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THE INFLUENCE OF RHIZOBACTERIA ON THE COMPETITION BETWEEN NATIVE AND INVASIVE *LESPEDEZA*

BY

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THESIS

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Abstract

In order to study the effect of bacteria from root nodules on plant competition, two greenhouse experiments were conducted. Seven rhizobia and five non-rhizobia rhizobacteria strains were used to determine how plant-microbe interactions affect the competition between invasive *Lespedeza cuneata* and its native relative, *Lespedeza virginica*. Different rhizobia strains showed different impacts on the competition between *L. cuneata* and *L. virginica*. A relatively fast-growing *Mesorhizobium* strain and one of the *Rhizobium* strains showed low nodulation rate and no significant effect on the two plant species. All three *Bradyrhizobium* strains and two of the *Rhizobium* strains showed positive effects on the growth of *L. cuneata*, but they do not have the effect on the growth of *L. virginica*, so I conclude that invasive *L. cuneata* benefited more from the plant-rhizobacteria symbiosis relationship. This result emphasizes the influence of belowground interactions on the aboveground community. The finding that rhizobacteria mutualism can support the invasion of *L. cuneata* provides another perspective to invasion studies of *L. cuneata*.

Key Words: *Lespedeza cuneata*, rhizobia, rhizosphere bacteria, plant invasion, plant competition, legume, symbiosis

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Introduction

Background

Over the last several decades, the tremendous impacts of plant invasions on native plant communities have been widely recognized [1,2,3]. Biological invasion has become the second leading threat to native species in the USA [4]. Understanding the mechanisms of plant invasion and how the native community regulates the effects from invasive plants is essential to control and manage the influence of plant invasions [1,5].

Lespedeza cuneata (Dum. Cours.) G. Don, is a long-lived perennial legume in the family Fabaceae and also one of the notorious invasive plants of North America. This plant was introduced from Japan to the U.S. in the 1800s, and since then it has become an invasive weed, causing ecological problems in its introduced range [6]. *L. cuneata* can tolerate high drought and shade conditions and survive in various habitats including prairies, woodlands, fields and borders of ponds and swamps [7]. It is known for its capability of causing changes in the species composition, structure and function of native habitats [6,8]. The symbiotic relationship between *L. cuneata* and nitrogen-fixing bacteria has the potential to alter soil nutrients in the ecosystem [9,10]. These soil nutrient changes might lead to vegetation community succession or microbial community alteration [10]. Due to its aggressive behavior and its harmful effects, *L. cuneata* has been put on the noxious weed list in several states [11], which calls for more insightful understanding of the invasion of *L. cuneata.*

Several possible ecological mechanisms of invasion success of *L. cuneata* have been studied. Studies by Smith and Allred find that its advantage of total and specific leaf area and

aggressive productivity allow *L. cuneata* to outcompete native species [12,13]. Additionally *L. cuneata* maintains constant net photosynthesis and gas exchange rates over the course of the day [12]. These characteristics build the foundation of the "shade-out" effect of *L. cuneata*. A study by Brandon et al. suggests that *L. cuneata* tends to lower the light availability, allowing it to take over the grassland community under favorable conditions [8]. In addition, *L. cuneata* can consistently maintain significant levels of both seed and vegetative reproduction over the three years of study with varied weather conditions, facilitating the spread of this species under a wide range of environmental conditions, including those that might otherwise affect flowering, pollination, seed dispersal, germination and establishment [14]. At the community level, it has been found that even though biological control is applied via leaf-chewing herbivores, *L. cuneata* can tolerate a large amount of leaf loss (80%) during the growing season while maintaining a similar growth rate to unclipped controls [15]. Furthermore, the release of phytotoxic compounds from plant residue of *L. cuneata* inhibits several crops and weeds [16].

What is more, invasive genotypes of *L. cuneata* exhibit enhanced aggressiveness [17] in accordance with the evolution of increased competitive ability hypothesis (EICA). This hypothesis predicts that under identical conditions successful invaded plants from the new invaded range tend to grow more biomass than individuals of the same plant species taken from its original native sites [18]. A study conducted by Beaton et al. found evidence that *L. cuneata* from its introduced sites is more competitive than native plants and its ancestral genotype [17]. However, none of these studies took microbial ecology processes into account, which may provide another pivotal explanation for successful invasion of *L. cuneata*.

An increasing number of studies indicate that interactions between plants and microorganisms can influence plant invasions [2,3,19,20,21,22]. One perspective to look at this is plant-soil feedback. Plant-soil feedback represents the alterations of soil properties by a particular plant species, and the consequential impact of these changes on the plant itself or other plant species. The properties of soil that may be changed by vegetation include physical and chemical soil characteristics and soil biota [23]. The plant-soil feedback is considered as positive when the altered soil enhances the performance of the plant that changed the soil relative to other plants, or negative when the reverse is true [24].

Several different hypotheses have been proposed that link soil microorganisms to plant-soil feedback and invasive plants. One hypothesis to account for this is that invasive plants suffer less negative soil feedback than native species, or even have neutral to positive feedback because they are released from their original pathogens [19,20]. "Novel Weapons" may be another mechanism of plant invasion related to microbial ecology. It means that allelochemicals exuded by invasive plants are hard to be detoxified by local microbial community, and thus, they can easily reach toxic levels to harm native plant species [25]. In addition, some invasive plants are able to disturb the invaded habitat by enhancing pathogen levels or disrupting mutualisms with arbuscular mycorrhizal fungi, thus suppressing native plants while non-mycorrhizal invaders suffer less from this process [26,27].

Mutualisms, for example, with rhizobacteria may also have a close relationship with plant invasion success. Most leguminous plants continue to form nodules after invading a new

habitat either by forming nodules with bacteria that were that were transported with them or by forming new mutualisms in the new range [28]. However, it was also found that the legume *Leucaena leucocephala* failed to survive in new range until their corresponding symbiotic rhizobia were introduced [29]. What is more, a threshold density of nitrogen fixing bacteria is required for nodule forming on some legumes [30]. Thus the symbiotic relationship with rhizobia still has important functions for plant growth after invading a new habitat.

Although it is known that gaining nitrogen from root nodules is important for invasive legumes in habitats with low levels of nitrogen, it is still not known how the mutualism with rhizobia may help invasive legumes to outcompete native vegetation. There are different means that nodule formation might influence plant competition, thereby impacting plant invasion. One of the possible mechanisms is that certain legumes (such as soybean and cowpea) may be superior hosts because they are relatively more adept at acquiring symbiotic partners [31]. Also, effectiveness of rhizobia varies dramatically from host to host indicating that certain plants will be favored if a site has more rhizobacteria that are effective for these plants [32].

Mechanisms by which nodule forming bacteria might influence plant competition may be operating in the spread of the invasive *L. cuneata* at the expense of its native congener, *L. virginica. L. cuneata* has thrived across the entire eastern part of US, while *L. virginica*, has been listed as a threatened native species [15]. Both legumes can take advantage of their ability of nitrogen fixation, but they are having different ecological success. This might be due to their physiological and morphological differences, but I hypothesize that it is also

related to their different responses to symbiosis with rhizobia under identical biotic and abiotic conditions. By forming nodules with *L. cuneata* and *L. virginica*, different rhizobial strains might benefit *L. cuneata* and *L. virginica* with different efficiency.

Although it has come to be recognized that soil microorganisms associated with plants (e.g. rhizobacteria and mycorrhizal fungi [22]) might play an important role in invasion success [3,19,21,22], few studies have reported how the interaction between *L. cuneata* and its associated microorganisms influence its invasion success. However, a recent study [33] about *L. cuneata* suggests that soil bacteria communities in heavily invaded and uninvaded sites were significantly different, which leads to the importance to investigate microorganisms associated with *L. cuneata* invasion.

Previous Study

In my previous study (unpublished), bulk soil, root ball soil (soil attached to plant root of invasive *L. cuneata* and native *L. virginica* when digging up the plant) and rhizosphere soil (soil remaining attached to the same plant root after shaking off the root ball soil) samples were collected from two Army base locations, Ft. Benning and Ft. Leonard Wood. DNA was extracted from soil samples and Automated Ribosomal Intergenic Spacer Analysis was conducted to generate bacteria community fingerprinting profiles. In the Non-metric Multi-dimension scaling plot, NMDS (Figure 1), each point represents a bacterial community generated by ARISA profiling. The closer the two points are plotted, the more similar the bacterial communities are. The plots of Ft. Benning and Ft. Leonerd Wood (Figure 1), show that the rhizosphere communities from invaded sites (light and dark blue points) are more clustered together than those from the native sites (green points), meaning that there was

less plant-to-plant variability in rhizosphere bacteria on *L. cuneata* than on the native *L. virginica* (ANOSIM: R=0.054, p=0.092). This might indicate that *L. cuneata* more tightly controlled its rhizosphere community composition (Figure 1). Although ARISA does not specifically identify which bacteria exist in the rhizosphere of *Lespedeza* or how they influence the invasion of *L. cuneata*, these results lead me to investigate further about the rhizosphere bacteria associated with *L. cuneata* in order to understand its invasion.

Objective and Design

In the present study, I investigated how the plant-associated rhizobacteria strains influence the plant growth and competition between *L. cuneata* and its native congener, *L. virginica*. Two greenhouse experiments were designed to test the effects of the chosen rhizobial and non-rhizobial strains on both invasive *L. cuneata* and native *L. virginica* when grown separately and in a common garden. The main question I address is: how do the different rhizobial and non-rhizobial strains influence the competition between *L. cuneata* and *L. cuneata* without adding nitrogen? For each greenhouse experiment, I evaluated several alternative hypotheses.

1) Rhizobia test

Hypothesis 1: Invasive *L. cuneata* benefits more from symbiosis with rhizobia than native *L. virginica* when the two plants exist together.

Hypothesis 2: Invasive *L. cuneata* benefits less from symbiosis with rhizobia than native *L. virginica* when the two plants exist together.

Hypothesis 3: Invasive *L. cuneata* and native *L. virginica* have similar benefits from symbiosis with rhizobia when the two plants exist together.

2) Non-rhizobia rhizobacteria test

Hypothesis 1: Invasive *L. cuneata* benefits more from symbiosis with non-rhizobia rhizobacteria than native *L. virginica* when the two plants exist together by directly affecting the plants or affecting legume-rhizobia symbiosis.

Hypothesis 2: Invasive *L. cuneata* benefits less from symbiosis with non-rhizobia rhizobacteria than native *L. virginica* when the two plants exist together by directly affecting the plants or affecting legume-rhizobia symbiosis.

Hypothesis 3: Invasive *L. cuneata* and native *L. virginica* have similar benefits from symbiosis with non-rhizobia rhizobacteria when the two plants exist together by directly affecting the plants or affecting legume-rhizobia symbiosis.

Methods

Isolation of bacterial strains and selection

My collaborator (R. Busby) obtained the nodules of invasive *L. cuenata*, native *L. virginica* and a native legume, *Chemaechrista fasciculata* from the field. Root nodules were surface sterilized with full strength of Clorox (6% sodium hypochlorite) and then rinsed with autoclaved DI water three times [34]. Root nodules were crushed after adding 500μl autoclaved DI water. 100μl bacteria liquid was put onto YM medium plate with a pipette, and spread gently to the whole plate. The YM-cultured bacteria were picked from their previous plate to a R2A medium plate until one single colony type was seen on the plate.

Each single colony was collected from isolated culture plates to enrich on a new R2A medium plate. After several days of growing in the incubator at 28°C, cultured bacteria were swabbed into 50% glycerol stock for longer storage at -80°C in a freezer. A portion of each culture was suspended in autoclaved DI water for polymerase chain reaction (PCR) and further analysis.

16S rRNA gene sequences were used to identify the isolates obtained from the field. The bacterial suspension was prepared for PCR by boiling in 100 °C water bath for 10 minutes to release DNA. 5μl of this liquid was used as a template for 16S rRNA gene PCR. The 16S rRNA gene PCR was done based on the following recipe: each 50μl reaction contained 5μl template, GoTaq (Promega, Madison, WI) buffer 1×, 0.25μg/μl BSA, 3mM $MgCl_2$, 0.25μM dNTPs, primer 8F (5'-GGGTTBCCCCATTCRG-3') 0.4μM, primer 1492R (5'-GGGTTBCCCCATTCRG-3') 0.4μM, Taq polymerase (Promega, Madison, WI) 0.05U/μl; program: 94°C 2min; 30 cycles of 94 °C 35s, 55°C 45s, 72°C 2 min; 72°C 2 min. The PCR products were purified using a Promega PCR clean-up kit and sent to the W.M. Keck Center for Comparative and Functional Genomics of University of Illinois at Urbana-Champaign for Sanger sequencing [35] for each strain. Then the strains were identified by 16S rRNA gene sequences [36]. This approach revealed that this isolate collection included not only rhizobia from the order Rhizobiales but also some other groups of bacteria not known to form symbioses with legumes. I refer to these other groups as non-rhizobia rhizosphere bacteria strains. These non-rhizobia rhizosphere bacteria would not necessarily have the ability to form nodules, but they may have the potential to influence plant growth.

Two approaches were used to screen strains in order to find the potential nitrogen fixers for greenhouse experiments: 1) PCR to confirm the presence of the *nifH* gene and 2) the ability of the strain to grown on nitrogen-free medium. PCR for the *nifH* gene was conducted from the boiled bacteria DNA liquid based on the following recipe: each 25μl reaction contained 4μl template, GoTaq buffer (Promega, Madison, WI) 1×, 0.25μg/μl bovine serum albumin (BSA), 2mM $MgCl_2$, 0.2μM dNTPs, primer polF (5'-TGCGAYCCSAARGCBGACTC-3') 0.5μM, primer polR (5'-ATSGCCATCATYTCRCCGGA-3') 0.5μM, Taq enzyme (Promega, Madison, WI) 0.05U/μl; program: 94°C 5min; 1 cycle of 94°C 45s, 64°C 45s, 72°C 45s, 2 cycles of 94°C 45s, 62°C 45s, 72°C 45s, 3 cycles of 94°C 45s, 60°C 45s, 72°C 45s, 4 cycles of 94°C 45s, 58°C 45s, 72°C 45s, 25 cycles of 94°C 45s, 56°C 45s, 72°C 45s, 72°C 10min. In order to verify the presence of the *nifH* gene, electrophoresis was done on the PCR products using 1.4% agarose gel under 135V condition for 30 minutes. In order to confirm the ability to grow without nitrogen, each strain was cultured again from glycerol stock on plates of AcD nitrogen-free medium [37]. I continued checking the plates to see if the bacteria strains grew on the plates for as long as two weeks.

Seven rhizobial strains and five non-rhizobial strains were selected for greenhouse experiments (Table 1 and Table A1). Thirteen rhizobial strains under the traditional nodule forming bacterial genera were able to grow on the nitrogen-free medium. By comparing the 16S rRNA gene sequences, I found that there were redundant strains. Thus, I narrowed down to seven different rhizobial strains used for a greenhouse experiment test of my hypotheses regarding bacterial influence on plant competition. Five non-rhizobial strains were also chosen according to its genera (based on 16S rRNA gene sequencing) and their potential to be plant-associated bacterial strains.

Greenhouse experiment

Two greenhouse experiments were conducted to find out how different rhizobial and non-rhizobial strains influence the competition between *L. cuneata* and *L. virginica.* The first experiment was a three-way factorial experiment to test the influence of rhizobial strain identity on plant competition. The plant factor had two levels, *L. cuneata* and *L. virginica*. The competition factor was assessed by growing two *L. cuneata* individuals alone (intra-specific competition), two *L. virginica* individuals alone (intra-specific competition), and each individual of the two species together (inter-specific competition). Seven different rhizobial strains were used to inoculate these plants, and a no-inoculum control was also used (8 levels total). Ten replicates were applied to each treatment.

The second greenhouse experiment was a four-way factorial experiment to test the influence of non-rhizobia rhizobacteria on legume-rhizobia symbiosis and plant competition. The plant factor had two levels, *L. cuneata* and *L. virginica*. The competition factor was assessed by growing two *L. cuneata* individuals alone (intra-specific competition), two *L. virginica* individuals alone (intra-specific competition), and each individual of the two species together (inter-specific competition). Half of the plants were inoculated with one rhizobial strain, R4 (Table 1), and the other half were not inoculated with any rhizobial strain. Five different individual non-rhizobial strains (Table 1 and A1) were used to inoculate these plants, and a no-inoculum control was also used (6 levels total). Ten replicates were applied to each treatment.

1. Preparation

Root wash soil (soil: calcinated clay: torpedo sand (1:1:1)) was autoclaved twice at 121 °C for 1hr. The soil was stirred to mix well between the two sterilizations. Then the soil was put into separated Leonard jars [38], which comprise two polycarbonate pots, one on top of the other, connected by a wick for each set. The lower box contained water and nutrients for plants, which were carried by the wick up to the upper box that contains soil and plants. All Leonard jars with soil were autoclaved for 25 min with lids on. Three Leonard jars of soil were left unplanted to test for the effectiveness of the sterilization treatments.

200ml half-strength of nitrogen-free Hoagland's solution [34] was diluted from autoclaved stock and put into each autoclaved Leonard jar to start with. The soil was moistened by nutrient solution for two days before dispersing seeds on the soil.

All seeds were sterilized with full strength of Clorox (6% sodium hypochlorite) for 5 min, and rinsed with Milli Q water three times [34]. Ten seeds were placed on an R2A medium plate for two weeks to confirm the effectiveness of this seed sterilization treatment. Three replicates for each plant species were conducted for the seed sterilization test. Eight seeds were put in each Leonard jar with moistened soil. Between the 9th to 11th day after germination, two seedlings were randomly selected and left in the jar as experimental subjects while others were removed. The seedlings were inoculated with strains 7-14 days after germination.

Twelve strains, including seven rhizobia and five non-rhizobial strains (Table 1 and Table A1 for more information), were selected and used to inoculate the plants. 10μl glycerol

stock was diluted in 1ml YM broth, and then 10μl of the diluted bacteria was added to 50ml YM broth. Strains were grown in an incubator, shaking at a speed of 160rpm, at 28°C. Strains were grown to the end of exponential stage based on a set of the growth curves built beforehand (Appendix Figure A1 and A2). The strains were diluted to 10^8 CFU/ml (two non-rhizobial strains are 10^7 CFU/ml) for inoculation. Every seedling was inoculated with 5ml bacteria suspension liquid.

During germination, lids with a small central vent covered with 0.2μm filter were put on Leonard jars to prevent contamination in the beginning phase. The lids were kept on after inoculation for 5-7 days to prevent cross contamination from the air.

Fresh, sterile nutrient supply was added weekly. At the end of experiment, the plants were so big that the frequency of watering increased to two to three times a week. The pots were randomized to different locations weekly. Each experiment was run for three months.

2. Harvest and data collection

Shoot and root biomass were collected to represent the response of plants to strains in comparison with the un-inoculated control. By comparing the shoot and root biomass with the same strains but under different competition treatments (inter-specific or intra-specific competition), I could tell whether the impact from the effect of strains differed under different competition conditions. Nodule number, nodule biomass and Acetylene Reduction Assay were used to help explain the alterations of plant performance.

After three months of growth, plants were harvested. The whole plants along with the soil were removed from the Leonard jars. The two plants from the same pot were gently

separated from each other and the soil. Plants were washed with tap water to get rid of soil. The clean plants were dried with a napkin and cut with scissors into shoot and root parts. Root nodule number was counted before each shoot and root was put into separated envelopes.

To estimate the rate of nitrogen-fixation under each experimental treatment, Acetylene Reduction Assays (ARA) were conducted after root nodule number was counted. Tubes were air-flushed and then roots were put into the tubes with rubber septa, leaving the root nodules intact. At the beginning of the assay, 7.5 ml air in the 75ml tube was replaced with acetylene. After 1hr incubation at room temperature in dark, 15ml was transferred into 10 ml pre-vacuumed Vacutainer tubes [34]. The Vacutainer tubes then were sealed with glass adhesive, and stored in the dark at room temperature until they could be analyzed. 1000μl of air samples was collected from each tube and injected into a gas chromatograph with a GS-Alumina column. The ethylene in the air sample was quantified with a flame ionization detector in comparison to a standard curve of ethylene [39,40].

After ARA, nodules were removed from roots and weighed on a three-digit scale to help explain the performance variation between plant individuals. The rest of roots were put back into the envelope. All envelops with shoots and roots were put into 60°C incubator. After three days, the biomass of shoot and root was measured on a three-digit scale.

3. Strain and contamination confirmation

After measuring root nodule biomass, nodules were put into 1.6ml micro tubes with 500μl PBS to maintain for at most one day. Root nodules were surface sterilized with full strength

of Clorox (6% sodium hypochlorite) and then rinsed with autoclaved DI water three times. All nodules were transferred to 96-well culture plate, one nodule per well. At most three nodules were picked for each root system. Root nodules were crushed with a sterile 48-pin replicator. The small amount of bacteria liquid adherent to the replicator was used directly to conduct 16S rRNA gene PCR for non-rhizobial strains and 16S+ITS rRNA for rhizobial strains. The 16S rRNA gene PCR was done based on the following recipe: each 50μl reaction contained 5μl template, GoTaq buffer (Promega, Madison, WI) 1×, 0.25μg/μl bovine serum albumin (BSA), 3mM $MgCl_2$, 0.25μM dNTPs, primer 8F (5'-GGGTTBCCCCATTCRG-3') 0.4μM, reverse primer (1492R (5'-GGGTTBCCCCATTCRG-3') for non-rhizobia, 23SR (5'-GGGTTBCCCCATTCRG-3') for rhizobia) 0.4μM, Taq enzyme (Promega, Madison, WI) 0.05U/μl; program: 94°C 2min; 30 cycles of 94 °C 35s, 55°C 45s, 72°C 2 min; 72°C 2 min. To confirm that there was no cross contamination and the root nodules on corresponding plants contained only the original inoculation, PCR products were digested using restriction enzymes and compared to the original strain's Restriction Fragment Length Polymorphism (RFLP) pattern [41]. RFLPs were generated by co-digestion with *Hha*I and *Bgl*II restriction enzymes using the following recipe: PCR product 10μl, RE buffer (Multi-core (Promega, Madison, WI) for rhizobial strains, Buffer 4 (NEB, Ipswich, MA) for non-rhizobia) 1×, Acetylene BSA 0.1 μg/μl, and restriction enzymes 0.25U/μl (*Hha*I and *Bgl*II for rhizobial strains, *Hha*I for non-rhizobial strains) [42].

4. Soil sterilization checking

10 cm^3 of soil were collected from one randomly chosen replicate of each treatment to determine if soil sterilization had been maintained over the course of the experiment. The

soil collected before experiment as control and from each treatment after the greenhouse experiments were freeze dried at -50°C. Total genomic DNA was extracted from 0.5 g freeze-dried soil samples with the FastDNA SPIN Kit for Soil (MP Biomedicals) using the protocol recommended by the manufacturer. Soil DNA extracts were purified using cetyltrimethylammonium bromide (CTAB) with 1:24 chloroform-isoamyl alcohol extraction, and then precipitated in 100% ethanol with two 70% ethanol washes to remove co-extracted humic acids before further molecular analyses [43]. Air-dried DNA was resuspended in 100 ml of DNA-free water and stored at -20C.

The soil sterilization effectiveness was checked by comparing the bacterial community among control and soil samples of each treatment. Automated Ribosomal Intergenic Spacer Analysis (ARISA) was used to characterize the bacterial composition in soil. The 16S rDNA PCR was done based on the following recipe: each 25μl reaction contained: 2μl template (10ng/μl), tris buffer 1×, 0.25μg/μl bovine serum albumin (BSA), 3mM $MgCl_2$, 0.25μM dNTPs, primer 1406F (5'-TGYACACACCGCCCGT-3') 0.4μM, 23SR (5'-GGGTTBCCCCATTCRG-3') 0.4μM, T.aq enzyme (Promega, Madison, WI) 0.05U/μl; program: 94°C 2min; 30 cycles of 94 °C 35s, 55°C 45s, 72°C 2 min; 72°C 2min. PCR products were sent to the W.M. Keck Center for Comparative and Functional Genomics of University of Illinois at Urbana-Champaign for capillary gel electrophoresis [33,44].

Molecular fingerprint profiles were analyzed with the GeneMarker v1.85 software program (SoftGenetics, State College, PA). Only fragments with signal intensities greater than 400 relative fluorescence units and within the size between 400-1000 bp were quantified. Fluorescence electropherograms were aligned by ROX-1000 internal size standard. Bin

positions and widths were automatically generated by the GeneMarker program and manually adjusted. The table of relative fluorescence data was used to measure the similarity of bacterial communities between all pairs of samples using the Bray-Curtis coefficient in PRIMER v6 (PRIMER-E Ltd., Plymouth, UK) to create similarity matrices. The degree of relative similarity within versus among treatment and control samples was computed using the analysis of similarity (ANOSIM) test implemented in PRIMER v6 (PRIMER-E Ltd., Plymouth, UK) [33,44].

Statistical Analysis

Statistical analyses were conducted in R software v2.14.1 (R Development Core Team, 2011). In order to evaluate the differences between plant treatments, strain treatments and the interaction, data from greenhouse experiments were analyzed using linear models in R to conduct Analysis of Variance (ANOVA). Post-hoc contrasts were used in R within the linear model in order to test the significant differences between specific treatment levels.

Results

Rhizobial strain Test

L. cuneata gained significantly more total biomass (Table 2), shoot biomass (Table 3), root biomass (Table 4), nodule biomass (Table 5), and nodule number (Table 6) than *L. virginica*.

It was also found that with R7 (*Bradyrhizobium*3) inoculation, plants had higher nodule biomass ($p=0.002$, Figure 3) than control, and more nodules when inoculated with R4 (*Bradyrhizobium*2) ($p=0.003$, Figure 3) or R7 (*Bradyrhizobium*3) ($p<0.001$, Figure 3) than

control. In addition, plants gained more nodule biomass under inter-specific competition than intra-specific competition (Table 5).

What is more, the two-way interaction of plant and competitor (Table 2 and Table 3) and the two-way interaction of plant and strain (Table 2 and Table 3) were significant in total biomass and shoot biomass, meaning that the growth of *L. cuneata* and *L. virginica* reacted differently to the competition treatment and the strain treatment. For *L. cuneata*, the total biomass ($p<0.001$, Figure 2) was significantly larger under inter-specific competition than under intra-specific competition, whereas there was no such difference for *L. virginica*. In addition, *L. cuneata* gained significantly more biomass than the no-inoculation control when inoculated with R2 (*Bradyrhizobium*1) ($p<0.001$, Figure 3), R3 (*Rhizobium*1) ($p=0.0113$, Figure 3), R4 (*Bradyrhizobium*2) ($p<0.001$, Figure 3), R5 (*Rhizobium*2) ($p=0.0126$, Figure 3) and R7 (*Bradyrhizobium*3) ($p=0.014163$, Figure 3). However, none of the strains yielded significant increases in *L. virginica* biomass in comparison to the no-strain control ($p=0.5541$, Figure 3).

The Shoot-to-root biomass ratio did not respond to any treatment except for plant species (Table A2). L. cuneata showed higher shoot-to-root ratio than *L. virginica*. Nitrogen fixation activity (Table A3), nitrogen fixation activity per nodule biomass (Table A4) did not respond to any treatments.

Although the concentration of the inoculated strain was intended to be more than the plants needed (according to our preliminary experiment), different strains were found to have significantly different nodulation rates ($p=<0.0001$ for *L. cuneata*, $p=0.03$ for *L. virginica*, Figure A3).

For *L. cuneata*, the nodule number (p<0.001, Figure 2) was significantly larger under inter-specific competition than under intra-specific competition, whereas there was no such difference for *L. virginica*.

Non-rhizobial strain Test

No significant differences were found between the control and any non-rhizobial strains in biomass (*L. cuneata*: p=0.1639 for shoot biomass, p=0.6529 for root biomass; *L. virginica:* p=0.3186 for shoot biomass, p=0.9786 for root biomass). I did not find any evidence that non-rhizobial strains nodulated with *L. cuneata* or *L. virginica*.

Discussion

Native Lespedeza was a better neighbor than invasive Lespedeza

L. cuneata gained lager total biomass when competing with *L. virginica* than itself (Figure 2). However, *L. virginica* showed no difference in total biomass in competition with *L. cuneata* as compared to competition with itself (Figure 2). If one assumes that alteration in plant performance reflect overall changes in plant reproductive capabilities, which eventually will affect population growth rates, then changes to plant biomass can be used as proxies for population growth rates. With this in mind, my results can be placed in the context of the Lotka-Volterra competition model [45]. In this case, *L. cuneata* competed with *L. virginica* at the same level of intensity as *L. virginica* competed with itself, whereas *L. virginica* did not show the same level of competition intensity on *L. cuneata* as *L. cuneata* affected on itself. Therefore according to the Lotka-Volterra competition model, *L. cuneata* is the superior competitor, and it should be predicted to exclude *L. virginica* under conditions similar to my experiment.

This conclusion is also supported by findings about the competitive features of *L. cuneata* at the population level [8,12,29], such as big leaf area and shade-out effect. It is also found that *L. cuneata* can maintain significant levels of seed and vegetative reproduction through the three years of study under varied weather conditions, [14]. The finding that *L. cuneata* is a more successful competitor than *L. virginica* may explain why to the fact that both *L. cuneata* and *L. virginica* can both fix nitrogen via the symbiotic relationship with rhizobial bacteria, but they are having different ecological success.

One way to view the aggressiveness of *L. cuneata* is that it is a superior competitor as mentioned above, and the other way is that *L. cuneata* has extremely intense intra-specific competition. *L. cuneata* suffered more under intra-specific competition than under inter-specific competition with *L. virginica*, regardless of which strain it encountered (Figure 2). The two *L. cuneata* individuals might still be under the stress of nitrogen limitation as they gained fewer nodules under intra-specific competition than inter-specific competition (Figure 2). This also suggests that *L. cuneata* plants have to compete with each other over effective rhizobacterial symbionts under intra-specific competition. The higher nodule number under inter-specific competition than intra-specific competition (Figure 2), in some degree, may account for the higher total biomass of *L. cuneata* under inter-specific competition (Figure 2). Another possibility is that with the limitation of nitrogen released, the intra-specific competition over other unknown resources, such as the pot space and other nutrient, might still remain intense, such that neither of the two *L. cuneata* individuals gained significantly more biomass than control.

Such aggressive characteristics of *L. cuneata* was also found in another intra-specific competition study [17], where the authors report that the aboveground biomass of the invasive genotypes of *L. cuneata* is larger than both native and ancestral genotypes regardless of the identity of the competitor, while both native and ancestral genotype gain less aboveground biomass when pair with the invasive genotype. It is also consistent with the weedy features that have been mentioned in other plant invasion studies about *L. cuneata*, such as big leaf area, high seed production and shade-out effect, [8,12,29]. This aggressiveness of *L. cuneata* explains the intense intra-specific competition found in my study, because larger leaf area and numerous seeds require the plant to obtain more resources, which causes intense competition between different individuals of the same species. What is more, adding rhizobial strains cannot change such aggressiveness.

Variation between different rhizobial strains

The nodulation effectiveness of the slow-growing rhizobial strains, R2 (*Bradyrhizobium*1), R4 (*Bradyrhizobium*2), and R7 (*Bradyrhizobium*3) were higher than others (Figure A3 and Figure A1), although the conditions were the same for all pots in regard to temperature, humidity, and nutrient levels. The variation between strains was also seen in the different effectiveness of rhizobial strains. For example, *L. cuneata* biomass was lower (Figure 3) when inoculated with strains R1 (*Mesorhizobium*) and R6 (*Rhizobium*3) in comparison to all of the other strains, even strains from the same genus (R3 (*Rhizobium*1) and R5 (*Rhizobium*2)). In Thrall's study [46] on the symbiosis of *Acacia* and rhizobia, they also report significant variation of the effectiveness of strains from different species within one genus. This was also consistent with the common findings that different strains have

different efficiency in forming nodules and fixing nitrogen [34,47,48]. My results provide evidence that such variation of strains in regards to effectiveness of nodulation and plant growth can apply to *L. cuneata* as well, suggesting that *L. cuneata* establishes at a site only if its proper symbionts are present.

Belowground affected aboveground

The aboveground community was closely related to the belowground. I found that strains R2 (*Bradyrhizobium*1), R3 (*Rhizobium*1), R4 (*Bradyrhizobium*2), R5 (*Rhizobium*2) and R7 (*Bradyrhizobium*3) aided *L. cuneata* but not *L. virginica* in general (Figure 3). Therefore, these bacteria can favor the *L. cuneata* in competition with *L. virginica* under nitrogen-limiting conditions. Thus I conclude that invasive *L. cuneata* benefits more from the plant-rhizobacteria symbiosis relationship than does its native congener. Additionally, plants inoculated with the poorly-nodulating strains, R1 (*Mesorhizobium*) and R6 (*Rhizobium*3), showed no increase in plant biomass. Therefore, neither plant species benefited from these two strains.

The discovery of the effective strains for *L. cuneata* confirmed the ability of invasive plants to form effective symbiotic relationship with rhizobia in its introduced range [9,29], which can help ensure the plant invasion success. The finding that rhizobacteria mutualism can support the invasion of *L. cuneata* provides another perspective to invasion studies of *L. cuneata*, emphasizing the influence of belowground interactions on the aboveground community. Such influences from belowground, in addition to “release from pathogens” and “disturbing the local microbial community”, extended the scope of how plant-microbe interaction influences plant invasion to a mutualism perspective [20,21,22,49].

Bradyrhizobium played an important role

The three *Bradyrhizobium* strains tested were all effective nodulators of invasive *Lespedeza* and they all significantly increased the total biomass of this plant (Figure 3). My results suggest that *Bradyrhizobium* might be related to the invasion of *L. cuneata*, and this is consistent with other previous observations of *Bradyrhizobium* and invasive nitrogen fixing plants. A study on the rhizobia diversity of different native and invasive legumes in New Zealand reports that all three invasive legumes studied are only associated with *Bradyrhizobium* [50]. Another study reports that all isolates from different plant invasion stages belong to the genus *Bradyrhizobium* [51]. However, another recent study reports that introduced plant species, *Acacia*, is more promiscuous in acquiring various symbionts than native *Acacia*. Introduced *Acacia* appeared to associate with not only fast-growing symbiotic bacteria, such as *Rhizobium* and *Mesorhizobium*, but also slow-growing *Bradyrhizobium*, whereas other *Acacia* exclusively associate with fast-growing symbiotic bacteria [52]. So although *Bradyrhizobium* strains in my study helped invasive *Lespedeza*, there is large variation within this genus in the effectiveness on different plant species.

Limitations

Soil biota contamination was a major limitation for this study. The first type of contamination was from soil bacteria resulting from insufficient sterilization. The soil used in the greenhouse experiments generated bacterial community fingerprints after sterilization. Thus the sterilization technique used in my study was not 100% effective. However, any bacterial communities present in the soil did not vary in any systematic way according to the treatments (Figure A4), and so these background communities may

contribute additional random error to my results. Although using root wash soil was closer to the natural condition than using a more easily sterilized sand vermiculite mix, this mix material may reduce the source of random error.

The second contamination was cross contamination. Based on the result of strain confirmation checking, 23% of the nodules produced RFLP patterns on the electrophoresis gel that did not match with the corresponding strains I added. Therefore, the replicates with cross contamination added inaccuracy to my conclusions. In order to prevent cross contamination, the pots should be located even further from each other and covered by the lids for even longer time.

The third contamination was from fungi. When I checked the sterilization efficiency of seeds, I found that fungi contaminated two out of the 60 seeds. This could be due to fungi spores present in the air. It is also possible that the seed sterilization was not completely effective since the fungal contamination was associated with the seed instead of random spot on the agar plate. The fungi contamination could directly harm the plant individuals, thus influencing the plant growth and confounding the effect from rhizobia and non-rhizobial strains. To overcome the trouble, I could culture the seed on sterilized agar and transplant the fungi-free seedlings into experiment pots.

Only twelve strains were chosen from 54 strains to be tested, which limited the interpretation of how these strains contribute to the competition between *L. cuneata* and *L. virginica*. The chosen strains were based on the 16S rRNA and experience. Any conclusion made should only apply to the specific strains obtained in my study, not to any natural plant invasion system, especially for the non-rhizobial strains. It is possible that some of

the strains that did not get tested might be able to influence plant performance or the effectiveness of the legume-rhizobium symbiosis. For example, some non-Rhizobium strains from the α and β and γ proteobacteria subgroups are reported to form nodules on legumes [53,54].

Conclusion

Although adding rhizobial strains did not change the aggressiveness of *L. cuneata*, different rhizobial strains showed different impacts on the competition between *L. cuneata* and *L. virginica*. A relatively fast-growing *Mesorhizobium* strain and one of the *Rhizobium* strains showed low nodulation rate and no significant effect on the two plant species. All three *Bradyrhizobium* strains and two of the *Rhizobium* strains showed positive effects on the growth of *L. cuneata*, but not *L. virginica*, so I conclude that invasive *L. cuneata* benefited more from the plant-rhizobacteria symbiosis relationship. This result emphasized the influence of belowground interactions on the aboveground community. The finding that rhizobacteria mutualism can support the invasion of *L. cuneata* highlights the importance of belowground perspective to invasion studies of *L. cuneata*.

Figures and Tables

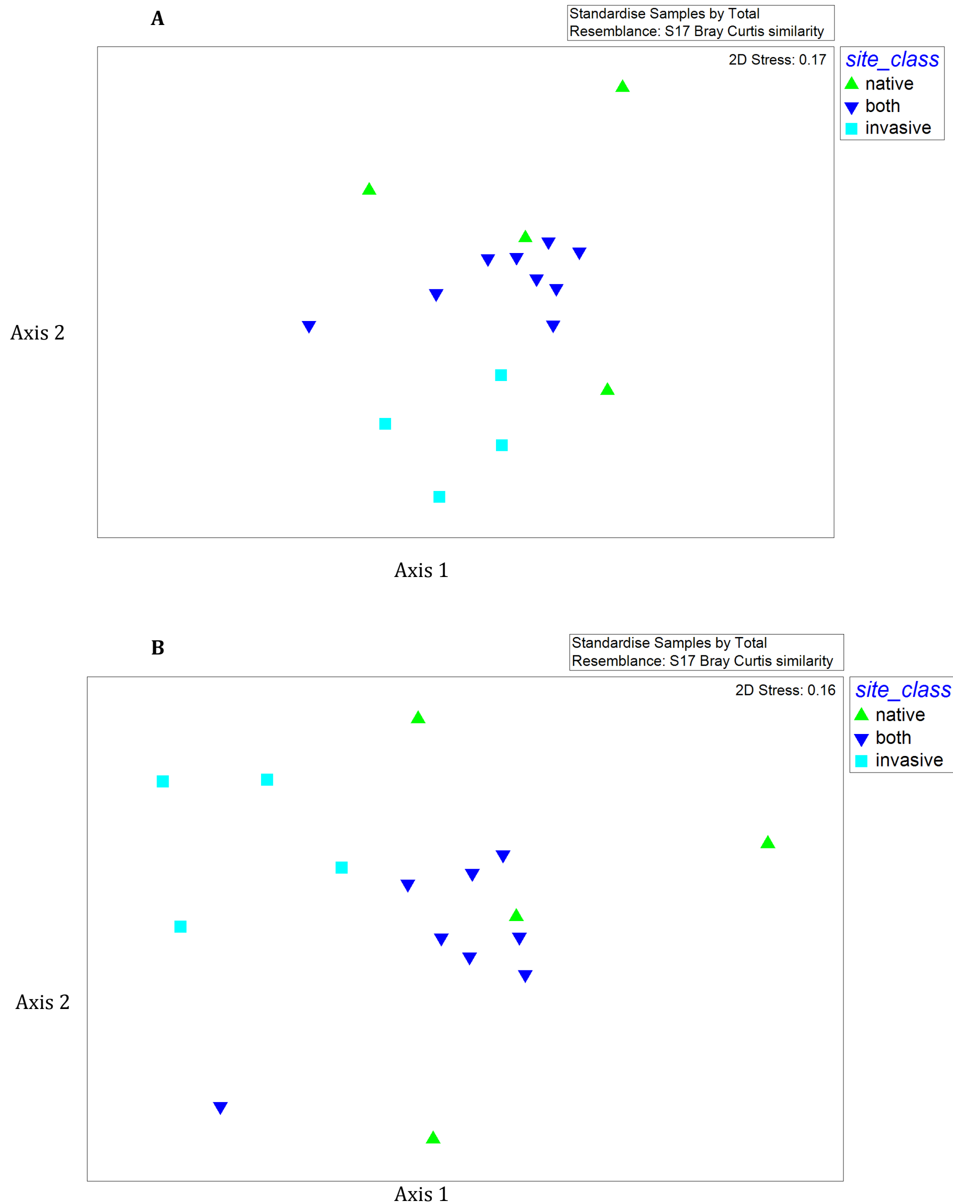

Figure 1 NMDS plot of ARISA data of rhizosphere soil from Facility Benning, West Georgia (A), and Facility Leonard Wood, Central Missouri (B); each point represents a bacterial community generated by ARISA fingerprinting from different sites (green triangle: sites without invasive *L. cuneata*; blue triangle: sites with a few invasive *L. cuneata*; sites with many invasive *L. cuneata*); the closer the two points are plotted, the more similar the bacterial communities are.

Table 1 Rhizobia and non-rhizobial strains for plant inoculums

Greenhouse ID[a]	**Origin[b]**	**Site[c]**	**Genus[d]**
R1	*L. cuneata*	LI1	*Mesorhizobium*
R2	*L. cuneata*	HI1	*Bradyrhizobium*
R3	*L. cuneata*	HI1	*Rhizobium*
R4	*L. cuneata*	HI2	*Bradyrhizobium*
R5	*L. cuneata*	HI2	*Rhizobium*
R6	*L. virginica*	NI	*Rhizobium*
R7	*C. fasciculata*	LI2	*Bradyrhizobium*
N1	*L. virginica*	LI2	Sphingomonas
N2	*L. cuneata*	HI2	Lysinibacillus
N3	*L. cuneata*	LI2	Pseudomonas
N4	*L. cuneata*	LI1	Burkholderia
N5	*L. cuneata*	LI1	Mycobacterium

[a]Greenhouse ID: the ID used for different strains in the greenhouse experiment
[b]Origin: different species of each strain collected from;
[c]Site: different sites of each strain collected from, LI for lightly invaded sites, HI for heavily invaded sites, NI for non-invaded sites
[d]Genus: the genus based on BLAST result of 16S rRNA sequences of each strain

Table 2 ANOVA table of rhizobial-strain test for total biomass

Response: total biomass[a]	Df	Sum Sq	Mean Sq	F value	[e]p value
plant[b]	1	139615316	139615316	176.5107	**< 2.2e-16**
competitor[c]	1	18708821	18708821	23.6529	**2.16E-06**
strain[d]	7	34758630	4965519	6.2777	**9.64E-07**
plant x competitor	1	13626955	13626955	17.2281	**4.69E-05**
plant x strain	7	13665808	1952258	2.4682	**0.01851**
competitor x strain	7	5034101	719157	0.9092	0.50002
plant x competitor x strain	7	4922963	703280	0.8891	0.51575
Residuals	227	179551036	790974		

[a]Response indicates the dependent variable, the total dry biomass (mg) of each individual plant
[b]plant: plant species as independent factor
[c]competitor: whether it is inter-specific or intra-specific competition as independent factor
[d]strain: different strain inoculated as independent factor
[e]p-values lower than 0.05 are indicated in **boldface**

Table 3 ANOVA table of rhizobial-strain test for shoot biomass

Response: shoot biomass[a]	Df	Sum Sq	Mean Sq	F value	[e]p value
plant[b]	1	120561102	120561102	235.5241	**< 2.2e-16**
competitor[c]	1	11392412	11392412	22.2558	**4.17E-06**
strain[d]	7	21658995	3094142	6.0446	**1.78E-06**
plant x competitor	1	8160448	8160448	15.942	**8.82E-05**
plant x strain	7	9282691	1326099	2.5906	**0.01372**
competitor x strain	7	2012220	287460	0.5616	0.78665
plant x competitor x strain	7	2373421	339060	0.6624	0.70377
Residuals	227	116197749	511884		

[a]Response indicates the dependent variable, the dry shoot biomass (mg) of each individual plant
[b]plant: plant species as independent factor
[c]competitor: whether it is inter-specific or intra-specific competition as independent factor
[d]strain: different strain inoculated as independent factor
[e]p-values lower than 0.05 are indicated in **boldface**

Table 4 ANOVA table of rhizobial-strain test for root biomass

Response: root biomass[a]	Df	Sum Sq	Mean Sq	F value	[e]p value
plant[b]	1	698663	698663	8.837	**0.00327**
competitor[c]	1	902699	902699	11.4178	**0.0008561**
strain[d]	7	2123104	303301	3.8363	**0.0005799**
plant x competitor	1	696926	696926	8.8151	**0.0033081**
plant x strain	7	784647	112092	1.4178	0.1990037
competitor x strain	7	899919	128560	1.6261	0.128984
plant x competitor x strain	7	736950	105279	1.3316	0.2362238
Residuals	227	17946779	79061		

[a]Response indicates the dependent variable, the dry root biomass (mg) of each individual plant
[b]plant: plant species as independent factor
[c]competitor: whether it is inter-specific or intra-specific competition as independent factor
[d]strain: different strain inoculated as independent factor
[e]p-values lower than 0.05 are indicated in **boldface**

Table 5 ANOVA table of rhizobial-strain test for nodule biomass

Response: nodule biomass[a]	Df	Sum Sq	Mean Sq	F value	[e]p value
plant[b]	1	36256	36256	10.5139	**0.001363**
competitor[c]	1	23155	23155	6.7147	**0.010182**
strain[d]	7	122741	17534	5.0848	**2.21E-05**
plant x competitor	1	172	172	0.0499	0.823463
plant x strain	7	38969	5567	1.6144	0.132258
competitor x strain	7	9350	1336	0.3874	0.909226
plant x competitor x strain	7	6493	928	0.269	0.965383
Residuals	227	782785	3448		

[a]Response indicates the dependent variable, the dry nodule biomass (mg) of each individual plant
[b]plant: plant species as independent factor
[c]competitor: whether it is inter-specific or intra-specific competition as independent factor
[d]strain: different strain inoculated as independent factor
[e]p-values lower than 0.05 are indicated in **boldface**

Table 6 ANOVA table of rhizobial-strain test for nodule number

Response: nodule number[a]	Df	Sum Sq	Mean Sq	F value	[e]p value
plant[b]	1	1018	1018.02	5.7735	**0.0170732**
competitor[c]	1	2073	2072.65	11.7547	**0.0007206**
strain[d]	7	9507	1358.19	7.7028	**2.37E-08**
plant x competitor	1	1128	1127.91	6.3968	**0.0121116**
plant x strain	7	2445	349.35	1.9813	0.0585906
competitor x strain	7	2270	324.22	1.8387	0.080937
plant x competitor x strain	7	737	105.26	0.597	0.7581142
Residuals	227	40026	176.33		

[a]Response indicates the dependent variable, the dry nodule number of each individual plant
[b]plant: plant species as independent factor
[c]competitor: whether it is inter-specific or intra-specific competition as independent factor
[d]strain: different strain inoculated as independent factor
[e]p-values lower than 0.05 are indicated in **boldface**

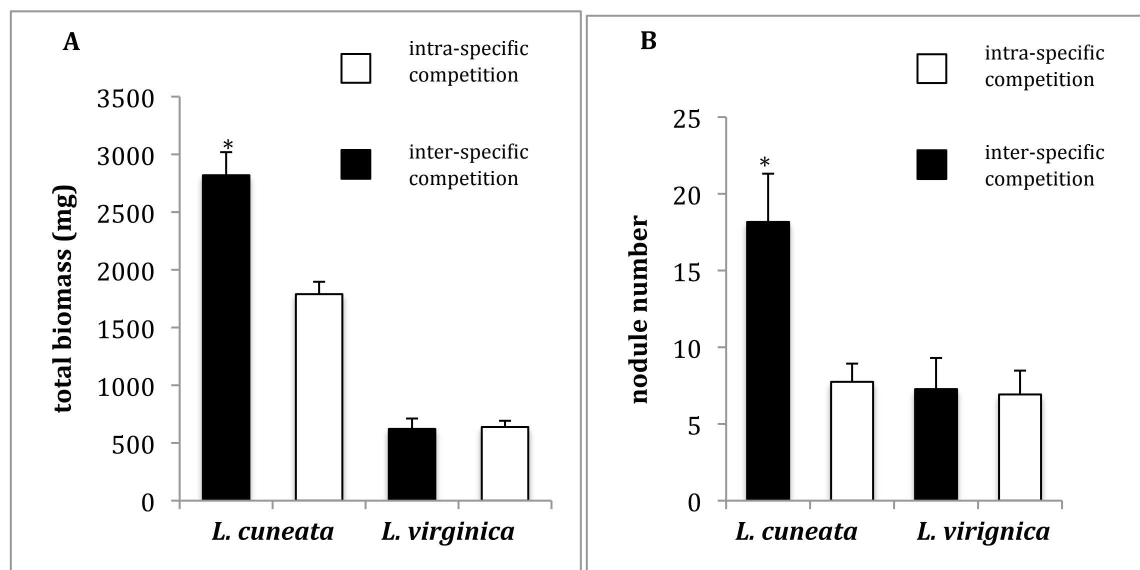

Figure 2 Total biomass (A) and nodule number (B) of different treatment levels (solid bar: inter-specific competition; empty bar: intra-specific competition; left two bars: *L. cuneata*; right two bars: *L. virignica*); columns with stars show significant larger value than the other competition conditions within one species at a 0.05 alpha level; error bar represents standard error

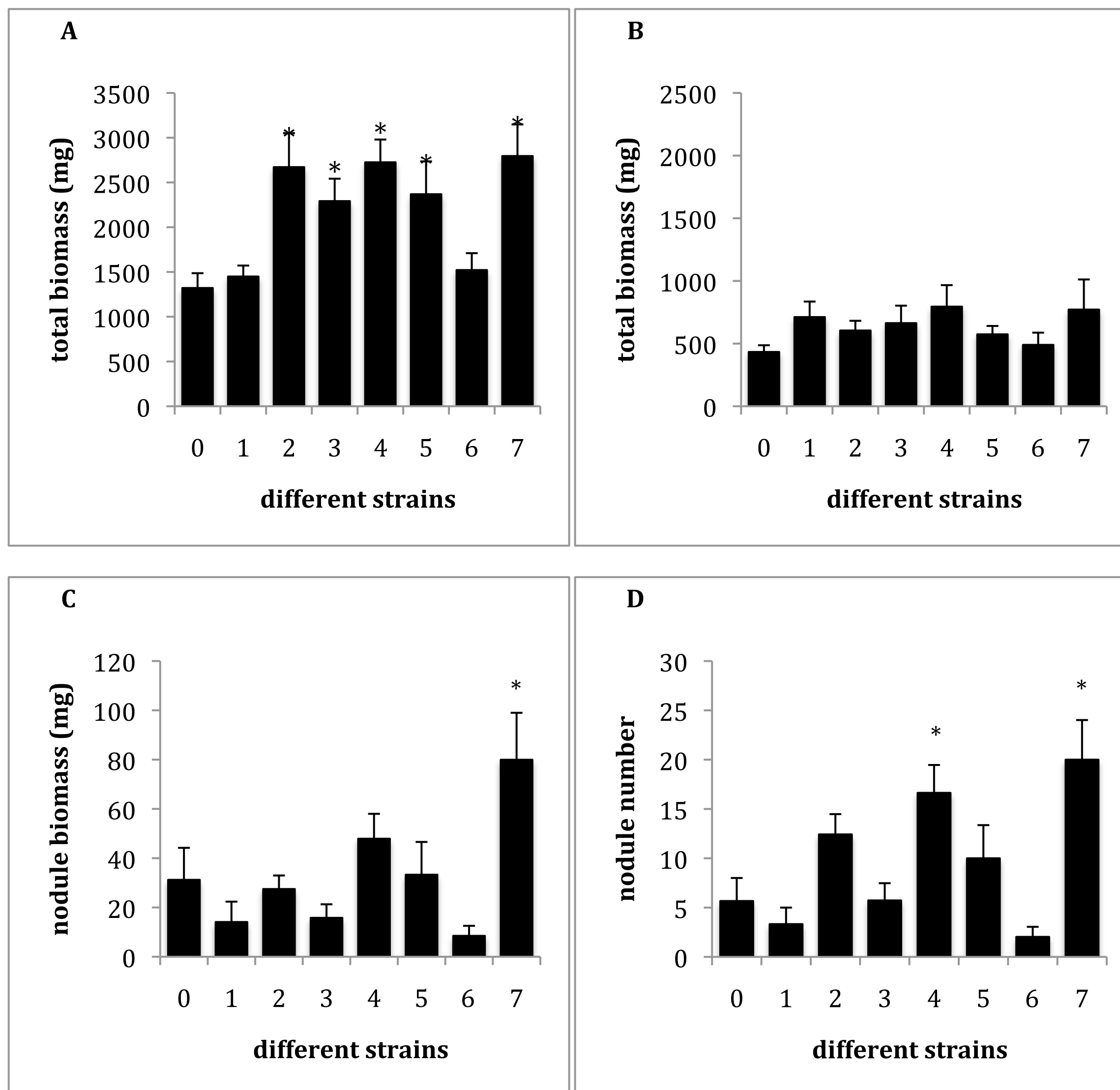

Figure 3 Total biomass of *L. cuneata* (A), total biomass of *L. virgnica* (B), nodule biomass of all plants (C) and nodule number of all plants (D) of different strains (0: no-inoculation control; 1: R1, *Mesorhizobium*; 2: R2, *Bradyrhizobium*1; 3: R3, *Rhizobium*1; 4: R4, *Bradyrhizobium*2; 5: R5, *Rhizobium*2; 6: R6, *Rhizobium*3; 7: R7, *Bradyrhizobium*3); columns with star show significant larger value than control at a 0.05 alpha level; error bar represents standard error

Appendix

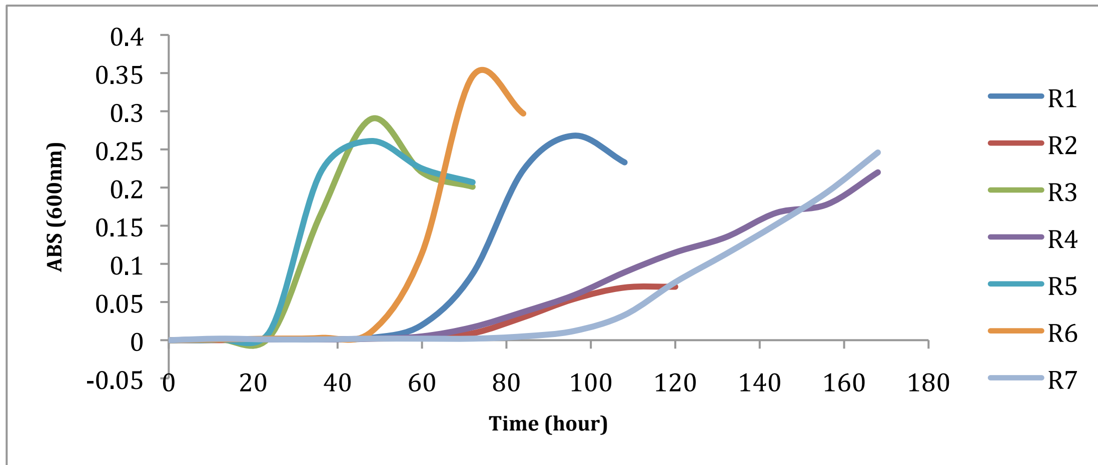

Figure A1 Growth curve of rhizobial strains (R1 to R7 are greenhouse ID, see also in Table A1)

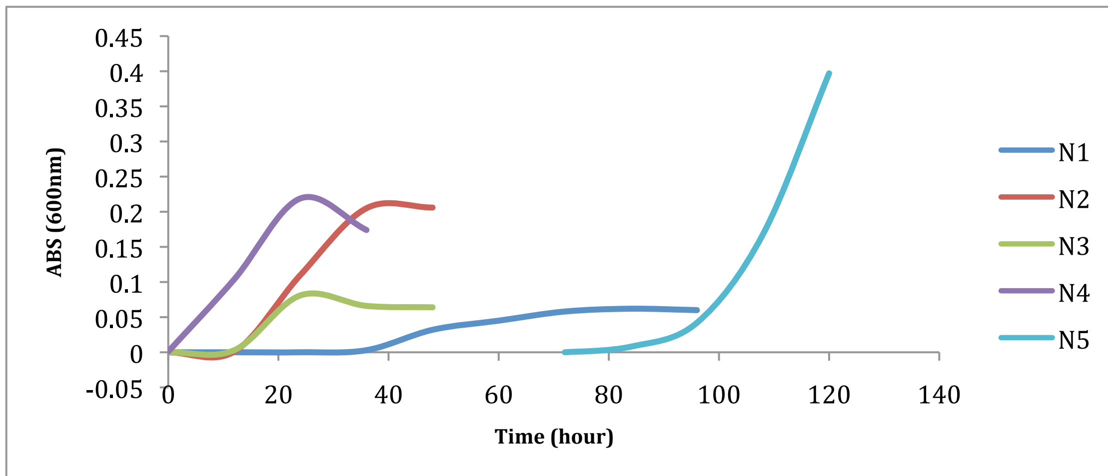

Figure A2 Growth curve of non-rhizobial strains (N1 to N5 are greenhouse ID, see also in Table A1)

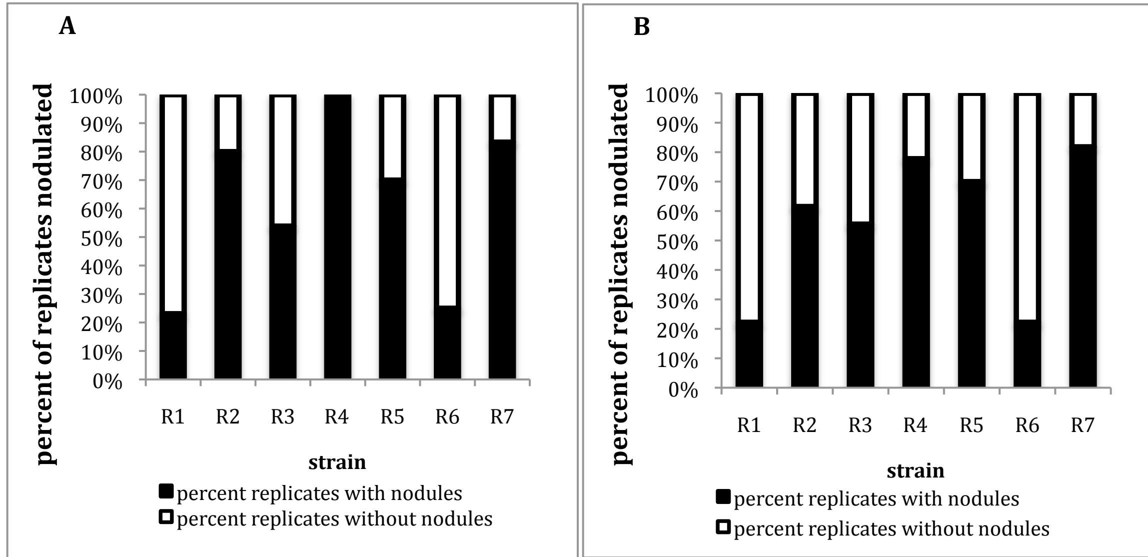

Figure A3 Colonization success of different strains (A: *L. cuneata*; B: *L. virginica*); R1 to R7 represents the seven strains used in the experiment, which stands for *Mesorhizobium*, *Bradyrhizobium1*, *Rhizobium1*, *Bradyrhizobium2, Rhizobium2, Rhizobium3, Bradyrhizobium3*

Table A1 Information about chosen strains

Strains[a]	Site[b]	Origin[c]	BLAST genus[d]	*nif*H band[e]	Description of colonies[f]	Invasion level[g]	Growth rate[h]
R1	BE2	*L. cuneata*	*Mesorhizobium*	weak	small, slimy white	medium	medium
R2	BE3	*L. cuneata*	*Bradyrhizobium*	strong	very small clear	high	slow
R3	BE3	*L. cuneata*	*Rhizobium*	weak	huge, slimy white	high	fast
R4	LW1	*L. cuneata*	*Bradyrhizobium*	strong	small, white, round	high	slow
R5	LW1	*L. cuneata*	*Rhizobium*	no	white, round, slimy	high	fast
R6	LW2	*L. virginica*	*Rhizobium*	no	slimy white, small	none	medium
R7	LW3	*C. fasciculata*	*Bradyrhizobium*	strong	white, small, round	medium	slow
N1	LW3	*L. virginica*	*Sphingomonas*	no	yellow, round, slimy	medium	slow
N2	LW1	*L. cuneata*	*Pseudomonas*	no	yellow, discolored media	high	fast
N3	LW3	*L. cuneata*	*Lysinibacillus*	no	small, white, smooth	medium	fast
N4	BE2	*L. cuneata*	*Burkholderia*	weak	large, slimy white	medium	fast
N5	BE2	*L. cuneata*	*Mycobacterium*	weak	smooth, milky white	medium	slow

[a]Greenhouse ID: the ID used for different strains in the greenhouse experiment
[b]Origin: different species of each strain collected from
[c]Site: different sites of each strain collected from, LI for lightly invaded sites, HI for heavily invaded sites, NI for non-invaded sites
[d]Genus: the genus based on BLAST result of 16S rRNA sequences of each strain
[e]*nif*H band: the presence of *nif*H band on electrophoresis gel (strong: I can see strong band; weak: I can see weak band; no: I did not see band)
[f]Description of colonies: visual features of colonies when growing on medium plate
[g]Invasion level: the invasion level of the site sampled (high: highly invaded; none: no invasion; medium: lightly invaded)
[h]Growth rate: how fast the strain grows (fast: grow relatively fast; slow: grow relatively slow; medium: growth rate in the middle; see also Figure A1 and A2)

Table A2 AONVA table of Rhizobial-strain test for shoot biomass to root biomass ratio

Response: shoot to root ratio[a]	Df	Sum Sq	Mean Sq	F value	[e]p value
plant[b]	1	495.57	495.57	224.1271	**<2e-16**
competitor[c]	1	0.15	0.15	0.069	0.7931
strain[d]	7	20.93	2.99	1.3526	0.2267
plant x competitor	1	0.02	0.02	0.0098	0.9211
plant x strain	7	12.17	1.74	0.7863	0.5994
competitor x strain	7	11.14	1.59	0.7195	0.6555
plant x competitor x strain	7	8.92	1.27	0.5766	0.7747
Residuals	227	501.92	2.21		

[a]Response indicates the dependent variable, the ratio of shoot over root biomass of each individual plant
[b]plant: plant species as independent factor
[c]competitor: whether it is inter-specific or intra-specific competition as independent factor
[d]strain: different strain inoculated as independent factor
[e]p-values lower than 0.05 are indicated in **boldface**

Table A3 AONVA table of Rhizobial-strain test for nitrogen fixation activity

Response: ARA[a]	Df	Sum Sq	Mean Sq	F value	[e]p value
plant[b]	1	3612	3612	0.1569	0.6924
competitor[c]	1	803	803	0.0349	0.8521
strain[d]	7	107801	15400	0.6688	0.6983
plant x competitor	1	58684	58684	2.5487	0.1118
plant x strain	7	271274	38753	1.6831	0.1141
competitor x strain	7	216839	30977	1.3454	0.2299
plant x competitor x strain	7	167802	23972	1.0411	0.4031
Residuals	227	5226643	23025		

[a]Response indicates the dependent variable, ARA (nmol/hour): nitrogen fixation activity of each plant individual
[b]plant: plant species as independent factor
[c]competitor: whether it is inter-specific or intra-specific competition as independent factor
[d]strain: different strain inoculated as independent factor
[e]p-values lower than 0.05 are indicated in **boldface**

Table A4 AONVA table of Rhizobial-strain test for standardized nitrogen fixation activity

Response: sARA[a]	Df	Sum Sq	Mean Sq	F value	[e]p value
plant[b]	1	37.1	37.077	2.401	0.1226
competitor[c]	1	19.3	19.318	1.251	0.2645
strain[d]	7	91.7	13.098	0.8482	0.5485
plant x competitor	1	17.9	17.936	1.1615	0.2823
plant x strain	7	99.4	14.194	0.9192	0.4923
competitor x strain	7	68.1	9.722	0.6296	0.7312
plant x competitor x strain	7	51.6	7.365	0.4769	0.8508
Residuals	227	3505.4	15.442		

[a]Response indicates the dependent variable, sARA (nmol/(hour*mg)): nitrogen fixation activity standardized by nodule biomass
[b]plant: plant species as independent factor
[c]competitor: whether it is inter-specific or intra-specific competition as independent factor
[d]strain: different strain inoculated as independent factor
[e]p-values lower than 0.05 are indicated in **boldface**

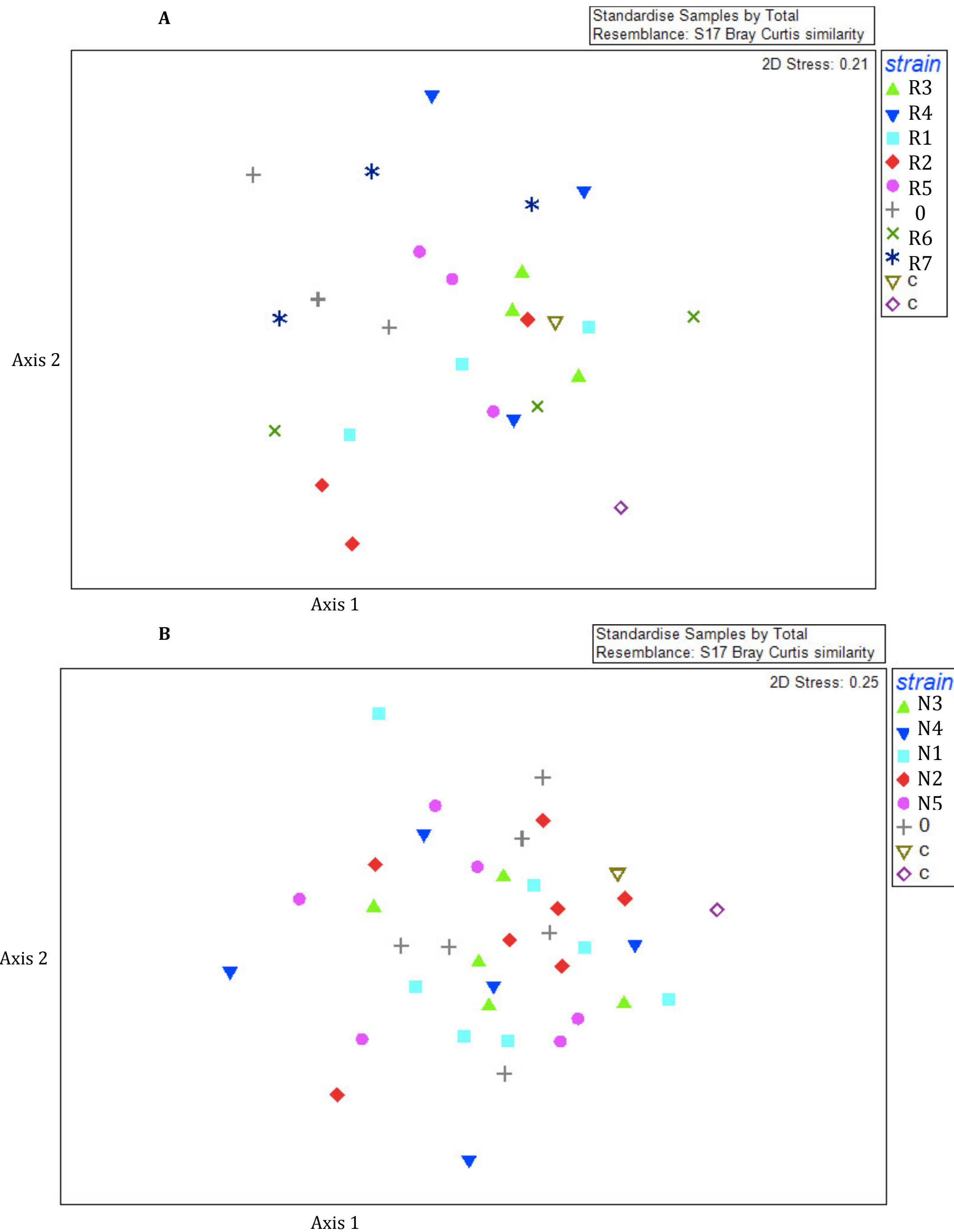

Figure A4 NMDS of Soil Sterilization Check for the first (A) and the second (B) Greenhouse Experiment: Different shapes of points represent the bacteria community of the soil with different strains treatment. Each point was generated by ARISA, a fingerprinting method for bacteria community analysis. Closer points in the NMDS plot represent more similar bacteria communities. “0” stands for the control in the greenhouse experiment. “c” stands for the soil that has been sterilized without being in the greenhouse.

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