A DROUGHT-TOLERANT NITROGEN-FIXING SYMBIONT FOR SUSTAINABLE SOYBEAN PRODUCTION

by
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ABSTRACT

A DROUGHT-TOLERANT NITROGEN-FIXING SYMBIONT FOR SUSTAINABLE SOYBEAN PRODUCTION

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Bradyrhizobia are an ideal model organism to study plant-microbe interactions due to dual lifestyles as free-living soil organisms and as endosymbionts for valuable cropping systems in agriculture. Farmers have been utilizing the Soybean-*Bradyrhizobium japonicum* mutualism for decades to revitalize marginal soils, increase yields, and establish a regenerative approach to farming by mitigating the use of synthetic nitrogenous fertilizers. The largest impediment to bio-fertilizer success is desiccation (e.g., drought) stress before the symbiosis can be established. Due to climate change, fluctuating high temperatures and drought occur seasonally in many growing regions and are becoming a growing concern for modern agriculture. Thus, alternative methods must be presented to farmers to enable sustainable production despite such abiotic extremes. To this end, we have bio-prospected drought-tolerant *Bradyrhizobia* from the root nodules of soybeans in south Texas and formulated an inoculant for farmers in drought prone regions. Field trials were conducted in the Mid-South for 3 years using the drought-tolerant inoculant *B. japonicum* TXVA and a non-inoculated control. The drought-tolerant inoculant dominated taproot and total root nodulation and imparted a 7% total grain yield across locations and years. In addition to drought field trials, we were given the opportunity to test the TXVA inoculant under heat stress using a novel temperature gradient greenhouse developed by Dr. Kent Burkey from the USDA in North Carolina. Lastly, whole genomes of two Texas native drought-tolerant isolates (i.e., *B. japonicum* TXVA & TXEA) were sequenced and the comparative genome analysis was performed among the Texas isolates, *B. japonicum* USDA 6 and SEMIA 5079. Phylogeny revealed similarities between the native strains and type strain USDA 6 which has been used as an inoculant. Our research allows for further understanding the application, function, and molecular characterization regarding biofertilizers to aid sustainable soybean production in the face of a changing climate.

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DEDICATION

I would like to dedicate this work to my entire family, but especially my late mother Gail Pauline Peterson. She is without a doubt the reason I became a scientist and has always pushed me to succeed. My father and stepmother, Don and Karen Peterson, have helped me through this program and given me role models to aspire to. My sister and her husband, Zack and Crystal Anderson, have shaped my morality and helped me become who I am today.

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CHAPTER 1

GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized into 5 chapters. The first chapter explores peer-reviewed literature that is relevant to comprehension and understanding of the research presented in the remainder of the dissertation. The second chapter covers the multi-state field trials of the Texas native drought-tolerant *Bradyrhizobium japonicum* TXVA strain, which was used as a biofertilizer for soybeans across the Mid-South in conjunction with a non-inoculated control. In the third chapter, I present greenhouse work completed in collaboration with the USDA and North Carolina State University using a novel temperature gradient greenhouse to test the effects of heat stress on the Soybean-*Bradyrhizobium* mutualism. In the following chapter, I describe the process of whole-genome sequencing for the drought-tolerant isolates TXVA and TXEA, and comparative genomics with closely related *B. japonicum* strains. The material presented in the fourth chapter was published in Microbial Resource Announcements in a condensed version. In the fifth chapter, conclusions and future directions are addressed for the translatory environmental molecular techniques presented in the dissertation.

Literature Review

Worldwide soybean production

Soybeans have become the 4th largest crop production system globally, with production for the last century dominated by the United States, Brazil, Argentina, China, and India, which provide 92% of the world's soybeans (Westcott et al., 2016). The first three countries produce the highest amount, with Brazil recently surpassing the United States as the top soybean producer in 2020 (137.0 vs. 116.3 million metric tons [mmt]), USDA, 2020). The extensive worldwide production of soybean has arisen due to multifaceted purposes, highlighting the diverse products derived from soybeans and their unique potential as a probiotic-like vessel for nutrient-poor soils. The former is attributed to the quality of oil and protein byproducts while the latter is due to the Soybean-*Bradyrhizobium* symbiosis and its climate-friendly and regenerative mode of action in rhizosphere soils.

The constituent breakdown products are more valuable than whole beans, with the seed composition being 37-39% crude protein, 18-20% fats, 35% carbohydrates, and the remaining 5% ash (Chen et al., 2012). Soy proteins are recognized as possessing all essential amino acids excluding methionine and tryptophan and have a Protein Digestibility Corrected Amino Acid Score (PDCAAS) of 1.00, which is equivalent to proteins from meat and dairy sources (Qin et al., 2022). Soy-foods rose to popularity alongside the evolution of processing techniques that purify the desired end-product and concentrate its respective health benefits. The bioavailability of different nutritional products can be increased by cooking, soaking, germination, extrusion, fermentation, protein concentration, and isolation (Guzeler & Yldrm, 2016). Antioxidants present in soybean known as polyphenols (i.e., isoflavones, anthocyanins, saponin, vitamin C, etc.) are shown to reduce the risk of cancer, cardiovascular disease, cancer, osteoporosis, Alzheimer’s, and obesity (Tepavčević et al., 2010; Chen et al., 2012; Dabbagh et al., 2014; Bintari & Nugraheni, 2017; Ramdath et al., 2017; Essawy et al., 2019).

Soybean is also widely recognized as the most cultivated and utilized oilseed crop worldwide and its oil is comprised of 5 fatty acids: palmitic acid (10%), stearic acid (4%), oleic acid (18%), linoleic acid (55%), and linolenic acid (13%). The high percentage of polyunsaturated fatty acids (i.e., linoleic, and linolenic acid) cause soybean oil to exhibit oxidative instability which limits use as a food oil and biodiesel due to the creation of rancidity and the buildup of viscous materials that clog oil filters (Clemente & Cahoon, 2009). Soybean oil use as biodiesel has typically been an afterthought of growers since protein and meal content are a higher percentage dry weight, but constant innovation in biodiesel production methods has improved performance to nearly equivalent to that of standard diesel with the USDA forecasting 10.35 billion pounds of soybean oil dedicated for biofuel production in the 2021-’22 year (USDA-WAP, 2022).

The versatile end-products command a respect of their own; however, the supreme economic and environmental benefit derived from soybean production stems from the multitude of environments in which the crop can be grown and the reduced need for yearly applications of synthetic nitrogen fertilizers due to the unique symbiotic relationship with the soil bacterium *Rhizobia.* Conventional breeding efforts have focused on high protein, oil content, and yield but these traits are controlled by multiple genes, show varied sensitivity to genotype x environment interactions, and often show inverse correlations (Chaudhary et al., 2015). A modern approach for phenotyping of germplasm under varying environments consists of marker-assisted selection techniques such as quantitative trait loci (QTL) mapping and genome-wide association studies

(GWAS). Using these molecular tools, breeders have expanded germplasm to contain numerous cultivars exhibiting elevated protein concentration, elevated seed oil content, resistance to multiple diseases, salt tolerance, drought-tolerance, germination in high-temperature environments, high yields, and even optimum nitrogen fixation capabilities (Shannon et al., 2022; Chen et al., 2022; Do et al., 2017; Carter et al., 2016; Smith et al., 2008; Chen et al., 2022; Muñoz et al., 2016). These advances in crop breeding have also spurred the corresponding marker-assisted techniques to be used to identify nodule populations of elite *Bradyrhizobium* to be used as plant growth-promoting inoculants in specific regions, indicating the importance of the soybean-*Bradyrhizobium* mutualism for maintaining sustainable agriculture in the face of climate change (Vinuesa et al., 2008; de Borja Reis et al., 2021; Freitas et al., 2022).

Biological nitrogen fixation (BNF)

Symbiotic BNF is the process that utilizes microbial symbionts to convert atmospheric nitrogen into ammonia via nitrogen-fixing nodules. The amount of fixed nitrogen varies among leguminous plant species, serovar of microsymbiont, and general environmental factors such as soil type, temperature, nutrients, and water regime. The symbiosis is established by a very sensitive process of chemoattraction in which plant-borne flavonoids induce stimulation of *nod* (nodulation) genes in rhizobia, beginning the signaling cascade that induces attachment. Expression of the *nod* genes induces synthesis of nodulation factors (Nod factors, NFs) by the bacterium which are mitogenic only to their specific mutualist partner through chemically unique lipochito-oligosaccharide structures (Schultze & Kondorosi, 1998). These NFs activate a series of events in the plant roots including root hair curling, which brings the bacterium into close proximity to the roots, and infection thread development (Zhukov et al., 2008). The trapped bacterium migrates intracellularly or intercellularly through these infection threads toward the dividing root cells where they are taken up by host plant cells, either entirely by phagocytotic vesicles or incompletely in plasma membrane invaginations, for root nodule formation that houses the bacteria that differentiate into bacteroids, the endosymbiotic vessels of N-fixing organelles maintained by the plant in return for fixed nitrogen as ammonia (Kijne, 1992). In the case of the Soybean-*Bradyrhizobium* symbiosis, this shift in form is followed by a shift in function with 34% of the gene expression being altered, as compared to the free-living state, to reflect efficient symbiotic maintenance and nitrogen-fixation in soybeans (Pessi et al., 2007). It is interesting that, in general, plant cells do not ingest large particles by phagocytosis; however, rhizobia form one of the few exceptions to this rule with complete endocytosis observed only in cells formed under the influence of rhizobial stimuli (Kijne, 1992).

Once differentiated into bacteroids, the cells inhabit a space termed the symbiosome which includes the baceroid, the symbiosome space, and the peribacteroid membrane. The compartmentalized fashion of these functionally unique areas indicates the close homology to other cell organelles, such as chloroplasts and mitochondria developed by independent endosymbiotic processes. Referring to the change in function, the following changes occur to the microsymbiont when differentiated into bacteroids; i) viability is slightly reduced (20-90%), ii) osmotic sensitivity is enhanced, exopolysaccharide (EPS) formation is reduced, iii) accumulation of the lipid polyhydroxybutyrate (PHB) as an energy source, iv) a special electron transport chain is utilized that includes bacteroid-specific cytochromes, v) heme-synthesis is increased, vi) nitrogenase represents up to 10% of the soluble protein formed, and vii) NifA-dependent processes (regulator of N-fixation) are put under oxygen control (Werner, 1992). Bacteroids house the oxygen-sensitive nitrogenase enzyme responsible for the conversion of di-nitrogen into ammonia, supplying this to the plant in exchange for carbohydrates, mainly sucrose, from photosynthesis. The leghemoglobin protein is expressed upon bacteroid formation and helps control nodule oxygen concentration as an oxygen-carrying phytoglobin, performing similar functions as hemoglobin in the bloodstream of humans. It has been confirmed that high nodule concentrations of leghemoglobin indicate an enhanced energy metabolism while if this protein is malfunctioning, BNF is severely inhibited (Ott et al., 2005). Once the di-nitrogen bonds are broken, the N product is “fixed” to the available hydrogen pool where the ammonia is rapidly excreted into the cytosol through the peribacteroid membrane of the infected cell to be assimilated into glutamine, glycine, and aspartate for purine synthesis. These purine nucleotides are transformed into uric acid, which is catabolized into allantoin and allantoic acid in the uninfected cells for transport to the rest of the plant via the shoot’s xylem vessels (Ohyama et al., 2017). The nitrogen-carrying compounds are important not only because they can precisely determine how much nitrogen was fixed from BNF (i.e., leaf/stem ureide content), but also because they provide a nutrient tracking system which is constantly in flux with changing evapotranspiration of the plant and allowing these metrics to be used as sensitive indicators that precede declining soybean growth due to environmental stressors such as drought and high air temperature (Sharaf et al., 2019).

With a number of feedback mechanisms for nitrogen fixation and the subsequent assimilation, tight regulations on the symbiosis have been established. For example, applied nitrogenous fertilizer increased levels of the amino acid asparagine while decreased levels of organic acids from photosynthates, especially malate, in xylem sap (Ono et al., 2021). Inhibition of nodulation in the presence of high soil nitrogen levels is well-known, molecular understanding of this phenomenon is still poorly understood. It is known that high nitrate supply completely inhibits accumulation of rhizobia on root hairs, root hair curling, infection thread development, and nodule formation (Truchet et al., 1982). The addition of indole-3-acetic acid (IAA) mitigates some effects of nitrate supply on root hair curling, infection thread formation, and nodulation which suggests that the presence of combined nitrogen impacts the root phytohormone balance (Valera et al., 1962). It is shown in soybean cultivar Bragg that increasing the concentration of nitrate from 0.5 mM to 7.5 mM reduced the nodule number from 100 to 50 nodules per plant (Day et al., 1989).

Use of nitrogen-fixing inoculants

Inoculant use is not a new development. One of the first documented cases comes from Hellriegel's report on the N nutrition of leguminous plants dating back to 1886 where he suggests transferring soil from a known legume field to a prospective location for cultivation (Hungria et al., 2005). Since then, application has become streamlined by using a carrier material, usually peat or some liquid formulation, in which the inoculant receives a bit of combined shelf-life and in-field protection from abiotic stress allowing for increased survivability. Despite the rising use of inoculants for leguminous crops, most inoculant products are still of relatively poor quality and do not perform consistently in the field due to lack of quality control in most countries, including the United States (García de Salamone et al., 2019). In Brazil 1,086 samples of inoculant products used for soybean were tested from 2010-2014 and showed a 2.21% contaminant rate and average concentrations of viable cells to be 1.1×10^{10} CFU/ml, which is higher than levels guaranteed by the manufacturers (de Souza et al., 2019). The high level of biofertilizer production and quality control shown in Brazil is one factor that contributes to its success as the newly appointed leading soybean-producing country in the world and should be held as an example of how governmental policy can contribute to climate-smart and sustainable agricultural practices. The global market share for bio-fertilizers is increasing, valued at $2.3 billion in 2020 with a projected compound annual growth rate (CAGR) of 11.6%, resulting in the expected growth to $3.9 billion by 2025 (Kodgire et al., 2022).

Despite the rapid growth of the inoculant industry, limitations of wide-scale adoption by smallholders and commercial farms alike are abundant. Smaller producers may not have the socioeconomic ability to obtain quality inoculants while large-scale farming operations face issues with storage conditions, application bottlenecks, and consistency of product when acquiring numerous batch cultures (Barman et al., 2017). Limitations that apply to all farmers include failure of inoculants under unfavorable environmental conditions (i.e., drought, flood, high temperature, etc.) and shelf-life concerns of the inoculant (Brar et al., 2012; Kihara, 2019; Bell et al., 2019). Therefore, optimizing an inoculant from an intrinsically drought-tolerant organism could aid in technology adoption for farmers by showing ease of use and return on investment.

Climate change and its effect on soybean production and BNF

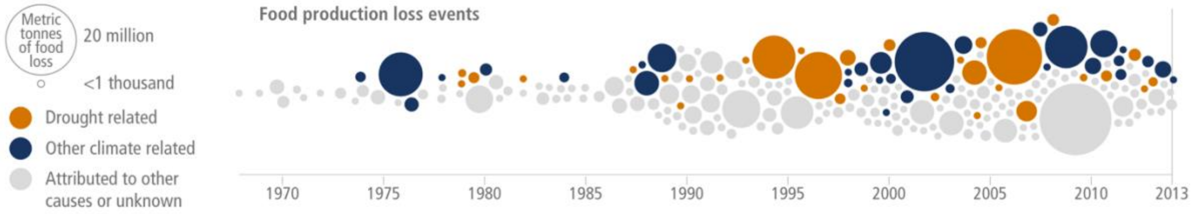
While the focus is commonly on carbon dioxide (CO_2) emissions, these comprised only about 72% of total global greenhouse gas (GHG) emissions in 2018 (Oliver et al., 2019). With the introduction of the United Nations Framework Convention on Climate Change (UNFCCC) and the Kyoto Protocol, the list of GHGs was expanded to include methane (CH_4), nitrous oxide (N_2O), perfluorocarbons (PFCs), hydrofluorocarbons (HFCs), and Sulphur hexafluoride (SF_6). The attention drawn to climate change in the last century has allowed for mitigation and GHG-capturing techniques to be developed across industries. However, widescale adoption is still being implemented since the production and lifetime of each respective GHG inhabits a complex web of biotic and abiotic interdependencies (Pörtner et al., 2022). The largest source of GHG emissions is energy production (mainly CO_2), the second largest being agriculture, forestry, and land-use with 25%, comprised primarily of CH_4 and N_2O (Oliver et al., 2019). While N_2O makes only about 6% of total GHG, the climate warming intensity of nitrous oxide is 300 times that of carbon dioxide over a period of 100 years (Menegat et al., 2022). Synthetic chemical nitrogen fertilizer additions to croplands have increased the global human-induced emissions of N_2O by 30% over the past four decades, exceeding the highest projected emission scenarios (Tian, 2020). Nitrogen use efficiency is one of the main tool's scientists can utilize to curb nitrous oxide emissions, shown by the "Nitrates Directive" established in Europe which reduced nitrate leaching by 16%, ammonia emissions by 3%, and nitrous oxide emissions by 6% (Velthof et al., 2014). Additionally, Gerber et al. (2016) showed that reducing N application rates by 5% in the Shandong province in China would reduce the province's share of global N_2O emissions by 9% and their global crop N_2O emissions by 0.35%. Besides focusing on N_2O emissions, net figures calculated across the aggregate global food chain estimate contributions of 19%-29% of total GHG emissions (Vermeulen et al., 2012). This estimation of the worldwide food chain considers practices like fertilizer and pesticide manufacture (preproduction), direct and indirect emissions from actual farming (production), and activities like processing, transport, and waste disposal (postproduction) (Holler Branco et al., 2022).

The Intergovernmental Panel on Climate Change (IPCC) utilizes the expertise of thousands of scientists worldwide who volunteer their time as authors to assess the drivers of global climate change, estimate its current and future effects, and outline the potential terms of adaptation or mitigation to avoid these scenarios. The terms used to denote consistent confidence in their findings reflect calibration of uncertainties from incomplete knowledge along with disagreement of what’s known, with findings classified into six categories of probability: virtually certain (i.e., 99-100%), very likely (90-100%), likely (66-100%), as likely as not (33-66%), unlikely (0-33%), and very unlikely (0-10%), and exceptionally unlikely (0-1%). In the most recently published 84-page technical summary, the word “drought” is mentioned 85 times, “production” 59 times, and “agriculture” 38 times, indicating pervasive climate impacts across production systems that are either ramifications of or exacerbated by agricultural instability. It is also noted that potential adaptation of food production systems decreases with increasing temperatures, while benefit in the short-term has many negative outcomes, including increased water demand, groundwater depletion, alteration of local/regional climates, increasing soil salinity, and widening inequalities for underserved producers (Pörtner et al., 2022). The effects of increased climate variability on food production systems have been occurring with more frequency, as shown in Figure 1-1, presenting longer and more expansive periods of drought interspersed with heavy precipitation events which damage crop production in numerous ways (Raza et al., 2019). Changes in seasonal duration are also projected as displayed by the extension of the warm season in Maine, USA, by two weeks over the past 100 years and doubling in this projected for the next 100 (Fernandez et al., 2015).

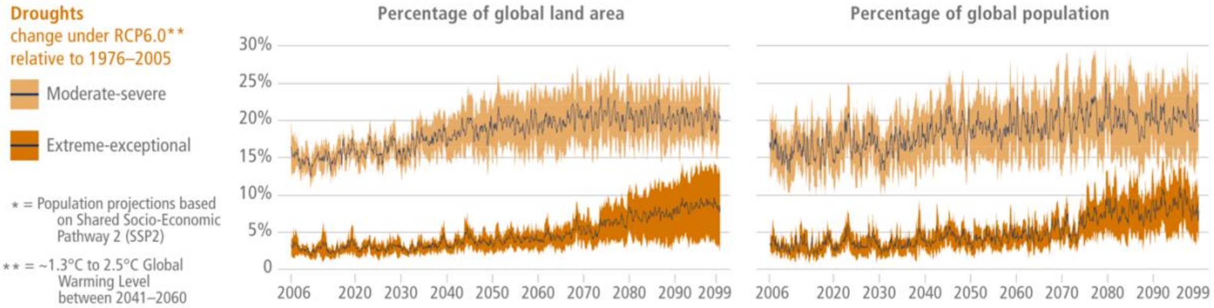
Climate change is affecting food security through pervasive water impacts

Its impacts are being felt in every water use sector, more so in agriculture which globally consumes over 80% of the total water.

(a) The frequency of climated-related food production losses in crops, livestocks, fisheries and aquacultures has been increasing over the last decades.



(b) By the late 21st century the share of the global land area and population* affected by combinations of agricultural, ecological and hydrological droughts is projected to increase substantially.



** RCP6.0 concentration pathways calculated via changes in terrestrial water storage.

Figure 1-1. Infographic sourced from IPCC depicting (a) the frequency of food-production losses across industries has increased since 1970 due to climate change and (b) the percentage of land area and population projected to be impacted by drought (IPCC, 2022, pg. 74).

Co-occurrence with yield variation, abiotic stressors are also known to cause a reduction in grain quality. While all abiotic stressors affect crop production, drought is the most pervasive in soybean, primarily due to the sensitivity of BNF to soil water deficits (Sinclair et al., 2007). BNF is one of the first plant physiological processes, showing responses under desiccation stress, indicating a matric sensitivity that deleteriously affects both members of the symbiosis.

Effects of drought stress on rhizobia and BNF

Drought-like conditions result from lack of rain, rising air temperature, or a combination of, resulting in increased evaporation from soils and decreased transpirable soil moisture for the plant. The effects of this lowered water potential are detrimental to the soybean-*Bradyrhizobium* mutualism by impacting plant development and symbiont maintenance/survival throughout the cycles of desiccation and subsequent rehydration. In the bacterium, the onset of desiccation initiates a cascade of genetic responses to stabilize and protect cellular integrity and function, such as the upregulation of oxidative stress response genes to protect from the reactive oxygen species (ROS) incurred by drought, increased EPS production to provide a polymeric buffer, and synthesis of the disaccharides sucrose and trehalose to stabilize DNA as compatible solutes (França et al., 2007; Cytryn et al., 2007; Benaroudj et al., 2001). Trehalose has been known to confer a level of protection for numerous organisms undergoing abiotic stress as an osmolyte, ensuring cell survivability through reinforcement of membrane and protein integrity when facing anhydrobiosis (Liu et al., 2008; Orozco-Mosqueda et al., 2019; Sharma et al., 2020). Besides the effects on bacterial cell stability, exogenously applied trehalose has been shown to aid desiccation and salt tolerance to plant roots of many species (Sarkar & Sadhukhan, 2022).

Soil water moisture induces morphological and ecological changes in *Bradyrhizobium*, with a dehydrating effect seen on cell surface morphology in as little as 10 min after being exposed to drought stress that allows for optimal membrane fluidity helping survive water deficit (Cesari et al., 2018). After incubation in soils, a greater percentage of introduced rhizobia became associated with soil particles when the moisture content of soils was lowered, also exhibiting a decrease in cell size and increase in EPS production which provide a means of biofilm-mediated water stress (Postma et al., 1989; Vanderlinde et al., 2010). This indicates a fine-tuned matric sensitivity that presents an ecological advantage to rhizobia by allowing them to occupy small pores to avoid the deleterious effects of water potential fluctuation that inevitably happens in soil microenvironments (Bottomley et al., 1992). Field isolates of *Rhizobium meliloti* displayed sensitivity to the re-wetting process after prolonged periods of low water potential (i.e., up-shock sensitivity), showing delayed nodulation on alfalfa seedlings (Busse et al., 1989). This same up-shock sensitivity is observed by increased levels of viable but non-culturable cells of *B. japonicum* 5038 at each rehydration timepoint (i.e., 0.5, 3, and 9 h) after periods of desiccation stress via comparison of live/dead fluorescence staining and plate counts (Zhu et al., 2022). Severe soil water restriction has shown reduced rhizobial survival by killing off the symbiont through desiccation stress but also imposes reduced diffusion of molecular signals, such as isoflavonoids and nodulation factors, hampering exudate efficacy and reducing the likelihood of symbiosis for any surviving rhizobia (Zilli et al., 2013).

From a plant standpoint, lowered soil moisture and/or air humidity levels initiate the stress by reducing evapotranspiration of soybean to such an extent that there is a visual rating system corresponding to drought-specific QTL molecular markers that allow breeders to identify slow-to-wilt and fast-to-wilt (i.e., drought-sensitive and drought-tolerant) cultivars of (Purdom et al., 2021; Zhou et al., 2021). The efficacy of this rating system is consistent due to physiological soil water conservation efforts from the plant where the stomata periodically close to decrease conductance at high vapor deficit, saving essential moisture as leaf turgidity for future increased biomass and reproductive growth (Sinclair et al., 2010). The soil and atmospheric vapor pressure deficit cause a series of drops in water potential that triggers a rapid biochemical response for stomatal closure, even before any change in leaf water potential (Mutava et al., 2015). Reduced meristem activity is typically the limiting factor in plant growth and is controlled by water restriction rather than the availability of photosynthate, indicated by increased non-structural carbohydrates which represent growth rate reductions before reductions of photosynthesis (Muller et al., 2011). This indicates that photosynthesis produces only as much carbon as sink activity (i.e., tissue growth) allows, validating that growth controls photosynthesis while available water pressure controls growth (Körner, 2015). The increase in these carbohydrates has displayed a deleterious effect on pest pressure, with Grinnan et al. (2012) showing 16-24% increased insect biomass when allowed to feed on plants recovering from drought stress and indicates the downstream effects of increased droughts on the worlds soybean production systems. This agrees with the Pulsed Stress Hypothesis that shows increased feeding on recovered plant tissue is due to the increase in stress-induced nitrogen (Huberty & Denno, 2004). The presence of non-structural carbohydrates accumulated by plant and bacterium alike when faced with desiccation represent an innate biological stress response to reallocate available resources to stabilizing cellular and genetic material with the goal of survivability.

Acquisition of nitrogen through BNF is a very energy-demanding process that requires more energy than if received as nitrate through the soil solution, exhibited by the rapid decline of N-fixation by 76% after application of 10 mol m^{-3} nitrate (Arrese-Igor et al., 1997). Yet, the subsequent sink of photoassimilates created by the symbiosis can stimulate photosynthetic rates up to 28% and even make this process more efficient, providing assimilated N from BNF to be integrated into numerous anabolic processes (Kaschuk et al., 2009). Therefore, the growth limitation of drought-stressed plants under successful inoculation changes from source limitation (i.e., photosynthates) to sink limitation, which means that during the later reproductive growth during pod fill the maximum photosynthetic activity can be achieved with only half the maximum sunlight (Werner, 1992). At this growth stage (R5-R6) the natural capacity of soybeans for nitrogen uptake is already significantly reduced to around 50%, giving plants with successful nodules a boost in late-season pod fill (Imsande, 1988).

Effects of heat stress on rhizobia and BNF

High-temperature stress results from the timing, magnitude, and duration of exposure to temperature extremes but can be managed differently depending on the soybean cultivar or species/strain of symbiont (Hungria & Franco, 1993; Asadi et al., 2009; Jumrani et al., 2018). Plants that experienced this stress at the vegetative phase displayed reduced photosynthesis, average leaf area, and biomass, but could recover if the duration was short. However, the stress incurred at reproductive stages causes more definitive damage with general cellular damage through the creation of reactive oxygen species leading to the termination of flowers and abortion of pods (Jumrani et al., 2017). Temperature is also one of the most important abiotic factors influencing bacterial growth and survival in natural environments, with vast changes in soil microbial populations displayed according to above-ground plants and other biotic and abiotic factors (Fierer, 2017).

Rhizobium exhibit a generalized response to elevated air or soil temperatures by the expression heat shock proteins (HSPs) which are highly conserved among both prokaryotes and eukaryotes, being involved in a plethora of crucial cellular functions such as the initiation of hormone signaling, protein synthesis, correct folding of proteins, assembly of multiprotein complexes, and protein degradation (Münchbach et al., 1999; Michiels et al., 1994; Netzer & Hartl, 1998). A more *genus*-specific response is also utilized that modifies the lipopolysaccharide (LPS) profile towards longer chain length, shown in *Bradyrhizobium* sp. BTAi1 to convey outer membrane stability and rigidity, leading to decreased sensitivity to stressful conditions (Zaharan et al., 1994; Silippo et al., 2014). The 60 kDa HSP GroEL is overproduced in *Bradyrhizobium* under high-temperature stress, but the overproduction does not confer tolerance to this stress, despite being a chaperonin required for the formation of a functional nitrogenase and involved in regulating the nitrogen fixation-specific *nif* gene. This indicates that temperature is a physiological shut-off switch for BNF (Rodrigues et al., 2006). Munevar and Wollum (1981) displayed the deleterious effects of this stress on symbiosis with a decline in the number of nodules, reduction in the specific nitrogenase activity, overall N content, and loss of biomass in roots and shoots when the overall temperature of the root system was raised from 28 to 40°C. However, they also displayed that those same conditions of increasing heat stress affected the initial infection and nitrogenase activity more than nodule organogenesis, indicating the evolutionary safety of the symbiotic niche (Munevar & Wollum, 1981). This was confirmed in the peanut-*Bradyrhizobium* symbiosis, with no nodules being formed at a constant 40°C root temperature; however, when faced with this same temperature stress in diurnal cycles, root nodules were formed that exhibited no nitrogen fixation (Kishinevsky et al., 1991). This temperature-dependency of nodule initiation, organogenesis, and functioning could arise from accelerated degradation of plant flavonoids in the rhizosphere, altered attachment to root hairs for infection, or induction of early senescence, which have all shown to be very sensitive to high and low temperatures alike (Guo et al., 2011; Abd-Alla et al., 2014; Shah & Smith, 2020). High temperatures in the rhizosphere cause depression in root hair formation as displayed at 37°C by spheroid, stunted, and even a complete absence of root hairs which correlated with the reduction of adsorbed *Bradyrhizobium* from 48% to 38% by a decrease in the root hair attachment sites (Dudeja & Khurana, 1989). Another reason for the restricted symbiosis is derived from nitrogenase function, with protein-mediated permeation of fixed N as ammonia (NH_3) through the peribacteroid membrane being temperature-mediated with an activation energy of 55 kJ/mol, rendering this process too costly under heat stress (Niemietz & Tyerman, 2000). As a part of microbiome recruitment and to overcome this stress, soybean and *Bradyrhizobium* produce the phytohormone IAA which stimulates root nodule formation, cell division, nodule differentiation (Mathesius, 2008; Liu et al., 2017). Exogenous application of 1 mM IAA increases survivability by 44% and 37% when exposed to temperatures of 42°C and 50°C for 10 min, respectively (Donati et al., 2013).

Temperature gradients are known to be one of the factors that define the spatial distribution of plant and animal species across latitudinal gradients; however, until recently, they were not correlated to microbial community abundance (Fierer & Jackson, 2006; Zhou et al., 2016). Altering this notion strengthens the evidence that ecological and evolutionary processes for eukaryotes and prokaryotes alike operate on similar spatiotemporal scales to influence local adaptation as a means of persistence (Chase et al., 2021). Saeki and collaborators (2013) displayed this geographical distribution of niche habitats for *Bradyrhizobium* clustering around latitude which correlated to climactic gradients in Japan. This clustering is also observed on local scales through the lens of competitiveness in the rhizosphere and nodule occupancy, indicating that nodule occupancy is temperature dependent (Adhikari et al., 2012). Individual and mixed inoculations of *B. elkanii* and *B. japonicum* were tested via growth chamber showing equal co-nodulation at 34°C initially but time-wise exclusion of *B. japonicum* from nodules by *B. elkanii* while nodules at 23°C were consistently dominant with *B. japonicum* (Suzuki et al., 2013). A key to this niche organization structure could be the ability to detect plant flavonoids and the subsequent expression of nodulation factors to initiate the symbiosis, since genistein is secreted at higher temperatures but in lower general abundance while daidzein is secreted at low temperatures with concentrations up to 5x that of the former (Pan & Smith, 2008). Shiro et al. (2016) performed microcosm studies with 5 type strains in the soybean-*Bradyrhizobium* genus at day/night cultivation temperatures of 23/18°C, 28/23°C, and 30°C which displayed competitiveness of nodule occupancy according to cultivation temperature, which agreed to the corresponding expression Nod factor biosynthesis by NodC.

High temperatures have shown negative correlation with nitrogenase functioning and thus CO_2 fixation rates since photosynthesis and nitrogen fixation are coupled due to the anabolism of photosynthesis, available nitrogen, and nitrogen demand generated through plant growth (Aranjuelo et al., 2007). A short-term and oft-used solution to overcoming heat stress is the application of nitrogen fertilizers, which overcome the thermal shock and continued heat stress partly by from uncoupling this BNF-photosynthesis feedback system and allowing easily accessible nitrates for plant growth (Hungria & Franco, 1993). Under severe heat stress, however, leaf senescence occurs due to protein degradation from reactive oxygen species as measured by malondialdehyde (MDA), a marker for lipid peroxidation (Xu & Zhou, 2006). This results in heat-stress specific physiological and chemical change in the leaf that has shown to positively influence the plants resistance to herbivores, as seen by a decrease in overall insect biomass when provided leaf disks from the stressed soybean leaf (Grinnan et al., 2012). Researchers have also shown that temperature, among other environmental factors, controls the fatty acid composition and protein content of soybean seed such that producers decide on regional varietal selections and planting dates to maximize seed quality just as much as yield (Bellaloui et al., 2015). In nature drought is often co-factored with elevated temperature thus sharing many of the same defense mechanisms, like production of ROS, reduction of CO_2/N_2 efficiency, accumulation of compatible solutes trehalose and sucrose, and upregulation of isocitrate lyase (*aceA*) which is reported to be significant under numerous stressors that depress carbon substrates from photosynthesis (Aranjuelo et al., 2007; Reina-Bueno et al., 2012; Benaroundj et al., 2001; Jeon et al., 2015). However, not all mechanisms for mitigation overlapped and thus presented an exponentially more deadly response in combination (Jumrani et al., 2018).

Bio-prospecting optimized strains for translational research

Surprisingly, a very few *Bradyrhizobium* strains have been utilized in inoculants worldwide (Hungria & Mendes, 2015). This is most likely due to the complexities of the soil microenvironments interacting with biotic and abiotic stressors that consistently befuddle the analysis of translatory research into biofertilizer use, often hiding the failed trials since these failures aren’t formally described throughout literature (Bashan et al., 2020). Up to 90% of applied microbes can be lost during field application and inoculant carrier formulations that provide favorable microenvironments can reduce this loss (Qiu et al., 2019). Once a pattern of soybean cropping with inoculation takes place, these soils are less apt to show a response to an applied inoculant because of high populations of indigenous rhizobia or an initial inoculant strain which is deteriorated in BNF efficiency, but still outcompetes applied strains in the rhizosphere and dominates nodulation (Sessitsch et al., 2002). The use of molecular techniques, such as molecular marker gene development, and genomic and metagenomic sequencing analysis have just now begun to reveal the nodule-bacterial diversity and phylogeny that will help better understand subverting environmental stressors and promoting positive rhizosphere microbiome interactions (Fierer, 2017). Rhizobial germplasm/training programs like USDA National Microbial Germplasm Program (USDA-ARS NMGP; Maryland, USA), Nitrogen Fixation in

Tropical Agricultural Legumes (NifTAL; Hawaii, USA), Brazilian Agricultural Research Corporation (EMBRAPA, Brasilia, Brazil), International Institute of Tropical Agriculture (IITA; Ibadan, Nigeria), International Crops Research Institute for the Semi-Arid Tropics (ICRISAT; Andhra Pradesh, India), etc., have been the building blocks for the integration of sustainable/regenerative agriculture and further outreach for these types of entities will only help the field continue to grow. The recent boom in sequencing has allowed for more in-depth genomic processing and phenotypic characterization of a wider association of symbionts for global cropping systems than ever before.

When bioprospecting for novel inoculant strains sampling should be from nodules in a region with similar edaphic properties and climate as the target environment, since rhizobial success in BNF can be significantly influenced by edaphoclimatic properties such as clay content, pH, cation exchange saturation, and rainfall (Hungria et al., 2016). Numerous studies have isolated strains of *Bradyrhizobia* to overcome specific environmental stressors, such as thermotolerance (Rahmani et al., 2009; Bansal et al., 2014), salt tolerance (Hashem et al., 1998; Dong et al., 2017), acid tolerance (Ozawa et al., 1999; Chinnaswamy & Dhar, 2006), and drought tolerance (Cerezini et al., 2020; Zhu et al., 2022). While overcoming each one of these stressors is important, drought-tolerant nitrogen fixation has been predicted to be the trait most pertinent to soybean yield (Sinclair et al., 2010). Despite efforts made to combat abiotic stressors, there has been little to no field validation of the efficacy of microbes are associated with salinity or desiccation tolerance (O'Callaghan et al., 2022).

With this aim, Chang Lab set out to bioprospect drought-tolerant *Bradyrhizobium* isolates to reveal how local adaptation to arid environments can impact genome structure and function, ultimately to test *in-vivo* efficacy and be used as a biofertilizer in drought-prone soybean growing regions. Cytryn and collaborators (2007) showed differential expression of 15-20% of the 8,480 open reading frames in *B. japonicum* USDA 110 (since reclassified as *B. diazoefficiens*) from non-solute mediated desiccation stress. Using a selection of 4 genes that displayed the highest expression across all time points as an indicator for desiccation stress, qRT-PCR was performed to determine the expression of the drought-specific genes across hundreds of isolates collected from multiple locations around Texas (Parks, 2018). The genes that make up the molecular marker are trehalose synthase (*treS*), a probable trehalose-6-phosphate synthase (*otsA*), a probable trehalose-phosphatase (*otsB*), and isocitrate lyase (*aceA*), all of which are heavily mentioned throughout literature to help *Bradyrhizobium* mitigate environmental stressors of numerous kinds (Benaroundj et al., 2001; Streeter, 2003; França et al., 2007; Iordachescu & Imai, 2011; Reina-Bueno et al., 2012; Jeon et al., 2015; Orozco-Mosqueda et al., 2019; Zhu et al., 2022). Using the top 15 native isolates selected from the molecular marker, filter-disk desiccation assays were performed as previously described (Van De Mortel & Halverson, 2004). Isolates *B. japonicum* TXVA & TXEA display the highest viability at the 24/72 h timepoints under desiccation stress with percentage survivability over type strain USDA 110 of 2.7/7.1% and 6.0/13.9%, respectively (Parks, 2018).

B. japonicum TXVA was isolated via soybean nodules from Victoria County, Texas (28.7713°N, 97.0068°) which has a mean average precipitation (MAP) of 99.9 cm and an average high temperature (AHT) of 27.0°C (NOAA, 2022). Classified as humid subtropical (Cfa) climate according to the widely used Köppen–Geiger climate classification system with main crops being corn, cotton, and sorghum, this region also displays the attributes of being semi-arid with climactic extremes; however, the bays and estuaries along the coastline buffer the full classification of an arid landscape (Uddameri, 2007; USDA-NASS, 2017; Beck et al., 2018).

B. japonicum TXEA was isolated via soybean nodules from Lubbock County, Texas (33.6754°N, 101.7980°W) with a MAP of 46.6 cm and an AHT of 23.8°C (NOAA, 2022). The Köppen–Geiger classification here is mid-latitude steppe/semi-arid cool climate (BSk) with the main production crops being cotton, sorghum, and corn (Beck et al., 2018; USDA-NASS, 2017). Since TXVA originates from a warmer region with moderate rainfall while TXEA comes from a region with less than half the cumulative rainfall and no coastline to maintain minimal soil moisture, the geographical locations agree with ecological fitness models and with the results of the desiccation assay that TXEA would exhibit higher desiccation tolerance due to its native environment (Zahran et al., 1999; Asadi Rahmani et al., 2009; Adhikari et al., 2012).

Proof of concept and expansion to multi-state field trials

While drought-tolerance was identified via our novel molecular marker system and measured *in-vivo* using a filter-disk desiccation assay, symbiotic BNF is the most important factor of strain selection to be applied as an inoculant. Thus, soybean yield trials were performed with different inoculants at Rio Farms Inc. in Monte Alto, Texas from 2016-2018. Rio Farms is one of the southernmost soybean fields in the nation and represents drought conditions well, being classified as a tropical savanna (Aw) climate with a MAP of 61.1 cm, MAT of 29.8°F, and soils classified as Willacy fine sandy loam which have low water retention (Beck et al., 2018; NOAA, 2022; USDA-NRCS, 2021). Parks (2018) has previously reported years 2016/2017, which consisted of hand-planted hilltop plots spaced 6 in apart in randomized complete block design (RCBD) with seed-coat inoculation treatments of the native strains, a commercial inoculant, type strain *B. japonicum* USDA 110, and a non-inoculated control. Soybean variety (cv.) Vernal was used for the first two years and was the first commercially available cultivar that showed success in south Texas (Hartwig, 1993). Overall, the application of all native strains was significant compared to all other treatments for both planting years (Parks, 2018).

For the field trials in 2018, commercial-sized planting was done via the split-plot method over 29 ac using in-furrow inoculation at the rate of 1 gal/7 ac. These two distinctions separate this trial from the previous years, with a drastically increased field size bringing spatial and edaphic variation while in-furrow inoculation technique presenting a more commercially accepted application method by leaving room on the seed for pre-fertilizer, pesticide, or fungicide seed treatments that may be deleterious to the inoculant (Santos et al., 2021). Inoculant treatments were the drought-tolerant inoculant TXVA, a commercial inoculant, and a non-inoculated control. The soybean cultivar used was cv. Capitan, an Asian rust-resistant line. The soybean field was maintained without irrigation. Combine harvest was performed, revealing that application of the native drought-tolerant inoculant showed a statistically significant yield increase of 54.9% and 24.2% over the non-inoculated control and commercial inoculant, respectively (Fig. 1-2).

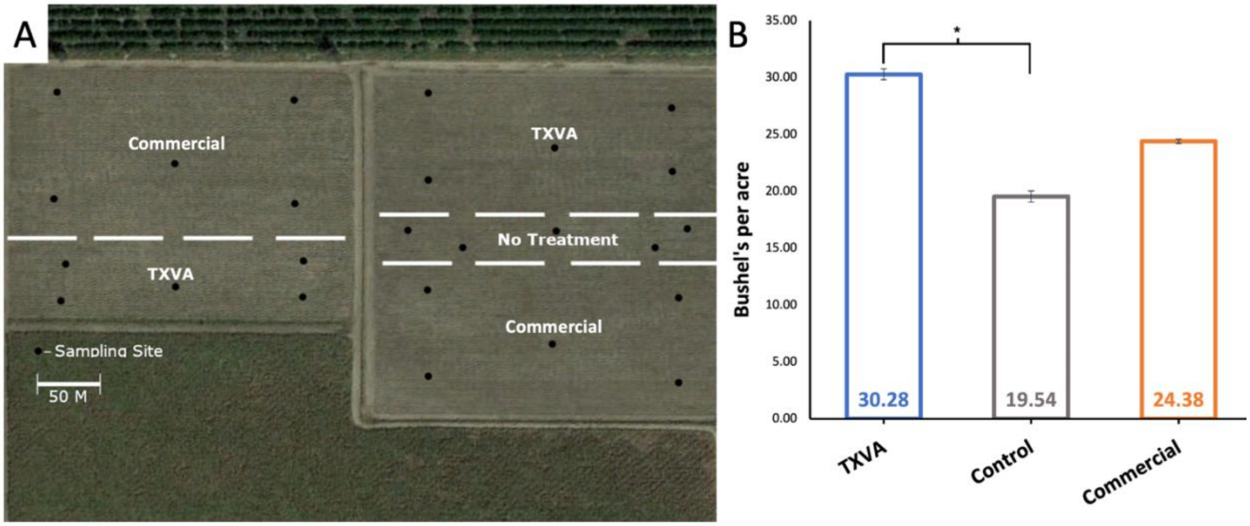


Figure 1-2. (A) Aerial photograph of the 2018 Monte Alto plant site. Dashed white lines indicate treatment delineations while black dots represent randomized mid-harvest sampling points. (B) Final soybean yield in bushels per acre. Statistical analysis performed was two-factor ANOVA without replication with * = p-value < 0.01.

The efficacy of the drought-tolerant TXVA inoculant was further evaluated across multiple regions, including commercial plots in Weslaco TX (Fig. A-1), Yoakum TX, Jackson TN, Clarkton/Portageville MO, Winnsboro LA, Stoneville MS, and Stuttgart AR. According to the most common climate classification system, Köppen-Geiger, all sites belong to Cfa apart from Weslaco, which belongs to Aw. While this climate classification may infer similar environmental characteristics, regional planting specifics still render various environments, soil types, and management conditions to test the native inoculant (Saracoglu & Sanli, 2021, Firth et al., 2022).

Heat stress using biologically relevant temperature gradients

The general scientific consensus regarding global climate patterns is that anthropogenic factors have expedited the fluctuation in earth's natural phases of warming and cooling, which creates an Abrupt Change in our Ecological System (i.e., ACES) (Turner et al., 2020). Common themes among ACES exist, like the differing impact of climate change upon ecosystems, events being typified by extremes rather than averages, and the compounding effect of numerous drivers; all are factors which befuddle current climate models and underestimate the risk of unforeseen interactions (Zscheischler et al., 2018). Global meteorological records display a 1°C rise in MAT specifically over croplands in the past century and with projected temperature increases of 2.5-5°C forecasted for the next 50 years (Afroz et al., 2021). The importance of high temperature as a contributor to drought has increased over time by displaying land-atmospheric feedbacks, like reduced snowmelt accumulation leading to reduced streamflow for downstream farmers and cities showing spatiotemporal and additive effects that would be detrimental to inter-basin transfer, which is the go-to solution for regional drought (Brunner et al., 2021). Development of local and region-specific climate-smart farming practices, like utilizing crop rotation/cover crops from leguminous species and using biofertilizers to increase nitrogen use efficiency, are necessary to preserve crop production, especially for underserved producers who are typically more vulnerable to these climatic events than commercial farms (Arnold, 2021).

Optimum growth of soybean occurs from 26°C to 30°C and with advances in breeding and crop production systems, such as early soybean production systems (ESPS) and high-yielding cultivars from different maturity groups; farmers across environments have been able to utilize this leguminous crop into their rotation (Grichar et al., 2010). Despite the accepted linear trend of increasing yields along temperature gradients up to 29°C for corn, 30°C for soybean, and 32°C for cotton, the decline beyond these points is multifactorial and thus presents a downward trend much steeper than the preceding incline of maximized yield potential up to this point (Schlenker & Roberts, 2009). It has been known that heat stress causes a decrease in germination potential, decrease in nodulation and function of successful nodules, reduction in photosynthesis and transpiration, and cumulatively these all lead to less economic and environmental yield (Kumagai & Sameshima, 2014; Rosenthal et al., 2014; Jumrani et al., 2017).

Bacterial inoculants have been shown to impart environmental tolerance to its mutualist partner, such as, *Achromobacter piechaudii* relieving water stress in tomatoes and peppers (Mayak et al., 2004), thermotolerant *Rhizobium* for mungbean (Bansal et al., 2014), ACC production for wheat by *Pseudomonanas palleroniana* DPB16 and *Variovorax paradoxus* RAA$_3$ (Chandra et al., 2019), *Paenibacillus yonginensis* DCY84^T to improve salt stress in *Panax ginseng* (Sukweenadhi et al., 2018), and *Bradyrhizobium* sp. under general edaphoclimatic stressors for many leguminous crops (Delamuta et al., 2017; Cerezini et al., 2020; Jovino et al., 2022). Among these, the most detailed studies involve the soybean-*Bradyrhizobium* symbiosis due to the downstream economic applications of the seed products and unique nutrient cycling ecological niche of the bacterium. *Bradyrhizobium* that elicits thermotolerance has been shown to utilize very similar survival mechanisms as salt and desiccation-tolerant isolates with increased production of the compatible solute trehalose (Sugawara et al., 2010; Asadi Rahmani et al., 2009). Thus, we tested the ability of *Bradyrhizobium japonicum* TXVA, a Texas native strain known to impart drought-tolerance to soybean, in its ability to relieve biologically relevant elevated heat stress (i.e., +2°C and +4°C) on the maturity group 4L soybean cv. TN16-520R1 in a novel temperature gradient greenhouse in collaboration with North Carolina State University and the USDA.

Genomic characterization of Texas native strains

Trends in science have supported more through sequencing depth with increasingly lower costs which has driven an abundance of omics data across industries, especially popular in the sustainable agriculture movement to develop higher yielding and more environmentally stable crop production systems with climate-smart practices (Li & Yan, 2020; Djemiel et al., 2022; Diwan et al., 2022). Increasing volatility in global climate across numerous crop production regions indicates the need for genomic characterization of the rhizosphere to view how above- and below-ground processes respond to environmental stressors (Qiu et al., 2019; Ashraf et al., 2019, Parks et al., 2022). Woese & Fox (1977) characterizing the rRNA gene as a molecular chronometer to determine phylogeny paved the way for marker development in model species so more in-depth analysis can be done for nutrient utilization pathways of different PGPR under environmental stressors with the goal of highlighting function and shedding light on community interactions. The International Nucleotide Sequence Database Collaboration (INSDC) is made up of the DNA databank of Japan (DDBJ; Mishima, Japan), European Nucleotide Archive (ENA; Hinxton, UK), and National Institute of Health's GenBank (NIH; Bethesda, Maryland) and represents the global standard for providing synchronous molecular data by working in combination with emerging sequencing technologies, genomics standards communities, and researchers from all sectors to uphold its concept of FAIR data principles of making data Findable, Accessible, Interoperable, and Reusable (Karsch-Mizrachi et al., 2018). The deposition of whole genome sequences in these databases allows for a more detailed phylogeny, functionality across niches, and communal interaction potential based on genes corresponding to cellular-functioning, virulence, pathogenicity, survival, antimicrobial resistance, and biocide resistance (Stevens et al., 2022).

The genus *Bradyrhizobium* was reported to contain 187 publicly available genomes in 2019 and have since added 19 strains, including the 2 drought-tolerant Texas isolates TXVA & TXEA (Ormeño-Orrillo & Martínez-Romero, 2019, Mousavi & Young, 2022, Peterson et al., 2022). Potential applications as an inoculant for sustainable crop production highlight these genomes as a bio-resource used to search for desirable traits, such as saprophytic ability and enhanced nitrogen fixation capability, that will benefit from surveying diverse geographical regions for elite strains (Mortuza et al., 2019). Texas-native strains (i.e., *Bradyrhizobium japonicum* TXVA & TXEA) were isolated using a desiccation-specific molecular marker and have been confirmed as successful inoculants in drought-prone agricultural regions. The focus of this study was to perform whole genome sequencing on the TXVA & TXEA isolates for phylogenetic characterization with the hopes of finding unique gene regions relating to the symbiosis, nitrogen fixation, desiccation tolerance, and quorum sensing, or to be used as identifying tags for future studies. Sequence Characterized Amplified Region (SCAR) markers present an opportunity to track persistence of applied inoculants with strain-level precision to determine ecological fitness and need of application (Ambreetha & Balachandar, 2022). By providing SCAR markers, which have already been developed for numerous inoculants, the behavior and fate of the native strains can be tracked to evaluate nodule occupancy, potential spread, and impact on local microbial communities.

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CHAPTER 2

Evaluation of a drought-tolerant *Bradyrhizobium* inoculant for nodulation and soybean production in the Mid-South

Abstract

The soybean-*Bradyrhizobium* symbiosis is fundamental to nutrient management systems in climate-smart agriculture by providing a multi-modal regeneration of sustainable crop production in light of the changing global climate. Improved plant and soil health and a reduction of the need for synthetic fertilizers along with increased yield quality and quantity are commonly observed results from application of a *Bradyrhizobium* biofertilizer (i.e., inoculant). In general, abiotic stressors present the greatest hinderance to consistency of inoculation efficacy, specifically with drought which inhibits both mutualist partners. Here, we evaluated the performance of the Texas-native drought-tolerant *B. japonium* TXVA inoculant compared to a non-inoculated control on plant growth, root nodulation, and final yield across varying soil profiles. Field trials were performed for three years (2019, 2020, and 2021) in the following locations: Yoakum, TX, Winnsboro, LA, Jackson, TN, Clarkton/Portageville, MO, Stoneville, MS, and Stuttgart, AR. Application of TXVA showed synergism with drought-tolerant soybean varieties in plant biomass and nodulation. Specifically, the best nodulation response was observed in Yoakum, TX, which is correlated to the highest average season temperature and greatest fluctuation in cumulative rainfall. Nodulation response was also significant in MO and AR. Overall, application of TXVA increased soybean yield 7% averaged across all locations and years. The results confirm successful translational application of a molecular marker-derived *Bradyrhizobium* inoculant in aiding drought-prone soybean production regions in the Mid-South.

Introduction

Soybean [*Glycine max* (L.) Merr] in North America grew to 34% of the world's production in 2019 (USDA, 2020). The size of this system reflects the diverse nature of soy-derived products and their evolving applications, ranging from human and livestock foods to biodiesel and seed oil production (Singh & Krishnaswamy, 2022). As increases in soybean acreage to meet the global demand for our growing society is limited, innovative techniques must be provided to farmers to maximize production systems. The health of soils and ecosystems should also be considered in balance while taking into account sustainable soybean production (Crookston et al., 1991; Reimer et al., 2017). Two key components to maintain soil health are a relationship between soil and microbes and plant-microbe interactions. The process called biological nitrogen fixation (BNF) is the best example of the plant-microbe symbiosis. It is facilitated through the mutualism where gram-negative *Bradyrhizobium* sp. infect the roots of compatible legumes, differentiate into compartments termed nodules, and fix atmospheric nitrogen into ammonia within nodules. This occurs via the oxygen-sensitive nitrogenase enzyme in return for photosynthates from the plant. Soybean production is calculated to fix around 16.4 Tg N globally and 5.7 Tg N in the United States, annually (Herridge et al., 2008). This process aids cropping systems by negating the need for excess synthetic nitrogenous fertilizers which are ecologically detrimental since their production relies on natural gas and their over-use causes leaching into groundwater, ammonia volatilization, and denitrification (Bashir, 2013).

Sustainable agriculture faces not only damaging concerns with the growth of the global food-supply chain, but also maintaining issues under increasingly fluctuating environmental conditions. The two environmental metrics temperature and precipitation in growing season control about 30% or more of yield variation for the world's six most important crops, including wheat, rice, maize, soybean, barley, and sorghum (Lobell et al., 2005; Leff et al., 2004; Kaufmann & Snell, 1997; Parry et al., 2005; Stone, 2001). For soybean specifically, it has been found that with one degree Celsius increase in growing season temperature, there was a 17% reduction in yield (Lobell & Field, 2007). Soybean has many physiological mechanisms to subvert drought stress, such as rooting depth extension and leaf area development, but the largest beneficial drought trait was found to be drought-tolerant nitrogen fixation (Sinclair et al., 2010). BNF is often inhibited in arid and semiarid soils due to the poor survival of rhizobia under the desiccation condition (Mary, 1985; Cytryn et al., 2007). This poor survival rate is because of the cascading effects of drought including reduced oxygen availability, reduction of carbon flux to nodules, a decline in nodule sucrose synthase activity, and an increase in ureides and free amino acids (Manavalan et al., 2009). These traits lead to a disbalanced symbiosis by limiting metabolic functioning and causing oxidative damage to cellular components, all with a negative impact on BNF. A difference in leaf and nodule water potential also holds the key to understanding this mutualism under drought, with a known 70% reduction in nitrogenase activity after four days of drought while only a 5% reduction in photosynthesis in the same period. This suggests that water stress influences the impaired respiratory condition of the nodule independent of photosynthesis (Naya et al., 2007).

Bell et al. (2019) outlined the limitations of growth for the inoculant (biofertilizer) industry to include failure of inoculants under unfavorable environmental conditions and shelf-life concerns of the inoculant. Therefore, developing and optimizing a drought-tolerant inoculant could aid in technology adoption by displaying the ease of use, beneficial results, and return on investment. To address these concerns, randomized sampling of soybean root nodules was performed across drought-prone sites in Texas with subsequent isolation of rhizobia from the nodules to bioprospect potential drought-tolerant isolates (Parks, 2018). Using a molecular marker system derived from a genome-wide transcriptional analysis of *B. japonicum* USDA 110 under desiccation stress, four primary isolates were selected to initiate preliminary field trials in 2016 (Parks, 2018). The isolate that exhibited the highest symbiotic efficacy, plant vitality, nodulation, and final yield at Rio Farms Inc., Monte Alto, Tx. was *B. japonicum* TXVA (Parks, 2018).

This study was conducted at six locations in three years (2019, 2022, and 2021) to test the TXVA inoculant against a non-inoculated control (Fig. 2-1). Taking into consideration that 94% of US farmland is rainfed, only non-irrigated plots were chosen for all sites (Purdom, 2021). Diverse soybean cultivars (cv.) were used to determine the cross-inoculation efficacy of the TXVA strain and determine if any synergism was present with fast-wilting (i.e., drought-sensitive) or slow-wilting (i.e., drought-tolerant) soybean lines. The bacteriome of soybean nodules has been shown to vary amongst cultivars under water stress, thus indicating a competitive niche that connects nodule occupants with optimum function and physiological response that ultimately enhance drought resilience (Sharaf et al., 2019).

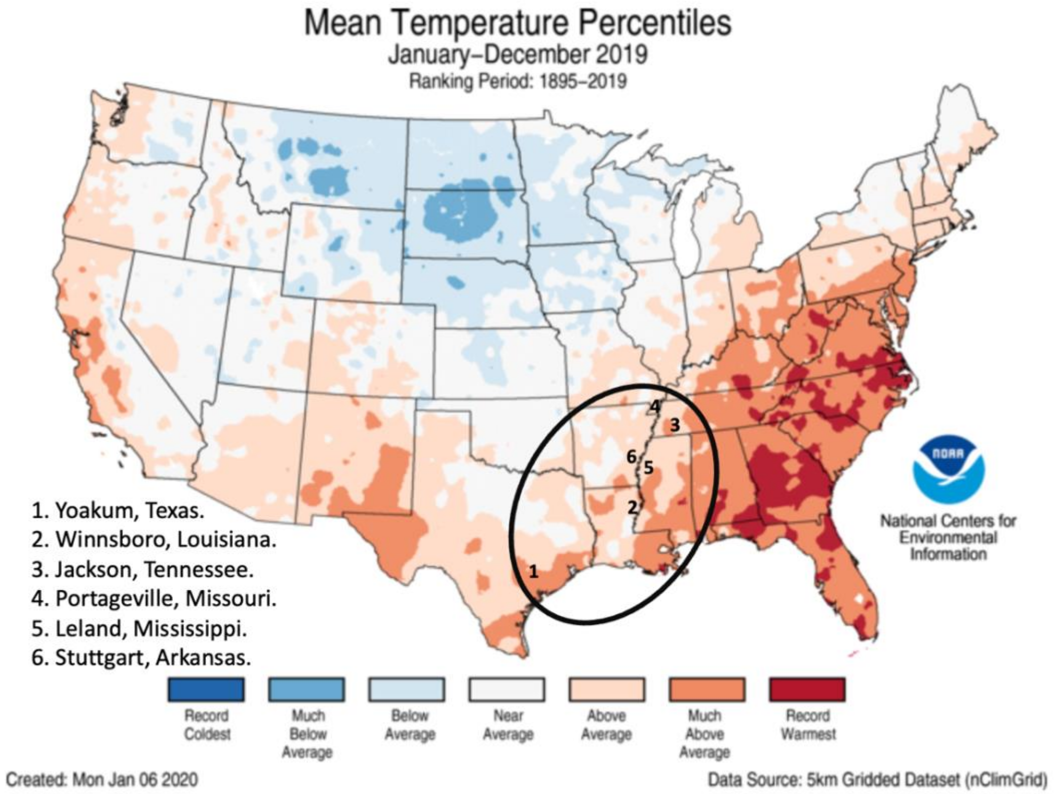

Figure 2-1. Increased mean temperature in 2019 as compared to 135-year temperature averages with numbered site locations (NCEI, 2020).

Materials and Methods

Location of field trials

In 2019, field trials were conducted at the following sites: i) Texas A&M AgriLife Research station in Yoakum, TX, ii) the University of Tennessee Institute of Agriculture Research site in Jackson, TN, iii) Rhodes farm at the University of Missouri in Clarkton, MO, iv) Macon Ridge Research station at the Louisiana State University College of Agriculture in Winnsboro, LA, v) the USDA Agricultural Research Station in Stoneville, MS, and vi) Dale Bumpers National Rice Research Center at the University of Arkansas in Stuttgart, AR.

In 2020, the only change in research stations was relocation of the trial from Rhodes farm in Clarkton to Lee Farm in Portageville in MO due to severe dicamba damage from surrounding farms and high southern root-knot nematode pressure (C. Vieira, personal communication). The other locations have remained the same as in 2019.

In 2021, all filed trials were carried out in the same locations as in 2020. In addition, all plots were moved to new fields on the same station each year to prevent carry-over of any inoculant that may have persisted from the previous year. However, rotation of the trial in Stoneville, MS to a new field was not possible due to land availability. We selected the six research sites because the climate types across all sites were humid subtropical based on the Koppen-Geiger climate classification system (Beck et al., 2018) (Fig. 2-1).

Experimental design and soil physio-chemical testing

2019 (Year 1)

The Yoakum field site had 5 replicates of 2 treatments and consisted of 6-row plots on 97 cm rows with a 6 m length using the cv. Dyna Grow S52RS86 (maturity group [MG] 5E, Reiter et al., 2018). Planting was performed with a 2-row vacuum planter (Monosem ATI, Inc., Lenoxa, KS, USA) set at a seeding rate of 33 seed m^{-1} (55,847 seeds ha^{-1}). The next location was Jackson with 4 replicates of 2 treatments and consisted of 4-row plots on 76 cm rows with a 3 m length. Starting in Jackson, the cv. TN16-520-R1 (MG4L; Boehm et al., 2019) was standardized across all locations for the remainder of the trial. Planting was carried out with a 4-row cone planter (Almaco, Nevada, IA, USA) set at a seeding rate of 30 seed m^{-1} (50,770 seeds ha^{-1}). Rhodes farm in Clarkton consisted of 5 replicates of 2 treatments with 4-row plots on 76 cm rows and a 3.7 m length. Planting was completed with an 8-row cone planter (Almaco, Nevada, IA, USA) set at a seeding rate of 40 seed m^{-1} (67,016 seeds ha^{-1}). The field site in Winnsboro had 4 replicates of 2 treatments with 4-row plots on 102 cm rows and a 7.6 m length. Planting was performed with a 4-row cone planter (Almaco, Nevada, IA, USA) set at a seeding rate of 33 seed m^{-1} (55,847 seeds ha^{-1}). The Stoneville location had 4 replicates of 2 treatments and consisted of 4-row plots on 97 cm rows with a 4.6 m length. This field site was planted with a 4-row cone planter (John Deere, Grovetown, GA, USA) set at a seeding rate of 30 seed m^{-1} (50,262 seeds ha^{-1}). Lastly, the field site in Stuttgart contained 5 replicates of 2 treatments and was placed on 4-row plots with 76 cm rows and a 4.6 m length. Planting was completed with a 4-row cone planter (Almaco, Nevada, IA, USA) set at a seeding rate of 33 seed m^{-1} (55,847 seeds ha^{-1}).

2020 (Year 2)

All treatments were maintained across site years with replicate and variety additions discussed below. Portageville, Stuttgart, and Stoneville were able to expand the trial to include 6 replicates while Yoakum, Winnsboro, and Jackson replicates were kept the same. The commercial variety CZ5515 (MG5, Bayer CropScience LP, Research Triangle NC, USA) was added in Yoakum and Winnsboro. A Texas-specific indeterminate cv. Otoño (Dr. A. Scott, personal communication, 2019) was also tested in Yoakum to make 3 cv. total. The fast-wilting variety USG-7496XTS (MG4L, Purdom 2021) was included at Jackson and Winnsboro to make a total of 2 and 3 cv., respectively. Additionally, the fast-wilting cv. S14-9017R was added to Stoneville and Stuttgart to make 2 varieties while Portageville received this cv. along with the slow-wilting S11-20242C, totaling 3 (MG5E, Chen et al., 2020).

2021 (Year 3)

Replicate count and plot sizes remained constant for the final year of the trial. Altered varieties include removing Otoño in Yoakum and swapping CZ5515 for the fast-wilting S14-9017R in both Yoakum and Louisiana totaling 2 and 3 varieties, respectively. The slow-wilting cv. S11-2024C was added at Stoneville and Stuttgart making a total of 3 varieties at each site. Jackson and Missouri had no variety changes.

Each field site was set up as a randomized complete block design (RCBD) to minimize for edaphic variation and were managed by their respective technicians according to their state extension recommendations. Soil cores were taken according to suggested USDA soil sampling protocols before planting at each site (USDA-NRCS, 2007). The soil cores were immediately submerged in ice, taken back to lab, sieved using a 2 mm sieve, and dried using a forced air oven at 65°C for 16 hr. or until dry. Processed soils were sent to the Soil, Water, and Forage Testing Laboratory at Texas A&M for physio-chemical property evaluation. All location year information including GPS coordinates, previous crop, planting, sampling, and harvest dates are listed below in Table 2-1.

Table 2-1. Summary of geographical coordinates, previous cropping information, planting date, sampling date, and harvest date for all location years. Note the changing GPS coordinates for each site year, with the largest change from 2019 to 2020 in MO representing approximately 20 miles between research stations.

Location	Year	Latitude	Longitude	Previous crop	Planting date	Sampling date (DAP[a])	Harvest date (DAP[a])
Yoakum, TX	2019	29.2761° N	97.1220° W	Peanuts	2-Apr	22-May (51)	6-Sep (158)
	2020	29.2774° N	97.1212° W	Corn	1-Apr	8-Jun (69)	2-Sep (155)
	2021	29.2769° N	97.1220° W	Corn	18-Mar	23-Jun (97)	20-Aug (156)
Winnsboro, LA	2019	32.1385° N	91.6885° W	Soybeans	16-May	12-Jul (58)	16-Sep (124)
	2020	32.1416° N	91.6959° W	Cotton	11-May	14-Jul (65)	16-Sep (129)
	2021	32.1387° N	91.7011° W	Corn	27-May	5-Aug (72)	4-Oct (131)
Jackson, TN	2019	35.6230° N	88.8495° W	Soybeans	6-May	25-Jun (51)	4-Oct (152)
	2020	35.6201° N	88.8487° W	Soybeans	15-May	27-Jul (74)	21-Oct (160)
	2021	35.6233° N	88.8491° W	Soybeans	19-May	22-Jul (64)	22-Oct (157)
Portageville, MO	2019	36.4885° N	89.9629° W	Cotton	7-May	10-Jul (65)	9-Oct (156)
	2020	36.4066° N	89.6157° W	Corn	2-Jun	28-Jul (57)	4-Nov (156)
	2021	36.4072° N	89.6162° W	Soybeans	19-May	20-Jul (62)	27-Oct (162)
Stoneville, MS	2019	33.4328° N	90.8996° W	Soybeans	17-May	11-Jul (56)	2-Oct (139)
	2020	33.4317° N	90.9099° W	Corn	29-Jun	9-Sep (73)	29-Oct (123)
	2021	33.4317° N	90.9099° W	Soybeans	3-Jun	4-Aug (62)	11-Oct (131)
Stuttgart, AR	2019	34.4749° N	91.4224° W	Soybeans	2-Jul	28-Aug (58)	23-Oct (114)
	2020	34.4683° N	91.4214° W	Rice	1-Jul	10-Sep (72)	7-Nov (130)
	2021	34.4702° N	91.4289° W	Soybeans	24-Jun	25-Aug (62)	24-Oct (122)

[a] DAP = Days after planting.

Inoculant preparation and application

The TXVA inoculant was cultured in arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of Na_2HPO_4, 250 mg of Na_2SO_4, 320 mg of NH_4Cl, 180 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2$, 4 mg of $FeCl_3$, 1.3 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1 g of 2-(N-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate per L (Sadowsky et al. 1987). Fresh cultures of TXVA were incubated aerobically at 30°C with vigorous shaking at 200 rpm until reaching an OD_{600} of 1.5, which results in a cell density of 4.5×10^9 cells/ml. Application of the TXVA inoculant was standardized across all sites at a rate of 10 ml per 1 lb. seed. The application rate was determined by ensuring complete coverage with the inoculant by observing that every seed was glistening and allowed absorption into the seedcoat within 5 min.

Planting techniques, physiology measurements, and harvesting

At each location, the control seed was planted first followed by seed treated with the drought-tolerant TXVA inoculant to avoid cross-contamination. Plants were harvested at 2 time points with the first targeted around the end of vegetation/beginning of reproductive stages (i.e., growth stage V6-R2), indicated by flowering of the plants, to measure above-ground plant biomass and root nodule location/enumeration. A second harvest was performed at full maturity (i.e., growth stage R8) for final harvest yield comparison.

During mid-season harvest, which usually occurred around 7-10 weeks of growth, 4 - 6 plants per treatment block were randomly selected from the outer 2 rows and labeled. The growth extremes were avoided, ensuring not to take the largest or smallest plants to minimize unnecessary variation. These plants were gently removed from the soil with a pointed digger shovel used to separate the rhizosphere/root-ball intact from the surrounding bulk soil without damaging either the deep taproots or wide-reaching lateral roots that serve as the vessels of nodulation. Plants were cut at the color change from green to brown, representing transition from stem to root. The labeled plants were taken back to lab and air dried at room temperature for 2 weeks to ensure that stems snapped easily before taking average above-ground plant biomass. The corresponding root sections were gently rinsed using distilled water from a 2-gal spray tank with a cone nozzle to prevent fungal contamination after storage. The root systems from each respective plot were jarred together using a cold 30% glycerol solution and kept on ice for transit back to lab where they were stored at 4°C for determination of nodulation parameters. Parameters examined include spatial distribution (i.e., presence on taproot and total root) and enumeration according to this distribution. The presence of taproot nodules is heavily correlated to success of applied inoculants by ensuring more viable cells from seed application time to root emergence indicating persistence in the rhizosphere microbiome (Singleton et al., 2009).

Harvesting for final plant yield was performed at full maturity (R8), marked by a loss of leaf biomass and all pods exhibiting a sandy brown color which indicates an optimum seed moisture content of around 14-15% (Delouche, 2021). A 2-row small plot harvest combine was used at most sites (Almaco SPC40, Nevada, IA, USA); however, at the Yoakum site for all years and Stuttgart site in 2019, a hand harvest was necessary due to lack of available equipment in Texas and the impending tropical storm Olga in Arkansas (NCEI, 2021). For combine harvest, the 2 whole functional rows were harvested while for hand harvest, only 2 select uninterrupted lengths of functional rows were harvested via hedge clipper. Yields in bushels per acre were determined by using the following formula:

$$Bushels\ per\ acre = \ (Harvested\ in\ lbs) \div Standard\ lbs\ per\ bushel\ \div plot\ area\ in\ acres$$

Statistical analysis

All data displayed a normal distribution pattern with no outliers, despite some sites showing high variability. Statistical analysis for significance was carried out in Microsoft Excel using two-factor ANOVA model without replication, as necessitated by randomized complete block design, and is defined by the equation $Yij = \mu + \eta i + \mathcal{E} j + eij$. Minimum significance for trials was considered $p \leq$0.05. Principle component analysis (PCA) was performed for each year to evaluate the interconnections between the edaphic physio-chemical properties (i.e., pH, conductivity, elemental nutrients, and organic matter), average seasonal high temperatures, and cumulative seasonal rainfall using factoextra_1.0.7 (Kassambara & Mundt, 2020) package in R version 4.2.0.

Results

Edaphic properties and nutrient levels

The measured physio-chemical characteristics from initial bulk soils sampled from each site-year are displayed in Table 2-2, revealing Yoakum to have the lowest average soil organic matter % (OM), while Winnsboro presented the highest and most consistent nitrate (N) and sodium (Na) levels, along with the most consistent OM%. The soil profile with the highest concentration of phosphorus (P) and potassium (K) throughout the 3-year study was Stoneville, along with the most consistent pH, conductivity, and PK values which reflect the fertile soils of that region. Lastly, the clay-rich profiles of Stoneville and Stuttgart presented the highest average iron concentration across all three years.

Table 2-2. Soil profile and physio-chemical characteristics from each site year. Values in parts per million (ppm) or percentage (%). OM = organic matter.

	Yoakum TX			Winnsboro LA			Jackson TN			Portageville MO			Stoneville MS			Stuttgart AR		
	2019	2020	2021	2019	2020	2021	2019*	2020	2021	2019	2020	2021	2019	2020	2021	2019	2020	2021
Soil Profile	Tremona loamy fine sand			Gigger-Gilbert silt loam			Iuka fine sandy loam			Malden fine sand	Portageville silty clay loam		Sharkey Clay			Dewitt silt loam		
pH	6.4	7.8	6.1	6.1	6.7	5.9	6.6	5.1	6.0	6.3	5.2	6.6	5.9	5.8	5.8	4.6	5.0	4.5
Conductivity	67	287	129	241	100	202	N/A	67	143	82	229	115	187	277	180	265	104	141
Nitrate (ppm)	7	19.3	14.0	30	5.5	30.0	N/A	1.5	14.0	10	25.5	11.0	7	30.0	19.0	19	7.5	7.0
Phosphorus (ppm)	23	63.7	32.0	50	63.0	49.0	16	57.0	117.0	78	46.5	55.0	60	88.0	94.0	44	19.5	27.0
Potassium (ppm)	103	345	179	73	123	150	37	98	165	89	159	170	214	328	258	102	111	140
Calcium (ppm)	803	5624	773	1210	1256	959	920	671	1656	654	1607	1908	2755	1786	2262	895	955	1000
Sodium (ppm)	33	37	16	18	41	66	7	1	12	3	1	11	25	1	18	50	1	27
Iron (ppm)	12	7.7	14.0	7.2	13.8	25.0	15	24.1	30.9	6.7	25.4	16.7	14.3	37.6	61.0	35.4	48.6	102.5
Manganese (ppm)	16.6	4.9	18.2	45.3	28.6	33.9	16.5	49.3	23.8	62.8	23.5	9.2	24.3	11.3	8.6	124.7	32.2	33.3
OM^a %	0.78	1.94	0.57	1.61	1.60	1.50	N/A	1.60	1.99	1.06	1.58	1.43	1.41	1.39	1.22	1.63	1.72	1.44

* For the 2019 trial in TN, conductivity, nitrate, and organic matter (OM) were not measured.

Seasonal weather conditions

The average high temperature (AHT; °C) and cumulative rainfall (CR; cm) from each month of each growing season site-year are displayed in Table 2-3, geographic differences in planting month, duration to maturity, and seasonal climate intensity are apparent. On average, AHT throughout the growing season was the most intensive in 2019 for Yoakum (32.8°C), Winnsboro (32.8°C), Jackson (29.3°C), and Clarkton/Portageville (28.9°C). Regarding Stoneville and Stuttgart, the hottest average seasonal high-temperatures occurred in 2021 with 31.6°C (MS) and 30.3°C (AR). Stoneville had the largest deviation in average high-temperature across years with a standard deviation (SD) of ± 0.9°C followed by Winnsboro with a SD of ± 0.6°C. Yoakum exhibited the largest deviation between cumulative seasonal rainfall amounts per year with a SD of ± 22.3 cm, then Winnsboro second with an SD of ± 12.1 cm. Stuttgart represented the most temperate environment displaying no temperatures above or precipitation below the 30-year averages. Finally, Yoakum broke AHT climate normals in 11/18 months of this trial.

PCA by year reflecting the soil physio-chemical characteristics, seasonal AHT, and cumulative rainfall display distinct clustering with percentage yield increase of applied inoculant (blue circle) suggesting yearly interactions of weather and edaphic conditions determining success of the drought-tolerant inoculant (Fig. 2-2).

Table 2-3. Average high temperature (AHT) and cumulative rainfall (CR) per month for each site-year

		April		May		June		July		August		September		October	
		AHT (°C)	CR (cm)	AHT (°C)	CR (cm)	AHT (°C)	CR (cm)	AHT (°C)	CR (cm)	AHT (°C)	CR (cm)	AHT (°C)	CR (cm)	AHT (°C)	CR (cm)
Yoakum TX	2019	26.2	5.50 [b]	29.4	13.9	32.9 [a]	15.6	34.9 [a]	4.30 [b]	37.3 [a]	0.40 [b]	35.8 [a]	7.70 [b]		
	2020	26.5 [a]	7.40 [b]	30.6 [a]	25.8	32.0	6.30 [b]	35.4 [a]	8.30	36.2 [a]	2.50 [b]	30.8	14.2		
	2021	27.2 [a]	0.70 [b]	29.7	46.0	32.9 [a]	12.8	32.9	21.0	34.7	9.30	33.4 [a]	1.80 [b]		
	30 yr [1]	*26.3*	*8.10*	*29.8*	*10.1*	*32.7*	*11.5*	*34.1*	*7.90*	*34.9*	*8.30*	*32.3*	*9.70*		
Winnsboro LA	2019			28.8	12.9 [b]	31.8	8.00 [b]	33.1	19.3	34.7 [a]	3.20 [b]	35.5 [a]	0.20 [b]		
	2020			28.2	9.30 [b]	32.0	8.30 [b]	34.0	7.50 [b]	33.8	7.00 [b]	30.5	14.4		
	2021			27.3	15.6	32.6	10.3 [b]	33.6	14.8	34.1	20.8	31.1	4.30 [b]		
	30 yr [1]			*29.3*	*13.4*	*32.8*	*12.0*	*34.3*	*10.0*	*34.4*	*9.00*	*31.3*	*8.60*		
Jackson TN	2019			28.2 [a]	9.30 [b]	29.6	10.4 [b]	31.5	32.5	31.6	10.3	32.6 [a]	3.80 [b]	22.4	8.50[b]
	2020			25.5	10.7 [b]	30.6	6.30 [b]	33.6 [a]	14.6	31.7	11.0	28.1	8.00 [b]	23.1	9.20[b]
	2021			24.8	9.70 [b]	29.8	6.40 [b]	31.3	11.4	31.4	23.0	28.4	6.90 [b]	24.2 [a]	11.9
	30 yr [1]			*26.4*	*14.5*	*30.7*	*11.4*	*32.6*	*11.1*	*32.6*	*7.70*	*28.9*	*9.40*	*23.1*	*10.2*
Portageville MO	2019			26.3 [a]	14.5	29.6	14.2	31.4	2.60 [b]	31.4	0.20 [b]	32.3 [a]	2.60 [b]	22.1 [a]	20.3
	2020			N/A	N/A	31.3 [a]	13.1	31.9	12.2	30.2	12.5	26.7	11.0	20.6	15.4
	2021			24.9	6.80 [b]	31.4 [a]	7.50 [b]	31.6	9.32	31.1	9.25	29.0 [a]	9.00	24.2 [a]	18.2
	30 yr [1]			*25.7*	*12.2*	*30.4*	*10.3*	*31.9*	*8.70*	*31.4*	*5.70*	*27.8*	*8.40*	*22.0*	*10.8*
Stoneville MS	2019			28.4 [a]	33.0	29.1	3.70 [b]	32.3	17.3	32	8.50	34.9 [a]	1.70 [b]	23.8	24.1
	2020			N/A	N/A	31.8 [a]	18.9	33.7 [a]	5.60 [b]	31.2	9.60	29.0	12.6	24.1	14.7
	2021			N/A	N/A	31.7 [a]	12.6	33.6 [a]	14.5	33.7 [a]	12.3	31.6 [a]	5.50 [b]	27.6 [a]	6.80[b]
	30 yr [1]			*28.2*	*12.6*	*31.6*	*10.2*	*33.1*	*9.10*	*32.9*	*7.00*	*30.2*	*8.30*	*24.6*	*11.1*
Stuttgart AR	2019					N/A	N/A	32.0	16.8	32.4	10.2	33.3 [a]	0.80 [b]	22.8	15.5
	2020					30.7	15.9	32.9	6.70 [b]	32	11.2	28.6	13.0	22.8	6.70
	2021					31.1	10.3	32.2	14.8	32.5	20.8	30.1 [a]	4.30 [b]	25.4 [a]	8.60
	30 yr [1]					*31.6*	*8.90*	*33.3*	*8.70*	*33.2*	*6.10*	*30.0*	*6.70*	*24.1*	*4.60*

[1] 30-year climate normal with italics (NOAA, 2022).

[a] AHT values above 30-year normal.

[b] CR values below 30-year normal.

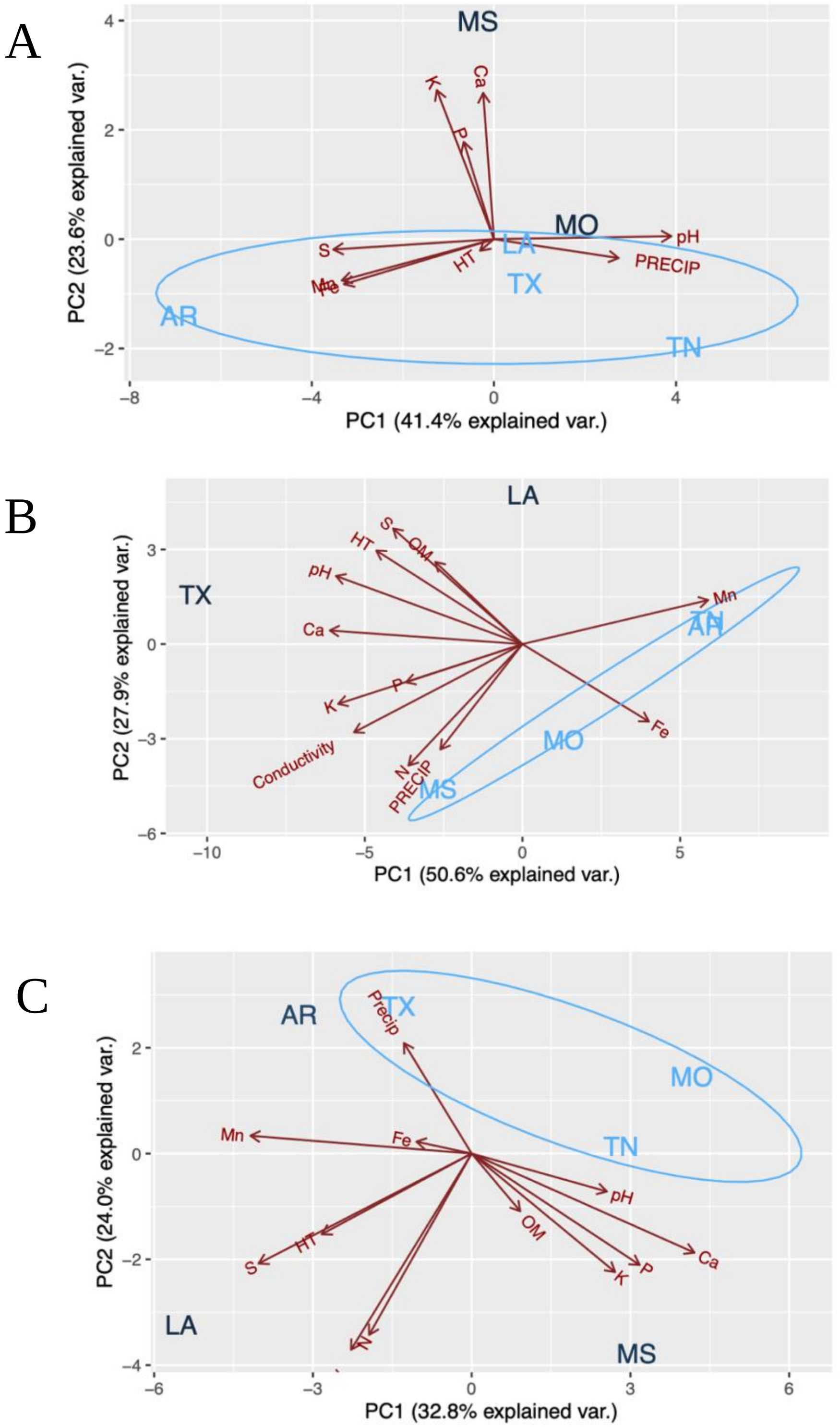

Figure 2-2. PCA analysis of soil physio-chemical properties, cumulative seasonal rainfall, seasonal average high-temperature, and soybean yield at each field site in 2019 (A), 2020 (B), and 2021 (C). Locations that experienced yield increase are circled in blue.

Effect of TXVA on nodulation, plant biomass, and soybean yield

2019

Application of the drought-tolerant TXVA inoculant on dry plant biomass showed non-significant increase at half of the sites in 2019, including Yoakum, Jackson, and Clarkton (Table 2-3). Nodulation efficacy displayed success of the TXVA inoculant via higher taproot nodule counts than the control at every site and higher total root nodule counts at every site excluding Stoneville. Significance was found for taproot and total root counts in Yoakum, Clarkton, and Stuttgart, with Yoakum and Stuttgart being extremely significant. Final yield was significant only at the Yoakum site with an increase of 4.6%. After discarding the data from MO due to planting inconsistencies and unequal replicates, the 2019 planting year average effect of inoculant was a 2.6% yield increase.

Table 2-3. Effect of inoculant treatment on soybean plant growth parameters for the 2019 growing season. Averaged values of 6 biological replicates per technical replicate are shown for each site. (p-value < 0.05 *, p-value < 0.001 **).

Location	**Cultivar**	**Treatment**[1]	**Plant dry Wt (g)**	**NTR**[2]	**NLR**[3]	**TRN**[4]	**Yield (bu/a)**	**ESY**[5] **(%)**
				nodules plant^{-1}				
Yoakum	Dyna Grow S52RS86	T	8.1	5.6^{**}	12.8	30.4^{**}	20.3^{*}	4.6
		C	6.6	1.7	4.5	27.4	19.4	
Winnsboro	TN16-520R1	T	4.3	9.5	11.8	44.6	31.6	3.9
		C	4.6	7.0	11.5	37.8	30.4	
Jackson	TN16-520R1	T	4.9	18.0	29.3	38.1	55.2	4.7
		C	3.8	15.2	28.9	34.5	52.7	
Clarkton	TN16-520R1	T	10.3	11.1^{*}	10.5	51.4^{*}	15.3	-29.2
		C	9.9	7.9	9.1	46.5	21.6	
Stoneville	TN16-520R1	T	8.8	11.6	10.5	52.5	33.8	-4.0
		C	9.8	6.4	17.9	26.3	35.2	
Stuttgart	TN16-520R1	T	7.8	14.8^{**}	12.5	54.2^{*}	23.5	3.5
		C	7.9	6.7	8.4	44.4	22.7	
						AVG ESY (%)		2.6

[1] T = TXVA inoculant treatment; C = non-inoculated control.
[2] NTR = Nodules located on tap root
[3] NLR = Nodules located on lateral roots.
[4] TRN = Total root nodules.
[5] ESY (%) = Effect of inoculant yield; ESY = $\left(\frac{T-C}{C}\right) \times 100$

2020

Data from 2020 shows better trends of plant biomass increase, with 11/15 cultivars possessing higher weights from application of the TXVA inoculant (Table 2-4). Every drought-sensitive variety planted in 2020 showed an increase in average biomass, with significance in cv. S14-9017R1 from Stoneville and Stuttgart. TXVA displayed domination of taproot counts with response in 14/15 varieties, eight of which were significant. Similarly, total root counts show inoculation response with 13/15 varieties and significance in eight. The S14-9017R1 variety at

Portageville displayed a significant effect of inoculant on taproot, total root counts, and final yield with a 13% increase. Stuttgart displayed this same trend with the drought-sensitive variety, having a 9% yield increase but no significance. Yoakum showed fluctuating high-temperature stress with concurrent rainfall deficits and flooding events, also having the most alkaline pH (7.8) and highest concentration of potassium (345 ppm) and calcium (5,624 ppm) of any site-year. After discarding the Yoakum site due to low/inconsistent yields from environmental and edaphic conditions, the 2020 planting year average effect of inoculant was a 4.8% yield increase.

Table 2-4. Effect of inoculant treatment on soybean plant growth parameters for the 2020 growing season. Averaged values of 6 biological replicates per technical replicate are shown for each site. (p-value $< 0.05^{*}$, p-value $< 0.01^{**}$, p-value $< 0.001^{***}$).

Location	Cultivar	Treatment[1]	Plant dry Wt (g)	NTR[2]	NLR[3]	TRN[4]	Yield (bu/a)	ESY[5] (%)
				nodules plant^{-1}				
Yoakum	TN16-520R1	T	17.5	5.6^{***}	10.7	16.3^{***}	19.4	-5.8
		C	17.2	0.0	0.10	0.10	20.6	
	CZ5515	T	23.9	1.6^{***}	3.15	4.75^{***}	12.4	0.0
		C	23.4	0.3	0.10	0.40	12.4	
	Otoño	T	28.7^{*}	4.4^{***}	7.10	11.5^{***}	10.7	245
		C	20.2	0.0	0.08	0.08	3.10	
Winnsboro	TN16-520R1	T	18.0	16.7^{***}	28.2	44.9^{***}	33.5	0.3
		C	18.7	10.8	15.0	25.8	33.4	
	CZ5515	T	11.7	22.5^{**}	28.9	51.4^{***}	16.1	-2.4
		C	12.6	14.4	14.3	28.7	16.5	
	USG-7496XTS[a]	T	31.0	13.6	27.5	41.1	24.4	-1.2
		C	26.1	12.7	22.2	34.9	24.7	
Jackson	TN16-520R1	T	14.6	5.1	18.9	24.0	53.3	-5.5
		C	14.7	3.8	14.9	18.7	56.4	
	USG-7496XTS[a]	T	23.8	6.6	24.2	30.8	51.9	11.9
		C	18.8	6.5	17.6	24.1	46.4	
Portageville	TN16-520R1	T	10.3	7.9^{*}	10.5	18.4^{*}	61.4	5.0
		C	9.70	6.6	7.70	14.3	58.5	
	S11-20242C[b]	T	10.1	8.0	12.8	20.8	64.4	0.3
		C	9.60	6.0	15.2	21.2	64.2	
	S14-9017R1[a]	T	9.90	9.9^{*}	15.2	25.1^{**}	67.4^{***}	13.1
		C	9.80	6.5	8.60	15.1	59.6	
Stoneville	TN16-520R1	T	36.1	4.1^{*}	16.2	20.3	46.8	-4.5
		C	26.3	2.6	15.9	18.5	49.0	
	S14-9017R1[a]	T	43.1^{**}	4.8	15.0	19.8	58.0	9.6
		C	29.6	5.7	15.4	21.1	52.9	
Stuttgart	TN16-520R1	T	15.1	3.1	12.7	15.8	27.5	21.7
		C	17.8	2.4	8.80	11.2	22.6	
	S14-9017R1[a]	T	19.1^{**}	4.3	18.7	23.0^{***}	24.4	9.4
		C	13.8	2.0	8.00	10.0	22.3	
						AVG ESY (%)		7.4

[a] Slow-wilting cultivar, [b] Fast-wilting cultivar

[1] T = TXVA inoculant treatment; C = non-inoculated control.

[2] NTR = Nodules located on tap root

[3] NLR = Nodules located on lateral roots.

[4] TRN = Total root nodules.

[5] ESY (%) = Effect of inoculant yield; ESY = $\left(\frac{T-C}{C}\right) \times 100$

2021

Effect of inoculant on dry plant matter trended slightly lower than the previous year with 10/16 cultivars showing beneficial biomass response, four of which were significant. Five of the seven drought-sensitive cultivars planted showed increased biomass. Inoculant efficacy agreed with previous years, showing increased taproot and total root counts in 13/16 cultivars. Less significance was observed in nodulation compared to the year prior, but still displayed significance at the Yoakum site. The drought-sensitive variety USG-7496XTS (i.e., USG) displayed very promiscuous nodulation at the Winnsboro site with more taproot and total root nodules in the non-inoculated control than the TXVA treated plots; however, the average plant biomass was significant over the control and yields showed a 1.9% increase in the TXVA treatment. The cv. S14-9017R1 that performed well in Portageville, Stoneville, and Stuttgart in 2020 was the worst performing variety in 2021, showing only positive plant growth patterns at the Yoakum site. Following in the flipped nodulation trends, the drought-tolerant variety S11-2024C displayed increased biomass, taproot, and total root nodule domination, but just a slight yield increase (0.3%) with application of TXVA in 2020. The same treatment in 2021 showed decreased biomass, almost equal taproot counts, decreased total root counts, yet a yield increase of 4.2%. This indicates environmental influence in the efficacy of symbiotic formation and functioning. After discarding the Stuttgart yields due to the clay-rich A horizon that caused waterlogging and an uneven stand early in the season, the 2021 planting year average effect of inoculant treatment was a 3.8% yield increase.

Table 2-5. Effect of inoculant treatment on soybean plant growth parameters for the 2021 growing season. Averaged values of 4 biological replicates per technical replicate are shown for each site. (p-value < 0.05 *, p-value < 0.01 **, p-value < 0.001 ***).

Location	**Cultivar**	**Treatment**[1]	**Plant dry Wt (g)**	**NTR**[2]	**NLR**[3]	**TRN**[4]	**Yield (bu/a)**	**ESY**[5] **(%)**
				nodules plant^{-1}				
Yoakum	TN16-520R1	T	17.8**	13.6***	30.0	43.6***	18.6	51.2
		C	11.7	0.1	0.7	0.80	12.3	
	S14-9017R1[a]	T	19.8	20.1**	20.5	40.6**	21.0	17.3
		C	16.4	2.70	6.8	9.50	17.9	
Winnsboro	TN16-520R1	T	41.6	18.9**	19.9	38.8**	52.0	-0.6
		C	38.8	12.6	19.5	32.1	52.3	
	S14-9017R1[a]	T	40.3	16.3	20.6	36.9	49.2	-1.8
		C	44.6	13.6	22.4	36.0	50.1	
	USG-7496XTS[a]	T	47.0*	16.0	23.8	39.8	52.3	1.9
		C	35.9	18.3	27.0	45.3	51.3	
Jackson	TN16-520R1	T	23.4	13.5	44.6	58.1	75.1	2.9
		C	22.5	12.1	44.4	56.5	73.0	
	USG-7496XTS[a]	T	24.3*	19.4	80.5	99.9	56.2	19.3
		C	17.0	19.9	60.0	79.9	47.1	
Portageville	TN16-520R1	T	12.1	15.2**	11.4	26.6	81.4	4.6
		C	13.8	11.4	15.1	26.5	77.8	
	S11-20242C2[b]	T	15.7	15.2	21.6	36.8	85.2	4.2
		C	16.2	14.3	24.3	38.6	81.8	
	S14-9017R1[a]	T	16.1	16.4	23.3	39.7	74.6	-0.8
		C	16.7	16.6	19.8	36.4	75.2	
Stoneville	TN16-520R1	T	32.1	12.3	27.0	39.3	43.0	-10.6
		C	31.3	12.1	23.6	35.7	48.1	
	S11-20242C2[b]	T	28.3	25.5	54.1	79.6	57.6	19.8
		C	29.2	24.5	7.1	31.6	48.1	
	S14-9017R1[a]	T	51.5*	14.8	49.3	64.1	47.2	-10.9
		C	34.7	11.9	95.9	108.0	53.0	
Stuttgart	TN16-520R1	T	13.1**	8.40*	10.4	18.8*	22.7	-4.2
		C	8.9	5.70	9.9	15.6	23.7	
	S11-20242C2[b]	T	13.9	11.8	18.8	30.6	30.0	-0.7
		C	14.0	10.5	17.4	27.9	30.2	
	S14-9017R1[a]	T	12.0	15.7	19.5	35.2	21.9	-28.0
		C	10.5	4.10	16.9	21.0	30.4	
							AVG ESY (%)	3.8

[a] Slow-wilting cultivar, [b] Fast-wilting cultivar

[1] T = TXVA inoculant treatment; C = non-inoculated control.

[2] NTR = Nodules located on tap root

[3] NLR = Nodules located on lateral roots.

[4] TRN = Total root nodules.

[5] ESY (%) = Effect of inoculant yield; ESY = $\left(\frac{T-C}{C}\right) \times 100$

Discussion

The heterogeneous microenvironments that diverse soil profiles host present keys to balancing proper nutrient flux, controlled by optimum water saturation and the related microbial survivability that comes with that. Population density of indigenous *Bradyrhizobia* has been positively correlated with organic matter, base saturation, mean annual rainfall, irradiance, presence of legumes, or vigor of legume growth (Woomer et al., 1988; Dudeja & Khurana, 1989; Giongo et al., 2008). It is of utmost importance that nutrient deficiencies not being targeted by the inoculant are addressed in conjunction with inoculant application because the essential nutrients required for a fully functioning symbiosis are wide ranging and not fully understood (O'Hara, 2001). Rainfall, the primary source of soil moisture, exhibits strong correlations with the productivity of cropping systems with variability being a major cause of fluctuations in crop yields and yield quality (Motha, 2011). Zipper and associates (2016) performed a 50-year study of meteorological drought across the United States, revealing that negative yield effects are most sensitive to short term drought (1-3 months) during critical development periods which causes between 13-50% of variability in final yield estimation.

In this study, we used dry plant biomass and nodulation information collected at the R2 (i.e., flowering) growth stage to assess the effects of our inoculant. Vegetative plant growth has been shown to be correlated with a successful inoculant by providing early-season nitrogen which increases above ground biomass (Chibeba et al., 2018). Formation of nodules is also an indirect indicator of biological nitrogen fixation since the symbiont fixes atmospheric nitrogen in exchange for photosynthates only if a microoxic environment is reached in the nodule, indicated by a dark red color from the oxygen-sequestering leghemoglobin (Fig. 2-3; Li et al., 2018; Du et al., 2020). While the presence or absence of nodules can indicate if there is an applicable indigenous rhizobium in the soil, the location can reveal how effective it is, and even gauge the competitiveness of the capable symbiont (Zubrod et al., 2022). Thus, taproot nodules are a key indicator of successful inoculation, with seedcoat introduced rhizobia immediately interacting with the germinating seed while lateral roots being more likely to collect indigenous rhizobia, unless the immediate microenvironment is favorable to growth of the introduced strain (McDermoti & Graham, 1989; Smith, 1992). Numerous species of bacterium are known endophytes, residing in plant tissues, and sometimes co-occupying a nodule with a symbiont, presenting competition or even synergism in the rhizosphere and nodule environment (Subramanian et al., 2015; Mendoza-Suárez & Anderson, 2021). To overcome this, the RCBD layout, randomized biological replicates, and spatial distance between sites enabled certainty in the knowledge that the nodulation response was due to the TXVA inoculant.

Figure 2-3. A cross-sectioned root nodule of cv. TN16-520R1 inoculated with the *B. japonicum* TXVA drought-tolerant isolate.

2019

Yoakum experienced high-temperatures and insufficient rainfall for 2/3 of the season, primarily around pod-fill (July/August) which corresponded to deteriorating conditions in the control plots, displaying less above ground biomass, significantly less nodules, and a subsequent significant yield increase for the TXVA treatment of 4.6% over control plots. Drought was edaphically confirmed at this site through the low conductivity value, which has shown to reduce the amount of water flow towards the root surface and impacts nutrient flux (Draye et al., 2010). Winnsboro did not receive similar early-season heat stress days, but August and September displayed temperatures higher than normal. However, cumulative season rainfall was below average, with a 9.4 cm deficit that occurred in May, June, and August, and correlated to the growing stages of emergence and pod-fill. These sites displayed yield increases of 4.6% and 3.9% under the TXVA treatment for Yoakum and Winnsboro, respectively.

Regarding the Jackson site, seasonal high temperatures only differed from climate normals in May and September with 6.4% and 11.4%, respectively. The early season high-temperature in May along with the rain deficit in May and June was realized at mid-harvest sampling with lower aboveground plant biomass and less taproot and total root nodules in the control plots. September also experienced a reduction of precipitation by 60%, presenting a total of 27 days without rainfall which exposed the plants to severe water restriction during the pod-filling stage, which has been shown to display the most sensitivity to drought stress leading to a drop in seed quality, protein and oil yield, and a yield loss of up to 28% (Demirtas et al., 2010). The subsequent yields displayed a 3.9% increase in TXVA plots.

Missouri showed seasonal average high-temperatures breaching climate normal slightly in the planting month of May but more extreme increases of 2.25% (4.5°C over normal) in

September. Cumulative seasonal rainfall was below average at Clarkton with monthly reductions of 70% in July, 96% in August, and 69% in September. Sampling was attempted in June immediately after sampling in Jackson, but even despite being planted on the same day the Clarkton plots were stunted and displayed severe dicamba damage (e.g., cupped and wilted leaves with no canopy coverage). As such, sampling was delayed two weeks which gave plants time to recover and showed the highest biomass among sites with 10.3 and 9.9 g, respectively. Increased biomass and statistically significant increases in taproot and total root nodulation indicate benefits from TXVA application. This increase of AHT, decrease of cumulative P, and herbicide damage was exacerbated by the reduced water-holding capacity of the soil profile in Clarkton, classified as Malden fine sand and having the qualities of coarse, nutrient poor (Li et al., 2021). The droughty soils are indicated by very low conductivity readings, the third lowest of the 3-year trial. Yields displayed a decrease of -29.2% in TXVA treated plots, however, planting mistakes, damage of functional rows during harvest, and the presence of severe dicamba damage caused this data to be discarded and not included in the study averages (C. Vieira, personal communication).

Stoneville displayed below average cumulative rainfall, but with only 3 cm less than the normal. Slightly elevated temperatures in the planting month with close to triple the normal rainfall followed immediately by a vegetative period in June that was 2.5°C cooler than average with below average rainfall presented a conducive environment for soybean production. September of that year had an average high temperature 4.7°C above and rainfall 6.6 cm below normal, but previous months provided plenty of moisture, with no above ground plant biomass increases displayed. Nodulation showed improved efficacy on taproot and total root systems, but no yield increase was observed with a -4% effect of inoculant. Water retention is much higher in soils with an increased clay content, such as the Sharkey clay profile found at this station and slow-draining Dewitt silt loam profile found at Stuttgart. The swelling of clay particles when reaching water-holding capacity restricts internal drainage and can be beneficial or detrimental to plant growth and rhizosphere microbiomes, depending on cumulative rainfall (Wesley et al., 2001).

Lastly for 2019, the Stuttgart site only displayed high-temperature stress in September with an 11% increase and concurrent lower seasonal precipitation of 88%. With no early-season stressors, above ground plant biomass was shown to be similar between treatments. The period indicative of intermittent drought stress aligned with the pod-filling stage, which has shown negative associations with final seed yield. The soil from the Arkansas field sites revealed an average pH of 4.7, making them the most acidic soils across locations and has shown negative associations with *Bradyrhizobium*. Taylor and colleagues tested 5 strains of *Bradyrhizobium japonicum* for tolerance to acid stress, aluminum stress, and low phosphorus levels revealing acidity as the having the strongest negative interaction with significant reduction of cell numbers, even with strains that are shown to be more tolerant (Taylor et al., 1991). Despite this, nodulation was significant in taproot and total root counts. A potential explanation for this is the fact that there is a high level of manganese here at 124.7 ppm, over double the next largest concentration of the trial, and is common in soils with such an acidic pH (Cline and Kaul, 1990). Soil application of manganese at rates of 10 and 20 ppm has shown to significantly increase nodule numbers and seed yield (Yanni 1990). Purcell et al. (2000) also displayed the effects of high manganese levels to assist the breakdown of ureides (the product of N-fixation) in leaves under drought-stress, prolonging the ability of BNF under water deficit. Final seed yield for the year was 3.6%, showing a positive response to the TXVA inoculant treatment.

2020

Above average temperatures and fluctuating rainfall were observed early in the season (April to May) at Yoakum, which also coincided with increased plant biomass in the inoculated plots and significance in the Texas variety, Otoño. Similarly, Stoneville had warm early season weather and a plant biomass increase in both cultivars, but significant increase only with the drought-sensitive cv. S14-9017R1l (i.e., S14). Nodulation for all Texas varieties displayed significant trends in taproot and total roots, with very low indigenous nodulation. However, nodulation for Stoneville varied with TN16-520R1 (i.e., TN16) showing increased counts with the TXVA treatment while the drought-sensitive variety displayed more nodules on control plants, indicating the presence of robust endophytes at Stoneville. Average temperatures were observed in Winnsboro and Stuttgart throughout the season, but a water deficit from May-August in Winnsboro posed some potential stress on the plants, with physiological results displaying an increase of above-ground biomass in the TXVA treatment only with the drought-sensitive strain USG. Nodule counts followed this upward trend and had significant increases in the other two varieties planted. Stuttgart rainfall was only slightly below normal in July, but above ground biomass was increased in the inoculant treatment with the drought-sensitive cultivar S14.

The same drought-sensitive cultivar, along with all others planted in Portageville, displayed increased plant biomass as well. The breakdown of monthly weather data shows AHT of 1°C over normal in the planting month, June. Nodulation data shows a significant increase of taproot and total root nodules in TXVA treated plants for cv. TN16 and S14, while drought-tolerant cv. S11-20242C showed increased taproot counts but not total root counts. This agrees with nodulation patterns in Stoneville and Stuttgart with trends following those at Stuttgart over

Stoneville, where control plants had more nodules. Jackson had moderate seasonal temperatures but faced a 44% reduction in seasonal cumulative rainfall in early and late-season, indicating lots of potential cumulative stress days but mostly during the vegetative stages of biomass accumulation (i.e., May, June, and July). This is reflected via soil physio-chemical data with the lowest conductivity values measured due to lower matric potential from the increased salinity of desiccated soils. Plant biomass was increased in the drought-sensitive cv. USG, with nodulation trends likewise agreeing with Winnsboro regarding the dominance of TXVA treatment in taproot and total root nodulation.

Drought-sensitive varieties displayed similar yield increases with application of the TXVA inoculant of 11.9%, 13.1% (P = 0.001), 9.6%, and 9.4% in Jackson, Portageville, Stoneville, and Stuttgart, respectively. Winnsboro broke this trend, with the drought-sensitive along with all other varieties yielding poorly despite positive mid-season sampling metrics (0.3%, -2.4%, and -1.2%). Yields were very low and inconsistent in Yoakum, showing yield increases of -5.8%, 0%, and 245%. The highest percentage increase was in Otoño and was due low yields (10.7 vs 3.1 bu/a). These values were discarded and resulted in an average inoculation response of 7.4% for this season.

2021

Yoakum revealed high temperatures throughout the season in April, June, and September with rainfall events 4x normal in May and 3x normal in July, which delayed sampling (97 DAP). TXVA plots displayed significant increased above ground plant biomass in cv. TN16 and in taproot and total root nodulation for that along with fast-wilting cv. S14, resulting in yield increases of 51.2% and 17.3%, respectively. This same fast-wilting cultivar planted at

Winnsboro, having above average precipitation and below average temperatures, showed reduced biomass despite the other fast-wilting variety, USG showing increase. Nodulation was significant in TN16 but was variable with the fast-wilting lines, displaying a promiscuity for indigenous symbionts with the USG line having more tap and total root counts in the control plots and the S14 line having about equal nodulation among treatments. Effect of inoculant on yield in Winnsboro were negligent at -0.6%, -1.8%, and 1.9% for TN16, S14, and USG.

Portageville and Stoneville both experienced seasonal high-temperatures while only Stoneville had reduced seasonal rainfall. Portageville had a 1°C increase in temperature and 27% reduction of rainfall for June, compounded by a 44% precipitation reduction in May that indicates a potential moisture deficit. No biomass increases were observed and only TN16 showed significance in nodulation, with yields being 4.6%, 4.2%, and -0.8% for TN16, S11, and S14. Stoneville had above high temperatures for every month of the growing season (3.8% above seasonal normal) and reduced rainfall for the last two months during pod-fill, indicating potential stress during the whole season. Plant biomass increase was observed for TN16 and S14 and nodulation promiscuity of S14 totaling over 108 nodules averaged per plant from the control plots. Despite this nodulation, yields were variable with only the drought-tolerant cv. S11 showing an increase of 19.8% with TXVA inoculant application.

Stuttgart and Jackson displayed slightly elevated temperatures and lowered rainfall compared to normal. Jackson showed reduced rainfall beginning and end-season with high temperatures only at the end during pod-fill. Similarly, Stuttgart had stress during the same growth stage, which has shown negative associations with final seed yield (Hungria & Franco, 1992). Plant biomass increased across all varieties from both of these sites, excluding the slow-wilting S11 in Stuttgart. Nodulation patterns were likewise trending towards success of the

TXVA inoculant regarding increased numbers on the whole root system. However, yields did not follow this pattern. Jackson showed 2.9% and 19.3% increases from inoculant application while Stuttgart showed -4.2%, -0.7%, and -28% with the largest decrease with the drought-sensitive S14 variety. When looking at physio-chemical data, it reveals an extremely high level of iron at 102.5 ppm, which is a little under double the amount observed at any other location-year and could be a factor of the resultant poor yields for this location. Also, this specific field site in Arkansas was located on a more clay-rich A horizon than previous years and, seeing that this station's primary research is rice production and flood-tests for soybean, soils prone to waterlogging are beneficial (Chen et al., 2018). These soils drive hypoxic conditions in the rhizosphere causing severe yield losses due to Fe toxicity from increased bioavailability while also displaying negative associations with nitrogen fixation efficiency (Ciampitti et al., 2021; Delias et al., 2022). Also, Stuttgart represented the lowest average pH across site-years with 2021 having the lowest of 4.5. Soil acidity has shown to be a major constraining factor for *Bradyrhizobium* survival and nodulation, with recommendations of double inoculation in areas of high acidity (Chueiri et al., 2005; Silva et al., 2011).

Conclusions

In summary, Yoakum, TX showed the most environmental variability with all 3 growing seasons defying the 30-year climate averages for the region and having the largest deviation in cumulative seasonal rainfall, correlating to better effectiveness of nodulation, plant growth, and yield with application of the drought-tolerant TXVA inoculant. Winnsboro, LA had the second largest deviation in seasonal rainfall and high-temperature across the study, with the highest sodium levels of any field station, the highest value being 66 ppm in 2021. This correlates to the edaphoclimatic profiling of the Macon Ridge research station considering their irrigation wells have naturally high levels of exchangeable sodium, which may account for minimal inoculation effects across years (Walthall, 1992). Winnsboro also had the least deviation regarding organic matter content, which is positively associated with water holding capacity and provides a food source for the rhizosphere microbial community to convert into plant-accessible nutrients and could explain variable results of inoculation (Meena et al., 2017). Jackson, TN displayed the highest levels of organic matter from their no-till land management and the most consistent averaged yield response of 6.7%. Missouri showed the least deviation in observed weather variables and still displayed cultivar-specific yield increases when early-season environmental stressors occur. Stoneville, MS had the highest average PK concentrations along with the least deviation in pH, conductivity, and PK values, indicating a high nutrient input system and fertile soils, thus displaying a varied response to inoculation. Stuttgart, AR presented the most temperate site, but the low pH, high iron, and waterlogging-prone soils inhibited inoculation response. Overall effects of the TXVA inoculant across location-years resulted in average yield increases of 7%, a greater efficacy at sites having higher environmental variation, and synergism in plant growth promotion with climate-smart cultivars. These results agree with an inoculant trial done at 11 locations in 4 northern US states which associated the lowest yields with conditions during the growing season warmer and drier than 30-year climate normals and recommended inoculation for locations that experience these stressful environmental conditions (Carciochi et al., 2019).

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CHAPTER 3

The effect of heat stress on soybean growth with a *Bradyrhizobium* biofertilizer using a novel temperature gradient greenhouse

Abstract

With a growing concern regarding the effects of climate variations on global and regional food supply, testing systems must be streamlined and verified for correctly estimating the effect of individual abiotic stressors on crop production and sustainability. In this particular study, increasing air temperature gradients were used to test the effects on viability, growth, and production of soybean [*Glycine max* (L.) Merr] inoculated with a drought-tolerant inoculant, *Bradyrhizobium japonicum* TXVA. In 2020, the newly established temperature gradient greenhouse was utilized in collaboration with North Carolina State University and the USDA's "Team Drought" project to test biologically relevant air temperature gradients of Ambient (T-1), ambient +2°C (T-2), and ambient +4°C (T-3) while maintaining field capacity water content of soils to ensure no drought effects skewed the results. Positive effect of inoculation was observed at T-2, with partitioning of resources to stem pods until the highest temperature gradient when inoculated plants deteriorated the most severely. The opposite trend was seen in nodulation, with higher temperature gradients lowering the nodule counts for control plants while TXVA overcame this temperature-dependent nodulation restriction and formed the most nodules in T-3.

Introduction

Since the industrial revolution, anthropogenic forces have caused increases in the three main greenhouse gasses (GHG), carbon dioxide (CO_2), methane (CH_2), and nitrous oxide (N_2O) by 40%, 150%, and 20%, respectively (Tian et al., 2016). Agriculture and land-use contributes to approximately 50% of CH_2 and 60% of N_2O which heavily influences global warming and establishes a level of unpredictability on our agricultural production systems via multi-factorial stressors such as floods, elevated ozone, drought, and erratic high temperatures (IPCC, 2013). While N_2O is a smaller fraction of monitored GHG, its climate impact is the most immense with the global warming potential (GWP) estimated to be 300 times that of CO_2 over a 100-year period (Forester et al., 2008). The Kyoto protocol regulates N_2O, however, the Montreal protocol does not recognize its stratospheric ozone depletion potential despite it being larger than any other anthropogenic-sourced substance, significantly impacting the Antarctic ozone hole and thus presenting a direct increase in GWP (Ravishankara et al., 2009).

A significant proportion of N_2O emissions stem from agriculture with the manufacture and use of both organic and synthetic fertilizers along with land management practices, thereby brecoming a dominant source of the resultant air pollution (Almaraz et al., 2018). Using the Hadley III climate model, a downward trend is predicted in area-weighted average crop yields, displaying yield losses of 30-46% by the turn of the century in the most conservative warming scenario and a loss of 63-82% under the most rapid scenario (Schlenker & Roberts, 2009). Growth of agribusiness across the continent due to the need to sustain a growing population stimulates the bio-economy, but this increase in production necessitates the integration of sustainable and even regenerative practices. To completely halt these agricultural emissions while sustaining production is unrealistic, thus mitigation tactics are necessary to avoid worsening the future modeled climate scenario. Clearly defined mitigation strategies of N_2O emissions exist and are being backed by multi-governmental entities with the support of research grants and climate-friendly agriculture (CFA) policies (McNunn et al., 2020).

The use of leguminous crops may aid CFA efforts of carbon and nitrogen nutrient management through their utilization into rotational cropping systems via cover and row cropping or providing additive benefits with reduced tillage systems (Muhammad et al., 2019). Financial incentives at Federal, State, and the private sector levels for research into these emerging farming practices have shown to work, with a 50% increase in cover crops being planted in the United States from 2012-2017 (Wallander et al., 2021). A 10-year study from Guardia et al. (2019) used a leguminous cover crop with integrated soil fertility management to show a 91.9% reduction of yield-scaled emissions, proposing the potentials of a carbon-neutral cropping system in semi-arid regions. Regarding row crop production, a thorough analysis of life-cycle impacts broken down into 16 categories displayed that, while the use of inoculants for soybean positively affected upstream and downstream production in minor ways, it had major impacts on the field and yield effects (Beltran et al., 2021). Impacts not considered in the above study are effects of indigenous soil microflora and water impacts, both of which have shown similar reduction in emissions through better nutrient availability from synergism with other beneficial organisms in the rhizosphere and improved yield quality/quantity from rainfed fields which lowers evapotranspiration (Abou-Shanab et al., 2017; Mekonnen & Hoekstra, 2011).

Microbial inoculants hold the key to fully unlocking the mitigation potential of climate-smart agriculture. Harnessing the diversity of the rhizosphere microbiome to buffer environmental stress has been well established, along with increasing yields and mitigation of further climate perturbations (Chibeba et al., 2018; Cerezini et al., 2020). Here we report the use of a Texas-native drought-tolerant inoculant (i.e., *Bradyrhizobium japonicum* TXVA) on soybean grown under biologically relevant heat stress (i.e., +2°C and +4°C) in a newly-developed temperature gradient greenhouse located in Raleigh, North Carolina. The objective of this research was to evaluate the effect of inoculation on soybean physiology and production under increasing heat stress by using the drought-tolerant inoculant with a non-inoculated control.

Materials and Methods

Research site and soil composition

The greenhouse experiment was performed from June 7th-October 14th in 2021 at a site located 5 km south of Raleigh, North Carolina, USA on the Lake Wheeler Farm of North Carolina State University (35°43′ N, 78°40′ W). The soil mixture used in this trial was 2/3rd topsoil from R.L. Bradsher Contracting, Inc. that was sourced from areas around Raleigh. The soil had been sieved by an approximately 1 cm^2 sieve and mixed via cement mixer with 1/3rd potting soil. Soil was wetted during the mixing process to aid homogenization and prevent loss of airborne soil organic particles. The potting soil consisted of primarily Canadian sphagnum peat moss with equal parts bark and coarse perlite (SunGro Metro-Mix® 820; SunGro Horticulture, Agawam, MA, USA). Two-liter pots were loosely filled and fertilized with slow-release Osmocote® plus synthetic fertilizer beads with hand mixing to ensure homogeneous distribution prior to topping off the pots (ICL group Ltd., Tel-Aviv, Israel). Fertilizer was applied at 1/6th the standard rate (10g/pot) to ensure no inhibition of nodulation by the addition of too much chemical nitrogen fertilizer but still ensure sufficient nutrients for plant growth (Lin et al., 2021). The levels of essential nutrients for Osmocote® are labeled as 15-9-12 (NPK), with nitrate being primarily supplied as ammoniacal nitrogen (N-NH_4) and lesser part nitrate (N-NO_3). Phosphate and Potassium, the other 2 essential nutrients for plant growth, were supplied as the common forms of available phosphate ((P_2O_5)^3) and soluble potash (K_2O)^3. Other micronutrients provided by the potting mix were 1.3% Magnesium, 6% Sulfur, 0.02% Boron, 0.05% Copper, 0.46% Iron, 0.06% Manganese, 0.02% Molybdenum, and 0.05% Zinc. All nutrients were coated with an organic alkyd-type resin bead that controls the release of the nutrients in this specific blend as a function of soil moisture content and growing medium temperature, predicted to display consistent release for 4-5 months at 26°C or 3-4 months at 32°C (Broschat, 2005). Half of the boarder pots were given the full dose of 60g pot^{-1} fertilizer which were used as a visual check to ensure adequate fertilization of all plants in our trial. An initial composite soil sample was taken from all batches of mixed soil prior to fertilizing to analyze starting nutrient levels and provide baseline values to determine inoculant effects. The pots were then wrapped with an insulated metalized polyethylene/nylon bubble roll to reduce thermal flux on soils before being placed in the temperature gradient greenhouse prior to planting (W.W. Grainger, Inc. Lake Forest, IL, USA).

Temperature gradient greenhouse description

The novel temperature gradient greenhouse was setup and maintained by Dr. Kent Burkey's group in NC State and the USDA. This study represents the first-time that soybean has been grown in a temperature gradient greenhouse specific for isolated heat stress, while examining *Bradyrhizobium* biofertilizer as a potential mitigation agent. The greenhouse specifications are 20 ft x 100 ft hoop-style with 6 ft sidewalls and a 14 ft peak, which provided room for 3 interior chambers that housed different temperature gradients (Fig. 3-1). On the intake side, bug netting was draped over the frame to prevent pest pressure from infiltrating the air intake manifold while

4 output fans at the end of the greenhouse allowed for equalized pull on the intake systems. The air was taken in and redistributed via a plexiglass wall with circular 2-inch holes that were shown to evenly heat each section to +2°C and +4°C, respectively (Fig. 3-2). Diurnal air temperature and relative humidity were recorded as 12 h averages across all treatments with sensors (HOBO U23, Onset Computer Corp., Bourne, MA, USA) adjusted to canopy height throughout the season. Drip irrigation hoses were placed into pots and automatically watered each morning to approximately 1-1.2-gal day^{-1} which was determined to be field capacity by monitoring initial irrigation such that water was leached from the bottom of each pot but not flushing the soil. Providing sufficient transpirable water moisture ensured that this trial only experienced high heat stress, not the multi-stress of high temperature and drought that typically follows suit.

Figure 3-1. A profile view of the temperature gradient greenhouse.

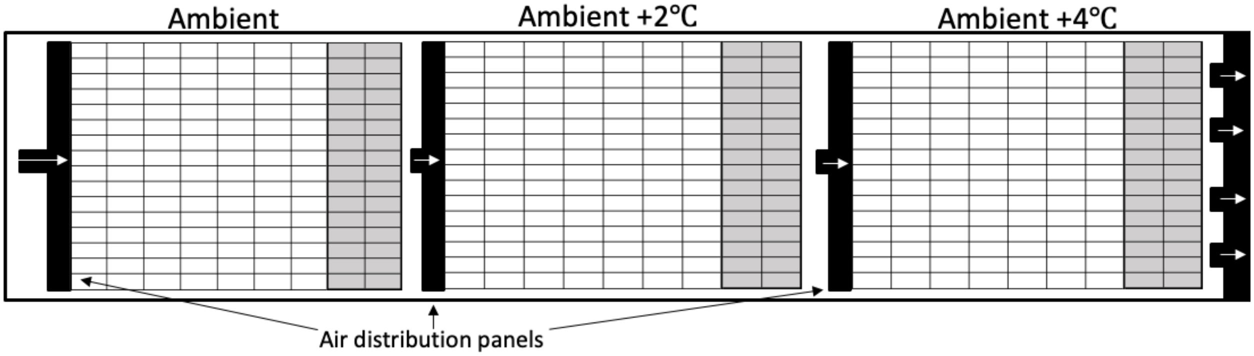

Figure 3-2. Aerial layout of the temperature gradient greenhouse depicting air distribution panels and outtake fans in black with grey blocks representing placement of our trial.

Experimental layout and treatment setup

The greenhouse setup consisted of 2 inoculant treatments across 3 temperature gradients with 6 replicates per gradient distributed in a randomized block design to minimize any unknown environmental variation. Using these 6 replicates, 3 were taken for mid-harvest analysis and 3 were taken for final harvest from each respective gradient. The cultivar planted was TN16-520R1 which shows resistance to stem canker and southern root knot nematode and is a glyphosate tolerant backcross derived selection from the popular high-yielding cultivar Ellis (Boehm et al., 2019). Pots were planted with 3 seeds each on June 8th before being thinned down to 1 viable plant per pot and caged for biomass support after 3 weeks. The inoculant treatments used were the Texas native drought tolerant *Bradyrhizobium japonicum* TXVA and a non-inoculated control. All bacterial cultures were utilized as liquid formulations and grown to a standardized cell density of 2×10^9 CFU/ml as verified by OD_{600} values as well as plate counts. Inoculation technique consisted of aseptically imbibing seeds at a rate of 1 ml/pot (1 ml applied on 3 seeds to be planted together) and mixed evenly for 5 min prior to hand planting at a 1-inch planting depth.

Crop management

Throughout the growing season, the greenhouse was maintained according to North Carolina research and extension guidelines, which included soil application of the insecticide Marathon granular 1% (Imidacloprid) and the fungicide Ridomil Gold SE (Mefenoxam) at a rate of 1 tbsp pot^{-1} after the emergence of the first trifoliate. No pest or disease pressure was observed all season, thus no additional applications were required.

Mid-harvest sampling

Sampling was performed 64 days after planting on August 10th which correlated with the R2 growth stage where plants are fully flowering and biological nitrogen fixation is at its peak (McWilliams et al., 1999). No pods were found on any of the plants indicating that all plants were maturing at the same rate. Sampling procedure consisted of removing two plants at a time from the greenhouse to ensure minimal leaf wilting took place prior to measurements of leaf area. Plant height was taken first, prior to being cut at the root/soil interface and taken inside where all leaves were removed and scanned for average leaf area with a LI-COR 3100 area meter (LI-COR Biosciences, Lincoln, NE, USA). The stem and branches were then bagged separately and placed in a tobacco drying barn at 70°C overnight to dry. Outside, after cutting plants at the root/soil interface, immediate bulk and rhizosphere soil samples were aseptically taken and stored at -80°C for downstream analysis. Bulk soils are defined as any loose soil that came off the root ball easily when taken out of each pot and was sampled using a metal ice scoop. Rhizosphere soils are defined as soil within the root ball itself and was sampled using a metal scoopula. Each sampling devise was sterilized between treatments by liberal cleaning with Clorox bleach wipes.

Harvest

Final harvest was performed 129 days after planting on October 15th. For harvest, 2 plants were removed from the greenhouse at a time where plant height was taken prior to being cut at the root/soil interface and the removal of all remaining foliage. The plants were then taken inside to count and classify branches before counting and removing all pods from each designated branch. Branch counts represent attempted pod-setting and were classified by the number of pod clustered attached, with large branches having two clusters or more while small branches only having one. Stems and branches were then bagged separately and placed in a tobacco drying barn to dry. After cutting plants at the root/soil interface, immediate bulk and rhizosphere soil samples were aseptically taken and stored at -80°C for downstream analysis. Total plant metrics for final harvest include plant height, stem biomass, large and small branch number, main stem and branch pod number, aborted pod number, and pod weights broken down by the same structural regions.

Data analysis

Data was analyzed in Microsoft Excel using a two-way ANOVA without replication. There were three replicates for each treatment and timepoint, excluding the TXVA T-3 gradient when n = 2.

Results

Soil nutrient levels and environmental conditions

Initial nutrient concentrations of the substrate mix were sampled prior to adding the reduced-rate (10 g) of fertilizer to the pots. The soil physio-chemical test for this trial revealed very high nutrient levels, with the Texas A&M soil testing lab only recommending a slight addition of potassium and boron (Table 3-1). The starting level of nitrate, sulfur, and manganese was determined to be between the critical levels of high and very high; high for iron, and moderate to high for phosphorus, calcium, magnesium, zinc, and copper. A very high amount of soil organic matter was also observed, which has shown to increase water holding capacity and provide a food source for the rhizosphere microbial community (Meena et al., 2017). The critical levels of all nutrient concentrations are determined by the fertilizer calculator provided online by the soil testing lab at Texas A&M University (AgriLife Extension Service, 2021). The fertilizer that was used, Osmocote® plus synthetic fertilizer beads, were coated with an organic resin that allows the controlled release of nitrogen, phosphorus, and potassium throughout the growing season. While their website details that release of nutrients is not affected by salinity, pH, microbial activity, water quality, or rain, but soil temperature has been shown through independent studies to have a strong impact on release rates, especially when incorporated into substrate and not applied to the soil surface (Husby et al., 2003; Broschat, 2005).

Targeted temperature gradients were maintained throughout the season as needed to achieve a balanced gradient of stress in each compartment (Table 3-2). Temperature gradient 1 is intended as the reference temperature and is notated as T-1. A two-degree seasonal difference presents from T-1 to T-2, but only 1.1-degree difference is observed between the T-2 and T-3 gradients. Seasonal fluctuations in day-time temperature are observed (Table 3-3) and display very high early-season temperatures from 33.2-36.8°C. Higher temperatures also meant less humidity in T-2 and T-3, with the largest deviations occurring at night. Cooling throughout the season is with September and October mostly being below 30°C based on 12 h averages.

Table 3-1. Soil physio-chemical characteristics from the combined substrate mix[a]

Properties	Values (ppm)
pH	5.1
Conductivity	265
Nitrate	46
Phosphorus	62
Potassium	127
Calcium	1,211
Magnesium	216
Sulfur	118
Sodium	21
Iron	59.56
Zinc	4.7
Manganese	17.38
Copper	0.81
Boron	0.02
Organic Matter (%)	3.22

[a] All nutrient values in ppm unless otherwise noted.

Table 3-2. Seasonal diurnal temperature and relative humidity at each temperature gradient[a]. Difference between day and night values are listed under T/RH$_{diff}$ column.

Temp. Gradient [b]	Temp (C)			RH [c] (%)		
	Day	Night	T$_{diff}$	Day	Night	RH$_{diff}$
AMB	28.6	20.7	*7.9*	67.9	93.1	*25.2*
T-1	30.0	21.3	*8.8*	67.4	91.3	*23.9*
T-2	32.1	23.0	*9.1*	65.1	86.3	*21.2*
T-3	33.2	23.8	*9.4*	64.7	83.2	*18.6*

[a] Data are expressed as 12 h averages.

[b] AMB = ambient temperature for Raleigh, N.C.
T - 1 = Baseline temperature gradient.
T - 2 = Temperature gradient T - 1 + 2°C.
T - 3 = Temperature gradient T - 1 + 4°C.

[c] RH (%) – Percentage relative humidity.

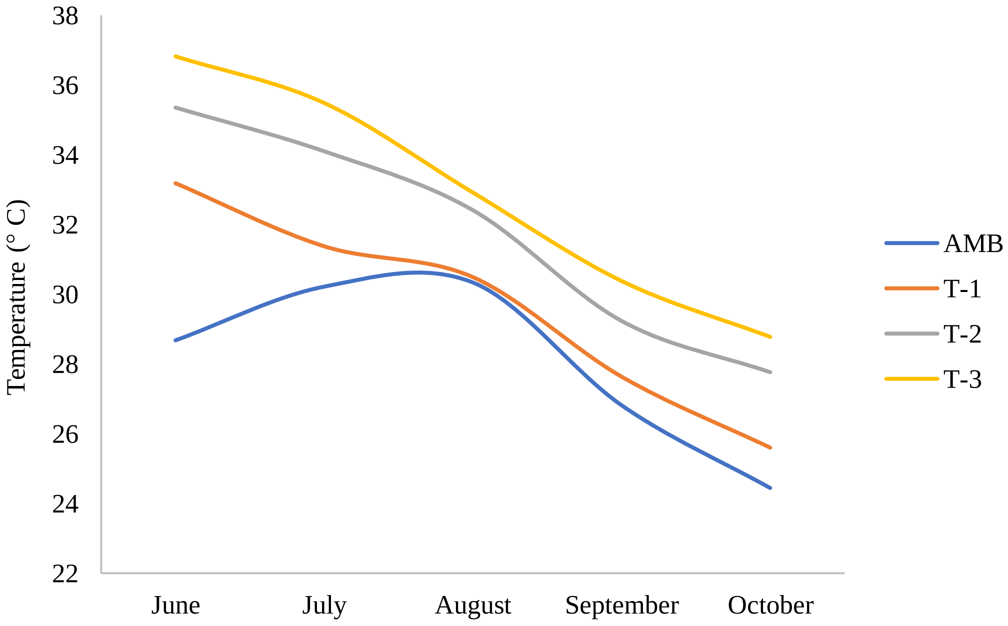

Figure 3-3. Daytime temperature throughout the season, reported in °C as 12 h averages.

Mid-season growth metrics

Mid-season sampling occurred at 64 days after planting when 90% of plants were flowering and thus shifting to reproductive growth. This partitioning of growth phases is slightly faster than normal for this variety due to the heat treatment but is very similar to growth stages at the same longitudinal gradient in Stoneville, Mississippi (Purcell et al., 2014). The results displayed on average taller plants across all gradients with the application of the TXVA strain, the largest height difference occurring at T-3 with 11 cm between inoculated and control (Table 3-3). The control plants showed a gradual height increase from T-1 to T-2 but is reduced at T-3. The effects of high-temperature stress were observed on leaf physiology, with the gradual reduction in leaf area and biomass from all treatments with increasing temperatures.

Table 3-3. Plant physiological response at full flowering (i.e., R2) at each temperature gradient[a]

Temp. Gradient[b]	Treatment	Height (cm)	Leaf Area (m^2)	Dry weight[c] (g)		
				Leaf	Stem	Total
T-1	T	100 ± 7	1.33 ± 0.09	39 ± 2.1	62 ± 3	103 ± 5
	C	99 ± 6	1.39 ± 0.15	42 ± 7.4	65 ± 12	108 ± 19
T-2	T	110 ± 11	0.96 ± 0.08	33 ± 3.8	49 ± 8	82 ± 12
	C	101 ± 2	1.27 ± 0.05	43 ± 3.8	61 ± 4	105 ± 7
T-3	T	105 ± 9	0.83 ± 0.20	25 ± 6.6	41 ± 12	67 ± 19
	C	94 ± 10	1.09 ± 0.13	32 ± 4.4	49 ± 8	83 ± 13

[a] Data are expressed as 12 h means ± standard errors of the means.
[b] T-1 = Baseline temperature gradient.
T-2 = Temperature gradient T - 1 + 2°C.
T-3 = Temperature gradient T - 1 + 4°C.
[c] Drying of plant biomass was done in a tobacco drying barn at 70°C

Nodulation revealed the plants to be pot-bound with root systems much larger than any field grown soybean (Fig. A-2). Roots were thoroughly rinsed with as little agitation as possible to prevent displacement of nodules before being put on paper plates to air out before being bagged and kept at 4°C for transport back to Texas. Enumeration of average nodulation between treatments reveals a steady reduction in nodules for the control treatment in a temperature-dependent fashion (Fig. 3-4). The TXVA inoculated plants exhibited the opposite effect, with a slight increase across each gradient. This indicates a strong indigenous rhizobial population in the substrate mixture since the control plants had over 1,000 nodules at the T-1 gradient, but also shows a temperature-dependent inhibition of nodulation for the indigenous nodule occupants, represented by reduced nodulation in the T-2 and T-3 gradients.

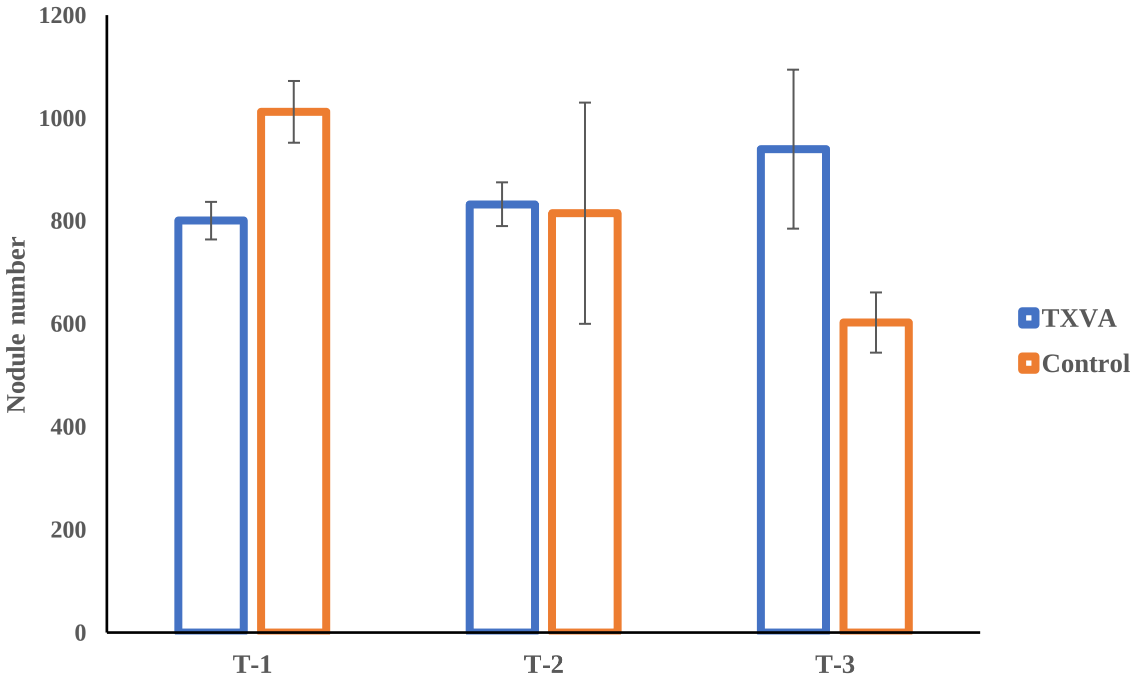

Figure 3-4. Nodulation patterns for the *Bradyrhizobium japonicum* TXVA inoculant and a non-inoculated control taken from soybean at the R2 growth stage grown in a temperature gradient greenhouse. T-1 = Baseline temperature gradient. T-2 = T-1 + 2°C. T-3 = T-1 + 4°C.

Plant height and number of branches

Harvest occurred at 129 days which agreed with the general maturation time for TN16-520R1 when grown at the same longitude in Stoneville, Mississippi, showing no large effect of temperature-induced maturation. Unfortunately, one of the TXVA treated plants in the T-3 gradient was accidentally deprived of water and killed so T-3 has a sample size of n=2 per treatment. Plant height and the allocation of branches in Table 3-4 display that respective to mid-harvest sampling, stem growth occurred in T-1 for all treatments, but in T-2 and T-3 only the non-inoculated plants showed stem growth. Across the temperature gradients for harvest, all plants showed a small reduction at T-2 and larger one at T-3 with a 7 and 5% reduction for TXVA and control, respectively. Control plants showed an increase in the number of large branches at the T-3 gradient while the inoculant treatment was relatively stable. For small branches, however, treated plants showed a reduction at T-2 and increase at T-3 while control pots followed the trend of TXVA with the large branches, maintaining steady until a reduction in T-3.

Table 3-4. Influence of heat stress and inoculation on plant height and number of branches at harvest[a]

Temp. Gradient[b]	Treatment	Height (cm)	Number of branches		
			Large	Small	Total
T – 1	T	110 ± 2	13 ± 1.2	4.3 ± 1.9	17 ± 1.7
	C	111 ± 6	11 ± 1.5	4.7 ± 1.7	16 ± 0.3
T – 2	T	109 ± 4	13 ± 2.4	3.0 ± 0.6	19 ± 3.0
	C	110 ± 6	11 ± 1.0	4.7 ± 0.9	18 ± 0.7
T – 3	T	101 ± 13	12 ± 1.0	4.5 ± 1.5	17 ± 0.5
	C	105 ± 10	15 ± 0.9	3.7 ± 0.9	18 ± 1.8

[a] Data are expressed as 12 h means ± standard errors of the means.
[b] T - 1 = Baseline temperature gradient.
T - 2 = Temperature gradient T - 1 + 2°C.
T - 3 = Temperature gradient T - 1 + 4°C.

Also measured at final harvest was the number, weight, type, and allocation of pods (Table 3-4 & 3-5). For the TXVA treated plants, total pod count goes up for T-2 and is heavily reduced by T-3, with pod allocation seeming to increase on the stem for T-2. TXVA also displays the lowest number of aborted pods under the T-2 and T-3 condition. The control plants see no allocation of pods to the stem, only a reduction under T-3. Conversely, the branch pods stay consistent between T-1 and T-2 and increase at T-3 to buffer the loss of pods on the stem at this condition.

Pod weights tell a similar story of resource allocation with weights of individual pods not changing much across gradients (Fig. 3-5). This is an averaging of partitioning trends occurring, with individual branch pods increasing in weight while the stem pods decrease in weight under the T-2 timepoint. In response to higher temperature gradients, weights are partitioned back to initial weights per pod.

Table 3-4. Influence of heat stress gradients and inoculation on pod-fill allocation and number of aborted pods[a]

Temp. Gradient[b]	Treatment	Number of Pods			
		Stem	Branch	Total	Aborted
T - 1	T	75 ± 6	500 ± 128	574 ± 134	8.3 ± 3.3
	C	73 ± 9	398 ± 30	471 ± 23	5.0 ± 1.7
T - 2	T	82 ± 5	368 ± 119	608 ± 124	3.5 ± 1.2
	C	73 ± 16	398 ± 92	456 ± 103	5.0 ± 2.4
T - 3	T	64 ± 1	329 ± 45	393 ± 44	5.0 ± 2.0
	C	58 ± 12	408 ± 20	467 ± 15	8.3 ± 4.9

[a] Data are expressed as means ± standard errors of the means.
[b] T - 1 = Baseline temperature gradient.
T - 2 = Temperature gradient T - 1 + 2°C.
T - 3 = Temperature gradient T - 1 + 4°C.

Table 3-5. Influence of heat stress gradients and inoculation on pod weight at harvest for the main stem, branch, and total[a]

Temp. Gradient[b]	Treatment	Stem	Branch	Total
		Weight of pods (g)		
T - 1	T	27 ± 2.3	187 ± 51	214 ± 53
	C	25 ± 2.7	143 ± 17	168 ± 14
T - 2	T	24 ± 2.4	190 ± 39	214 ± 40
	C	19 ± 6.0	153 ± 37	171 ± 40
T - 3	T	23 ± 0.9	124 ± 27	148 ± 27
	C	21 ± 4.5	149 ± 12	170 ± 8

[a] Data are expressed as means ± standard errors of the means.
[b] T - 1 = Baseline temperature gradient.
T - 2 = Temperature gradient T - 1 + 2°C.
T - 3 = Temperature gradient T - 1 + 4°C.

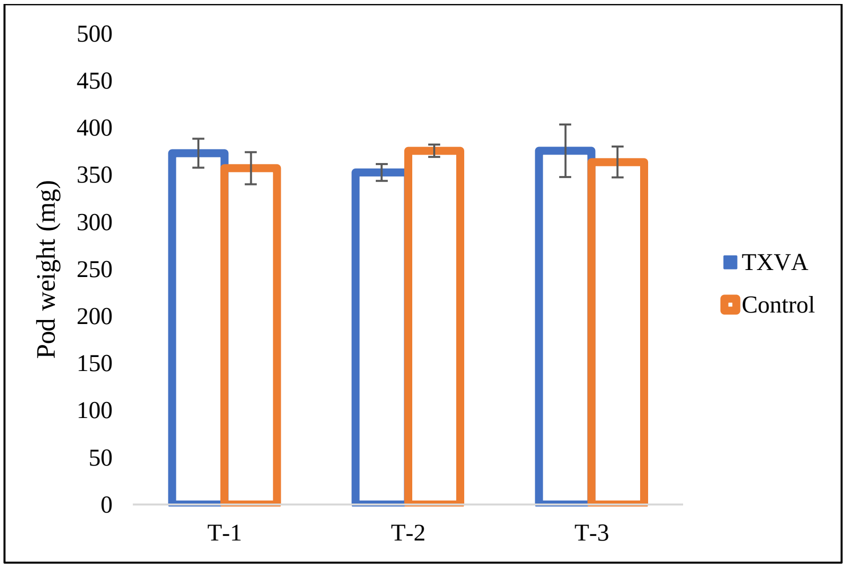

Figure 3-5. Combined individual pod weights.

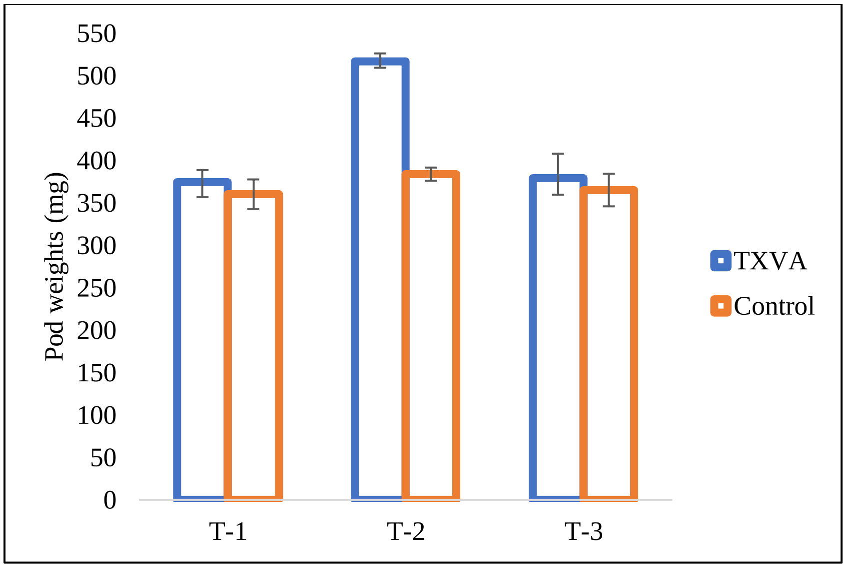

Figure 3-6. Individual pod weights from all branch pod sets, in mg.

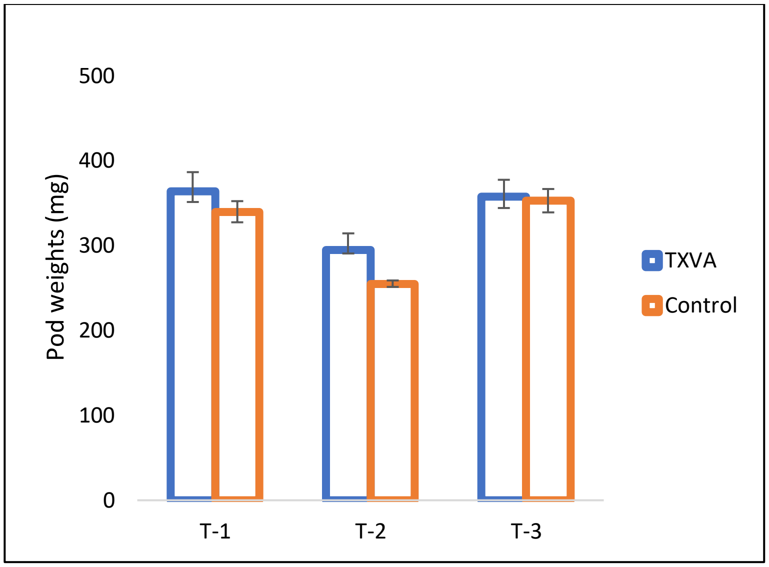

Figure 3-7. Individual pod weights from all stem pod sets, in mg.

Discussion

Environmental conditions

Soil nutrient levels were very high prior to adding the reduced rate of fertilizer. Since soil composition was made customized for the trial on-site the day of planting, prior nutrient testing was not able to be done. The high levels of nitrogen in the soils potentially present a mitigation technique for non-inoculated plants since it has been shown that providing plants a nitrogen solution under abiotic stress helps with their physiological response to the stressor (Wang et al., 2019). Likewise, high levels of phosphorus and iron have shown to increase nitrogen fixation rates and the subsequent ability to export the ureides from the nodules, providing enhanced ability for indigenous rhizobia to aid plant growth (Rotaru & Sinclair, 2009). Sulfur and manganese have also shown positive effects regarding stimulating rhizobial activity in soybean under environmental stressors (Hussain et al., 2011; Purcell et al., 2000). Release of nutrients from Osmocote® plus synthetic fertilizer beads could have been unnaturally increased through the daily soil saturation and high-temperatures causing a flux of NPK to already nutrient rich soils, presenting a very nutrient rich environment for natural microbial abundance and plant growth. Being strongly temperature dependent, the rates of osmotic release in the T-3 gradient present the highest potential of nutrient overabundance, which is shown to inhibit nitrogen fixation by subverting the need of plant investment into the symbiosis by readily available soil nitrate and could reflect why nodulation was reduced in the non-treated plots as temperature gradients increased (Zhang et al., 2021).

Greenhouses present opportune environments to control specific growth parameters but are also in turn affected by environmental conditions of the resident location. This phenomenon was observed in this trial with increased monthly ambient temperatures in Raleigh reaching peak conditions of 30/23°C in August when temperatures in the greenhouse were 30/23°C, 32/25°C, and 33/25°C for T1-T3, respectively. Peak conditions in all temperature gradients (T1-T3) were in June with temperatures of 33/22°C, 35/23°C, and 36/24°C. While plants were not established at the ambient conditions due to size restraint, these differential temperature gradients insinuate that T-1 (the gradient used as ambient in this study) was already at an increased temperature compared to the normal conditions of the region. Gradients were averaged over the season to show a +2.1°C increase of the T-2 gradient while the T-3 gradient was only +3.2°C instead of the targeted +4°C, with averages reaching the target goal in T-3 only in July of the growing season. Concordantly with increased daytime temperatures there comes greater cooling effect of nighttime conditions, showing higher temperature differentials and thus more temperature flux on the plant and rhizosphere which are shown to contribute to changes in carbon source allocation to minimize deleterious impacts of limiting resources on growth (Franklin et al., 2012). Taking into consideration that these are 12 h averages and that heating/cooling is a difficult process to standardize, especially with growing plants that are respiring at high rates, the microclimates of plant location introduce an aspect of background noise into this trial that can only be overcome with randomized locations (as performed) and high biological replicates (Ma et al., 2019).

Mid-season growth metrics

Mid-harvest sampling for both plant and rhizobial fitness is always performed around the shift from vegetative stage to reproductive stage, indicated by flowering, because this stage correlates with the termination of vegetative growth (for determinate varieties of soybean, as was used) and beginning the process of pod-setting (Fehr & Caviness, 1977). This transitioning of growth also coincides with increased nodulation showing maximum rates of nitrogen fixation occurring around pod formation (i.e., R3) due to the increased nitrogen sink that seed formation presents (Ciampitti et al., 2021). Ciampitti et al. (2021) also displayed the detrimental effect of high-temperature on nitrogen fixation in the vegetative period, which is clearly shown in the T-3 gradient with plants that heavily invested in nodulation performing poorly compared to non-inoculated plants meeting their need directly from soil nitrate levels. However, inoculated plants show more stem and nodule growth with increasing temperatures (excluding height in T-3, still taller than non-inoculated plants) while the control displays the opposite trend despite having more above ground plant biomass under each condition.

Final yield metrics

A reduction of reproductive development in soybean is shown with mean temperatures above 30°C; however, starting in September (i.e., pod-fill) average temperatures were at that temperature for T-3 and below that in all other gradients, indicating that more heat stress was exhibited early season compared to late (Thomas et al., 2010). While heat did not significantly impact maturity date, positive effects were observed on pest pressure since no application of pesticides were required in the growing season, which is unusual due to the high prevalence of tobacco thrips in the production systems of North Carolina (Grinnan et al., 2013; Brandenburg et al., 2021).

Physiological growth progression from sampling to harvest in the T-1 gradient displayed continued growth after flowering for both treatments, but only the non-inoculated plants continued growth under the upper two temperature gradients. Similarly, when viewed at harvest across temperature gradients all plants show a height reduction, with TXVA treated plants displaying the largest negative difference at T-3. Large branches maintained consistent for each treatment at T-2 while the control increased and TXVA decreased at T-3. The opposite trend was observed with the number of small branches, being highest across gradients in T-3 for TXVA and lowest in the non-inoculated plants. Total pod numbers were maximum at the T-1 and T-2 gradients in TXVA treated plants with 25.6% and 33.3% increases over the control at each gradient but it showed a reduction of 30.8% at T-3 (as compared to T-1). Non-inoculated plants did not deviate much and even had a 1.2% yield increase at T-3. The inoculated plants allocated more pods on the stem at T-2 than T-1, but both treatments showed reductions in stem pods at T-3. Non-inoculated branch pods were steady across the first two gradients before a slight increase at T-3 while TXVA branch pods numbered the highest at 500 in T-1 before decreasing with temperature. It has been shown that high-temperature stress applied at early reproductive stages causes chlorophyll disfunction and back-up of starches and sugars, resulting in nutrient imbalance and reduction in number of pods (Siebers et al., 2015). This partitioning of pods to the stem at T-2 in TXVA is of interest because it has been documented that late-season pod-abortion occurred 15% higher on branches then the stem (Egli & Dennis, 2006). The inoculation treatment also reduced the number of aborted pods from 8 for the control to 5 for TXVA in T-3, but this cultivar showed a lesser amount compared to other varieties in the greenhouse that were grown by collaborators so was not significant, confirming that there is a varietal response to pod abortion (Junior et al., 2017).

It is interesting to report that when looking at individual pod weights to see allocation of resources via seed-fill, derived from dividing weight by number from each partitioning. The total weights of individual pods did not deviate much, ranging from 352-375 mg per pod. However, partitioning of weights displayed heavier pods on branches with 38.2% and 6.7% over T-1 in

TXVA and non-inoculated, respectively. Concurrently, there were lower weight pods on the stem under the T-2 gradient before redistributing under the T-3 gradient, with TXVA having slightly heavier branch pods and the non-inoculated plants having heavier branch and stem pods. This investment of resources under stressors could indicate the costs and benefits regarding allocation of C/N. The plants inoculated with the drought-tolerant strain were still able to form nodules even with increased infectivity under the early-season high temperatures, however, the symbiosis was rendered ineffective at the highest temperature gradient through breakdown of photosynthesis or inhibition of nitrogenase, both being the driving force behind plant growth from primarily biological nitrogen fixation (Araniuelo et al., 2007). Nitrogen fertilizers have shown to uncouple photosynthesis from the nodule-centric feedback loop and help plants overcome shock from high-temperature stress, in turn rendering the investment of nodule mass useless (Hungria & Franco, 1993). Cultivar response to abiotic stressors is known to vary in severity regarding its effect on final seed weight (Blanco et al., 2022). Maturity, consistent base yield of control plants, and very low aborted pod numbers indicate TN16-520R1 has tolerance to high-temperatures, which has since been confirmed by Purdom et al. (2022) with TN16-520R1 displaying drought-tolerance tested via rainout shelter.

Conclusions

The results of this study show a partitioning of plant resources in a temperature-dependent manner and show negative physiological responses with varying effect on yield. High initial levels of nutrients with added input led to pot-bound plants that varied in stress-response (as determined by the large standard deviations), which have shown to be an issue in greenhouse production (Ray & Sinclair, 1998). Zhang et al. (2021) displayed that high nitrogen levels also inhibit the utilization of BNF and how flood-irrigation exacerbates this problem, showing the potential of reduced nodule activity but not nodule formation for the TXVA strain and indicates the saprophytic ability of the inoculant to persist even when not being fully utilized metabolically. This trade-off has been displayed in *Rhizobium* before, with higher tolerance to temperature stress generally showing a lower symbiotic efficacy (Rodriguez et al., 2005). The changes in allocation of carbon and nitrogen that occur under increased heat stress impact soil carbon sequestration, the rhizosphere microbiome, and ultimately the soybean seed yield (Milcu et al., 2011; Hopkins et al., 2013). The use of the TN16-520R1 line under biologically relevant temperature gradients confirmed its cross-tolerance to drought and heat stress, showing promise for future studies to elucidate functionality behind this trait to determine effect on seed quality, plant metabolism, and biological nitrogen fixation (Blanco et al., 2022). The inoculant treatment displayed increased pod weights for plants in T-1 and T-2 and a heavier distribution of weights towards pods that are less likely to terminate due to environmental conditions in T-2, with deteriorated symbiosis seen at T-3 despite increased initial nodule formation.

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CHAPTER 4

Whole-genome sequencing of two desiccation-tolerant strains, *Bradyrhizobium japonicum* TXVA & TXEA, isolated from the root nodules of soybean grown in Texas

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Abstract

Two *Bradyrhizobium japonicum* strains, TXVA and TXEA, were isolated for their desiccation tolerance and symbiotic performance with soybean as biofertilizers. Their genomes were sequenced and annotated using the Department of Energy Joint Genome Institute annotation pipeline. Using comparative genomics, the TXVA and TXEA strains were compared to the model organisms *B. japonicum* USDA 6 along with the “elite” inoculant used in Brazil, *B. japonicum* SEMIA 5079. Sequencing yielded chromosomes of 9,193,770 and 9,339,455 bp for TXVA and TXEA, respectively. Phylogeny showed 100% similarity between the native

Bradyrhizobium and USDA 6 with genome comparisons displaying highest synteny between TXVA and USDA 6 at 99.9977% average nucleotide identity (ANI).

Introduction

Bradyrhizobium japonicum is a soil-dwelling nitrogen-fixing symbiont and has been widely applied as an inoculant in soybean (*Glycine max*) fields (Legget et al., 2017). However, drought presents a huge impediment to the application of this inoculant due to inhibition of the symbiosis caused by poor survival of the symbiont under desiccation stress (Parnell et al., 2016). Thus, the primary aim of this research was to bio-prospect for desiccation tolerant *Bradyrhizobium* strains. Previously, soybean root systems from drought-prone agricultural fields in Texas were collected and stored at 4°C until isolation. Randomly selected nodules from each respective plant root were surface sterilized, crushed, and isolated using techniques established in our lab (Parks, 2018). To test isolates for desiccation tolerance, a molecular marker was developed from transcriptomic data of highly expressed genes from *B. japonicum* USDA 110 when placed under non-solute mediated desiccation stress (Cytryn et al., 2007). Using qRT-PCR methods previously described, the expression of this molecular marker was examined across isolates to perform initial selections to be tested via polycarbonate membrane filter survivability assay (Chang et al., 2007). Increased survivability was found for sp. TXVA and TXEA with 7.1% and 13.9% more viable cells, respectively, after being exposed to desiccation for 72 h. (Parks, 2018).

While survival of the symbiont in soil is necessary there are a multitude of other factors that affect the fitness of the mutualism, such as cross-inoculation specificity, competitiveness in the rhizosphere microbiome, and capability as a nitrogen-fixing partner. With *in-vitro* desiccation tolerance shown in the Texas isolates, the next step was to apply these organisms as inoculants in drought-prone soybean fields to test their response *in-vivo*. To date, a total of 7-years of field trials spanning multiple agriculture-centric biomes have been performed; the application of TXVA as a biofertilizer consistently gave soybean plants of numerous cultivars an average increase in biomass accumulation, nodule location, number, size, and ultimately final yield (Beck et al., 2018; Parks, 2018).

Expansion of fieldwork has continued; however, success has been validated with intrinsic drought tolerance and translational applications as a climate-smart biofertilizer in regions of variable environmental extremes. Further characterization of the *B. japonicum* TXVA strain was necessary to determine phylogeny and find a molecular tag that differentiates this strain from native rhizobia for nodule occupancy studies as well as to track persistence in soils. Here, we report the genome sequences of drought tolerant *B. japonicum* TXVA and TXEA isolated from soybean root nodules sampled in Victoria County and Lubbock County TX, respectively. Victoria has more acres devoted to soybean, warmer average temperatures, and slightly over double the rainfall compared to Lubbock (NOAA, 2022; USDA-NASS, 2017). General genome statistics were analyzed along with the comparison of symbiotic, desiccation-specific, and quorum sensing gene regions between the Texas isolates, *B. japonicum* USDA 6, and SEMIA 5079.

USDA 6 is the type strain for the *B. japonicum* species and has been utilized for research applications since being isolated from soybean nodules in Japan around 1929 (Kaneko et al., 2011). Comparative genomic hybridization (CGH) has revealed three distinct genome types in *B. japonicum* strains, USDA 110, 112, and 6 (Itakura et al., 2009). SEMIA 5079 was isolated in 1992 from Brazilian Cerrado soils containing no compatible indigenous rhizobia that had been previously inoculated with parent strain SEMIA 566 (Hungria & Mendes, 2015). It is considered an “elite” strain in Brazil due to its well-characterized competitiveness and saprophytic nature and is one of the most common to be utilized in inoculants, comprising around 70 million yearly doses (Ferreria et al., 2022).

The 16S rRNA gene is used as the universal genetic marker for the classification of bacteria but has been shown to be a poor marker of phylogeny in the *Bradyrhizobium* genus due to the high conservation among this species (Risal et al., 2012). Willems et al. (2003) describes wider variation in the ITS sequences compared to 16S, with ITS results matching DNA-DNA hybridization data. Other markers of phylogeny in the *Bradyrhizobium* genus are the housekeeping genes *dnaK*, *gyrB*, *recA*, and *rpoB* (Avontuur et al., 2019). Using these along with the canonical nodulation (nod) and nitrogen fixing (fix) genes, as well as genes specific to drought, the genomes of native strains *B. japonicum* TXVA & TXEA will be compared.

Materials and Methods

Bacterial growth conditions

Initially, strains were aseptically isolated from nodules and dilutions were plated onto YEM plates with Congo red and actidione (cycloheximide). The YEM agar plates (pH 6.8) consist of 0.5 g of K2HPO4, 0.2 g of MgSO4·7H2O, 0.1 g of NaCl, 10 g of mannitol, 0.5 g of yeast extract, and 15 g of agar per liter with congo red to confirm the presence of rhizobia and 0.002% actidione (cycloheximide) to inhibit fungal growth (Vincent 1970). Plates were incubated at 30°C and checked daily for growth with 4-5 days being typical of slow-growing rhizobia. Colonies confirmed to be rhizobia were then re-streaked on the nutrient rich arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of Na2HPO4, 250 mg of Na2SO4, 320 mg of NH4Cl, 180 mg of MgSO4·7H2O, 10 mg of CaCl2, 4 mg of FeCl3, 1.3 g of 4- (2-hydroxyethyl)-

1-piperazineethanesulfonic acid (HEPES), 1.1 g of 2-(N-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate, and 15 g of agar per L (Sadowsky et al., 1987).

DNA extraction and sequencing

After 4 days of growth at 30°C with shaking at 200 rpm, genomic DNA (gDNA) extraction was performed using the GeneJET genomic DNA purification kit (Thermo Fisher Scientific). The concentration (>100 ng/μL) and purity (260/280 ratio, 1.8) of the gDNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Library prep and sequencing were performed at the University of Texas at Austin's Genomic Sequencing and Analysis Facility (GSAF). Libraries were prepared using the NEBNext Ultra II DNA kit (New England BioLabs) for Illumina according to the manufacturer's instructions. Prior to the library prep the genomic DNA was sheared using the Covaris S2 instrument for an average insert size of 400 bp. The resulting libraries tagged with unique dual indices were checked for size and quality using the Agilent High Sensitivity DNA kit (Agilent). Library concentrations were measured using the KAPA SYBR Fast qPCR kit and loaded for sequencing on an Illumina MiSeq v3 (paired-end [PE] 2 × 300-bp read format) platform.

Quality filtering of reads, assembly, and annotation

The raw reads were quality filtered, and trace adapter sequences were subsequently removed using BBDuk (Bushnell), a program developed at the DOE Joint Genome Institute (DOE-JGI), with the parameters k = 23, mink = 11, hdist = 1, tpe tbo qtrim = rl, trimq = 15, ftl = 5, ftr = 294, ftm = 5, maq = 15, and minlen = 100. The filtered reads were quality checked using FastQC

(Andrews, 2010). Default parameters were used for all software, unless otherwise specified. Assembly of the filtered reads was performed using SPAdes 3.15.3 (Bankevich et al., 2012), and the assembly quality was assessed using QUAST 5.0.2 (Gurevich et al., 2013). Functional annotation and gene prediction were performed using the DOE-JGI Microbial Genome Annotation Pipeline (Huntemann et al., 2015).

Genome analysis and comparison

All genomic analysis and functional comparison tools such as general genome statistics, dot plot synteny, average nucleotide identity (ANI) calculations, and identification of shared and unique genes were performed through DOE-JGI's Integrated Microbial Genomes – Expert Review (IMG-ER) platform (Markowitz et al., 2009). The IMG-ER serves as an online workstation in which registered users can review new and publicly available microbial genomes for comparative genome analysis. Default parameters were used for all analysis tools in IMG-ER. The 16S rRNA sequences were amended to 1481 bp in the final dataset. Multiple sequence alignments were done using the ClustalW algorithm (Thompson et al., 1994). Evolutionary analyses were performed using the maximum-likelihood method (1000 bootstrap re-sampling) in MEGA X (Stecher et al., 2020).

Results

Sequence and assembly quality

Sequencing of TXVA & TXEA produced 6,950,108 and 6,968,744 reads with approximately 227× and 224× coverage, respectively. The assembled genome sequence for TXVA contained 70 contigs (≥1,000 bp) totaling ~9.2 Mbp, with an N_{50} value of 621,423 bp. The assembled genome sequence for TXEA contained 71 contigs (≥1,000 bp) totaling ~9.3 Mbp, with an N_{50} value of 528,185 bp (Table 4-1).

Table 4-1. Quality scores for the assembly of *Bradyrhizobium japonicum* TXVA and TXEA assessed via QUAST.

Contigs and Length profiles	**TXVA**	**TXEA**
Total Contig #	93	103
Contig # (> = 0 bp)	222	237
Contig # (> = 1000 bp)	70	71
Contig # (> = 5000 bp)	51	54
Contig # (> = 10000 bp)	39	42
Contig # (> = 25000 bp)	28	32
Contig # (> = 50000 bp)	23	28
Total Length	9,154,139	9,292,995
Total Length (> = 0 bp)	9,195,799	9,340,585
Total Length (> = 1000 bp)	9,139,612	9,272,207
Total Length (> = 5000 bp)	9,099,722	9,238,939
Total Length (> = 10000 bp)	9,013,494	9,160,096
Total Length (> = 25000 bp)	8,826,178	8,989,013
Total Length (> = 50000 bp)	8,826,178	8,867,797
Largest Contig	1,418,997	1,095,834
N50[a]	621,423	528,185
N70[a]	345,423	231,420
L50[b]	5	6
L75[b]	10	13

[a] N50 and N75 values represent contigs totaling 50 and 75% of total assembly length, respectively.

[b] L50 and L75 represent the ranking of contigs that provide L50 and L75 length, respectively.

Genome structure and statistics

JGI's Integrated Microbial Genomes (IMG) system revealed that the assembled genome sequence of strain TXVA consists of 9,193,770 bp, with 8,980 protein coding genes (72.95% with predicted functions), 4 rRNA genes, 64 tRNA genes, and an average G+C content of

63.67%. The complete genome of strain TXEA consists of 9,339,455 bp, with 9,158 protein coding genes (72.99% with predicted functions), 3 rRNA genes, 63 tRNA genes, and an average G+C content of 63.64%. Genome information for *B. japonicum* USDA 6 and SEMIA 5079 is listed in Table 4-2. *B. japonicum* SEMIA 5079 has the largest genome with 9,583,027 bp while TXEA contains the most protein coding genes. TXVA and TXEA have more tRNA coding genes while USDA 6 and SEMIA 5070 have more rRNA gene clusters.

Table 4-2. Genome statistics for each *Bradyrhizobium* strain involved in this study.

	TXVA	TXEA	USDA 6	SEMIA 5079
Size (bp)	9,193,770	9,339,455	9,207,384	9,583,027
G + C content (%)	63.67	63.64	63.67	63.54
tRNA coding genes	64	63	51	51
rRNA gene clusters	4	3	6	6
Protein-encoding Genes	8,980	9,158	8,655	8,648
Genes assigned to COG	6,641	6,776	5,482	5,576
KEGG Orthology	3,882	3,959	3,867	3,841

Genome comparison of TXVA, TXEA, USDA 6, and SEMIA 5079

Average nucleotide identity displays a high genomic similarity of all strains in this study with the highest nucleotide-level similarity between the genomes of TXVA and USDA 6 (Table 4-3). TXVA and TXEA are similarly related to SEMIA 5079 with an ANI of 98.7128% and 98.7102%, respectively. The least similar genomes are USDA 6 and SEMIA 5079.

Table 4-3. Average nucleotide identity (ANI) for *Bradyrhizobium japonicum* TXVA and TXEA as compared to each respective strain.

Genome 1	Genome 2	ANI
TXVA	USDA 6	99.9977
TXVA	SEMIA 5079	98.7128
TXVA	TXEA	99.9899
TXEA	USDA 6	99.9857
TXEA	SEMIA 5079	98.7102
USDA 6	SEMIA 5079	98.6932

Synteny of genomes was assessed using dot plots generated from IMG-ER using Mummer (Fig. 4-1). In Figure 4-1A, TXEA displays numerous frameshift mutations and insertions with a major duplication event. The asterisk notates insertion of a gene in TXVA as compared to TXEA, an *arsR* gene showing 100% homology and insertion into the same gene region as USDA 6. When comparing TXVA and SEMIA 5079 there is one large and one small translocation (i.e., reverse complement match regions) while many insertions are scattered throughout the genomes (Fig. 4-1B). The highest synteny belongs to TXVA and USDA 6 showing a pattern of co-linearity with a few frameshift insertions and deletions (Fig. 4-1C). The asterisk in Figure 4-1C indicates the insertion of the *dnaC* gene in the TXVA genome in the same region that contains *nodM* in *B. japonicum* USDA 6, gene region shown in Figure 4-2.

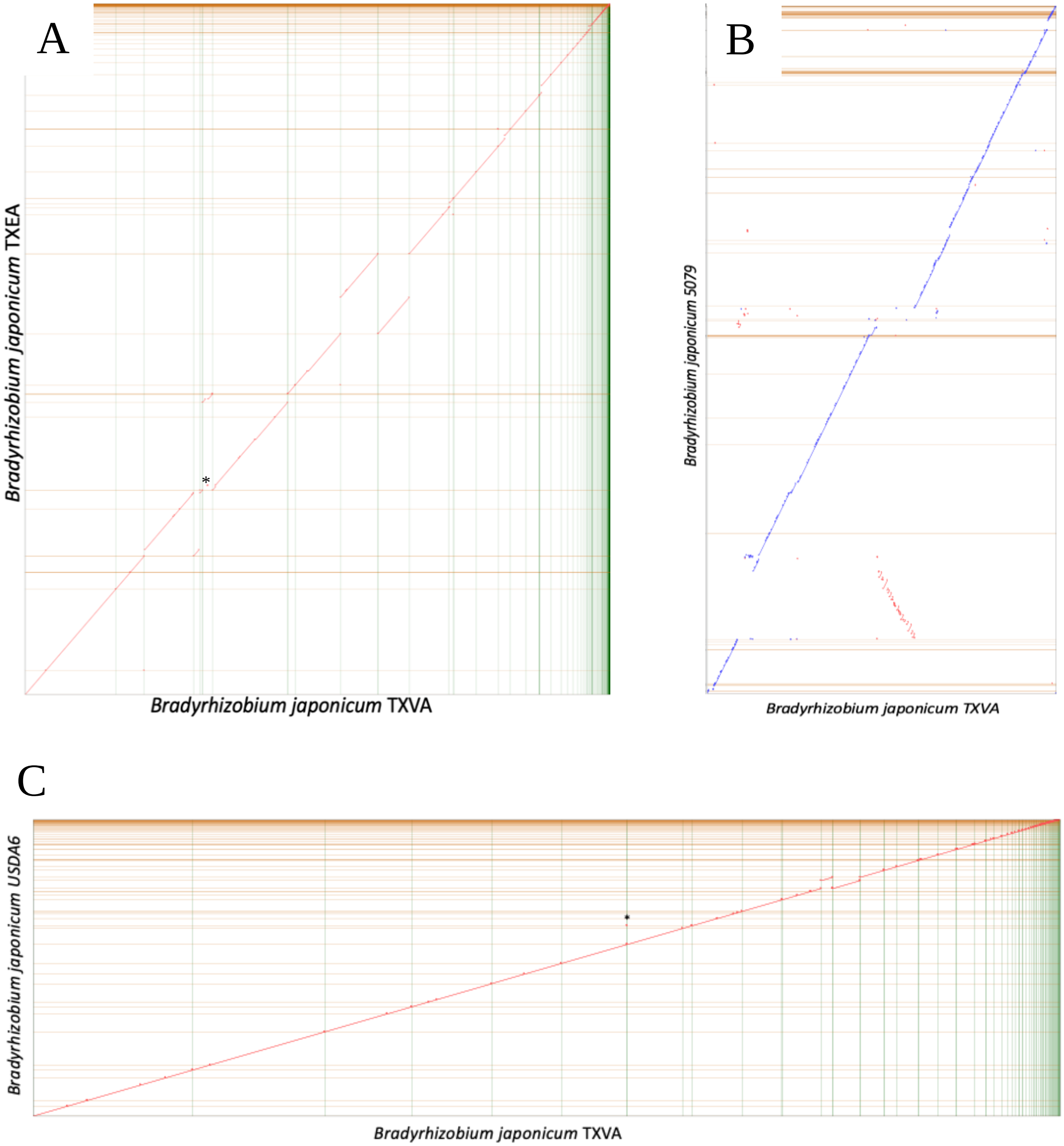

Figure 4-1. Syntenic dot plot between the genomes of *Bradyrhizobium japonicum* TXVA with the 3 closely related strains. (A) TXVA vs. TXEA, (B) TXVA vs. SEMIA 5079, and (C) TXVA vs. USDA 6. The plot represents the set of all maximal unique matches (MUMs) that exist between the two genomes. Gridlines represent scaffolds from each microorganism.

Figure 4-2. Insertion of a regulator of replicative DNA helicase in the TXVA genome around the same gene neighborhood as glutamine-fructose-6-phosphate transaminase (NodM) in USDA 6, indicating a potential insertion in a genetically conserved region.

Phylogeny via 16S rRNA and ITS gene sequence

The 16S rRNA sequences show 100% similarity between the four strains compared in this study (Fig. 4-2). Other strains used include *B. japonicum* USDA 110 and *B. japonicum* USDA 122, which have since been reclassified as *B. diazoefficiens* and thus group together with species phylogeny. *Bradyrhizobium* sp. BTAi1 & ORS278 which represent members of the nod-independent stem-nodulating photosynthetic clade (Giraud et al., 2007). The last strain included was *B. elkanni* USDA 76, which is the type strain for the *elkanii* species and is known to be more endemic in subtropical and tropical regions (Saeki et al., 2010; Reeve et al., 2017). Figure 4-3 shows the phylogeny according to the housekeeping genes *atpD, dnaK, glnII, gyrB, recA, and rpoB* which highlights the differences between the native strains and SEMIA 5079, but still reflects high similarity with USDA 6 does not differentiate the native strains from the type strain.

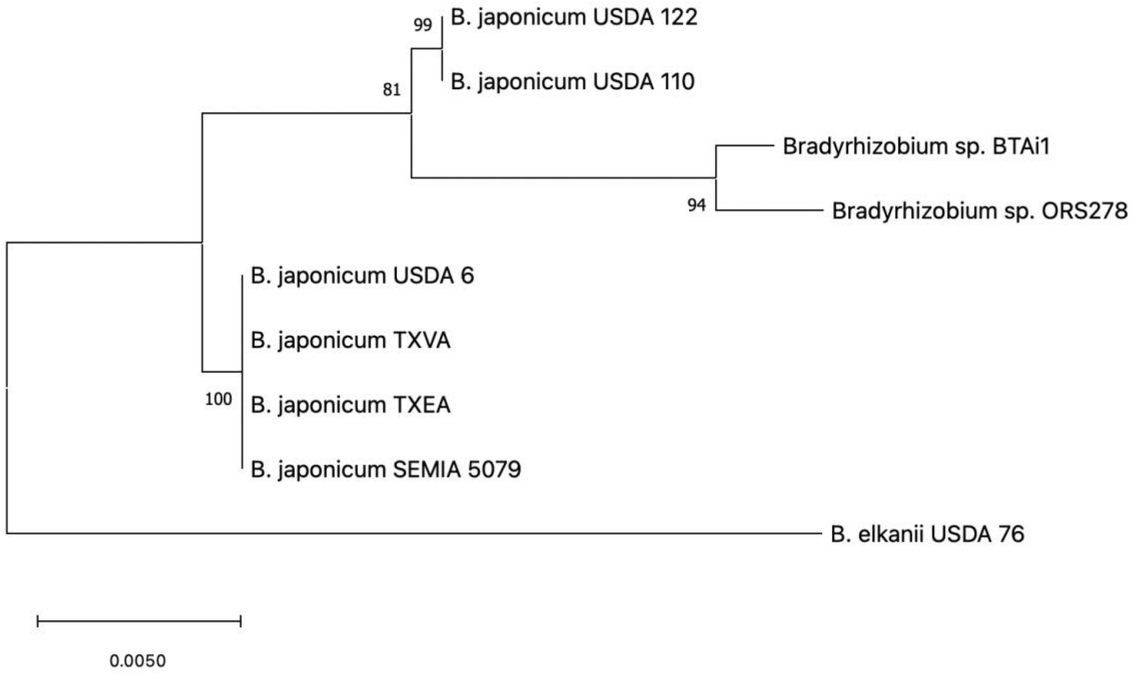

Figure 4-3. Phylogenetic tree generated using the 16S rRNA sequences from native isolates among type strains from each of the major *Bradyrhizobium* clades using MEGA X. ClustalW alignment was performed prior to tree rendering. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

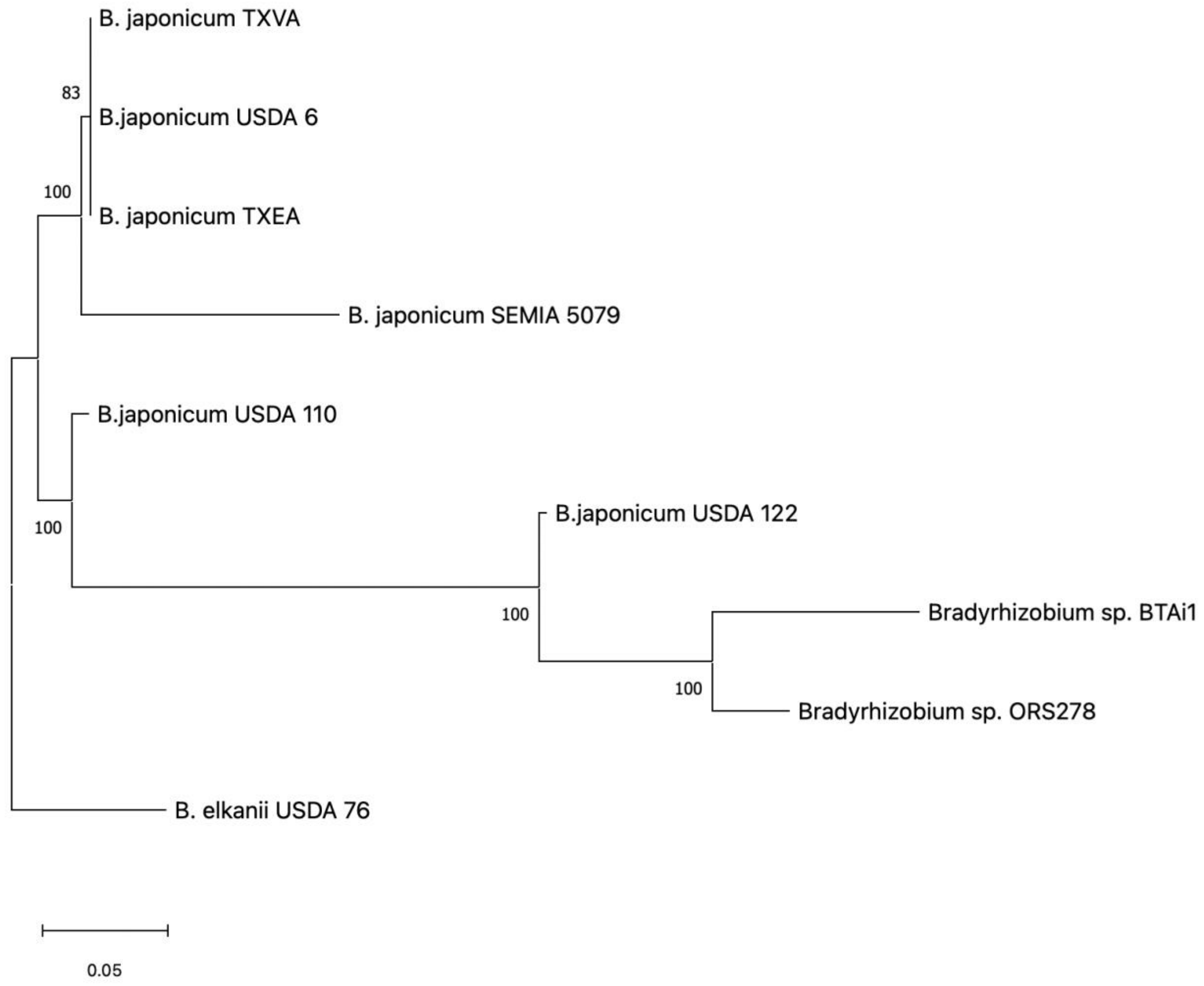

Figure 4-4. Phylogenetic tree generated using the housekeeping genes *atpD, dnaK, glnII, gyrB, recA, and rpoB* from native isolates among type strains from each of the major *Bradyrhizobium* clades using MEGA X. ClustalW alignment was performed prior to tree rendering. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Nodulation and nitrogen fixation genes

Nodulation is indispensable for a viable symbiosis with all *Bradyrhizobium,* excluding the nod-factor independent photosynthetic clade, and therefore present a conserved marker for evolution studies. These genes are clustered on a conserved symbiosis island that contains all essential functions for symbiosis and are shown to be mostly conserved between compared strains, but key differences exist between *B. japonicum* SEMIA 5079 and others (Fig. 4-3). NodD is the initial transcriptional activator of the nodulation pathway (Ferreira et al., 2022). This gene was shown to be divergent in *B. japonicum* TXVA & SEMIA 5079, as shown in the phylogenic tree rendering of the concatenated 860 bp *nodD* sequences (Fig. 4-4). The *nifH* gene is highly conserved and encodes for dinitrogenase reductase which makes it crucial for the assembly of the nitrogenase enzyme. Shown in Figure 4-5, the gene neighborhoods of NifH are shown in all four organisms and display similarity among the genomes.

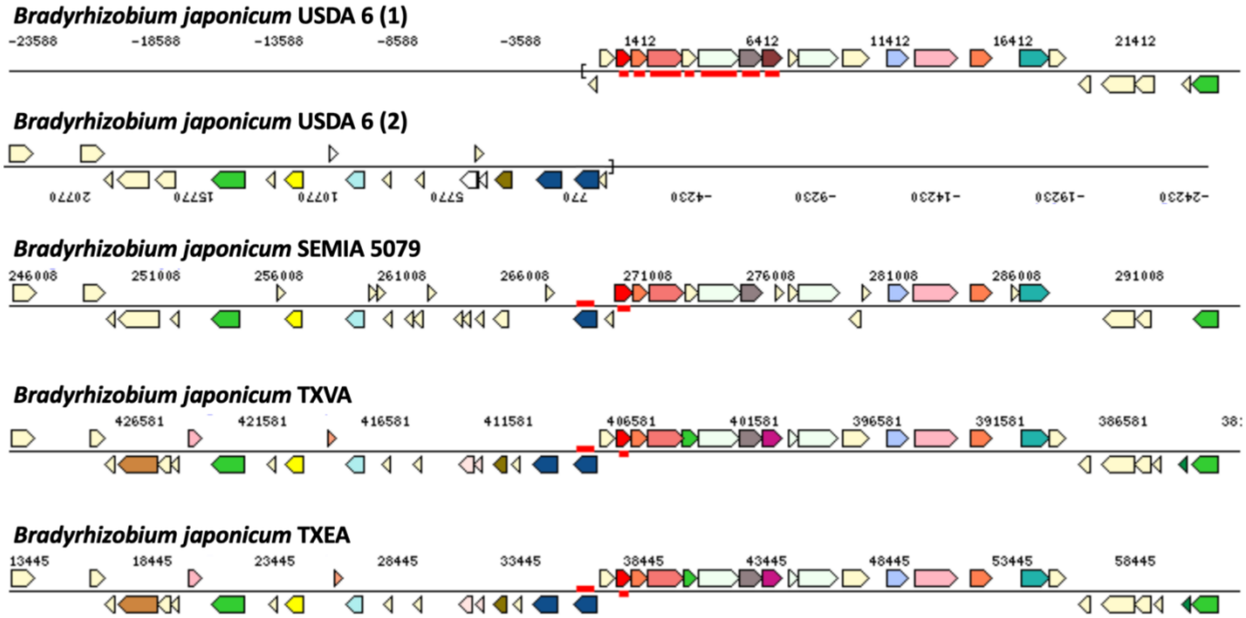

Figure 4-5. Gene neighborhood representations rendered from IMG-ER for each respective strain. Red colored gene in center represents *nodA*. *nodY* is shown immediately before *nodA* which is flipped in SEMIA 5079. A split scaffold is shown in USDA 6 (1 & 2).

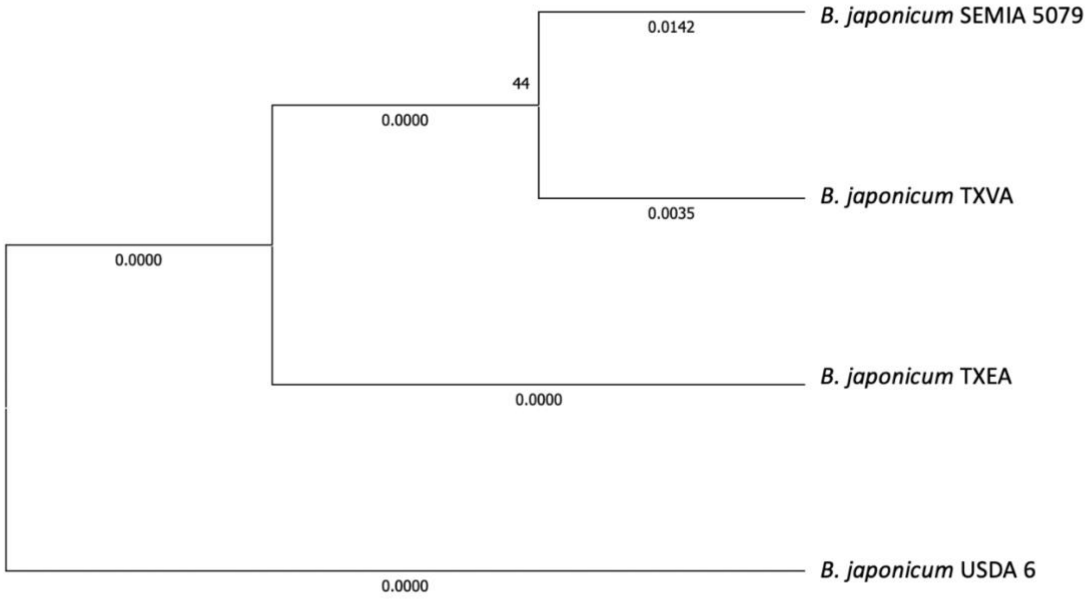

Figure 4-6. Phylogenetic tree generated using *nodD* sequences accessed from NCBI. ClustalW alignment algorithm was used to align sequences and tree generation was performed by MEGA X. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

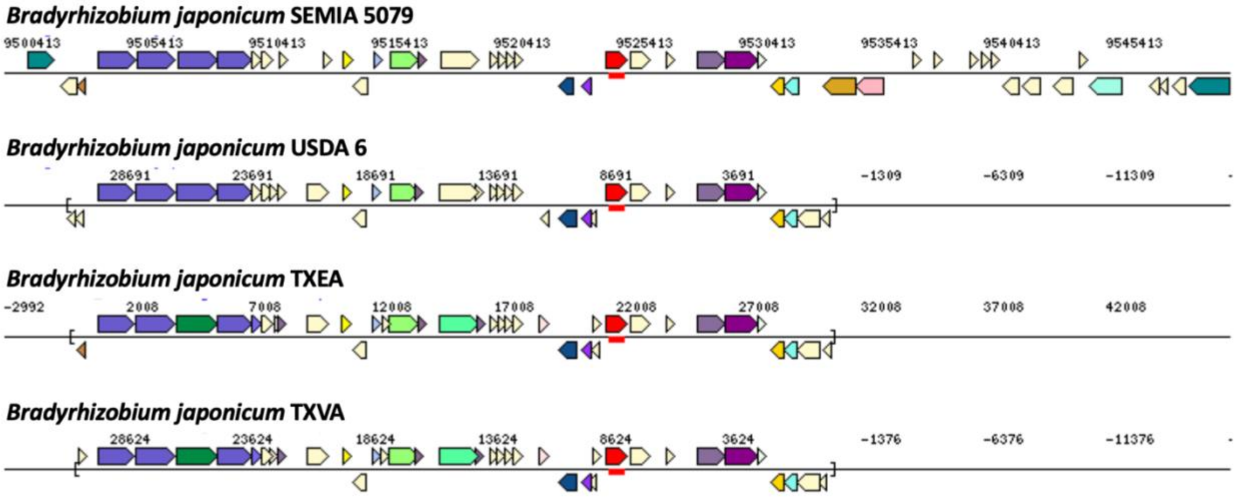

Figure 4-7. Gene neighborhoods of *nifH* in all four *Bradyrhizobium* strains, highlighted in red.

Discussion

The diverse bio-resource of the *Bradyrhizobium* genome presents insights into the symbiotic potential and evolution of this genus. Extremely high conservation among the 16S rRNA gene is displayed among the native strains TXVA and TXEA with USDA 6 and SEMIA 5079, showing 100% homology. Therefore, multilocus sequences alignment (MLSA) was done using the housekeeping genes *atpD, dnaK, glnII, gyrB, recA, and rpoB* to determine speciation between the native isolates and the compared strains (Menna et al., 2009). This displayed separation of SEMIA 5079 from the others, but still did not differentiate the native strains from type strain USDA 6, supporting that MLSA provides more phylogenic clarity as compared to 16S rRNA, but the high similarity between the USDA 6 type strain and native strains did not allow either one of these tools to identify differences in sequences (Martens et al., 2007).

Syntenic dot plots represent genomic comparisons on a nucleotide-to-nucleotide basis that quickly show repetitions, mutations, breaks, and inversions of compared sequences. The interactive tool on IMG-ER provides dot plots based on genome alignments via MUMMER (Marçais et al., 2018). Highest synteny is observed with TXVA and USDA 6, with only a few frameshift insertions and deletions. One of these insertion events, indicated by the asterisk on Fig. 4-1C, represents insertion of a *dnaC* gene annotated as a DNA replication protein which initiates DNA replication (Sandler et al., 1996). Homologs of this gene are 100% in TXEA, 99.86% in SEMIA 5079, and is not present in USDA 6, indicating potential assistance of symbiotic functioning since the strains which encode for this protein display better symbiotic efficiency. Another interesting facet of this insertion is the location on the chromosome, showing insertion downstream of the *nodM* gene when compared to the USDA 6 scaffold which has shown to be essential for efficiency of nod signaling and bacteroid maturation (Baev et al., 1992). When differentiating between native strains, an insertion is shown (asterisk, Fig. 4-1A) notating insertion of a gene in TXVA as compared to TXEA, an *arsR* gene showing 100% homology in USDA 6. ArsR has shown to be its own transcription regulator in *Agrobacterium tumefaciens* influencing numerous cellular functions, such as, arsenic resistance, sugar transport, phosphate metabolism/acquisition, chemotaxis, copper tolerance, and iron homeostasis (Rawle et al., 2021). The presence of this *arsR* gene in TXVA and USDA 6 may convey competitive advantage in symbiosis with soybean.

Nodulation (*nod*) genes have been shown to be indispensable with symbiosis, with rare exception being the photosynthetic clade (Giraud et al., 2007). These genes play key roles in the detection of plant flavonoid compounds which stimulate NodD, a LysR-type regulator, and induce expression of the remaining *nod* genes starting with *nodA*, shown in Fig. 4-5 with the red bar indicating regulatory relationships (Göttfert et al., 1992). Multiple isoforms of NodD have been detailed that suggest an expansion of flavonoid recognition and therefore host-range compatibility (Loh & Stacey, 2003). The differentiation of SEMIA 5079 is observed when at the *nodD* sequence, most notably an inversion and size reduction of *nodD2* which is present in native strains and USDA 6 represented by the blue box on the left (*nodD2)* and right (*nodD1)*. Besides, numerous small alterations are observed between USDA 6 and the native strains, all labeled as hypothetical proteins with unknown function. While NodBC are 100% similar across all four strains, NodA shows 99.84% homology in SEMIA 5079. NifH is known to be essential for assembly of the nitrogenase enzyme and for a functioning symbiosis. While there is 100% homology across compared strains, gene neighborhoods display difference mostly with inversions/insertions of hypothetical proteins upstream of the *nifH* gene.

For desiccation-specific regions, all strains contain the desiccation-specific molecular marker genes (i.e., *otsA*, *otsB*, *treS*, and *aceA*). Homology between native isolates and USDA 6 shows 100% of all markers, but SEMIA 5079 displays similarities of 99.26%, 98.59%, 99.18%, and 99.51%, respectively. Additionally, *B. japonicum* TXVA was shown to display three copies of the *lexA* gene while all other strains only had two. The COG description shows 21% homology to an "SOS-response transcriptional repressor LexA", however, the Pfam hit (protein family) is 100% identity for the LexA DNA binding domain. These *lexA* genes provide SOS response to aid DNA repair, enabling persistence in unsuitable environments (Podlesk & Bertok, 2020). This transcriptional repressor was also shown to be induced when water stress-tolerant *Bradyrhizobium japonicum* 5038 underwent the rehydration condition after dehydration, potentially displaying an attempt to rescue regulation of genes up-regulated under desiccation stress (Zhu et al., 2022). The presence of this additional transcriptional regulator in TXVA indicates a potential mechanism of cell function recovering and thus mechanism of increased survivability. The sequence length of the additional *lexA* is only 129 bp (43 aa) but recent attention to the regulatory power of small proteins has been noted such as the 30-aa MgtR protein in *Salmonella* which interacts with magnesium regulators in the cell membrane under low concentrations (Gray et al., 2022). When looking for homologous genes, it shows 100% to seven different strains of *Burkholdia contaminans* which are known plant growth promoting organisms found in the rhizosphere that provide protection against pathogenic fungi and even have a patent filed in this regard (Lu et al., 2009; Lu et al., 2011).

Whole genome sequencing of the Texas-native drought-tolerant isolates showed close phylogeny with *Bradyrhizobium japonicum* USDA 6 and SEMIA 5079, which are both known to be used as inoculants for soybean production with SEMIA 5079 comprising one of the most widely used in Brazil (Chibeba et al., 2018). Comparative genomics revealed unique insertions in the native strains with some of those genetic elements being recruited in the desiccation/hydration response.

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CHAPTER 5

CONCLUSIONS

The primary aim of the current project is to validate *in-vivo* and *in-vitro* success of the drought-tolerant inoculant *B. japonicum* TXVA on soybean production in the Mid-South with multiple management/input systems, as well as to characterize the phylogeny of the Texas-native isolates to probe their genome for molecular insights into their ecological functioning. This was achieved by the performance of multi-state trials to evaluate the drought-tolerant inoculant in the drought-prone areas in the 6 states, displaying improved plant health and overall soybean yield increase of 7%. Furthermore, the native strain was utilized in a novel temperature gradient greenhouse to test heat stress in biologically relevant temperature gradients. This project displayed the partitioning of resources that comes with inoculated plants, showing higher seed yield under ambient and ambient +2°C temperature gradients, with a reduction seed at ambient +4°C. Lastly, whole genome sequencing was performed to genetically characterize the native strains *B. japonicum* TXVA and TXEA, showing high similarity to type strain USDA 6, the "elite" Brazilian inoculant SEMIA 5079, and displaying unique gene regions that could potentially serve as molecular tags for ecological persistence studies.

The second and broader aim of this study is to highlight the role of translatory research that is combined molecular techniques (i.e., desiccation-specific molecular marker and whole genome sequencing) with application-driven science (i.e., greenhouse and field trials), with the goal of increasing sustainable farming systems through a joint concern for soil fertility and mitigation of our changing climate. Occurring in the medical sector with multi-omics analysis allowing precision medicine and in the environmental sector with the "pot to plot" transition from genetic pathway specifics to agricultural realizations, both sectors stand to benefit from this interdisciplinary communication of continual research and application (De Maria Marchiano et al., 2021, Nelissen et al., 2014). The key to being successful in this collaborative dialog requires engaging in questions and seeking out research, as well as utilizing it to bridge inherent differences present in knowledge systems (Ingram et al., 2018). For commercial operations or producers that are involved in co-ops, access to supplies, equipment, and knowledge may be readily available but for smallholder farms less resources present larger barriers to overcome (Valdivia et al., 2014).

Increasing availability and comprehension of peer-reviewed agricultural studies, along with outreach such as speaking at crop tours or giving seminars, is becoming more of a standard for sustainable food systems being developed with the goal of mitigating and reversing the damage done to the environment from overcropping, overapplication of fertilizers, and over-use of tillage. The improvements in crop science, environmental microbiology, and molecular biology have created a foundation for building climate-smart agricultural practices where small variations between inputs can be replicated, repeated, and vetted for consistency before being communicated to the public and private sector for full-scale adoption. Nitrogen fertilizer has been a go-to solution as it provides the most limited essential nutrient and helps plants overcome less than optimum growing conditions (Hettiarachchi et al., 2021). This easy solution often has far-reaching and thus ignored consequences, like air/water acidification, eutrophication of water systems, and environmental risk from producer to consumer (Kaur et al., 2018; Itakura et al., 2013). By using climate-smart practices like inoculation, biochar addition, cover-cropping, crop rotation, and reduced-tillage, environmental impacts can become regenerative in nature (Guardia et al., 2019; Ashiq et al., 2020, Niraula et al., 2021). The nitrogen fixation rate for soybean is upwards of 100 kg N per hectare per year and with the use of cover cropping legumes like alfalfa and clover, fixation can be up to 250 kg N per hectare per year (Werner, 1922). Evaluation of life cycle impacts on the inoculant industry in Argentina have shown a reduction of GHG emissions from the field, yield, upstream, and downstream processes (Mendoza Beltran et al., 2021). Thus, a combination of these efforts presents even more mitigation potential.

The leguminous symbiosis stands to aid global-scale environmental efforts by curbing GHG emissions, regional scale impacts from reduced run-off, and producer-level with essentially a higher net income from reduced inputs or increased yields from acres planted. An all-around great scenario that has lacked large-scale adoption or translation into meaningful production (Ingram et al., 2018). This is due to a lack of communication, as mentioned above, or lack of research on the increasingly complex edaphic niche and how microbial inoculants of varying formulations impact this microbiome. Microbial inoculants are being utilized in a variety of ways by researchers to provide ecologically-sound solutions to climate-change and its subsequent abiotic stressors, with co-inoculations of *Rhizobium* and arbuscular mycorrhizal fungi shown to avoid drought-induced senescence while increasing yield, size, and quality of soybean seed (Takács et al., 2018; Igiehon et al., 2021). Even lesser-known genera of endophytic bacteria, such as, *Gluconacetobacter, Ralstonia, Sphingomonas, Lactobacillus,* etc. are showing promise as microbiome modulators to increase beneficial associations with plants through known and yet-undiscovered methods of nutrient utilization (Subramanian et al., 2015; Daranas et al., 2019; Fadiji et al., 2020). The impact on soil microbiomes is mostly temporary in nature, but with more and more microbial consortia being used as synthetic microbial communities (SynComs) to modulate biotic and abiotic factors with regard to plant, soil, and ecosystem health, there is an ever-growing need to fill in the gaps regarding the communal interactions in the rhizosphere (Marin et al., 2021; Garcia et al., 2021; Picoli et al., 2022).

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Appendix A

Figure A-1. A drone image of an irrigated 30 ac split-plot trial comparing *B. japonicum* TXVA to a commercial inoculant at Vanderpool farm in the Rio Grande Valley, Texas.

Figure A-2. A pot-bound root ball from the soybeans grown in the temperature gradient greenhouse in Raleigh, North Carolina. Nodule counts of up to 1,100 have been reported on these root systems.