ENDOPHYTIC BACTERIA ISOLATED FROM AESCHYNOMENE INDICA PLANTS AND THEIR ROLE IN NOD-FACTOR INDEPENDENT NODULE FORMATION AND NITROGEN FIXATION

By

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ABSTRACT

ENDOPHYTIC BACTERIA ISOLATED FROM AESCHYNOMENE INDICA PLANTS AND THEIR ROLE IN NOD-FACTOR INDEPENDENT NODULE FORMATION AND NITROGEN FIXATION

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The University of Texas - Arlington, 2023

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The formation of specialized root organs known as nodules is the most defining characteristic of leguminous plants due to the fixation of atmospheric nitrogen to bioavailable forms enabled by rhizobial endosymbionts. This relationship has played an integral role in the agricultural practice of using such crop plants as biofertilizers to reduce the use of and dependency on synthetic chemical fertilizers. Tropical legumes, such as members of the genus *Aeschynomene*, are particularly recognized for their ability to form stem nodules through an unknown mechanism independently of the canonical Nod Factor signaling pathway used in standard root nodulation. The works in this study have identified the significance of the *Bbta_p0110* gene from the NF-independent nodulating *Bradyrhizobium* sp. BTAi1 in nodulation of the plant *Aeschynomene indica* under laboratory conditions. In addition to nitrogen fixing nodule symbionts, these organs house a range of plant growth promoting endophytic bacteria that confer traits such as nutrient acquisition, disease resistance, phytohormone production, and abiotic stress tolerance. These endophytic bacteria are specific to the regions in which the host plant is cultivated. There have been many studies demonstrating host/symbiont specificity depending on species and region. However, none have demonstrated host specificity of the same

plant species, particularly *A. indica* depending on geographical origin. In this study, we show a clear difference in host plant response to endophytic bacteria obtained from varying geographical regions and plant growth promotion indicating biogeographic importance on host/endophyte symbiosis. Additionally, we isolated and identified a novel nodule promoting *Leifsonia* bacterial species and, through whole genome sequencing, have postulated its role as an abiotic stress mitigator for *A. indica* and other significant crop plants. Insights to the dynamics of endophyte and host plant relationships regarding nodulation, nutrient cycling, and stress tolerance leads to further advancements and understanding behind the use of bacterial inoculants as biological fertilizers for agricultural purposes.

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DEDICATION

This work is dedicated to the honor and glory of the Heavenly Father, without whom I would not have the great pleasure of studying His natural world. Without the comfort of Christ, the strength of God, and the guidance of the Holy Spirit, I surely would not have seen the end of these works. I strive to make Him proud in all that I do and I give my endless thanks for the opportunity to experience and study His creation. IX IC NIKA

"A little science estranges men from God, but much science leads them back to Him."

"The more I study nature, the more I stand amazed at the work of the Creator."

- Louis Pasteur

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CHAPTER 1

INTRODUCTION

Biological significance of bacteria in nutrient cycling and plant growth

Bacteria are known to form beneficial symbiotic relationships with nearly every type of complex life and can contribute to the overall health and survival of their host through aid in defense, growth, nutrition, and many more attributes. These relationships can be from a distance, such as the surrounding soil, water, or air, and they can be even more intimate such as on the surface or within the host tissues themselves. In the plant world, bacteria are teeming within the surrounding soils and on the surfaces of every plant organ (root, stem, leaves, flowers, & fruit). Free living bacteria within plant-surrounded soils in close association with root systems, or within the "rhizosphere" have been shown time and time again to confer beneficial characteristics to their host plants (Compant et al., 2010; Glick, 2012; Olanrewaju et al., 2017). These organisms, generally known as plant growth promoting rhizobacteria (PGPR) are of great interest to agricultural researchers due to the assistance they provide to their host plants through nutrient cycling, hormone production, and defense (Aeron et al., 2011; Podile & Kishore, 2007). There are many genera of PGPR documented (Table 1-1) and more are being reported every year, with a few being *Agrobacterium, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Burkholderia, Bacillus, Bradyrhizobium, Cyanobacteria, Gluconacetobacter, Enterobacter, Herbaspirillum, Klebsiella, Pseudomonas, Paenibacillus, Rhizobium, Serratia, Xanthomonas,* and *Streptomyces* (Basit et al., 2021). Those that are found within plants are referred to as "endophytes" and, by definition, cause no harm to their host plant (Christina et al., 2013). Like

their free-living PGPR counterparts, endophytic bacteria are incredibly diverse, providing the opportunity for host plants to capitalize on a whole host of metabolic capabilities to gain advantages against biotic and abiotic challenges (Table 1-2). In most cases, these endophytic bacteria form mutualistic relationships with the plant and play a vital role in plant growth, nutrient cycling, and plant defense against pathogens in exchange for environmental stability and supplemental nutrient sources (Brader et al., 2014; Liu et al., 2017). These relationships have proven to be a crucial area of study particularly regarding the enhancement of agricultural practices leading to increased crop survivability, yield, and zone habitability through abiotic stress mitigation (Malik & Arora, 2022; Senthikumar et al., 2011; White et al., 2021). Endophytic bacteria contribute to these factors in that, even in their free-living state, they promote plant growth by fixing nitrogen, solubilizing phosphorus, producing plant growth promoting hormones, and suppressing plant pathogens through the production antibiotics and their precursors (Chebotar et al., 2015; Ferreira da Silva et al., 2017; Tiwari et al., 2010). Additionally, these organisms play important roles in nutrient cycling through the decomposition of organic materials, making these nutrients further available for all forms of life.

The five most abundant elements making up an organism are carbon, oxygen, hydrogen, phosphorus, and nitrogen (Frieden, 1972). Nitrogen is one of the most limiting factors on the proliferation of life due to its limited bioavailability (Shridhar et al., 2012). Most of the nitrogen here on Earth is trapped in the form of atmospheric nitrogen, consisting of two nitrogen atoms held together with an incredibly strong triple bond (Zhang & Zindler, 1993). This bond must be broken through nitrogen fixation, either naturally or, most commonly for agricultural purposes, artificially. Artificial nitrogen fixation is the process of producing ammonia (NH_3) and other nitrogen-containing compounds from atmospheric nitrogen (N_2) using industrial processes such

as the Haber-Bosch process (Modak, 2002). This system uses high temperature and pressure to force atmospheric nitrogen and hydrogen gas together in order to produce ammonia, a common bioavailable form of nitrogen. This process revolutionized the production of fertilizers and greatly increased the availability of nitrogen for crop growth, leading to a massive increase in agricultural productivity. As of today, the Haber-Bosch process remains the primary method for artificial nitrogen fixation and is currently an indispensable practice allowing for enough food production to sustain the ever-growing human population. However, this form of nitrogen fixation is extremely costly, not only energetically but monetarily as well (Chen et al., 2021). Additionally, the resulting products are often inefficiently applied leading to disastrous environmental effects such as eutrophication, where excess nutrients in water bodies can cause harmful algal blooms and other ecological disruptions (Carpenter, 2005; Erisman et al., 2011; Mulvaney et al., 2009; Rabalais et al., 2007). Issues such as these are driving research into alternative methods of nitrogen fixation, through artificial means such as low temperature plasma nitrogen fixation and through natural methods such as biological nitrogen fixation (BNF) facilitated by symbiotic bacteria, to reduce agricultural dependencies on synthetic fertilizers (Chen et al., 2021; Raymond et al., 2004). This method of nitrogen fixation is growing in popularity for agricultural purposes thanks to the usable forms of nitrogen produced and subsequently utilized by the host plant. Similarly, endophytic bacteria can solubilize phosphorus, another expensive and widely used synthetic fertilizer, making it available to the plant for growth (Matos et al., 2017; Oteino et al., 2015).

In addition to growth promotion through nutrient acquisition, these symbiotic bacteria often aid their host plant in survival by hormonal means. Some endophytic bacteria can produce plant hormones, as well as their precursors, such as auxins, cytokinins, and gibberellins that

promote plant growth and development (Etminani & Harighi, 2018; Ishak et al., 2016; Ismail et al., 2021; Shi et al., 2009). These naturally occurring compounds are responsible for the regulation of plant growth and development through the control of cell differentiation, elongation, and division. They are also utilized in metabolic processes within the host plant to control growth direction, root and shoot growth, fruit maturation, seed development, and environmental stress response (Bottini et al., 2004; Luo, 2012; Song et al., 2013; Teale et al., 2006). Within the auxin hormone class, indole-3-acetic acid (IAA) is the most common and well-studied due to its importance in cell elongation and division. Through the induction of these activities, plant tissue growth is facilitated and expanded. Additionally, this hormone plays a vital role in signaling, being responsible for growth and development of plant organs. Various genera of endophytic bacteria have been documented to synthesize this hormone, or promote its production, such as *Arthrobacter, Azotobacter, Azospirillum, Bacillus, Enterobacter, Klebsiella, Pseudomonas, Sphingopyxis,* and *Streptomyces* (Anugrah et al., 2020; Dias et al., 2009; Khamna et al., 2010). Cytokinins, responsible for root and shoot growth through mediation of cell division and differentiation, are also produced naturally by both free-living and endophytic bacteria (Eid et al., 2021). Some examples of bacterial genera that have been reported to produce cytokinins include *Acenitobacter, Azospirillum, Paenibacillus, Pseudomonas*, and *Rhizobium* (Bhore et al., 2010; Hita et al, 2020). While auxins and cytokinins typically have roles involving cell division, elongation, and differentiation, hormones under the gibberellin class play a more unique role involving the control of seed germination, fruit maturation, and flower development which are incredibly important factors when it comes to agricultural production (Bottini et al., 2004; Eid et al., 2021). Examples of common endophytic bacteria, some of which are already used as crop inoculants, capable of producing such hormones include *Bacillus, Pseudomonas,*

Rhizobium, Sphingomonas (Kaur & Karnwal, 2023; Khan et al., 2014). The incorporation of natural fertilizers such as beneficial bacterial endophyte inoculums can not only reduce our dependencies on synthetic fertilizers which are heavy in cost, economically and environmentally, but could also lead to a decrease in use of synthetic herbicides, pesticides, and other toxic compounds utilized for crop protection.

Endophytic bacteria aid in the defense against herbivory and plant pathogen induced damage by competing for space and resources, producing antimicrobial and insecticidal compounds, inducing systemic resistance, and creating anti-pathogenic compounds and their metabolic precursors. A few notable genera of beneficial endophytic bacteria that aid host plants in general defense that have been or are currently being investigated include *Bacillus*, *Enterobacter, Paenibacillus, Pseudomonas,* and *Streptomyces* (Castillo et al., 2002; D'Alessandro et al., 2013; Gao et al., 2017; Massawe et al., 2018; Miller et al., 1998; Pleban et al., 1997; Sheoran et al., 2015; Zhao et al., 2015) In the natural setting, host plants produce antimicrobial compounds which endophytic bacteria have developed resistance to, allowing them a distinct advantage against potential plant pathogens. This allows the beneficial endophytes to colonize the surfaces and surround areas of the host plant root systems; taking control of not only the spatial resource, but also the metabolic resources available before invading pathogenic strains can develop a foot hold (Basit et al., 2021; Xu et al., 2021). Interestingly, some endophytes will even produce insecticidal compounds, such as jasmonic acids and glucosinolates, that prevent host plant destruction by regulating the core insect-chewing systemic response elucidated from insect grazing that comes from a wide variety of insect taxonomic orders, including but not limited to, Coleoptera (beetles), Lepidoptera (moths), Orthoptera (grasshoppers), and Hemiptera (planthoppers) (Basit et al., 2021; Khare et al., 2018; Pangesti et al., 2016). This principal is

already being investigated for incorporation into agricultural systems as seen with a study conducted in 2012 where researchers were able to genetically engineer a naturally occurring bacterial endophytic strain, *Bacillus subtilis* WH2 to produce the insecticidal *Pinellia termata* agglutinin which was shown to be effective in protecting the rice plant hosts from whitebacked plant hopper herbivory (Qi et al, 2012). Host plant response to invading pathogens is also induced or enhanced by beneficial endophytic bacteria, live or dead, and their metabolic products whose effects can even be found expressing in later plant generations (Miliute et al, 2015; Oukala et al., 2021; Portieles et al., 2021; Tiwari et al., 2010). For example, wheat plants displayed increased survival from the fungal pathogen *Mycospharella graminicola* after being inoculated with *Paenibacillus* and *Curtobacterium* strains which were able to promote the production of pathogenesis-related proteins (PR) and chitinase enzymes vital to combating pathogenic fungal infections (Samain et al, 2017). There are many endophytic bacterial strains that contribute to host immune response as well as holding dual or multipurpose inoculum characteristics to contribute to host plant health and growth that possess great potential in innovating current agricultural maintenance and enhancement techniques without causing disastrous environmental harm.

Agricultural and ecological importance of root and stem nodulation

Members of the legume plant family *Fabaceae*, such as peas, soybeans, and beans are most well known for their specialized symbiotic relationships they form with nitrogen fixing endosymbionts, rhizobia, and are used as forms of green manure in agricultural systems. This relationship is characterized by the formation of unique organs known as nodules which develop in response to the colonization of root tissues by rhizobacteria (Spaink, 2000). Nodules allow for a controlled, anoxic environment within which the endosymbionts can safely and effectively

convert atmospheric nitrogen into forms that are then utilized by the host plant. This environment is crucial due to the oxygen sensitivity of the nitrogenase enzyme used by the rhizobacteria to convert atmospheric nitrogen to ammonia (Becana et al., 2010). With nitrogen being one of the most limiting factors on plant growth, this symbiotic relationship has given the host plants a distinct advantage over other non-nodule forming plant families particularly concerning crop cultivation. The incorporation of leguminous crops as green manure has provided an alternative method of fertilization that returns nutrients to soils in a controlled manner that lasts longer and doesn't leach off into the environment as is seen with synthetic fertilizers (Talgre et al., 2012). As farmers focus on specific cash crops, such as cotton or corn, they over cultivate these crops and over time, deplete the soil of vital nutrients, particularly nitrogen and phosphorus. Green manure through crop rotation will capture atmospheric nitrogen and slowly release it into surrounding soils, helping to combat nutrient depletion seen with overzealous cash crop cultivation (Becker et al., 1995; Studdert & Echeverria, 2000). Additionally, this increase in nutrient availability allows for enhanced food productivity necessary to keep up with the high demand of the growing global human population.

The unique ability to form root nodules is at the heart of the utilization of leguminous plants for crop rotation and therefore the reduction of synthetic fertilizer application. Although nitrogenous fertilizers are applied in the form of ammonia, almost a third of this material is released into the atmosphere as nitrous oxide (Reay et al., 2012). Nitrous oxide is quickly becoming one of the most notable greenhouse gasses being added to the atmosphere as a result of agricultural practices due to the fact that it is almost 300 times more insulative than CO2, retaining radiated thermal energy and increasing atmospheric temperatures (Lammel & Grafll, 1995; Webb et al., 2019; Yung et al., 1976). Growing concerns over ballooning global

atmospheric and surface temperatures are pushing researchers now, more than ever, to seek out alternative agricultural practices that increase crop yield and survivability while reducing environmental impacts. Both nitrogen and carbon levels within the environment can be controlled through the use of leguminous crops and their nutrient symbionts. In addition to nitrogen fixation, some rhizobia are also photosynthetic, taking in atmospheric CO2 and converting it metabolically for further incorporation as supplemental carbon sources for host plant growth resulting in increased biomass and yield (Chaintreuil et al., 2000; Promyou et al., 2015). Photosynthetic rhizobia are not the typical nitrogen fixing symbiont documented in association with leguminous crops. In fact, this rare relationship is primarily reserved to stem-nodulating plants such as those in the genera *Aeschynomene, Neptunia,* and *Sesbania* (Bonaldi et al., 2011; Giraud et al., 2007; Molouba et al., 1999). Stem nodulation itself is a highly unique process that is only more recently being studied in depth due to the unusual nodulation mechanisms found in these symbiotic relationships.

Mechanisms and morphology of nodule formation

Nodulation has been best characterized by the soybean (*Glycine max*) and *Bradyrhizobium japonicum* partnership due to the agricultural significance of this crop. In this system, the plant releases root exudates containing flavonoids and amino acids during growth to which the naturally occurring soil *Bradyrhizobium* and *Rhizobium* species respond to and gravitate toward via positive chemotaxis (Aguilar et al., 1988; Armitage et al., 1988; Barbour et al., 1991; Gaworzewska & Carlile, 1982). The detection of the plant derived flavonoids initiates a response in the bacteria, no matter the host/symbiont specifics, that activates the genes that start the process specialized signal molecule production. Rhizobia then adhere to the root surface to initiate colonization. While colonization does not occur in any one area of the rhizosphere, the

rhizobia populated near emergent root hairs and lateral roots are able to gain entry (Caetano-Anolles et al., 1992; Rhijn & Vanderleyden, 1995; Smit et al., 1992). This attachment stage does not demonstrate host or symbiont specificity for only rhizobia and therefore, many of the surrounding soil dwelling bacteria may colonize the rhizosphere not limiting it to just rhizobia (Lopes et al., 2016; Mills & Bauer, 1985). However, to gain entry in this specific situation, the rhizobia release specialized lipo-chitooligosaccharide signal molecules, known as Nod factors (NFs), which cause branching and deformation of the root hair (Bhuvaneswari & Solheim, 1985; Brussel et al., 1986; Yao & Vincent, 1969). The *nod* genes that synthesize the various components of NFs and play other roles such as NF modification or NF excretion, range from *nodA* to *nodZ*; *nolA* to *nolZ* with differences being specific to each host/symbiont mechanism (Table 1-3) (Gottfert, 1993; Halverson & Stacey, 1986; Kondorosi et al., 1984; Lugtenberg, 1989). Despite so many nodulation specific genes being characterized and identified, there are still variations being discovered, demonstrating the age, specificity, and variety of this special symbiotic relationship (Gottfert, 1993; Heidstra & Bisseling, 1996).

The *nodD* gene is the most conserved and crucial gene to this process as it responds to the plant released flavonoids and initiates the transcription of the entire NF signal molecule by turning on *nodABC* which is highly conserved as well, serving as the backbone for which NFs may be tailored to each relationship (Hong et al., 1987; Horvath et al., 1987; Rostas et al., 1986). Depending on the host/symbiont relationship, the NFs are further modified by a variety of genes that have evolved over millions of years to allow host plant access to only a select few nitrogen-fixing microbes. Once the NFs are released, root hair curling occurs at the site of young root hair cells, which entrap the rhizobia within a cell wall surrounded pocket. Subsequently, the cell wall is disintegrated via hydrolysis, allowing rhizobia to invade and penetrate the root hair through

plasma membrane invagination (Newcomb, 1976; Newcomb et al., 1979; Turgeon & Bauer, 1982). The host plant responds to this invasion by generating new cell wall material inwardly, resulting in a tube enclosing the rhizobia (Callaham & Torrey, 1981; Fournier et al., 2015). While the tube is being formed, cortical cells within the root cortex begin to redifferentiate into nodule primordia cells as a response to the infection through NF recognition (Ding & Oldroyd, 2009; Gage, 2004; Heckmann et al., 2011). This tube, known as the infection thread, continues to generate inward, transporting the dividing rhizobia to the site of the nodule primordium (Vasse & Truchet, 1984; Wood & Newcomb, 1989). Upon reaching the nodule primordia, bacterial cells are deposited into the now active nodule meristematic cells. As the nodule meristematic cells divide, rhizobia are distributed among the resulting daughter cells which then differentiate to form tissue housing the nitrogen-fixation cites of the nodule (Brewin, 1991; Newcomb, 1981). As the host plant grows, the nodule organs swell over time, becoming mature and functioning to facilitate the biological nitrogen-fixation process (Hirsch, 1992; Stougaard, 2000). Mature nodules will continue to fix nitrogen until senescence is initiated, triggered by age or abiotic stressors such as drought, temperature fluctuations, or excess environmental nitrates (Cam et al., 2012; Chen & Phillips, 1977; Dart & Mercer. 1966; Dupont et al. 2012). This method of infection, known as NF-dependent nodulation, is the most well studied mechanism for nodulation and was believed to be the dominant nodulation process. However, it was recently discovered that certain rhizobia, which are photosynthetic, can induce nodulation independently of these NF signal molecules in a system aptly dubbed, NF-independent nodulation.

While NF-dependent nodulation is widely documented among leguminous plants, NF-independent nodulation is limited to the stem-nodulating family of *Aeschynomene*. The unusual mechanism was first documented in this genus of semi-aquatic legumes when stem nodulating

photosynthetic *Bradyrhizobium* strains ORS278 and BTAi1 were found to lack the canonical *nodABC* genes integral to NF synthesis (Giraud et al., 2007). Since this discovery, researchers have been searching to identify the molecular mechanism behind NF-independent nodulation in *Aeschynomene* plants but have yet to make a clear determination on what signal molecule(s) could be directing this system (Bonaldi et al., 2010; Chaintreuil et al., 2018; Fabre et al., 2015; Gully et al., 2018). Although the molecular key behind NF-independence is still hidden, there have been great efforts to reveal the anatomical changes and differences between this system and the standard NF-dependent nodule formation process. As opposed to the highly specialized infection process of root hair curling and infection thread formation, the semi-aquatic legumes are colonized via crack entry, wherein the rhizobia enter the plant tissues through micro fissures on the dermal tissue of the plant stem and root systems (Arora, 1954; Boogerd & van Rossum, 1997). This entry method accounts for how stem nodules can form in that these plants live in environments where flooding is frequent, thus allowing access to the stem regions by native soil dwelling rhizobia. In the case of NF-independent root nodulation, colonization will typically centralize near the emergence points of lateral roots, instead of root hairs, and begin entry at the micro fissures surrounding the emerging lateral root and axillary root hairs (Alazard, 1985; Alazard & Duhoux, 1988; Arora, 1954; Eaglesham & Szalay, 1983; Loureiro et al, 1995; Yatazawa et al, 1984). The bacteria then spread intracellularly, gaining access to cortical cells, penetrating through them via invagination of the cell wall, resulting in cell wall pockets within the cells housing rhizobia which subsequently begin to degrade. From this point, the surrounding cortical cells respond to this invasion and cell destruction in an unknown mechanism that results in their own infection with the bacteria. After this infection spreading occurs, surviving cells begin to quickly divide, passing the encapsulated bacteria on to daughter cells, forming nodule

primordia. As the nodule primordia divides, the bacteria within also continue to divide and are then evenly distributed among the resulting daughter cells. In typical nodule formation manner, the nodule primordia continue to divide giving rise to an enlarging nodule organ until maturation is achieved (Bonaldi et al., 2011). This entire process is completed without the use of NFs yet still results in fully functioning nodules capable of facilitating the fixation of atmospheric nitrogen. However, the bacteria responsible for this unique symbiosis also have the added advantage of being photosynthetic, allowing for more energy production, assisting in more efficient nitrogen fixation.

Significance of nod-factor (NF)-independent nodulation in agriculture and environmental ecology

Although the mechanisms are not yet fully understood behind the success of NF-independent nodulation, it is currently recognized that these symbiotic relationships form without the use of complex signal molecules like NFs (Bonaldi et al., 2010). This system also utilizes a more simplistic mode of root colonization, via crack entry or intracellular entry that does not involve the root hair curling or infection thread formation (Boogerd & van Rossum, 1997). However, there may still be important signal molecules specific to NF-independent nodulation being produced, although not as complex as the NF system, to illicit the immune response required for nodule primordia formation and infection to occur. The fact that colonization of root cortical tissues can occur through such a straightforward method bears great importance for the potential induction of nitrogen fixing symbioses in non-leguminous cash crops that could revolutionize current agricultural systems. Although complex NFs are not used in this system, there may still be an important signal molecule, or molecular cascade involving multiple signals, at play here even if it is simple in nature that is recognized by legumes specifically, inducing

nodule formation. If such were the case, the identification and characterization of this simple signal molecule or signaling pathway could lead to the incorporation of the gene(s) responsible for the molecule's transcription into other bacteria that offer a diverse array of beneficial endophytic traits such as host defense, protection against abiotic stress, increased growth, and yield in legumes (Aeron et al., 2011). This theoretical signal molecule or signaling system would be responsible for eliciting the necessary immune response in the host plant that generates the nodule primordium. Therefore, to truly incorporate this NF-independent nodulation system, gene expressions analyses, such as those completed by Chaintreuil et al. in 2016, would need to be completed to determine what plant specific genes are being turned on when exposed to these NF-independent nodulation bacteria that would be releasing this theoretical signal molecule(s) as this would be crucial information required to transfer nodulation capabilities across plant families, removing the legume limitation. Once the lock and key system behind this special immune response found in leguminous plants is identified, these genes could be inserted into our current major cash crops through transgenic practices already used in genetically engineered crop seed manufacturing methods and thereby increase crop yield and survival while reducing synthetic fertilizer use (Ahmad et al., 2012; Shankar et al., 2023). Although these genetic engineering practices hold a social stigma, cash crops with this incorporated immune response system, generating nitrogen fixing nodule organs (with other potential endophytic benefits), would be able to allow farmers a way to produce the crops they want, where they want, without the depletion of soil nutrients and without the added cost of synthetic fertilizer application. Studying this NF-independent nodulation mechanism is *Aeschynomene* plants provides an excellent model for potentially unlocking the secret to this host immune response, in part, due to the ancestral

history and geographical distribution regarding host nodulation capabilities within this plant genus (Alazard, 1985; Chaintreuil et al., 2016 & 2018).

Geographical impacts on nodule endophyte populations

Endophytes can colonize and proliferate within every plant tissue type, all the way from the roots to the very seeds, but determining what organisms have the chance to become such a successful symbiont first requires a look into the host's surrounding environmental conditions. The diversity of the endophytic organisms, be it bacterial or fungal, within a plant depends on quite a few factors including, but not limited to, host species, specific tissue type, growth conditions, and location (Chen et al, 2021; Harrison & Griffin, 2020). This holds true, even for the composition of the endophytic community within nodule organs found on leguminous plants (van Dijk, 1978). When people think of the nodules, they are typically focused only on the nitrogen fixing rhizobia symbionts and don't take time to consider the possibility that there are other potentially beneficial microbes inhabiting the same tissues (Boukhatem et al., 2017). We have seen that endophytes will readily colonize the internal tissues of plants, free of complex signaling systems such as NF-dependent nodulation, through crack or intracellular entry, simply by squeezing between the plant cells through small openings in the epidermis (Boogerd & van Rossum, 1997; Kandel et al., 2017). It should go without saying that the bacteria thriving within the plant tissues had to have originated from outside the plant at some point in their symbiotic history. Some endophytes begin their initial colonization by hiding out in the seeds and dispersing throughout the growing and rapidly dividing plant cells once germination occurs (Walitang et al., 2018). However, even the seed endophytes would have needed to gain entry into the host plant at some point before the seeds were even developed (Nelson, 2018). The most logical conclusion would be that essentially all endophytic species originated from the external

environment including air and water, but primarily from the soil (Compant et al., 2012). There have even been suggestions of endophyte colonization resulting from the gut microflora of seed dispersing animals, as they digest the fruits and release the seeds back to the environment for later germination (Martinez-Romero et al., 2020). Additionally, the same theory has been applied in reverse in that the diversity of the gut microflora in animals is impacted by the fruit or vegetative materials and thereby the endophytes they consume (Hardoim, 2019). Nevertheless, the primary source, historically and evolutionarily speaking, of potential endophytic symbionts would be derived from the free-living microbes within soils (Compant et al., 2012). Therefore, the composition of the soil bacterial communities is a crucial aspect to understand when seeking a better understanding regarding the selection of those microbes that would go on to be successful symbionts as endophytes.

There have been countless studies discussing the importance of abiotic factors and biogeographic differences on the composition soil microbial communities around the world (Andrews et al., 1987; Bahram et al., 2018; Chu et al., 2020; Fierer & Jackson, 2006; Wang et al., 2017). Some of the most important factors that control the level or composition of microbial diversity include human activity, pH, nutrient resources, elevation, temperature, salt, and moisture content (Shen et al., 2013 & 2014; Tripathi et al., 2018). For example, in soils that have had their pH levels drastically altered by human related practices such as industrial and agricultural pollution, there are lower levels of bacterial diversity (Konopka et al., 1999; Marcin et al., 2013). Instead, you see more bacterial genera that are adapted to mitigating the damages of heavy metal contents, pH extremes, and restricted complex carbon sources (Sheik et al., 2012). Similarly, desolate soils, barren in neighboring complex life forms such as plants and animals, almost entirely absent of nutrient cycling, there are limited microbial organisms (Delgado-

Baquerizo et al., 2016; de Vries et al., 2012). Yet even that in itself is a remarkable testament to the hardiness and adaptability of bacteria, particularly extremophilic examples, in their ability to fill any niche that may arise, despite a lack of massive diversity (Aszalos et al., 2016; Chu, et al., 2010). Additionally, factors such as drought or salt levels have proven to be great challenges faced by farmers attempting to cultivate costal or equatorial lands to achieve the food yields that are so highly demanded (Mandal et al., 2018; Wilhite, 2000). Studies have shown that plants inoculated with particular endophytes adapted to those abiotic stressors are able to mitigate those negative effects and permit the host plant an opportunity to thrive in such hostile conditions (Byregowda et al., 2022; John et al., 2023; Suryanarayanan & Shaanker, 2021; West, 1994). With these environmental conditions playing such a role in shaping soil microbial communities, it should go without question that the resulting endophytic diversity within a plant is directly dependent on the environment they are cultivated within.

Geographical distributions of plants and microbes are a key factor in the determination of the composition of soil microbial communities clearly through abiotic factors affected by latitude, altitude, biome, and human activities. However, even the biotic factors can be affected by the geographical distribution as well. Plants and microbes suited to each specific biome or environmental condition will inhabit those areas, proliferate, and further speciate out over time (Li et al., 2023; Nottingham et al., 2018). Both organism types are constantly in contact with one another throughout the course of history, growing or diminishing as a result of their interactions with each other (Prober et al., 2015; de Vries et al., 2012). Special relationships form under these conditions, allowing for host or symbiont genetic modifications specific to the facilitation of each partnership (Tayung et al., 2011; Tedersoo et al., 2007). Thus, the diversity of endophytic communities will change, depending on the location, environmental conditions, and biotic

stressors of their origin, despite sharing a similar lineage (Christian et a., 2016; Delgado-Baquerizo et al., 2017). A plant species, widely distributed around the world, will have a unique microbial community depending on the site of cultivation and observation.

Complexities of bacterial populations within nodules

Soils are teeming with microscopic life, each individual competing with the other for resources and space while developing unique metabolic systems to give themselves the upper hand through metabolic boosts and diversity or through the amassing of antimicrobial arsenals (Fierer & Jackson, 2006; Torsvik et al., 1996). Many bacteria, some pathogenic, thrive near the root systems of plants and feed off the surrounding organic materials (Shigyo et al., 2019). Those that have evolved to live endophytically are attracted to plant rhizospheres and will actively gravitate towards them, following the root exudate trail (Bacilio-Jimenez et al., 2003; Tsai et al., 2020). Nitrogen fixing and non-nitrogen fixing bacteria alike are drawn toward plant root systems and adhere to the surface, either through the production of their own extracellular polysaccharides or by physically being ensnared in those produced by the plant (Clua et al., 2018; Kandel et al., 2017). The diversity of bacteria colonizing a plant's rhizosphere depends on a variety of factors, including location, environment, and species of the host plant (Zhang et al., 2017; Zhang et al., 2018). Additionally, the composition of the rhizosphere community depends heavily up the competition derived from nutrient availability and antimicrobial production/resistance (Bever, 2003; Blagodatskaya et al., 2014). Soil bacteria are in such constant turmoil, fighting off one another, that these bacteria are one of the primary sources of antibiotic bioprospecting, providing new methods of infection mitigation used in modern day medicine (Ceylan et al., 2008; Chandra & Kumar, 2017; Kumar et al., 2010). Those that survive and successfully populate plant surfaces could either be plant growth promoting, or potentially

pathogenic, causing harm or even death to the host plant (Mendes et al., 2013). It has already been established that soil microbes can successfully enter the inner layers of plant tissues simply by squeezing into small cracks of the dermal layers. Therefore, a wide variety of bacteria, beneficial and harmful, have the opportunity to colonize plant tissues either beneficially or pathogenetically (Hassan et al., 2019; Li et al., 2021). Upon entry and establishment into the plant cortex, nodule inducing symbiotic bacteria could be living alongside potential friends or foes (Deng et al., 2011). This means that during nodule development, bacteria other than the stereo-typical nitrogen-fixing symbionts can hitch a ride and colonize nodule primordia during the early stages of invasion (Clua et al., 2018). Once inside the nodule primordia, just like the nitrogen fixers, non-nitrogen fixing bacteria begin to proliferate and distribute among the quickly maturing nodule organs. In the case of plant growth promoting bacteria, this provides a great opportunity for the host plant to incorporate other metabolic capabilities, such as carbon fixation, phosphate uptake, and hormone production in addition to the dominant nitrogen fixation dynamic.

Plants with a wide diversity of beneficial bacteria working to fight infection, produce growth hormones, acquire nutrients, mitigate abiotic stressors like drought or heavy metal contamination, in addition to the nitrogen-fixing symbionts have a much greater chance of success, allowing for further distribution of wild populations or greater production in agricultural crop settings (Emmanuel & Babalola, 2020; Le et al., 2015; Mahgoub et al., 2021). With the potential for such diversities within nodule tissues, it is important that we understand the population dynamics within these organs and find ways to combine various plant growth promoting bacteria in a manner that provides a wide array of metabolic capabilities to the host plant instead of focusing solely on the nitrogen-fixing symbionts. More importantly,

investigating plant growth promoting bacteria from local plant populations provides the opportunity to identify organisms already suited to survival in that environment, allowing for specialized plant inoculum specifically tailored and optimized to the specific growing conditions. Additionally, understanding the region-specific needs for each crop could provide insights on the best inoculum to provide. While endophytes can live harmoniously with their host plants, providing nutrients, metabolites, hormones, etc., recent studies have also shown that plants actively attract, “consume”, and degrade soil bacteria through exposure to radical oxygen species for direct nutritional sources (Paungfoo-Lonhienne et al., 2010). This “rhizophagy” process is still only in the early stages of discovery but, once fully understood, could perhaps lead to a whole new understanding and application of biological fertilizers.

Table 1-1. Examples of plant growth promoting rhizobia (non-symbiotic) that aid in plant survival and growth.

Plant Growth Promoting Trait	Mechanism or Activity	PGPR Examples	Reference
Nutrition & Growth Stimulation			
	Nutrient Acquisition (N & P)	*Advenella kashmirensis, Azoarcus, Azotobacter, A. chroococcum, Azospirillum, Bacillus, B. subtilis, Beijerinckia, Bradyrhizobium, B. japonicum, Klebsiella, Mesorhizobium, Micrococcus luteus, Pantoea, Psudomonas, P. fluorescens, Rhizobium, R. leguminosarum, Seratia*	(Basit et al., 2021)(Khan et al., 2008)(Yasmeen et al., 2021)
	Plant Growth Hormone Production	*Azotobacter, A. chroococcum, Azospirillum, Bacillus, B. subtillis, B. megaterium, Bradyrhizobium, Enterobacter, Enterobacter cloacae, Mesorhizobium, Moraxella, Paenibacillus illinoisensis, P. polymyxa, Pseudomonas, P. extremaustralis, P. moraviensis, P. putida, P. stutzeri, Rhizobium, Streptomyces, Stenotrophomonas maltophilia, Variovorax paradoxus*	(Basit et al., 2021)(Kahn et al., 2008)(Kudoyarova, 2019)(Yasmeen et al., 2021)
Abiotic Stress Mitigation			
	Heavy Metal Remediation	*Acinetobacter, Bacillus, B. subtilis, B. licheniformis, Bradyrhizobium* sp. RM8, *Burkholderia, Halobacillus, Mesorhizobium, M.* sp. RC3, *Methylobacterium oryzae, Ochrobacterium intermedium, Pseudomonas, P. aeruginosa, P. maltophilio, Proteus vulgaris, Ralstonia, Rhizobium, R.* sp. RP5, *R.* sp. RL9, *Serratia marscecens, Streptoccus faecalis, S. aureus,*	(Khan et al., 2008)(Kudoyarova, 2019)(Monteiro et al., 2021)
	Salinity Tolerance	*Agrobacterium, Bacillus, B. pumilus, Enterobacter, E. aurantiacum, Klebsiella, Ochromobacter, Pseudomonas, P. fluorescens, P. moraviensis, Streptomyces,*	(Monteiro et al., 2021)(Nawaz et al., 2020)(Yasmeen et al., 2021)

Table 1-1. Continued

Plant Growth Promoting Trait	Mechanism or Activity	PGPR Examples	Reference
Biotic Stress Mitigation			
	Biological Control Activity	*Ananbaena, Bacillus, B. velezensis, B. amyloliquefaciens, B. subtilis, Calothrix, Chlorella, Microcytis, Nostoc, Pseudomonas, P. flourescens, P. aeruginosa, P. chlororaphis, Rhizobium leguminosarum,*	(Hamid et al., 2021)(Meyer et al., 2016)(Smitha et al., 2017)
	Biopesticidal/Nematocidal Activity	*Bacillus, B. popilliae, B. thuringiensis, Chromobacterium substugae, Paenibacillus popilliae, Pasteuria penetrans, P. fluprescens, Pseudomonas, Serratia entomophila*	(Ahmad et al., 2021)(Shah et al., 2021)

Table 1-2. General list of beneficial endophytic (symbiotic) bacteria and some of their symbiotic roles

Symbiotic Role	Mechanism or Activity	Endophytic Examples	Reference
Nutrition & Growth Stimulation			
	Nitrogen Fixation	*Anabaena, Azobacter, Azospirillum, Bradyrhizobium, B. japonicum, Burkholderia, Enterobacter, Herbaspirillum, H. seropedicae, Klebsiella, Nostoc, Pantoea, Pelomonas, Rhizobium*	(Bashan & de-Bashan, 2005)(Goren-Saglam, 2021)(Muangthong et al., 2015)(Qin et al., 2022)
	Phosphate Solubilization	*Azobacter, Bacillus, Bacillus circulans, B. edaphicus, B. megaterium, B. methylotrophicus, B. mucilaginosus, B. subtilis, Burkholderia, Enterobacter, Erwinia, Kushneria, Paenibacillus, Paenibacillus mucilaginosus, P. glucanolyticus, Pseudomonas, Ralstonia, Rhizobium, Serratia,*	(Bashan & de-Bashan, 2005)(Goren-Saglam, 2021)(Mohamed et al., 2021)(Zhu et al., 2011)
	Plant Growth Hormone Production	*Azobacter, Bacillus, B. megaterium, B. subtilis, Bradhyrhizobium, Burkholderia, B. phytofirmans, Glucoacenatobacter diazotrophicus, Pseudomonas, P. putida, P. syringae, P. tolaasii, Rahnella, R. aquatilis, Rhizobium, Serratia, Streptomyces, S. coelicolor, S. olivaceus, S. geysiriensis*	(Etminani & Harighi, 2018)(Khan et al., 2012)(Phetcharat & Duangpaeng, 2012)
Abiotic Stress Mitigation			
	Heavy Metal Remediation	*Acidobacteria, Actinobacteria, Aeromonans, Agrobacterium, Alcaligenes, Aureobasidium, Bacillus, Burkholderia, Enterobacter, Exiguobacterium, Firmicutes, Klebsiella, Leifsonia, Lysobacter, Microbacterium, Micrococcus, Ochrobactrum, Paenibacillus, Paracoccus, Pantoea, Proteobacteria, Psudomonas, Rhodococcus, Sphingobium, Sphingomonas, Streptomyces*	(Han et al., 2016)(Prasher & Sharma, 2021)(Xu et al., 2016)
	Drought Tolerance	*Azospirillum, A. lipoferum, Bacillus, B. subtillis, Burkholderia, B. phytofirmans, Bradyrhizobium, Glucoacenatobacter diazotrophicus, Pseudomonas, P. azotoformans*	(Cohen et al., 2009)(Gagne-Bourgue et al., 2015)(Ullah et al., 2019)

Table 1-2. Continued

Symbiotic Role	Mechanism or Activity	Endophytic Examples	Reference
	Salt Tolerance	*Achromobacter xylosoxidans ,Altererythrobacter, Bacillus licheniformis, B. pumilus, B. subtilis, Brevibacterium halotolerans, Hoeflea, Labrenzia, Lysinibacillus fusiformis, Marinilactibacillus, Pseudomonas putida*	(Fidalgo et al., 2016; Sgroy et al., 2009)
Biotic Stress Mitigation			
	Biological Control Activity	*Aureobacterium saperdae, Bacillus megaterium, B. methylorophicus, B. pumilus, B. subtillus, B. velezensis, Burkholderia solanacearum, Paenibacillus polymyxa, Phyllobacterium rubiacearum, Pseudomonas, P. choloroaphis, P. putida, P. orientalis, Streptomyces, S. palmae, S. rubrogriseus, S. badius, S. pactum,*	(Almoneafy et al., 2021; Senthilkumar et al., 2011)
	Biopesticidal Activity	*Bacillus, B. subtilis, B. safensis, B. thuringiensis, Pseudomonas, P. chlororaphis, Serratia, S. marcescens, Streptomyces*	(Akutse et al., 2020)(Pliego et al., 2011)(Sutio et al., 2023)

Table 1-3. A list of nodulation genes, relative protein names, and functions regarding nodulation and host specificity.

Gene	Protein	Function	Reference
nodA	Nodulation protein A	N-acetyltransferase	(Gonzalez et al., 2003; Vazquez et al., 1991)
nodB	Chitooligosaccharide deacetylase	de-N-acetylase; hydrolase; metal-binding	(Dobert et al., 1994: Rossen et al., 1984)
nodC	N-acetylglucosaminyltransferase	UDP-GlcNac transferase; glycosyltransferase	(Rossen et al., 1984)
nodD1	Nodulation protein D 1	DNA-binding transcription factor; Regulates *nodABCFE* genes; binds flavonoids	(Egelhoff et al., 1985; Goettfert et al., 1986)
nodD2	Nodulation protein D 2	DNA-binding transcription factor; Regulates *nodABCFE* genes; binds flavonoids	(Davis & Johnston, 1990; Goettfert et al., 1986)
nodD3	Nodulation protein D 3	DNA-binding transcription factor; Regulates *nodABCFE* genes; binds flavonoids	(Davis & Johnston, 1990)
nodE	Nodulation protein E	beta-ketoacyl synthase; 3-oxoacyl-[acyl-carrier-protein] synthase activity; partial host specific NF fatty acyl chain synthesis	(Davis & Johnston, 1990)
nodF	Nodulation protein F (Host-specificty of nodulation protein A)	trans-2,trans-4,trans-6,cis-11-octadecatetraenoic acid synthase; partial host specific NF fatty acyl chain synthesis	(Schofield & Watson, 1986)
nodG	Nodulation protein G	3-oxoacyl-[acyl-carrier-protein] reductase (NADPH) activity; NF fatty acyl chain modification	(Delledonne et al., 1990; Vieille & Elmerich, 1992)
nodH	Nodulation protein H	sulfotransferase activity; transfers activated sulfate to N-acetylgucosamine on NF	(Luaeremans et al., 1996)
nodI	Nod factor export ATP-binding protein I	transmembrane transporter and ATP hydrolysis activity; part of NF export system	(Suominen et al., 1999)
nodJ	Nodulation protein J	ABC-type transporter activity; part of NF export system	(Evans & Downie, 1986; Suominen et al., 1999)
nodK	Nodulation protein K	Unknown function. Part of highly conserved *nodKABC* operon. *Bradyrhizobium* specific.	(Scott, 1986)
nodL	Nodulation protein L	O-acetyltransferase; actetyltransferase of NFs for host specificity	(Surin & Downie, 1988)
nodM	Gultamine--fructose-6-phosphate aminotransferase	root hair deformation factor (*Medicago* genus specific)	(Surin & Downie, 1988)
nodN	Nodulation protein N	root hair deformation factor (*Medicago* genus specific)	(Surin & Downie, 1988)
nodO	Nodulation protein O	Calcium binding protein; interracting with root hair or surface; exported protein	(Economou et al., 1990; de Maagd et al., 1989)

Table 1-3. A list of nodulation genes, relative protein names, and functions regarding nodulation and host specificity. (Continued)

Gene	Protein	Function	Reference
nodP	Sulfate adenylytransferase subunit 2	Putatively provides activated sulfate to NFs	(Vieille & Elmerich, 1990)
nodQ	Bifunctional enzyme NodQ	sulfate adenylytransferase activity; ATP/GTP binding; Transfers activated sulfate to NFs	(Vieille & Elmerich, 1990)
nodR	NodR	unknown function; *Rhizobium* specific; regulated by nodD	(Schlaman et al., 2006)
nodS	Nodulation protein S	S-adenosylmethionine-dependent methyltransferase activity; host specific NF modification	(Waelkens et al., 1995)
nodT	Nodulation protein T	unknown function; specific to *Rhizobium leguminosarum* nodulation	(Surin et al., 1990)
nodU	Nodulation protein U	6-O-carbamoylation of NF; host specific NF modification	(Krishna et al., 1992)
nodV	Nodulation protein V	part of NodV/NodW regulatory system; putative nod gene transcription regulator; putative membrane kinase for NodW phosphorylation	(Goettfert et al., 1990)
nodW	Nodulation protein W	nod gene transcription regulation; activated by NodV	(Goettfert et al., 1990)
nodX	Nodulation protein 10	Mechanism unknown; facilitates *R. leguminosarum* to nodulate Afghanistan peas	(Davis et al., 1988; Solovev et al., 2021)
nodY	NodY	unknown function; primarily specific to *Bradyrhizobium* sp.	(Bracellos et al., 2007)
nodZ	Nodulation protein Z	Fucosyltransferase; adds fucose moiety reducing N-acetylglucosamine end of NF	(Stacey et al., 1994)
nolA	Nodulation protein NolA	DNA-binding transcription factor specific to soybean symbiosis	(Dobert et al., 1994)
nolB	Nodulation protein NolB	Soybean cultivar-specific nodulation regulation	(Meinhardt et al., 1993)
nolC	Protein NolC	Soybean cultivar-specific nodulation regulation; heat-shock gene homology	(Krishnan & Pueppke, 1991)
nolE	Nodulation protein NolE	Exported protein not critical to nodulation or nitrogen fixation. Putative host specific NF	(Davis & Johnston, 1990)
nolF	Nodulation protein NolF	Transmembrane transporter specific to Medicago nodulation	(Baev et al., 1991; Barnett et al., 2001)
nolG	Nodulation protein NolG	Transmembrane transporter specific to Medicago NF	(Baev et al., 1991; Galibert et al., 2001)
nolJ	Nodulation protein NolJ	Controls nodulation efficiency and delay in soybean	(Boundry-Mills et al., 1994; Sadowsky et al., 1988)
nolK	GDP-L-fucose synthase	Converts GDP-4-dehydro-6-deoxy-D-mannose to GDP-fucose for NF modulation	(Kaneko et al., 2002)

Table 1-3. A list of nodulation genes, relative protein names, and functions regarding nodulation and host specificity. (Continued)

Gene	Protein	Function	Reference
nolL	Nodulation protein NolL	acetyltransferase; modifies NF fucose	(Scott et al., 1996)
nolM	Nodulation protein NolM	unknown function	(Kaneko et al., 2002; Yu et al., 2014)
nolN	NolN protein	Catalytic activity involved in NF synthesis	(Luka et al., 1993; Luka & Stacey, 1997)
nolO	Nodulation protein NolO	6-O-carbamoylation of NF; host specific NF modification	(Goettfert et al., 2001)
nolP	Nodulation protein NolP	Unknown mechanism; specific to genus *Rhizobium*	(Gonzalez et al., 2008)
nolR	Nodulation protein NolR	DNA-binding transcription factory activity; regulation of nod regulon (*nodD1*, *nodD2, nodD3, nodABC*)	(Kondorosi et al., 1991)
nolS	Nodulation protein NolS	Host-specific nodulation of *M. lupulina* by *Sinorhizobium meliloti*	(Plazanet et al., 1995; Sallet et al., 2013)
nolT	Nodulation protein NolT	Soybean-cultivar specific nodulation regulation	(Meinhardt et al., 1993)
nolU	Nodulation protein NolU	Soybean-cultivar specific nodulation regulation; flavonoid induced	(Meinhardt et al., 1993)
nolV	Type 3 secretion system stator protein	Protein secretion type III system exporting NFs	(Freiberg et al., 1997; Schmeisser et al., 2009)
nolW	Nodulation protein NolW	Soybean-cultivar specific nodulation regulation of *Rhizobium fredii*	(Freiberg et al., 1997; Meinhardt et al., 1993)
nolX	Nodulation outer protein X	Extracellular protein involved in NF transportation through type III secretion system; *Rhizobium* specific	(Saad et al., 2008; Viprey et al., 1998)
nolY	NolY	Host specific nodulation gene for *Bradyrhizobium* symbiosis; mechanism unknown	(Dockendorff et al., 1994; Goettfert et al., 2001)
nolZ	NolZ	Host specific nodulation gene for *Bradyrhizobium* symbiosis; mechanism unknown	(Dockendorff et al., 1994; Kaneko et al., 2002)
syrM	HTH-type transcriptional regulator	DNA-binding transcription factor; stimulates nod gene expression via *nodD3*	(Michiels et al., 1993)

REFERENCES

Aeron A., Kumar, S., Pandey, P., & Maheshwari D.K. 2011. Emerging role of plant growth promoting rhizobacteria in agrobiology. *Bacteria in Agrobiology: Crop Ecosystems*. 1-36.

Aguilar, J.M., Ashby, A.M., Richards, J.M., Loake, G.J., Watson, M.D., & Shaw, C.H. 1988. Chemotaxis of Rhizobium leguminosarum biovar phaseoli towards flavonoid inducers of the symbiotic nodulation genes. *J. Gen. Microbiol.* **134**, 2741-2746.

Ahmad, G., Nishat, Y., Ansari, M.S., Khan, A., Haris, M., & Khan, A.A. 2021. Eco-friendly approaches for the alleviation of root-knot nematodes. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management.* **20,** 557-575.

Ahmad, P., Ashraf, M., Younis, M., Hu, X., Kumar, A., Akram, N.A., & Al-qurainy, F. 2012. Role of transgenic plants in agriculture and biopharming. *Biotechnol. Adv.* **30,** 524-540.

Akutse, K.S., Subramanian, S., Maniania, N.K., Dubois, T., & Ekesi, S. 2020. Biopesticide research and product development in Africa for sustainable agriculture and food security – experiences from the international centre of insect physiology and ecology (icipe). *Front. Sustain. Food Syst.* **4,** 1-14.

Alazard, D. 1985. Stem and root nodulation in Aeschynomene spp. *Appl. Environ. Microbiol.* **50(3)**, 732-734.

Alazard, D., & Duhoux, E. 1988. Diversity of stem nodulation sites in Aeschynomene spp. *J. Plant. Physiol.* **132**, 123-125.

Almoneafy, A.A., Moustafa-Farag, M., & Mohamed, H.I. 2021. The auspicious role of plant growth promoting rhizobacteria in the sustainable management of plant disease. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management.* **10**, 251-283.

Andrews, J.H., Kinkel, L.L., Berbee, F.M., & Nordheim, E.V. 1987. Fungi, leaves, and the theory of island biogeography. *Microb. Ecol.* **14,** 277-290.

Anugrah, F.A., Fanany, R., Putra, S.A., Masita, R., & Safitri, D.Y. 2020. Indole acetic acid (IAA) hormone production by endophytic bacteria isolate from cinchona plant (Cinchona ledgerina moens.) root. *Proc. Int. Conf. Life Sci. Technol.* **2353**, 030082-1-030082-4.

Armitage, J.P., Gallagher, A., & Johnston, A.W.B. 1988. Comparison of the chemotactic behaviour of Rhizobium leguminosarum with and without the nodulation plasmid. *Mol. Microbiol* **2**, 743-748.

Arora, N. 1954. Morphological development of the root and stem nodules of Aeschynomene indica l. *Phytomorph.* **4**, 211-216.

Aszalos, J.M., Krett, G., Anda, D., Marialigeti, K., Nagy, B., & Borsodi, A.K. 2016. Diversity of extremophilic bacteria in the sediment of high-altitude lakes located in the mountain desert of ojos del saldo volcano, dry-andes. *Extremophiles.* **20,** 603-620.

Bacilio-Jimenez, M., Aguilar-Flores, S., Ventura-Zapata, E., Perez, Campos, E., Bouquelet, S., & Zenteno, E. 2003. Chemical characterization of root exudates from rice (Oryza sativa) and their effects on the chemotactic response of endophytic bacteria. *Plant Soil.* **249,** 271-277.

Baev, N., Endre, G., Petrovics, G., Banafalvi, Z., & Kondorosi, A. 1991. Six nodulation genes of nod box locus 4 in Rhizobium meliloti are involved in nodulation signal production: nodM codes for D-glucosamine synthetase. *Mol. Gen. Genet.* **228,** 113-124.

Bahram, M., Hildebrand, F., Forslund, S.K., Anderson, J.L., Soudzilovskaia, N.A., Bodegom, P.M., Bengtsson-Palme, J., Anslan, S., Coelho, L.P., Harend, H., Huerta-Cepas, J., Medema, M.H., Maltz, M.R., Mundra, S., Olsson, P.A., Pent, M., Põlme, S., Sunagawa, S., Ryberg, M., Tedersoo, L., & Bork, P. 2018. Structure and function of the global topsoil microbiome. *Nature* **560**, 233–237.

Barbour, W.M., Hattermann, D.R., & Stacey, G. 1991. Chemotaxis of Bradyrhizobium japonicum to soybean exudates. *Appl. Environ. Microbiol.* **57(9)**, 2635-2639.

Barnett, M.J., Fisher, R.F., Jones, T., Komp, C., Abola, A.P., Barloy-Hubler, F., Bowser, L., Capela, D., Galibert, F., Gouzy, J., Gurjal, M., Hong, A., Huizar, L., Hyman, R.W., Kahn, D., Kahn, M., L., Kalman, S., Keating D.H., Palm, C., Peck, M.C., Surzycki, R., Wells, D.H., Yeh, K.C., Davis, R.W., Federspiel, N.A., & Long, S.R. 2001. Nucleotide sequence and predicted functions of the entire Sinorhizobium meliloti psyma megaplasmid. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 9883-9888.

Bashan, Y., & de-Bashan, L.E. 2005. Bacteria: plant growth-promoting. *Encyclopedia of soils in the environment*. **1**, 103-115.

Basit, A., Shah, S.T., Ullah, I., Ullah, I, & Mohamed, H.I. 2021. Plant growth-promoting rhizobacteria (PGPR) as biocontrol agents for viral protection. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management*. **8,** 188-225.

Basit, A., Shah, S.T., Ullah, I., Ullah, I, & Mohamed, H.I. 2021. Microbial bioactive compounds produced by endophytes (bacteria and fungi) and their uses in plant health. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management*. **11,** 285-318.

Becana, M., Matamoros, M.A., Udvardi, M., & Dalton, D.A. 2010. Recent insights into antioxidant defenses of legume root nodules. *New Phytol.* **188**, 960-976.

Becker, M., Ladha, J.K., & Ali, M. 1995. Green manure technology: potential, usage, and limitations. A case study for lowland rice. *Plant and Soil*. **174**, 181-194.

Bever, J.D. 2003. Soil community feedback and the coexistence of competitors: conceptual frameworks and empirical tests. *New Phytol.* **157,** 465-473

Bhore, S.J., Nithya, R., & Loh, C.Y. 2010. Screening endophytic bacteria isolated from leaves of Sambung nyawa [Gynura procumbens (lour.) merr.] for cytokinin-like compounds. *Bioinformation*. **5(5)**, 191-197.

Bhuvaneswari, T.V., & Solheim, B. 1985. Root hair deformation in the white clover/Rhizobium trifolii symbiosis. *Physiol. Plant.* **68**, 1144-1149.

Blagodatskaya, E., Littschwager, J., Lauerer, M., & Kuzyakov, Y. 2014. Plant traits regulating N capture define microbial competition in the rhizosphere. *Eur. J. Soil Biol.* **61,** 41-48.

Bonaldi, K., Gherbi, H., Franche, C., Bastien, G., Fardoux, J., Barker, D., Giraud, E., & Cartieaux, F. 2010. The nod factor-independent symbiotic signaling pathway: development of Agrobacterium rhizogenes-mediated transformation for the legume Aeschynomene indica. *Mol. Plant Microbe Interact.* **23**, 1537-1544.

Bonaldi, K., Gargani, D., Prin, Y., Fardoux, J., Gully, D., Nouwen, N., Goormachtig, S., & Giraud, E. 2011. Nodulation of Aeschynomene afraspera and A. indica by photosynthetic Bradyrhizobium sp. Strain ors285: the nod-dependent versus the nod-independent symbiotic interaction. *Mol. Plant. Microbe. Interact.* **24(11)**, 1359-1371.

Boogerd, F.C., & van Rossum, D. 1997. Nodulation of groundnut by Bradyrhizobium: a simple infection process by crack entry. (*Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* **21**, 5-27.

Bottini, R., Cassan, F., & Piccoli, P. 2004. Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. *Appl. Microbiol. Biotechnol.* **65**, 497-503.

Boukhatem, Z.F., Merabet, C., Bekki, A., Sekkour, S., Domergue, O., Dupponois, R., & Galiana, A. 2017. Nodular bacterial endophyte diversity associated with native Acacia spp. in desert region of algeria. *Afr. J. Microbio. Res.* **10(18),** 634-645.

Boundy-Mills, K.L., Kosslak, R.M., Tully, R.E., Pueppke, S.G., Lohrke, S.M., & Sadowsky, M.J. 1994. Induction of the Rhizobium fredii nod box-independent nodulation gene nolj requires a functional nodd1 gene. *Mol. Plant Microbe. Interact.* **7**, 305-308.

Brader, G., Compant, S., Mitter, B., Trognitz, F., & Sessitsch, A. 2014. Metabolic potential of endophytic bacteria. *Curr. Opin. Biotechnol.* **27**, 30-37.

Bracellos, F.G., Menna, P., da Silva Batista, J.S. & Hungria, M. 2007. Evidence of horizontal transfer of symbiotic genes from a Bradyrhizobium japonicum inoculant strain to indigenous diazotrophs Sinorhizobium (Ensifer) fredii and Bradyrhizobium elkanii in a brazilian savannah soil. *Appl. Environ. Microbiol.* **73,** 2635-2643.

Brewin, N.J. 1991. Development of the legume root nodule. *Annu. Rev. Cell. Biol.* **7**, 191-226.

Brussel, A.A.N., Zaat, S.A.J., Canter-Cremers, H.C.J., Wijfelman, C.A., Pees, E., & Lugtenberg, B.J.J. 1986. Role of plant root exudate and sym plasmid-localized nodulation genes in the synthesis by Rhizobium leguminosarum of trs factor, which causes thick and short roots on common vetch. *J. Bacteriol.* **165**, 517-522.

Byregowda, R., Prasad, S.R., Oelmuller, R., Nataraja, K.N., & Kumar, M.K.P. 2022. Is endophytic colonization of host plants a method of alleviating drought stress? Conceptualizing the hidden world of endophytes. *Int. J. Mol. Sci.* **23(16),** 1-33.

Caetano-Anolles, G., Wrobel-Boerner, E., & Bauer, W.D. 1992. Growth and movement of spot inoculated Rhizobium meliloti on the root surface of alfalfa. *Plant Physiol.* **98**, 1181-1189.

Callaham, D.A. & Torrey, J.G. 1981. The structural basis for the infection of root hairs in trifolium repens by Rhizobium. *Can. J. Bot.* **59**, 1647-1664.

Cam, Y., Pierre, O., Boncompagni, E., Herouart, D., Meilhoc, E., & Bruand, C. 2012. Nitric oxide (no): a key player in the senescence of Medicago truncatula root nodules. *New Phytol.* **196**, 548-560.

Carpenter, S.R. 2005. Eutrophication of aquatic ecosystems: bistability and soil phosphorus. *Proc. Natl. Acad. Sci. U. S. A.,* **102**, 10002–10005.

Castillo, U., Strobel, G.A., Ford, E.J., & Hess, W.M. 2002. Munumbicins, wide-spectrum antibiotics produced by Streptomyces nrrl 30562, endophytic on Kennedia nigriscans. *Microbiol*. **148(9)**, 2675-2685.

Ceylan, O., Okmen, G., & Ugur, A. 2008. Isolation of soil Streptomyces as source antibiotics active against antibiotic-resistant bacteria. *EurAsia J. BioSci.* **2,** 73-82.

Chaintreuil, C., Giraud, E., Prin, Y., Lorquin, J., Ba, A., Gillis, M., de Lajudie, P., & Dreyfus, B. 2000. Photosynthetic bradyrhizobia are natural endophytes of the African wild rice Oryza breviligulata. *Appl. Envrion. Microbiol.* **66(12)**, 5437-5447.

Chaintreuil, C., Perrier, X., Martin, G., Fardoux, J., Lewis, G.P., Brottier, L., Rivallan, R., Gomez-Pachecco, M., Bourges, M., Lamy, L., Thilbaud, B., Ramanankierana, H., Randriambanona, H., Vandrot, H., Mournet, P., Giraud, E., & Arrighi, J.F. 2018. Naturally occurring variations in the nod-independent model legume Aeschynomene evenia and relatives: a resource for nodulation genetics. *BMC Plant Biol.* **18(54)**, 1-15.

Chaintreuil, C., Rivallan, R., Bertioli, D.J., Klopp, C., Gouzy, J., Courtois, B., Leleux, P., Martin, G., Rami, J.F., Gully, D., Parrinello, H., Severac, D., Patrel, D., Fardoux, J., Ribiere, W., Boursot, M., Cartieaux, F., Czernic, P., Ratet, P., Mournet, P., Giraud, E., & Arrighi, J.F. 2016. A gene-based map of the nod factor-independent Aeschynomene evenia sheds new light on the evolution of nodulation and legume genomes. *DNA Res.* **23(4),** 365-376.

Chandra, N., & Kumar, S. 2017. Antibiotics producing soil microorganisms. *Antibiotics and Antibiotics Resistance Genes in Soils.* **51,** 1-18.

Chebotar, V.K., Malfanova, N.V., Shcherbakov, A.V., Ahtemova, G.A., Borisov, A.Y., Lugtenberg, B., & Tikhonovich, I.A. 2015. Endophytic bacteria in microbial preparations that improve plant development (review). *Appl. Biochem. Microbiol.* **51(3)**, 271-277.

Chen, D., Jia, L., Hou, Q., Zhao, X., & Sun, K. 2021. Analysis of endophytic diversity of Rheum palmatum from different production areas in gansu province of china and the association with secondary metabolite. *Microorganisms*. **9,** 1-12.

Chen, H., Yuan, D., Wu, A., Lin, X., & Li, X. 2021. Review of low-temperature plasma nitrogen fixation technology. *Waste Dispos. Sustain. Energy.* **3**, 201-217.

Chen, P.C., & Phillips, D.A. 1977. Induction of root nodule senescence by combined nitrogen in Pisum sativum l. *Plant Physiol*. **59**, 440-442.

Christian, N., Sullivan, C., Visser, N.D., & Clay, K. 2016. Plant host and geographic location drive endophyte community composition in the face of perturbation. *Microb. Ecol.* 1-12.

Christina, A., Christapher, V., & Bhore, S.J. 2013. Endophytic bacteria as a source of novel antibiotics: an overview. *Pharmacol. Rev.* **7(13)**, 11-16.

Chu, H., Fierer, N., Lauber, C.L., Caporaso, J.G., Knight, R., Grogan, P. 2010. Soil bacterial diversity in the arctic is not fundamentally different from that found in other biomes. *Environ. Microbiol.* **12,** 2998-3006.

Chu, H., Gao, G.F., Ma, Y., Fan, K., & Delgado-Baquerizo, M. 2020. Soil microbial biogeography in a changing world: recent advances and future perspectives. *mSystems Ecol. Evol.* **5,** 1-18.

Clua, J., Roda, C., Zanetti, M.E., & Blanco, F.A. 2018. Compatibility between legumes and Rhizobia for the establishment of successful nitrogen-fixing symbiosis. *Genes*. **9,** 1-21.

Cohen, A.C., Travaglia, C.N., Bottini, R., Piccoli, P.N. 2009. Participation of abscisic acid and gibberellins produced by endophytic Azospirillum in the alleviation of drought effects in maize. *Bot*. **87**, 455–462.

Compant, S., Clement, C., & Sessitsch, A. 2010. Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* **42(5),** 669-678.

Compant, S., Sessitsch, A., & Mathieu, F. 2012. The 125th anniversary of the first postulation of the soil origin of endophytic bacteria – a tribute to M.L.V. Galippe. *Plant Soil.* **356,** 299-301.

D'Alessandro, M., Erb, M., Ton, J., Brandenburg, A. 2013. Volatiles produced by soil-borne endophytic bacteria increase plant pathogen resistance and affect tritophic interactions. *Plant Cell Environ.* **37**, 813-826.

Dart, P.J., & Mercer, F.V. 1966. The influence of ammonium nitrate on the fine structure of nodules of Medicago tribuloides desr. And Trifolium subterraneum l. *Arch. Mikrobiol*. **51**, 233-257.

Davis, E.O., Evans, I.J., & Johnston, A.W.B. 1988. Identification of noxX, a gene that allows Rhizobium leguminosarum bivar viciae strain tom to nodulate Afghanistan peas. *Mol. Gen. Genet.* **212,** 531-535.

Davis, E.O. & Johnston, A.W.B. 1990. Analysis of three nodD genes in Rhizobium leguminosarum biovar phaseoli; nodD1 is preceded by nolE, a gene whose product is secreted from the cytoplasm. *Mol. Microbiol.* **4**, 921-932.

Delgado-Baquerizo, M., Maestre, F.T., Reich, P.B., Trivedi, P., Osanai, Y., Liu, Y.R., Hamonts, K., Jeffries, T.C., Singh, B.K. 2016. Carbon content and climate variability drive global soil bacterial diversity patterns. *Ecol. Monogr.* **86,** 373–380.

Delagdo-Baquerizo, M., Bissett, A., Eldridge, D.J., Maestre, F.T., He, J.Z., Wang, J.T., Hamonts, K., Liu, Y.R., Singh, B.K., & Fierer, N. 2017. Paleoclimate explains a unique proportion of the global variation in soil bacterial communities. *Nat. Ecol. Evol.* **1,** 1339-1347.

Delledonne, M., Porcari, R., & Fogher, C. 1990. Nucleotide sequence of the nodG gene of Asopsirillum brasilense. *Nucleic Acids Res.* **18,** 6435-6435.

Deng, Z.S., Zhao, L.F., Kong, Z.Y., Yang, W.Q., Lindstrom, K., Wang, E.T., & Wei, G.H. 2011. Diversity of endophytic bacteria within nodules of Sphaerophysa salsula in defferent regions of loess plateau in china. *FEMS Microbiol. Ecol.* **76,** 463-475.

Dias, A.C.F., Costa, F.E.C., Andreote, F.D., Lacava, P.T., Teixeira, M.A., Assumpcao, L.C., Araujo, W.L., Azevedo, J.L., & Melo, I.S. 2009. Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. *World J. Microbiol. Biotechnol.* **25**, 189-195.

van Dijk, C. 1978. Spore formation and endophyte diversity in root nodules of Alnus glutinosa (l.) vill. *New Phytol.* **81,** 601-615.

Ding, Y., & Oldroyd, G.E.D. 2009. Positioning the nodule, the hormone dictum. *Plant Signal. Behav.* **4(2)**, 89-93.

Dobert R.C., Breil, B.T., & Triplett, E.W. 1994. DNA sequence of the common nodulation genes of Bradyrhizobium elkanii and their phylogenetic relationship to those of other nodulating bacteria. *Mol. Plant Microbe. Interact.* **7**, 564-572.

Dockendorff, T.C., Sharma, A.J. & Stacey, G. 1994. Identification and characterization of the nolYZ genes of Bradyrhizobium japonicum.

Dupont, L., Alloing, G., Pierre, O., Msehli, S.E., Hopkins, J., Herouart, D., & Frendo, P. 2012. The legume root nodule: from symbiotic nitrogen fixation to senescence. *Senescence ISBN.* 137-168.

Eaglesham, A.R.J., & Szalay, A.A. 1983. Aerial stem nodules on Aeschynomene spp. *Plant Sci. Lett.* **29**, 265-272.

Economou, A., Hamilton, W.D.O., Johnston, A.W.B., & Downie, J.A. 1990. The Rhizobium nodulation gene nodO encodes a Ca2(+)-binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. *EMBO J.* **9,** 349-354.

Egelhoff, T.T., Fisher, R.F., Jacobs, T.W., Mulligan, J.T., & Long, S.R. 1985. Nucleotide sequence of Rhizobium meliloti 1021 nodulation genes: nodD is read divergently from nodABC. *DNA*. **4,** 241-248.

Eid, A.M., Fouda, A., Abdel-Rahman, M.A., Salem, S.S., Elsaied, A., Oelmuller, R., Hijri, M., Bhowmik, A., Elkelish, A., & Hassan, S.E.D. 2021. Harnessing bacterial endophytes

for promotion of plant growth and biotechnological applications: an overview. *Plants*. **10(935)**, 1-33.

Emmanuel, O.C., & Babalola, O.O. 2020. Productivity and quality of horticultural crops through co-inoculation of arbuscular mycorrhizal fungi and plant growth promoting bacteria. *Microbiol. Res.* **239**, 1-11

Erisman, J.W., Galloway, J., Seitzinger, S., Bleeker, A., & Butterbach-Bahl, K. 2011. Reactive nitrogen in the environment and its effect on climate change. *Curr. Opin. Environ. Sustain*., **3**, 281–290.

Etminani, F., & Harighi, B. 2018. Isolation and identification of endophytic bacteria with plant growth promoting activity and biocontrol potential from wild pistachio trees. *Plant Pathol. J.* **34(3)**, 208-217.

Evans, I.J., & Downie, J.A. 1986. The nodI gene product of Rhizobium leguminosarum is closely related to the ATP-binding bacterial transport proteins; nucleotide sequence analysis of the nodI and nodJ genes. *Gene*. **43,** 95-101.

Fabre, S., Gully, D., Poitout, A., Patrel, D., Arrighi, J.F., Giraud, E., Czernic, P., & Cartieaux, F. 2015. Nod factor-independent nodulation in Aeschynomene evenia required the common plant-microbe symbiotic toolkit. *Plant Physiol.* **169**, 2654-2664.

Ferreira da Silva, S., Olivares, F.L., & Canellas, L.P. 2017. The biostimulant manufactured using diazotrophic endophytic bacteria and humates is effective to increase sugarcane yield. *Chem. Biol. Technol. Agric.* **4(24)**, 1-6.

Fidalgo, C., Henriques, I., Rocha, J., Tacao, M., & Alves, A. 2016. Culturable endophytic bacteria from the salt marsh plant Halimione portulacoides. *Engiron. Sci. Pollut. Res.* **23(10),** 10200-10214.

Fierer, N., & Jackson, R.B. 2006. The diversity and biogeography of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 626-631.

Fournier, J., Teillet, A., Chabaud, M., Ivanov, S., Genre, A., Limpens, E., Carvalho-Niebel, F., & Barker, D.G. 2015. Remodeling of the infection chamber before infection thread formation reveals a two-step mechanism for rhizobial entry into the host legume root hair. *Plant. Physiol.* **167**, 1233-1242.

Freiberg, C.A., Fellay, R., Bairoch, A., Broughton, W.J., Rosenthal, A., & Perret, X. 1997. Molecular basis of symbiosis between rhizobium and legumes. *Nature*. **387,** 394-401.

Frieden, E. 1972. The chemical elements of life. *Scientific American.* **227(1)**, 52-64.

Galibert, F., Finan, T.M., Long, S.R., Puehler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M.J., Becker, A., Boistard, P., Bother, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R.W., Dreano, S., Federspiel, N.A., Fisher, R.F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R.W., Jones, T., Kahn, D., Kahn, M.L., Kalman, S., Keating, D.H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M.C., Pohl, T.M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorhoelter, F.J., Weidner, S., Wells, D.H., Wong, K., Yeh, K.C., & Batut, J. 2001. The composite genome of the legume symbiont Sinorhizobium meliloti. *Science*. **29**, 668-672.

Gage, D.J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.* **268**, 280-300.

Gagné-Bourque, F., Mayer, B.F., Charron, J.B., Vali, H., Bertrand, A., Jabaji, S. 2015. Accelerated growth rate and increased drought stress resilience of the model grass Brachypodium distachyon colonized by Bacillus subtilis B26. *PLoS One*. **10(6),** 1-23.

Gao, Z., Zhang, B., Liu, H., & Han, J. 2017. Identification of endophytic bacillus velezensis zsy-1 strain and antifungal activity of its volatile compounds against Alternaria solani and Botrytis cinerea. *Biol. Control.* **105**, 27-39.

Gaworzewska, E.T., & Carlile, M.J. 1982. Positive chemotaxis of Rhizobium leguminosarum and other bacteria towards root exudates from legumes and other plants. *J. Gen. Microbiol.* **128,** 789-798.

Giraud, E., Moulin, L., Vallenet, D., Barbe, V., Cytryn, E., Avarre, J.C., Jaubert, M., Simon, D., Cartieaux, F., Prin, Y., Bena, G., Hannibal, L., Fardoux, J., Kojadinovic, M., Vuillet, L., Lajus, A., Cruveiller, S., Rouy, Z., Mangenot, S., Segurens, B., Dossat, C., Franck, W.L., Chang, W.S., Saunders, E., Bruce, D., Richardson, P., Normand, P., Dreyfus, B., Pignol, D., Stacey, G., Emerich, D., Vermeglio, A., Medigue, C., & Sadowsky, M. 2007. Legumes symbioses: absence of nod genes in photosynthetic bradyrhizobia. *Sci.* **316**, 1307-1312.

Glick, B.R. 2012. Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*. **963401,** 1-15.

Goettfert, M., Horvath, B., Kondorosi, E., Putnoky, P., Rodriguez-Quinones, F., & Kondorosi, A. 1986. At least two nodD genes are necessary for efficient nodulation of alfalfa by Rhizobium meliloti. *J. Mol. Biol.* **191,** 411-420.

Goettfert, M., Grob, P., & Hennecke, H. 1990. Proposed regulatory pathway encoded by the nodV and nodW genes, determinants of host specificity in Bradyrhizobium japonicum. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2680-2684.

Gonzalez, V., Acosta, J.L., Santamaria, R.I., Bustos, P., Hernandez-Gonzalez, I.L., Fernandez, J.L., Diaz, R., Flores, M., Mora, J., Palacios, R., & Davila, G. 2008. Genome diversity and DNA divergence of Rhizobium etli. *EMBL GenBank DDBJ*

Gonzalez, V., Bustos, P., Ramierz-Romero, M.A., Medrano-Soto, A., Salgado, H., Hernandez-Gonzalez, I., Hernanzed-Celis, J.C., Quintero, V., Moreno-Hagelsieb, G., Girard, L., Rodriguez, O., Flores, M., Cevallos, M.A., Collado-Vides, J., Romero, D., Davila, G. 2003. The mosaic structure of the symbiotic plasmid of Rhizobium etli CFN42 and its relation to other symbiotic genome compartments. *Genome Biol.* **4,** R36.1-R36.13.

Goren-Saglam, N. 2021. Ch. 18 Cyanobacteria as biofertilizer and their effect under biotic stress. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management*. 485-504.

Gottfert, M. 1993. Regulation and function of rhizobial nodulation genes. *Microbiol. Revs.* **104**, 39-64.

Gottfert, M., Roethlisberger, S., Kuendig, C., Beck, C., Marty, R., & Hennecke, H. 2001. Potential symbiosis-specific genes uncovered by sequencing a 410-kb DNA region of the Bradyrhizobium japonicum chromosome. *J. Bacteriol.* **183,** 1405-1412.

Gully, D., Czernic, P., Cruveiller, S., Mahe, F., Longin, C., Vallenet, D., Francois, P., Nidelet, S., Rialle, S., Giraud, E., Arrighi, J.F., DasGupta, M., & Cartieaux, F. 2018. Transcriptome profiles of nod factor-independent symbiosis in the tropical legume Aeschynomene evenia. *Sci. Rep.* **8**, 1-12.

Halverson, L.J., & Stacey, G. 1986. Signal exchange in plant-microbe interactions. *Microbiol. Rev.* **50**, 193-225.

Hamid, S., Lone, R., & Mohamed, H.I. 2021. Production of antibiotics from PGPR and their role in biocontrol of plant diseases. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management.* **16,** 441-461.

Han, Y.H., Fu, J.W., Chen, Y., Rathinasbapathi, B., Ma, L.Q. 2016. Arsenic uptake, arsenite efflux and plant growth in hyperaccumulator Pteris vittata: role of arsenic-resistant bacteria. *Chemosphere.* **144,** 1937–1942.

Hardoim, P. 2019. The ecology of seed microbiota. *Seed Endophytes.* 103-125

Harrison, J.G., & Griffin, E.A. 2020. The diversity and distribution of endophytes across biomes, plant phylogeny and host tissues: how far have we come and where do we go from here? *Environ. Microbiol.* **22,** 2107-2123.

Hassan, M.K., McInroy, J.A., & Kloepper, J.W. 2019. The interactions of rhizodeposits with plant growth-promoting rhizobacteria in the rhizosphere: a review. *Agriculture*. **9(7),** 1-13.

Heckmann, A.B., Sandal, N., Bek, A.S., Madsen, L.H., Jurkiewicz, A., Nielson, M.W., Tirichine, L., Stougaard, J. 2011. Cytokinin induction of root nodule primordia in Lotus japnicus is regulated by a mechanism operating in the root cortex. *Mol. Plant Microbe. Interact.* **24(11)**, 1385-1395.

Heidstra, R., & Bisseling, T. 1996. Nod factor-induced host responses and mechanisms of Nod factor perception. *New Phytol.* **133,** 25-43

Hirsch, A.M. 1992. Developmental biology of legume nodulation. *New Phytol.* **122**, 211-237.

Hita, D.D., Fuentes, M., Zamarreno, A.M., Ruiz, Y., & Garcia-Mina, J.M. 2020. Culturable bacterial endophytes from sedimentary humic acid-treated plants. *Front. Plant Sci.* **11(837)**, 1-12.

Hong, G.F., Burn, J.E., & Johnston, A.W. 1987. Evidence that DNA involved in the expression of nodulation (nod) genes in Rhizobium binds to the product of the regulatory gene nodD. *Nucleic Acids. Res.* **15(23)**, 9677-9690.

Horvath, B., Bachem, C.W.B., Schell, J., & Kondorosi, A. 1987. Host-specific regulation of nodulation genes in Rhizobium is mediated by a plant-signal, interacting with the nod gene product. *EMBO J.* **6(4)**, 841-848.

Ishak, Z., Iswadi, M.K.M., Nizam, A.H.R., Kamil, M.J.A., Eileen, R.R.E., Syaidatul, A.W., & Anion, H. 2016. Plant growth hormones produced by endophytic Bacillus subtilis strain lkm-bk isolated from cocoa. *Malays. J. Cocoa.* **9(1)**, 127-133.

Ismail, M.A., Amin, M.A., Eid, A.M., Hassan, S.E.D., Mahgoub, H.A.M., Lashin, I., Abdelwahab, A.T., Azab, E., Gobouri, A.A., Elkelish, A., & Fouda, A. 2021. Comparative study between exogenously applied plant growth hormones versus metabolites of microbial endophytes as plant growth-promoting for Phaseolus vulgaris l. *Cells*. **10**, 1-26.

John, J.E., Maheswari, M., Kalaiselvi, T., Prasanthrajan, M., Poornachandhra, C., Rakesh, S.S., Gopalakrishnan, B., Davamani, V., Kokiladevi, E., & Ranjith, S. 2023. Biomining Sesuvium portulacastrum for halotolerant pgpr and endophytes for promotion of salt tolerance in Vigna mungo l. *Front. Microbiol.* 1-18.

Kaneko, T., Nakamura, Y., Sato, S., Minamisawa, K., Uchiumi, T., Sasamoto, S., Watanabe, A., Idesawa, K., Iriguchi, M., Kawashima, K., Kohara, M., Matsumoto, M., Shimpo, S., Tsuruoka, H., Wada, T., Yamada, M., & Tabata, S. 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium Bradyrhizobium japonicum usda110. *DNA Res.* **9,** 189-197.

Kandel, S.L., Joubert, P.M., & Doty, S.L. 2017. Bacterial endophyte colonization and distribution within plants. *Microorganisms.* **5(4),** 1-26.

Kaur, M., & Karnwal, A. 2023. Screening of plant growth-promoting attributes bearing endogenous bacteria from abiotic stress resisting high altitude plants. *J. Agric. Food. Res.* **11**, 1-7.

Khamna, S., Yokota, A., Peberdy, J.F., Lumyong, S. 2010. Indole-3-acetic acid production by Streptomyces sp. Isolated from some thai medicinal plant rhizosphere soils. *EurAsia J. BioSci.* **4**, 23-32.

Khan, A.L., Wawas, M., Kang, S.M., Al-Harrasi, A., Hussain, J., Al-Rawahi, A., Al-Khiziri, S., Ullah, I., Ali, L., Jung, H.Y., & Lee, I.J. 2014. Bacterial endophyte Sphingomonas sp. Lk11 produces gibberellins and iaa and promotes tomato plant growth. *J. Microbiol.* **52(8)**, 689-695.

Khan, M.S., Zaidi, A., Wani, P.A., & Oves, M. 2008. Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. *Environ. Chem. Lett.* **7,** 1-19.

Khan, Z., Guelich, G., Phan, H., Redman, R., & Doty, S. 2012. Bacterial and yeast endophytes from poplar and willow promote growth in crop plants and grasses. *ISRN Agron.* 1-11.

Khare, E., Mishra, J., & Arora, N.K. 2018. Multifaceted interactions between endophytes and plant: developments and prospects. *Front. Mircobiol.* **9(2732)**, 1-12.

Kondorosi, E., Banfalvi, Z., & Kondorosi, A. 1984. Physical and genetic analysis of a symbiotic region of Rhizobium meliloti: identification of nodulation genes. *Mol. Gen. Genet.* **193**, 445-452.

Kondorosi, E., Pierre, M., Cren, M., Haumann, U., Buire, M., Hoffman, B., Schell, J., & Kondorosi, A. 1991. Identification of NolR, a negative transacting factor controlling nod regulon in Rhizobium meliloti. *J. Mol. Biol.* **222,** 885-896.

Konopka, A., Zakharova, T., Bischoff, M., Oliver, L., Nakatsu, C., & Turco, R.F. 1999. Microbial biomass and activity in lead-contaminated soil. *Appl. Environ. Microbiol.* **65(5),** 2256-2259.

Krishnan, H.B., Lewin, A., Fellay, R., Broughton, W.J., & Pueppke, S.G. 1992. Differential expression of nodS accounts for the varied abilities of Rhizobium fredii usda257 and Rhizobium sp. strain ngr234 to nodulate Leucaena spp. *Mol. Microbiol.* **6,** 3321-3330.

Krishnan, H.B., & Pueppke, S.G. 1991. nolC, a Rhizobium fredii gene involved in cultivar-specific nodulation of soybean, shares homology with a heat-shock gene. *Mol. Microbiol.* **5,** 737-745.

Kudoyarova, G., Arkhipova, T., Korshunova, T., Bakaeva, M., Loginov, O., & Dodd, I.C. 2019. Phytohormone mediation of interactions between plants and non-symbiotic growth promoting bacteria under edaphic stresses. *Front Plant Sci.* **10,** 1-11

Kumar, N., Singh, R.K., Mishra, S.K., Sing, A.K., & Pachouri, U.C. 2010. Isolation and screening of soil Actinomycetes as source of antibiotics active against bacteria. *Int. J. Microbiol. Res.* **2,** 12-16.

Laeremans, T., Caluwaerts, I., Verreth, C., Rogel, M.A., Vanderleyden, J., & Martinez-Romero, E. 1996. Isolation and characterization of Rhizobium tropici nod factor sulfation genes. *Mol. Plant Microbe Interact.* **9,** 492-500.

Lammel, G., & Grafll, H. 1995. Greenhouse effect of NO_X. *Environ. Sci. & Pollut. Res.* **2**, 40-45.

Le, X.H., Ballard, R.A., & Franco, C.M.M. 2016. Effects of endophytic Streptomyces and mineral nitrogen on Lucerne (Medicago sativa l.) growth and its symbiosis with rhizobia. *Plant Soil.* **405,** 25-34.

Li, J., Wang, C., Liang, W., & Liu, S. 2021. Rhizosphere microbiome: the emerging barrier in plant-pathogen interactions. *Front. Microbiol.* **12,** 1-9.

Li, Q., Fu, Q., Zhu, J., Sun, Y., He, H., & Hu, H. 2023. Endophytic bacteria in Ricinus communis l.: diversity of bacterial community, plant-growth promoting traits of the isolates and its effects on Cu and Cd speciation in soil. *Agronomy.* **13(2),** 333.

Liu, H., Carvalhais, L.C., Crawford, M., Singh, E., Dennis, P.G., Pieterse, C.M.J., & Schenk, P.M. 2017. Inner plant values: diversity, colonization and benefits from endophytic bacteria. *Front. Microbiol.* **8**, 1-17.

Lopes, L.D., Pereira e Silva, M.C. & Andreote, F.D. 2016. Bacterial abilities and adaptation toward the rhizosphere colonization. *Front. Microbiol.* **7**, 1-13.

Loureiro, M.F., James, E.K., Sprent, J.I & Franco, A.A. 1995. Stem and root nodules on the tropical wetland legume, Aeschynomene fluminensis. *New Phytol.* **130**, 531-544.

Lugtenberg, B.J.J. 1989. Signal molecules in plants and plant-microbe interactions. *NATO ASI.* **36**

Luka, S., Sanjuan, J., Carlson, R.W., & Stacey, G. 1993. nolMNO genes of Bradyrhizobium japonicum are co-transcribed with nodYABCSUIJ, and nolO is involved in the synthesis of the lip-oligosaccharide nodulation signals. *J. Biol. Chem.* **268,** 27053-27059.

Luka, S., & Stacey, G. 1997. Molecular studies on a new genetic locus linked to the common nodulation genes in Bradyrhizobium japonicum. *FEMS Microbiol. Lett.* **148,** 145-151.

Luo, L. 2012. Plant cytokine or phytocytokine. *Plant Signal. Behav.* **7(12)**, 1513-1514.

de Maagd, R.A., Wijfjes, A.H.M., Spaink, H.P., Ruiz-Sainz, J.E., Wijffelman, C.A., Okker, R.J.H., Lugtenberg, B.J.J. 1989. nodO, a new nod gene of the Rhizobium leguminosarum bivar viciae sym plasmid pRL1JI, encodes a secreted protein. *J. Bacteriol.* **17,** 6764-6770.

Mahgoubm H.A.M., Fouda, A., Eid, A.M., Ewais, E.E.D., & Hassan, S.E.D. 2021. Biotechnological application of plant growth-promoting endophytic bacteria isolated from halophytic plants to ameliorate salinity tolerance of Vicia faba l. *Plant Biotechnol. Rep.* **15.** 819-843.

Malik, G. & Arora, R. 2022. Chapter 2 endophytic bacteria: mitigating abiotic stress from inside. *Bacterial Endophytes for Sustainable Agriculture and Environmental Management*. 23-43.

Mandal, S., Raju, R., Kumar, A., Kumar, P., & Sharma, P.C. 2018. Current status of research, technology response, and policy needs of salt-affected soils in india – a review. *J. Indian Soc. Coastal Agric. Res.* **36(2),** 40-53.

Marcin, C., Marcin, G., Justyna, M.P., Katarzyna, K., & Maria, N. 2013. Diversity of microorganisms from forest soils differently polluted with heavy metals. *Appl. Soil. Ecol.* **64,** 7-14.

Martinez-Romero, E., Aguirre-Noyola, J.L., Bustamante-Brito, R., Gonzalez-Roman, P., Hernandez-Oaxaca, D., Hiragareda-Alvear, V., Montes-Carreto, L.M., Martinez-Romero, J.C., Rosenblueth, Monica, & Servin-Garciduenas, L.E. 2020. We and herbivores eat endophytes. *Microbiol. Biotechnol.* **14(4),** 1282-1299.

Massawe, V.C., Hanif, A., Farzand, A., Mburu, D.K., Ochola, S.O., Wu, L., Tahir, H.A.S., Gu, Q., Qu, H., & Gao, X. 2018. Volatile compounds of endophytic Bacillus spp. have biocontrol activity against Sclerotinia sclerotiorum. *Phytopathology*. **108(12)**,1373-1385.

Matos, A.D.M., Gomes, I.C.P., Nietsche, S., Xavier, A.A., Gomes, W.S., Dos Santos Neto, J.A., & Pereira, M.C.T. 2017. Phosphate solubilization by endophytic bacteria isolated from banana trees. *An. Acad. Bras. Cienc.* **89(4)**, 2945-2954.

Meinhardt, L.W., Krishnan, H.B., Balatti, P.A., & Pueppke, S.G. 1993. Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by Rhizobium fredii usda257. *Mol. Microbiol*. **9**,17-29.

Mendes, R., Garbeva, P., & Raaijmakers, J.M. 2013. The rhizosphere microbiome: significance of plant beneficial, plant, pathogenic, and human pathogenic microorganisms. *FEMS Microbiol. Rev.* **37,** 634-663.

Meyer, S.L.F., Halbrendt, J.M., Carta, L.K., Skantar, A.M., Liu, T., Abdelnabby, H.M.E., Vinyard, B.T. 2009. Toxicity of 2,4-diacetylphloroglucinol (DAPG) to plant-parasitic and bacterial-feeding nematodes. *J Nematol* **41**, 274–280.

Michiels, J., de Wilde, P., & Vanderleyden, J. 1993. Sequence of Rhizobium leguminosarum biovar phaseoli syrM gene. *Nucleic Acids Res.* **21,** 3893-3893.

Miliute, I., Buzaite, O., Baniulis, D., & Stanys, V. 2015. Bacterial endophytes in agricultural crops and their role in stress tolerance: a review. *Zemdirbyste*. **102(4)**, 465-478.

Miller, R.V., Miller, C.M., Kinney, D.G., Redgrave, B., Sears, J., Condron, M., Strobel, T. 1998. Ecomycins, unique antimycotics from Pseudomonas viridiflava. *J. Appl. Microbiol.* **84**, 937-944.

Mills, K.M. & Bauer, W.D. 1985. Rhizobium attachment to clover roots. *J. Cell. Sci. Suppl.* **2**, 333-345.

Modak, J.M. 2002. Haber process for ammonia synthesis. *Resonance*. 69-77.

Mohamed, H.I., Sofy, M.R., Almoneafy, A.A., Abdelhamid, M.T., Basit, A., Sofy, A.R., Lone, R., & Abou-El-Enain, M.M. 2021. Ch. 4 role of microorganisms in managing soil fertility and plant nutrition in sustainable agriculture. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management*. 93-114.

Molouba, F., Lorquin, J., Willems, A., Hoste, B., Giraud, E., Dreyfus, B., Gillis, M., de Lajudie, P., & Masson-Boivin, C. 1999. Photosynthetic bradyrhizobia from Aescynomene spp. are specific to stem-nodulated species and form a separate 16S ribosomal dna restriction fragment length polymorphism group. *Appl. Environ. Microbiol. Plant Microbiol.* **65(7)**, 3084-3094.

Monteiro, P.H.R., da Silva, F.B., de Abreu, C.M., & da Silva, G.J. 2021. Plant growth promoting rhizobacteria in amelioration of abiotic stress: a functional interplay and prospective. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management.* **2,** 25-49.

Muangthong, A., Youpensuk, S., & Rerkasem, B. 2015. Isolation and characterization of endophytic nitrogen fixing bacteria in sugarcane. *Trop. Life Sci. Res.* **26(1)**, 41-51.

Mulvaney, R.L., Khan, S.A., & Ellsworth, T.R. 2009. Synthetic nitrogen fertilizers deplete soil nitrogen: a global dilemma for sustainable cereal production. *J. Environ. Qual*., **38**, 2295–2314.

Nawaz, A., Shahbaz, M., Asadullah, Imran, A., Marghoob, M.U., Imatiza, M., & Mubeen, F. 2020. Potential of salt tolerant PGPR in growth and yield augmentation of wheat (Triticum aestivum l.) under saline conditions). *Front. Microbiol.* **11,** 1-12.

Nelson, E.B. 2018. The seed microbiome: origins, interactions, and impacts. *Plant Soil.* **422,** 7-34.

Newcomb, W. 1976. A correlated light electron microscopic study of symbiotic growth and differentiation in Pisum sativum root hair nodules. *Can. J. Bot.* **54**, 2163-2186.

Newcomb, W. 1981. Nodule morphogenesis and differentiation. *Int. Rev. Cytol. Suppl.* **13**, 246-298.

Newcomb, W., Spippel, D., Peterson, R.L. 1979. The early morphogenesis of Glycine max and Pisum sativum root nodules. *Can. J. Bot.* **57**, 2603-2616.

Nottingham, A.T., Fierer, N., Turner, B.L., Whitaker, J., Ostle, N.J., McNamar, N.P., Bardgett, R.D., Leff, J.W., Salinas, N., Silman, M.R., Kruuk, L.E.B., & Meir, P. 2018. Microbes follow Humboldt: temperature drives plant and soil microbial diversity patterns from the amazon to the andes. *Ecology.* **99,** 2455-2466.

Olanrewaju, O.S., Glick, B.R., & Babalola, O.O. 2017. Mechanisms of action of plant growth promoting bacteria. *World J. Microbiol. Biotechnol.* **33,** 1-16.

Oukala, N., Aissat, K., & Pastor, V. 2021. Bacterial endophytes: the hidden actor in plant immune response against biotic stress. *Plants.* **10**, 1-24.

Oteino, N., Lally, R.D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K.J., & Dowling, D.N. 2015. Plant growth promotion induced by phosphate solubilizing endophytic Pesudomonas isolates. *Front. Microbiol.* **6(745)**, 1-9.

Pangesti, N., Reichelt, M., van de Mortel, J.E., Kapsomenou, E., Gershenzon, J., van Loon, J.J.A., Dicke, M., & Pineda, A. 2016. Jasmonic acid and ethylene signaling pathways regulate glucosinolate levels in plants during rhizobacteria-induced systemic resistance agains a leaf-chewing herbivore. *J. Chem. Ecol.* **42**, 1212-1225.

Paungfoo-Lonhienne, C., Rentsch, D., Robatzek, S., Webb, R.I., Sagulenko, E., Nasholm, T., Schmidt, S., & Lonhienne, T.G. 2010. Turning the table: plants consume microbes as a source of nutrients. *PLoS One.* **5(7),** 1-11.

Phetcharat, P., & Duangpaeng, A. 2012. Screening of endophytic bacteria from organic rice tissue for indole acetic acid production. *Procedila. Eng.* **32,** 177-183.

Piromyou, P., Greetatorn, T., Teamtisong, K., Okubo, T., Shinoda, R., Nuntakij, A., Tittabutr, P., Boonkerd, N., Minamisawa, K., & Teaumroong, N. 2015 Preferential association of endophytic bradyrhizobia with different rice cultivars and its implications for rice endophyte evolution. *Appl. Environ. Microbiol.* **81(9)**, 3049-3061.

Plazanet, C., Refregier, G., Demont, N., Truchet, G., & Rosenberg, C. 1995. The Rhizobium meliloti region located downstream of the nod box n6 is involved in the specific nodulation of Medicago lupulina. *FEMS Microbiol. Lett.* **133,** 285-291.

Pleban, S., Chermin, L., & Chet, I. 1997. Chitinolytic activity of an endophytic strain of Bacillus cereus. *Lett. Appl. Microbiol.* **25**, 284-288.

Pliego, C., Kamilova, F., & Lugtenberg, B. 2011. Plant growth-promoting bacteria: fundamentals and exploitation. *Bacteria in Agrobiology: Crop Ecosystems*. **11,** 295-343.

Podile, A.R., & Kishore, G.K. 2007. Plant growth-promoting rhizobacteria. *Plant-Associated Bacteria.* 195-230.

Portieles, R., Xu, H., Yue, Q., Zhao, L., Zhang, D., Du, L., Gao, X., Gao, J., Gonzalez, N.P., Bermudez, R.S., & Borras-Hidalgo, O. 2021. Heat-killed endophytic bacterium induces robust plant defense response against important pathogens. *Sci. Rep.* **11**, 1-14.

Prasher, P., & Sharma, M. 2021. Role of endophytic bacteria in the alleviation of heavy metals from an ecosystem. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management*. **5,** 115-131.

Prober, S.M., Leff, J.W., Bates, S.T., Borer, E.T., Firn, J., Harpole, W.S., Lind, E.M., Seabloom, E.W., Adler, P.B., Bakker, J.D., Cleland, E.E., DeCrappeo, N.M., DeLorenze, E., Hagenah, N., Hautier, Y., Hofmockel, K.S., Kirkman, K.P., Knops, J.M.H., La Pierre, K.J., MacDougall, A.S., McCulley, R.L., Mitchell, C.E., Risch, A.C., Schuetz, M., Stevens, C.J., Williams, R.J., & Fierer, N. 2015. Plant diversity predicts beta but not alpha diversity of soil microbes across grasslands worldwide. *Ecol. Lett.* **18**, 85–95.

Qi, G., Zhang, X., & Zhao, X. 2013. Endophytic Bacillus subtilis wh2 contianing Pinellia ternate agglutinin showed insecticidal activity against whitebacked planthopper Sogatella furcifera. *Bio. Control.* **58**, 233-246.

Qin, Y., Xie, X.Q., Khan, Q., Wei, J.L., Sun, A.N., Su, Y.M., Guo, D.J., Li, Y.R., & Xing, Y.X. 2022. Endophytic nitrogen-fixing bacteria dx120e inoculation altered the carbon and nitrogen metabolism in sugarcane. *Front. Microbiol*. **13**, 1-15.

Rabalais, N.N., Turner, R.E., Sen Gupta, B.K., Boesch, D.F., Chapman, P., & Murrell, M.C. 2007. Hypoxia in the northern gulf of mexico: does the science support the plan to reduce, mitigate, and control hypoxia? *Estuaries and Coasts*, **30**, 753–772.

Raymond, J., Siefert, J.L., Staples, C.R., & Blankenship, R.E. 2004. The natural history of nitrogen fixation. *Mol. Biol. Evol.* **21(3)**, 541-554.

Reay, D.S., Davidson, E.A., Smith, K.A., Smith, P., Melillo, J.M., Dentener, F., & Crutzen, P.J. 2012. Global agriculture and nitrous oxide emissions. *Nat. Clim. Change*. **2(6)**, 410-416.

Rhijn, P., & Vanderleyden, J. 1995. The Rhizobium-plant symbiosis. *Microbiol. Rev.* **59(1)**, 124-142.

Rossen, L., Johnston, A.W.B., & Downie, J.A. 1984. DNA sequence of the rhizobium leguminosarum nodulation genes nodAB and C required for root hair curling. *Nucleic Acids Res.* **12,** 9497-9508.

Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A., & Kondorosi, A. 1986. Conservation of extended promoter regions of nodulation genes in Rhizobium. *Proc. Natl. Acad. Sci. USA.* **83**, 1757-1761.

Saad, M.M., Staehelin, C., Broughton, W.J., & Deakin, W.J. 2008. Protein-protein interactions within type III secretion system-dependent pili of Rhizobium sp. strain NGR234. *J. Bacteriol.* **190,** 750-754.

Sadowsky, M.J., Olson, E.R., Foster, V.E., Kosslak, R.M., & Verma, D.P.S. 1988. Two host-inducible genes of Rhizobium fredii and characterization of the inducing compound. *J. Bacteriol.* **170,** 171-178.

Sallet, E., Roux, B., Sauviac, L., Jardinaud, M.F., Carrere, S., Faraut, T., de Carvalho-Niebel, F., Gouzy, J., Gamas, P., Capela, D., Bruand, C., & Schiex, T. 2013. Next-generation annotation of prokaryotic genomes with EuGene-P: application to Sinorhizobium meliloti 2011. *DNA Res.* **20,** 339-354.

Samin, E., van Tuinen, D., Jeandet, P., Aussenac, T., & Selim, S. 2017. Biological control of Septoria leaf blotch and growth promotion in wheat by Paenibacillus sp. Strain B2 and Curtobacterium plantarum strain EDS. *Biol. Control*. **114**, 87-96.

Schemeisser, C., Liesegang, H., Krysciak, D., Bakkou, N., Le Quere, A., Wollherr, A., Heinemeyer, I., Morgenstern, B., Pommerening-Roeser, A., Flores, M., Palacios, R., Brenner, S., Gottschalk, G., Schmitz, R.A., Broughton, W.J., Perret, X., Strittmatter, A.W., & Streit, W.R. 2009. Rhizobium sp. Strain ngr234 possesses a remarkable number of secretion systems. *Appl. Environ. Microbiol.* **75,** 4035-4045.

Schlaman, H.R., Olsthoorn, M.M., Harteveld, M., Dorner, L., Djordjevic, M.A., Thomas-Oates, J.E. & Spaink, H.P. 2006. The production of species-specific highly unsaturated fatty acyl-containing LCOs from Rhizobium leguminosarum bv. trifolii is stringently regulated by nodD and involves the nodRL genes. *Mol Plant Microbe Interact.* **19,** 215-226.

Schofield, P.R. & Watson, J.M. 1986. DNA sequence of Rhizobium trifolii nodulation genes reveals a reiterated and potentially regulatory sequence preceding nodABC and nod FE. *Nucleic Acids Res.* **14,** 2891-2903.

Scott, K.F. 1986. Conserved nodulation genes from the non-legume symbiont Bradyrhizobium sp. (Parasponia). *Nucleic Acids Res.* **14,** 2905-2919.

Scott, D.B., Young, C.A., Collins-Emerson, J.M., Terzaghi, E.A., Rockman, E.S., Lewis, P.E., & Pankhurst, C.E. 1996. Novel and complex chromosomal arrangement of Rhizobium loti nodulation genes. *Mol. Plant Microbe. Interact.* **9,** 187-197.

Senthilkumar, M., Anandham, R., Madhaiyan, M., Venkateswaran, V., & Sa, T. 2011. Endophytic bacteria: perspectives and applications in agricultural crop production. *Bacteria in Agrobiology: Crop Ecosystems*. **3,** 61-96.

Sgroy, V., Cassan, F., Masciarelli, O., Del Papa, M.F., Lagares, A., & Luna, V. 2009. Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte Prosopis strombulifera. *Appl. Microbiol. Biotechnol.* **85(2),** 371-381.

Shah, S.T., Basit, A., Ullah, I., & Mohamed, H.I. 2021. Cyanobacteria and algae as biocontrol agents against fungal and bacterial plant pathogens. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management*. **1,** 1-23.

Shah, S.T., Basit, A., Ullah, I., & Mohamed, H.I. 2021. Microorganism: a potent biological tool to combat insects and herbivores. *Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management*. **19,** 506-555.

Shankar, A., Kaushik, P., Alyemeni, M.S., Alansi, S., & Yousuf, P.Y. 2023. Transcriptional alterations associated with overexpression of a chlorogenic acid pathway gene in eggplant fruit. *J. King Saud Univ. Sci.* **35,** 1-6.

Sheik, C.S., Mitchell, T.W., Rizvi, F.Z., Rehman, Y., Faisal, M., Hasnain, S., McInerney, M.J., & Krumholz, L.R. 2012. Exposure of soil microbial communities to chromium and arsenic alters their diversity and structure. *PLoS One.* **7(6),** 1-13.

Shen, C., Xiong, J., Zhang, H., Feng, Y., Lin, X., Li, X., Liang, W., Chu, H. 2013. Soil pH drives the spatial distribution of bacterial communities along elevation on changbai mountains. *Soil. Biol. Biochem.* **57,** 204-211.

Shen, C., Liang, W., Shi, Y., Lin, X., Zhang, H., Wu, X., Xie, G., Chain, P., Grogan, P., & Chu, H.. 2014. Contrasting elevational diversity patterns between eukaryotic soil microbes and plants. *Ecology* **95**, 3190 –3202.

Sheoran, N., Nadakkakath, A.V., Munjal, V., Kundu, A., Subaharan, K., Venugopal, V., Rajamma, S., Eapen, S.J., & Kumar, A. 2015. Genetic analysis of plant

endophytic Pseudomonas putida bp25 and chemo-profiling of its antimicrobial volatile organic compounds. *Microbiol. Res.* **173**, 66-78.

Shi, Y., Lou, K., & Li, C. 2009. Promotion of plant growth by phytohormone-producing endophytic microbes of sugar beet. *Biol. Fertil. Soils.* **45**, 645-653.

Shigyo, N., Umeki, K., & Kirao, T. 2019. Plant functional diversity and soil properties control elevational diversity gradients of soil bacteria. *FEMS Microbiol. Ecol.* **95,** 1-10.

Shridhar, B.S. 2012. Review: nitrogen fixing microorganisms. *Int. J. Microbiol. Res*., **3**, 46–52.

Smit, G., Swart, S., Lugtenberg, B.J.J. & Kijne, J. 1992. Molecular mechanisms of attachment of Rhizobium bacteria to plant roots. *Mol. Microbiol.* **6,** 2897-2903.

Smitha, K., Mohan, R., Devadason, A., Raguchander, T. 2017. Exploiting novel rhizosphere Bacillus species to suppress the root rot and wilt pathogens of chickpea. *Afr. J. Microbiol. Res.* **9**, 1098–1104.

Solovev, Y.V., Igolkina, A.A., Kuliaev, P.O., Sulima, A.S., Zhukov, V.A., Porozov, Y.B., Pidko, E.A., & Andronov, E.E. 2021. Towards understanding Afghanistan pea symbiotic phenotype through the molecular modeling of the interactions between lykx-sym10 recepter heterodimer and nod factors. *Front. Plant Sci.* **12**, 1-12.

Song, X.F., Xu, T.T., Ren, S.C., & Liu, C.M. 2013. Individual amino acid residues in clv3 peptide contribute to its stability in vitro. *Plant Signal. Behav.***8(9)**, e25344-1-7.

Spaink, H.P. 2000. Root nodulation and infection factors produced by rhizobial bacteria. *Ann. Rev. Microbiol*. **54**, 257-288.

Stacey, G., Luka, S., Sanjuan, J., Banfalvi, Z., Bieuwkoop, A.J., Chun, J.Y., Forsberg, L.S., & Carlson, R. 1994. nodZ, a unique host-specific nodulation gene, is involved in fucosylation of the lipooligosaccharide nodulation signal of Bradyrhizobium japonicum. *J. Bacteriol.* **17**, 620-633.

Stougaard, J. 2000. Regulators and regulation of legume root nodule development. *Plant Physiol.* **124**, 531-540.

Studdert, G.A., & Echeverria, H.E. 2000. Crop rotations and nitrogen fertilization to manage soil organic carbon dynamics. *Soil Sci. Soc. Am. J.* **64**, 1496-1503.

Suominen, L., Paulin, L., Saano, A., Saren, A.M., Tas, E., & Lindstroem, K. 1999. Identification of nodulation promoter (nod-box) regions of Rhizobium galegae. *FEMS Microbiol. Lett.* **177,** 217-223.

Surin, B.P. & Downie, J.A. 1988. Characterization of the Rhizobium leguminosarum genes nodLMN involved in efficient host-specific nodulation. *Mol. Microbiol.* **2,** 173-183.

Surin, B.P., Watson, J.M., Hamilton, W.D.O., Economou, A., & Downie, J.A. 1990. Molecular characterization of the nodulation gene, nodT, from two biovars of Rhizobium leguminosarum. *Mol. Microbiol.* **4,** 245-252.

Suryanarayanan, T.S. & Shaanker, R.U. 2021. Can fungal endophytes fast-track plant adaptations to climate change? *Fungal Ecol.* **101039**

Sutio, G., Afifah, A.N., Maharani, R., & Basri, M. 2023. Serratia marcescens strain NPKC3_2_21 as endophytic phosphate solubilizing bacteria and entomopathogen: promising combination approach as rice biofertilizers and biopesticide. *Biodiversitas*. **24(2),** 901-909.

Talgre, L., Lauringson, E., Roostalu, H., Astover, A., & Makke, A. 2012. Green manure as a nutrient source for succeeding crops. *Plant Soil Environ*. **58**, 275-281

Tayung, K., Barik, B.P., Jagadev, P.N., & Mohapatra, U.B. 2011. Phylogenetic investigation of endophytic Fusarium strain producing antimicrobial metabolie isolated from Himalayan yew bark. *Malays. J. Microbiol.* **7(1),** 1-6.

Teale, W.D., Paponov, I.A., & Palme, K. 2006. Auxin in action: signalling, transport and the control of plant growth and development. *Nat. Rev. Mol. Cell Biol.* **7**, 847-859

Tedersoo, L., Pellet, P., Koljalg, U., & Selosse, M.A. 2006. Parallel evolutionary paths to mycoheterotrophy in understorey Ericaceae and Orchidaceae: ecological evidence for mixotrophy in pyroleae. *Ecophysiol.* **151,** 206-217.

Tiwari, R., Kalra, A., Darokar, M.P., Chandra, M., Aggarwal, N., Singh, A.K. & Khanuja, S.P.S. 2010. Endophytic bacteria from Ocimum sanctum and their yield enhancing capabilities. *Curr. Microbiol*. **60**, 167-171.

Torsvik, V., Sorheim, R., & Goksoyr, J. 1996. Total bacterial diversity in soil and sediment communities – a review. *J. Ind. Microbiol. Biotechnol.* **17,** 170-178.

Tripathi, B.M., Stegen, J.C., Kim, M., Dong, K., Adams, J.M., Lee, Y.K. 2018. Soil pH mediates the balance between stochastic and deterministic assembly of bacteria. *ISME J.* **12,** 1072-1083.

Tsai, A.Y.L., Oota, M., & Sawa, S. 2020. Chemotactic host-finding strategies of plant endoparasites and endophytes. *Front. Plant Sci.* **11,** 1-12.

Turgeon, B.G., & Bauer, W.D. 1982. Early events in the infection of the soybean by Rhizobium japonicum. Time course cytology of the initial infection process. *Can. J. Bot.* **60**, 152-161.

Ullah, A., Nisar, M., Ali, H., Hazrat, A., Hayat, K., Keerio, A.A., Ihsan, M., Laiq, M., Ullah, S., Fahad, S., Khan, A., Khan, A.H., Akbar, A., & Yang, X. 2019. Drought tolerance improvement in plants: and endophytic bacterial approach. *Appl. Microbiol. Biotechnol.* **103,** 7385-7397.

Vazquez, M.V., Davalos, A., Las Penas, A., Sanchez, F., & Quinto, C. 1991. Novel organization of the common nodulation genes in Rhizobium leguminosarum bv. phaseoli strains. *J. Bacteriol.* **173,** 1250-1258.

Vasse, J., & Truchet, G. 1984. The Rhizobium-legume symbiosis: observation of root infection by bright-field microscopy after staining with methylene blue. *Planta*. **161**, 487-489.

Vieille, C., & Elmerich, C. 1990. Characterization of two Azospirillum brasilense sp7 plasmid genes homolgous to Rhizobium meliloti nodPQ. *Mol. Plant Microbe Interact.* **3,** 389-400.

Vieille, C., & Elmerich, C. 1992. Characterization of an Azospirillum brasilense sp7 gene homologous to Alcaligenes eutrophus phbB and to Rhizobium meliloti nodG. *Mol. Gen. Genet.* **231,** 375-384.

Viprey, V., Del Greco, A., Golinowski, W., Broughton, W.J., & Perret, X. 1998. Symbiotic implications of type III protein secretion machinery in Rhizobium. *Mol. Microbiol.* **28,** 1381-1389.

de Vries, F.T., Manning, P., Tallowin, J.R.B., Mortimer, S.R., Pilgrims, E.S., Harisson, K.A., Hobbs, P.J., Quirk, H., Shipley, B., Comelissen, J.H.C., Kattge, J., & Bardgett, R.D. 2012. Abiotic drivers and plant traits explain landscape-scale patterns in soil microbial communities. *Ecol. Lett.* **15,** 1230-1239.

Waelkens, F., Voets, T., Vlassak, K., Vanderleyden, J., & van Rhijn, P. 1995. The nodS gene of Rhizobium tropici strain ciat899 is necessary for nodulation on Phaseolus vulgaris and on Leucaena leucocephala. *Mol. Plant Microbe Interact.* **8,** 147-154.

Wang, J., Meier, S., Soininen, J., Casamayor, E.O., Pan, F., Tang, X., Yang, X., Zhang, Y., Wu, Q., Zhou, J., & Shen, J. 2017. Regional and global elevational patterns of microbial species richness and evenness. *Ecography (Cop)*. **40,** 393-402.

Webb, J.R., Hayes, N.M., Simpson, G.L., Leavitt, P.R., Baulch, H.M., & Finlay, K. 2019. Widespread nitrous oxide undersaturation in farm waterbodies creates an unexpected greenhouse gas sink. *PNAS*. **116(20)**, 9814-9819.

West, C.P. 1994. Physiology and drought tolerance of endophyte-infected grasses. *Biotechnology of Endophytic Fungi of Grasses*. 87-99.

Wilhite, D.A. 2000. Drought as a natural hazard: concepts and definitions. *Drought: A Global Assessment*. **1(1),** 3-18.

White J.F., Chang, X., Kingsley, K.L., Zhang, Q., Chiaranunt, P., Micci, A., Velazquez, F., Elmore, M., Crane, S., Li, S., Lu, J., Monila, M.C., Gonzalez-Benitez, N., Beltran-Garcia, M.J., & Kowalski, K.P. 2021. Endophytic bacteria in grass crop growth promotion and biostimulation. *Grass Research*. **1(5)**, 1-9.

Wood, S.M., & Newcomb, W. 1989. Nodule morphogenesis: the early infection of alfalfa (Medicago sativa) root hairs by Rhizobium meliloti. *Can. J. Bot.* **67**, 3108-3122.

Xu, J.Y., Han, Y.H., Chen, Y., Zhu, L.J., Ma, L.Q. 2016. Arsenic transformation and plant growth promotion characteristics of as-resistant endophytic bacteria from as-hyperaccumulator Pteris vittata. *Chemosphere* **144**, 1233–1240.

Xu, H., Chen, Z., Huang, R., Cui, Y., Li, Q., Zhao, Y., Wang, X., Mao, D., Luo, Y., & Ren, H. 2021. Antibiotic resistance gene-carrying plasmid spreads into the plant endophytic bacteria using soil bacteria as carriers. *Envrion. Sci. Technol*. **55(15)**, 10462-10470.

Yao, P.Y. & Vincent, J.M. 1969. Host specificity in the root hair "curling factor" of Rhizobium sp. *Aust. J. Biol. Sci.* **22**, 413-423.

Yasameen, T., Aziz, A., Tariq, M., Arif, M.S., Shahzad, S.M., Riaz, M., Javed, A., Ali, S., & Rizwan, M. 2021. Pseudomonas as plant growth-promoting bacteria and its role in alleviation of abiotic stress. *Plant Growth-Promoing Microbes for Sustainable Biotic and Abiotic Stress Management.* **7,** 157-185.

Yatazawa, M., Yoshida, S., & Maeda, E. 1984. Fine structure of root nodules of Aeschynomene indica l. *Soil Sci. Plant. Nutr.* **30**, 405-441.

Yu, X., Cloutier, S., Tambong, J.T., & Bromfield, E.S.P. 2014. Bradyrhizobium ottawaense sp. nov., a symbiotic nitrogen fixing bacterium from root nodules of soybeans in Canada. *Int. J.Syst. Evol. Microbiol.* **64,** 3202-3207.

Yung, Y.L., Wang, W.C., & Lacis, A.A. 1976. Greenhouse effect due to atmospheric nitrous oxide. *Am. Geophys. Union.* **3(10)**, 619-621.

Zhang, J., Zhang, B., Liu, Y., Guo, Y., Shi, P., & Wei, G. 2018. Distinct large-scale biogeographic patterns of fungal communities in bulk soil and soybean rhizosphere in china. *Sci. Total Environ.* **644,** 791-800.

Zhang, K., Adamns, J.M., Shi, Y., Yang, T., Sun, R., He, D., Ni, Y., & Chu, H. 2017. Environment and geographic distance differ in relative importance for determining fungal community of rhizosphere and bulk soil. *Environ. Microbiol.* **19,** 3649-3659.

Zhang, Y. & Zindler, A. 1993. Distribution and evolution of carbon and nitrogen in Earth. *Earth Planet. Sci. Lett.* **117**, 331-345.

Zhao, L., Xu, Y., Lai, X.H., Shan, C.Z., & Yuliang, D.J. 2015. Screening and characterization of endophytic Bacillus and Paenibacillus strains from medicinal plant Lonicera japonica for use as potential plant growth promoters. *Braz. J. Microbiol.* **46(4)**, 977-989.

Zhu, F., Qu, L., Hong, X., & Sun, X. 2011. Isolation and characterization of a phosphate-solubilizing halophilic bacterium Kushneria sp. ycwa18 from daqiao saltern on the coast of yellow sea of china. **2011**, 1-6.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF TEXAS STEM NODULE ENDOPHYTES: OBSERVATIONS ON BIOGEOGRAPHIC IMPACTS FOR HOST SPECIFICITY

ABSTRACT

Stem nodulation is a rare symbiotic relationship between a host plant and nitrogen fixing bacteria that is largely limited to members of the tropic leguminous plant genus *Aeschynomene*. While this phenomenon is interesting thanks to its unusual nodule formation phenotype, it is of particular interest to researchers due to its ability to form these symbiotic relationships independently of the canonical nod factor synthesizing genes. These plants are widely distributed around the globe and have been used as an important biofertilzer in the form of green manure. Agricultural researchers have been investigating this nod factor (NF)-independent nodulation relationship in hopes of finding ways of inducing this nitrogen fixing relationship in non-leguminous crops to reduce dependencies on synthetic chemical fertilizers. Many plant growth promoting endophytic bacteria have been isolated and identified from these stem nodules, not just nitrogen fixing rhizobia. However, there are no current studies regarding the biogeographic limitations on a host plant's capability to form such NF-independent relationships and how this could impact crop inoculum selection for region specific challenges. In this study, we observed the phenotypic response of *A. indica* plants collected from North America and South Korea upon inoculation with known NF-independent nodulating organisms. Additionally, we isolated and characterized Texas native stem nodule endophytes for their nodule promotion characteristics

and host specificity. We found that the region of origin for a host plant plays a significant role in its symbiotic response regarding endophytic inoculation for nodule formation and nitrogen fixing capacity. We identified four unique stem nodule isolates, *Bacillus megaterium, Leifsonia shinshuensis, Streptomyces* sp., and *Paenibacillus* sp. and observed host specific nodulation response. To enhance biofertilizer practices in agriculture, it is important we understand how biogeographic factors interact with both bacterial and plant responses in order to tailor make region and crop specific inoculums, allowing for the increase in crop survivability, yield, and nutrient cycling.

INTRODUCTION

The genus of semiaquatic legumes, *Aeschynomene*, are highly cosmopolitan, with over 170 species thriving in a vast range, encompassing tropical (0 - 23° N & S) and subtropical (23 - 35° N & S) regions around the world (Martins et al; 2021; Rudd, 1955). As global temperatures continue to climb and the habitable zone pushes further and further from the equator, it can be expected that so too will this plant genus spread into temperate zones (Wilson et al., 2005). These tropical legumes have gained great interest for over 70 years due to their potential agricultural applications and most importantly, their ability to form stem nodules in addition to the standard root nodules (Arora, 19554). These plants naturally occur and thrive in frequently flooded conditions, hence the classification as semi-aquatic, providing the opportunity to establish their populations within the commonly inundated fields used in rice farming (Chaintreuil et al., 2016). Members of the *Aeschynomene* genus have been incorporated into agricultural systems, acting as green manure to aid in nutrient cycling and nitrogen availability for rice crops in Africa, Asia, Brazil, and the United States (Alazard & Becker, 1987; Diekmann et al., 1996; Lawal et al., 2015: Pitman et al., 1992). Some studies insist these plants act as

invasive weeds and are harmful to agricultural practices, taking nutrients away from the desired crop plants (Conceno et al., 2018; Martins et al., 2021). However, this argument is only raised in setting wherein no-till practices are used. The idea of using the *Aeschynomene* plants as green manure is most successful when the plants are allowed to populate the fields during the off season, collecting and storing nitrogen and other nutrients, then subsequently the tops are removed and the plant materials reincorporated into the soil to act as natural fertilizer (Nilanthi et al., 2015). In a study conducted by Alazard and Becker in 1987, they demonstrated the potential nitrogen cycling capabilities of these plants as a green manure source, which resulted in an increased rice yield by more than double compared to the control and about a 33% N content increase compared to those treated with N-urea fertilizers. In addition to being a widely available, naturally occurring green manure source, these plants have also proven to be useful in textile production, medicinal practices, and as a source of livestock fodder (excluding swine) (Aiken et al., 1991; Hodges et al., 1982; Kalmbacker et al., 1988). It has been demonstrated that bahia grass fields in Florida used for cattle grazing that have been rotated with *Aeschynomene* plants produced higher average daily gains for yearling cattle compared to fields treated with synthetic nitrogenous fertilizers (Pitman et al., 1992). This genus of semiaquatic legumes harbor great potential for reshaping tropical and subtropical agricultural areas due to their ability to form nitrogen-fixing stem and root nodules independently of NF signal molecules for soil nutrient cycling in addition to the possibilities of complex endophytic populations providing added benefits such as carbon fixation, salinity mitigation, drought tolerance, disease deterrence, and growth hormone production (Maliki et al., 2017; Noisangiam et al., 2012; Sturz et al., 1997; Yapi & Kimse, 2019). Understanding the endophytic organisms within these plants and their plant growth promoting characteristics combined with knowledge gained from NF-independent

nodulation systems could lead to revolutionizing agricultural practices wherein nutritional, hormonal, and stress resistance traits can be introduced to crop plants that would otherwise be dependent on synthetic chemical fertilizers.

It is believed that the semiaquatic lifestyle these plants are accustomed to is what opened the door to the formation of stem nodules, in addition to the already present, root nodules typical of leguminous plants (Alazard & Duhoux, 1987 & 1990; Eaglesham & Szalay, 1983). While the phenomenon of stem nodulation is already highly unusual, the fact that the first documented Nod-Factor independent nodulating bacteria (*Bradyrhizobium* sp. ORS78 & BTAi1) were isolated from these organs, further adds to the mystery and allure of these plants (Giraud et al., 2007). The predominant attitude toward nodule forming endosymbionts and leguminous plants revolves around the nitrogen fixation aspect of the relationship. However, nodule organs, just like all other plant tissue types, are capable of housing a variety of endophytic organisms that confer beneficial characteristics, not just nitrogen fixation, such as plant growth promoting, abiotic stress tolerance, and disease suppression (Palaniappan, et al., 2010; Pandya et al., 2015; Tariq et al., 2013; Zhao et al., 2018). Could there also be co-habitants within these nodules that are capable of stimulating nodule formation or facilitating nitrogen fixation at a greater efficiency than depending on rhizobia alone? Additionally, would these endophytic co-habitants be specific to the geographical region of origin for their host or would they be able to illicit the same endophytic response in the host plant, no matter the regional distribution? Evolutionarily speaking, the point of origin for endophytic organisms comes from either the seeds, which pass the beneficial symbionts on to the next generation, or most primitively, from the surrounding soils (Compant et al., 2012; Walitang et al., 2018; White, 1988). Soil conditions, from pH, organic material constitution, nutrient availability, salinity, temperature, pollution levels, all play

important roles in determining the native microbial populations (Andrews et al., 1987; Bahram et al., 2018; Chu et al., 2020; Marcin et al., 2013; Shen et al, 2013; Tripathi et al., 2018). The diversity of endophytic bacteria within plant tissues is a clear reflection of the environmental conditions and therefore geographic distribution of the parent plant (Li et al, 2012; Xu et al., 2014; Zhang et al, 2018). Additionally, the response of the plant to the surrounding microbial populations will be dependent on that genera or species they are historically accustomed to interacting with (Hahl et al., 2019; Singh et al., 2004; Westover et al., 1997). The aim of this study is dual pointed: (1) will nodule derived endophytes interact with their host plant in a similar manner if the seeds have different geographical origins? (2) can region specific nodule endophytes promote plant growth and nodule formation? To answer these questions, we collected *Aeschynomene indica* seeds from two parts of the world: Asia and North America. Previously, it has already been established that *Bradyrhizobium* sp. BTAi1 will induce nodule formation and conduct nitrogen fixation with *Aeschynomene indica* plants grown from African-origin seeds (Molouba et al., 1999). Additionally, this lab specifically has isolated the strain *B.* sp. RDA-1 from South Korean native *A. indica* nodules, demonstrating their ability to induce nodule formation and conduct nitrogen fixation. To answer aim 1 of this study, we collected Texas native *A. indica* seeds and inoculated them, along with South Korean native seeds using established nodule endophytes BTAi1 and RDA-1. In this case, seeds from two different geographical regions, Asia, and North America, were inoculated with organisms known to establish relationships with *A. indica* plants. The resulting nodulation frequency and nitrogenase activity were observed, noting any differences based on geographic origins of host and symbiont. To answer aim 2 of this study, Texas native stem nodule isolates were obtained and used to inoculate seeds from South Korea and Texas while the resulting nodulation and nitrogenase

activity were observed. In this case, the host plant response is being observed for differences depending on seed origin (Texas vs South Korea) when inoculated with the same, Texas native endophytic bacteria alongside established symbionts RDA-1 and BTAi1. Will Texas native isolates stimulate a plant growth promoting response in the plant they are most accustomed to interacting with, from an evolutionary standpoint at the same level of intensity as compared to plants that they are unfamiliar with? The goal in answering these questions is to provide a better understanding of how the geographical origin of a host plant can affect the endophytic relationships they form as well as how region-specific endophytes perform when introduced to plants that have evolved outside their ancestral areas. Identifying Texas native co-habiting nodule endophytic bacteria that can promote plant growth as well as stimulate or otherwise not hinder nodule formation and function could lead to important discoveries regarding region specific crop inoculums and the practice of creating tailor-made inoculums to handle area specific challenges faced by farmers such as disease, drought, salinity, pollution, etc. On a more specific level, better understanding these relationships could lead to an increased attention of modern agricultural systems toward *A. indica* and their endophytes as an alternative to expensive and disastrous synthetic fertilizers through the practice of green manuring via crop rotation (Pitman et al., 1992). Additionally, it is significant to recognize the host plant's role in this relationship and how their response to potential endophytic organisms could depend on their region of origin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bradyrhizobium* sp. BTAi1 and RDA-1 were cultured in the arabinose-gluconate (AG) medium adjusted to pH 6.8 containing 125mg Na_2HPO_4, 250mg Na_2SO_4, 320mg NH_4Cl, 180mg $MgSO_4{\cdot}7H_2O$, 10mg $CaCl_2$, 4mg $FeCl_3$, 1.3g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1g 2-(N-morpholino) ethanesulfonic acid (MES), 1g yeast extract, 1g L-arabinose, and 1g D-gluconic acid sodium sulfate per L of distilled water (Sadowsky et al., 1987). For the initial isolation, stem endophytes were cultured in YEM media (pH 6.8) consisting of 0.5g K_2HPO_4, 0.2g $MgSO_4{\cdot}7H_2O$, 0.1g NaCl, 10g mannitol, 0.5g yeast extract, with 0.002% actidione (cycloheximide) to inhibit fungal growth (Vincent, 1970) and 2.5mL (1% aqueous) Congo Red per liter. Once pure cultures of stem isolates were obtained, they are grown on AG media for the duration of the study. For both media types, 15g agar were added per L to create solid media plates. All strains in this study, when grown in liquid media, were incubated aerobically at 30°C while shaking at 200rpm.

Isolation of Texas strains from *A. indica* stem nodules. A population of Texas native *Aeschynomene indica* plants were identified in Tarkington Prairie, TX (30°18'37.4"N 94°57'02.1"W) and whole plant samples were collected and transported to the lab in a cooler within 12 h. Stem and root systems were separated and washed thoroughly with sterile distilled water, removing all soil and debris. Nodules were excised from the stems using a sterile razor blade and place into clean 1.5mL microcentrifuge tubes. The nodules were then rinsed with 0.9% NaCl and inverted for 1 min before being rinsed three times with sterile distilled water. After removing the final rinse, the nodules were treated with 70% ethanol and inverted for 1 minute. Again, they were rinsed three times with sterile distilled water. Following this, the nodules were washed with 10% bleach and left to sit for 10 min, inverting frequently. A final rinse with sterile

distilled water was completed a total of 6 times. From this final rinse, 50µL were transferred to 5mL of AG media and 100µL spread on solid AG media and the final rinse aliquots were incubated at 30°C for 2 days to ensure the nodules were properly sterilized. Once surface sterilization was confirmed, nodule endophytes were extracted by aseptically crushing them with a sterile 1mL pipette tip. Following this initial step, 1mL liquid YEM was added and the tubes were vortex vigorously for 30 min, to further break down the nodule tissue. From this mixture, 100µL were taken and serially diluted (1/10 and 1/100) before being spread on solid YEM + Congo Red + cycloheximide agar plates. Plates were incubated at 30°C and observed for colony formation and growth. All unique colonies were re-streaked on solid AG media. For re-isolation of Texas nodule strains from *A. indica* inoculated during plant growth experiments, the same surface sterilization and endophyte extraction steps were taken. The resulting colonies were phenotypically compared to the original inoculum by the obvious pigmentation to determine if the isolates could indeed be recovered from the nodules they were used to induce.

Identification of Texas strains. Each unique stem isolate was cultured overnight in liquid AG media at 30°C before their genomic DNA was extracted using the Thermo Fisher ScientificTM genomic DNA extraction kit. Purified gDNA was then quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher ScientificTM, Wilmington, DE). The 16S rRNA gene was amplified via PCR using the universal bacterial forward and reverse primers 8F (5'AGAGTTTGATCCTGGCTCAG 3') and 1541R (5' AAGGAGGTGATCCAGCCGCA 3') (Loffler et al., 2000) and sequenced via Sanger sequencing completed at the University of Texas at Arlington's Life Sciences Core Facility (3130xL Sanger Sequencer). Raw sequences were aligned and analyzed using the NCBI Nucleotide BLAST Sequence Analysis Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Identification of nodulation regulating genes. Sequences specific to the region within the *nodD* gene were targeted through PCR using gDNA purified from each unique stem nodule isolate with the primer pair NODD2PH678 (5' TGAGTTGCAAGGGCCTTGATC 3') and NODD3PH2152' (5' AGATGACTGCGCCCCCGATAG 3') (Laguerre et al., 1996). The *nodAB* region was targeted using the forward primer nodA-1 (5' TGCRGTGGAARNTRNNCTGGGAAA 3') and reverse primer nodA-2 (5' GGNCCGTCRTCRAAWGTCARGTA 3') which yield a fragment around 660bp long (Haukka et al., 1998). PCR amplification was completed using an Eppendorf 5331 Mastercycler gradient 96-well thermal cycler (Eppendorf, Hamburg, Germany). The products were then visualized via gel electrophoresis using an EPS 301 (Amersham Biosciences [GE], Chicago, IL) to ensure appropriate amplification was achieved.

Comparison of nodule formation and plant growth promotion of Texas stem isolates with geographically differing host origins. *Aeschynomene indica* seeds collected from Tarkington Prairie, TX (30°18'37.4"N 94°57'02.1"W) and Jeju Island, South Korea (33°23'46.8"N 126°32'00.5"E) were surface sterilized through an initial wash with 70% ethanol, shaking by hand for 5 min, followed by 3 washes of sterile distilled water. The seeds were then washed with a 10% bleach solution and shaken vigorously (via vortexer) for 30 min. Finally, the seeds were rinsed with sterile distilled water 6 times and given one final rinse with 70% ethanol before drying on sterile filter paper overnight under a clean bench. Once dried, three seeds were randomly selected and aseptically transferred to liquid AG media and incubated for 2 days to ensure successful sterilization. Sterile seeds were germinated using 0.8% water agar with sterile distilled water for 2 days at 30°C in the dark until radicle emergence occurred. After germination, 3 seeds were aseptically transferred to sterile CYG growth pouches (Mega

International, Roseville, MN) with a sterile straw inserted on either side of the pouch. Once loaded, two pouches were attached using sterile paper clips to sterile hanging paper folders placed into metal folder racks. Each pouch was inoculated with 1mL overnight cultures of the desired bacterial strain (*Bradyrhizobium* sp. BTAi1, RDA-1, Texas stem nodule isolates) normalized to 0.1 OD_{600} by wash and resuspension in half strength nitrogen-free Broughton and Dilworth (1/2 B&D) media at pH 6.8 containing 500μM $CaCl_2$, 250μM KH_2PO_4, 250μM K_2HPO_4, 5μM Fe-citrate, 125μM $MgSO_4 \cdot 7H_2O$, 125μM K_2SO_4, 0.5μM $MnSO_4 \cdot H_2O$, 1μM H_3BO_3, 0.25μM $ZnSO_4 \cdot 7H_2O$, 1μM $CuSO_4 \cdot 5H_2O$, 0.05μM $CoSO_4 \cdot 7H_2O$, and 0.05μM $Na_2MoO_4 \cdot 2H_2O$ (Lee et al., 2012). Plants were then incubated at 27°C with a 16h/8h day/night cycle for 40 days, watering with ½ B&D media every other day. After 40 days, the plants were removed from the pouches and the plant growth parameters were measured (nodule pigmentation, nodule number, nodule dry weight, and plant dry weight). Root systems were visualized using a Nikon SMZ-U dissection microscope.

Comparison of nitrogenase enzymatic activity. Gas chromatography (Shimadzu GC2014) was used to conduct an acetylene reduction assay, measuring the activity of the nitrogenase enzyme from control *Bradyrhizobium* sp. BTAi1/RDA-1 and the obtained Texas stem nodule strains in converting acetylene to ethylene. *Aeschynomene indica* seeds collected from Tarkington Prairie, TX (30°18'37.4"N 94°57'02.1"W) and Jeju Island, South Korea (33°23'46.8"N 126°32'00.5"E) were sterilized, germinated, and inoculated with either bacterial strain and harvested after 40 days as previously described. Root systems were removed from the stems and transferred to a sterile 50mL glass vial sealed with a serum cap. Ten percent of the total air within the vial was removed and replaced with an equal volume of pure acetylene gas using a gas-tight 5mL syringe (Hamilton Co., Reno, NV). After mixing each vial by vigorous shaking, gas samples 250μL in

volume were taken and injected into the gas chromatograph port using nitrogen as the carrier gas and a hydrogen flame detector. Glass vials containing root systems lacking nodules and vials containing no root systems were used as controls. Using standard curves calculated before the experiment with pure ethylene and pure acetylene, the concentration of these gases for each vial could be determined using the obtained peak areas. Samples were measured for two consecutive hours, yielding time points T0, T1, and T2. The average change in ethylene concentration was then calculated. Upon completion of the acetylene reduction assay, root systems and their original stems were dried and measured together to obtain a total plant dry weight. The nodules were then removed, and the root and stem systems measured again, to obtain root nodule dry weights.

RESULTS

Isolation and identification of Texas *Aeschynomene indica* stem nodule endophytes. A healthy population of *Aeschynomene indica* plants was identified in the ditch along a roadside in Texas, growing about 4ft tall with apparent root and stem nodules (Figure 2-1). A total of 4 unique culturable isolates were obtained from the Texas native *Aeschynomene indica* stem nodules. Although 8 colony types were obtained initially, further investigation through microscopy, PCR, and 16S rRNA sequencing, it was determined that there were only 4 unique identities. After running the 16S rRNA sequence results for each isolate through the NCBI BLAST database, the following closest identities were obtained: *Bacillus megaterium* (TSN1) with 100% identity similarity, *Leifsonia shinshuensis* (TSN2) with a 99%+ identity similarity, *Streptomyces* sp. (TSN5) with a 99%+ identity similarity, and *Paenibacillus* sp. (TSN7) with a 99%+ identity similarity. For colony morphology, TSN1 develops as circular colonies, pale in appearance within the first 24 hours of growth (Figure 2-2A). However, after 24 hours, colonies

begin to develop internalized concentric rings of dark and light shades of brown. Isolate TSN2 also begins as a pale colony within the first 24 hours of growth but begins to display a vibrant yellow color beyond the 24-hour threshold. (Figure 2-2B). The isolate TSN5 stood out the most in that it was the slowest grower (taking 48hours or more to appear), developed white (blue/green in the center) fuzzy, imbedded colonies on the media, and have a pungent scent of "fertile soil" that could be detected upon opening the parafilmed plates (Figure 2-2C.). The final unique isolate obtained in this study, TSN7,. will develop very slimy colonies with white pigmentation dispersed among translucent regions within the first 24 hours of incubation and will overtake the solid media if given the opportunity (Figure 2-2D).

Determination of *nod* gene presence in Texas stem nodule isolates. Each stem nodule isolate underwent genomic DNA isolation for further molecular purposes such as PCR amplification. The purpose in obtaining and analyzing stem nodule isolates is to determine if they are forming or stimulating the formation of nodules in the absence of NFs. The most important NF related gene is *nodD* which transcribes the NodD protein, responsible for detection of plant flavonoids and initiating *nodABC* NF gene transcription, as well as other symbiotic genes. To determine if this gene was present in either of the Texas stem nodule isolates, PCR amplification was conducted using *Bradyrhizobium* sp. USDA110, a known NF-dependent nodule symbiont, as a positive control (expected amplification size ~1.2-1.3kb). Upon gel visualization of the PCR products, it was clear none of the Texas stem nodule isolates possessed the *nodD* gene, with only non-specific binding and amplification appearing in each isolate to some degree (Figure 2-3). TSN1 demonstrated no significant amplification, with only smears and only a few faint bands appearing at the 0.5kb, 0.3kb, and just below the 0.2kb marks, indicating a lack of the *nodD* gene in this organism. Both TSN2 and TSN5 showed banding around the 75bp mark, indicating

nonspecific binding and a clear absence of the *nodD* gene in these two organisms. TSN7 also showed nonspecific binding with amplification between the 75bp and 200bp marks. This organism also demonstrated similar amplification of a fragment ~250bp in length that was also amplified in the positive control. However, this amplified fragment is significant smaller than the expected length for the successful amplification of the *nodD* region, indicating again that nonspecific bind has occurred in this situation and TSN7 lacks this nodulation gene as well. The negative control, *Bradyrhizobium* sp. BTAi1, displayed no amplification as was expected. The region within *nodAB* was also targeted through PCR amplification with *Bradyrhizobium* sp. USDA110 as the positive control (expected amplification size ~660bp) (Figure 2-4). Gel visualization of these PCR products for TSN1 (lane 3), TSN2 (lanes 4 & 5), TSN5 (lane 6), and TSN7 (lane 7) displayed no amplification of the *nodAB* internal region while the positive control (lane 8) showed the expected banding pattern. Again, the negative control, *Bradyrhizobium* sp. BTAi1 (lane 2), displayed no amplification as was expected. All PCR reactions were run simultaneously depending on primer pair, to ensure consistent lack or presence of amplification with each reaction.

Characteristics of nodule formation.

i) Nodule dry weights. In *A. indica* plants from germinated Korean seeds inoculated BTAi1 vs RDA-1, the total nodule dry weights were slightly higher in those inoculated with BTAi1 with an average mass of 0.59 (±0.06) mg as compared to those inoculated with RDA-1 0.51 (±0.08) mg, a difference between the two treatments of 0.8 mg. A difference of about half this was observed in *A. indica* plants from germinated Texas seeds inoculated with either strain, yielding the following averages: BTAi1 of 0.71 (±0.1) mg and RDA-1 of 0.68 (±0.06) mg (Figure 2-5). A clear difference in host plant reaction to bacterial inoculum can be seen in terms of nodule dry

weights with Texas *A. indica* showing a higher average when inoculated with both TSN1 & TSN2, respective averages being 1.2 (±0.2) mg and 0.9 (±0.02) mg, whereas the average nodule dry weights in Korean *A. indica* plants inoculated with these same isolates were 0.37 (±0.05) mg and 0.61 (±0.08) mg respectively. While compared to the known nodule inducing strains (BTAi1 & RDA-1), TSN1 in the Korean *A. indica* plants showed a decreased total nodule dry weight (0.14-0.22mg difference) whereas this same parameter was increased by 0.52-0.49mg in Texas plants inoculated with TSN1. This demonstrates a difference in host plant reaction to inoculum depending on region of origin for seeds despite the plant species remaining the same. Additionally, TSN2 showed a very slight increase in total nodule dry weight in Korean seeds (0.1-0.02mg) while the increase was more than doubled this amount in the Texas seeds (0.19-0.22mg) compared again to the known nodulating strains, BTAi1 & RDA-1. Surprisingly, the nodule dry weight averages obtained for Korean plants inoculated with TSN7, 0.38 (±0.06) mg, while still lower than the values for BTAi1 & RDA-1 in the plants (0.13-0.21 mg difference), were drastically higher than the apparent nodule inhibition that occurred in the Texas plants inoculated with TSN7, average nodule dry weight being 0.05 (±0.01) mg, a decrease of 0.66-0.63mg compared to Texas plants with BTAi1 & RDA-1.

ii) Pigmented nodule frequency. In Korean *A. indica* plants inoculated with BTAi1 vs RDA-1, the formation of pigmented nodules was essentially the same with BTAi1 forming 3.23 (±0.49) pigmented nodules on average while RDA-1 yielded 3.26 (±0.66) pigmented nodules on average, a minute difference of 0.03 between the two averages. A difference 10-fold this amount was observed (0.37) in the Texas plants inoculated with either strain, where BTAi1 yielded 4.05 (±0.74) pigmented nodules on average and RDA-1 an average of 4.42 (±0.53) pigmented nodules (Figure 2-6). For the TSN isolates, Korean plants displayed decreased pigmented nodulation

rates with all isolates except for TSN2, which had an average of 4.33 (±0.63) pigmented nodules, a 1.07-1.1 difference in average pigmented nodules as compared to treatment with BTAi1 or RDA-1. The same pattern was displayed in Texas plants where all isolates displayed lower pigmented nodules except for TSN2, with an average of 6.11 (±1.16), a difference of 1.69-2.06 average pigmented nodules comparted to BTAi1 and RDA-1. Most surprisingly was the absolute lack of pigmented nodules in Texas TSN7 treated plants whereas Korean plants treated with the same bacteria produced an average of 2.73 pigmented nodules.

iii) Pale nodule formation. The formation of pale nodules in Korean plants inoculated with BTAi1 was slightly higher than those inoculated with RDA-1, 2.85 (±0.52) and 2.20 (±0.54) average pale nodules respectively (Figure 2-7). The same pattern could be seen with average pale nodule formation in Texas plants with the same inoculation treatments. Again, BTAi1 induced slightly higher pale nodule formation compared to RDA-1, 2.65 (±0.49) and 1.84 (±0.28) averages respectively. For the Texas stem nodule isolates, there was a clear difference in host plant response to TSN1 with Korean plants showing subdued pale nodule formation while Texas plants had severely amplified pale nodule formation. Additionally, TSN7 once again showed nodulation suppression in Texas plants and slight inhibition in Korean plants.

iv) Emergent lateral root pigmentation. The average pigmented sites of emerging lateral roots were very unremarkable in Korean plants with most have less than 1 on average (Figure 2-8). However, Texas plants had higher ELR pigmentation rates in all treatments except, of course, for TSN7 treated plants. Additionally, Texas plants treated with TSN1 had four times the amount of pigmented ELR sites compared to BTAi1 and RDA-1 treated plants. While not as extreme, TSN2 treated plants also showed an increased average in ELR pigmentation compared to the formerly mentioned treatment groups.

Confirmation of Koch's Postulates. To confirm that the TSN isolates were responsible for the nodulation phenotypes observed, they had to be reisolated from the nodules they formed. The reisolation was confirmed visually, due to the obvious color patterns for each isolate (TSN1 = brown, TSN2 = yellow, TSN7 = slimy clear and white). Each isolate was successfully obtained from the nodules they formed, except for Texas plants inoculated with TSN7. These plants did not form nodules, so it was impossible to recover the isolate.

Comparison of nitrogenase activities. To infer the nitrogen fixation capabilities of each plant after being inoculated with either strain, an acetylene reduction assay was performed. The resulting amount of acetylene converted to ethylene was measured and normalized to determine the amount converted per minute per gram of total nodule material (Figure 2-9). Texas plants inoculated with BTAi1 performed better than those inoculated with RDA-1, reflecting the nodulation pattern seen for this treatment group in Texas plants throughout the study. While Korean plants inoculated with RDA-1 did not perform well in the nodulation department, they did display greater nitrogenase efficiency as compared to those inoculated with BTAi1. Regarding the Texas stem nodule isolates, Texas plants treated with either TSN1 or TSN2, while demonstrating increased nodulation frequencies, had very low nitrogenase activity with TSN1 barely outperforming TSN2. Conversely, TSN7 treated plants performed horribly in terms of nitrogenase activity, as would be expected with the nodule inhibition demonstrated throughout the study.

DISCUSSION

It has been established that the endophytic diversity within nodules (as well as any other plant organ) depends heavily on host plant species, endophytic organisms, and environmental

conditions including geographical distribution. However, it has not yet been addressed if a specific plant species that occurs in different geographical regions will react in the same manner towards potential endophytic organisms regarding nodule formation and phenotype. The purpose in this study was to determine if *Aeschynomene indica* plants from Texas harbor their own, region specific, NF-independent nodulating bacteria that would potentially serve as crop inoculants with inherent environmental adaptations in Texas. We also aimed to determine if Texas and Korean *A. indica* display the same symbiotic phenotypes (nodulation and nitrogen fixation) when exposed to known and novel symbionts of these plants, demonstrating biogeographical limitations to host plant response. Additionally, would they react the same depending on the host origin of the potential endosymbiont. For example, would Korean *A. indica* plants have the same interaction with Texas stem nodule isolates that is displayed in the original host? Understanding host specificity in relation to geographic distribution of the host plant is an important concept to developing region specific crop inoculums to address local environmental challenges faced in agriculture.

Texas *A. indica* plants were located and 4 unique bacterial isolates were obtained from their stem nodules (Figure 2-1). Through molecular (16S rRNA sequencing) and phenotypic (colony morphology) analysis, the Texas native stem nodule isolates were identified as: TSN1 – a brown *Bacillus megaterium* strain; TSN2 – a yellow *Leifsonia shinshuensis* strain; TSN5 – a white (later green and white) *Streptomyces* sp.; and TSN7 – a slimy clear and white *Paenibacillus* sp. (Figure 2-2). All 4 organisms are documented as plant endophytes and have even shown plant growth promoting characteristics. *B. megaterium* and *Paenibacillus* are common phosphate solubilizing endophytes that have also been shown to aid in the production of plant growth promoting hormones. For example, *B. megaterium* strains have been isolated from

root nodules of the common weed *Medicago polymorpha* as well as from those of the Mediterranean bush *Retama monosperma* in addition to other leguminous plant species (Chinnaswamy et al., 2018; Dahmani et al., 2020; Khalifa & Almalki, 2015). Previous studies have demonstrated the plant growth promotion conferred by this endophytic organism, individually and as a co-inoculant, displaying increased nodule occurrence, salt stress tolerance, and phytohormone production (Knezevic et al., 2021; Rios-Ruiz et al., 2019; Shah et al., 2019; Subramanian et al., 2015). The results obtained in this study support this claim in that the Texas native stem nodule isolate, TSN1 (tentatively identified as *B. megaterium*), increased total nodule dry weight (Figure 2-5), pale nodule formation (Figure 2-7), and pigmented emergent lateral root sites (Figure 2-8) in their original host plant. Although this isolate did not display a significant change in nitrogenase activity (Figure 2-9) as the sole inoculant, this does not mean it cannot enhance these capabilities as a co-inoculant to known nitrogen fixing endosymbionts such as *Bradyrhizobium* sp.

Similarly, *Paenibacillus* species have been repeatedly isolated from legume root nodules and is often touted as a plant growth promoting endophyte and rhizosphere inhabitant (Carro et al., 2013 & 2014; Ferchichi et al., 2019; Khan et al., 2020; Valverde et al., 2010). They have even been isolated from soybean nodules and root systems and characterized as plant growth promoting (Annapurna et al., 2013; Wang et al., 2021). In terms of its role as an endophyte, this organism is highly regarded for its intrinsic antifungal capabilities, phosphate solubilization, phytohormone production, and nitrogen fixation enhancement (Costa et al., 2022; Ham et al., 2022; Lai et al., 2015; Padda et al., 2017; Paul et al., 2013; Zhao et al., 2015). Additionally, strains of this organism have even been shown to increase grain crop yield and nutrient values (Ferchichi et al., 2020). Despite numerous studies supporting *Paenibacillus* as a plant growth

and nodulation promoting endophyte, the results obtained from this study display evidence to the contrary for the specific Texas native stem nodule isolate TSN7. Both Korean and Texas *A. indica* plants inoculated with this isolate did not show remarkable increases in total nodule dry weights (Figure 2-5), pigmented emergent lateral root sites (Figure 2-8), nor enhanced nitrogenase activity (Figure 2-9). Most surprisingly, Texas plants inoculated with TSN7 displayed an outright absence of pigmented nodule formation (Figure 2-6) and severe inhibition of pale nodule formation (Figure 2-7) when used as the sole inoculant. Firstly, these results demonstrate a clear difference in host plant response, depending on geographical origin, to inoculants isolated from specific localities. The Texas plants displayed an apparent negative response to this isolate, from a nodulation standpoint, while Korean plants showed only a slightly impaired or otherwise unaffected nodulation phenotype. These surprising results hint that something larger is at play here, other than the effects caused by one single organism. Previous studies have suggested *Paenibacillus* strains play an important role in population dynamics of root nodules and rhizospheres through their antimicrobial products (Ali et al., 2021; Hansen et al., 2020). It is important to remember that the applied inoculum are not interacting with the host plant alone and are, at least for the initial stages of colonization, interacting with the native seed endophytes as well. Seed endophytes are at a disadvantage when it comes to competing for colonization establishment against freshly cultured inoculum, such as was the case in this study (Ridout et al., 2019; Sasan et al., 2012). The seed dwelling organisms must take time to awaken from their dormant state and, if faced with a potential inhibitory microbe, they will not be able to successfully establish the predesigned symbiotic relationship. To further investigate this theory, Texas plants were inoculated with fresh cultures of TSN7 and the known symbiont, BTAi1 and the resulting nodulation phenotypes were observed. This experiment showed a rescue of

nodulation and nitrogenase activity in the Texas plants (data not shown). While *Paenibacillus* may hold potential as a plant growth promoting endophyte, researchers must be cognizant of their role in population dynamics and native organisms they might be paired with. Additionally, seed origin and the environment in which the mother plant was cultivated will play a significant role in what seed endophytes are passed on. For example, the Texas seeds used in this study were from a population which grew in the ditch beside a highway, being exposed to harsh conditions and excessive pollutants from litter and vehicular activity. Their native seed endophytic population would be significantly different than that of the Korean seeds used in this study, which were collected from an undisturbed ecological park on Jeju Island. While the information gained from nodule endophytic studies have provided advances to the argument of bacterial use as biofertilizers, we must also exert the same enthusiasm to better understanding the composition and dynamics of seed endophytic populations if we wish to optimize this practice.

L. shinshuensis is another known endophyte particularly recognized for its heavy metal remediation in addition to plant growth hormone production has been isolated from root, rhizosphere, and nodule samples (Assad et al., 2021; Favero et al., 2021; Jiang et al., 2022; Li et al., 2019; Madhaiyan et al., 2010). However, to our knowledge, no studies have been conducted regarding this organism's ability to stimulate nodule formation or its effects on nitrogenase activity. Therefore, the results we have obtained are the first indication of a *Leifsonia* isolate promoting nodule formation in a NF-independent manner. This isolate, TSN2, was found increase total nodule dry weight (Figure 2-5) and pigmented nodule occurrence (Figure 2-6) with moderate pale nodule formation (Figure 2-7) in both Texas and Korean *A. indica* plants. We do see elevated pigmented emergent lateral root sites in Texas plants inoculated with this isolate (Figure 2-8) but not in the Korean plants. These findings suggest beneficial nodule promotion

induced by this Texas native isolate, particularly in its original host plant. However, the subpar nitrogenase activity seen in the Texas plants inoculated with TSN2 (Figure 2-9) suggest no enhancement in the activity of this enzyme by this isolate in relation to the Texas native seed endophytes. Again, this further enforces the idea that geographic origins of host plants and their subsequent seed endophyte populations have dynamic relationships concerning outside inoculation and plant growth phenotype.

While *Streptomyces* are known plant growth promoters and are the biggest source of antibiotics both from the soil and from plants, this isolate did not undergo the same cross-inoculation studies as the other isolates due to time constraints (Alekya & Gopalakrishnan, 2017; Gopalakrishnan et al., 2015; Olanrewaju & Babalola, 2018; Procopio et al., 2012; Quinn et al., 2020; Schrey & Tarkka, 2008; Watve et al., 2001). Nevertheless, this isolate was still obtained from the Texas native *A. indica* stem nodules and should be studied for its nodulation promotion capabilities as a potential co-inoculant with specific emphasis on host plant disease resistance. These bacteria commonly form endophytic relationships and have been used to fight off various crop killing diseases in laboratory settings. However, there have never been any studies regarding these organisms and their relationship to either NF-dependent or NF-independent nodulation. Since they were obtained from plants famous for NF-independent nodule formation, we investigated the presence of nodulation related genes in these organisms. Here we have included the molecular analysis results demonstrating a lack of *nod* or *nodAB* in any of the 5 organisms (Figures 2-3 & 2-4). The *nodD* gene is integral to NF transcription, so it was the primary target for molecular confirmation of NF-independence. However, previous studies have demonstrated NF-independent nodulation to occur under the presence of but not the transcription

of this gene (Nouwen et al., 2016). Therefore, we also targeted the *nodAB* genes which are responsible for the transcription of the actual NF signal molecule backbone.

The Texas native isolates along with known NF-independent nodulating bacteria (*Braadyrhizobium* sp. BTAi1 & RDA-1) were characterized for their nodulation abilities depending on host plant region of origin. *A. indica* seeds from two very different geographical locations, South Korea (Jeju Island) and North America (Texas) were inoculated with *Bradyrhizobium* sp. BTAi1 & RDA-1, TSN1 (tentatively identified as *Bacillus megaterium*), TSN2 (tentatively identified as *Leifsonia shinshuensis*), and TSN7 (tentatively identified as *Paenibacillus* sp.). The first organism, *Bradyrhizobium* sp. BTAi1 has been documented to initiate nodulation in *A. indica* plants from Africa. The second organism, *Bradyrhizobium* sp. RDA-1 was previously isolated in this lab from Korean stem nodules and has been shown to initiate nodulation in seeds from this same location. However, neither one of these strains have yet been demonstrated to induce nodulation in Texas native *A. indica* seeds. In this study, the nodulation patterns of Texas and Korean *A. indica* seeds inoculated with BTAi1, RDA-1, and TSN NF-independent isolates were observed.

There was a clear difference between the way the host plant, depending on region of origin, reacted to either inoculating bacterial strain. Although the plants are the same species, their nodulation reactions varied depending on the inoculum. Both plant types displayed a nodulation response, although varied in intensity, no matter the bacterial strain. There were stark differences in either plants reaction to the isolates regarding all nodulation and nitrogenase activity parameters measured in this study. These differences could perhaps be attributed to the inoculum interactions with the native seed endophytes specific to each host plant's region of origin. The Texas *A. indica* seeds were collected from a roadside population that would have

been exposed to very different environmental conditions and potential endophytes as those that would have interacted with the Korean plant populations which were thriving in an undisturbed natural area of Jeju island. In this study, we were able to demonstrate clear differences in host plant response to endophytic bacteria with biogeographical dependencies. Additionally, we were able to isolate and characterize NF-independent endophytes that are capable of supporting nodulation and have great potential as co-inoculants for agricultural systems, acting as forms of biofertilizers. To understand how to best incorporate these practices as solid agricultural fertilization methods, it is important that researchers investigate the host plant's side of the story, and not just the inoculant's.

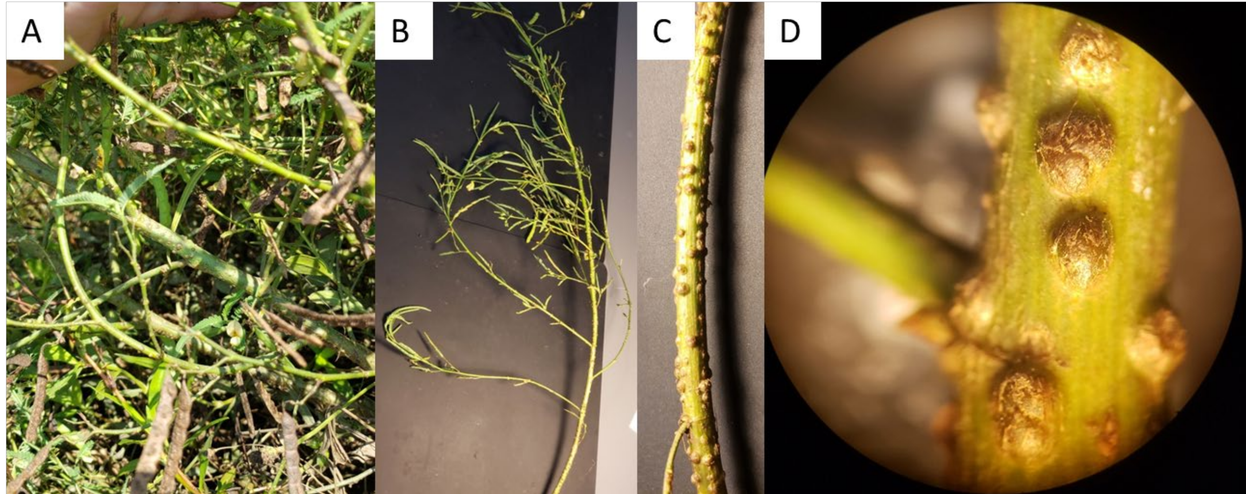

Figure 2-1. Field and laboratory photos of collected Texas (Tarkington Prairie, TX) *Aeschynomene indica* plants showing (A & B) leaf, stem, flower, and pod morphology in addition to (C & D) stem nodulation.

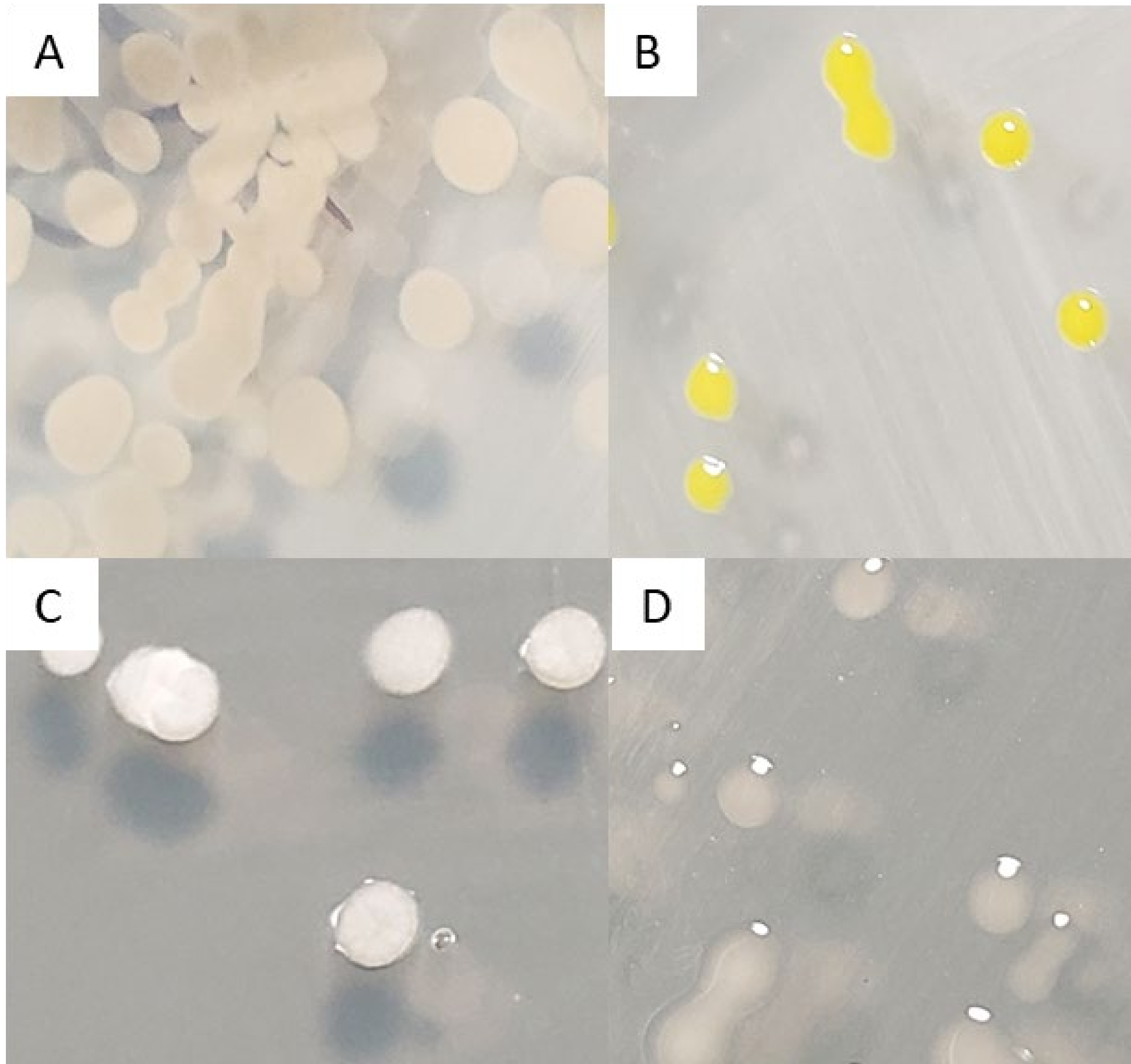

Figure 2-2. Colony morphology for unique obtained Texas native stem nodule isolates (A) TSN1 – *Bacillus megaterium* (B) TSN2 – *Leifsonia shinshuensis* (C) TSN5 – *Streptomyces* sp. (D) TSN7 – *Paenibacillus* sp.

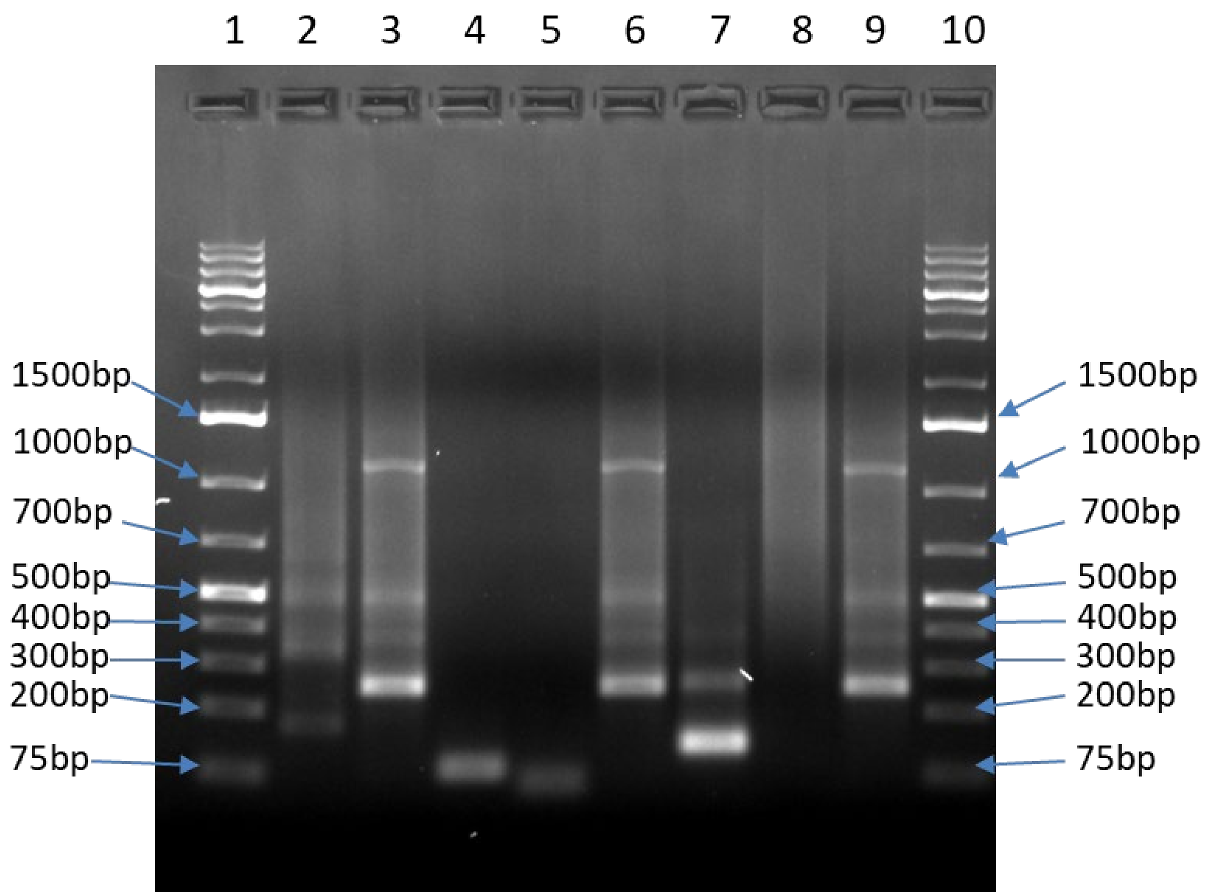

Figure 2-3. Gel electrophoresis of PCR amplicons targeting the inner portions of the *nodD* gene in the positive control *Bradyrhizobium* sp. USDA110 (lanes 3, 5, & 9), *Bacillus megaterium* – TSN1 (lane 2), *Leifsonia shinshuensis* – TSN2 (lane 4), *Streptomyces* sp. – TSN5 (lane 5), *Paenibacillus* sp. – TSN7 (lane 7), and *Bradyrhizobium* sp. BTAi1 (8) as negative control. Thermo Scientific GeneRuler 1kb plus DNA ladder was used (lanes 1 & 10).

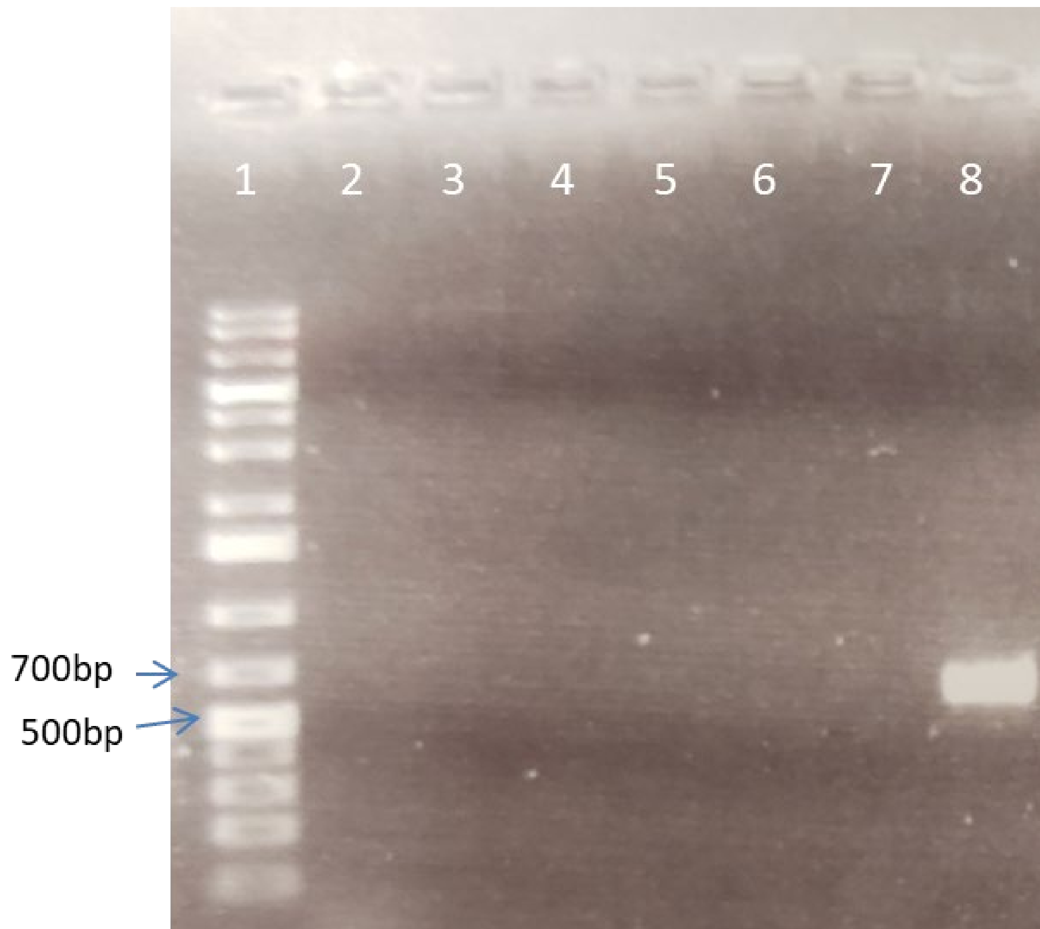

Figure 2-4. Gel electrophoresis of PCR amplicons targeting the inner portions of the *nodAB* genes in the positive control *Bradyrhizobium* sp. USDA110 (lane 8), negative control *Bradyrhizobium* sp. BTAi1 (lane 2), *Bacillus megaterium* – TSN1 (lane 3), *Leifsonia shinshuensis* – TSN2 (lanes 4 & 5), *Streptomyces* sp. – TSN5 (lane 6), and *Paenibacillus* sp. – TSN7 (lane 7). Thermo Scientific GeneRuler 1kb plus DNA ladder was used (lane 1).

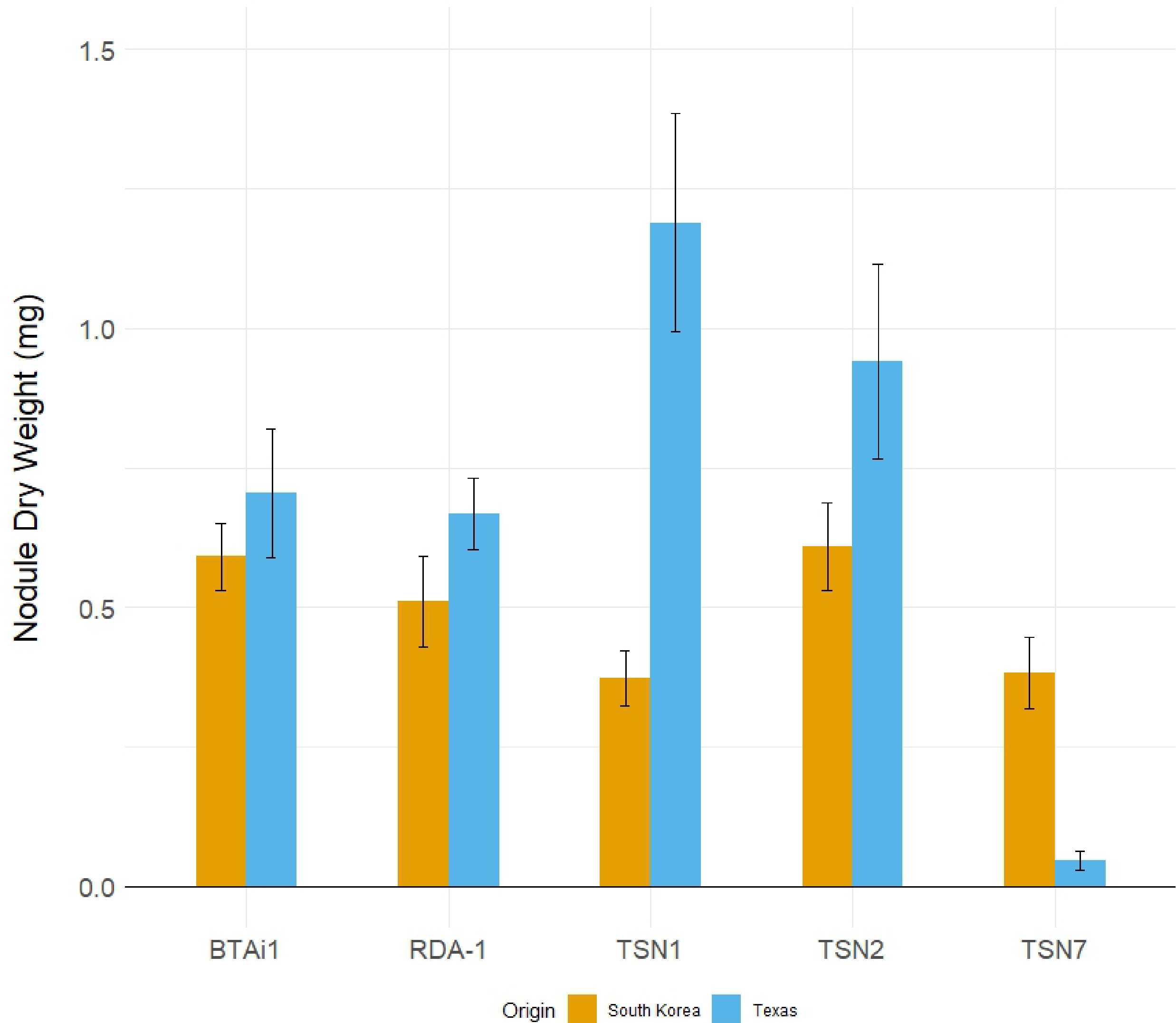

Figure 2-5. Average total nodule dry weights observed in seeds from South Korean (Jeju Island) and Texas (Tarkington Prairie) *A. indica* plants inoculated with either *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* sp. RDA-1, TSN1 – *Bacillus megaterium*, TSN2 – *Leifsonia shinshuensis*, or TSN7 – *Paenibacillus* sp.

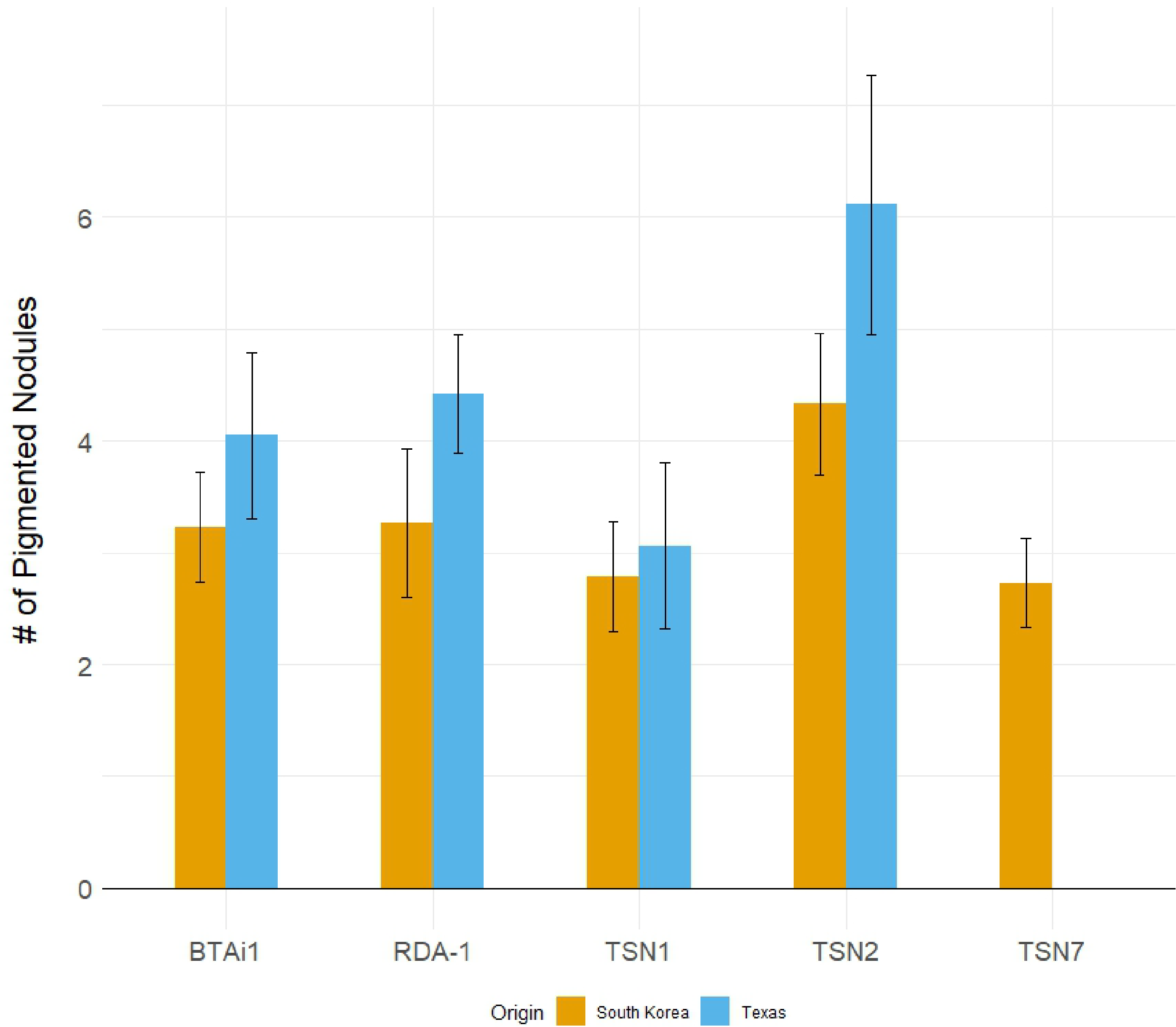

Figure 2-6. Average pigmented nodules observed in seeds from South Korean (Jeju Island) and Texas (Tarkington Prairie) *A. indica* plants inoculated with either *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* sp. RDA-1, TSN1 – *Bacillus megaterium*, TSN2 – *Leifsonia shinshuensis*, or TSN7 – *Paenibacillus* sp.

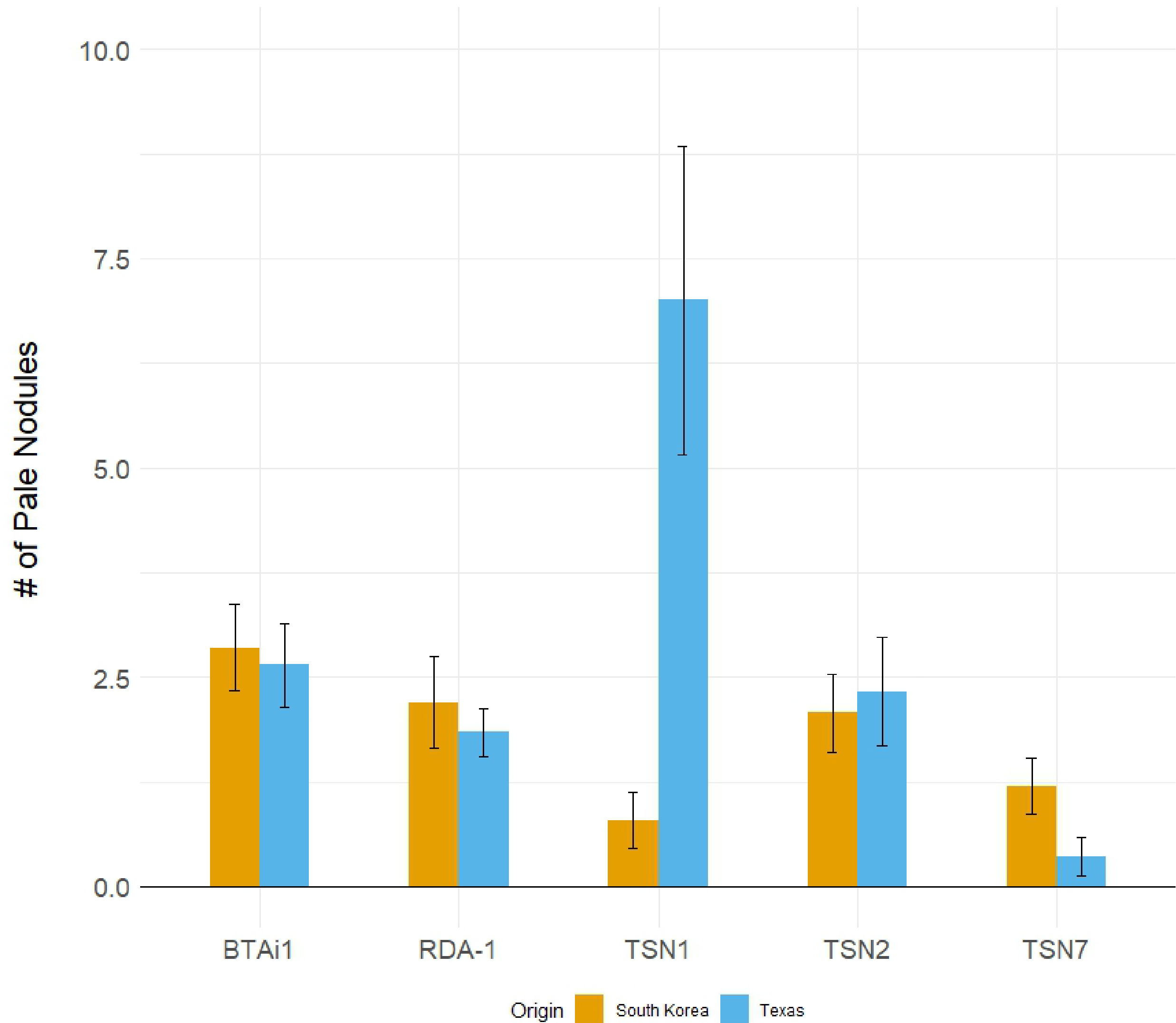

Figure 2-7. Average pale nodules observed in seeds from South Korean (Jeju Island) and Texas (Tarkington Prairie) *A. indica* plants inoculated with either *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* sp. RDA-1, TSN1 – *Bacillus megaterium*, TSN2 – *Leifsonia shinshuensis*, or TSN7 – *Paenibacillus* sp.

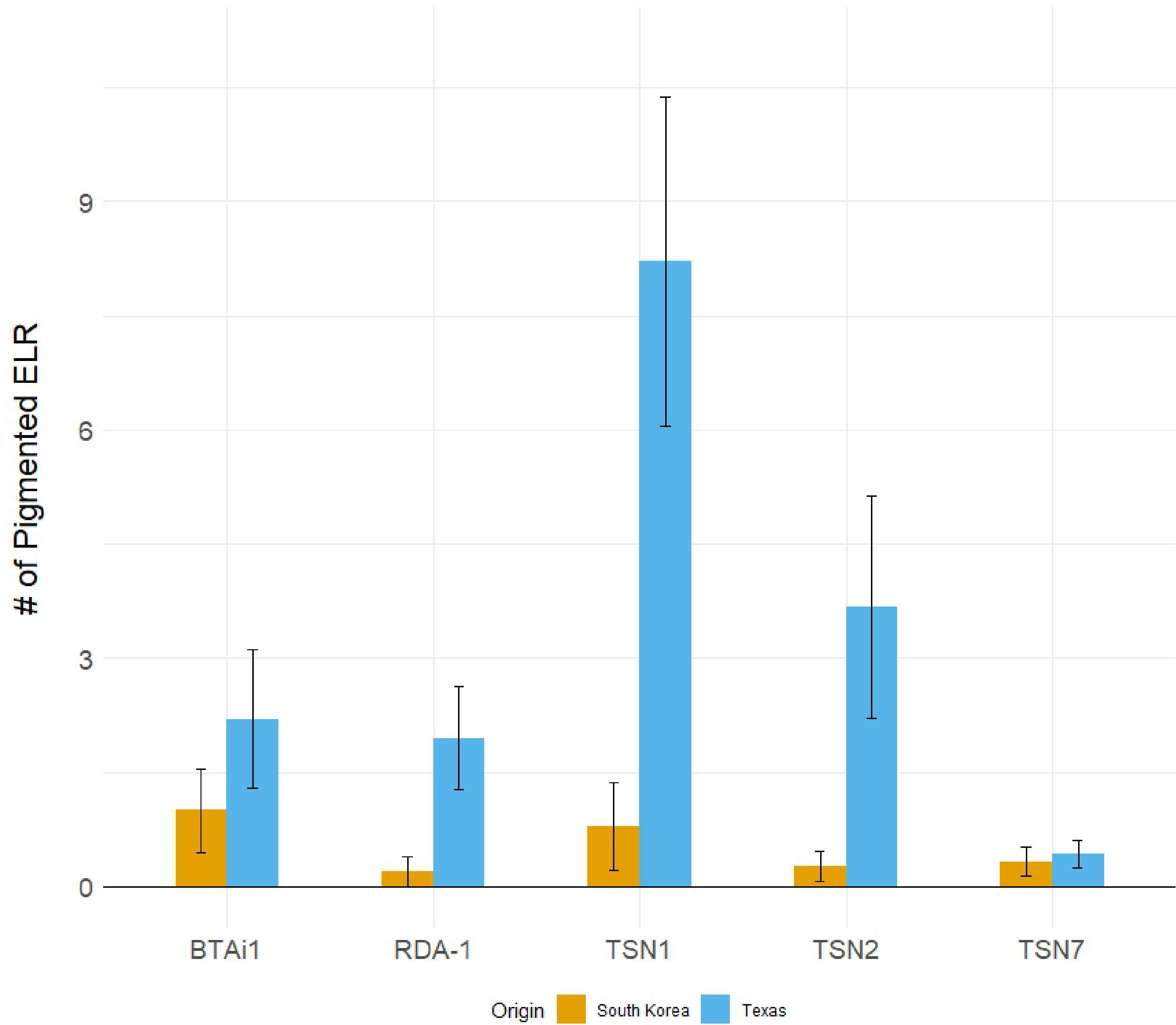

Figure 2-8. Average pigmented sites of emerging lateral roots observed in seeds from South Korean (Jeju Island) and Texas (Tarkington Prairie) *A. indica* plants inoculated with either *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* sp. RDA-1, TSN1 – *Bacillus megaterium*, TSN2 – *Leifsonia shinshuensis*, or TSN7 – *Paenibacillus* sp.

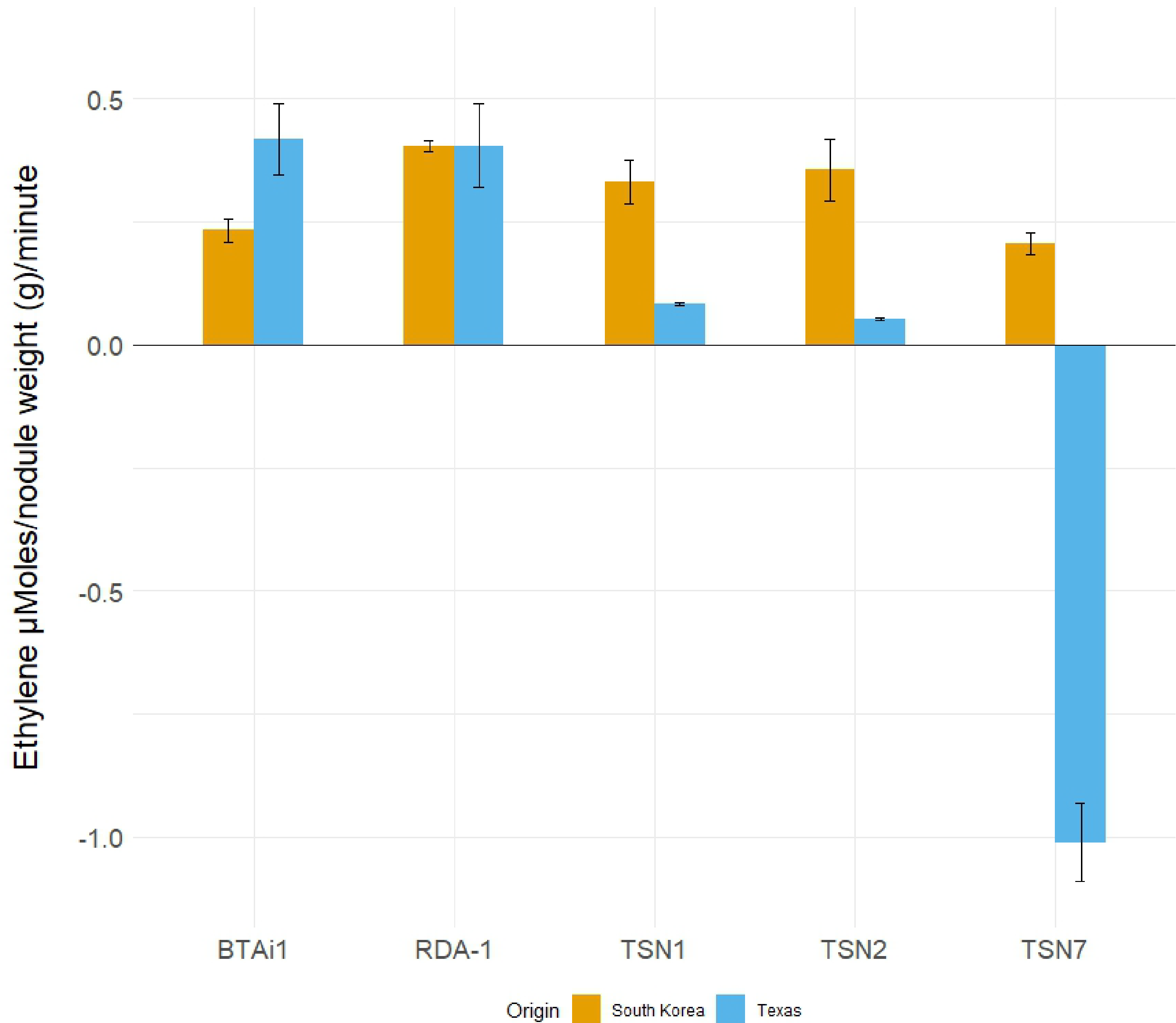

Figure 2-9. Average changes in ethylene surrounding root systems in seeds from South Korean (Jeju Island) and Texas (Tarkington Prairie) *A. indica* plants inoculated with either *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* sp. RDA-1, TSN1 – *Bacillus megaterium,* TSN2 – *Leifsonia shinshuensis,* or TSN7 – *Paenibacillus* sp.

REFERENCES

Aiken, G.E., Pitman, W.D., Chambliss, C.G., & Portier, K.M. 1991. Responses of yearling steers to different stocking rates on a subtropical grass-legume pasture. *J. Anim. Sci.* **69,** 3348-3356.

Alazard, D., & Becker, M. 1987. Aeschynomene as green manure for rice. *Plant Soil.* **101,** 141-143.

Alazard, D., & Duhoux, E. 1987. Nitrogen-fixing stem nodules on Aeschynomene afraspera. *Biol. Fert. Soils.* **4,** 61-66.

Alazard, D., & Duhoux, E. 1990. Development of stem nodules in tropical forage legume, Aeschynomene afraspera. *J. Exp. Bot.* **41,** 1199-1206

Alekhya, G., & Gopalakrishnan, S. 2017. Biological control and plant growth-promotion traits of Streptomyces species under greenhouse and field conditions in chickpea. *Agri. Res.* **6,** 410-420.

Ali, M.A., Lou, Y., Hafeez, R., Li, X., Hossain, A., Xie, T., Lin, L., Li, B., Yin, Y., Yan, J., & An, Q. 2021. Functional analysis and genome mining reveal high potential of biocontrol and plant growth promotion in nodule-inhabiting bacteria within Paenibacillus polymyxa complex. *Front. Microbiol.* **11,** 1-16.

Andrews, J.H., Kinkel, L.L., Berbee, F.M., & Nordheim, E.V. 1987. Fungi, leaves, and the theory of island biogeography. *Microb. Ecol.* **14,** 277-290.

Annapurna, K., Ramados, D., Bose, P., & VithalKumar, L. 2013. In situ localization of Paenibacillus polymyxa HKA-15 in roots and root nodules of soybean (Glycine max. l.). *Plant Soil*. **373,** 641-648.

Arora, N. 1954. Morphological development of the root and stem nodules of Aeschynomene indica l. *Phytomorph.* **4**, 211-216.

Assad, B.M., Savi, D.C., Biscaia, S.M.P., Mayrhofer, B.F., Iantas, J., Mews, M., de Oliveira, J.C., Trindade, E.S., & Glienke, C. 2021. Endophytic Actinobacteria of Hymenachne amplexicaulis from Brazilian Pantanal wetland produce compounds with antibacterial and antitumor activities. *Microbiol. Res.* **248,** 1-15.

Bahram, M., Hildebrand, F., Forslund, S.K., Anderson, J.L., Soudzilovskaia, N.A., Bodegom, P.M., Bengtsson-Palme, J., Anslan, S., Coelho, L.P., Harend, H., Huerta-Cepas, J., Medema, M.H., Maltz, M.R., Mundra, S., Olsson, P.A., Pent, M., Põlme, S., Sunagawa, S., Ryberg, M., Tedersoo, L., & Bork, P. 2018. Structure and function of the global topsoil microbiome. *Nature* **560**, 233–237.

Carro, L., Flores-Felix, J.D., Cerda-Castillo, E., Ramirez-Bahena, M.H., Igual, J.M., Tejedor, C., Velazquez, E., & Peix, A. 2013. Paenibacillus endophyticus sp. nov., isolated from nodules of Cicer arietinum. *Int. J. Syst. Evol. Microbiol.* **63,** 4433-4438.

Carro, L., Flores-Felix, J.D., Ramirez-Bahena, M.H., Garcia-Fraile, P., Martinez-Hidalgo, P., Igual, J.M., Tejedor, C., Peix, A., & Velazquez, E. 2014. Paenibacillus lupine sp. nov., isolated from nodules of Lupinus albus. *Int. J. Syst. Evol. Microbiol.* **64,** 3028-3033.

Chaintreuil, C., Gully, D., Hervouet, C., Tittabutr, P., Randriambanona, H., Brown, S.C., Lewis, G.P., Bourge, M., Cartieaux, F., Boursot, M., Ramanankierana, H., D'Hont, A., Teaumroong, N., Giraud, E., & Arrighi, J.F. 2016. The evolutionary dynamics of ancient and recent polyploidy in the African semiaquatic species of the legume genus Aeschynomene. *New Phytol.* **211,** 1077-1091.

Chinnaswamy, A., de la Pena, C., Rojo, D.P., Rincon, B.A., Lucas, M.M., & Pueyo, J.J. 2018. A nodule endophytic Bacillus megaterium strain isolated from Medicago polymorpha enhances growth, promotes nodulation by Ensifer medicae and alleviates salt stress in alfalfa plants. *Ann. Appl. Biol.* **172(3),** 295-308.

Chu, H., Gao, G.F., Ma, Y., Fan, K., & Delgado-Baquerizo, M. 2020. Soil microbial biogeography in a changing world: recent advances and future perspectives. *mSystems Ecol. Evol.* **5,** 1-18.

Compant, S., Sessitsch, A., & Mathieu, F. 2012. The 125th anniversary of the first postulation of the soil origin of endophytic bacteria – a tribute to M.L.V. Galippe. *Plant Soil.* **356,** 299-301.

Costa, A., Corallo, B., Amarelle, V., Stewart, S., Pan, D., Tsicornia, S., & Fabiano, E. 2022. Paenibacillus sp. strain UY79, isolated from a root nodule of arachis villosa, displays a broad spectrum of antifungal activity. *Appl. Environ. Microbiol.* **88(2)**

Dahmani, M.A., Desrut, A., Moumen, B., Verdon, J., Mermouri, L., Kacem, M., Coutos-Thevenot, P., Kaid-Harche, M., Berges, T., & Vriet, C. 2020. Unearthing the plant growth-promoting traits of Bacillus megaterium RmBm31, an endophytic bacterium isolated from root nodules of Retama monosperma. *Front. Plant. Sci.* **27,** 1-15.

Diemann, K.H., Ottow, J.C.G., & de Datta, S.K. 1996. Yield and nitrogen response of lowland rice (Oryza sativa l.) to Sesbania nod100tate and Aeschynomene afraspera green manure in different marginally productive soils in the Philippines. *Biol. Fertil. Soils.* **21,** 103-108.

Eaglesham, A.R.J., & Szalay, A.A. 1983. Aerial stem nodules on Aeschynomene spp. *Plant Sci. Lett.* **29,** 265-272.

Favero, V.O., de Carvalho, R.H., Leite, A.B.C., de Freitas, K.M., Zilli, J.E., Xavier, G.R., Rumjanek, N.G., & Urquiaga, S. 2021. Characterization and nodulation capacity of native bacteria isolated from mung bean nodules used as a trap plant in Brazilian tropical soils. *Appl. Soil Ecol.* **167,** 104041

Ferchichi, N., Toukabri, W., Boularess, M., Samoui, A., Mhamdi, R., & Trabelsi, D. 2019. Isolation, identification and plant growth promotion ability of endophytic bacteria associated with lupine root nodule grown in Tunisian soil. *Arch. Microbiol.* **201,** 1333-1349.

Ferchichi, N., Toukabri, W., Vrhovsek, U., Angeli, A., Masuero, D., Mhamdi, R., & Trabelsi, D. 2020. Inoculation of Lupinus albus with the nodule-endophyte Paenibacillus glycanilyticus LJ121 improves grain nutritional quality. *Arch. Microbiol.* **202,** 283-291.

Giraud, E., Moulin, L., Vallenet, D., Barbe, V., Cytryn, E., Avarre, J. C., Jaubert, M., Simon, D., Cartieaux, F., Prin, Y., Bena, G., Hannibal, L., Fardoux, J., Kojadinovic, M., Vuillet, L., Lajus, A., Cruveiller, S., Rouy, Z., Mangenot, S., Segurens, B., Dossat, C., Franch, W. L., Chang, W. S., Sauders, E., Bruce, D., Richardson, P., Normand, P., Dreyfus, B., Pignol, D., Stacey, G., Emerich, D., Vermeglio, A., Medigue, C., Sadowsky, M. 2007. Legumes symbioses: absence of nod genes in photosynthetic Bradyrhizobia. *Science*. **316(5829)**, 1307-1312.

Gopalakrishnan, S., Srinivas, V., Alekhya, G., Prakash, B., Kudapa, H., & Varshney, R.K. 2015. Evaluation of Streptomyces sp. obtained from herbal vermicompost for broad spectrum of plant growth-promoting activities in chickpea. *Org. Agric.* **5,** 123-133.

Hahl, T., van Moorsel, S.J., Schmid, M.W., Zuppinger-Dingley, D., Schmid, B., & Wagg, C. 2019. Plant responses to diversity-driven selection and associated rhizosphere microbial communities. *Funct. Ecol.* **34,** 707-722.

Ham, Y.J., Jeong, J.W., Lee, D.H., & Kim, S.B. 2022. Paenibacillus artemisiicola sp. nov. and Paenibacillus lignilyticus sp. nov., two new endophytic bacterial species isolated from plant roots. *Int. J. Syst. Evol. Microbiol.* **72(3)**

Hansen, B.L., Pessotti, R.C., Fischer, M.S., Collins, A., El-Hifnawi, L., Liu, M.D., & Traxler, M.F. 2020. Cooperation, competition, and specialized metabolism in a simplified root nodule microbiome. *Ecol. Evol. Sci.* **11**

Haukka, K., Lindstrom, K., & Young, J.P.W. 1998. Three phylogenetic groups of nodA and nifH genes in Sinorhizobium and Mesorhizobium isolates from leguminous tress growing in Africa and latin America. *Appl. Environ. Microbiol.* **64(2),** 419-426.

Hodges, E.M., Kretschmer, A.E., Jr., Mislevy, P., Roush, R.D., Ruelke, O.C. & Snyder, G.H. 1982. Prodution and utilization of the tropical legume Aeschynomene. *Florida Agricultural Experiment Station Circular S-290,* Gainesville, Florida.

Jiang, X., Li, W.W., Han, M., Chen, G., Wu, J., Lai, S., Fu, Z., Zhang, S., Deng, W.W., Gao, L., & Xia, T. 2022. Aluminum-tolerant, growth-promoting endophytic bacteria as

contributors in promoting tea plant growth and alleviating aluminum stress. *Tree Physiol.* **42,** 1043-1058.

Kalmbacher, R.S., Mislevy, P., Martin, F.G., Prevatt, J.W., Chambliss, C.G., & Kidder, G. 1988. Establishment of Aeschynomene in bahia grass sod. *Florida Agricultural Experiment Station Circular S-355,* Gainesville, Florida.

Khalifa, A.Y.Z., & Almalki, M.A. 2014. Isolation and characterization of an endophytic bacterium, Bacillus megaterium BMN1, associated with root-nodules of Medicago sativa l. growing in al-ashaa region, Saudi arabia. *Ann. Microbiol.* **65,** 1017-1026.

Khan, M.S., Gao, J., Chen, X., Zhang, M., Yang, F., Du, Y., Moe, T.S., Munir, I., Xue, J., & Zhang, X. 2020. Isolation and characterization of plant growth-promoting endophytic bacteria Paenibacillus polymyxa SK1 from Lilium lancifolium. *Biomed Res. Int.* 1-17.

Knezevic, M., Beric, T., Buntic, A., Delic, D., Nikolic, I., Stankovic, S., & Stajkovic-Srbinovic, O. 2021. Potential of root nodule nonrhizobial endophytic bacteria for growth promotion of Lotus corniculatus l. and Dactylis glomerata l. *J. Appl. Microbiol.* **131(6),** 2929-2940.

Lai, W.A., Hameed, A., Lin, S.Y., Hung, M.H., Hsu, Y.H., Liu, Y.C., Shahina, M., Shen, F.T., & Young, C.C. 2015. Paenibacillus medicaginis sp. nov. a chitinolytic endophyte isolated from a root nodule of alfalfa (Medicago sativa l.). *Int. J. Syst. Evol. Microbiol.* **65,** 3853-3860.

Languerre, G., Mavingui, P., Allard, M., Charnay, M., Louvrier, P., Mazurier, S., Rigottier-Gois, L., & Amarger, N. 1996. Typing of Rhizobia by PCR DNA fingerprinting and

PR-Restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to Rhizobium leguminosarum and its different biovars. *Appl. Environ. Microbiol.* **62(6)**, 2029-2036.

Lawal, B.A., Adeboye, M.K.A., Usman, A., Afolabi, S.G., & Adekanmbi, A.A. 2015. Rotation effect of Aeschynomene histrix on soil carbon and nitrogen and maize grain yield at minna in the southern guinea savanna of Nigeria. *J. Agriculture Agri. Technol.* **6(1),** 1-9.

Lee, H.I., Lee, J.H., Park, K.H., Sangurdekar, D., and Chang, W.S. 2012. Effect of soybean coumestrol on Bradyrhizobium japonicum nodulation ability, biofilm formation, and transcriptional profile. *Appl. Environ. Microbiol*. **78**, 2896-2903.

Li, L., Sinkko, H., Montonen, L., Wei, G., Lindstrom, K., & Rasanen, L.A. 2012. Biogeography of symbiotic and other endophytic bacteria isolated from medicinal Glycyrrhiza species in china. *FEMS Microbiol. Ecol.* **79,** 46-68.

Li, Q., Guo, R., Li, Y., Hartman, W.H., Li, S., Zhang, Z., Tringe, S.G., & Wang, H. 2019. Insight into the bacterial endophytic communities of peach cultivars related to crown gall disease resistance. *App. Environ. Microbiol.* **85**

Loffler, F.E., Sun, Q., Li, J., & Tiedje, J.M. 2000. 16S rRNA Gene-Based Detection of Tetrachloroethene-Dechlorinating Desulfurmonas and Dehalococcoides Species. *Appl. Environ. Microbiol.* **66(4)**, 1369-1374.

Madhaiyan, M., Poonguzhali, S., Lee, J.S., Senthilkumar, M., Lee, K.C., & Sundaram, S. 2010. Leifsonia soli sp. nov., a yellow-pigmented actinobacterium isolated from teak rhizosphere soil. *Int. J. Syst. Evol. Microbiol.* **60,** 1322-1327

Maliki, R., Sinsin, B., Parrot, L., Lancon, J., Floquet, A., & Lutaladio, N. 2017. Econometric analysis of the determinants of adoption of yam-based cropping systems in rotation with the mixed intercropping Aeschynomene histrix and Maize in the guinea-sudan zone of benin. *Int. J. Agric. Innov. Res.* **6(2),** 2319-1473.

Marcin, C., Marcin, G., Justyna, M.P., Katarzyna, K., & Maria, N. 2013. Diversity of microorganisms from forest soils differently polluted with heavy metals. *Appl. Soil. Ecol.* **64,** 7-14.

Martins, M.B., Agostinetto, D., Fogliatto, S., Vidotto, F., & Andres, A. 2021. Aeschynomene spp. Identification and weed management in rice fields in southern brazil. *Agronomy.* **11,** 1-17.

Molouba, F., Lorquin, J., Willems, A., Hoste, B., Giraud, E., Dreyfus, B., Gillis, M., de Lajudie, P., & Masson-Boivin, C. 1999. Photosynthetic Bradyrhizobia from Aeschynomene spp. Are specific to stem-nodulated species and form a separate 16s ribosomal dna restriction fragment length polymorphism group. *Appl. Environ. Microbiol.* **65,** 3084-3094.

Nilanthi, D., Inoka, K.P.I., Madhushani, P.A., Dissanayaka, D.M.P., & Kumarasinghe, S. 2015. Effect of weeds Diya siyambala (Aeschynomene indica), Kudamatta (Fimbristylis miliacea) and Thunassa (Cyperus iria) on growth and yield of Rice (Oryza sativa l.) variety bg 379-2, ld 365, bg 357. *Int. J. Sci. Res. Publ.* **5,** 473-475.

Noisangiam, R., Teamtisong, K., Tittabutr, P., Boonkerd, N., Toshiki, U., Minamisawa, K., & Teaumroong, N. 2012. Genetic diversity, symbiotic evolution, and proposed infection process of Bradyrhizobium strains isolated from root nodules of Aeschynomene nod104tate104 l. in Thailand. *Evol. Genom. Microbiol.* **78**

Nouwen, N., Fardoux, J., & Giraud, E. 2016. NodD1 and NodD2 are not required for the symbiotic interaction of Bradyrhizobium ORS285 with Nod-Factor-independent Aeschynomene legumes. *PloS One.* 1-12.

Olanrewaju, O.S., & Babalola, O.O. 2019. Streptomyces: implications and interactions in plant growth promotion. *Appl. Microbiol. Biotechnol.* **103,** 1179-1188.

Palaniappan, P., Chauhan, P.S., Saravanan, V.S., Anandham, R., & Sa, T. 2010. Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodules of Lesedeza sp. *Biol. Fert. Soils.* **46,** 807-816.

Pandya, M., Rajput, M., & Rajkumar, S. 2015. Exploring plant growth promoting potential of non rhizobial root nodules endophytes of Vigna nod105tat. *Microbiol.* **84,** 80-89.

Padda, K.P., Puri, A., Zeng, Q., Chanway, C.P., & Wu, X. 2017. Effect of GFP-tagging on nitrogen fixation and plant growth promotion of an endophytic diazotrophic strain of Paenibacillus polymyxa. *Botany.* **95,** 933-942.

Paul, T., Halder, S.K., Das, A., Bera, S., Maity, C., Mandal, A., Das, P.S., Mohapatra, P.K.D., Pati, B.R., & Mondal, K.C. 2013. Exploitation of chicken feather waste as a plant growth promoting agent using keratinase producing novel isolate Paenibacillus woosongensis TKB2. *Biocatal. Agric. Biotechnol.* **2(1),** 50-57.

Pitman, W.D., Portier, K.M., Chambliss, C.G., & Kretschmer Jr., A.E. 1992. Performance of yearling steers grazing bahia grass pastures with summer annual legumes or nitrogen fertilizer in subtropical Florida. *Trop. Grassl.* **26,** 206-211.

Procopio, R.E.L., da Silva, I.R., Martins, M.K., de Azevedo, J.L., & de Araujo, J.M. 2012. Antibiotics produced by Streptomyces. *Braz. J. Infect. Dis.* **16(5),** 466-471.

Quinn, G.A., Banat, A.M., Abdelhameed, A.M., & Banat, I.M. 2020. Streptomyces from traditional medicine: sources of new innovations in antibiotic discovery. *J. Med. Microbiol.* **69(8),** 1040-1048.

Ridout, M.E., Schroeder, K.L., Hunter, S.S., Styer, J., & Newcombe, G. 2019. Priority effects of wheat seed endophytes on rhizosphere symbiosis. *Symbiosis.* **78,** 19-31.

Rios-Ruiz, W.F., Valdez-Nunez, R.A., Bedmar, E.J., & Castellano-Hinojosa, A. 2019. Utilization of endophytic bacteria isolated from legume root nodules for plant growth promotion. *Field Crops: Sustainable Management by PGPR.* 145-176.

Rudd, V. 1955. The American species of Aeschynomene. *Cont. US Natl. Herb.* **32,** 1-176.

Sadowsky, M.J., Tully, R.E., Cregan, P.B., Keyser, H.H. 1987. Genetic diversity in Bradyrhizobium japonicum serogroup 123 and its relation to genotype-specific nodulation of soybean. *Appl. Environ. Microbiol*. **53(11)**, 2624-2630.

Sasan, R.K., & Bidochka, M.J. 2012. The insect-pathogenic fungus Metarhizium robertsii (Clavicipitaceae) is also an endophyte that stimulates plant root development. *Botany.* **99(1),** 101-107.

Schrey, S.D., & Tarkka, M.T. 2008. Friends and foes: streptomycetes as modulators of plant disease and symbiosis. *Antonie van Leeuwenhoek*. **94,** 11-19.

Shah, A.A., Liu, Z., Qian, C., Wu, J., Zhong, X., & Kalsoom, U. 2019. Effect of endophytic Bacillus megaterium colonization of structure strengthening, microbial community, chemical composition and stabilization properties of hybrid pennisetum. *J. Sci. Food. Agric.* **100(3),** 1164-1173.

Shen, C., Xiong, J., Zhang, H., Feng, Y., Lin, X., Li, X., Liang, W., Chu, H. 2013. Soil pH drives the spatial distribution of bacterial communities along elevation on changbai mountains. *Soil. Biol. Biochem.* **57,** 204-211.

Singh, B.K., Millard, P., Whiteley, A.S., & Murrell, J.C. 2004. Unravelling rhizosphere-microbial interactions: opportunities and limitations. *Trends Microbiol.* **12,** 386-393.

Sturz, A.V., Christie, B.R., Matheson, B.G., & Nowak, J. 1997. Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biol. Fert. Soils.* **25,** 13-19.

Subramanian, P., Kim, K., Krishnamoorthy, R., Sundaram, S., & Sa, T. 2015. Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with Bradyrhizobium japonicum MN110. *Plant Growth Regul.* **76,** 327-332.

Tariq, M., Hameed, S., Yasmeen, T., Zahid, M., & Zafar, M. 2013. Molecular characterization and identification of plant growth promoting endophytic bacteria isolated from the root nodules of pea (Pisum sativum l.). *World J. Microbiol. Biotechnol.* **30,** 719-725.

Tripathi, B.M., Stegen, J.C., Kim, M., Dong, K., Adams, J.M., Lee, Y.K. 2018. Soil pH mediates the balance between stochastic and deterministic assembly of bacteria. *ISME J.* **12,** 1072-1083.

Valverde, A., Fterich, A., Mahdhi, M., Ramirez-Bahena, M.H., Caviedes, M.A., Mars, M., Velazquez, E., & Rodriguez-Llorente, I.D. 2010. Paenibacillus prosopidis sp. nov., isolated from the nodules of Prosopis farcta. *Int. J. Syst. Evol. Microbiol.* **60,** 2182-2186.

Vincent, J.M. 1970. A manual for the practical study of root nodule bacteria. *IBP Handbook N°15*. Blackwell, Oxford.

Walitang, D.I., Kim, C.G., Jeon, S., Kang, Y., & Sa, T. 2018. Conservation and transmission of seed bacterial endophytes across generations following crossbreeding and repeated inbreeding of rice at different geographic locations. *Microbiologyopen.* **8,** 1-13.

Wang, H., Hu, D., Wang, Z., Yang, C., Zhu, S., Gu, C., & Wang, E.T. 2021. Paenibacillus glycinis sp. nov., an endophytic bacterium isolated from the nodules of soybean (Glycine max (l.) merr). *Curr. Microbiol.* **78,** 1678-1685.

Watve, M.G., Tickoo, R., Jog, M.M., & Bhole, B.D. 2001. Homany antibiotics are produced by the genus Streptomyces? *Arch. Microbiol.* **176,** 386-390.

Westover, K.M., Kennedy, A.C., & Kelley, S.E. 1997. Patterns of rhizosphere microbial community structure associated with co-occuring plant species. *J. Ecol.* **85,** 863-873.

White Jr., J.F. 2018. Endophyte-host associations in forage grasses. XI. A proposal concerning origin and evolution. *Mycologia*. **80,** 442-446.

Wilson, R.J., Gutierrez, D., Gutierrez, J., Martinez, D., Agudo, R., & Monserrat, V.J. 2005. Changes to the elevational limits and extent of species ranges associated with climate change. *Ecol. Lett.* **8,** 1138-1146.

Xu, L., Zhang, Y., Wang, L., Chen, W., & Wei, G. 2014. Diversity of endophytic bacteria associated with nodules of two indigenous legumes at different altitudes of the qilian mountains in china. *Syst. Appl. Microbiol.* **37,** 457-465.

Yapi, Y.M., & Kimse, M. 2019. Association of Aeschynomene histrix with Andropogon gayanus or Pancium maximum: a comparative study of pasture quality. *Am. J. Agric. For.* **7(2),** 36-43.

Zhang, B., Du, N., Li, Y., Shi, P., & Wei, G. 2018. Distinct biogeographic patterns of rhizobia and non-rhizobial endophytes associated with soybean nodules across china. *Sci. Total Environ.* **643,** 569-578.

Zhao, L., Xu, Y., Lair, X.H., Shan, C., Deng, Z., & Ji, Y. 2015. Screening and characterization of endophytic Bacillus and Paenibacillus strains for medicinal plant Lonicera japonica for use as potential plant growth promoters. *Braz. J. Microbiol.* **46,** 977-989.

Zhao, L.F., Xu, Y.J., & Lai, X.H. 2018. Antagonistic endophytic bacteria associated with nodules of soybean (Glycine max l.) and plant growth-promoting properties. *Braz. J. Microbiol.* **49,** 269-278.

CHAPTER 3

IDENFICATION OF A NOVEL NOD FACTOR-INDEPENDENT NODULE PROMOTING *LEIFSONIA* SPECIES AND ITS WHOLE GENOME SEQUENCING

ABSTRACT

The nodule organs of leguminous plants house a diverse endophytic community that is not only limited to nitrogen fixing rhizobacteria. Just like all other plant tissues, plant growth promoting bacteria can be isolated from nodule tissues and have been shown to confer beneficial traits to the host plant involving growth and general health. These endophytic communities are composed of locally obtained soil dwelling bacteria that are specifically adapted to survival in their respective environmental conditions. Such endophytes have been isolated and demonstrate metabolic capabilities unique to their original environment and can confer those abilities to aid in host plant nutrient acquisition, growth, and survival. A nod factor (NF)-independent nodule promoting *Leifsonia* isolate (TSN2) was obtained from stem nodules of a Texas native *Aeschynomene indica* and its whole genome was sequenced and analyzed for plant growth promotion related genes. It was found that this isolate possesses numerous genes involved in heavy metal remediation (such as cadmium, cobalt, copper, & nickel) and drought tolerance. Given that these plants were located on the side of a Texas highway, the presence of heavy metal remediation and drought tolerance genes can be attributed to this organism's efficient adaptation to these harsh environmental conditions. Additionally, the presence of typical root colonization genes used by endophytic and rhizospheric bacteria involved in motility and chemotaxis suggest

a specialized evolutionary path leading this soil dwelling bacteria to become a mutualistic plant endosymbiont. The average nucleotide identity results obtained for this isolate find no taxonomic match greater than 84% (*L. aquatica*). Given the standard cutoff of 95% ANI, we propose this Texas native stem nodule isolate be taxonomically placed into its own species under the genus *Leifsonia*. Additionally, we propose the use of this organism as a NF-independent co-inoculant for agricultural purposes to aid in metal remediation, nutrient cycling, and drought tolerance of *A. indica* plants currently used as greenmanure through crop rotation.

INTRODUCTION

Endophytic bacteria can inhabit every type of plant tissue and often confer beneficial traits to their host plant such as phytohormone production, disease resistance, abiotic stress tolerance, and increases to crop yields. Many such plant growth promoting bacteria have been isolated and characterized from legume nodules as well, other than the typical nitrogen fixing endosymbionts *Rhizobium* and *Bradyrhizobium*. Even stem nodules have been found to harbor diverse endophytic communities with these specialized organs. The environmental conditions in which a plant is grown play a significant role in endophyte populations in large part due to the pressures imposed on the surrounding soil bacteria. There have been studies demonstrating the biogeographic impacts on the endophytic diversity due to such differences in soil condition, temperature, moisture, and heavy metal pollutants. Members of the bacterial genus *Leifsonia* have been isolated from environments where industrial pollutions have impacted the pH and metal content of surrounding soils (Egidi et al., 2017; Rodriguez-Rodriguez et al., 2021; Tan et al., 2020). These bacteria have been found in such environments, living in tandem with host plants and aiding them in growth and survival by heavy metal remediation in both the rhizosphere and within the plant as endophytes (Jiang et al., 2022; Kang et al., 2017; Madhaiyan

et al., 2010). Some strains of this bacterial genus have been demonstrated to combat host plant infection and disease through antibiotic production while others, such as *Leifsonia xyli* are notorious sugar cane pathogens, causing ratoon stunting disease (Assad et al., 2021; Brumbley et al., 2006; Faria et al., 2020; Li et al., 2019). Despite this, *Leifsonia* species and strains have been found to produce plant growth hormones such as gibberellin and indole-3-acetic acid (IAA), known for controlling cell division and elongation as well as aiding in abiotic stress mitigation (Kang et al., 2017; Khang et al, 2014; Nordstedt et al., 2021). These endophytic bacteria have been isolated from a variety of host plants including, but not limited to, grasses, legumes, and even tubers such as potatoes (Battu & Ulaganathan, 2020; Mills et al., 2001; Liu et al., 2020). In addition to this, *Leifsonia* sp. have also been isolated from legume root nodules, particularly from forage legumes such as mung beans (*Vigna radiata)*, blue peas (*Clitoria ternatea)*, and pencilflowers (*Stylosanthes biflora*) (Favero et al., 2021; Nunes et al., 2018). However, there are no previous studies reporting the isolation and characterization of a *Leifsonia* species from stem nodules, much less those of *Aeschynomene indica*, and their role in nodule formation. The results obtained in the chapter prior to this section inspired the whole-genome sequencing and analysis of the *Leifsonia* Texas native stem nodule isolate, TSN2. This isolate was tentatively identified through 16S rRNA sequencing as a *Leifsonia shinshuensis* strain. We inoculated Texas *A. indica* plants with this unique isolate and noted a clear increase in total nodule dry and pigmented nodule formation, despite a lack of *nodD2-3* regions determined by target PCR. This gene is responsible for the detection of the plant released flavonoids as well as the transcription of the *nodABC* genes which produce the base of the nod factor (NF) signaling molecule, specific to NF-dependent nodulation. Based on these initial experiments, we set out to sequence the entire genome of this organism to determine if any known nodulation, nitrogen fixation, or other

beneficial symbiotic genes were present in this *Leifsonia* sp. genome. Additionally, we wanted to provide this genome as a reference for use in future studies, particularly those concerning gene expression analysis of this organism as it interacts with its host plant during the initial phases of root colonization and nodule formation to better understand NF-independent nodulation. We hope to further characterize this isolate, as well as many others, to optimize multi-inoculum systems as forms of biofertilizers for crop protection and production enhancement.

MATERIALS AND METHODS

Bacterial strain and growth conditions. TSN2, once isolated from Texas *Aeschynomene indica* stem nodules, was cultured in YEM media (pH 6.8) consisting of 0.5g K_2HPO_4, 0.2g $MgSO_4 \cdot 7H_2O$, 0.1g NaCl, 10g mannitol, 0.5g yeast extract, with 0.002% actidione (cycloheximide) to inhibit fungal growth (Vincent, 1970) and 2.5mL (1% aqueous) Congo Red per liter were cultured in arabinose-gluconate (AG) medium normalized to pH 6.8 containing 125mg Na_2HPO_4, 250mg Na_2SO_4, 320mg NH_4Cl, 180mg $MgSO_4 \cdot 7H_2O$, 10mg $CaCl_2$, 4mg $FeCl_3$, 1.3g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1g 2-(N-morpholino) ethanesulfonic acid (MES), 1g yeast extract, 1g L-arabinose, and 1g D-gluconic acid sodium sulfate per L of distilled water (Sadowsky et al., 1987). For both media types, 15g agar were added per L to create solid media plates. TSN2, when grown in liquid media, was incubated aerobically at 30°C while shaking at 200rpm.

DNA isolation and whole genome sequencing of TSN2. TSN2 was cultured overnight in liquid AG media at 30°C before genomic DNA was extracted using the Thermo Fisher ScientificTM genomic DNA extraction kit. Purified gDNA was then quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher ScientificTM, Wilmington, DE). Whole

genome sequencing was completed by SeqCenter, LLC (Pittsburgh, PA) using a combined assembly system which incorporated Nanopore sequencing yielding 600Mbp long reads in addition to 1Gbp Illumina reads to form a single assembled and annotated genome. The Nanopore samples used for library sequencing were prepared using an Oxford Nanopore Technologies (ONT) Native Barcoding Kit 24 V14 (SQK-NBD114.24). Samples were run on Nanopore R10.4.1 flow cells on a MinION, Mk1B, or GridION according to SeqCenter's standard operation procedure. For Illumina sequencing, samples were prepared using an Illumina DNA Prep kit and IDT 10bp UDI indices. These were sequenced on an Illumina NovaSeq 6000, yielding 2x151bp reads, according to SeqCenter's operational procedure.

Read quality filtering, assembly, and annotation. Quality control and adapter trimming was performed by SeqCenter using their combined methods of bcl-convert version 4.0.3 developed by Illumina (https://support-docs.illumina.com/SW/BCL_Convert/Content/SW/FrontPages/BCL_Convert.htm) and porechop version 0.2.3_seqan2.1.1 (https://github.com/rrwick/Porechop). Both Illumina and Nanopore reads were assembled and ONT reads performed with Unicylcer version 0.4.8 (Wick et al., 2017). Assembly statistics were observed using QUAST version 5.0.2 (Gurevich et al., 2013). Annotation of the assembly was completed using Prokka version 1.14.5 (Seeman, 2014). Annotated genome, GC content (version 1.0.1), and GC skew (version 1.0.1) were visualized using Proksee (https://proksee.ca/) to generate high-quality navigable maps of the whole annotated genome.

Genome analysis and comparative genomics. The whole genome and the 16S rRNA assemblies were characterized using Protologger version 0.99 (www.protologger.de) to generate average nucleotide identity (ANI), GC percentage, number of coding sequence, functional

analysis, number of CAZymes, glcoside hydrolase families, glycoside transferase families, polysaccharise lyase families, carbohydrate esterase families, carbonhydrate-binding molecule familes, ecological analysis, pairwise percentage of conserved proteins (POCP), 16S rRNA phylogenetic tree, and genomic phylogenetic tree (Hitch et al., 2021). Whole genome sequence alignment of TSN2, *Leifsonia shinuensis* INR9 (Accession CP043641.1) and *L. aquatica* DSM 20146 (Accession JACHVP000000000.1) using the pairwise DNA sequence aligner LASTZ (version 1.04.15), samtools (version 1.14), r-base (version 3.6.3), and bzip2 (version 1.0.8) from the bio.tools: lastz package (https://github.com/lastz/lastz). These alignments were used as links for the syntenic analysis between all three genomes using the Circos plotting tool version 0.69.8 (Kryzwinski et al., 2009; Rasche & Hiltemann, 2020).

RESULTS AND DISCUSSION

Whole genome sequence and assembly quality check. TSN2 whole genome sequencing was completed using a hybrid Nanopore and Illumina technique practiced at SeqCenter (https://www.seqcenter.com/). The Nanopore sequencing generated a total of 147,382 reads with a total of 366,278,126 bps which had an average read length of 2,485.2 bp and a total Q15 score of 89.2%. The Illumina NovaSeq 6000 generated a total of 10,414,162 read pairs (2x151 bp) for a total of 20,828,324 reads, 3,079,049,125 bps and a Q30 score of 93%. The combined assembly of the hybrid sequencing technique produced a total of 2 contigs with lengths of 4,156,614 bp and 66,724 bp respectively for a total genome length of 4.22 Mbp and an N50 value of 4,156,614 (Table 3-1).

Genome annotation and functional analysis. Annotation through Prokka and Protologger revealed TSN2 to have a total genome size of 4.22 Mbp with a genome completion

of 99.49% and genome contamination of 0.76% (Table 3-2) and GC content of 69.51%. Additionally, a total of 4081 coding sequences were identified with 152 transporters, 19 secretion genes, 687 unique enzymes, and 348 CAZymes. The annotated genome was also visualized using Proksee, as seen in Figure 3-1, where the outer most markings indicate all CDS of predicted and unpredicted functions followed by all predicted RNA types as colored arrows and the outer most ring displaying the location of these CDS with predicted function in dark blue, and unpredicted function in light blue. The following inner ring in dark gray represents the GC% content for each specific CDS region followed by the GC skews in red and green. Additionally, Protologger was used to determine the occurrence of carbohydrate-active enzyme families within the TSN2 genome as reported (but not exclusive) in Table 3-3. A total of 10 glycoside hydrolase families with ≥5 occurrences were reported with the GH1 and GH13 being the most predominant. A total of 5 glycosyltranserase and 4 carbohydrate binding module families with ≥5 occurrences were reported. Within the TSN2 genome, we noted 106 potential plant growth promoting or otherwise symbiotically important genes or copies of genes with 12 relating to phytohormone product, 21 for heavy metal remediation, 21 that aid in salinity and drought tolerance, 17 involved in potassium and phosphate uptake, 19 involved in cytochrome synthesis and metabolism, and 16 responsible for chemotactic response and motility (Table 3-4).

Comparative genomics of TSN2, *Leifsonia shinshuensis* INR9, and *L. aquatica* DSM 20146. The 16S rRNA sequencing results were analyzed using sequence alignment tools such as NCBI BLASTn and Protologger with the largest % sequence similarities being for *L. shinshuensis* and *L. soli* at 99.2% and 98.9% respectively (Table 3-5). Additionally, these same results are reflected in the phylogenetic tree distribution for the 16S rRNA sequence alignments with bootstrap confidence values of 0.944 for the *L. shinshuensis* branch and 0.867 for the *L. soli*

branch (Figure 3-2). However, the whole genome average nucleotide identity (ANI) reported suggested *L. aquatica* as the closest relative to TSN2 with an 83% ANI value (Table 3-6). Using these combined results, a circular syntenic plot comparing the genomes of TSN2, *L. shinshuensis*, and *L. aquatica* was generated with blue ribbons indicating conserved regions between TSN2 vs *L. aquatica*, green ribbons for TSN2 vs *L. shinshuensis*, black ticks representing the genomic location of annotated CDS in multiple layers to avoid overlap and enhance readability, and colored arcs for each contig of a particular genome (Figure 3-3). A phylogenetic tree was also constructed for the whole genome and demonstrated a close relation between TSN2 and *L. aquatica* (Figure 3-4). The comparison of potential genes and copies of genes relating to plant growth promotion and symbiosis between the three genomes revealed 12 in TSN2, 13 in *L. shinshuensis*, and 17 in *L. aquatica* regarding phytohormone synthesis (Table 3-7). In relation to cytochrome regulation, 19 in TSN2, 19 in *L. shinshuensis*, and 21 in *L. aquatica* genes or copies of genes were identified (Table 3-8). A total of 21 in TSN2, 34 in *L. shinshuensis*, and 38 in *L. aquatica* genes or copies of genes regarding metal sequestration were identified (Table 3-9). For genes or copies of genes relating to salt and drought tolerance through maltose or trehalose production, a total of 21 in TSN2, 19 in *L. shinshuensis*, and 17 in *L. aquatica* were identified (Table 3-10). Regarding genes or copies of genes involved in phosphate or potassium acquisition, a total of 17 in TSN2, 21 in *L. shinshuensis*, and 17 in *L. aquatica* were identified (Table 3-11). For potential symbiotically important genes relating to motility and chemotaxis, a total of 16 in TSN2, 16 in *L. shinshuensis*, and 14 in *L. aquatica* were identified (Table 3-12). Of all the functional categories examined, the genome of TSN2 held more emphasis on salt and drought mitigation in addition to motility and chemotaxis. Additionally, TSN2 has sacrificed, or lost through evolutionary processeses, genes relating to heavy metal

remediation, phytohormone production, nutrient uptake, and cytochrome regulation in favor of a large, unidentified genetic island between 0.2 and 0.3 Mbp long not found in these two closely related *Leifsonia* strains. While the content of this genetic island has not yet been determined due to time constraints, it is postulated that it may contain genes involved in the NF independent nodulation stimulation exhibited by TSN2 particularly regarding the host plant *Aeschynomene indica*.

Molecular confirmation of NF independence. In the previous chapter, TSN2 demonstrated nodule formation stimulation independently of canonical Nod Factors. We observed a lack of the *nodD* and *nodAB* genes through PCR on TSN2 colonies and gDNA. However, upon annotation and analysis of the TSN2 genome, the presence of *nodD2* was detected. Despite this, no other *nod* or *nol* genes relating to NF synthesis were observed in the genome, supporting the claim that this isolate is inducing or supporting nodulation in a NF-independent manner. The presence of *nodD2* alone does not demonstrate a dependency on the NF signaling mechanism for the induction of this symbiotic relationship as the product of this gene has been shown to not only detect plant flavonoids and induce *nodABC* transcription, but also induces transcription of other, non-NF related symbiotic genes (Nouwen et al., 2016). Additionally, our previous experiment showed a distinct lack of enhanced nitrogenase activity upon inoculation of TSN2 to *A. indica* plants. After further investigation of the whole TSN2 genome, no *nif* genes or other nitrogen fixation genes were observed, supporting these results we obtained. TSN2 alone can enhance nodulation independent of NFs but does not play a significant role in nitrogen fixation individually.

Plant growth promotion potential via phytohormone production. After annotation of the TSN2 genome, numerous plant growth promoting genes were documented with a notable

amount being responsible for heavy metal remediation, salinity and drought tolerance, cytochrome, and phytohormone synthesis (Table 3-4). A few previous studies have noted the biosynthesis of plant growth hormone by *Leifsonia* strains living endophytically (Kang et al., 2014; Nordstedt et al., 2021). Evidence of such capabilities were also observed in this study with the presence of genes involved in tryptophan synthesis and modification as well as hormone precursor production (*kynA* and *trpABCGS*), crucial to Indole-3-acetic acid (IAA) plant hormone production and regulation (Donoso et al., 2017; Simm et al., 2016; Zhao et al., 2002). We also observed genes involved in synthesis of cytokinins (*idi*), gibberellic acid (*cpsY*), and auxin (*sdrM*), phytohormones that play major roles in stress mitigation, cell division, cell elongation, and plant growth in general (Hirano et al., 2018; del Orozco-Mosqueda et alk., 2023; Yang et al., 2020). Although roughly 12 genes or copies of genes have been dedicated to the synthesis and regulation of these phytohormones combined, TSN2 had a total of 19 genes or copies of genes responsible for cytochrome management alone, one of the most important proteins involved in plant stress response and mitigation (Jun et al., 2015; Minerdi et al., 2023; Pandian et al., 2020).

Heavy metal remediation capabilities. Following the same trend seen in literature, 21 genes or copies of genes relating to heavy metal remediation were also observed in the TSN2 genome (Table 3-4). Genes regarding transport or utilization of cadmium and cobalt (*czcD, cadA,* & *corA*), copper (*cutC*, *csoR, copAB*), arsenic (*arsBC*), nickel (*nikB*), mercury (*merA*), and zinc (*ftsH, rip1, zitB, znuB, zupT,* & *zur*), all of which are among the most common heavy metal environmental pollutants particularly originating from high traffic roadways (Diels et al., 1995; Hao et al., 2021; Henao & Ghneim-Herrera, 2021; Liu et al., 2021; Pande et al., 2022; Salam, 2020).

Abiotic stress mitigation potential. Previous studies have attributed a conference of salt and drought tolerance to plants inoculated with *Leifsonia* strains yet the genetic factors involved have not yet been identified. The annotation results of TSN2's genome revealed 11 genes or copies of genes involved in maltose synthesis, modification, and metabolism (*malILT, glgE1,* & *glgM*) which has been shown to play a significant role in salt, cold, and drought tolerance in plants (Table 3-4) (Kaplan & Guy, 2004 & 2005; Kaplan et al., 2004; Patel et al., 2020; Purdy et al., 2013; Thalmann & Santelia, 2017). Another important sugar involved in plant abiotic stress, particularly heat and salt stress, tolerance is trehalose for which 10 related genes or copies of genes (*otsAB, sugAB*, *treS, & treYZ*) were identified in the TSN2 genome (Garg et al., 2002; Iordachescu & Imai, 2008; Kosar et al., 2019; Sharma et al., 2020; Yang et al., 2022). The identification of these and other endophytic symbiosis related genes in the TSN2 genome coupled with the observed nodule promotion suggest great potential of this organism for incorporation as a co-inoculant in agricultural practices seeking increased nutrient acquisition and abiotic stress tolerance.

ANI evidence of novel *Leifsonia* species. While the 16S rRNA sequencing results of TSN2 indicate a high similarity to *L. shinshuensis* (>95%) (Table 3-5), the pairwise comparison and average nucleotide identity (ANI) of this isolate suggest the taxonomic placement as a novel *Leifsonia* species, matching no previously documented bacterial genomes more than 84% (Table 3-6). The current standard threshold ANI value of 2 whole genome sequence alignments is 96% to define a new operational species, which is well above the ANI of TSN2 reported here (Ciufo et al., 2018). With all the reported data provided in this study, we propose the identification of the strain TSN2 isolated from stem nodules of Texas native *Aeschynomene indica* plants to be a novel member of the *Leifsonia* genus with potential applications as a plant growth promoting

biofertilizer. This Texas native stem nodule isolate could be co-inoculated with other, known nitrogen fixing endophytic bacteria to promote nutrient cycling and plant growth particularly in environments like Texas roadsides that experience drought and heavy metal contamination. Such an inoculant would be especially useful for the cultivation of crops in land along the high traffic highways found throughout Texas.

Table 3-1. Genome assembly metrics obtained for whole genome sequence of TSN2 as reported by QUAST.

	TSN2 Assembly
# contigs (>= 0 bp)	2
# contigs (>= 1000 bp)	2
# contigs (>= 5000 bp)	2
# contigs (>= 10000 bp)	2
# contigs (>= 25000 bp)	2
# contigs (>= 50000 bp)	2
Total length (>= 0 bp)	4223338
Total length (>= 1000 bp)	4223338
Total length (>= 5000 bp)	4223338
Total length (>= 10000 bp)	4223338
Total length (>= 25000 bp)	4223338
Total length (>= 50000 bp)	4223338
# contigs	2
Largest contig	4156614
Total length	4223338
GC (%)	69.51
N50	4156614
N75	4156614
L50	1
L75	1

Statics for GC% calculated for content of entire assembly.
N50 and N75 values represent the length for which all contigs of that length or longer cover at least 50% and 75% the assembly.
L50 and L75 values represent the number of contigs equal to or longer than N50 or N75 respectively.

Table 3-2. Genomic assembly and annotation statistics for TSN2 as reported by Protologger.

	TSN2
Genome Size (bp)	4,223,338
Genome Completeness (%)	99.49
Genome Contamination (%)	0.76
GC %	69.51
Coding Sequences	4081
# of Transporters	152
# of Secretion Genes	19
# of Unique Enzymes	687
# of CAZymes	348

Table 3-3. Carbohydrate-active enzyme families with an occurrence greater than or equal to 5 observed in the whole genome of TSN2 compared to CAZyme database.

Glycoside Hydrolase (GH)	Occurrence	Glycosyltransferase (GT)	Occurrence	Carbohydrate-Binding Module (CMB)	Occurrence
GH0	6	GT0	7	CBM2	7
GH1	27	GT1	20	CBM5	10
GH3	8	GT2	40	CBM48	9
GH6	5	GT4	33	CBM50	13
GH13	21	GT51	6		
GH16	8				
GH23	5				
GH32	5				
GH105	5				
GH130	10				

Table 3-4. List of plant growth promoting endophytic genes observed in the TSN2 genome.

Symbiotic Role	Gene	Symbiotic Role	Gene
Phytohormone Synthesis	*cpsY*	Phosphate Uptake	*pstB1*
	cypB		*pstC2*
	idi		*pstP*
	kynA		*pstS2*
	sdrM	Potassium Uptake	*chaA*
	trpABCDEGS		*kdpABCD*
Heavy Metal Sequestration	*cadA*		*kefB*
	corA		*kimA*
	czcD		*ktrAB*
	copAB		*kup*
	csoR		*nhaK*
	cutC		*nhaP2*
	arsBC	Motility & Chemotaxis	*flgCEL*
	merA		*flhAB*
	nikB		*fliDEFGMRWY*
	ftsH		*motB*
	rip1		*pomA*
	zitB	Cytochrome Regulation	*appC*
	znuB		*ccs1*
	zosA		*ccsA*
	zupT		*ctaABCDE*
	zur		*cydABD*
Salinity & Drought Tolerance	*glgE1*		*petBC*
	glgM		*qcrABC*
	malI		
	malLT		
	otsAB		
	sugAB		
	treSYZ		

Table 3-5. The 16S rRNA nucleotide sequence similarities to closest 10 matches for TSN2.

Matching species	Similarity (%)
Leifsonia shinshuensis--Microbacteriaceae--DQ232614 (Valid)	99.23077
Leifsonia soli--Microbacteriaceae--EU912483 (Valid)	98.95678
Leifsonia lichenia--Microbacteriaceae--AB278552 (Valid)	98.74126
Leifsonia xyli--Microbacteriaceae--AB016985 (Valid)	98.73773
Leifsonia naganoensis--Microbacteriaceae--DQ232612 (Valid)	98.04196
Leifsonia poae--Microbacteriaceae--AF116342 (Valid)	97.9021
Leifsonia aquatica--Microbacteriaceae--D45057 (Valid)	97.13287
Humibacter albus--Microbacteriaceae--AM494541 (Valid)	96.71329
Homoserinibacter gongjuensis--Microbacteriaceae--JQ639055 (Valid)	96.71099
Diaminobutyricibacter tongyongensis--Microbacteriaceae--JX876865 (Valid)	96.42857

Table 3-6. Average nucleotide identity % of TSN2 compared to the closest matching relatives.

Matching species	Similarity (%)
Leifsonia_aquatica--GCF_000633535.1	83.1244
Leifsonia_xyli--GCF_000007665.1	81.3897
Microterricola_viridarii--GCF_900104895.1	79.4599
Agromyces_cerinus--GCF_900142065.1	79.2527
Herbiconiux_solani--GCF_001571005.1	79.2507
Diaminobutyricimonas_aerilata--GCF_002797715.1	78.9296
Herbiconiux_ginsengi--GCF_900107435.1	78.9141
Cnuibacter_physcomitrellae--GCF_002096055.1	78.7862
Humibacter_albus--GCF_000421825.1	78.7803
Curtobacterium_citreum--GCF_001475775.1	78.4969
Curtobacterium_luteum--GCF_001475545.1	78.2751
Agreia_bicolorata--GCF_000938265.1	78.2588
Agreia_pratensis--GCF_900177685.1	78.1203
Agrococcus_casei--GCF_900163565.1	77.7808

Table 3-7. Genes and copies of genes concerning pytohormone synthesis in the genomes of TSN2, *Leifsonia shinshuensis* INR9, and *Leifsonia aquatica* DSM 20146.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
cpsY	Exopolysaccharide phosphotransferase CpsY	OBKDJCNG_02622	-	MEBNKEKO_02431
cypB	Peptidyl-prolyl cis-trans isomerase B	OBKDJCNG_02214	LEDPJBKC_02891	MEBNKEKO_02288
cypC	Fatty-acid peroxygenase	-	-	MEBNKEKO_02735
cypM	Cypemycin N-terminal methyltransferase	-	-	MEBNKEKO_03660
idi	Isopentenyl-diphosphate Delta-isomerase	OBKDJCNG_01893	LEDPJBKC_03193	MEBNKEKO_03630
kynA	Tryptophan 2%2C3-dioxygenase	OBKDJCNG_01791	LEDPJBKC_03326	MEBNKEKO_03494
kynB	Kynurenine formamidase	-	LEDPJBKC_03427	MEBNKEKO_00222
kynU	Kynureninase	-	LEDPJBKC_03324	MEBNKEKO_03496
sdrM	Multidrug efflux pump SdrM	OBKDJCNG_03181	LEDPJBKC_01814	MEBNKEKO_03157
trpA	Tryptophan synthase alpha chain	OBKDJCNG_00453	LEDPJBKC_00483	MEBNKEKO_01359
trpB	Tryptophan synthase beta chain	OBKDJCNG_00454	LEDPJBKC_00482	MEBNKEKO_01360
trpC	Indole-3-glycerol phosphate synthase	OBKDJCNG_00455	LEDPJBKC_00481	MEBNKEKO_01361
trpD	Anthranilate phosphoribosyltransferase	OBKDJCNG_00615	LEDPJBKC_00304	MEBNKEKO_01505
trpE	Anthranilate synthase component 1	OBKDJCNG_00458	LEDPJBKC_00478	MEBNKEKO_01107
trpF	N-(5'-phosphoribosyl)anthranilate isomerase	-	-	MEBNKEKO_02649
trpG	Anthranilate synthase component 2	OBKDJCNG_02219	LEDPJBKC_02886	MEBNKEKO_02283
trpS	Tryptophan--tRNA ligase	OBKDJCNG_03311	LEDPJBKC_01672	MEBNKEKO_00128

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-8. Genes and copies of genes concerning cytochrome synthesis in the genomes of TSN2, *Leifsonia shinshuensis* INR9, and *Leifsonia aquatica* DSM 20146.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
appA	Oligopeptide-binding protein AppA	-	LEDPJBKC_02460	-
appC	Cytochrome bd-II ubiquinol oxidase subunit 1	OBKDJCNG_00313	LEDPJBKC_00622	-
ccs1_1	Cytochrome c biogenesis protein Ccs1	OBKDJCNG_02361	LEDPJBKC_02735	MEBNKEKO_02130
ccs1_2	Cytochrome c biogenesis protein Ccs1	-	-	MEBNKEKO_04043
ccsA_1	Cytochrome c biogenesis protein CcsA	OBKDJCNG_02362	LEDPJBKC_02574	MEBNKEKO_02129
ccsA_2	Cytochrome c biogenesis protein CcsA	-	LEDPJBKC_02734	MEBNKEKO_04042
cssA_3	Cytochrome c biogenesis protein CcsA	-	-	MEBNKEKO_04246
ctaA	Heme A synthase	OBKDJCNG_00390	LEDPJBKC_00541	MEBNKEKO_01305
ctaB	Protoheme IX farnesyltransferase	OBKDJCNG_00392	LEDPJBKC_00539	MEBNKEKO_01307
ctaC	Cytochrome c oxidase subunit 2	OBKDJCNG_00623	LEDPJBKC_00295	MEBNKEKO_01515
ctaD_1	putative cytochrome c oxidase subunit 1	OBKDJCNG_00622	LEDPJBKC_00296	MEBNKEKO_01514
ctaD_2	putative cytochrome c oxidase subunit 1	-	-	MEBNKEKO_02855
ctaE	Cytochrome c oxidase subunit 3	OBKDJCNG_00616	LEDPJBKC_00303	MEBNKEKO_01506
ctaF	Cytochrome c oxidase polypeptide 4	-	LEDPJBKC_00297	MEBNKEKO_01513
cydA	Cytochrome bd-I ubiquinol oxidase subunit 1	OBKDJCNG_01034	-	MEBNKEKO_01102
cydB_1	Cytochrome bd-I ubiquinol oxidase subunit 2	OBKDJCNG_00312	LEDPJBKC_00623	MEBNKEKO_01103
cydB_2	Cytochrome bd-I ubiquinol oxidase subunit 2	OBKDJCNG_01035	-	-
cydD	ATP-binding/permease protein CydD	OBKDJCNG_00311	LEDPJBKC_00624	MEBNKEKO_01104

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-8. Continued.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
petB	Cytochrome bc complex cytochrome b subunit	OBKDJCNG_01404	LEDPJBKC_03782	MEBNKEKO_02675
petC_1	Cytochrome b6-f complex iron-sulfur subunit	OBKDJCNG_00110	LEDPJBKC_00849	MEBNKEKO_00968
petC_2	Cytochrome b6-f complex iron-sulfur subunit	OBKDJCNG_02066	-	MEBNKEKO_02459
qcrA	Cytochrome bc1 complex Rieske iron-sulfur subunit	OBKDJCNG_00618	LEDPJBKC_00301	MEBNKEKO_01508
qcrB_1	Cytochrome bc1 complex cytochrome b subunit	OBKDJCNG_00619	LEDPJBKC_00300	MEBNKEKO_01509
qcrB_2	Cytochrome bc1 complex cytochrome b subunit	OBKDJCNG_01979	LEDPJBKC_03151	-
qcrC	Cytochrome bc1 complex cytochrome c subunit	OBKDJCNG_00617	LEDPJBKC_00302	MEBNKEKO_01507

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-9. Genes and copies of genes concerning heavy metal sequestration in the genomes of TSN2, *Leifsonia shinshuensis* INR9, and *Leifsonia aquatica* DSM 20146.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
arsB	Arsenical pump membrane protein	OBKDJCNG_03997	LEDPJBKC_01048	MEBNKEKO_00770
arsC_1	Arsenate reductase	OBKDJCNG_03443	LEDPJBKC_01558	MEBNKEKO_01990
arsC_2	Arsenate reductase	-	LEDPJBKC_02589	MEBNKEKO_01991
arsC_3	Arsenate reductase	-	LEDPJBKC_02590	MEBNKEKO_01992
arsC_4	Arsenate reductase	-	LEDPJBKC_02591	MEBNKEKO_01996
arsC_5	Arsenate reductase	-	LEDPJBKC_02597	MEBNKEKO_02383
arsC1	Arsenate-mycothiol transferase ArsC1	-	-	MEBNKEKO_01999
arsC2	Arsenate-mycothiol transferase ArsC2	-	LEDPJBKC_02600	-
cadA	Cadmium%2C zinc and cobalt-transporting ATPase	OBKDJCNG_01773	-	-
corA	Cobalt/magnesium transport protein CorA	OBKDJCNG_03321	LEDPJBKC_01660	MEBNKEKO_00141
czcD	Cadmium%2C cobalt and zinc/H(+)-K(+) antiporter	OBKDJCNG_03923	LEDPJBKC_01120	MEBNKEKO_00729
actP	Copper-transporting P-type ATPase	-	-	MEBNKEKO_00592
copA	Copper-exporting P-type ATPase	OBKDJCNG_00982	-	MEBNKEKO_00593
copB	Copper-exporting P-type ATPase B	OBKDJCNG_00983	LEDPJBKC_02608	MEBNKEKO_00831
copZ	Copper chaperone CopZ	-	LEDPJBKC_04225	-
csoR	Copper-sensing transcriptional repressor CsoR	OBKDJCNG_03797	LEDPJBKC_01258	MEBNKEKO_00591
ctpA	Copper-exporting P-type ATPase	-	LEDPJBKC_02606	MEBNKEKO_04023
ctpG	putative cation-transporting ATPase G	-	LEDPJBKC_02567	MEBNKEKO_02008

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-9. Continued.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
cutC_1	Copper homeostasis protein CutC	OBKDJCNG_02795	LEDPJBKC_01035	MEBNKEKO_00782
cutC_2	Copper homeostasis protein CutC	OBKDJCNG_04010	LEDPJBKC_02277	MEBNKEKO_02922
mmcO	Multicopper oxidase MmcO	-	LEDPJBKC_02612	MEBNKEKO_04031
ricR	Copper-sensing transcriptional repressor RicR	-	LEDPJBKC_02609	MEBNKEKO_04025
merA_1	Mercuric reductase	OBKDJCNG_03717	LEDPJBKC_01343	MEBNKEKO_00508
merA_2	Mercuric reductase	-	LEDPJBKC_03642	MEBNKEKO_02670
merA_3	Mercuric reductase	-	-	MEBNKEKO_04009
merB_1	Alkylmercury lyase	-	LEDPJBKC_03641	MEBNKEKO_01980
merB_2	Alkylmercury lyase	-	-	MEBNKEKO_02669
merB_3	Alkylmercury lyase	-	-	MEBNKEKO_04010
merR1_1	Mercuric resistance operon regulatory protein	-	LEDPJBKC_03643	MEBNKEKO_02671
merR1_2	Mercuric resistance operon regulatory protein	-	-	MEBNKEKO_04008
nikB_1	Nickel transport system permease protein NikB	OBKDJCNG_01451	LEDPJBKC_01778	MEBNKEKO_03636
nikB_2	Nickel transport system permease protein NikB	OBKDJCNG_01487	LEDPJBKC_03729	-
ftsH_1	ATP-dependent zinc metalloprotease FtsH	OBKDJCNG_01395	LEDPJBKC_03574	MEBNKEKO_00332
ftsH_2	ATP-dependent zinc metalloprotease FtsH	OBKDJCNG_01575	-	MEBNKEKO_03272
ftsH_3	ATP-dependent zinc metalloprotease FtsH	OBKDJCNG_02520	-	-

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-9. Continued.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
rip1	Zinc metalloprotease Rip1	OBKDJCNG_00328	LEDPJBKC_00607	MEBNKEKO_01086
zitB	Zinc transporter ZitB	OBKDJCNG_01513	LEDPJBKC_00762	MEBNKEKO_01268
zntB	Zinc transport protein ZntB		LEDPJBKC_03244	MEBNKEKO_03298
znuB	High-affinity zinc uptake system membrane protein ZnuB	OBKDJCNG_02164	LEDPJBKC_02948	MEBNKEKO_02344
zosA	Zinc-transporting ATPase	-	LEDPJBKC_03338	-
zupT	Zinc transporter ZupT	OBKDJCNG_03077	LEDPJBKC_02051	-
zur	Zinc uptake regulation protein	OBKDJCNG_02163	LEDPJBKC_02949	MEBNKEKO_02345

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-10. Genes and copies of genes concerning salt and drought stress tolerance in the genomes of TSN2, *Leifsonia shinshuensis* INR9, and *Leifsonia aquatica* DSM 20146.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
glgE1	Alpha-1%2C4-glucan:maltose-1-phosphate maltosyltransferase 1	OBKDJCNG_00054	LEDPJBKC_00905	MEBNKEKO_00908
glgM	Alpha-maltose-1-phosphate synthase	OBKDJCNG_00377	LEDPJBKC_00554	MEBNKEKO_01292
maa	Maltose O-acetyltransferase	-	-	MEBNKEKO_02556
malI	Maltose regulon regulatory protein MalI	OBKDJCNG_03063	-	-
malL_1	Oligo-1%2C6-glucosidase	OBKDJCNG_00989	LEDPJBKC_00776	MEBNKEKO_01044
malL_2	Oligo-1%2C6-glucosidase	OBKDJCNG_02803	LEDPJBKC_02233	MEBNKEKO_02930
malL_3	Oligo-1%2C6-glucosidase	OBKDJCNG_02839	LEDPJBKC_02269	MEBNKEKO_02978
malT_1	HTH-type transcriptional regulator MalT	OBKDJCNG_00915	LEDPJBKC_00407	MEBNKEKO_01204
malT_2	HTH-type transcriptional regulator MalT	OBKDJCNG_00941	LEDPJBKC_00717	MEBNKEKO_01825
malT_3	HTH-type transcriptional regulator MalT	OBKDJCNG_01537	LEDPJBKC_00996	MEBNKEKO_01863
malT_4	HTH-type transcriptional regulator MalT	OBKDJCNG_01709	LEDPJBKC_03393	MEBNKEKO_03726
malT_5	HTH-type transcriptional regulator MalT	OBKDJCNG_01995	LEDPJBKC_04270	-
malT_6	HTH-type transcriptional regulator MalT	-	LEDPJBKC_04299	-
otsA	Trehalose-6-phosphate synthase	OBKDJCNG_04003	LEDPJBKC_01042	MEBNKEKO_00776
otsB	Trehalose-6-phosphate phosphatase	OBKDJCNG_04004	LEDPJBKC_01041	MEBNKEKO_00777
sugA_1	Trehalose transport system permease protein SugA	OBKDJCNG_02898	LEDPJBKC_01846	MEBNKEKO_03137
sugA_2	Trehalose transport system permease protein SugA	OBKDJCNG_03157	-	-
sugB_1	Trehalose transport system permease protein SugB	OBKDJCNG_01470	LEDPJBKC_01847	MEBNKEKO_03136

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-10. Continued.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
sugB_2	Trehalose transport system permease protein SugB	OBKDJCNG_03156	-	-
treS_1	Trehalose synthase/amylase TreS	OBKDJCNG_00372	LEDPJBKC_00559	MEBNKEKO_01287
treS_2	Trehalose synthase/amylase TreS	OBKDJCNG_01361	LEDPJBKC_03811	-
treY	Maltooligosyl trehalose synthase	OBKDJCNG_02741	LEDPJBKC_02325	MEBNKEKO_02876
treZ	Malto-oligosyltrehalose trehalohydrolase	OBKDJCNG_02740	LEDPJBKC_02326	MEBNKEKO_02875

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-11. Genes and copies of genes concerning uptake of the nutrients phosphate and potassium in the genomes of TSN2, *Leifsonia shinshuensis* INR9, and *Leifsonia aquatica* DSM 20146.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
pstB1	Phosphate import ATP-binding protein PstB 1	OBKDJCNG_01147	LEDPJBKC_04053	MEBNKEKO_03952
pstB3	Phosphate import ATP-binding protein PstB 3	-	-	MEBNKEKO_02511
pstC2_1	Phosphate transport system permease protein PstC 2	OBKDJCNG_01145	LEDPJBKC_04055	MEBNKEKO_02502
pstC2_2	Phosphate transport system permease protein PstC 2	-	-	MEBNKEKO_03954
pstP	PP2C-family Ser/Thr phosphatase	OBKDJCNG_02224	LEDPJBKC_02881	MEBNKEKO_02278
pstS_1	Phosphate-binding protein PstS	-	LEDPJBKC_02198	-
pstS_2	Phosphate-binding protein PstS	-	LEDPJBKC_02199	-
pstS_3	Phosphate-binding protein PstS	-	LEDPJBKC_02204	-
pstS_4	Phosphate-binding protein PstS	-	LEDPJBKC_02223	-
pstS2	Phosphate-binding protein PstS2	OBKDJCNG_01144	LEDPJBKC_04056	MEBNKEKO_03955
chaA	Sodium-potassium/proton antiporter ChaA	OBKDJCNG_01161	LEDPJBKC_04037	MEBNKEKO_03945
kdpA	Potassium-transporting ATPase potassium-binding subunit	OBKDJCNG_01931	LEDPJBKC_00321	MEBNKEKO_01487
kdpB	Potassium-transporting ATPase ATP-binding subunit	OBKDJCNG_01930	LEDPJBKC_00320	MEBNKEKO_01488
kdpC	Potassium-transporting ATPase KdpC subunit	OBKDJCNG_01929	LEDPJBKC_00319	MEBNKEKO_01489
kdpD	Sensor protein KdpD	OBKDJCNG_01928	LEDPJBKC_00318	MEBNKEKO_01490

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-11. Continued.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
kefB_1	Glutathione-regulated potassium-efflux system protein KefB	OBKDJCNG_01231	LEDPJBKC_00857	MEBNKEKO_03879
kefB_2	Glutathione-regulated potassium-efflux system protein KefB	-	LEDPJBKC_03966	-
kimA_1	Potassium transporter KimA	OBKDJCNG_01743	LEDPJBKC_03368	MEBNKEKO_03445
kimA_2	Potassium transporter KimA	OBKDJCNG_01954	-	-
ktrA	Ktr system potassium uptake protein A	OBKDJCNG_02817	LEDPJBKC_02255	MEBNKEKO_02945
ktrB	Ktr system potassium uptake protein B	OBKDJCNG_02818	LEDPJBKC_02254	MEBNKEKO_02946
kup	Low affinity potassium transport system protein kup	OBKDJCNG_03003	LEDPJBKC_02113	-
nhaK	Sodium%2C potassium%2C lithium and rubidium/H(+) antiporter	OBKDJCNG_03086	LEDPJBKC_02043	-
nhaP2_1	K(+)/H(+) antiporter NhaP2	OBKDJCNG_02349	LEDPJBKC_02749	MEBNKEKO_00156
nhaP2_2	K(+)/H(+) antiporter NhaP2	-	-	MEBNKEKO_02144

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Table 3-12. Genes and copies of genes concerning motility and chemotaxis in the genomes of TSN2, *Leifsonia shinshuensis* INR9, and *Leifsonia aquatica* DSM 20146.

Gene Name	Product	TSN2 Locus Tag (ID)	*L. shinshuensis* INR9 Locus Tag (ID)	*L. aquatica* DSM 20146 Locus Tag (ID)
flgB	Flagellar basal body rod protein FlgB	-	LEDPJBKC_02972	MEBNKEKO_02370
flgC	Flagellar basal-body rod protein FlgC	OBKDJCNG_02141	LEDPJBKC_02973	MEBNKEKO_02371
flgE	Flagellar hook protein FlgE	OBKDJCNG_02131	LEDPJBKC_02983	MEBNKEKO_02381
flgL	Flagellar hook-associated protein 3	OBKDJCNG_02150	-	-
flhA_1	Flagellar biosynthesis protein FlhA	OBKDJCNG_02104	LEDPJBKC_02994	MEBNKEKO_02402
flhA_2	Flagellar biosynthesis protein FlhA	OBKDJCNG_02939	-	-
flhB	Flagellar biosynthetic protein FlhB	OBKDJCNG_02105	LEDPJBKC_02993	MEBNKEKO_02401
fliD	B-type flagellar hook-associated protein 2	OBKDJCNG_02145	LEDPJBKC_02969	MEBNKEKO_02367
fliE	Flagellar hook-basal body complex protein FliE	OBKDJCNG_02140	LEDPJBKC_02974	MEBNKEKO_02372
fliF	Flagellar M-ring protein	OBKDJCNG_02139	-	-
fliG	Flagellar motor switch protein FliG	OBKDJCNG_02138	LEDPJBKC_02976	MEBNKEKO_02374
fliM	Flagellar motor switch protein FliM	OBKDJCNG_02118	LEDPJBKC_02987	MEBNKEKO_02394
fliQ	Flagellar biosynthetic protein FliQ	-	LEDPJBKC_02991	-
fliR	Flagellar biosynthetic protein FliR	OBKDJCNG_02106	LEDPJBKC_02992	MEBNKEKO_02400
fliS	Flagellar secretion chaperone FliS	-	LEDPJBKC_02970	MEBNKEKO_02368
fliW	Flagellar assembly factor FliW	OBKDJCNG_02151	LEDPJBKC_02964	MEBNKEKO_02361
fliY	L-cystine-binding protein FliY	OBKDJCNG_02893	LEDPJBKC_04246	-
motB	Motility protein B	OBKDJCNG_02126	LEDPJBKC_02986	MEBNKEKO_02391
pomA	Chemotaxis protein PomA	OBKDJCNG_02125	LEDPJBKC_02985	MEBNKEKO_02392

gene_# Indicates respective gene copy number
- Indicates the absence of the respective gene within the indicated genome

Figure 3-1. Circular representation of the whole TSN2 genome with the outer most labels indicating annotation of the CDS regions. The rings, starting from the outside indicate the tRNA, rRNA, misc_RNA, and tmRNA regions of the genome, direction of arrow representing strand location. This is followed by the entire genome annotation including all CDS regions with dark blue marks representing predicted CDS and light blue for unpredicted CDS. The next ring represents the GC% throughout the genome in dark gray. The inner most ring represents the GC skew with green being positive and red being negative

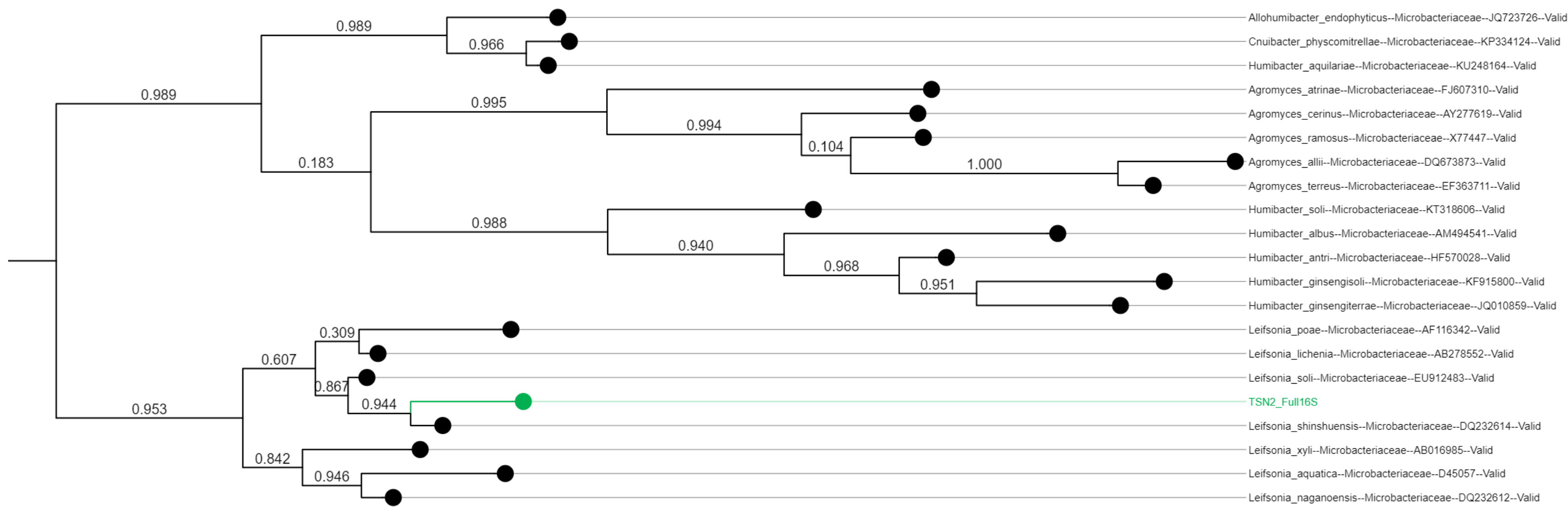

Figure 3-2. Phylogenetic tree of TSN2 (green) and its closest relatives based on 16S rRNA sequences with bootstrap confidence values displayed created using Protologger.

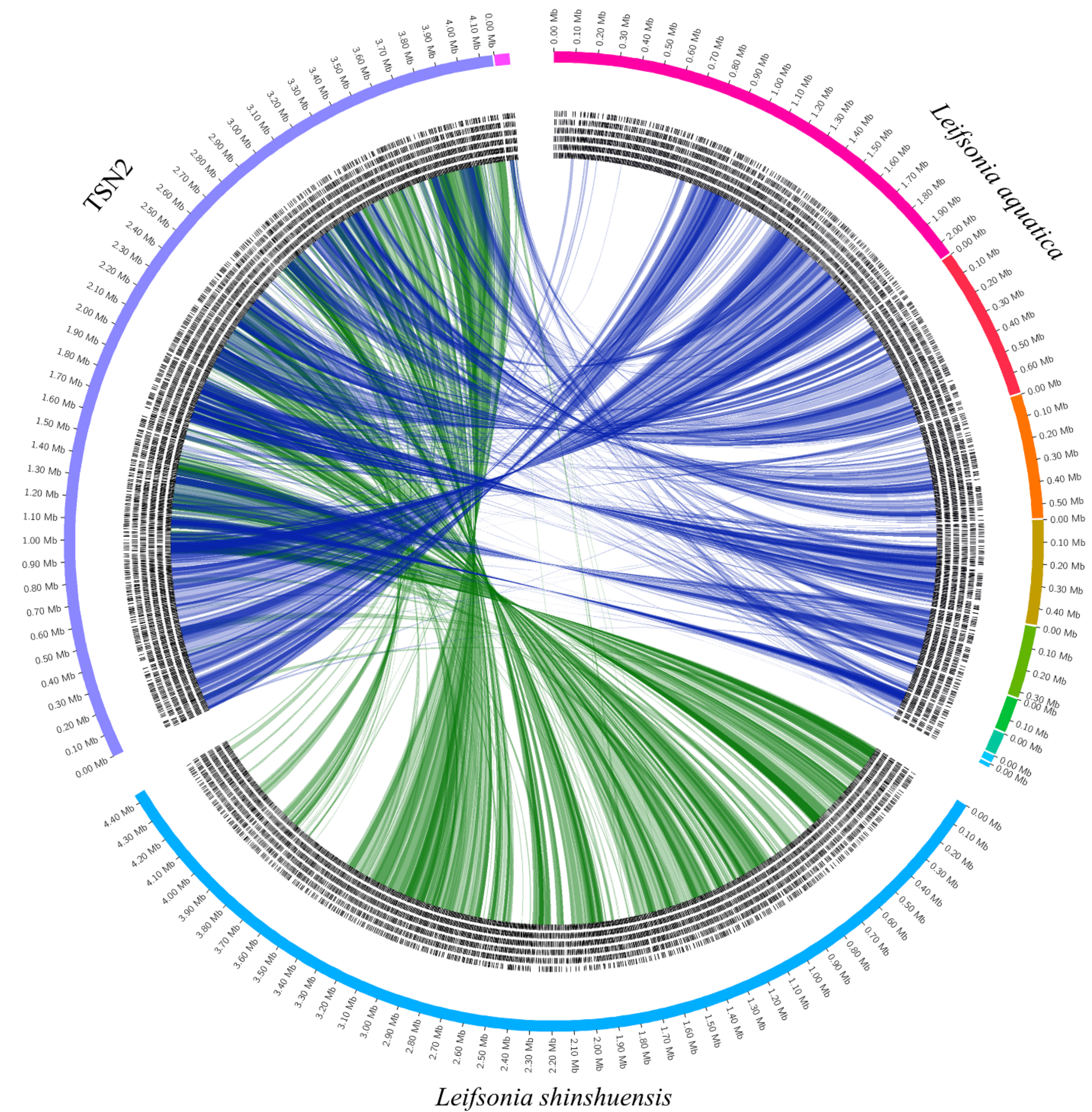

Figure 3-3. Circular synteny plot depicting the conserved regions between *Leifsonia shinshuensis* INR9 (green ribbons), *L. aquatica* DSM 20146 (blue ribbons), and TSN2.

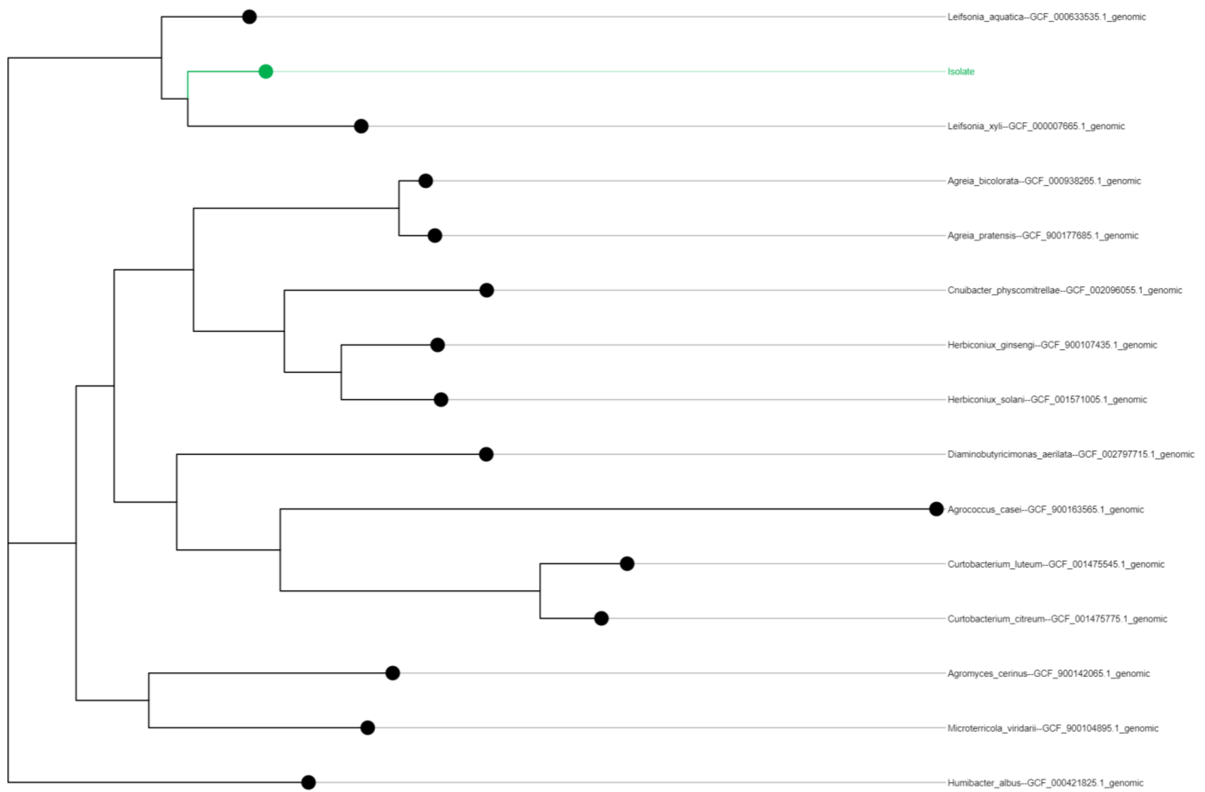

Figure 3-4. Phylogenetic tree of TSN2 (green) and its closest relatives based on whole genomic sequences with created using Protologger.

REFERENCES

Assad, B.M., Savi, D.C., Biscaia, S.M.P., Mayrhofer, B.F., Iantas, J., Mews, M., de Oliveira, J.C., Trindade, E.S., & Glienke, C. 2021. Endophytic Actinobacteria of Hymenachne amplexicaulis from Brazilian Pantanal wetland produce compounds with antibacterial and antitumor activities. *Microbiol. Res.* **248,** 1-15.

Battu, L., & Ulaganathan, K. 2020. Whole genome sequencing and identification of host-interactive genes in the rice endophytic Leifsonia sp. ku-ls. *Funct. Integr. Genom.* **20,** 237-243.

Brumbley, S.M., Petrasovits, L.A., Hermann, S.R., Young, A.J., & Croft, B.J. 2006. Recent advances in the molecular biology of Leifsonia xyli subsp. xyli, causal organism of ratoon stunting disease. *Australas. Plant Pathol.* **35,** 681-689.

Ciufo, S., Kannan, S., Sharma, S., Badretdin, A., Clark, K., Turner, S., Brover, S., Schoch, C.L., Kimchi, A., & DiCuccio, M. 2018. Using average nucleotide identity to improve taxonomic assignments in prokaryotic genomes at the NCBI. *Int. J. Syst. Evol. Microbiol.* **68(7),** 2386-2392.

Diels, L., Dong, Q., van der Lelie, D., Baeyens, W., & Mergeay, M. 1995. The czc operon of Alcaligenes eutrophus CH34: from resistance mechanism to the removal of heavy metals. *J. Ind. Microbiol.* **14(2),** 142-53.

Donoso, R., Leiva-Novoa, R., Zuniga, A., Timmermann, T., Recabarren-Gajardo, G., & Gonzalez, B. 2017. Biochemical and genetic bases of indole-3-acetic acid (auxin

phytohormone) degradation by the plant-growth-promoting rhizobacterium Parabukholderia phytofirmans PsJn. *Appl. Environ. Microbiol.* **83(1),** 1-20.

Egidi, E., Wood, J.L., Fox, E.M., Liu, W., & Franks, A.E. 2017. Draft genome sequence of Leifsonia sp. strain NCR5, a rhizobacterium isolated from vadmium-contaminated soil. *Genome Announc.* **5(23)**

Faria, R.S.C.A., Cia, M.C., Monteiro-Vitorello, C.B., Azevedo, R.A., & Camargo, L.E.A. 2020. Characterization of genes responsive to osmotic and oxidative stress of the sugarcane bacterial pathogen Leifsonia xyli subsp. xyli. *Braz. J. Microbiol.* **51,** 77-86.

Favero, V.O., de Carvalho, R.H., Leite, A.B.C., de Freitas, K.M., Zilli, J.E., Xavier, G.R., Rumjanek, N.G., & Urquiaga, S. 2021. Characterization and nodulation capacity of native bacteria isolated from mung bean nodules used as a trap plant in Brazilian tropical soils. *Appl. Soil Ecol.* **167,** 104041

Garg, A.K., Kim, J.K., Owens, T.G., & Wu, R.J. 2002. Trehalose accumulation in rice plants concers high tolerance levels to different abiotic stresses. *App. Biol. Sci.* **99,** 15898-15903.

Gurevich, A., Savelieve, V., Vyahhi, N., & Tesler, G. 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics*. **29(8),** 1072-1075.

Hao, X., Zhu, J., Rensing, C., Liu, Y., Gao, S., Chen, W., Huang, Q., & Liu, Y.R. 2021. Recent advances in exploring the heavy metal(loid) resistant microbiome. *Comput. Struct. Biotechnol. J.* **19,** 94-109.

Henao, S.G., & Ghneim-Herrera, T. 2021. Heavy metals in soils and the remediation potential of bacteria associated with the plant microbiome. *Front. Environ. Sci.* **9,** 1-17.

Hirano, K., Aya, K., Hobo, T., Sakakibara, H., Kojima, M., Shim, R.A., Hasegawa, Y., Ueguchi-Tanaka, M., & Matsuoka, M. 2008. *Plant Cell Physiol.* **49(10),** 1429-1450.

Hitch, T.C.A., Riedel, T., Oren, A., Overmann, J., Lawley, T.D., & Clavel, T. 2021. Automated analysis of genomic sequences facilitates high-throughput and comprehensive description of bacteria. *ISME Comm.* **16,** 1-16

Ibrahim, H.A., & Abdellatif, Y.M.R. 2016. Effect of maltose and trehalose on growth, yield, and some biochemical components of wheat plant under water stress. *Ann. Agric. Sci.* **61,** 267-274.

Iordachescu, M., & Imai, R. 2008. Trehalose biosynthesis in response to abiotic stresses. *J. Integr. Plant Biol.* **50,** 1223-1229.

Jiang, X., Li, W.W., Han, M., Chen, G., Wu, J., Lai, S., Fu, Z., Zhang, S., Deng, W.W., Gao, L., & Xia, T. 2022. Aluminum-tolerant, growth-promoting endophytic bacteria as contributors in promoting tea plant growth and alleviating aluminum stress. *Tree Physiol.* **42,** 1043-1058.

Jun, X., Xin-yu, W., & Wang-zhen, G. 2015. The cytochrome p450 superfamily: key players in plant development and defense. *J. Integr. Agric.* **14(9),** 1673-1686.

Kang, S.M., Waqas, M., Hamayun, M., Asaf, S., Khan, A.L., Kim, A.Y., Park, Y.G., & Lee, I.J. 2017. Gibberellins and indole-3-acetic acid producing rhizospheric bacterium Leifsonia xyli SE124 mitigates the adverse effects of copper-mediated stress on tomato. *J. Plant Interact.* **12,** 373-380.

Kaplan, F., Guy, C.L. 2004. Beta-amylase induction and the protective role of maltose during temperature schock. *Plant Physiol.* **135,** 1674-1684.

Kaplan, F., & Guy, C.L. 2005. RNA interference of Arabidopsis beta-amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. *Plant J.* **44,** 730-743.

Kaplan, F., Kopka, J., Haskell, D., Zhao, W., Schiller, K.C., Gatzke, N., Sung, D.Y., & Guy, C.L. 2004. Exploring the temperature stress metabolome of Arabidopsis thaliana. *Plant Physiol.* **136,** 4159-4168.

Khang, S.M., Khan, A.L., You, Y.H., Kim, J.G., Kamran, M., & Lee, I.J. 2014. Gibberellin production by newly isolated strain leifsonia soli SE134 and its pontential to promote plant growth. *J. Microbiol. Biotechnol.* **24,** 106-112.

Kosar, F., Akram, N.A., Sadiq, M., Al-Qurainy, F., & Ashraf, M. 2019. Trehalose: a key organic osmolyte effectively involved in plant abiotic stress tolerance. *J. Plant Growth Regul.* **38,** 606-618.

Kryzwinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S.J., & Marra, M.A. 2009. Circos: an information aesthetic for comparative genomics. *Genom. Res.* **19(9),** 1639-1645.

Li, Q., Guo, R., Li, Y., Hartman, W.H., Li, S., Zhang, Z., Tringe, S.G., & Wang, H. 2019. Insight into the bacterial endophytic communities of peach cultivars related to crown gall disease resistance. *App. Environ. Microbiol.* **85**

Liu, H., Zhang, Y., Wang, Y., Xie, X., & Shi, Q. 2021. The connection between czc and cad systems involved in cadmium resistance in Pseudomonas putida. *Int. J. Mol. Sci.* **22(18),** 1-14.

Liu, Y., Ma, X., Helmann, T.C., McLane, H., Stodghill, P., Swingle, B., Filiatrault, M.J., & Perry, K.L. 2020. Complete genome sequence of a gram-positive bacterium, Leifsonia sp. strain PS1209, a potato endophyte. *Microbiol. Resour. Announc.* **9**

Madhaiyan, M., Poonguzhali, S., Lee, J.S., Senthilkumar, M., Lee, K.C., & Sundaram, S. 2010. Leifsonia soli sp. nov., a yellow-pigmented actinobacterium isolated from teak rhizosphere soil. *Int. J. Syst. Evol. Microbiol.* **60,** 1322-1327

Mills, L., Leaman, T.M., Taghavi, S.M., Shackel, L., Dominiak, B.C., Taylor, P.W.J., Fegan, M., & Teakle, D.S. 2001. Leifsonia xyli-like bacteria are endophytes of grasses in eastern Australia. *Australas. Plant. Pathol.* **30,** 145-151.

Minerdi, D., Savoi, S., & Sabbatini, P. 2023. Role of cytochrome p450 enzyme in plant microorganisms communication: a focus on grapevine. *Int. J. Mol. Sci.* **24(5),** 1-13.

Nordstedt, N.P., Roman-Reyna, V., Jacobs, J.M., & Jones, M.L. 2021. Comparative genomic understanding of gram-positive plant growth-promoting Leifsonia. **5,** 263-274.

Nunes, G.F.O., Menezes, K.A.S., Sampaio, A.A., Leite, J., Fernandes-Junior, P.I., Seido, S.L., Zilli, J.E., & Martins, L.M.V. 2018. Polyphasic characterization of forage legumes root nodule bacteria isolated from semiarid region in brazil. *Rev. Cienc. Agr.* **41,** 612-624.

Nouwen, N., Fardoux, J., & Giraud, E. 2016. NodD1 and NodD2 are not required for the symbiotic interaction of Bradyrhizobium ORS285 with Nod-Factor-independent Aeschynomene legumes. *PloS One.* 1-12.

del Orozco-Mosqueda, M., Santoyo, G., & Glick, B.R. 2023. Recent advances in the bacterial phytohormone modulation of plant growth. *Plants.* **12(3),** 606.

Pande, V., Pandey, S.C., Sati, D., Bhatt, P., & Samant, M. 2022. Microbial interventions in bioremediation of heavy metal contaminants in agroecosystem. *Front. Microbiol.* **13,** 1-16.

Pandian, B.A., Sathishraj, R., Djanaguiraman, M., Prasad, P.V.V., & Jugulam, M. 2020. Role of cytochrome p450 enzymes in plant stress response. *MDPI Antioxidants*. **9(5),** 1-15.

Patel, M.K., Kumar, M., Li, W., Luo, Y., Burrit, D.J., Alkan, N., & Tran, L.S.P. 2020. Enhancing salt tolerance of plants: from metabolic reprogramming to exogenous chemical treatments and molecular approaches. *Cells.* **9(11),** 1-26.

Purdy, S.J., Bussell, J.D., Nunn, C.P., & Smith, S.M. 2013. Leaves of the Arabidopsis maltose exporter1 mutant exhibit a metabolic profile with features of cold acclimation in the warm. *PLoS One.* **8,** 1-12.

Rasche, H., & Hiltemann, S. 2020. Galactic circose: user-friendly circus plots within the galaxy platform. *GigaScience.* **9(6).**

Rodriguez-Rodriguez, R.M., Guimaraes, A.A., de Castro, J.L., Siqueira, J.O., Carneiro, M.A.C., & Moreira, F.M.S. 2021. Rhizobia and endophytic bacteria isolated from

rainforest fragments within an iron ore mining site of the eastern Brazilian amazon. *Braz. J. Microbiol.* **52,** 1461-1474.

Sadowsky, M.J., Tully, R.E., Cregan, P.B., Keyser, H.H. 1987. Genetic diversity in Bradyrhizobium japonicum serogroup 123 and its relation to genotype-specific nodulation of soybean. *Appl. Environ. Microbiol*. **53(11)**, 2624-2630.

Salam, L.B. 2020. Unravelling the antibiotic and heavy metal resistome of a chronically polluted soil. *3 Biotech.* **10(6),** 1-23.

Seeman, T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics.* **30(14),** 2068-2069.

Sharma, P.M., Grover, M., Chourasiya, D., Bharti, A., Agnihotri, R., Maheshwari, H.S., Pareek, A., Buyer, J.S., Sharma, S.K., Schutz, L., Mathimaran, N., Singla-Pareek, S.L., Grossman, J.M., & Bagyaraj. D.J. 2020. Deciphering the role of trehalose in tripartite symbiosis among rhizobia arbuscular mycorrhizal fungi, and legumes for enhancing abiotic stress tolerance in crop plants. *Front. Microbiol.* **11,** 1-12.

Simm, S., Scharf, K.D., Jegadeesan, S., Chiusano, M.L., Firon, N., & Schleiff, E. 2016. Survey of genes involved in biosynthesis, transport, and signaling of phytohormones with focus on Solanum lycopersicum. *Bioinform. Biol. Insights.* **10,** 185-207.

Tan, W.F., Li, Y., Guo, F., Wang, Y.C., Ding, L., Mumford, K., Lv, J.W., Deng, Q.W., Fang, Q., & Zhang, X.W. 2020. Effect of Leifsonia sp. on retardation of uranium in natural soil and its potential mechanisms. *J. Environ. Radioact.* **217,** 106202.

Thalmann, M., & Santelia, D. 2016. Starch as a determinant of plant fitness under abiotic stress. *New Phytol.* **214,** 943-951.

Vincent, J.M. 1970. A manual for the practical study of root nodule bacteria. *IBP Handbook N°15*. Blackwell, Oxford.

Wick, R.R., Judd, L.M., Gorrie, C.L., & Holt, K.E. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* **13(6),** e1005595.

Yang, X.Y., Zhao, T.Y., Rao, P., Lei, B.Q., Khan, W.U., Gao, K., Chen, Z., & An, X.M. 2020. Transcriptiome profiles of phytohormone-related genes in Populus tomentosa vegetative buds from endodormancy to ecodormancy. *S. Afr. J. Bot.* **130,** 430-439.

Yang, Y., Yao, Y., Li, J., Zhang, J., Zhang, X., Hu, L., Ding, D., Bakpa, E.P., & Xie, J. 2022. Trehalose alleviated salt stress in tomato by regulating ros metabolism, photosynthesis, osmolyte synthesis, and trehalose metabolic pathways. *Front. Plant Sci.* **13,** 1-18.

Zhao, Y., Hull, A.K., Gupta, N.R., Goss, K.A., Alonso, J., Ecker, J.R., Normanly, J., Chory, J., & Celenza, J. 2002. Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome p450s cyp79B2 and cyp79B3. *Genes Dev.* **16(23),** 3100-3112.

CHAPTER 4

CHARACTERIZATION OF A REGULATORY GENE IN NOD FACTOR INDEPENDENT NODULATION

ABSTRACT

Nodulation is one of the most defining characteristics of legume plants that has been studied for more than a century yet new discoveries regarding this phenomenon are made almost every few years. The discovery that nodulation of a plant can occur without the use of complex nod factor (NF) signaling molecules was groundbreaking and turned the world of nodulation research on its head. For the longest time, until almost 15 years ago, it was firmly believed and recognized that the special nodule organs housing the nitrogen fixing endosymbiont would only be able to form due to a signaling cascade between host plant and bacteria involving molecules referred to as NFs. It was discovered that the tropical legume *Aeschynomene* harbor stem nodulating endophytes that induce this unique organogenesis in the absence of NFs and their numerous, well-documented genes. One of the isolated identified in this system, *Bradyrhizobium* sp. BTAi1 has served as a model organism for understanding this relationship, particularly due to the presence of a potential symbiosis plasmid within its genome. If the genes responsible for NF-independent nodulation are found on the plasmid, it is believed that this ability can then be transferred to other plant growth promoting bacteria, providing additional benefits to the already established nitrogen fixing nodule relationship. To identify what genes are potential involved in the establishment of this relationship, a gene expression analysis was previously performed and from this information, the gene *Bbta_p0110* was selected for disruption through site-specific mutagenesis. Once this gene was disrupted, *Aeschynomene indica* plants inoculated with the

mutant strain were unable to form mature, pigmented nodules. Instead, only pale, immature nodules formed, even at a decreased rated as opposed to the wild type. The results obtained from this study indicate the importance of *Bbta_p0110* in bacterial assimilation and resulting nodule maturation of *A. indica* plants, independent of NFs. This gene, located in *Bradyrhizobium* sp. BTAi1's plasmid could be transformed to non-nodulating bacteria to determine if NF-independent nodulation can be conferred across bacterial genera.

INTRODUCTION

The traditional and most well documented method of root nodule induction involves the production of bacterially derived signal molecules referred to as od-Factors (NFs). Nodule organs typically form on the root systems of leguminous plants (e.g., peas, beans, soybeans, etc.) in response to the NF released by these specialized rhizobacteria as a result of a complex cascade of molecular communication system that has taken millions of years to evolve (Newcomb, 1981; Wood & Newcomb, 1989). NFs are released by rhizobacteria in response to the uptake of plant released root exudates containing flavonoids released during the natural progression of root system growth and expansion (Aguilar et al., 1988; Spaink, 2000). These flavonoids attract the surrounding rhizobia and are detected by a protein transcribed by the *nodD* gene which in turn initiates the transcription of *nodABC* genes, comprising the backbone of the NF signal molecule (Aguilar et al., 1988; Armitage et al., 1988; Brussel et al., 1986; Hong et al., 1987). This molecule is further modulated using strain/host specific *nod* genes, facilitating the invasion and nodule organ formation depending on the bacterial strain and host plant relationship (Bhuvaneswari & Solheim, 1985; Gottfert, 1993; Halverson & Stacey, 1986; Horvath et al., 1987). After colonizing the root surface, rhizobia release NFs specific to their host plant, initiating the deformation and curling of root hair cells (Caetano-Anolles et al., 1992; Rhijn &

Vanderleyden, 1995; Rossen et al., 1984). These root hairs curl upon themselves, encapsulating the surface-dwelling rhizobia (Smit et al., 1992; Yao & Vincent, 1969). This leads to plant cell wall degradation via hydrolysis and subsequent invagination and invasion of the bacteria into the root hair cell (Bhuvaneswari & Solheim, 1985; Brussel et al., 1986). This invasion, directed by various NF molecules, results in the formation of a cell wall lined infection thread that eventually deposits the rhizobia into the inner cortex of the plant root (Vasse & Truchet, 1984; Wood & Newcomb, 1989). The cortical tissues, in preparation for the arrival of rhizobia cells, respond to yet more specialized NFs, initiating the formation of root nodule primordial cells and the subsequent colonization is distribution of the endosymbionts within nodule tissues (Brewin, 1991; Ding & Oldroyd, 2009; Gage, 2004; Heckmann et al., 2011; Newcomb, 1981). This highly specific relationship provides an advantage to both parties in that, the host plant receives a direct source of biologically available nitrogen for further incorporation into metabolic processes while the endosymbiont receives a stable environment with reduced oxygen levels and supplemental carbon sources, providing the necessary conditions for amplified nitrogen fixation (Becana et al., 2010; Rhijn & Vanderleyden, 1995).

For over two centuries, this NF-dependent nodule formation process was believed to be the only method in which symbiotic nitrogen fixing relationship between bacteria and plants could be formed, despite being so complex (Masson-Boivin & Sachs, 2018; Vasse & Truchet, 1984). About a century ago, it was discovered that root nodules are not the only form of nodulation to occur in leguminous plants, or plants in general, with the documentation of stem nodule formation in tropical semi-aquatic legumes of the genus *Aeschynomene* (Arora, 1954; Alazard, 1985; Eaglesham & Szalay, 1983; Ladha et al., 1990). From these unusual stem nodules, a remarkable discovery came in that among the endosymbionts isolated from these

organs there were photosynthetic *Bradyrhizobium* sp. strains which had never been documented in this nodule forming symbiotic relationship before (Giraud & Fleischman, 2004; Madigan, 1995). In fact, photosynthetic rhizobia strains, while are free-living soil dwelling bacteria, if nodule forming, are solely found within stem nodules (Molouba et al., 1999). In 2000, Chaintreuil et al. identified photosynthetic rhizobia living endophytically within African wild rice (*Oryza breviligulata*) that naturally grow in the same areas as populations of *Aeschynomene* species even though rice is a non-leguminous plant. This finding supports the idea that NF-independent photosynthetic rhizobia are free-living within soils but will effectively form symbiotic relationships with plants and successfully invade host tissues should the opportunity arise, without strict host limitation. Upon further investigation of the unique stem nodulation system, two bacterial strains, *Bradyrhizobium* sp. ORS278 & BTAi1, isolated from *Aeschynomene* stem nodules were found to induce nodulation despite lacking the critical *nod* genes (Giraud et al., 2007). This discovery led to a flurry, relatively speaking, of nodulation research attempting to unlock the secret behind NF-independent nodulation and how it could possibly occur in the absence of the once believed vital NF signal molecule. While this effort has made great progress in proposing potential molecular mechanisms for the signaling involved and has provided great insights into plant phenotypic response and nodule formation in this system, the exact signal molecule(s) inducing NF-independent nodulation has yet to be elucidated (Alazard & Duhoux, 1988; Armitage et al., 1988; Bonaldi et al., 2010 & 2011; Chaintreuil et al., 2018; Gully et al., 2018).

As opposed to the highly complex invasion method implemented by NF-dependent nodulation, involving root hair curling and infection thread formation, it is becoming apparent that a more simplistic method of invasion, via crack-entry and intracellular dispersion, is the

standard mechanism utilized for NF-independent nodulation (Loureiro et al., 1995;. Yatazawa et al., 1984). From an evolutionary standpoint, NF-independent nodulation through crack-entry is most likely the ancestral process that led to the eventual development of specialized NFs and the resulting symbiotic relationship we predominantly notice. Crack-entry is such a common mode of bacterial invasion, this method has been documented in many endophytic/plant relationships, regardless of nodule formation (Boogerd & van Rossum, 1997; Dudeja & Giri, 2014; Liu et al., 2017). In this method, bacteria adhere to the rhizosphere, proliferating near points of lateral root emergence (Lopes et al., 2016). As the root systems develop, lateral roots emerge from within the tap root, bursting through the epidermis, leaving small cracks or micro fissures open for short periods of time before the plant can seal them (Mills & Bauer, 1985). These small openings allow the established bacterial populations from the rhizosphere to enter and continually multiply within the intracellular spaces of the root cortex (Compant et al., 2005; Iyer & Rajkumar, 2017). Once inside, in short, the invading bacteria illicit an immune response from the host plant which encapsulates the bacteria and disperses them among developing nodule primordia cells. Once bacteria have penetrated nodule primordia, nodule development occurs as normal (Alazard & Duhoux; 1990; Arora, 1954). However, the NF-independent system completes this goal without a documented dedicated signal molecule or signaling system. According to previous studies, the primary step in this process that lacks explanation is what tells surrounding nodule primordia tissue to form, accept, and house the invading bacteria upon the death of initially infected primordial cells (Bonaldi et al., 2011; Madsen et al, 2010). This step involves the sudden assimilation of the invading bacteria into nodule primordial cells, an unusual immune response that would be counterintuitive to the typical response to a potential pathogen yet similar on a surface level to NF-dependent nodulation (Lancelle & Torrey, 1984).

To better understand what is occurring during this infection process, in searching for some signal molecule alternative to NFs, from the molecular bacterial standpoint, a gene expression experiment utilizing RNA-Seq was previously conducted in this lab. Briefly, this experiment exposed *Bradyrhizobium* sp. BTAi1 to root exudates from *Aeschynomene indica* plants in the early stages of BTAi1 infection, hoping to stimulate a response in the bacteria that would be seen within the inner cortical layers. The resulting gene expression of the bacteria in response to these root exudates, containing things like flavonoids, proteins, and amino acids, was observed with the top 3 upregulated genes being shown here (Table 1). From these data, the gene encoding a putative exported protein (*Bbta_p0110*), a potential signal molecule, was selected for knock-out mutagenesis. The primary aim in this study is to determine if the putative exported protein encoded by *Bbta_p0110* gene in *Bradyrhizobium* sp. BTAi1 plays a role in nodule formation of *Aeschynomene indica* plants to shed some light on what controls this specific NF-independent relationship. It is possible that the exported p0110 protein elicits the immune response in the host plant necessary to induce initial cortical cell death and subsequent nodule primordia development. If this were the case, the inhibition of p0110 protein production would result in an inability to form mature root nodules in *A. indica* that house nitrogen-fixing BTAi1 bacterioids. Alternatively, if nodulation capabilities are not affected at all by the inhibition of this protein synthesis, then it is likely some other gene(s) would be responsible for controlling this specialized immune response. Both *Bradyrhizobium* sp. BTAi1 and *A. indica* have served as one of the primary model systems for better understanding NF-independent nodulation and the signaling mechanism behind it. We find this unique nodulation system important to understand so that these mechanism principles may one day be applied to non-nodulating endophytic

bacteria or even non-nodulating crop plants, resulting in crops with greater capacity for self-sufficiency in yield and survivability that doesn't depend on costly synthetic fertilizers.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains and plasmids used in this study can be found in Table 2. Wild type *Bradyrhizobium* sp. BTAi1 was cultured in the complete arabinose-gluconate (AG) medium normalized to pH 6.8 containing 125mg Na_2HPO_4, 250mg Na_2SO_4, 320mg NH_4Cl, 180mg $MgSO_4 \cdot 7H_2O$, 10mg $CaCl_2$, 4mg $FeCl_3$, 1.3g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1g 2-(N-morpholino) ethanesulfonic acid (MES), 1g yeast extract, 1g L-arabinose, and 1g D-gluconic acid sodium sulfate per L of distilled water (Sadowsky et al., 1987). *Bradyrhizobium* sp. BTAi1, when grown in liquid media, was incubated aerobically at 30°C while shaking at 200rpm. The following antibiotics were used for selection of the BTAi1 strains specifically: chloramphenicol (50 µgml-1 for wild type and mutant strains of *Bradyrhizobium* sp. BTAi1) and kanamycin (150 µgml-1 for the mutant strain). All *Escherichia coli* strains and plasmids were cultured using Luria-Bertani (LB) media at pH 7.0 containing 10g tryptone, 5g yeast extract, and 10g NaCl per L distilled water (Bertani, 1951). For the preparation of all solid media types, 15g of agar were added per L of liquid media. *E. coli* strains and plasmids (except for DH5α pKD78 which was incubated at 30°C) were cultured at 37°C overnight with agitation at 200rpm in liquid media. Antibiotics specific to each strain and plasmid were added accordingly: gentamicin (15µgml-1 for pJQ200SK), chloramphenicol (15µgml-1for pKD78) and kanamycin (50µgml-1 for pRK2013 and pKD4).

Comparison of growth rate. *Bradyrhizobium* sp. BTAi1 (wild type) and Δp0110 mutant strains were grown in 10 ml of AG medium (pH 6.8) at 30°C with shaking at 200 rpm for 2 days

with proper antibiotics. Strains were grown under these conditions until mid-log phase was reached. The strains were then subcultured to 50 mL of AG media (pH 6.8) with appropriate antibiotics respectively. Three replicates for each strain cultured in 50 mL were incubated at 30°C with shaking at 200 rpm for 7 days until the cultures reach the stationary phase. Culture turbidity OD600 measurements were performed at 12-hour intervals throughout the experiment.

Selection of *Bbta_p0110* as a potential regulatory gene in NF-independent nodulation. Previously, gene expression of *Bradyrhizobium* sp. BTAi1 exposed to *A. indica* root exudates was determined by genome-wide transcriptomic analysis through RNA-Seq in this lab. Briefly, BTAi1 cultures were exposed to purified root exudates and the total RNA was extracted and purified for sequencing. *A. indica* plants were grown in sterilized containers at 28°C for 7 days with roots suspended in sterile ½ B&D media. The root exudates released into the media were collected and filter sterilized. After sterilization, 100µL aliquots were taken and spread on solid AG plates for incubation and sterilization confirmation. Filter sterilized root exudate solution was then used to resuspend washed and pelleted BTAi1 cells, cultured to an OD_{600} = 0.8 in a volume of 100mL AG media. The root exudate and bacterial mixture were then incubated at 30°C for 24 h. The resulting bacterial total RNA was extracted and purified using the RNeasy mini kit (Qiagen) and RNase-free DNase (Qiagen) according to the manufacturer's protocol. Purified total RNA samples were then further processed by the Department of Energy Joint Gnome Institute (DOE-JGI), Walnut Creek, CA where library preparation, sequencing and analysis were completed. Differential gene expression between BTAi1 exposed to root exudate and BTAi1 grown only in buffer (negative control) were used to generate normalized read counts via DESeq2 (Love et al., 2014). Table A-1 shows differentially expressed genes in response to plant root exudates.

Construction of a mutant strain. The mutant strain was constructed using site-specific mutagenesis to disrupt the Bbta_p0110 gene which is upregulated by *Bradyrhizobium* sp. BTAi1 in response to purified *Aeschynomene indica* root exudate. An 827 bp fragment containing the Bbta_p0110 sequence encoding a putative exported protein with suggested signaling function was amplified through PCR using the forward primer p0110F (5' AATTGGATCCCCGAATTCCAGGATCACTATCG 3') and reverse primer p0110R (5' AATATGAGCTCTCTAGGTGCTACCGACATTCT 3') each including restriction enzyme recognition linkers for BamHI and SacI, respectively, at their 5' end.

PCR amplification was completed using an Eppendorf 5331 Mastercycler gradient 96-well thermal cycler (Eppendorf, Hamburg, Germany). The products were then visualized via gel electrophoresis using an EPS 301 (Amersham Biosciences [GE], Chicago, IL) to ensure appropriate amplicon size was achieved. The desired p0110 amplicon and gentamycin resistant suicide vector plasmid pJQ200SK (Quandt & Hynes, 1993) were digested with both restriction enzymes, BamHI and SacI, before being ligated using T4 DNA ligase (Promega, Madison, WI), facilitating the insert of the p0110 amplicon into pJQ200SK. Using heat shock transformation, the recombinant pJQ200SK suicide vector plasmid containing the p0110 amplicon (clone) was then introduced into competent DH5α *E. coli* cells allowing for replication and storage of the recombinant vector plasmid. This was then used to introduce the vector plasmid + clone (pJQ200SK + p0110) into electrocompetent, chloramphenicol resistant, DH5α pKD78 *E. coli* through electroporation using a Micro PulserTM Electroporator (Biorad, Hercules, CA) set at 2.5 kV in a 0.2cm cuvette. The pKD78 plasmid, housed within DH5α *E. coli* cells, contains the Lambda Red Recombinase system which is expressed later on during the triparental mating. Separately, the kanamycin resistance cassette (1.4kb) was amplified from the pKD4 plasmid

through PCR with the following forward and reverse primers: PS1 (5' GTGTAGGCTGGAGCTGCTTC 3') and PS2 (5' CATATGAATATCCTCCTTAG 3')(Datsenko & Wanner, 2000). The resulting product was then used as a template for further amplification using 47-bp and 60-bp primers having 27-bp and 40-bp linkers specific to the flanking regions of the deletion target, housing the p0110 gene sequence, in addition to the flanking regions (PS1 & PS2) of the kanamycin resistance cassette. This was completed using Phusion Taq polymerase (Promega, Madison, WI) with the following primers specific to the p0110 gene: p0110UP_47 (5' CCGAATTCCAGGATCACTATCGTGCGGGTGTAGGCTGGAGCTGCTTC 3') and p0110DWN_60 (5' CTAAGGAGGATATTCATATGTCCCGTTTCTCCTGTTTGAACTAACCAATCTAAAGAC AAT 3'). The resulting PCR product was purified and introduced into previously created electrocompetent DH5α pKD78 *E. coli* cells containing the vector plasmid + clone via electroporation (Micro PulsterTM Electroporator) at 2.5kV in a 0.2cm cuvette. This step induced the Lambda Red Recombinase genes, facilitating homologous recombination. After incubation, kanamycin resistant recombinants were selected and vector constructs were confirmed via colony PCR using the forward and reverse primers from the initial PCR cloning (p0110F & p0110R) in combination with primers PSk1 (5' CAGTCATAGCCGAAT AGCCT 3') and PSkt′ (5' GGATTCATCGACTGTGGCCG 3'), targeting regions within the kanamycin resistance gene (Datsenko & Wanner, 2000). The confirmed vector constructs were transferred from the housing strain (DH5α pKD78) to the wild type *Bradyrhizobium* sp. BTAi1 strain via tri-parental mating with assistance from the helper strain pRK2013. Successful transconjugants resulting from double-crossover homologous recombination were selected through the use of the *sacB* marker

and kanamycin and chloramphenicol resistance. Resulting mutant strains were confirmed visually (pink hue of BTAi1) and molecularly through PCR using the above-mentioned primer combinations used to confirm the vector constructs).

Comparison of nodule formation and plant growth. *Aeschynomene indica* seeds were surface sterilized through an initial wash with 70% ethanol, shaking by hand for 5 min, followed by 3 washes of sterile distilled water. The seeds were then washed with a 10% bleach solution and shaken vigorously (via vortexer) for 30 min. Finally, the seeds were rinsed with sterile distilled water 6 times and given one final rinse with 70% ethanol before drying on sterile filter paper overnight under a clean bench. Once dried, three seeds were randomly selected and aseptically transferred to liquid AG media and incubated for 2 days to ensure successful surface sterilization. Sterile seeds were germinated using 0.8% water agar with sterile distilled water for 2 days at 30°C in the dark until radicle emergence occurred. After germination, 3 seeds were aseptically transferred to sterile CYG growth pouches (Mega International, Roseville, MN) with a sterile straw inserted on either side of the pouch for future inoculation and watering purposes. Once loaded, two pouches were attached using sterile paper clips to sterile hanging paper folders placed into metal folder racks. Each pouch was inoculated with 1mL overnight cultures of the desired bacterial strain (wild type or mutant *Bradyrhizobium* sp. BTAi1) normalized to 0.1 OD_{600} by wash, resuspension, and dilution in half strength nitrogen-free Broughton and Dilworth (1/2 B&D) media at pH 6.8 containing 500µM $CaCl_2$, 250µM KH_2PO_4, 250µM K_2HPO_4, 5µM Fe-citrate, 125µM $MgSO_4 \cdot 7H_2O$, 125µM K_2SO_4, 0.5µM $MnSO_4 \cdot H_2O$, 1µM H_3BO_3, 0.25µM $ZnSO_4 \cdot 7H_2O$, 1µM $CuSO_4 \cdot 5H_2O$, 0.05µM $CoSO_4 \cdot 7H_2O$, and 0.05µM $Na_2MoO_4 \cdot 2H_2O$ (Lee et al., 2012). Plants were then incubated at 27°C with a 16h/8h day/night cycle for 40 days, watering with ½ B&D media every other day. After 40 days, the plants were removed from the

pouches and the plant growth parameters were measured (nodule pigmentation, nodule number, nodule dry weight, and plant dry weight). Root systems were visualized using a Nikon SMZ-U dissection microscope.

Comparison of nitrogenase enzymatic activities. Gas chromatography (Shimadzu GC2014) was used to conduct an acetylene reduction assay, measuring the activity of the nitrogenase enzyme from both the wild type and mutant (Δp0110) *Bradyrhizobium* sp. BTAi1 strains in converting acetylene to ethylene. *Aeschynomene indica* seeds were sterilized, germinated, and inoculated with either bacterial strain and harvested after 40 days as previously described. Root systems were removed from the stems and transferred to a sterile 50mL glass vial sealed with a serum cap. Ten percent of the total air within the vial was removed and replaced with an equal volume of pure acetylene gas using a gas-tight 5mL syringe (Hamilton Co., Reno, NV). After mixing each vial by vigorous shaking, gas samples 250μL in volume were taken and injected into the gas chromatograph port using nitrogen as the carrier gas and a hydrogen flame detector. Glass vials containing root systems lacking nodules and vials containing no root systems were used as controls. Using standard curves calculated before the experiment with pure ethylene and pure acetylene, the concentration of these gases for each vial could be determined using the obtained peak areas. Samples were measured for two consecutive hours, yielding time points T0, T1, and T2. The average change in ethylene concentration was then calculated. Upon completion of the acetylene reduction assay, root systems and their original stems were dried and measured together to obtain a total plant dry weight. The nodules were then removed using a sterile razor blade with visual aid through the Nikon SMZ-U dissection microscope, and the root and stem systems measured again, to obtain root nodule dry weights.

RESULTS AND DISCUSSION

Identification of *p0110* as a potential gene encoding signaling molecule. For selection of potential signal molecule encoding genes involved in the NF-independent nodulation mechanism between *Bradyrhizobium* sp. BTAi1 and *A. indica*, gene expression analyses were conducted previously in this lab. Briefly, BTAi1 cultures were exposed to root exudates from *A. indica* plants and the resulting transcriptome was obtained. The transcriptomes of two conditions, BTAi1 exposed to root exudate and BTAi1 not exposed to root exudate, were compared to one another and genes that were up-regulated were reviewed for potential signal molecule characteristics. Among the top 3 up-regulated (Table 4-1), *Bbta_p0110* was initially selected due to its proposed function as a putative exported protein and positive fold change value of 6.76 while the other top up-regulated genes were hypothetical or already characterized as metabolically significant. The reasoning behind this selection was such that if we are looking for a signal molecule produced by the bacteria in response to the host plant root exudates (containing flavonoids), then we expect such a molecule to be exported and recognized by the host plant.

Comparison of growth kinetics between the wild type and mutant (Δp0110). Growth curve analysis conducted between wild type and the Δp0110 mutant demonstrate that while this gene was up-regulated in response to host plant root exudates, it plays no significant role in the growth of this organism under normal conditions (Figure 4-1). Being responsible for the transcription of a putative exported protein, this gene may not play a role in general metabolic processes with BTAi1 and might be specified more towards cell-to-cell signaling or host-symbiont interactions.

Nodule formation. In *A. indica* plants inoculated with *Bbta_p0110* disrupted BTAi1 cells (Δp0110), total nodule counts were significantly decreased with only a 2 out of more than 40

plants forming functioning pigmented nodules, non-nitrogen fixing pale nodules, or pigmented lateral root emergence sites. For total nodule dry weights, Δp0110 inoculated plants had an average mass of 0.058 (±0.01) mg while wild type inoculated plants had an average mass of 0.6 (±0.07) mg, an almost 10-fold difference (Figure 4-2A). The number of pigmented nodules of 1mm or larger in size formed in Δp0110 inoculated plants averaged to be less than 1 at 0.04 (±0.02) and 3.5 (±0.5) in wild type inoculated plants (Figure 4-2B). Similarly, Δp0110 inoculated plants averaged 0.6 (±0.1) pale nodules while wild type displayed an average of 2.6 (±0.3) pale nodules (Figure 4-2C). Additionally, pigmented initial colonization sites, or emerging later roots, were lower in Δp0110 inoculated plants, average of 0.09 (±0.06), compared to wild type inoculated plants with an average of 1.6 (±0.5) (Figure 4-2D). The distinct differences in root phenotypes among the two plant treatment groups can be seen in Figure 4-3, depicting root systems from both groups.

Host plant biomass. After inoculation with either bacterial strain, total plant dry weights were recorded. The average plant dry masses were recorded before the nodules were excised for nodule numeration analysis. *A. indica* plants inoculated with Δp0110 BTAi1 have an average mass of 10.03 (±0.35) mg whereas wild type inoculated plants had an average mass of 22.39 (±4.05) mg (Figure 4-4A). The wild type inoculated plants had an average mass more than twice that of those inoculated with p0110 deficient BTAi1 cells.

Nitrogenase activity. The enzymatic ability of nitrogenase from wild type and p0110 deficient BTAi1 strains was determined through an acetylene reduction assay of the entire root systems for either plant treatment group harvested at 40 days of growth. Root nodules formed on *Aeschynomene indica* plants, even at full maturation, are small (1-2mm diameter) so to accurately measure the nitrogenase activity of the nodules without harming their structural

integrity, the entire root system is used in this analysis. As mentioned, Δp0110 BTAi1 inoculated plants did not form a comparable amount of pigmented, nitrogen fixation capable nodules. However, to ensure the bacteria were not supplying or readily fixing atmospheric nitrogen, these root systems were analyzed through this technique. The average nitrogenase activity in converting ethylene to acetylene across two hours for wild type inoculated plants was found to be 0.417 (±0.07) ethylene μMoles/nodule weight (g)/minute and an average rate of -1.081 (±0.19) ethylene μMoles/nodule weight (g)/minute in Δp0110 BTAi1 inoculated plants, consistent with the natural rate of ethylene degradation in nature (Figure 4-4B).

NF-independence and pathway to cross-crop nodulation. Nodulation is an integral part of the utilization of leguminous crops as a method of nutrient acquisition and cycling in the effort to reduce dependence on costly synthetic nitrogenous fertilizers (Stagnari et al., 2017). The unfortunate perspective of this relationship is that it is restricted only to crops of the *Fabaceae* family, in the agricultural aspect (Triplett & Sadowsky, 1992). These leguminous crops and their endosymbionts responsible for nitrogen fixation are restricted and highly dependent on the production of NF signal molecules and the nodules these induce (Vijn et al., 1993). The discovery of and potential insights gained through studying the mechanisms behind this symbiotic relationship, with or without these NF signal molecules, could lead to revolutionary agricultural practices such as the induction of nodulation or growth promotion on non-nodule forming cash crops such as corn, canola, cotton, etc. (Marsh et al., 2007; Prithiviraj et al., 2003). Investigations into NF-independent nodulation hold high hopes of providing methods for transferring this nodulation ability across plant families due to the simplistic method of endophyte introduction to plant tissues coupled with the ability to fix atmospheric nitrogen and

produce a more robust crop in a natural way (Guha et al., 2022; Okazaki et al., 2016; Patra & Mandal, 2022).

***Bradyrhizobium* sp. BTAi1 model for NF-independent nodulation.** Tropical legumes such as *Aeschynomene* and their endosymbionts are being studied for this exact reason (Bonaldi et al., 2011). One such endosymbiont, *Bradyrhizobium* sp. BTAi1 provides an excellent model for studying this NF-independent nodulation system particularly because it has a plasmid that, if housing symbiosis related genes, could be transformed to other potentially endophytic species, allowing for a more diverse arsenal of molecular mechanisms to be utilized benefiting the host plant in growth, yield, defense, and survivability under various abiotic stressors (Cytryn et al., 2008; Giraud et al., 2007). In this study, the plasmid located gene *Bbta_p0110* in *Bradyrhizobium* sp. BTAi1 was selected for disruption and consequential nodulation morphology, due to its upregulation in response to *A. indica* root exudates. This gene encodes a putative, uncharacterized exported protein that may play a role in the induction of the host immune response required for nodule formation. Determining the role of this gene could play a key part in unlocking the pathway to nodule formation abilities in non-nodule forming bacteria through plasmid transformation. Additionally, understanding this relatively primitive nodulation mechanism will provide insights into how we can one day induce nodulation in non-leguminous crop plants.

***Bbta_p0110* directed regulation of NF-independent nodulation and nitrogen fixation in *A. indica* plants.** The *Bbta_p0110* gene was disrupted with a kanamycin resistance gene, allowing a look into the resulting *A. indica* nodulation and BTAi1 nitrogenase behaviors that follow, in the absence the synthesized p0110 protein. To ensure the disruption of the *p0110* gene did not have an inhibitory effect on the growth of BTAi1, a growth curve was completed and demonstrated

almost no difference in growth rate between the two strains (Figure 4-1). After inoculation with p0110 disrupted BTAi1 cells, *A. indica* plants displayed a severe inhibition of root nodule formation and a decrease in total plant biomass by half. Total nodule formation, in terms of dry mass, was reduced by 10-fold in the mutant treatment group as compared to treatment with the wild type train (Figure 4-2A). This includes the mass of all nodule types (pale & pigmented) as well as the removal of pigmented lateral root emergence points if present. Additionally, the occurrence of pale and pigmented nodules was drastically reduced in plants treated with Δp0110 cells (Figures 4-2B&C) as compared to the wild type. Interestingly, the plants in this treatment group still had the ability to form pale nodules (Figure 4-2D), albeit at a lower rate than that of the wild type. In contrast to this and most importantly, pigmented nodule formation was extremely rare, only cropping up on a couple out of more than 40 plants treated with the Δp0110 mutant strain. There was also a distinct lack of pigmented points of emerging lateral roots along the tap root even though the bacteria were able to penetrate into cortical tissues and persist at least temporarily, clearly indicated by the pale nodule formation (Figure 4-2C). There was also an absence of nitrogenase activity in root systems inoculated with p0110 disrupted BTAi1 as compared to the wild type, as would be expected considering the lack of pigmented nodule formation, typically housing the nitrogen-fixation reaction (Figure 4-4B). The decrease in ethylene across the two-hour measured time frame indicates the natural decomposition of ethylene not being overcome by nitrogenase activity. This suggests that even if the p0110 disrupted BTAi1 cells are able to temporarily colonize the rhizosphere and enter the subdermal tissue layers, they are not able to properly establish an anoxic, energy rich environment conducive to the fixation of atmospheric acetylene to ethylene. However, the fact that very little pigmentation, resulting from leghemoglobin produced by the plant in response to endosymbiosis

establishment, indicates that while Δp0110 BTAi1 may penetrate to the cortical tissues and initiate nodule primordium development, successful proliferation of the bacteria was halted.

Role of molecular regulation in NF-independent nodulation. It is known that nodules typically form as a result of a complex, modified immune response geared toward forming symbiotic nodule organs to facilitate the nitrogen-fixation process. While the initial steps of rhizosphere colonization are similar no matter to nodulation method (NF-dependent vs NF-independent), wherein soil dwelling or seed deposited bacteria are attracted to the root surface through detection of plant released root exudates during growth, the location of colonization and proliferation differ (Barbour et al., 1991; Gaworzewska & Carlile, 1982; Lopes et al., 2016; Mills & Baver, 1985). In the preferred method employed for the NF-independent nodulation system, bacteria congregate along the root system at points of lateral root emergence (Alazard, 1985; Arora, 1954; Yatazawa et al., 1984). These bacteria may then enter through micro fissures, crack entry, intracellularly making their way to the cortical tissues (Boogerd & van Rossum, 1997; Eaglesham & Szalay, 1983). Therefore, the first signs of potential nodulation would include increased microbial populations in the subdermal layers surrounding an emerging lateral root (Alazard & Duhoux, 1988; Loureiro et al., 1995; Yatazawa et al, 1984). If the invading bacteria do not progress beyond this point, they will instead proliferate in these subdermal tissues, resulting in pigmentation from leghemoglobin to assist in the nitrogen fixation process in response to endosymbiont presence (Appleby, 1984; Chihaoui et al., 2022; Navascues et al., 2012). These first steps, if never extended further into the cortical tissues, could provide insight into the initial evolutionary steps to develop the processes behind this symbiotic relationship by drawing a clear distinction between endophytes capable or incapable of penetration beyond the outer most subdermal tissue layers. Those that are able to penetrate further into the root cortex

but do not successfully invade nodule primordial tissues or cannot establish a growing population within these spaces would not be assimilated and passed on to primordial nodule daughter cells, resulting in pale, non-nitrogen fixing nodules. However, if successful penetration and further distribution into dividing primordial nodule cells is achieved, the result is mature, pigmented nodules capable of nitrogen fixation (Bonaldi et al., 2011). This whole process is completed in the absence of the canonical signal molecules, NFs, under the NF-independent system. However, it is still believed that there must be some signal molecule produced by these bacteria to elicit the required immune response of initial cortical cell death and primordia infection, though it may not be as complex of a system as the formerly mentioned NF-dependent mechanisms.

Role of *Bbta_p0110* in successful root colonization and nodule formation. The obtained results, after knocking out the *Bbta_p0110* gene function, demonstrated a clear inability for Δp0110 BTAi1 cells to (1) establish a successful and growing population neither within the subdermal tissue layers nor on the root surface (2) fully assimilate within nodule primordia cells. The mutant was not able to perpetuate its root colonization as indicated by the decreased lateral root emergence sites (Figure 4-2D). However, they clearly were able to penetrate to the inner cortical tissues and induce the early stages of root nodule formation as indicated by the appearance, although diminished amounts, of pale nodules. The steps that would typically follow this root nodule primordia formation would be the successful assimilation of the bacteria into the resulting nodule daughter cells. This crucial step in nodule organogenesis did not occur, as indicated by the distinct lack of pigmented nodule formation that would arise with successful endosymbiont establishment in nodule primordial tissue (Figure 4-2B). These results support the hypothesis that the hypothetical, putative exported protein synthesized from *Bradyrhizobium* sp.

BTAi1's *Bbta_p0110* gene plays a role in nodule formation within the host plant *Aeschynomene indica.* While the bacteria were able to initially colonize the root surface and penetrate to the inner cortical layers to start the very early stages of nodule primordia formation, they were not able to successfully assimilate within primordial cells and drive nodule maturation, leaving only small, pale nodules behind. Furthermore, the Δp0110 BTAi1 cells were not able to maintain their colonization of the root surface or subdermal layers as indicated by the lack of nitrogenase activity that would otherwise be expected from these organisms (Figure 4-4B). With all the obtained observations and data, one pertinent question remains: what happened to the bacteria? If they were able to initialize colonization but it did not persist, where did they go? To answer this question, be reminded that studies have shown plants will participate, such as ones grown in these conditions, will participate in rhizophagy and actively destroy and absorb endophytic bacteria as supplemental nutrient sources. These plants are grown strictly in a nitrogen free environment to encourage the formation of this symbiotic relationship else they suffer nutritionally (Figure 4-4A). However, if the bacteria are unable to participate in this relationship through nodule formation, the plant has no other option than to destroy and consume the microscopic inhabitants, taking in the only nutritional source available to them. Further studies need to be completed to fully claim the nodulation role of the *Bbta_p0110* gene, such as transformation of the plasmid housing this gene to a non-nodulating endophyte and observing the resulting nodulation response. Additionally, from the plant perspective, studies involving the plant gene expression when exposed to p0110 protein should be conducted to better understand the host immune response involved in this relationship.

The complex dynamics involved in plant-microbe interactions are fascinating and only further show just how intricate the roles in nature have become since the beginning. Bacteria and

plants are in constant communication, interacting with one another in the natural world. These interactions have led to the development of remarkable pathways and relationships that either support or hinder one another. The findings obtained from this study indicate that *Bbta_p0110* certainly plays a significant role in the assimilation and further distribution of *Bradyrhizobium* sp. BTAi1 cells throughout nodule tissues. While this gene may not the be only signal used in this NF-independent nodulation system, understanding its function helps shed light on the potential molecular cascade involved in this symbiotic relationship. Additionally, the results obtained suggest an interesting and complex battle for survival by both the host plant and endosymbiont with a relationship that could be broken at any time should the host find it is not receiving the required nutritional support. Understanding these amazingly complex and diverse endophyte/plant interactions will undoubtedly lead to new discoveries and advancements to our current agricultural systems. The global population is growing, environmental conditions are becoming more hostile, and we need to use the tools already available to us to combat and overcome these challenges.

Table 4-1. Top 3 up regulated unique gene expression of BTAi1 exposed to root exudate from *A. indica* plants.

GeneID	GeneName	log2FC	p-adj	FC	Function
640555469	Bbta_0450	3.95	0.05	15.43	fructose-bisphosphate aldolase (EC 4.1.2.13)
640539027	Bbta_p0110	2.76	0.05	6.76	putative exported protein of unknown function
640555536	Bbta_0522	0.65	0.05	1.56	hypothetical protein

Table 4-2. List of bacterial strains and plasmids used in this study.

Strain or Plasmid	Genotype or phenotype	Reference
***Escherichia coli* strains**		
DH5α	supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 F' (traD36, proAB+ lacIq, Δ(lacZ)M15) endA1 recA1 hsdR17 mcrA supE44λ gyrA96 relA1	Bethesda Research Laboratories
***Bradyrhizobium* sp. strains**		
BTAi1	Cm^R Wild type	(Giraud et al.)
Δ*p0110* mutant	Cm^R Km^R *p0110* :: Km	This work
Plasmids		
pKD4	Km^R expression vector	(Datsenko and Wanner 2000)
pKD78	Cm^R Lambda Red recombinase expression plasmid	(Datsenko and Wanner 2000)
pRK2013	Km^R cloning vector	(Ditta et al.1980)
pJQ200SK	Gm^R *sacB* suicide vector	(Quandt and Hynes 1993)
pJQ200SK-*p0110*	Gm^R suicide vector containing *p0110* gene	This work
pKD78-pJQ200SK-*p0110*-λ	Gm^R Cm^R suicide vector containing *p0110* gene with Lambda Red recombinase expression plasmid	This work
pKD78-pJQ200SK-*p0110*-Km	Gm^R Km^R suicide vector containing *p0110* gene substituted with kanamycin cassette	This work

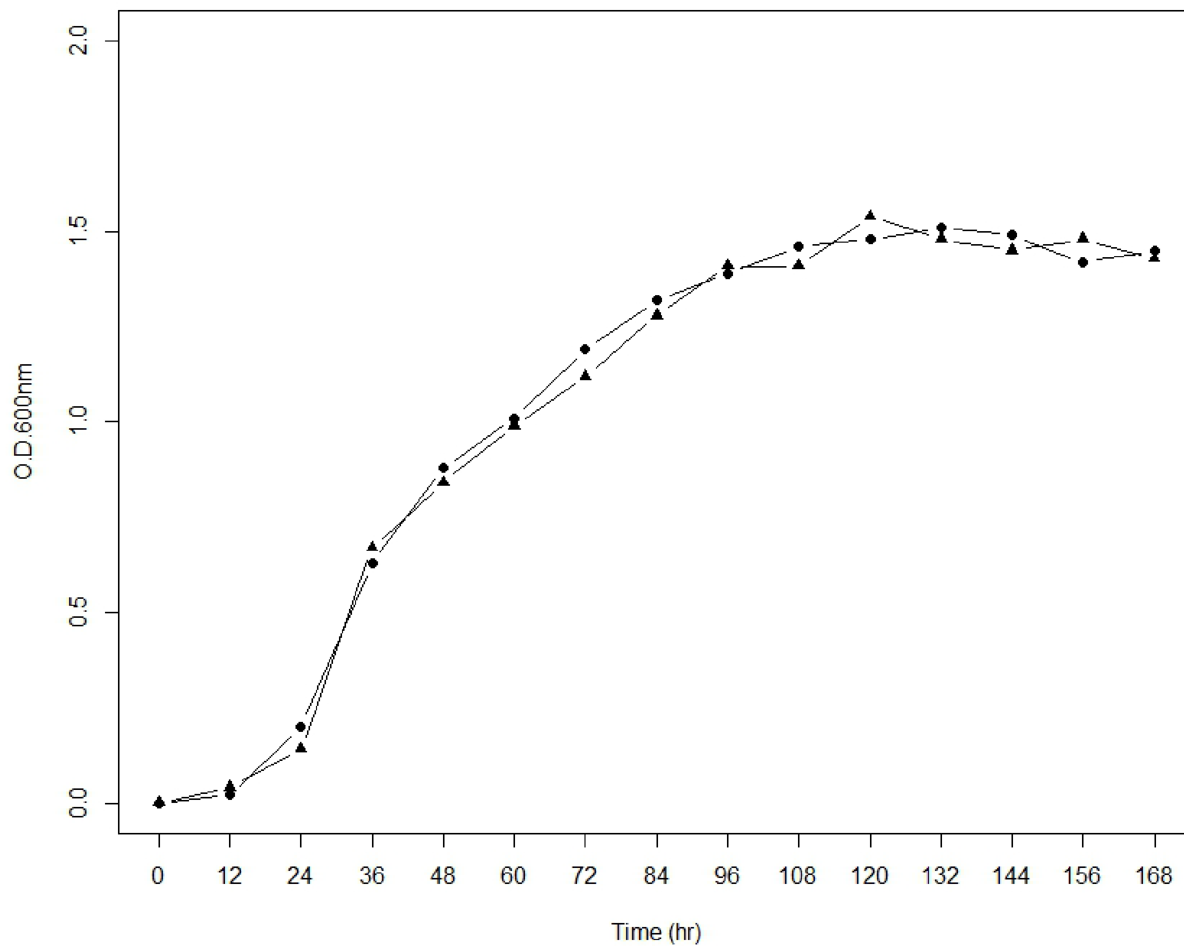

Figure 4-1. Growth of *Bradyrhizobium* sp. BTAi1 (wild type)(● symbol) and Δp0110 mutant (▲ symbol) strains over 7 days. Each time point represents the average OD_{600} value for three replicates.

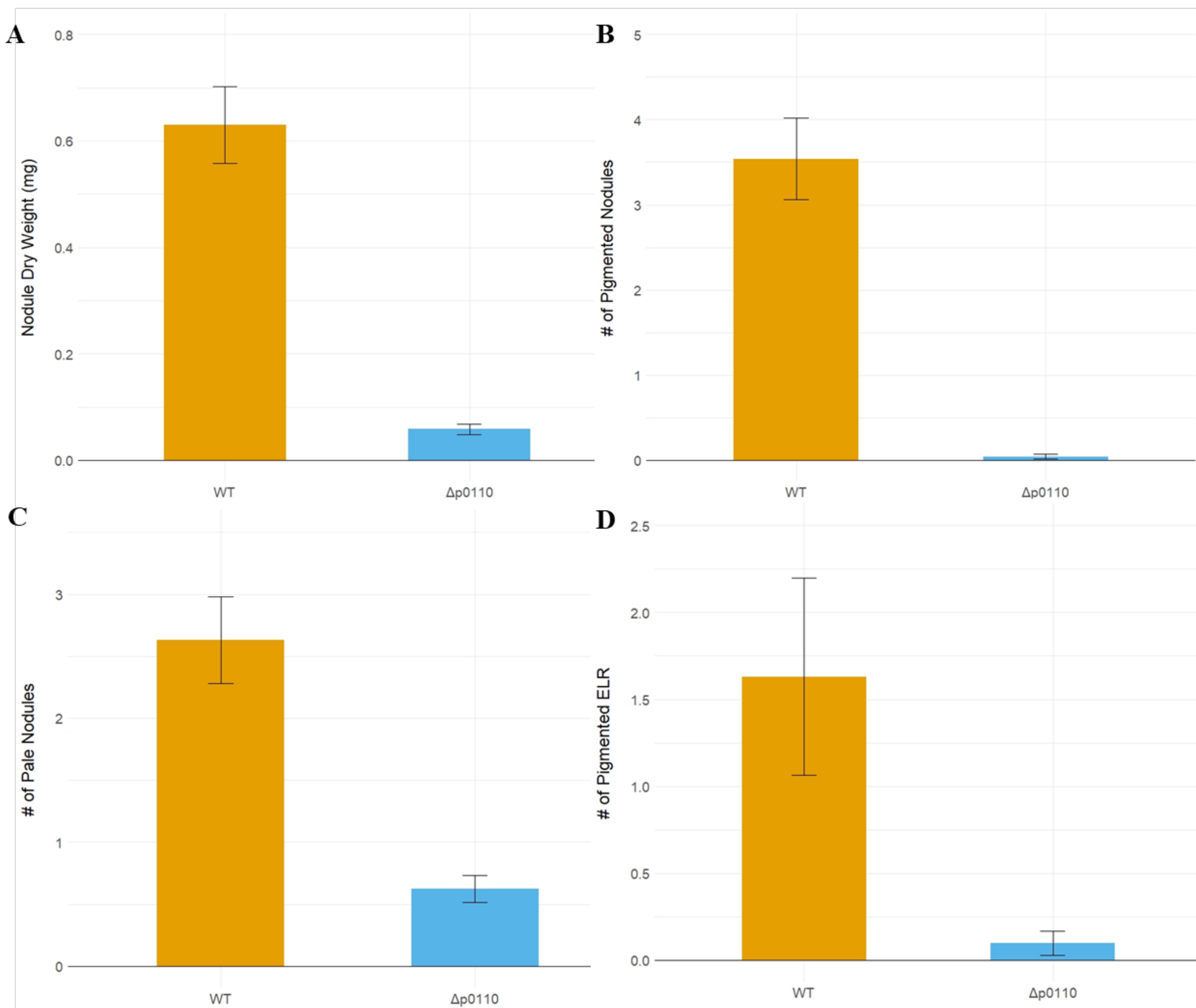

Figure 4-2. *A. indica* plant growth parameters after inoculation with either wild type or Δp0110 *Bradyrhizobium* sp. BTAi1 strains. (A) Average nodule dry weights reported in mg (B) Average number of pigmented nodules observed (C) Average number of pale nodules observed (D) Average number of pigmented emerging later root sites observed**.**

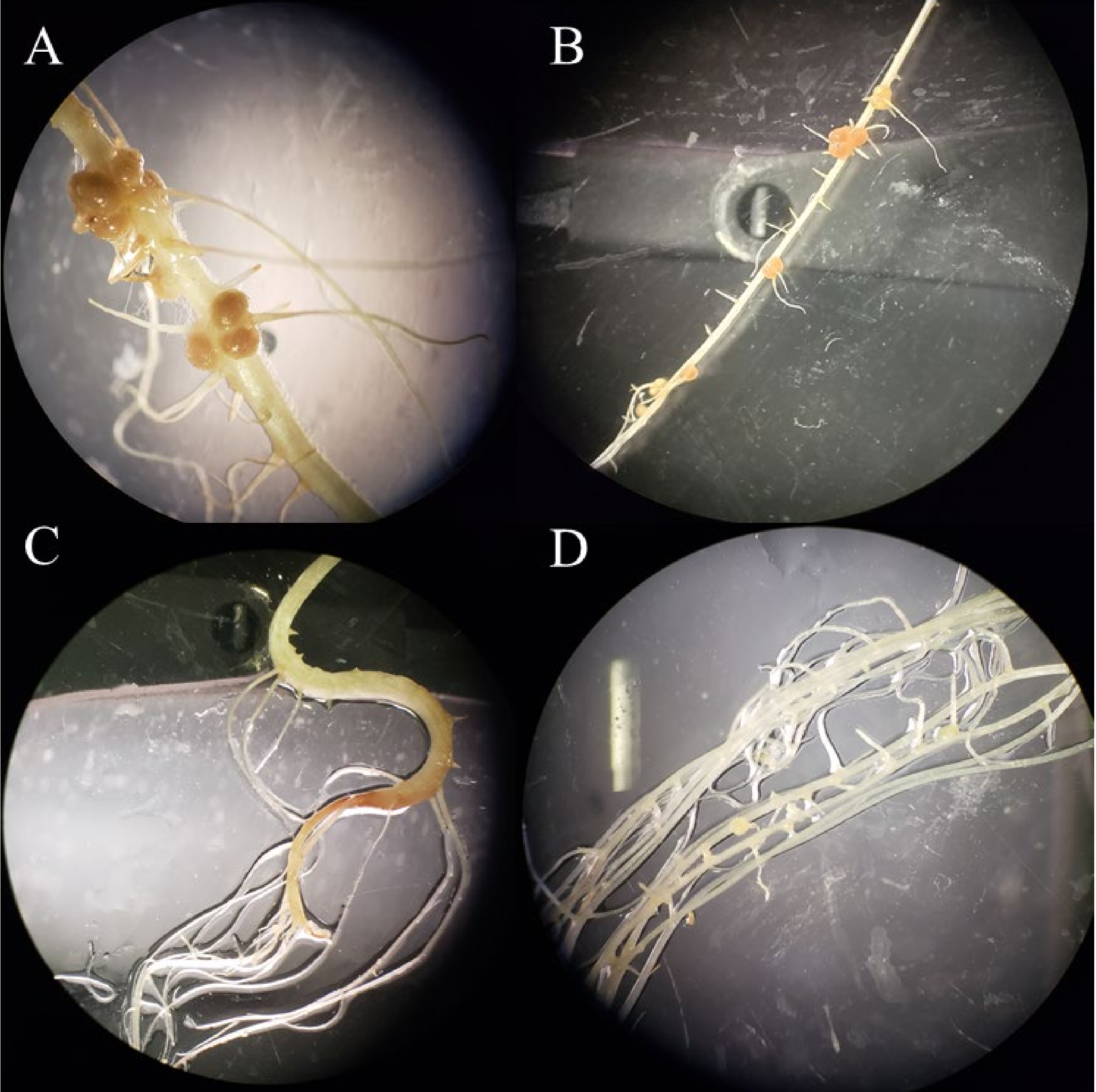

Figure 4-3. Dissection microscopy images depicting root systems of *A. indica* inoculated with (A & B) wild type *Bradyrhizobium* sp. BTAi1 or (C & D) Δp0110 *Bradyrhizobium* sp. BTAi1.

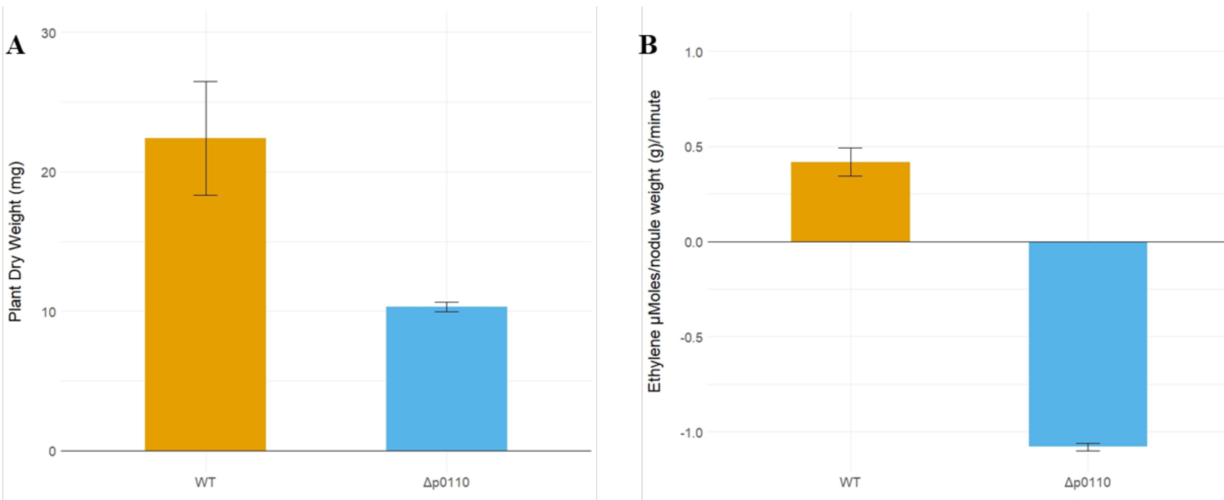

Figure 4-4. Average total plant dry weights (A) and average changes in ethylene (B) observed in *A. indica* plants inoculated with either wild type or Δp0110 *Bradyrhizobium* sp. BTAi1 bacteria after 40 days

REFERENCES

Aguilar, J.M., Ashby, A.M., Richards, J.M., Loake, G.J., Watson, M.D., & Shaw, C.H. 1988. Chemotaxis of Rhizobium leguminosarum biovar phaseoli towards flavonoid inducers of the symbiotic nodulation genes. *J. Gen. Microbiol.* **134**, 2741-2746.

Alazard, D. 1985. Stem and root nodulation in Aeschynomene spp. *Appl. Environ. Microbiol.* **50(3)**, 732-734.

Alazard, D., & Duhoux, E. 1988. Diversity of stem nodulation sites in Aeschynomene spp. *J. Plant. Physiol.* **132**, 123-125.

Alazard, D., & Duhoux, E. 1990. Development of stem nodules in a tropical forage legume, Aeschynomene afraspera. *J. Exp. Bot.* **41**, 1199-1206.

Appleby, C.A. 1984. Leghemoglobin and Rhizobium respiration. *Ann. Rev. Plant Physiol.* **35,** 443-478.

Armitage, J.P., Gallagher, A., & Johnston, A.W.B. 1988. Comparison of the chemotactic behaviour of Rhizobium leguminosarum with and without the nodulation plasmid. *Mol. Microbiol* **2**, 743-748.

Arora, N. 1954. Morphological development of the root and stem nodules of Aeschynomene indica l. *Phytomorph.* **4**, 211-216.

Barbour, W.M., Hattermann, D.R., & Stacey, G. 1991. Chemotaxis of Bradyrhizobium japonicum to soybean exudates. *Appl. Environ. Microbiol.* **57(9)**, 2635-2639.

Becana, M., Matamoros, M.A., Udvardi, M., & Dalton, D.A. 2010. Recent insights into antioxidant defenses of legume root nodules. *New Phytol.* **188**, 960-976.

Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. *J. Bacteriol.*, **62**, 293–300.

Bhuvaneswari, T.V., & Solheim, B. 1985. Root hair deformation in the white clover/Rhizobium trifolii symbiosis. *Physiol. Plant.* **68**, 1144-1149.

Bonaldi, K., Gherbi, H., Franche, C., Bastien, G., Fardoux, J., Barker, D., Giraud, E., & Cartieaux, F. 2010. The nod factor-independent symbiotic signaling pathway: development of Agrobacterium rhizogenes-mediated transformation for the legume Aeschynomene indica. *Mol. Plant Microbe Interact.* **23**, 1537-1544.

Bonaldi, K., Gargani, D., Prin, Y., Fardoux, J., Gully, D., Nouwen, N., Goormachtig, S., & Giraud, E. 2011. Nodulation of Aeschynomene afraspera and A. indica by photosynthetic Bradyrhizobium sp. Strain ors285: the nod-dependent versus the nod-independent symbiotic interaction. *Mol. Plant. Microbe. Interact.* **24(11)**, 1359-1371.

Boogerd, F.C., & van Rossum, D. 1997. Nodulation of groundnut by Bradyrhizobium: a simple infection process by crack entry. (*Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* **21**, 5-27.

Brewin, N.J. 1991. Development of the legume root nodule. *Annu. Rev. Cell. Biol.* **7**, 191-226.

Brussel, A.A.N., Zaat, S.A.J., Canter-Cremers, H.C.J., Wijfelman, C.A., Pees, E., & Lugtenberg, B.J.J. 1986. Role of plant root exudate and sym plasmid-localized nodulation

genes in the synthesis by Rhizobium leguminosarum of trs factor, which causes thick and short roots on common vetch. *J. Bacteriol.* **165**, 517-522.

Caetano-Anolles, G., Wrobel-Boerner, E., & Bauer, W.D. 1992. Growth and movement of spot inoculated Rhizobium meliloti on the root surface of alfalfa. *Plant Physiol.* **98**, 1181-1189.

Chaintreuil, C., Giraud, E., Prin, Y., Lorquin, J., Ba, A., Gillis, M., de Lajudie, P., & Dreyfus, B. 2000. Photosynthetic bradyrhizobia are natural endophytes of the African wild rice Oryza breviligulata. *Appl. Envrion. Microbiol.* **66(12)**, 5437-5447.

Chaintreuil, C., Perrier, X., Martin, G., Fardoux, J., Lewis, G.P., Brottier, L., Rivallan, R., Gomez-Pachecco, M., Bourges, M., Lamy, L., Thilbaud, B., Ramanankierana, H., Randriambanona, H., Vandrot, H., Mournet, P., Giraud, E., & Arrighi, J.F. 2018. Naturally occurring variations in the nod-independent model legume Aeschynomene evenia and relatives: a resource for nodulation genetics. *BMC Plant Biol.* **18(54)**, 1-15.

Chihaoui, S.A., Barhoumi, F., Taamalli, W., Wahab, M.A., Mhamdi, R., & Mhadhbi, H. 2022. Contrasting effects of the inoculation time with passenger endophytic Agrobacterium sp. 10c2 on the nodule functioning and growth of Medicago truncatula. *Rhizosphere.* **22,** 100505.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clement, C., Barka, E.A. 2005. Endophytic colonization of Vitis vinifera l. by plant growth-promoting bacterium Burkholderia sp. strain psjn. *Appl. Environ. Microbiol. Plant Mircobiol.* **71(4)**, 1685-1693.

Cytryn, E.J., Jitacksorn, S., Giraud, E., & Sadowsky, M.J. 2008. Insights learned from pBTAi1, a 229-kb accessory plasmid from Bradyrhizobium sp. strain BTAi1 and prevalence of accessory plasmids in other Bradyrhizobium sp. strains.

Datsenko, K. A., & Wanner, B. L. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.,* **97**, 6640–6645.

Ding, Y., & Oldroyd, G.E.D. 2009. Positioning the nodule, the hormone dictum. *Plant Signal. Behav.* **4(2)**, 89-93.

Ditta, G., Stanfield, S., Corbin, D., Helinski, D. 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. *Proc. Natl. Acad. Sci.* **77(1),** 7347-7351.

Dudeja, S.S., & Giri, R. 2014. Beneficial properties, colonization, establishment and molecular diversity of endophytic bacteria in legumes and non legumes. *Afr. J. Microbiol. Res.* **8(15)**, 1562-1572.

Eaglesham, A.R.J., & Szalay, A.A. 1983. Aerial stem nodules on Aeschynomene spp. *Plant Sci. Lett.* **29**, 265-272.

Gage, D.J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.* **268**, 280-300.

Gaworzewska, E.T., & Carlile, M.J. 1982. Positive chemotaxis of Rhizobium leguminosarum and other bacteria towards root exudates from legumes and other plants. *J. Gen. Microbiol.* **128,** 789-798.

Giraud, E., & Fleischman, D. 2004. Nitrogen-fixing symbiosis between photosynthetic bacteria and legumes. *Photosynth. Res.* **82**, 115-130.

Giraud, E., Moulin, L., Vallenet, D., Barbe, V., Cytryn, E., Avarre, J. C., Jaubert, M., Simon, D., Cartieaux, F., Prin, Y., Bena, G., Hannibal, L., Fardoux, J., Kojadinovic, M., Vuillet, L., Lajus, A., Cruveiller, S., Rouy, Z., Mangenot, S., Segurens, B., Dossat, C., Franch, W. L., Chang, W. S., Sauders, E., Bruce, D., Richardson, P., Normand, P., Dreyfus, B., Pignol, D., Stacey, G., Emerich, D., Vermeglio, A., Medigue, C., Sadowsky, M. 2007. Legumes symbioses: absence of nod genes in photosynthetic Bradyrhizobia. *Science*. **316(5829)**, 1307-1312.

Gottfert, M. 1993. Regulation and function of rhizobial nodulation genes. *Microbiol. Revs.* **104**, 39-64.

Guha, S., Molla, F., Sarkar, M., Ibanez, F., Fabra, A., & DasGupta, M. 2022. Nod factor-independent 'crack-entry' symbiosis in dalbergoid legume Arachis hypogaea. *Environ. Microbiol.* **24,** 2732-2746.

Gully, D., Czernic, P., Cruveiller, S., Mahe, F., Longin, C., Vallenet, D., Francois, P., Nidelet, S., Rialle, S., Giraud, E., Arrighi, J.F., DasGupta, M., & Cartieaux, F. 2018. Transcriptome profiles of nod factor-independent symbiosis in the tropical legume Aeschynomene evenia. *Sci. Rep.* **8**, 1-12.

Halverson, L.J., & Stacey, G. 1986. Signal exchange in plant-microbe interactions. *Microbiol. Rev.* **50**, 193-225.

Heckmann, A.B., Sandal, N., Bek, A.S., Madsen, L.H., Jurkiewicz, A., Nielson, M.W., Tirichine, L., Stougaard, J. 2011. Cytokinin induction of root nodule primordia in Lotus japnicus is regulated by a mechanism operating in the root cortex. *Mol. Plant Microbe. Interact.* **24(11)**, 1385-1395.

Hong, G.F., Burn, J.E., & Johnston, A.W. 1987. Evidence that DNA involved in the expression of nodulation (nod) genes in Rhizobium binds to the product of the regulatory gene nodD. *Nucleic Acids. Res.* **15(23)**, 9677-9690.

Horvath, B., Bachem, C.W.B., Schell, J., & Kondorosi, A. 1987. Host-specific regulation of nodulation genes in Rhizobium is mediated by a plant-signal, interacting with the nod gene product. *EMBO J.* **6(4)**, 841-848

Iyer, B., & Rajkumar, S. 2017. Host specificity and plant growth promotion by bacterial endophytes. *Curr. Res. Microbiol. Biotechnol.* **5(2)**, 1018-1030.

Ladha, J.K., Pareek, R.P. So, R., Becker, M. 1990. Stem-nodule symbiosis and its unusual properties. *Nitrogen Fixation*. 633-640.

Lancelle, S.A., & Torrey, J.G. 1984. Early development of Rhizobium-induced root nodules of Parasponia rigida. II Nodule morphogenesis and symbiotic development. *Can. J. Bot.* **63**, 25-35.

Lee, H.I., Lee, J.H., Park, K.H., Sangurdekar, D., and Chang, W.S. 2012. Effect of soybean coumestrol on Bradyrhizobium japonicum nodulation ability, biofilm formation, and transcriptional profile. *Appl. Environ. Microbiol*. **78**, 2896-2903.

Liu, H., Carvalhais, L.C., Crawford, M., Singh, E., Dennis, P.G., Pieterse, C.M.J., & Schenk, P.M. 2017. Inner plant values: diversity, colonization and benefits from endophytic bacteria. *Front. Microbiol.* **8**, 1-17.

Lopes, L.D., Pereira e Silva, M.C. & Andreote, F.D. 2016. Bacterial abilities and adaptation toward the rhizosphere colonization. *Front. Microbiol.* **7**, 1-13.

Loureiro, M.F., James, E.K., Sprent, J.I & Franco, A.A. 1995. Stem and root nodules on the tropical wetland legume, Aeschynomene fluminensis. *New Phytol.* **130**, 531-544.

Love, M. I, Huber, W., & Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. **15,** 550.

Madigan, M.T. 1995. Microbiology of nitrogen fixation by anoxygenic photosynthetic bacteria. *Anoxygenic Photosynthetic Bacteria. Advances in Photosynthesis and Respiration*. **2**, 915-928.

Madsen, L.H., Tirichine, L., Jurkiewiez, A., Sullivan, J.T., Heckmann, A.B., Bek, A.S., Ronson, C.W., James, E.K., & Stougaard, J. 2010. The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. *Nat. Commun.* **1**, 1-10.

Marsh, J.F., Rakocevic, A., Mitra, R.M., Brocard, L., Sun, J., Eschstruth, A., Long, S.R., Schultze, M., Ratet, P., & Oldroyd, G.E. 2007. Medicago truncatula nin is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiol.* **144,** 324-335.

Masson-Boivin, C., & Sachs, J.L. 2018. Symbiotic nitrogen fixation by rhizobia – the roots of a success story. *Curr. Opin. Plant Biol.* **44**, 7-15.

Mills, K.M. & Bauer, W.D. 1985. Rhizobium attachment to clover roots. *J. Cell. Sci. Suppl.* **2**, 333-345.

Molouba, F., Lorquin, J., Willems, A., Hoste, B., Giraud, E., Dreyfus, B., Gillis, M., de Lajudie, P., & Masson-Boivin, C. 1999. Photosynthetic bradyrhizobia from Aescynomene spp. are specific to stem-nodulated species and form a separate 16S ribosomal dna restriction fragment length polymorphism group. *Appl. Environ. Microbiol. Plant Microbiol.* **65(7)**, 3084-3094.

Navascues J., Perez-Rontome, C., Gay, M., Marcos, M., Yang, F., Walker, F.A., Desbois, A., Abian, J., & Becana, M. 2012. Leghemoglobin green derivatives with nitrated hemes evidence production of highly reactive nitrogen species during aging of legume nodules. *PNAS.* **109,** 2660-2665.

Newcomb, W. 1981. Nodule morphogenesis and differentiation. *Int. Rev. Cytol. Suppl.* **13**, 246-298.

Okazaki, S., Tittabutr, P., Teulet, A., Thouin, J., Fardoux, J., Chaintreuil, C., Gully, D., Arrighi, J.F., Furuta, N., Miwa, H., Yasuda, M., Nouwen, N., Teaumroong, N., & Giraud, E. 2016. Rhizobium-legume symbiosis in the absence of Nod factors: two possible scenarios with or without the T3SS. *ISME J.* **10,** 64-74.

Patra, D., & Mandal, S. 2022. Nod-factors are dispensable for nodulation: a twist in bradyrhizobia-legume symbiosis. *Symbiosis.* **86,** 1-15.

Prithiviraj, B., Zhou, X., Souleimanov, A., Kahn, W., & Smith, D. 2003. A host-specific bacteria-to-plant signal molecule (nod factor) enhances germination and early growth of diverse crop plants. *Planta*. **216,** 437-445.

Quandt, J., & Hynes, M.F. 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene*, **127**, 15–21.

Rhijn, P., & Vanderleyden, J. 1995. The Rhizobium-plant symbiosis. *Microbiol. Rev.* **59(1)**, 124-142.

Rossen, L., Johnston, A.W.B., & Downie, J.A. 1984. DNA sequence of the rhizobium leguminosarum nodulation genes nodAB and C required for root hair curling. *Nucleic Acids Res.* **12,** 9497-9508.

Sadowsky, M.J., Tully, R.E., Cregan, P.B., Keyser, H.H. 1987. Genetic diversity in Bradyrhizobium japonicum serogroup 123 and its relation to genotype-specfic nodulation of soybean. *Appl. Environ. Microbiol*. **53(11)**, 2624-2630.

Smit, G., Swart, S., Lugtenberg, B.J.J. & Kijne, J. 1992. Molecular mechanisms of attachment of Rhizobium bacteria to plant roots. *Mol. Microbiol.* **6,** 2897-2903.

Spaink, H.P. 2000. Root nodulation and infection factors produced by rhizobial bacteria. *Ann. Rev. Microbiol*. **54**, 257-288.

Stagnari, F., Maggio, A., Galieni, A., & Pisante, M. 2017. Multiple benefits of legumes for agriculture sustainability: an overview. *Chem. Biol. Technol. Agric.* **4,** 1-13.

Triplett, E.W., & Sadowsky, M.J. 1992. Genetics of competition for nodulation of legumes. *Annu. Rev. Microbiol.* **46,** 399-428.

Vasse, J., & Truchet, G. 1984. The Rhizobium-legume symbiosis: observation of root infection by bright-field microscopy after staining with methylene blue. *Planta*. **161**, 487-489.

Vijn, I., Neves, L.D., Kammen, A.B., Franssen, H., & Bisseling, T. 1993. Nod factors and nodulation in plants. *Science.* **260,** 1764-1765.

Wood, S.M., & Newcomb, W. 1989. Nodule morphogenesis: the early infection of alfalfa (Medicago sativa) root hairs by Rhizobium meliloti. *Can. J. Bot.* **67**, 3108-3122.

Yatazawa, M., Yoshida, S., & Maeda, E. 1984. Fine structure of root nodules of Aeschynomene indica l. *Soil Sci. Plant. Nutr.* **30**, 405-441.

CHAPTER 5

CONCLUSIONS

Endophytic bacteria confer many beneficial characteristics to host plants including, but not limited to, nutrient acquisition, antibiotic production for disease suppression, and overall plant growth and survivability. The formation of nodule organs is one of the most notable results of an endophytic symbiotic relationship, allowing for the fixation of atmospheric nitrogen into forms usable by the host plant. This unique relationship has been incorporated into agricultural systems in the form of crop rotation as means of natural nutrient cycling independent of synthetic chemical fertilizers. Such fertilizers are expensive to produce, and these costs are transferred to the farmers and subsequently the consumers of their products. Additionally, these compounds often leach into the surrounding environment, devastating most often to marine life. Understanding and weaving the special symbiotic relationship between plants and microorganisms into modern agricultural practices will lead to decreased food costs, increased crop production, and reduced negative environmental impacts. While soybean may be the most well-known leguminous crop to utilize such a relationship, there are other, lesser-known plants like *Aeschynomene* that are used as green manure to return nutrients to soil and improve crop production. These plants are particularly special due to their stem nodules and their formation independently of Nod Factor signal molecules. This discovery has lead researchers on a mission to understand and translate this unique relation to non-nodule forming crop plants in hopes of achieving complete independence from synthetic chemical fertilizers.

Using these plants as a model host for NF-independent nodulation, we set out to explore the biogeographical dependencies regarding a host plant's response to endophytic bacteria even

if the host species remains the same. The origin of seeds and how this plays a role in host plant/bacterial inoculant symbiosis is important to understanding how tailor-made crop inoculum might succeed or fail in particular regions of cultivation. Upon inoculation with known NF-independent nodulating bacteria that have formed symbiotic relationships with host *A. indica* plants from Africa and South Korea, we saw a clear difference in how Texas and South Korean plants react to the bacteria. Additionally, there were marked differences in Texas and South Korean plant reactions to Texas native stem nodule isolates, particularly regarding the *Paenibacillus* sp. isolate. Nodule formation is the result of a highly evolved immune response, and the survival and subsequent successful colonization of an endophytic organism depends on their adaptations to the specific host plant.

A total of 4 unique stem nodule isolates were obtained from Texas native *A. indica* plants and were tentatively identified as *Bacillus megaterium* (TSN1), *Leifsonia shinshuensis* (TSN2), *Streptomyces* sp. (TSN5), and *Paenibacillus* sp. (TSN7) through 16S rRNA sequencing. All obtained isolates are known plant symbionts and are documented as promoting plant growth through the production of phytohormones, nutrient acquisition, abiotic stress tolerance, and disease suppression. Of those identified, TSN1 and TSN2 demonstrated the most consistent and notable benefits regarding nodule promotion.

After further investigation, TSN2 was identified as a novel *L.* species through whole genome sequencing. Additionally, the whole genome analysis of this organism revealed a total of 106 plant growth promoting genes with 21 of these being involved in the remediation of heavy metal pollutants such as cadmium, cobalt, copper, arsenic, mercury, zinc, and nickel. There were 21 genes identified that are involved in salt and drought stress tolerance for plants as well as 19 genes for cytochrome production, aiding in other abiotic stress mitigation. This stem nodule

isolate shows great potential for future use as a co-inoculant for the plant growth promotion of legumes in hostile, nutrient poor conditions. Further analysis of the beneficial symbiotic characteristics of this organism must be conducted, particularly on non-leguminous crop plants such as corn or cotton under detrimental environmental conditions to evaluate its role as a potential crop inoculant. Additionally, investigation in the the potential function of the unidentified genetic island unique to TSN2 and not found in the two closest relatives, *L. shinshuensis* and *L. aquatica* is currently underway. It is postulated that this unique genomic region may play an important role in NF-independent nodulation and plant-microbe symbiosis in *Aeschynomene indica* plants.

In addition to the isolation and characterization of new NF-independent stem nodule endophytic bacteria, we also worked to better understand the molecular mechanisms involved in this unique nodulation system. There are many NF-independent rhizobia that have been isolated from *Aeschynomene* plants around the world that are used as model organisms for studying this symbiotic relationship. One such NF-independent symbiont, *Bradyrhizobium* sp. BTAi1 was the subject of study for this work to identify bacterial genes involved in NF-independent nodule formation. Our results from plant growth experiments using a *Bbta_p0110* disrupted mutant demonstrated the significance in this gene regarding NF-independent nodulation in *A. indica* plants. Further experiments must be done to characterize the protein encoded by this gene to better understand the exact mechanisms involved. Results from the experiments suggest a role of this gene involved in the colonization and distribution of the bacteria within nodule primordia. This gene may play a role in the cell death and uptake of the bacteria into the initial nodule tissues and thus the subsequent assimilation of these bacteria into mature nodule organs.

Interestingly, this gene was not identified within the genome of the Texas native *A. indica* stem nodule isolate, TSN2, despite the elevated pigmented nodule formation stimulated by this organism independently of NF signal molecules. With crack entry being such a simplistic method of endophytic colonization within host corticial tissues, it is possible that throughout the evolutionary history of these symbiotic relationships, the bacteria have developed unique methods of communication with the host that is independent from one another. While these nodulation mechanisms occur in the absence of NF signal molecules, there most likely are other signal molecules being produced and released by the bacteria that, at some point, allows them to gain entry to the host plant nodule primordia or perhaps even interact with the pericycle, from which the cortical cells arise. These NF independent nodulation mechanisms are relatively new and have not yet been fully characterized. They provide a wealth of interesting research subjects that, in the end, will help to further our understanding of and enhance our current agricultural practices.

If we want to elevate our current agricultural methods through biological means, it is imperative that we understand how microorganisms can be incorporated as means of crop enhancement. Bacteria play important metabolic roles in every single documented niche around the world. There have even been suggestions of these microorganisms thriving in extraterrestrial environments as well. With mankind quickly advancing towards the colonization of celestial objects, such as the Moon or Mars, it would be an impossible feat with our current agricultural practices. Not only can bacteria improve our current, Earth-bound crop cultivation, perhaps they could also be the key to supporting and sustaining agricultural systems and terraforming practices outside of this planet.

APPENDIX

Table A-1. Significant gene expression report for BTAi1 exposed to *A. indica* root exudates.

GeneID	log2FC	p-adj	FC	Function
640555469	3.95	0.05	15.43	fructose-bisphosphate aldolase (EC 4.1.2.13)
640539027	2.76	0.05	6.76	putative exported protein of unknown function
640559454	2.44	0.05	5.44	hypothetical protein
640559545	1.66	0.00	3.16	hypothetical protein
640560691	1.07	0.00	2.11	Methanol dehydrogenase large subunit
640561915	1.03	0.05	2.04	hypothetical protein
640555536	0.65	0.05	1.56	hypothetical protein
640555143	-0.76	0.05	-1.69	putative small heat shock protein
640560236	-1.21	0.05	-2.32	putative exported protein of unknown function

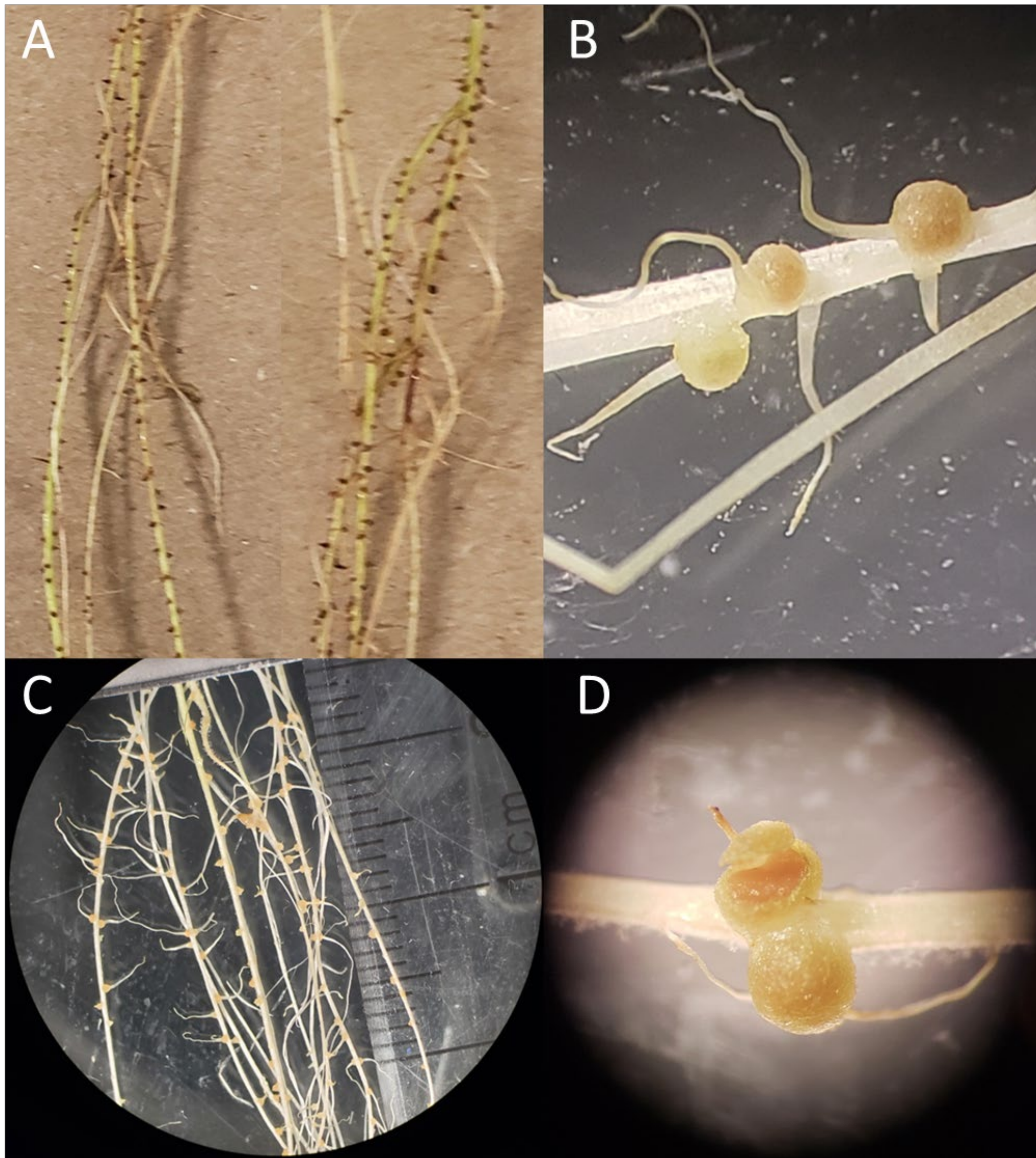

Figure A-1. Examples of nodule morphology observed through the course of this study. (A & C) Depicting pigmented emergent lateral root (ELR) sites. (B) Depicting one pale (left) nodule compared to two neighboring pigmented nodules. (D) Opened pigmented nodule demonstrating the inner nodule organ with pink leghemoglobin color.