>Bacillus bataviensis</i>	MC2	1	99
<i>Sphingobium yanoikuyae</i>	MC2	1	99
<i>Sphingomonas koreensis</i>	MC1	1	99
<i>Arthrobacter phenanthrenivorans</i>	MC2	1	99
<i>Pseudomonas psychrotolerans</i>	MC1	1	99

^a Site stands for the location of each bacterium that was isolated. ^b Range is the match range found in BLAST database

Table 5 Mean nodule number, seed number, leaf number, shoot length and root length of greenhouse grown *L. cuneata* plants inoculated with *B. liaoningense*, *B. betae* or no rhizobia (control). Values are mean \pm SE. Significant differences between treatments are indicated by different letters (a, b, c)

Treatment	<i>n</i>	Nodule number	Seed number	Leaf number	Shoot length (cm)	Root length (cm)
<i>B. betae</i>	17	4.47 \pm 0.57a	3.77 \pm 1.54	85.41 \pm 10.64	15.34 \pm 2.09a	20.39 \pm 1.15
<i>B. liaoningense</i>	14	2.21 \pm 0.76b	2.25 \pm 1.52	85.50 \pm 23.22	14.91 \pm 1.49ab	21.01 \pm 1.13
Control	22	0 c	0.5 \pm 0.31	58.18 \pm 6.51	10.42 \pm 1.03b	21.89 \pm 1.01

Table 6 Mean shoot mass, root mass and total mass of greenhouse grown *L. cuneata* plants inoculated with *B. liaoningense*, *B. betae* or no rhizobia (control). Values are mean \pm SE. Significant differences between treatments are indicated by different letters (a, b, c)

Treatment	<i>n</i>	Shoot mass (g)	Root mass (g)	Total mass (g)
<i>B. betae</i>	17	0.26 \pm 0.03a	0.12 \pm 0.02	0.38 \pm 0.05
<i>B. liaoningense</i>	14	0.23 \pm 0.03ab	0.13 \pm 0.02	0.35 \pm 0.04
Control	22	0.15 \pm 0.01b	0.11 \pm 0.01	0.28 \pm 0.06

Table 7 Mean shoot mass ratio, root mass ratio and shoot root mass ratio of greenhouse grown *L. cuneata* plants inoculated with *B. liaoningense*, *B. betae* or no rhizobia (control).

Values are mean \pm SE. Significant differences between treatments are indicated by different letters (a, b, c)

Treatment	<i>n</i>	Shoot mass ratio	Root mass ratio	Shoot to root mass ratio
<i>B. betae</i>	17	0.68 \pm 0.02a	0.32 \pm 0.02a	2.41 \pm 0.30a
<i>B. liaoningense</i>	14	0.64 \pm 0.03ab	0.36 \pm 0.03ab	2.04 \pm 0.21ab
Control	22	0.57 \pm 0.02b	0.40 \pm 0.02b	1.46 \pm 0.10b

Fig. 1 Amplification of 16S rRNA genes isolated from bacteria in the root nodules of *L. cuneata*. This is a representative agarose gel of my PCR products. Lane M is DNA ladder maker; Lane 1- 4 contain 4 PCR amplifications of approximately 1500 bp for 16S rRNA genes.

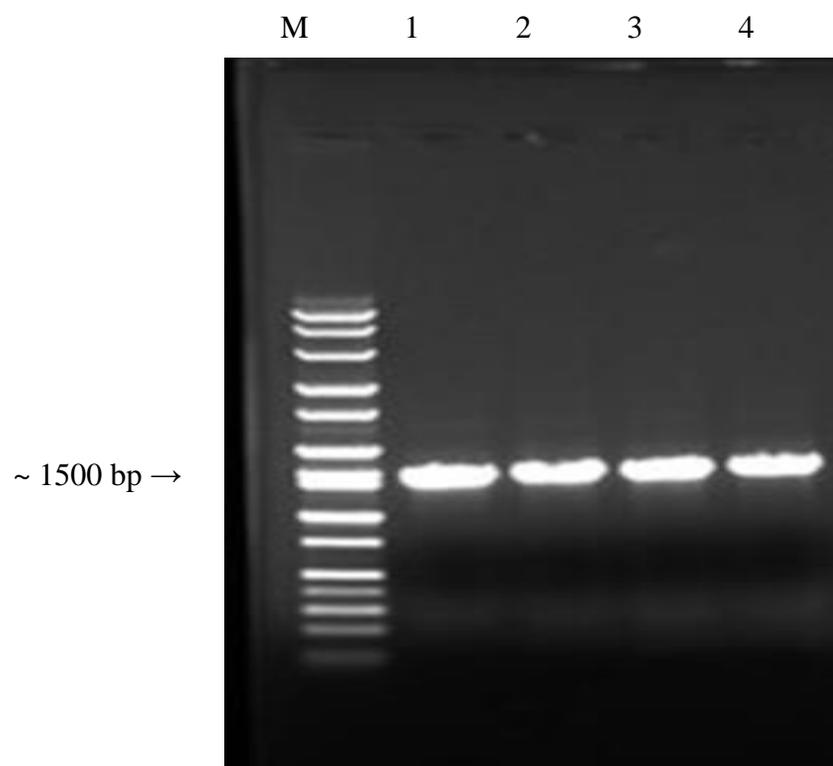


Fig. 2 Percentage of nodules containing culturable bacteria from *L. cuneata* roots. Plants were collected from 6 restored sites (FHNWR, RNHR, KD, SB RF, HB) and 4 native sites (FHNWR, HB, MC1, MC2) in Kansas. Bars are means ($n_1 = 6$, $n_2 = 4$) \pm standard deviation. No significant differences were found between site types ($P = 1.000$).

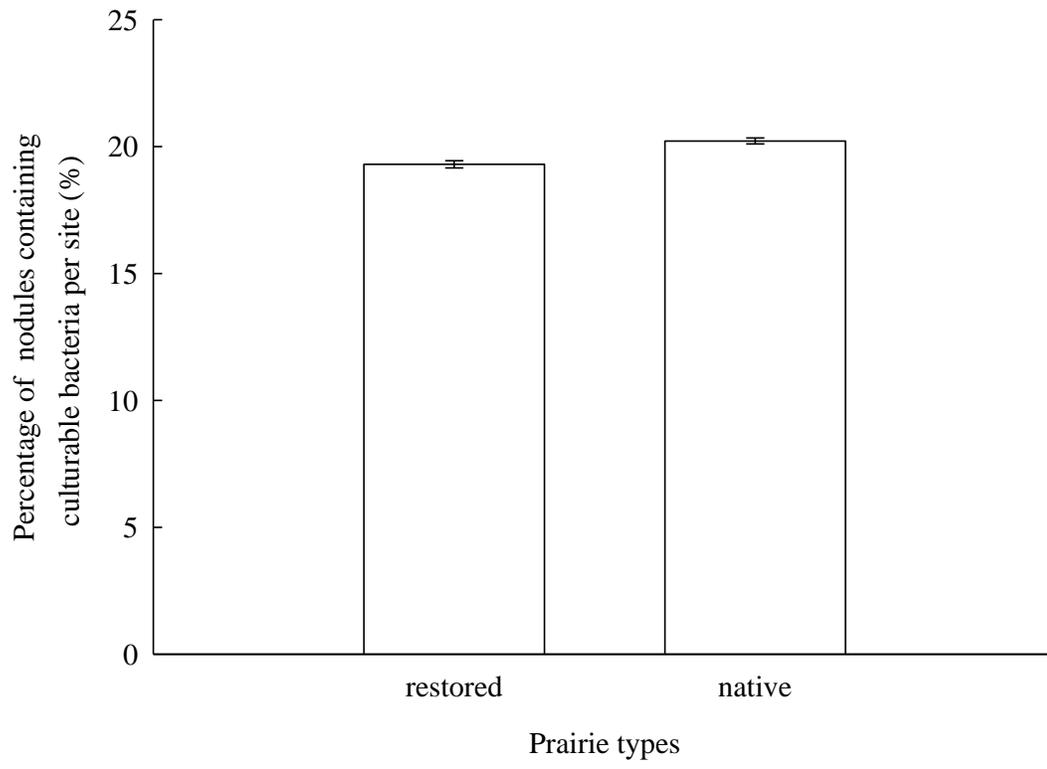
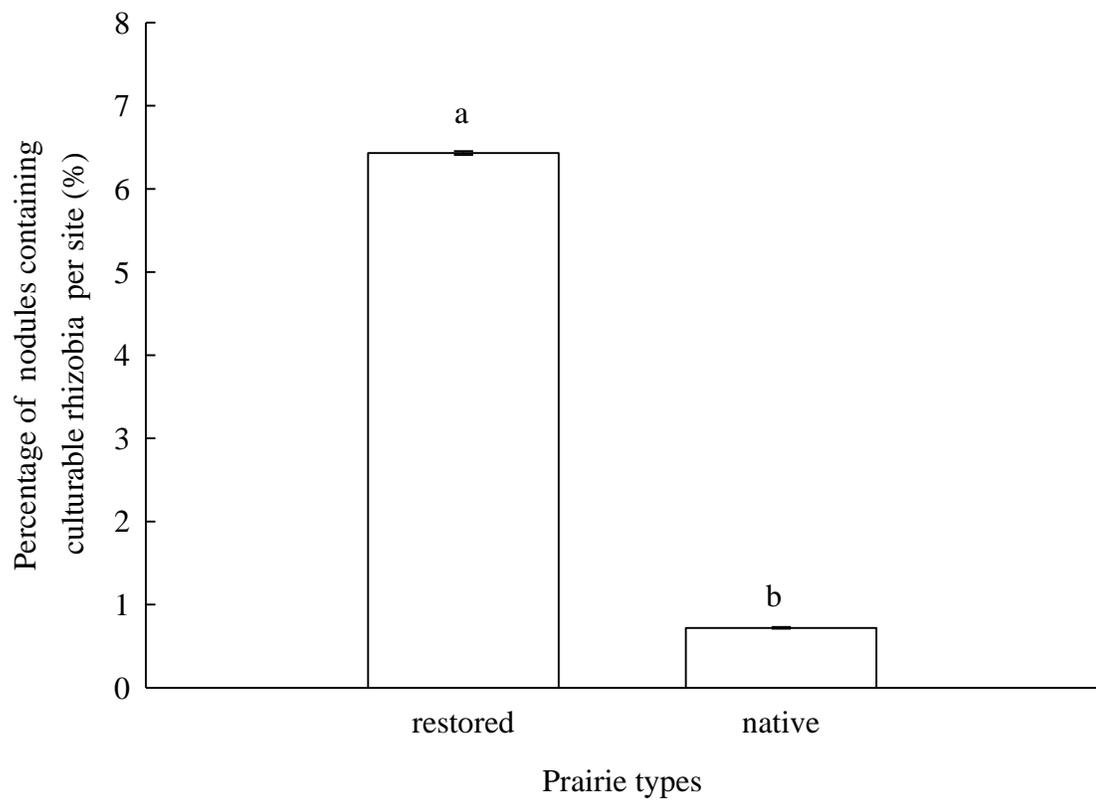


Fig. 3 Percentage of nodules containing culturable rhizobia from *L. cuneata* roots. Plants were collected from 6 restored sites (FHNWR, RNHR, KD, SB RF, HB) and 4 native sites (FHNWR, HB, MC1, MC2) in Kansas. Bars are means ($n_1 = 6$, $n_2 = 4$) \pm standard deviation. Significant difference ($P = 0.009$) between them are represented by bars with different lowercase letters a, b.



Appendix 1. Representative DNA sequences of bacteria identified in this study

***Bradyrhizobium liaoningense* (100% homology to GenBank NR 041785.1)**

5'-TAGCGCACCGTCTTCAGGTAAAGCCAACCTCCCATGGTGTGACGGGCGGTGT
 GTACAAGGCCCGGGAACGTATTCACCGTGGCGTGCTGATCCACGATTACTAG
 CGATTCCAACCTTCATGGGCTCGAGTTGCAGAGCCCAATCCGAACTGAGACGG
 CTTTTTGAGATTTGCGAAGGGTCGCCCTTAGCATCCCATTGTCACCGCCATT
 GTAGCACGTGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCC
 CCACCTTCCTCGCGGCTTATCACCGGCAGTCTCCTTAGAGTGCTCAACTAAAT
 GGTAGCAACTAAGGACGGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATC
 TCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCCAGGCTCCGAA
 GAGAAGGTCACATCTCTGCGACCGGTCCTGGACATGTCAAGGGCTGGTAAGG
 TTCTGCGCGTTGCGTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCC
 CGTCAATTCCTTTGAGTTTTAATCTTGCAGCCGTAATCCCCAGGCGGAATGCT
 TAAAGCGTTAGCTGCGCCACTAGTGAGTAAACCCACT-3'

***Bradyrhizobium betae* (100% homology to GenBank NR029104.1)**

5'-TAAAACCAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC
 GTATTCACCGTGGCGTGCTGATCCACGATTACTAGCGATTCCAACCTTCATGGGC
 TCGAGTTGCAGAGCCCAATCCGAACTGAGACGGCTTTTTGAGATTTGCGAAGG
 GTCGCCCTTAGCATCCCATTGTCACCGCCATTGTAGCACGTGTGTAGCCCAGC

CCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCGCGGCTTATCA
 CCGGCAGTCTCCTTAGAGTGCTCAACTAAATGGTAGCAACTAAGGACGGGGGT
 TCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAG
 CCATGCAGCACCTGTGTTCCAGGCTCCGAAGAGAAGGTCACATCTCTGCGACC
 GGTCCTGGACATGTCAAGGGCTGGTAAGGTTCTGCGCGTTGCGTCGAATTAAA
 CCACATGCTCCACCGCTTGTGCGGGCCCCGTC AATTCCTTTGAGTTTTAATCT
 TCGGACCGTACTCCCCAGGCGGAATGCTTAAAGCGTTAGCTGCGCCACTAGTG
 AGTAAACCCACTAACGGCTGGCATTTCATCGTTTACGGCGTGGACTACCAGGGT
 ATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGCGTCAGTATCGGGCCA
 GTGAGCCGCCT-3'

***Mesorhizobium caraganae* (99% homology to GenBank NR044118.1)**

5'-AGCGCCTTCGGGTAAAACCAACTCCCATGGTGTGACGGGCGGTGTGTACAA
 GGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCC
 AACTTCATGCACTCGAGTTGCAGAGTGCAATCCGAACTGAGATGGCTTTTGG
 GATTAGCTCGACCTCGCGGTCTCGCTGCCACTGTCACCACCATTGTAGCACG
 TGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTC
 TCTCGGCTTATCACCGGCAGTCCCCTTAGAGTGCCCAACTTAATGCTGGCAACT
 AAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
 AGCTGACGACAGCCATGCAGCACCTGTCACCGGTCCAGCCGAACTGAAGGTT
 CCCATCTCTGGAAACCGCGACCGGGATGTCAAGGGCTGGTAAGGTTCTGCGC

GTTGCTTCGAATTAACACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTC
CTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGGAAGCTTAATGCGTTA
GCTGCGCCACCGACAAGTAAACTTGCCAACGGCTAGCTTCCATCGTTTACAGC
GTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGC
GTCAGTACCGAGCCAGTGAGCCGCCTTCGCCACTGGTGTTCCCTCCGAATATCT
ACGAATTCACCTCTACACTCGGAATTTCCACTCACCTCTCTCGGACTCGAGAT
ACCCAGTATCAAAGGCAGTTCCCGGGGTTGAGCCCCGGGATTACCCCTGACT
TAAATATCCCGCCTACGTGCGCTTTACGCCAGTAATTCCGAACAACGCTAGCC
CCTTCGTTATTACCGCGCCTGCTGGCACGAAGTAGGCCGGGCTCTCGTACG-3'

Bacillus sp. LMG 20238 (100% homology to GenBank NR042083.1)

5'-TCGGGTGTTACAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGG
GAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCAT
GCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGCTTTATGGGATTTCGC
TTACCTTCGCAGGTTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGC
CCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTG
TCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA
CAACCATGCACCACCTGTCACTCTGTCCCCGAAGGGGAAAGCCCTATCTCTA
GGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATT
AAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCA

GCCTTGCGGCCGTACTCCCCAG-3'

***Bacillus megaterium* (100% homology to GenBank NR043401.1)**

5'-TACGGTTACTCCACCGACTTCGGGTGTTACAACTCTCGTGGTGTGACGGG
 CGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATT
 ACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAG
 AATGGTTTTATGGGATTGGCTTGACCTCGCGGTCTTGCAGCCCTTTGTACCATC
 CATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCAT
 CCCACCTTCCTCCGGTTTGTACCCGGCAGTCACCTTAGAGTGCCCAACTAAA
 TGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATC
 TCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCCGA
 AGGGGAACGCTCTATCTCTAGAGTTGTCAGAGGATGTCAAGACCTGGTAAGGT
 TCTTCGC-3'

***Bacillus luciferensis* (100% homology to GenBank NR025511.1)**

5'-TTCGGGTGTTACAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCG
 GGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGTGATTCCGGCTTCA
 TGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATAGTTTTCTGGGATTAG
 CTCCACCTCGCGGTCTTGCAACCCTTTGTACTATCCATTGTAGCACGTGTGTAG
 CCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTT
 GTCACCGGCAGTCACCTTAGAGTGCCCAACTAAATGCTGGCAACTAAGATCAA

GGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG
ACAACCATGCACCACCTGTCACTCTGTCCCCGAAGGGAACGCCCTATCTCTAG
GGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTA
AACCACATGCTCCACCACTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAG
TCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTA
AAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACAGCGTGGACTACC
AGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTA-3'

***Bacillus acidiceler* (100% homology to GenBank NR043774.1)**

5'-ACCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTG
TACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGTG
ATTCCGGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATAGTTT
TCTGGGATTAGCTCCACCTCGCGGTCTTGCAACCCTTTGTACTATCCATTGTAG
CACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACC
TTCCTCCGGTTTGTACCGGCAGTCACCTTAGAGTGCCCAACTAAATGCTGGC
AACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAC
ACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCGAAGGGAACG
CCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTT
GCTTCGAATTAAACCACATGCTCCACCACTTGTGCGGGCCCCCGTCAATTCCTT
TGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAAC
TTCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGC-3'

***Bacillus subtilis subsp. subtilis* (100% homology to GenBank NR027552.1)**

5'-CTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGAC
GGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCG
ATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACT
GAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCT
GTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGT
CATCCCCACCTTCCTCCGTTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACT
GAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGG-3'

***Bacillus flexus* (100% homology to GenBank NR024691.1)**

5'-AAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCA
CCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTT
GCAGCCTACAATCCGAACTGAGAATGGTTTTATGGGATTGGCTTGACCTCGCG
GTCTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAA
GGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGTTTTGTCACCGGCAGT
CACCTTAGAGTGCCCAACTAAATGCTGGCAACTAAGATCAAGGGTTGCGCTCG
TTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCAC
CACCTGTCACTCTGTCCCCGAAGGGGAACGCTCTATCTCTAGAGTTGTCAGA
GGATGTCAAGACCTGG-3'

***Bacillus funiculus* (99% homology to GenBank NR028624.1)**

5'-AGGTTACCCACCGACTTCGGGTGTTACAACTCTCGTGGTGTGACGGGCG
 GTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTAC
 TAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAA
 CGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCGACTCTTTGTACCGTCCA
 TTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC
 CCACCTTCCTCCGGTTTGTACCGGCAGTCACCTTAGAGTGCCCAACTTAATG
 ATGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC
 ACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCGAAGG
 GGAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTC-3'

***Bacillus nealsonii* (100% homology to GenBank NR044546.1)**

5'-GTTACCCACCGACTTCGGGTGTTACAACTCTCGTGGTGTGACGGGCGGT
 GTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTA
 GCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATG
 GTTTTATGGGATTGGCTCGACCTCGCGGTTTTGCTGCCCTTTGTACCATCCATT
 GTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCC
 CACCTTCCTCCGGTTTGTACCGGCAGTCACCTTAGAGTGCCCAACTAAATGC
 TGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCA
 CGACACGAGCTGACGACAACCATGCACCACCTGTCATCCTGTCCCCGAAGG
 GGAACGTCCTATCTCTAGGATTGTCAGGAGATGTCAAGACCTGGTAAGGTTC

TCGCGTTGCTTCGAATTAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTC
 AATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAAT
 GCGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCG
 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGC
 GC-3'

***Bacillus drentensis* (99% homology to GenBank NR029002.1)**

5'-TACGGTTACCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGG
 CGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATT
 ACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAG
 AATGGTTTTATGGGATTGGCTAAACCTCGCGGTCTTGCAGCCCTTGTACCATC
 CATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCAT
 CCCACCTTCCTCCGGTTTGTACCGGCAGTCACCTTAAAGTGCCCAACTAAA
 TGCTGGCAACTAAAATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATC
 TCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCCGA
 AGGGGAACGTCCTATCTCTAGGAGTGTCAGAGGATGTCAAGACCTGGTAAGGT
 TCTTCGCGTTGCTTCGAATTAACCACATGCTCC-3'

***Bacillus bataviensis* (99% homology to GenBank NR036766.1)**

5'-CACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACA
 AGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTC

CGGCTTCATGTAGGCCAGTTGCAGCCTACAATCCGAACTGAGAATGGTTTTATG
 GGATTGGCTAGGCCTCGCGGCTTTGCTGCCCTTTGTACCATCCATTGGAGCACG
 TGTGTAGCCCAAGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCT
 CCGGTTTGTACCGGCAGTCACCTTAAAGTGCCCAACTGAATGCTGGCAACTA
 AAATCAAGGGTTGCGCTCGTTGCGG-3'

***Rubidibacter lacunae* (99% homology to GenBank NR044104.1)**

5'-TGACGGGCGGTGTGTACAAGGCCCGGGAACGAATTCACCGCCGTATGGCTG
 ACCGGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTACAAT
 CCGAACTGAGGACGGGTTTTTGGAGTTAGCTCACCTCGCGGGATCGCGATCC
 TTTGTCCCGTCCATTGTAGCACGTGTGTGCGCCAGGGCATAAGGGGCATGATG
 ACTTGACGTCATCCTCACCTTCCTCCGGCTTATCACCGGCAGTCTGCTCAGGGT
 TCCAAACTCAACGTTGGCAACTAAACACGAGGGTTGCGCTCGTTGCGGGACT
 TAACCCAACACCTTACGGCACGAGCTGACGACAGCCATGCACCACCTGTGTCC
 GCGTTCCCGAAGGCACCCCTCTCTTTCAAGAGGATTCGCGGCATGTCAAGCCC
 TGGTAAGGTTCTTCGCTTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGC
 GGGCCCCGTCAATTCCTTTGAGTTTCATTCTTGCGAACGTACTCCCCAGGCG
 GGATACTTAACGCGTTAGCTACAGCATTGCACGGGTCGATACGCACAGCGCCT
 AGTATCCATCGTTTACGGCTAGGACTACTGGGGTATCTAATCCCATTGCTCCCC
 TAGCTTTCGTCTCTCAGTGTCAGTGTCGGCCCAGCAGAGTGCTTTCGCCGTTG
 GTGTTCTTTCCGATCTCTACGCATTTACCGCTCCACCGGAAATTCCCTCTGCC

CCTACCGTACTCCAGCTTGGTAGTTT-3'

***Brevibacillus brevis* (100% homology to GenBank NR041524.1)**

5'-CCTTGCGGTTACCTCACCGACTTCGGGTGTTGCAAACCTCCCGTGGTGTGAC
GGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCG
ATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTG
AGATTGGTTTTAAGAGATTGGCGTCCTCTCGCGAGGTAGCATCCCGTTGTACCA
ACCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGT
CATCCCCGCCTTCTCCGTCTTGTCGACGGCAGTCTCTCTAGAGTGCCCAACT
GAATGCTGGCAACTAAAGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAAC
ATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCGCTGCCCC
GAAGGGAAGCTCTGTCTCCAGAGCGGTCAGCGGGATGTCAAGACCTGGTAAG
GTTCTTCGCGTTGCTTCGAATTAACCACATGCTCCACCGCTTGTGCGGGCCCC
CGTCAATTCCTTTGAGTTTCACTCTTGCGAGCGTACTCCCCAGGCGGAGTGCTT
ATTGCGTTAGCTGCGGCACTGAGGGTATTGAAACCCCAACACCTAGCACTCA
TCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTT
CGCGCCTCAGCGTCAGTTACAGACCAGAAAGCCGCCTTCGCCACTGGTGTTC
TCCACATCTCTACGCATTTACCGCTACACGTGGAATACCGCTTTCTCTTCTG
CACTCAAGCTACACAGTTTCCGATGCGAACCGGGGTTGAGCCCCGGGCTTTAA
CACCAGACTTACATAGCCGCCTGCGCGCGCTTTACGCCCAATAAATC-3'

***Brevibacillus panacihumi* (100% homology to GenBank NR044485.1)**

5'-TGCGGTTACCTCACCGACTTCGGGTGTTGCAAACCTCCCGTGGTGTGACGGG
 CGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGAT
 TACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTG
 AGATTGGTTTTAAGAGATTGGCGTCCTCTCGCGAGGTAGCATCCCGTTGTACC
 AACCATTTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGAC
 GTCATCCCCGCCTTCCTCCGTCTTGTCGACGGCAGTCTCTCTAGAGTGCCCAA
 CTGAATGCTGGCAACTAAAGATAAGGGTTGCGCTCGTTGCGGGACTTAACCC
 AACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCGCTGC
 CCCGAAGGGAAGCTCTGTCTCCAGAGCGGTCAGCGGGATGTCAAGACCTGGT
 AAGGTTCTTCGCGTTGCTTCGAATTAACCACATGCTCCACCGCTTGTGCGGG
 CCCCCGTCAATTCCTTTGAGTTTCACTCTTGCGAGCGTACTCCCCAGGCGGAG
 TGCTTATTGCGTTAGCTGCGGCACTGAGGGTATTGAAACCCCCAACACCTAGC
 ACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCA
 CGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAAAGCCGCCTTCGCCACTG
 GTGTTCCCTCCACATCTCTACGCATT-3'

***Arthrobacter phenanthrenivorans* (100% homology to GenBankNR074770.1)**

5'-GCCACCGGCTTCGGGTGTTACCAACTTTCGTGACTTGACGGGCGGTGTGTA
 CAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCG
 ACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACTGAGACCGGC

TTTTTGGGATTAGCTCCACCTCACAGTATCGCAACCCTTTGTACCGGCCATTG
 TAGCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGACGTCGTCCC
 CACCTTCCTCCGAGTTGACCCCGGCAGTCTCCTATGAGTCCCCGCCATCACGC
 GCTGGCAACATAGAACGAGGGTTGCGCTCGTT-3'

***Sphingobium yanoikuyae* (99% homology to GenBank NR036767.1)**

5'-CTTACGGTTACGCTCAACGCCTTCGAGTGAATCCAACCTCCCATGGTGTGAC
 GGGCGGTGTGTACAAGGCCTGGGAACGTATTCACCGCGGCATGCTGATCCGCG
 ATTACTAGCGATTCCGCCTTCACGCTCTCGAGTTGCAGAGAACGATCCGAACT
 GAGACGACTTTTGGAGATTAGCTCCCTCTCGCGAGGTGGCTGCCCACTGTAGT
 CGCCATTGTAGCACGTGTGTAGCCCAACGCGTAAGGGCCATGAGGACTTGACG
 TCATCCCCACCTTCCTCCGGCTTATCACCGGCGGTTTCCTTTAGAGTACCCAACT
 AAATGCTGGCAACTAAAGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAA
 CATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTCACCTATCCAGC
 CGAACTGAAGG-3'

***Sphingomonas koreensis* (99% homology to GenBank NR024998.1)**

5'-CTCTCTTGCGAGTTAGCGCAACGCCTTCGGGTGAACCCAACTCCCATGGTG
 TGACGGGCGGTGTGTACAAGGCCTGGGAACGTATTCACCGCGGCATGCTGATC
 CGCGATTACTAGCGATTCCGCCTTCATGCTCTCGAGTTGCAGAGAACAATCCG
 AACTGAGACAACCTTTTGGAGATTAGCTCACCTCGCGGGATTGCTGCCCACTG

TAGTTGCCATTGTAGCACGTGTGTAGCCCAGCGCGTAAGGGCCATGAGGACTT
 GACGTCATCCCCACCTTCCTCCGGCTTATCACCGGCGGTTCCCTTTAGAGTACCC
 AACTAAATGATGGTAACTAAAGGCGAGGGTTGCGCTCGTTGCGGGACTTAACC
 CAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCCAGT
 CCCC GAAGGGAAGAAATCCATCTCTGGAAATCGTCCGGACATGTCAAACGCT
 GGTAAGGTTCTGCGCGTTGCTTCGAATTAACCACATGCTCCACCGCTTGTGC
 AGGC-3'

***Pseudomonas psychrotolerans* (99% homology to GenBank NR042191.1)**

5'-CCCGANGGTTAGACTTAGCTACTTCTGGTAGCAACCCACTCCCATGGTGTG
 ACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGACGTTCTGATTCA
 CGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGA
 CTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTA
 CCGACCATTGTACCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGA
 CGTCATCCCCACCTTCCTCCGTTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAC
 CATAACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACC
 CAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGT
 TCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGT
 AAGGTTCTTCGCGTTGCTTCGAATTAACCACATGCTCCACCGCTTGTGCGGG
 CCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCA
 ACTT-3'

CHAPTER 3

GENERAL CONCLUSION

Since *L. cuneata* is an introduced legume, it must utilize the rhizobia available in its new environments. The invasive capacity of *L. cuneata* may partly be due to its symbiotic relationship with available rhizobia.

L. cuneata is relatively conservative in the rhizobia species it utilizes. Only two species of *Bradyrhizobium* were found to form mutualisms with it. *B. liaoningense* likely originated in China (Yao et al. 2002), and is commonly associated with *Glycine max* (L.) Merrill (soybean) root nodules (Yang and Zhou 2008), but may also form symbiotic relationships with other legumes. The agricultural production of soybeans may have introduced the necessary rhizobia for *L. cuneata*. *B. betae* was first found to initiate tumor-like root formations on some varieties of *Beta vulgaris* (sugar beet) (Rivas et al. 2004). Its N-fixation capacity is unknown, and it is unknown if *B. betae* associates with any other native or agricultural legume. A greenhouse experiment showed that only *B. betae* could improve *L. cuneata* growth and similar trend was found in the group treated with *B. liaoningense*. It is likely that these two rhizobia species work together to improve *L. cuneata* growth. An additional rhizobium found in a single nodule was *Mesorhizobium caraganae*, which is a novel rhizobial species nodulating *Caragana* spp. in China that was identified in 2008 (Guan et al. 2008). The relationship between *M. caraganae* with *L. cuneata* is still unknown. These compatible rhizobia may improve the N status of *L.*

cuneata. The availability of these compatible rhizobia may be part of the reason that why *L. cuneata* has become invasive. The critical tipping point may be the presence or quantity of suitable rhizobia present in the soil.

The majority of the rest of the bacteria isolated from root nodules of *L. cuneata* at both site types belonged to seven *Bacillus* species. Some *Bacillus* species have been shown to improve the growth of certain legumes (Chanway et al. 1990; Srinivasan et al. 1997) which may also be the case for *L. cuneata*. The functional relationships between the *Bacillus* bacteria identified in this study and *L. cuneata* are still unknown. This is a relationship that should be further investigated.

Other possible factor like mycorrhizae and enzymes produced by some soil bacteria and fungi may also contribute to the invasive capacity of *L. cuneata* by improving nodule formation (Ghazal and Azzazy 1994). Additionally, *L. cuneata* appear to be most successful at restoration sites, possibly as a result of a modified soil microbial community and reduced plant competition.

The specificity between *L. cuneata* and rhizobia suggests that a targeted future control method for this invasive plant could be developed by creating a mechanism to block the infection pathway between *L. cuneata* and *Bradyrhizobium* spp. This study helps improve the understanding of why some introduced legumes either naturalize or become highly invasive. By increasing our understanding of why *L. cuneata* is able to succeed in new environment, and the role of symbiotic rhizobia play in the success of it, new control measures may be found.

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