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BIOLOGICAL NITROGEN FIXATION IN *AESCHYNOMENE INDICA*: A COMPARISON BETWEEN BTAi1 AND A NATIVE STRAIN ISOLATED IN TEXAS

by

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ABSTRACT

BIOLOGICAL NITROGEN FIXATION IN *AESCHYNOMENE INDICA*:
A COMPARISON BETWEEN BTAi1 AND A NATIVE
STRAIN ISOLATED IN TEXAS

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Host-microbe interactions between legumes and rhizobia exemplify a symbiotic relationship; the rhizobia provide fixed nitrogen to leguminous plants via symbiotic nitrogen fixation, while they are safely harbored in plant root nodules. In this study, the nodulation of the legume *Aeschynomene indica* inoculated with BTAi1, a known rhizobium, was monitored and compared when inoculated with TSN2, a newly isolated rhizobium in Texas. Upon maturation, the physical characteristics of the plant were examined, and nitrogen fixation abilities were measured. In addition, the rhizobium isolated from these plants was sequenced and named "TSN2". It was found that there were no significant differences in physical characteristics or nitrogen fixation ability between *A. indica* inoculated with TSN2 and *A. indica* inoculated with BTAi1. Taken together, TSN2 is as a good of a symbiont to *A. indica* growth compared to its counterpart.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1

INTRODUCTION

One major limiting nutrient to plant growth is nitrogen, and as a result, farmers are forced to use synthetic nitrogen fertilizers to meet product demand. Although necessary, these fertilizers can lead to serious environmental consequences, including groundwater contamination, land degradation, and the formation of aquatic dead zones (14). In fact, it is estimated that greater than 50% of chemical nitrogen fertilizers applied to fields are lost via groundwater leaching and runoff (7). One notable example of the impact of nitrogen runoff has been observed where the mouth of the Mississippi River meets the Gulf of Mexico. During the summer months, the Mississippi River carries runoff from the fields of the Mississippi River Basin to the Gulf Coast, where the nutrients deposited in the waters create massive eutrophication events (5). The algal blooms induce hypoxic conditions below the ocean surface, leading to massive dead zones that can span thousands of miles wide (5). The result is the disruption of the ecosystem with a lengthy environmental and economic disaster.

To combat such serious environmental consequences, scientists are turning toward studying biological nitrogen fixation (BNF) to reduce the need for synthetic fertilizers. BNF is the process in which atmospheric nitrogen (N_2), is converted into a form that plants can use (e.g., NH_3) (10). Because breaking the N_2 triple bond requires a considerable amount of energy and specialized enzymes, eukaryotes are incapable of this form of metabolism. Instead, this ability is limited to bacteria and some archaea (10). As a result,

plants will often form mutualistic relationships with soil bacteria, or rhizobia; in return for fixed nitrogen, the plant will harbor the rhizobia in specialized plant organs called nodules (10). This type of mutualism is primarily observed in the legumes. Interestingly, if BNF could be optimized or engineered to apply to other non-leguminous plants (e.g., corn and wheat), it could help decrease the need for synthetic nitrogen fertilizers.

Rhizobia are spread throughout the classes of Alphaproteobacteria and Betaproteobacteria, but the genus within Alphaproteobacteria called *Bradyrhizobium* is most well-known for establishing symbiotic relationships with legumes (9). Photosynthetic Bradyrhizobia, including *Bradyrhizobium sp.* BTAi1 and ORS278 are unique in both their infection process and nodulation. More specifically, BTAi1 can form both stem and root nodules without exhibiting *nod* genes (9). The *nod* genes are genetic factors that interact with a plant host to instigate the infection process. Nod factors are present in almost all other rhizobia, suggesting that somehow BTAi1 can circumvent canonical nodulation processes and initiate a Nod factor-independent pathway during infection (15). Furthermore, even though it bypasses Nod factors, BTAi1 can still form both stem and root nodules, with stem nodules being characteristic of only photosynthetic Bradyrhizobia (9).

Investigating BTAi1 and other potential nodulators is useful because it can help provide a greater understanding of the types of rhizobia that can uniquely infect *Aeschynomene* plants. It is likely that some other rhizobia than BTAi1 and ORS278 could form root and stem nodules via the Nod factor-independent pathway. Better understanding of the physical ways by which nodulators impact the legume plant could help lead to further studies on potential BNF in non-legume plants via the Nod factor-independent nodulation.

In this study, physical characteristics from nodulation of BTAi1 is compared to that of a bacterial isolate (TSN2) from stem nodules found on Texas *Aeschynomene*. This bacterial sample was isolated by Meaghan Rose, a graduate student of Chang Lab. The purpose is to compare both the physical characteristics of the nodules and the nitrogenase capability of the symbionts. Additionally, the 16S rRNA gene of TSN2 was sequenced to identify the TSN2 microorganism at the species level.

CHAPTER 2

METHODOLOGY

2.1*Aeschynomene indica* germination (13)

A. indica seeds were surface sterilized via soaking in sulfuric acid for 20 min. They were then washed with six changes of sterile water and soaked in sterile water over night in a shaking incubator at 30 °C and 200 rpm. After soaking, they were aseptically transferred onto water agar, wrapped in foil, and incubated at 30 °C until germination. Once the germinated seeds reached a root length of 1.5-2.0 cm, they were aseptically transferred into autoclaved seed pouches (Mega International) with three seeds to a pouch. A straw was placed on one end of the pouch to serve as a watering tube and was arranged such that the bottom of the straw was about 5 cm above the bottom of the pouch. Two pouches were paperclipped to a hanging folder, and each folder of pouches was placed on a folder rack. These folder racks are what supported the pouches during plant growth (Figure 3.1).

2.2 Bacterial preparation and plant inoculation (13)

To make the inoculant, TSN2 and *Bradyrhizobium* sp. BTAi1 were grown to mid-log phase (~0.8 OD_{600}) in 10 mL of AG medium in a shaking incubator at 30 °C and 200 rpm. The cells were centrifuged at 4,000 rpm for 10 min and washed with half strength Boughton and Dilworth (B&D) medium. The strain suspension in half strength B&D medium was adjusted to an OD_{600} of 1.0. Each pouch was given 20 mL of half strength B&D to help cultivate the germinated seeds. Then, half of the plants were inoculated with 200µL of the TSN2 suspension and the other half of the plants were inoculated with 200µL

of the BTAi1 suspension. The plants were grown in a growth chamber with 16 h of light at 32°C and 8 h of dark at 28 °C. They were watered every two days with 10 mL of half strength B&D medium over the course of 10 weeks.

2.3 Acetylene reduction assay (3, 13)

The acetylene reduction assay (ARA) was used to study the nitrogen fixation activity of BTAi1 and TSN2 by using gas chromatography (GC) to detect ethylene production. Six mature plant samples were harvested, and their root nodules were aseptically transferred into a sterile jar. The jar was covered with a septum cap, and using a syringe (Hamilton Co., Reno, NV) 10% of the air inside of the jar was replaced with acetylene gas. After light shaking, 250µL of gas from the jar was drawn with a small syringe (Hamilton Co., Reno NV) and inserted into the gas chromatograph (GC-2014; Shimadzu). The areas of the acetylene and ethylene peaks were recorded. This experiment was repeated three times, and after each repetition, the nodules from each sample were dried in the oven and weighed (Denver Instruments APX-60).

2.4 Nodule surface sterilization and bacterial reisolation

Root nodules from each group were placed into respective 2 mL microcentrifuge tubes. Next, they were soaked in 20% ethanol solution (20% EtOH) for 15 sec and rinsed with three changes of sterile water. They were then rinsed with 0.9% NaCl solution and then washed with another change of sterile water. The nodules were soaked for 5 min in 3% NaOCl solution, with frequent inverting of the tubes. Finally, they were rinsed with three changes of sterile water, completing sterilization. To reisolate the bacteria, the tubes were vortexed at maximum speed for 2 min, serially diluted, and plated on Yeast Mannitol Agar (YEM) with congo red.

2.5 Glycerol stocks

Bradyrhizobium sp. BTAi1 and TSN2 were growth to late log phase in AG medium. After growing them, 500 µL of each culture was transferred into a respective stock tube with 500 µL of 50% glycerol. They have been stored at -80°C.

2.6 DNA extraction

For all DNA extractions performed in this experiment, Gene JET genomic DNA purification kits were used. The instructions that follow reflect the instructions in the kit. Exactly 1.5 mL of bacterial culture grown in AG was pelleted via centrifugation at 8,000 rpm for 5 min. The supernatant was disposed and 180 µL of digestion solution and 20 µL of proteinase K was added to the reaction mixture. The solution was soaked in a hot water bath at 56°C for 4 h, and then 30 µL of RNase A was added to the reaction mixture. The mixture was vortexed and incubated at room temperature for 2 min, before 200 µL of lysis solution and 200 µL of 100% ethanol was added. The mixture was transferred to a filter column in a 2 mL collection tube and centrifuged at 8,000 rpm for 1 minute. The flowthrough was disposed and after placing the filter into a fresh column, 500 µL of wash buffer 1 was added to the filter column. The apparatus was centrifuged at 8,000 rpm for 1 minute. The flowthrough was disposed of and 500 µL of wash buffer 2 were added to the filter column and the apparatus was centrifuged again at 14,000 rpm for 3 min. Next, 30 µL of water was added and centrifuged through the tube for 1 minute at full speed. This last step was repeated with another 30 µL aliquot of water. The final flow through was transferred into a fresh microcentrifuge tube and stored at -20°C.

2.7 Polymerase chain reaction (PCR)

A master reaction mixture was made with 35.5µL of nano pure water, 1.0 µL of forward primer, 1.0 µL of reverse primer, 1.0 µL of dNTP mixture, 10.0 µL of Taq buffer and 0.5 µL of Taq polymerase. This mixture was scaled up such that the number of samples desired to be made was added to 0.5 and multiplied by the basal volumes presented above. For 16S rRNA PCR, 8F and 1492R primers were used. After vertexing the reaction mixture, aliquots of 49 µL were added into each PCR reaction tube. Then, 1.0 µL of the DNA template were added to each tube, increasing the total volume of each reaction tube to 50 µL. The PCR tubes were placed in a C1000 Touch Thermo Cycler (Bio-Rad) such that pre-denaturation was 95°C for 5 min, 35 cycles of denaturation at 90°C for 1 minute, annealing at 55°C for 30 sec, and extension at 72°C for 1 minute and 30 sec, a final extension at 72°C for 10 min, and storage set at 4°C indefinitely.

2.8 Gel electrophoresis

In an Erlenmeyer flask, 0.7g of agarose powder was added with 70mL of 1x TAE buffer solution. The mixture was heated in a microwave for 1 minute and 10 sec and left to cool to handling temperature. Once cool, 0.5 µL of 1% EtBr was added to the mixture. The solution was swirled and poured into a gel mold fit with a comb. This was allowed to harden for thirty minutes, until it was an opaque white hue. Then, 1× TAE buffer was poured over the gel to submerge it and the comb was carefully removed. The dye and DNA samples were mixed in a 1:4 ratio and loaded into the wells. The gel was covered and left to run at 160V for 45 min, and the final product was viewed in a gel doc under UV light.

CHAPTER 3

RESULTS

It was found that *A. indica* had germination rate of approximately 37% (Figure 3.2). The roots tended reach 1.5 – 3.0 cm after 2 days incubating on water agar. After inoculation, root nodulation began at day 12 (Figure 3.3), and by day 20, some plants began to express stem nodulation. Root nodules also began to exhibit a pink color by day 20. This characteristic was present in both plant groups inoculated with TSN2 and BTAi1.

Figure 3.1: An image of A. indica in the growth chamber

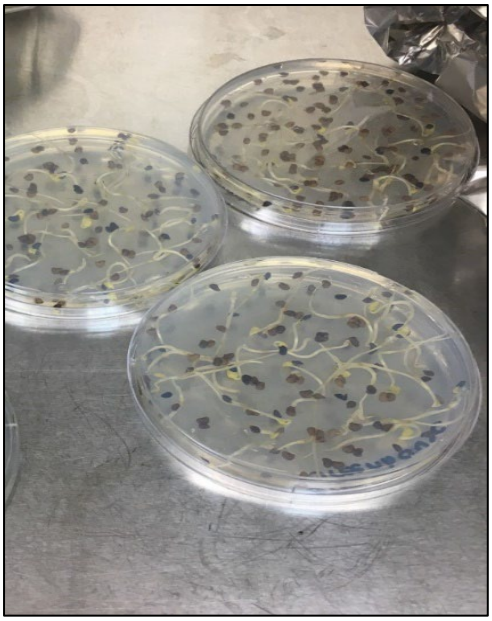

Figure 3.2: *A. indica* seeds germinated on water agar

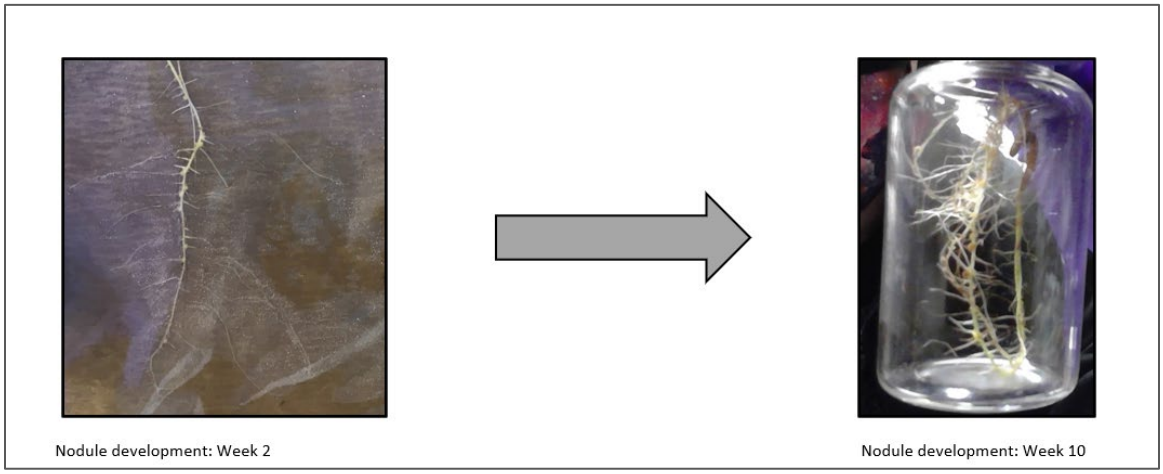

Figure 3.3: Maturing root nodules of *A. indica* inoculated with TSN2

After maturation, physical characteristics, including plant height, nodule number, stem dry weight, root dry weight, and root length, were compared. It was found that there was no statistically significant difference in these characteristics between *A. indica* inoculated with TSN2 and *A. indica* inoculated with BTAil (Figure 4A-F). Furthermore,

there was no significant difference when comparing nitrogenase activities between the two groups (Figure 4G).

In this experiment, both the bacteria used to inoculate the plant and the bacteria re-isolated from the plants underwent the 16S rRNA analysis. To clone 16S rRNA genes, DNA extraction, PCR, and gel electrophoresis were performed. A total of 4 bacterial samples were investigated: the two inoculants (BTAi1 and TSN2) and the two reisolates (BTAi1* and TSN2*). After DNA extraction, BTAi1 and TSN2 exhibited 260/280 values of 1.91 and 1.92 respectively, and both BTAi1* and TSN2* exhibited 260/280 values of 1.86. After PCR, 16S rRNA genes (i.e., 1.5 kb fragments) were confirmed as shown in Figure 5. Through the sequencing analysis with alignment and BLAST (11), TSN2 was identified as *Leifsonia shinshuensis*.

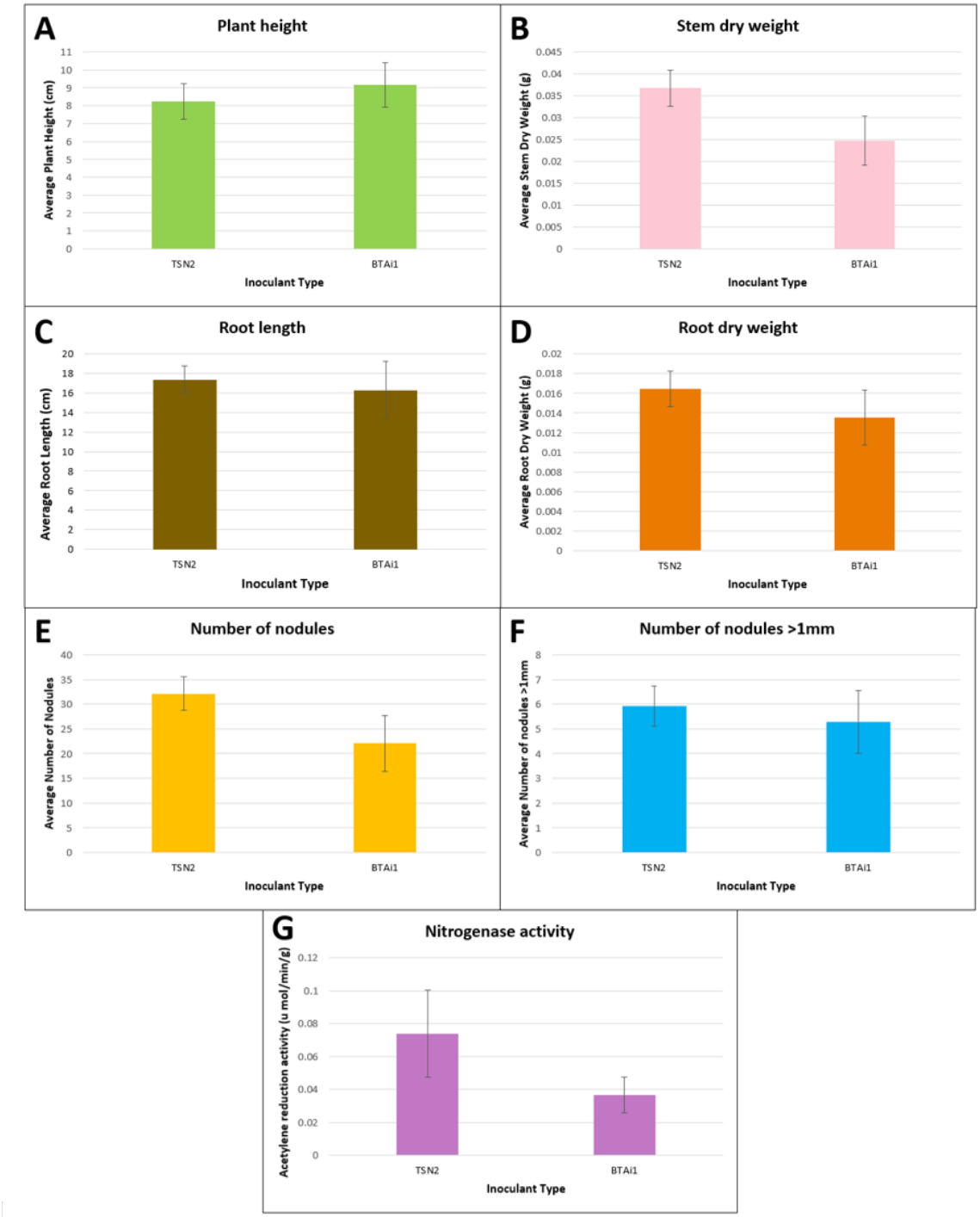

Figure 3.4: Comparative plots of physical characteristics of inoculated plants and the nitrogenase activity of their root nodules. Comparison of plant height (A), stem dry weight (B), root length (C), root dry weight (D), nodule numbers (E, F) and nitrogenase activity (G) between A. indica inoculated with TSN2 and A. indica inoculated with BTAi1

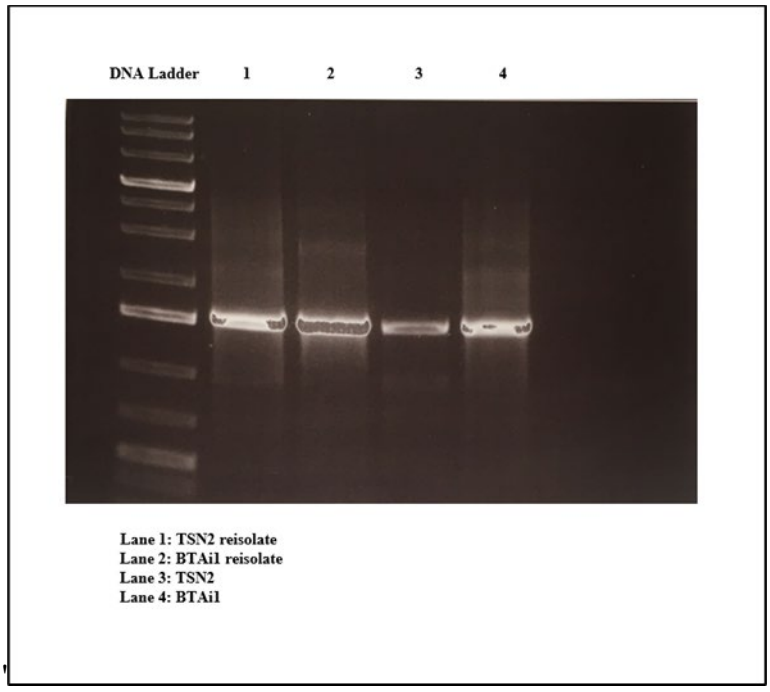

Figure 3.5: An image from gel electrophoresis of 16S rRNA genes from PCR amplification

CHAPTER 4

DISCUSSION

Stem nodulation and Nod factor-independent infection are two unusual ways that BTAi1 interacts with *A. indica*. Since stem nodulation is only characteristic of photosynthetic Bradyrhizobia (9), it is important to understand any rhizobia that can initiate this form of nodulation. Understanding of this mechanism could also help us research into ways symbiotic nitrogen fixation can be optimized in non-leguminous plants such as corn and wheat.

A. indica seeds stored and grown in typical ambient conditions express a germination rate of 42% (4). In this study, these seeds expressed a germination rate of 37%. This likely has to do with the surface sterilization process of the seeds (13). Because they were soaked in concentrated sulfuric acid, seeds with a weaker seed coat did not germinate. Approximately 12 days after germination and inoculation, root nodulation was expressed in both groups, and by day 20, stem nodulation had developed in both groups. While stem nodulation was expected, the observation of root nodules in the TSN2 group confirms Koch's postulates: the reinfection of TSN2 led to stem nodule development, and TSN2 could be reisolated from nodules of the infected host.

This research provides a comparison between the physical characteristics and nitrogen fixation ability between plants infected with BTAi1 and plants infected with a Texas stem nodule isolate. It was found that there was no significant difference between the two groups. Both plants exhibited similar stem heights, nodule counts and dry weights,

indicating that TSN2 promoted growth just as well as its counterpart BTAi1. Additionally, after maturation, both plants exhibited a pink color in their nodules, suggesting the presence of Leghemoglobin. Leghemoglobin is plant heme proteins that facilitate the anoxic conditions necessary in root nodules for symbionts to perform nitrogenase activity (2). Formation of a strong pink color in nodules is characteristic of successful nitrogen fixation between the rhizobia and host plant. This successful nitrogen fixation was confirmed in the acetylene reduction assay (ARA), where the reduction of acetylene to ethylene was measured via gas chromatography (3,5). Through ARA, TSN2 showed effective nitrogenase activity as much as BTAi1 did.

After 16S rRNA sequencing of TSN2 and BTAi1 reisolates, the microorganisms were identified as *Leifsonia shinshuensis* and *Bulkolderia stagnalis,* respectively. *B. stagnalis* is a form of β-proteobacteria that could act as a contaminant in this study (1). The presence of this contamination likely occurred during a solution mix-up in the bacterial re-isolation process of the BTAi1 sample. Due to challenges presented by the pandemic, time was too limited to go back and reperform this part of the experiment on the BTAi1 sample. However, sequencing analysis confirmed the presence of *L. shinshuensis* DNA in both the inoculum and reisolate of TSN2, effectively revealing its identity. *L. shinshuensis* is known as a root nodule endophyte, or a type of microorganism that normally presents a supporting role in root nodule development (8). Not only is *L. shinshuensis* found in legume root nodules, along with other common rhizobia (8), but it can also be found in a variety of plants, including fruit trees. This indicates that they can exhibit a diverse range of mutualistic activities, including nitrogen fixation ability (8). Although we identified TSN2 using the 16S rRNA sequencing analysis, this part of the experiment should be further

investigated because *L. shinsheuensis* is considered a non-rhizobium and this could be the first observation in the rhizobia-*Aeschynomene* symbiosis (2,6,8).

Although the identification of TSN2 is still under further evaluation, it has proven to be just as successful as BTAi1 to *A. indica*. Both the physical characteristics of the plants and their nitrogenase activities were not significantly different, indicating that the two bacteria were similar in terms of function. Furthermore, TSN2 could be used as an inoculant to enhance BNF in leguminous plants, as well as potentially in non-legumes.

REFERENCES

1. Bahulikar RA, Torres-Jerez I, Worley E, Craven K, Udvardi MK. 2014 Diversity of nitrogen-fixing bacteria associated with switchgrass in the native tallgrass prairie of northern Oklahoma. *Applied Environmental Microbiology*. 80(18): 5636-5643
2. Cardoso JD, Hungria M, Andrade DS. 2012. Polyphasic approach for the characterization of rhizobial symbionts effective in fixing N2 with the common bean (Phaselous vulgaris L.). Applied Environmental Biotechnology. 93: 2035-2049.
3. Hardy RW, Holsten RD, Jackson EK, Burns RC. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiology*. 43(8): 1185-1207
4. Jin CH, Romij Uddin, Pyon JY. 2010. Seed germination and seedling emergence of Indian jointvetch (Aeschynomene indica L.) in different conditions. *Korean Journal of Weed Science.* 30(1).
5. Joyce S. 2000. The dead zones: oxygen-starved coastal waters. *Environmental Health Perspective.* 108(3): A129-A125Laiqat F, Eltem R. 2016. Identification and characterization of endophytic bacteria isolated from in vitro cultures of peach and pear rootstocks. *3 Biotech*. 6(120).
6. Mahmud K, Makaju S, Ibrahim R, Missaoui A. 2020. Current Progress in Nitrogen Fixing Plants and Microbiome Research. *Plants*. 9(1): 97

7. Meyer SED, Beuf KD, Vekeman B, Willems A. 2015. A large diversity of non-rhizobial endophytes found in legume root nodules in Flanders (Belgium). *Soil Biology and Biochemistry.* 82:1-11.
8. Mornico D, Miché L, Béna G, Nouwen N, Verméglio A, Vallenet D, Smith AAT, Giraud E, Médigue C, Moulin L. 2012. Comparative genomics of Aeschynomene symbionts: insights into the ecological lifestyle of non-independent photosynthetic Bradyrhizobia. *Genes*. 3(1):35-61.
9. Mus F, Crook MB, Garcia K, Garcia Costas A, Geddes BA, Kouri ED, Paramasivan P, Ryu M-H, Oldroyd G, Poole P, Udvardi M, Voigt C, Ane J-M, Peters J. 2016. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. *Applied Environmental Microbiology*. 82(13): 3698-3710.
10. National Center for Biotechnology Information (NCBI)[Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988] – [accessed 2020 Nov 19]. Available from: https://www.ncbi.nlm.nih.gov/
11. Ott, T., van Dongen, J. T., Gunther, C., Krusell, L., Desbrosses, G., Vigeolas, H., Bock, V. Czechowski, T., Geigenberger, P., Udvardi, M. K. 2005. Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Current Biology*. 15: 531-535.
12. Parks D. 2018. Circadian Orchestration of symbiotic genes in photosynthetic nitrogen-fixing bacteria [dissertation]. 18–21.
13. Ward M. 2011. Too much of a good thing? Nitrate from nitrogen fertilizers and cancer. 2009. *Reviews on Environmental Health*. 24(4): 357-363.

14. Zhang Z, Li Y, Pan X, Shao S, Liu W, Wang E-T, Xie Z. 2019. Diversity and evolution of Aeschynomene indica nodulating rhizobia without the nod factor synthesis genes in Shandong Peninsula, China. *Applied and Environmental Microbiology*. AEM.00782-19.

BIOGRAPHICAL INFORMATION

Abbey Raymond began her attendance at the University of Texas in Arlington in Fall 2017. In addition to her love of biology, she has a passion for engineering and physics. During her time as an undergraduate, she has worked in three research laboratories and served as vice president of an on-campus competitive robotics team.

As well as studying biological nitrogen fixation under Dr. Chang, she has also studied neutrinos and neutrinoless double beta decay under Dr. Asaadi and Dr. Jones. In her work in physics, she has designed and built a sputter deposition chamber, kickstarted a project on novel circuit boards for neutrino detection and has worked on simulations of particle flight patterns along radiofrequency carpets.

Although she loves physics and engineering, Abbey is passionate about environmentalism and microbiology. After college, she hopes to apply these two concepts to help engineer a more sustainable future.