Pseudomonas spp. Isolated from the Soybean Nodule Interior Promote Soybean Growth upon Field Amendment

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Abstract (Academic)

Diazotrophic microbes reside in soybean nodules; however, other non-nitrogen fixing bacteria are a part of the interior nodule microbiome. Results from a previous greenhouse study show that a novel species of *Pseudomonas* associates with soybean nodules as a plant-growth promoting rhizobacteria (PGPR). This study observes the soybean growth promoting potential of *Pseudomonas* spp. in a field setting. Additionally, this study observed differences in soybean growth promotion based on amending the plant with isolated strains or a mixed culture of the species' strains. Two cultivars of soybean (Asgrow AG46X6 and Pioneer P48A60X) were either amended with isolated strains of the novel *Pseudomonas* spp. (referred to as PAMW1 and BUMW2 in this study), a mix of the two strains, or an uninoculated control. The study recorded measurements to observe growth, yield, and nitrogen fixation differences. The study uses two-way factorial ANOVAs and non-parametric, multivariate analyses to determine differences in growth promotion among samples. Soybean amended with PAMW1 has greater shoot mass, biomass, and height than other treatments. Through nonmetric multidimensional scaling (NMS), samples amended with a mixed culture or PAMW1 may be different regarding growth promotion relative to the non-amended samples. Univariate results support the hypothesis that the novel *Pseudomonas* spp. benefit soybean in a field setting. However, it is inconclusive whether a mixed culture amendment of multiple strains alters the overall growth promotion of soybean compared to samples amended with isolated strains.

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Abstract (General Abstract)

Soil hosts a relatively abundant and diverse community of microorganisms. Moreover, the area of soil that interacts closely with plant roots and their associated exudates, called the rhizosphere, has a significantly greater microbial abundance than surrounding bulk soil. Interactions between microbes and the plant often promote plant growth because of secondary metabolites produced by these beneficial microbes. One particular bacterial species, belonging to the *Pseudomonas* genus, was discovered and extracted from the soybean nodule interior. Nitrogen-fixing bacteria predominantly reside in the soybean nodule, yet this microorganism cannot fix nitrogen. Although trace amounts of non-nitrogen-fixing bacteria reside in the soybean nodule, this novel species has a relatively high abundance. This study determines the benefits of this species in the soybean nodule. Following positive results in a greenhouse study, this field experiment observes variance in soybean growth and productivity based on their received bacterial amendment. For this study, two soybean cultivars were either amended with an isolated strain of this species, a mix of the two strains, or left uninoculated to serve as a control. Numerous recorded measurements serve as indices of soybean growth and productivity. The results suggest that this novel *Pseudomonas* species benefits the plant by significantly improving biomass. With further research, this species can potentially serve as an environmentally sensitive and sustainable alternative to fertilizers through its ability to promote soybean growth.

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Section 1: Literature Review

1.0 Preface

The study in this thesis describes the role of a novel species of *Pseudomonas*, most closely related to *Pseudomonas azotoformans*, as a growth promoter in *Glycine max* (soybean). The novel species was isolated from the interior of soybean nodules. To determine the role of this microbial species in the nodule, two of its strains were amended into the root zone of numerous soybeans to measure changes in plant growth and productivity. This study hypothesizes that the strains of the novel species of *Pseudomonas* benefit soybean growth upon its amendment. With a focus on the *Pseudomonas* genus, this literature review highlights the interactions between microbes in the rhizosphere and introduces plant growth-promoting rhizobacteria (PGPR). There is intent to acquaint the reader with possible microbial functions responsible for promoting soybean growth. Furthermore, since this microbial species originates from the soybean nodule interior, this literature review explains microbial improvements toward nutrient availability to understand the effects of an interaction between *Pseudomonas* and *Bradyrhizobia*, the latter of which is responsible for soybean nodulation.

1.1 Plant-Microbial Interactions in the Rhizosphere

Microbes are highly abundant in the rhizosphere, the region of soil associated with plant roots and their exudates where bacterial numbers can be 10 to 1,000 times greater compared to surrounding non-rhizosphere bulk soil (Hiltner, 1904; B. Lugtenberg & Kamilova, 2009). Available plant-root exudates are a reason for a relatively high microbial abundance within this layer. Root exudates are nutrient-rich metabolites secreted from the plant. Among other components, the root exudate composition includes fatty acids, amino acids, organic acids, sugars, and vitamins (Uren, 2007). The microbes utilize these secretions as nutrients for microbial growth, resulting in large populations growing along the plant's epidermal cells and lateral roots (Rovira, 1956).

One could expect plant-microbial interactions in the rhizosphere because the microbes are in direct contact or closely associated with the plant root. These interactions can lead to positive or antagonistic effects on the plant (Newman & Banfield, 2002). Plant growth promotion through microbial interactions is a notably positive outcome in the rhizosphere. As microbes uptake root exudates, microbes also secrete compounds into the rhizosphere. Microbes secrete hormones such as gibberellins and auxin, inorganic nitrogen such as ammonia, and solubilized phosphorus, iron, and other micronutrients, which may benefit plant growth (Bais et al., 2006). Through plant-nutrient and phytohormone uptake, bacteria can directly promote plant growth. Bacteria capable of influencing plant growth beneficially are plant growth-promoting rhizobacteria (PGPR).

Not all plant-microbial interactions in the rhizosphere are positive, as some lead to adverse or antagonistic effects on the plant. Certain pathogenic microbes may invade the rhizosphere and interact with the plant. Common bacterial pathogens include members of *Pseudomonas syringae* pathovars, *Agrobacterium tumefaciens*, and *Ralstonia solanacearum* (Mansfield et al., 2012). Interactions between *P. syringae* and tomato (*Solanum lycopersicum*) plants, for example, result in bacterial speck (Shenge et al., 2007). This microbe can cause bleeding cankers in horse chestnut (*Aesculus hippocastanum*), resulting in foliar discoloration and cankers along stems and branches, ultimately leading to plant death (Green et al., 2009, 2010). The interaction between numerous crops and *A. tumefaciens* has resulted in crown gall tumors. Plants infected by this pathogen appear stunted in growth from an overgrowth gall on the roots or base of plants. Since it can infect 93 families of plants, this microbe is now commonly genetically modified for trans-kingdom gene transfer (Chilton et al., 1977; Kado, 2002; E. F. Smith & Townsend, 1907). Additionally, *R. solanacearum* infects numerous plants through the root tips or cracks in the lateral root, which results in plant wilting by a build-up of microbes in the xylem. This plant pathogen is relatively lethal and has a broad host range (Denny, 2006; Mansfield et al., 2012).

Beneficial interactions between microbes can benefit the plant by suppressing the biological functions of soil pathogens. For example, *Pseudomonas denitrificans* and *Pseudomonas putida* reduced infection of disease-causing *Ceratocustic fagacerum* on oak (*Quercus* spp.), which decreased the potential of the pathogen to affect the plant crown (Brooks et al., 1994). Whether the effect was due to direct suppression or competition, the net result shows that *Pseudomonas* can indirectly benefit plant growth. In this case, the microbes serve as disease suppressing biocontrol agents.

Depending on the specific species or strain, *Pseudomonas* can suppress fungal pathogens. *Fusarium oxysporum* and *Aspergillus niger* are examples of pathogens negatively influenced by *Pseudomonas* (S. K. Pandey & Chandel, 2014). The *Pseudomonas* genus produces antifungal metabolites, including phenazines, 2,4-diacetylphloroglucinol (DAPG), and pyrrolnitrin (Bloemberg & Lugtenberg, 2001). The production of antifungal or antibiotics by *Pseudomonads* and other bacteria residing in the rhizosphere exemplifies biocontrol agents.

Induced systemic resistance (ISR) is a common mechanism for biocontrol in the rhizosphere. For example, an interaction between the *Bacillus* genus and tomato offers protection against the cucumber mosaic virus. The results of this study suggest that an interaction between the plant and virus can result in some species of *Bacillus* eliciting induced resistance for the plant. (Murphy et al., 2003). Upon pathogenic infection or insect herbivory, airborne or vasculature signals are released by the plant and interpreted by beneficial bacteria to increase protection in healthy components of the plant (Pieterse et al., 2014).

In soils with a relatively high microbial diversity, microbe-microbe interactions may lead to synergistic effects on microbial growth and function (Anderson & Coats, 1995; Chaudhry et al., 2005). A synergistic interaction enhances microbial growth or biological function so profoundly that it is not observable without the interaction (Schink, 2002). Thus, two microbes may interact synergistically if their combined effects are greater than the sum of their separated effects. Synergistic interactions may enhance the degradation rates of carbohydrates, leading to microbial proliferation increases.

For example, two isolates grew in media containing glucose or lignocellulose. The turbidity of each culture quantified differences in microbial growth based on the available carbon source. Isolates inoculated in media containing lignocellulose appeared to have minimal growth because the degradation of this carbohydrate has greater energetic costs than glucose. Alternatively, the co-inoculation of these two microbes in media containing lignocellulose resulted in a significant increase in growth because of differences in the microbe's capabilities to degrade the carbohydrate. Thus, the microbe's combined effects are greater than the sum of their separate effects. These results show that comparing microbial growth in media with different degradable carbon sources can provide insight into a mixed culture's diversity regarding degradation functions (Deng & Wang, 2016). Additionally, available substrates may induce synergistic interactions among microbes.

Numerous studies have described increases in microbial proliferation through synergistic interactions. Synergistic interaction between two microbes is observable in a laboratory by comparing culture with isolated growth to co-inoculated media. Co-inoculated cultures showing more significant growth than the cultures containing their respective isolates represents a synergistic interaction. Labs will often manipulate carbon sources in media to observe synergistic microbial proliferation.

Furthermore, syntrophic degradation of amino acids requires the coupling of microbes, resulting in a complete enzymatic pathway that would not exist without interaction. (Schink, 2002; Morris et al., 2003). For example, the coupling of redox reactions that transform amino acids to carboxylic acids is known as Stickland fermentation (Schink & Stams, 2006). If a Stickland acceptor does not exist in the microbial community, hydrogen will be produced instead of carboxylic acids (Nisman, 1954). For improved degradation of amino acids, mesophilic and thermophilic bacteria are often paired with methanogenic bacteria to achieve syntrophic degradation (Schink & Stams, 2006). The coupling of microbes satisfies the requirements of Stickland fermentation, which may increase microbial growth (Neumann & Römheld, 1999; Nisman, 1954).

Microbial consortia may demonstrate synergism by increasing the rate or extent of digestion. For example, the degradation rate of a microbial species can accelerate in the presence of a

secondary member as it removes inhibitory products produced by the former. A study involving the interactions of rumen bacteria observed synergistic effects in cellulose degradation. This study compared the rates of cellulose degradation by cellulolytic and non-cellulolytic species. Adding non-cellulolytic species to cultures inoculated with cellulolytic species enhances degradation rates synergistically (Dehority, 1997). Although the non-cellulolytic microbe cannot degrade cellulose independently, this microbial combination can increase degradation by up to 5%, depending on the substrate used.

Differences in secondary metabolite production among microbes can lead to synergistic interactions. A microbe's secondary metabolite profile may vary at a strain level. Secondary metabolites do not have a clear definition, but these compounds are diverse, complex, and nonessential for microbial growth (Challis & Hopwood, 2003). Contrary to their role in microbes, secondary metabolites may significantly impact plant growth. A diverse rhizosphere has more significant potential to host microbes with varying functions. Consequently, the secondary metabolites present in the rhizosphere are diversified. Greater diversity of produced secondary metabolites may enhance the effectiveness of microbial plant growth promotion compared to isolated strains. Thus, diverse secondary metabolites in the rhizosphere may promote significant enhancements of biological function characteristic of synergism.

The enhancement of microbial functions through synergistic interactions in soil is often observable through changes in plant growth. For example, one beneficial function of mycorrhizae is plant growth promotion by increasing nutrient availability through hyphal extension. The dual inoculation of *Bacillus amyloliquefaciens* and *Rhizophagus irregularis* results in a greater shoot weight and photosynthesis efficiency in white clover (*Trifolium repens)* and wild strawberry (*Fragaria vesca)* (Xie et al., 2018). The presence of *B. amyloiquefaciens* significantly increased mycorrhizal association in white clover. As a result, the biological function of mycorrhizae enhanced synergistically, which deemed *B. amyloliquefaciens* as a "mycorrhiza helper bacterium."

Similarly, one study observed that the co-inoculation of a specific strain of *Streptomyces kanamyceticus* and *Bradyrhizobium japonicum* led to increased nodulation (Gregor et al., 2003). Initially, inoculating *S. kanamyceticus* killed *B. japonicum* through antibiotic production, which restricted nodulation. However, Gregor et al. (2003) observed that the subsequent antibiotic-resistant members of *B. japonicum* interacted with *S. kanamyceticus* leading to an increase in nodulation greater than the independent effects of *B. japonicum*. Thus, an interaction between two microbes led to the enhanced biological function, which ultimately characterizes *S. kanamyceticus* as a PGPR capable of synergism upon interaction with *B. japonicum.*

In summary, many microbial interactions have a neutral effect on plant growth. Nonetheless, interactions can also lead to beneficial, antagonistic, or synergistic effects on plant growth and

productivity. Plant-microbe interactions that consequently benefit plant growth often include plant growth-promoting rhizobacteria (PGPR). The application of PGPR into the rhizosphere results in various functions influencing the plant.

1.2 Application of Plant Growth Promoting Rhizobacteria (PGPR)

Plant-growth-promoting rhizobacteria (PGPR) are microorganisms capable of influencing plant growth directly or indirectly (Lugtenberg & Kamilova, 2009). Direct applications of PGPR can achieve biofertilization, rhizoremediation, and plant hormone production, consequently influencing plant growth and productivity (B. Lugtenberg & Kamilova, 2009). Indirect methods may refer to microbial competition or biocontrol. These interactions directly alter the microbial community of the rhizosphere, which may indirectly promote plant growth.

Biofertilization is the microbial increase of nutrient availability creating a fertilized rhizosphere. Often, the nutrient that becomes more bioavailable upon a microbe's interaction with the rhizosphere characterizes the microorganism. Classifications may include nitrogen-fixing, phosphate-solubilizing, potassium-solubilizing, iron-solubilizing, and zinc-solubilizing microbes (S. Singh et al., 2014; Thomas & Singh, 2019). Before amending crops, considering PGPR classifications can achieve biofertilization based on the rhizosphere's conditions and the plant's needs (B. Lugtenberg & Kamilova, 2009).

Through these classifying functions, microbes can increase nutrients in the rhizosphere and remove limitations to plant growth. For example, the amendment of a phosphate-solubilizing strain of *Pseudomonas*, PS-32, increased maize shoot dry weight by 42% compared to unamended maize plants (Iqbal Hussain et al., 2013). Similarly, Hussain observed that the amendment of phosphate-solubilizing *Bacillus* sp. PS-12 resulted in 33% greater maize grain yield than its respective control. The effects of *Pseudomonas sp.* PS-32 and *Bacillus* sp. PS-12 may be consequential to increases in available phosphorus through phosphate solubilizing mechanisms. Of course, increasing the availability of other nutrients besides phosphorus by applying growth-promoting microbes can also result in biofertilization. A study observed that the amendment of *Pantoea anatis* increased the dry root weight of rice crops by 80.1%. *P. anatis* can solubilize potassium, increasing rice root weight (Bakhshandeh et al., 2017). Along with mechanisms that result in nitrogen, iron, zinc, and other micronutrient increases, the application of PGPR can promote biofertilization and plant growth.

Another direct result of PGPR application can be rhizoremediation. As organic pollutants enter the rhizosphere, microbes and other biological systems tolerate these pollutants through active efflux systems or sequestration (Lima et al., 2006; Nies, 2003). Microbes can transform pollutants into a less toxic form, reducing plant stress (Bruins et al., 2000; Caplan, 1993; Dua et al., 2002; S. Liu & Suflita, 1993). For example, in soil subjected to copper stress, the amendment

of *Providencia vermicola*, a copper-resistant microbe, significantly increased height by 31.7%, root length by 52.7%, biomass by 37.5%, and leaf area by 31.2% in lentil plants (Islam et al., 2016). *P. verimicola* immobilizes copper through phosphorus solubilization, resulting in the accumulation of phosphorus, which can decrease the uptake of copper by plants. Absorption and desorption functions may have also reduced the concentration of copper in the rhizosphere. Ultimately, an amendment of *P. verimicola* promotes plant growth through rhizoremediation and other PGPR s, including phytohormone production.

An increase of phytohormones in the rhizosphere can be another direct result of PGPR application affecting plant growth. Phytohormones act as signaling or messenger molecules synthesized in defined plant organs. The plant's reception of phytohormones can positively influence plant growth by impacting plant metabolism and mitigating abiotic stress (Egamberdieva, Wirth, Jabborova, et al., 2017; Y. F. Hu et al., 2013; Kazan, 2013; Teale et al., 2006). PGPR commonly produce phytohormones like pyrroloquinoline quinone (PQQ) or auxin, indole-3-acetic acid (IAA) in the absence of pathogens (Choi et al., 2008; Egamberdieva, Wirth, Alqarawi, et al., 2017). For example, a strain of *Pseudomonas putida* promotes chickpea growth through its ability to produce 25.65 µg/mL of IAA at 100µg/mL of L-tryptophan, which contributed to a significant increase in shoot length by 64.1%, root length by 117%, and biomass by 47.4% (Yadav et al., 2010).

Beneficial microbes may produce other hormones that influence plant growth. For example, the production of cytokinins encourages cellular proliferation and differentiation in plants (Schmülling, 2002). *Citrococcus zhacaiensis* exemplifies growth promotion through cytokinin production as its amendment under normal and osmotic stress resulted in the increase of dry root weight by 98.2% and yield by 39.5% in tomato (Selvakumar et al., 2018). In addition, microbes can produce abscisic and salicylic acid, which improve plant responses to stress. Also, the microbial production of gibberellic acid can result in improved lateral shoot growth in plants (P. Ahmad et al., 2011; Egamberdieva, Wirth, Alqarawi, et al., 2017; Mendes et al., 2013; Olszewski et al., 2002; Wilkinson & Davies, 2002). One study shows the effects of gibberellic acid production in one variety of spring wheat amended with either *Azospirillum brasilense* or gibberellic acid. A similar promotion in shoot weight by 32.5% occurred in both treatments relative to the control (R. m. n. Kucey, 1988). Overall, the direct effect of PGPR can produce various phytohormones that influence plant growth.

The application of PGPR capable of siderophore production can either directly or indirectly promote plant growth. Siderophore production may directly affect plant-available micronutrients in the rhizosphere (Choi et al., 2008; Leong, 1986; Scott, 1995). Siderophores are metal-chelating agents produced by microbes that solubilize and sequester inaccessible micronutrients under limiting conditions (Behnsen & Raffatellu, 2016; Schwyn & Neilands, 1987). Primarily, catechol siderophores, including aminochelin, azotochelin, and protochelin, promote iron uptake

in plants because of their high affinity for this micronutrient (Kraepiel et al., 2009). The high affinity of siderophores for iron allows for interaction with mineralized iron, promoting dissolution in soil and plant accessibility. For example, a mutant strain of *Pseudomonas fluorescens* ATCC 13525 that produces siderophores at a 1,500% greater rate than the wild-type increased root and length by 35.4% and 28.8%, respectively, when amended in the mung bean rhizosphere (Katiyar & Goel, 2004). The effects of the mutant strain of *P. fluorescens* ATCC 13525 on mung bean growth exemplify the direct effect of applying siderophore-producing PGPR.

Although the production of siderophores is commonly associated with the increase of plant-available iron in the rhizosphere, these metal-chelating agents are also a means of biocontrol, an indirect means of promoting plant growth. Biocontrol through siderophore production occurs through antibiotic activity or iron chelation, affecting microbial competition and survivability (Shah et al., 1992). For example, the growth of numerous species, including *Azotobacter*, *Pseudomonas*, *Bacillus*, *Rhizobia*, and *Staphylococcus*, were antagonized by the production of siderophores by a strain of *Azospirillum lipoferum* M. In the rhizosphere, siderophore-mediated antagonism of *A. lipoferum* M may indirectly promote plant growth by its subsequent proliferation following the reduction in size of the local microbial community. Its proliferation may benefit plant growth through increases in plant-available nitrogen or iron as this diazotrophic microbe continues producing siderophores.

Furthermore, *Azotobacter* sp. SUB-III AB742373 exemplifies the indirect effects of siderophore-producing PGPR on plant growth. *Azotobacter* sp. SUB-III AB742373 produces catecholate-type siderophores, providing plant-available iron and antimicrobial properties into the rhizosphere. Its siderophore production can inhibit the growth of numerous microbial species like *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Salmonella typhimurium* (Supanekar et al., 2021). This study does not amend *Azotobacter* sp. SUB-III AB742373 into the rhizosphere to observe potential plant growth promotion. However, its antimicrobial effects on well-studied growth promoters like *P. aeruginosa* or *B. subtilis* could indirectly affect plant growth by limiting these bacterial species' positive effects on biofertilization or rhizoremediation (Adesemoye et al., 2008; Islam et al., 2014; S. Pandey et al., 2013).

Several bacterial genera are commonly applied to the rhizosphere, directly or indirectly promoting observable plant growth. For example, *Pseudomonas* and *Bacillus* affect various amounts of phosphate, zinc, and potassium solubilization, iron sequestration, and phytohormone production. *Azotobacter*, *Azospirillum*, and *Cyanobacteria* are free-living diazotrophs that live endophytically in the roots of cereals. Most well known, *Rhizobia* form a symbiotic relationship with legumes that results in high levels of nitrogen fixation within the root nodule (Verma, 2019). In addition, *Rhizobia* promotes phosphate solubilization and phytohormone production

among various crops. Thus, a diverse group of bacterial genera influences the rhizosphere of various crops, which may consequently promote plant growth upon their application.

1.3 PGPR Improves Nutrient Availability: Nitrogen

Nitrogen is a crucial requirement for plant growth and productivity (Mengel et al., 2001). This nutrient is essential for synthesizing plant proteins, chlorophyll, and nucleic acids. Additionally, this nutrient promotes carbohydrate utilization and is a component of enzymes. Nitrogen stimulates root development and activity while supporting the plant's ability to uptake other nutrients (Olson & Kurtz, 1982). Its versatility in function and requirement for high yields rationalizes the relatively high nitrogen requirements for many plants (Pang & Letey, 2000). Indeed, nitrogen availability is the most common limiting nutrient for plant biomass accumulation and growth.

The bacterial influence on nitrogen availability is essential for the growth and productivity of plants. Although nitrogen is usually abundant from sources of atmospheric nitrogen, this nutrient is commonly limited in a form unavailable for plant uptake in the rhizosphere (Warembourg, 1993). Nitrogen may exist within dead organic matter in the rhizosphere, which can only be made accessible through microbial degradation (Lewis & Lewis, 1991). The transformation of nitrogen into a plant-available form occurs through biological nitrogen fixation, which reduces atmospheric nitrogen into ammonia (Teng et al., 2015). Nitrogen as ammonia or nitrate is plant-available since plant roots can uptake these compounds for biological functions. In soil, microorganisms, bacteria and archaea, serve an important role in the nitrogen cycle as diazotrophs through the enzyme responsible for nitrogen fixation, nitrogenase (Young, 1992; Zehr et al., 2003).

Nitrogenase contains an active site consisting of two metalloprotein components, Fe and MoFe protein, which contain metal clusters within the complex (Allen et al., 2019). The Fe protein is a reductase component, while the MoFe protein is the catalytic component of the nitrogenase complex (Y. Hu & Ribbe, 2014). The enzyme requires ATP and an electron donor to catalyze nitrogen fixation (Dixon & Kahn, 2004). The ATP-binding sites are within each subunit of the bridging Fe_4S_4 cluster within the Fe protein. Electron donors indirectly utilize the structure of MoFe protein to reach its FeMo cofactor. The MoFe protein contains Fe_8S_7 at each subunit interface, termed P-cluster. The electron transfer pathway extends from the Fe_4S_4 cluster of the Fe protein to the P-cluster to reach the FeMo cofactor of the MoFe protein. Upon ATP binding and electron transfer, atmospheric nitrogen reduces to ammonia at the active FeMo cofactor (Y. Hu & Ribbe, 2014).

Siderophores of free-living diazotrophs incorporate metals into the nitrogenase complex, Fe, Mo, and V, which serve as cofactors of the enzymatic active site (Castellano-Hinojosa et al., 2016;

Sigel & Sigel, 2002; Zeng et al., 2022). For example, a diazotrophic bacteria, *Azotobacter vinelandii*, produces catecholate siderophores, which results in a sufficient uptake of Mo or V cations to support rapid nitrogen fixation and microbial growth (Bellenger et al., 2008; Wichard et al., 2009). However, diazotrophs residing in legume nodules may rely on the production of siderophores by PGPR to incorporate metallic cofactors into the nodule (Kramer et al., 2020; Sukul et al., 2021). For example, the co-inoculation of *Pseudomonas fluorescens* P-39, a siderophore-producing bacteria, and *Rhizobia sp.* Rb-133 results in significant increases in kidney bean nodulation and nitrogen fixation. An increase in kidney bean nitrogen fixation may result from greater metallic sequestration through siderophore production by *P. flourenscens* P-39, along with the microbe's ability to produce IAA and induce phytoalexins (van Peer, 1991; Yadegari et al., 2010). PGPR significantly influence the growth and productivity of plants when interacting with nodulating *Rhizobia*.

Nodules notably host nitrogen-fixing bacteria because their microaerophilic conditions support the productivity of nitrogenase. In fact, once oxygen levels rise to 10% of the atmospheric level, nitrogenase will be inhibited, and nitrogen-fixing bacteria will no longer grow (Allen et al., 2019). These plant organs provide low oxygen conditions within their interior and exist as spherical, tumor-like plant root organs. Microorganisms perform nitrogen fixation within the nodule. Specifically, these diazotrophs are likely either *Rhizobia* or *Bradyrhizobia*, and initially exist as free-living organisms within the rhizosphere (Oldroyd et al., 2011). These bacteria can exist independently, but if the legume is in low nitrogen conditions, the plant will signal for these diazotrophs to establish a mutualistic relationship (Ohyama et al., 2011). Once legumes signal for compatible nitrogen-fixing bacteria to interact with their root hairs and begin the nodulation process, a symbiotic relationship can achieve greater plant-available nitrogen. Within the nodule, the plant will receive sources of nitrogen, such as glutamate, in exchange for sources of carbon (D. Wang et al., 2012).

Nodules form once flavonoids are secreted into the soil by the legume, which act as signaling molecules for the bacteria. In turn, the bacteria begin interacting with root receptors of the signaling plant. These flavonoids are a part of a large and diverse group of phenolics, which result in an interaction with a specific genus of bacteria dependent on the signaling plant (D. Wang et al., 2012). The flavonoids interact with the NodD protein, and the bacteria release nodulation factors. The nod factors bind to receptors along the plant root. Additionally, bacterial surface polysaccharides bind to plant root receptors. This interaction serves as a cue for the legume, indicating that the correct genera of bacteria are close to the plant (Laranjo et al., 2013).

Root curling begins while plant cortical cells divide to incorporate the surrounding bacteria (Oldroyd et al., 2011). Next, an infection thread forms through the root hair leading the bacteria to the cortex cells. Once the infection thread releases the bacterial cells, the bacteria stop dividing and form bacteroides surrounded by cell membranes. Next, bacterial cytokinins result in

the plant cortical cells dividing (Crespi & Frugier, 2008). Now, a root nodule forms from rapidly dividing cortical cells with bacteroides performing nitrogen fixation mutualistically (Brussel et al., 1992).

Once established, a nodule with low oxygen is maintained by an oxygen diffusion barrier (ODB) and leghemoglobin. The ODB is flexible and responds appropriately to external oxygen concentration changes (Avenhaus et al., 2016). Its response ensures that there is no surplus of oxygen diffusing through the nodule membrane. Additionally, plant leghemoglobin is a compound containing iron for oxygen transport. This compound is responsible for the nodule's healthy red color. Leghemoglobin binds oxygen and transports it to cytochrome oxidases of the bacteroid (Mylona et al., 1995). Also, this compound acts as a buffer to lower the oxygen concentration when the content is relatively high (Appleby, 1984). These mechanisms ensure that the nodule is relatively anaerobic for prokaryotic nitrogen fixation.

A few diazotrophs demand aerobic conditions for cellular metabolism (Z. Dong et al., 2002). The functionality of nitrogenase can still be maintained under aerobic conditions. A surplus of carbon consumption meets the environment's higher concentration of oxygen to decrease the accessibility for oxygen to interact with nitrogenase (Lery et al., 2010). Bacteria growing in a high amount of extracellular polysaccharides can achieve a carbon surplus. These secreted compounds produce a microaerobic environment for bacterial growth within colonies (Lery et al., 2010).

Although PGPR most commonly increases available plant nitrogen through nitrogen fixation within nodules, it is important to emphasize that free-living diazotrophs exist within the rhizosphere. For example, *Azotobacter spp.* and *cyanobacteria* freely fix nitrogen in terrestrial or aquatic systems (DeLuca et al., 1996). In fact, *cyanobacteria* can annually fix about 3-5kg of N/h in cropping systems (Paul, 2014). Free-living diazotrophs, like *Azotobacter*, are most abundant in soil treated with manure, an organic fertilizer (Abd-El-Malek, 1971). Although the fixed nitrogen produced by free-living diazotrophs may not be in as close proximity to the plant as the products of nodule endophytes, free-living diazotrophs are impactful toward the biofertilization of the rhizosphere and the plant.

Regarding the nitrogen cycle, the role of soil microbial communities is not limited to nitrogen fixation. Similar to the diazotrophic function of transforming atmospheric nitrogen to ammonium, a process of ammonification, nitrifying and denitrifying bacteria also influence the plant-available nitrogen in the rhizosphere (Barnard et al., 2005). Nitrifying bacteria transform ammonium, a form of usable nitrogen, into nitrite. *Nitrosomonas* and *Nitrococcus* are some genera of soil bacteria responsible for converting ammonium to nitrite (Fumasoli et al., 2017). At optimal pH, daily chemolithotrophic nitrification occurs at a rate of 0.55 g of N / biomass (Tarre & Green, 2004). As seedlings, forbes, including annual grass and weeds, exhibit inhibitory

effects towards the nitrification of *Nitrosomonas*, which may benefit plant growth during a stage that requires a high amount of nitrogen for development (Rice, 1964). Nitrite is found at very low concentrations relative to nitrate as nitrifying bacteria continue to convert nitrite to nitrate. Bacterial species belonging to the *Nitrobacter* genus are commonly responsible for nitrite oxidation to nitrate (Winkler et al., 2015). Upon plant invasion, observations show that the bacterial community shifts, increasing the relative abundance of nitrifiers, which promotes the success of invasive species through increases in nitrogen turnover (Rodrigues et al., 2015). Alternatively, the application of nitrifiers can promote plant growth through increases in available nitrogen and phosphorus. For example, the treatment of *Nitrobacter*, *Nitrosomonas*, and *Azotobacter* onto tomato seeds promotes height, stem width, and root length (Ibiene et al., 2012).

PGPR are a crucial component in nutrient availability in the rhizosphere. Through stimulated nitrogenase activity and mutualistic interactions, plants have readily available nitrogen (Sulieman & Schulze, 2010). However, the rate of nitrogen fixation will be hindered by phosphorus deficiency, especially in legumes (Chaudhary et al., 2008). Thus, phosphate-solubilizing bacteria in the rhizosphere can provide bioavailable phosphorus to the plant.

1.4 PGPR Improves Nutrient Availability: Phosphorus

Phosphorus (P) is a primary nutrient for plant growth and development; however, it often limits plant growth in cropping soils (Raghothama, 1999). Low phosphorus availability can be a detrimental constraint for crops, which may be a common circumstance in cropping systems as only about 0.1% of phosphorus in soil is readily available for plant use. Its low bioavailability results from its insolubility and fixation to soil (Mahdi et al., 2012).

Plants may experience phosphorus deficiency because of the high concentrations of insoluble organic or inorganic phosphorus in soil. A phosphorus deficiency will limit photosynthesis rates, energy transfer, signal transduction, and macromolecular biosynthesis (Khan et al., 2010). The exemplified cellular functions require this nutrient to synthesize nucleic acids, proteins, sugars, lipids, and adenylate (Z. Zhang et al., 2014). As a result, during phosphorus deficiency, plants may experience significant decreases in root-to-shoot ratio, hydration, and leuco-anthocyanin content (Atkinson, 1973). Since many biological functions are dependent on phosphorus availability, its deficiency hinders plant growth and yield (Hammond & White, 2008).

Solubilization of phosphorus also occurs by interactions between the rhizosphere and bulk soil. Root exudates, including organic acids, sugars, phosphatases, and hydrogen ions, can diffuse into the bulk soil from the rhizosphere. Once present in bulk soil, these compounds acidify the soil. In turn, organic phosphorus is mineralized and solubilized. Upon solubilization, the phosphorus soil solution increases and diffuses into the rhizosphere (Khan et al., 2007). Upon entry to the

rhizosphere, the phosphorus deficiency will be less severe, and phosphorus will be more bioavailable for plant use.

Phosphorus solubilization is a common characteristic among many microorganisms, including bacteria, fungi, actinomycetes, and algae (Sharma et al., 2013). Within the rhizosphere, phosphate solubilizing bacteria account for 1-50% of the microbial community, while phosphate solubilizing fungi account for 0.1%-0.5%. Generally, phosphate-soluble bacteria are 2-150 times more abundant than phosphate-solubilizing fungi in the rhizosphere (Kucey, 2011).

In terms of bacteria, a few genera of PGPR are phosphate-solubilizing microorganisms (PSM). While this characteristic is most apparent in *Pseudomonas*, *Bacillus*, and *Rhizobia*, species belonging to *Enterobacter*, *Chrysemonas*, *Arthrobacter*, *Micrococcus*, and *Penibacillus* exhibit capabilities of phosphate solubilization (Banik & Dey, 1982; Vazquez et al., 2000). Bacteria of the *Pseudomonas*, *Bacillus*, and *Azospirillium* genus commonly secrete gluconic acid, leading to phosphate solubilization (Otieno et al., 2015; Rodriguez et al., 2004; Tahir et al., 2013). The ability of *P. fluorescens* CHA0 to solubilize mineral phosphate is strongly dependent on gluconic acid production (de Werra et al., 2009). The production of organic acids, like gluconic acid, permits a temporary reduction in soil pH, promoting phosphorus solubilization and plant growth. Other species may produce oxalic, lactic, 2-ketogluconic, and succinic acid, making phosphate more available in the rhizosphere (Khan et al., 2007).

Gram-negative PGPR produces organic acids at its periplasm within the oxidation pathway of glucose (Anthony, 2004). The most efficient means of phosphorus solubilization by organic acid is the extracellular oxidation of glucose to gluconic acid (Rodriguez et al., 2000). The enzyme glucose dehydrogenase (GCD), which requires the cofactor pyrroloquinoline quinone (PQQ), biosynthesizes gluconic acid (Sharma et al., 2013). This organic acid efficiently chletes to cations bound to phosphate with its hydroxyl and carboxyl groups, which results in solubilized phosphorus available for plant use (Kpomblekou-A & Tabatabai, 1994).

There are feedback mechanisms for the production of organic acids by microbes. For example, high soluble phosphate in the soil inhibits the microbial glucose oxidation pathway. In turn, microbes do not release phosphorus-solubilizing organic acids. This negative regulation occurs in *E. herbicola*, *R.leguminosarum*, and *S. marcescens* (Ben Farhat et al., 2013; Liu et al., 1992).

Alternatively, organisms, like *B. multivorans*, constitutively rely on the direct oxidative pathway of glucose. Therefore, the method of inhibiting glucose oxidation pathways does not occur as a response to high phosphorus concentrations. However, these organisms are still affected by relatively high concentrations of soluble phosphate through changes in glycerate kinase and 2-oxoglutarate gene expression (Zeng et al., 2017). These enzymes are essential components of the phosphorylative pathway of glucose metabolism (Reher et al., 2006). For species like *B.*

multivorans, feedback mechanisms are practical under low phosphorus conditions. Feedback regulates the expression of glycerate kinase and 2-oxoglutarate genes to prevent the accumulation of surplus phosphorus. In the presence of phosphorus deficiency, these genes upregulate, which increases phosphate solubility by the increase of organic acids produced. Kinase upregulation halts once the environment is no longer deficient in phosphorus (Buch et al., 2008).

It is common for some species to use alternative phosphate solubilization mechanisms rather than releasing organic acids. Although plant growth-promoting microorganisms can secrete organic acids, this may not be energetically favorable. A product of ammonium assimilation or respiration is protons pumped out of the cell, which would consequently acidify the soil (Krishnaraj et al., 1998). For *Pseudomonas,* proton release is more significant from respiration, while members of *Penicillium* and *Azospirillum* have greater proton release following ammonium assimilation (Asea et al., 1988; Carrillo et al., 2002; Park et al., 2009).

Phosphate solubilizing bacteria may increase available phosphorus by releasing enzymes that interact with insoluble phosphorus within soil. Specifically, these phosphomonoesterases, or simply phosphatases, will dephosphorylate phophoster or phosphoanhydride bonds of organic matter (Nannipieri et al., 2011). Phosphatase depends on the soil's pH, as acid or alkaline phosphatase will exist in their corresponding soil pH (Sharma et al., 2013). Plant roots may rarely release alkaline phosphatase, but it has been observed that microbial-produced phosphatase has a greater affinity for phosphate (Tarafdar et al., 2001).

The accumulation of bioavailable phosphorus largely depends on the concentration of microbes within the rhizosphere. PGPR are necessary to release organic acids or enzymes into soil that make phosphorus more available within the rhizosphere. Therefore, PGPR are an essential component for nutrient availability for the two most important plant nutrients, nitrogen and phosphorus.

1.5 PGPR Improves Nutrient Availability: Potassium

The application of PGPR for biofertilization is not limited to increases in available nitrogen or phosphorus, as many studies report improvements in plant potassium content through bacterial potassium solubilization (Esitken et al., 2006; Omara et al., 2017; Zare et al., 2011). The application of diverse potassium solubilizers results in an increase in available potassium, improving plant germination percentage, growth, and yield across numerous crops. The diversity of potassium solubilizing bacteria include members of the α-, β-, and γ-, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* phylum. More relevantly, the γ-, *Proteobacteria* phylum includes the genus *Pseudomonas*, which has shown to improve potassium availability in sorghum, maize, rize, and tea (P. Verma et al., 2017). Bacteria across several genera solubilize

potassium, including *Bacillus* spp., *Enterobacter* sp., *Burkholderia* sp., and *Pseudomonas* sp. (X. Dong et al., 2019; Etesami et al., 2017). Similar to the mechanisms resulting in phosphorus solubilization, microbial organic acid production increases potassium in the soil solution. (Keshavarz Zarjani et al., 2013; Saiyad et al., 2015).

Potassium (K) is one of three primary macronutrients necessary for plant growth; however, only 1-2% of it is readily available for plant uptake (D. l. Sparks & Huang, 1985). Its role in plant functionality results from about 80 enzymes that use potassium as a cofactor to catalyze plant processes like starch synthesis, sugar metabolism, nitrate reduction, and photosynthesis (Almeida et al., 2015; Hussain et al., 2016). Additionally, potassium is crucial for plant resistance against herbivory and abiotic stress like low temperatures or drought (Kaur et al., 2021). Its involvement in water regulation promotes drought resistance (Oosterhuis et al., 2014). Ultimately, this nutrient stimulates flowing, promoting yield (P. Pal & Ghosh, 2010).

In addition to potassium in soil solution, sources of potassium available for plant uptake are in either a mineral, exchangeable, or non-exchangeable phase (Etesami et al., 2017). Mineral potassium is often unavailable or non-exchangeable as it exists within insoluble minerals of soil. It is slowly available to plants depending on several factors, including the level of potassium in soil solution or in different mineral phases (D. L. Sparks, 1987). Non-exchangeable, insoluble sources of potassium include zeolites, vermiculite minerals, potassium taranakite, potassium alunite, and certain aluminosilicate, which account for 1 to 10% of soil potassium (D. l. Sparks & Huang, 1985).

Alternatively, other phases of soil potassium are more accessible to plants. Exchangeable potassium is more accessible to plants than other potassium phases because the negative charges of organic matter hold exchangeable potassium to clay minerals (D. L. Sparks, 1987). Cation ion exchange desorbs potassium from minerals. Specifically, th H^+ ions disorbs potassium into soil solution from exchangeable sources containing Mg^{2+}, Ca^{2+}, or Mn^{2+} through the cation-exchange complex in soil (Huang et al., 2013). Thus, a greater cation exchange capacity in the soil promotes the availability of exchangeable potassium for plant use (Huang et al., 2013; Mukherjee, 2022). The sources of exchangeable potassium include organic matter, clay minerals, and sesquioxides (D. l. Sparks & Huang, 1985).

Similarly, micas, potassium-feldspars, and other potassium-bearing minerals are sources of non-exchangeable potassium. Potassium solubilizing bacteria lower soil pH by releasing organic acids that solubilize non-exchangeable minerals into soil solution. The produced organic acids chelate various cations associated with non-exchangeable phases of potassium, ultimately allowing the nutrient to enter soil solution. Sources of potassium containing Si^{4+}, Al^{3+}, Fe^{2+}, and Ca^{2+} are commonly affected by chelating (Meena et al., 2014). In comparison, non-exchangeable phases of potassium become readily available to plants under different conditions

than exchangeable phases because the soil cation exchange capacity decreases as soil acidifies (Mukherjee, 2022).

Potassium-solubilizing bacteria acidify the soil by secreting byproducts like tartaric, malic, a-ketogluconic succinic, and glycolic acid to release non-exchangeable potassium from minerals (Keshavarz Zarjani et al., 2013; Meena et al., 2014; Prajapati & Modi, 2012; P. Verma et al., 2017). Studies more commonly report that citric and oxalic acid production produces solubilized potassium (Prajapati & Modi, 2012). For example, the known production of oxalate acid by *Bacillus edaphicus* NBT results in increased potassium uptake and growth in cotton plants (X. F. Sheng, 2005; X. Sheng & Huang, 2002).

Bacterial proximity with sources of potassium can achieve greater rates of potassium solubilization. Extracellular polymers, including proteins and polysaccharides, release potassium from minerals in the soil through microbial attachment. The microbial exopolysaccharide attachment releases a high concentration of organic acids to interact with potassium minerals, allowing for increased mineral dissolution (W. Liu et al., 2006). The interaction between microbial exopolysaccharides and bytownite exemplifies potassium desorption through microbial attachment. Exopolysaccharide production on bytownite increases its dissolution compared to controls because organic compounds catalyze bytownite dissolution by producing potassium-organic complexes at the mineral surface that weaken potassium-oxygen bonds with the mineral (Welch & Vandevivere, 1994).

Chelated compounds comprise the previously described complexes formed by microbial attachment through exopolysaccharide production (M. Ahmad et al., 2016). These compounds result from bacterial acidolysis upon interactions with potassium minerals, including illite and feldspar (Römheld & Kirkby, 2010; X. F. Sheng & He, 2006). As a result, these complexes form strong bonds with potassium or the mineral itself (Uroz et al., 2009). In turn, the chelated compounds form potassium complexes, increasing mineral dissolution rates and its availability in soil solution.

The bacterial mechanisms responsible for potassium solubilization consequently affect plant growth. For example, the amendment of two strains of *Burechulderia glathei*, PMB (7) and PML1 (12), to pine roots significantly affected the plant's root morphology by releasing potassium from mineral sources (Calvaruso et al., 2006). The application of PGPR co-inoculations show improvements in plant available potassium, resulting in plant growth promotion. A study exemplifies the application of solubilizing bacteria by creating a consortium comprising of two potassium-solubilizing bacteria, *Bacillus mucilaginous* and *Bacillus subtilis*, and one strain of *Bacillus megaterium* var. *phosphaticum*, capable of both potassium and phosphorus solubilization. Upon treating rice plants with this consortium and mineralized potassium and phosphorus, there were significant increases in shoot and root growth through

increases in potassium and phosphorus availability and uptake (Abou-el-Seoud & Abdel-Megeed, 2012).

There are fewer reports of effective potassium solubilizing PGPR in the literature compared to nitrogen and phosphorus (G. Gupta et al., 2015; Schutz et al., 2018). Lugtenberg claims PGPR, including potassium-solubilizers, may not beneficially affect the plant upon application because of insufficient rhizosphere colonization (B. J. J. Lugtenberg et al., 2001). The amendment of *B. mucliaginous* MCRCp1 to the rhizosphere resulted in increased proliferation by 10,000 fold after 90 days compared to the controls; however, few studies have reported adequate proliferation and survivability of potassium-solubilizing bacteria in soil (Pachaiyappan & Janarthanam, 2007).

1.6 *Pseudomonas* Interactions with Soybean and *Bradyrhizobium*

The numerous mechanisms of the *Pseudomonas* genus that result in improved biofertilization, phytohormone production, and biocontrol support their classification as plant-growth promoting rhizobacteria. These observations are consistent with the characterization of *Pseudomonas* growth promoters in the soybean rhizosphere. For example, one study isolated 115 *Pseudomonas spp.* from the soybean rhizosphere to were then screened for phytostimulation and biocontrol capabilities. This study observed 113 isolates able to produce IAA, 62 phosphate solubilizers, and 17 isolates capable of promoting seed germination (Wahyudi, 2011).

Studies show that these functions translate into observable soybean growth promotion. For example, inoculating *Pseudomonas koreensis* KT2440 onto soybean seed subjected to saline stress significantly promotes plant vigor, height, and root length relative to uninoculated controls. The study speculates attributing observed growth promotion to the strain's ability to produce IAA, solubilize phosphate, and produce siderophores (Costa-Gutierrez et al., 2020). Furthermore, an additional study bacterized soybean seed with fluorescent *Pseudomonas* sp. RBT 13, significantly increased shoot and root length, biomass, and yield. The study considers that observed siderophore production is a possible means of soybean growth promotion by the applied microbe (B. S. D. Kumar & Dube, 1992). Similarly, applying fluorescent *Pseudomonas* sp. FP1 and FP2, which can produce IAA, gibberellins, siderophores, and exopolysaccharides, can significantly increase soybean germination and shoot length (Sayyed et al., 2007).

With a trend in *Pseudomonas* significantly influencing the growth of soybean, it is necessary to recognize the interaction between *Pseudomonas* and *Bradyrhizobia*, the genus responsible for soybean nodulation (D. Wang et al., 2012). A meta-analysis using 42 studies from 13 countries observed that the co-inoculation of these two genera significantly increases nodule biomass and root mass for soybean. In fact, of the 16 genera analyzed, *Pseudomonas* was the only genus responsible for significant increases in those growth parameters (Zeffa et al., 2020). Indeed, the

effects of *Pseudomonas* and *Bradyrhizobia* on soybean nodulation and root mass justify their application in consortia.

The effects of an applied co-inoculation of *Pseudomonas* and *Bradyrhizobia* on nodulation often translate to significant increases in soybean nitrogen content. For example, the co-inoculation of *P. aeruginosa* LSE-2 and *Bradyrhizobium* LSBR-3 results in a significant increase in soybean height, nodulation, nodule biomass, and shoot dry weight during its flowering stage. This co-inoculation results in a 17.9% increase in soybean shoot nitrogen content (K. C. Kumawat et al., 2019). These results are consistent with the effects of *Pseudomonas* sp. 54RB and *Bradyrhizobia* sp TAL 337 amendment, which significantly increases soybean nodulation and seed nitrogen content compared to the controls (Afzal et al., 2010)

Arguably, there is more literature documentation on the effects of co-inoculating *Pseudomonas putida* and *Bradyrhizobium* compared to other *Pseudomonas* species. For example, a dual inoculation of *P. putida* SP21 or SP22 with *B, japonicum* TIIIB results in significant increases in soybean nodulation and biomass (Rosas et al., 2006). Additionally, the co-inoculation of *P. putida* TSAU 1 and *B. japonicum* USDA 110 significantly affects nodulation and results in a 19% increase in soybean shoot mass relative to non-amended plants. In addition, following the application of co-inoculation, this study observed significant altercations in root architecture by quantifying significant increases in total root length, surface area, diameter, and volume (Jabborova et al., 2018). Furthermore, a separate study shows that *P. putida* TSAU1 significantly increases soybean growth under salt stress when co-inoculated with *B. japonicum* USDA 110. The co-inoculation alleviated salt stress by significantly increasing the shoot mass by 35% and root mass by 20%. The ability of *P. putida* TSAU1 to solubilize phosphorus in a medium containing 200mM NaCl may be an attribution for observed plant growth promotion (Egamberdieva, Wirth, Jabborova, et al., 2017).

Ten days following the co-inoculation of *B. japonicum* A107 and *P. fluorescens* 2137 or WCS365 to the rhizosphere, the population density of *B. japonicum* was significantly greater from both co-inoculations than its isolated population density. From a *Pseudomonas* perspective, the population density of 2137 ten days after co-inoculation was significantly lower than its population density following an isolated amendment; however, the population density of WCS365 was not significantly affected by co-inoculation (Chebotar et al., 2001). Therefore, the co-inoculation of PGPR and *Bradyrhizobia* may offer further effects on soybean growth promotion compared to their respective isolated effects; however, microbial interactions within the rhizosphere affect the inoculations' survivability, and considering a consortia's compatibility or survivability may support their practical application.

Section 2: Experimental Introduction, Justification, and Background

Few plant growth-promoting rhizobacteria associate with defined organs of the plant. Instead, microbes may often translocate to other sites, resulting in biochemical, physiological, and morphological changes that contribute to plant growth and development (Hayat et al., 2012; Hayat & Ali, 2010). For example, applying *Pseudomonas putida* to the rhizosphere often results in changes in plant-root architecture (Hall et al., 1996; Matilla et al., 2010; Patten & Glick, 2002). Alternatively, *Azotobacter, Azospirillum, Bacillus, and Paenibacillus* are associated with nitrogen fixation because these microbes possess a *nif* gene cluster responsible for the nitrogen fixation enzyme, nitrogenase (Goswami et al., 2016; Seldin et al., 1984). However, these microbes are not often associated with the nodule.

Rhizobiaceae induces nodulation through a mutualistic interaction with legumes (Mahmud et al., 2020; Martinez-Hidalgo & Hirsch, 2017). *Rhizobiaceae* resides in the nodule as a primary nitrogen fixer, exchanging usable nitrogen sources for carbohydrates (Elhady et al., 2020). *Bradyrhizobiaceae* is associated explicitly with soybean nodulation (Jaiswal & Dakora, 2019; Stacey, 1995). Despite numerous non-rhizobial PGPR with the *nif* gene cluster, few observations show the incorporation of these microbes into the nodule interior (Dhole et al., 2016; Masson-Boivin et al., 2009). Thus, the nodule interior microbiome was explored across numerous soybean cultivars to observe families and genera that may also have high abundance in the nodule.

Pseudomonadaceae were found in relatively high abundances in the nodules of some cultivars of soybean. How these bacteria invade and survive in nodules, and how they function in nodules is not known. Invasion by this genus may result in plant-growth promotion or consequential loss of plant resources. For clarification, the residency of *Pseudomonas* in soybean nodules subjects the genus to available carbohydrates provided by the plant; however, if these bacteria cannot reciprocate with resources, then the relationship is costly to the plant. Indeed, *Bradyrhizobium* reciprocates by providing usable nitrogen in exchange for plant-derived carbohydrates. Microbes unable to reciprocate are cheaters, an ecological term classifying microbes that may benefit as a third party residing in an environment that depends on mutualistic interactions (Jones et al., 2015; P. Smith & Schuster, 2019).

Genotypes belonging to several vaired microbial genera classify as ecological cheaters. For example, *Pseudomonas fluorescens* form biofilms, which promote adhesion, cohesion, cellular communication, and metabolite exchange; however, cheater genotypes unable to form biofilms independently incorporate themselves into the formation and receive benefits without cost (Rainey & Rainey, 2003; Santos et al., 2018). Similarly, *Saccharomyces cerevisiae*, fungi known as brewer's yeast, excrete extracellular enzymes for nutrient acquisition, potentially providing nutrients for bacterial cheater that cannot release similar enzymes (Greig & Travisano, 2004). Surprisingly, *Rhizobia* genotypes may exemplify cheating by limiting nitrogen fixation rates in

the nodule to allocate energy for reproduction. However, *Rhizobia* that are inefficient in nitrogen fixation may meet sanctions from the host plant, resulting in a loss of carbohydrate input (Oono et al., 2009). In the short term, natural selection favors cheaters (Gano-Cohen et al., 2019; Travisano & Velicer, 2004). Thus, *Pseudomonads* may reside in the nodules to gain carbohydrates from soybean without providing nutrients.

Alternatively, observations show that the presence of *Pseudomonas* in the nodule may benefit the plant. For example, *Pseudomonas* rely on quorum sensing, allowing members of the genus to estimate population density and express genes based on their surroundings accordingly (Venturi, 2006). Quorum sensing may be advantageous as *Pseudomonads* express genes necessary to carry out beneficial functions in the nodule, including exoprotease, auxin, and siderophore production (Castañeda-Tamez et al., 2018). Additionally, the role of *Pseudomonas* in the nodule interior may not be limited to nutrient acquisition or phytohormone production. For example, *Pseudomonas* species can reduce the number of ineffective nodules in lotus plants by antagonizing cheater *Rhizobia* (Crosbie et al., 2022).

To further understand the role of *Pseudomonadaceae* in soybean nodules, a sample of nodules from an individual soybean plant was collected and surface sterilized. The soybean nodule interior microbiome was extracted onto KBC media, a King's B media modification. KBC media is semi-selective for *Pseudomonas*, resulting in numerous *Pseudomonas* colonies exhibiting growth with distinct morphological differences (Mohan & Schaad, 1987). Following extraction, four isolated colonies were selected and applied to the soybean rhizosphere in a greenhouse to evaluate their effects on soybean growth (Griggs et al., 2022).

The whole genome of each isolate was sequenced following their application into the rhizosphere. The genomic sequences of all isolates were identical at a species level; however, records do not indicate a taxonomic match to any known species of *Pseudomonas*. NCBI genome records show the closest known taxonomic relative to these isolates is *Pseudomonas* azotoformans LMG 21611. This strain originates as a rice plant endophyte and shares a 96% match with the isolates applied in the greenhouse study (Hesse et al., 2018). Thus, it was determined that these *Pseudomonads* appear to belong to a novel species. After observing morphological differences among isolates, and significant differences within their average nucleotide identity (ANI), these isolates are considered to be four distinct strains of the same species.

The strains were temporarily named to recognize their colony morphology. Pancake was named after its similar appearance to batter on a skillet, Bullseye for its center ring that resembles a dart board, Starfish which appears to be surrounded by an extracellular matrix, and Jellyfish for its globular appearance. Now, with more information obtained regarding their growth-promoting potential and genomic sequence, these members are referred to as strains of a novel species of

Pseudomonas spp.*:* PAMW1, BUMW2, STMW3, and JEMW4, respectively (*Figure 2.0.1; 2.0.2)*.

A greenhouse experiment led to observable results in soybean growth promotion. Observations show that PAMW1 was the most effective growth promoter in soybean. The interaction between one cultivar and PAMW1 resulted in a significant increase in root length by 59%. Additionally, the interaction between PAMW1 and another soybean cultivar significantly increased nodule mass by 141% and individual nodule mass by 146% (Griggs et al., 2022).

Following greenhouse results indicating that these novel strains are potential soybean growth promoters, there is an inquiry regarding the effects of PAMW1 on soybean growth in a setting that resembles soybean cultivation. It is hypothesized that soybean growth promotion occurs upon amending *Pseudomonas* strains in a field setting, consistent with the previous greenhouse study. A greenhouse setting may limit plant growth because of potting effects that restrict root growth (NeSmith & Duval, 1998). Thus, moving the experiment to a field setting reduces restrictions in growth, which may increase variance in growth promotion by *Pseudomonas.* Additionally, replicating the study in a setting more like that of a farm is consistent with the roadmap to PGPR development and commercialization (Backer et al., 2018).

It is important to emphasize that these strains were derived from a nodule sample from an individual soybean plant because there is a possibility that these strains initially interacted in the nodule. This scenario justifies testing the growth-promoting potential of these isolates by applying a mixed culture to the soybean rhizosphere. A mixed culture would more closely resemble their original environmental interactions. Conserving these interactions could potentially result in additive or synergistic effects in soybean growth because of differences in each strain's PGPR functions or secondary metabolite profiles. Here, it is hypothesized that a mixed culture amendment will offer synergistic or additive effects toward soybean growth promotion compared to individual bacterial strains.

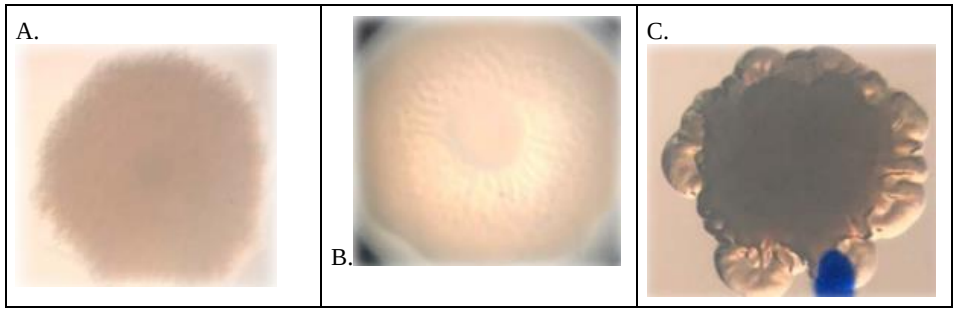

Figure 2.0.1: Colony morphologies of the strains applied to the rhizosphere of greenhouse soybean: (A.) "Pancake" PAMW1, (B.) "Bullseye" BUMW2, (C.) "Starfish" STMW3 grown on modified LB agar (blue mark is permanent marker on the outside of the plate, not part of the colony).

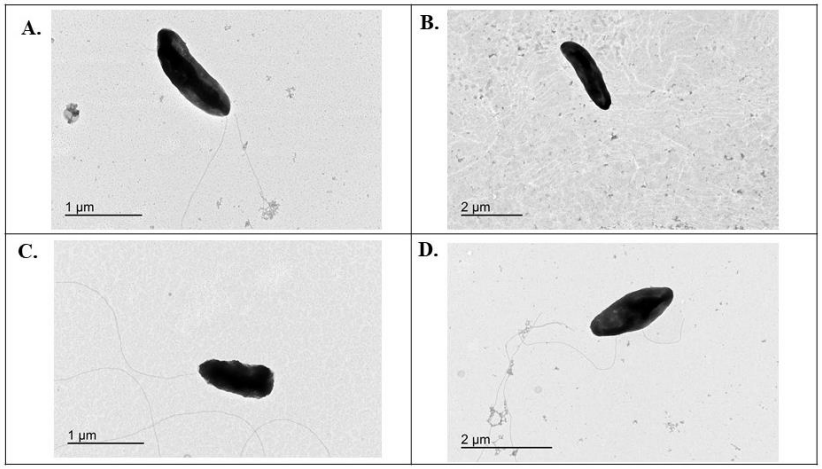

Figure 2.0.2: Negative stained transmission electron microscopy (TEM) images of Pseudomonas spp. strains: (A.) PAMW1, (B.) BUMW2, (C.) STMW3, (D.) JEMW4. These images were produced using a carbon formvar grid and 3% w/v ethanolic uranyl acetate stain with a 90s incubation period. Microscopy was performed in collaboration with Dr. Nathalie del Pilar Becerra Mora of the Virginia Tech MedVet Institute.

Section 3: Materials and Methods

3.1 Soybean Planting and Establishing Plot Conditions

Our field study took place at Virginia Tech's Kentland Farms in Blacksburg, VA (37.19919634299051, -80.57980415155241). The experimental plot, 9.75 m x 13.72 m (32 ft x 45 ft) was established on Unison and Braddock soil (Fine, mixed, semiactive, mesic Typic Hapludults) with a 2-7% slope (*Web Soil Survey*). The plot experienced normal temperatures and rainfall compared to previous growing seasons at this location.

Two soybean cultivars were planted for this experiment, Asgrow AG46X6 and Pioneer P48A60X. These cultivars have a relative maturity group rating of 4.6 and 4.8, respectively. This rating is most appropriate for Southwest Virginia's day length as maturity groups account for the photosensitivity of the cultivar (Major et al., 1975). Furthermore, these cultivars were selected for this study because they are representative of cultivars with high *Pseudomonaceae* abundancy within their nodule interior (Sharaf et al., 2019).

Experimental soybean plants (60 per cultivar) were randomly assigned 120 sites on the plot. The randomization for this first treatment level was created using Excel. A random number was assigned to each of the 60 Asgrow plants and 60 Pioneer plants. Each assigned number was organized from least to greatestand used to designate a plant's location on the plot. For example, the plant with the lowest assigned number was planted on the first available site on the plot, while the plant with the greatest assigned number was planted on the last available site. As a result, 120 soybeans were randomly distributed throughout the plot, preventing the influence of location on the cultivar treatment level.

On June 10, 2020, soybean seeds were planted 76.2 cm (2.5 ft) apart in all directions. Each site received three seeds to ensure there would be 120 experimental soybean plants. The seeds were planted about 2.5cm (1in) deep. Once the seed was placed, soil was returned to the hole and lightly compacted. Two borders of soybean plants were added, surrounding the experimental plants. The borders, or edge plants, were spaced in the same manner as the experimental plants. Adding edge plants ensures equal competition between experimental plants in all directions.

On June 26, 2020, the emergence of each experimental site was evaluated. For sites that had two or more emerged seedlings, surplus plants were pinched off so that only one remained in the site. In the event that a site had no emergence, an extra plant belonging to the same cultivar was transplanted to the site without emergence. Twelve sites required transplants (6 per cultivar). A trowel was used to move a plant to a site without emergence. The plant and all of the soil within a 3-inch circumference and 6-inch depth of the seedling remained intact and moved from one site

to the other. All plants received water following transplants and each site that received a transplant was documented.

Upon emergence, soil belonging to a previous summer's soybean plot was added to each site. Soil associated with soybean cultivation may have a high abundance of *Bradyrhizobium*. The addition of soil belonging to a recent soybean plot theoretically ensures that *Bradyrhizobium* is present at each site. The lack of *Bradyrhizobium* would hinder nodulation in soybean.

Soybean plants were allowed a 12-week growing period before harvest on September 5, 2020. For each site, a shovel was placed about a foot away from the plant's stem. The shovel was inserted into the soil slowly to feel for lateral roots. If it felt like the shovel would not damage roots, the shovel was angled at its complete depth to loosen the soil and expose the direction of lateral roots. This also allowed us to understand the depth of the roots. If the soybean would not move from angling the shovel, we were able to continue digging deeper in the same location. After considering the plant's root system, the plant was harvested by digging in areas away from the roots to loosen the plant out of the soil.

Once the plant was removed from the soil, the stem and root were clipped apart at the first lateral root, regardless of length. The roots were placed in sealable storage bags and temporarily stored at 10℃. The shoots were placed in brown bags and dried for 1 week at 37 ℃.

3.2 Bacterial Amendments

PAMW1 and BUMW2 were chosen for this experiment after considering the results of the preliminary greenhouse study regarding each strain's effectiveness in soybean growth promotion. As previously mentioned, the amendment of PAMW1 to soybean resulted in significant nodule mass and root length increases. Therefore, PAMW1 and BUMW2 were the more effective strains of the four original extractants resulting in their inclusion for this field study.

All soybean sites received their bacterial amendments in equal concentrations to avoid a potential confounding factor in the experimental design. The addition of treatments at different cell concentrations may directly affect survivability and colonization. Serial dilutions were performed before amending soybean with their respective bacterial treatments to quantify each strain's growth rate in media. Serial dilutions determine the concentration of cells within each culture by reducing the density of cells over several dilutions. Bottles containing 500mL of LB-Miller liquid media were inoculated with either PAMW1 or BUMW2 and incubated for 48 hours at 28℃. From the original culture, 1 mL was aseptically removed and added to 9 mL of sterile LB-Miller broth, making a 1/10 dilution. Then, 1 mL was aseptically removed from the dilution and added to another tube with 9 mL of sterile LB-Miller broth, making a 1/100 dilution.

Dilutions continued, reaching a 10^{-8} dilution factor. Each dilution was inoculated onto LB-Miller agar plates to record countable growth. The countable growth was related to the dilution factor to determine the concentration of the original culture in colony forming units (CFUs) / mL (Chen et al., 2003).

Once the concentration of culture was determined through serial dilutions, the original cultures were aliquoted into cuvettes and their absorbance at 600 nm wavelength was measured by spectrophotometer (Thermo Scientific, Genesys 20). Each culture's optical density at 600 nm (OD600) was related to itscountable growth and the slope of the relationship was used to determine the culture's concentration at different absorbances. Based on this relationship, cultures were diluted to an equal concentration before treating experimental soybean. Serial dilutions and OD600 measurements were taken for cultures over numerous incubation periods to obtain enough data to create an accurate slope for this relationship (*Figure 3.2.1*).

To amend the soybean with bacterial treatments, bottles containing 500mL of LB-Miller liquid media were inoculated with a single strain and incubated for 48 hours at 28°C. The OD600 was measured for each culture to determine their concentrations and compare one culture's concentration with the other. Dilutions were performed by adding sterile LB-Miller broth to the culture with greater growth; therefore, all cultures were of the same concentration before amending the soybean plants (OD 0.150 +/- 0.001).

Four bacterial treatments were applied: PAMW1, BUMW2, a mix of PAMW1 and BUMW2, and a control. Following dilutions, a mixed culture was made by mixing equal amounts of the PAMW1 and BUMW2 cultures in a sterile bottle. Sterile LB-Miller broth was used as an unamended control to ensure all soybean sites received the same amount of additional nutrients from a bacterial amendment without receiving bacteria.

The bacterial treatments were amended to the soybean plants using a completely randomized design consistent with the random distribution of soybean cultivar. For each bacterial treatment, 15 sites containing Asgrow soybean were randomly selected to receive the same bacterial amendment. The bacterial treatment assignments for the Pioneer cultivar were done similarly. This randomization was performed using separate random number rankings for Asgrow and Pioneer through Excel. The complete experimental design can be visualized in *Figure 3.2.2*.

On June 29th, 2020, each soybean plant received its assigned bacterial treatments. Prior to amending its root zone, the bottle of culture was mixed to ensure homogeneity. Each of the 120 soybean sites was amended with 5mL of culture. The amendments were applied as a drench using a 5mL pipette positioned at the base of each soybean and aimed towards the roots. At the time of the amendment, soybeans were in the VC growth stage. All plants received 0.5L of water after receiving an amendment.

Treatments were reapplied in the same manner on July 10th, 2020. Soybean growth stage was between late V1 and early V2 at the time of the second amendment. This study prioritized adding the bacterial amendment prior to complete nodulation. According to Pioneer, this occurs once soybean reaches R2, the reproductive growth stage representative of full flowering (*Soybean Nodulation*, 2022). The second bacterial amendment was applied between V2 and V3, allowing the incoulumn to interact with the plant during potential peak nodulation. Additionally, it is necessary for the incorporation of the bacterial amendment to occur during early soybean growth stages because nodule activity and nitrogen fixation are restricted between R2 and R5 **(**Lofton & Arnell, 2017).

Two applications may not have beem necessary. Additional bacterial amendments may not be effective if bacteria were already incorporated for nodulation. The double amendment was applied to promote the likelihood that the bacteria of interest were incorporated into the nodule.

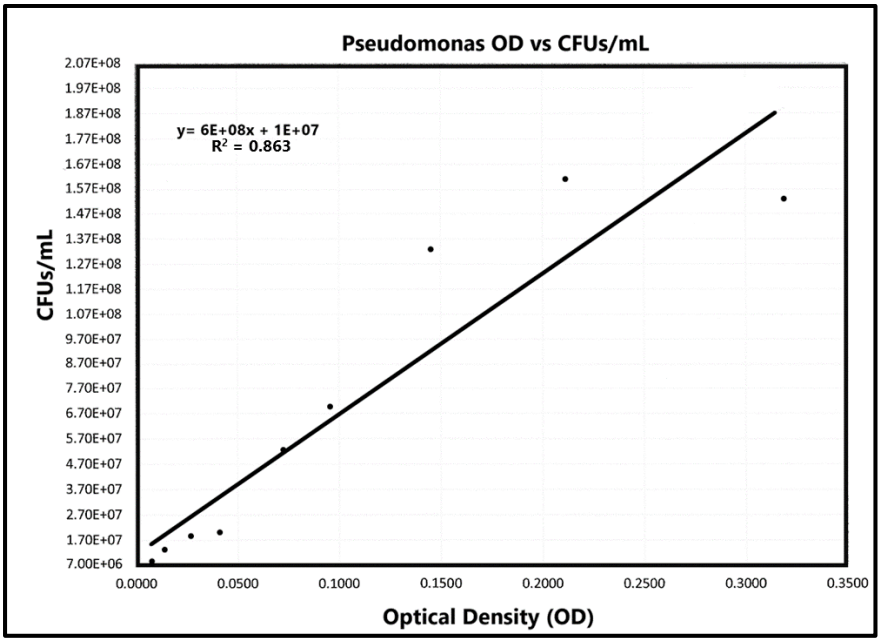

Figure 3.2.1: Determination of Pseudomonas growth by optical density. CFUs/mL were determined by serial dilution and absorbance values were measured by spectrophotometry.

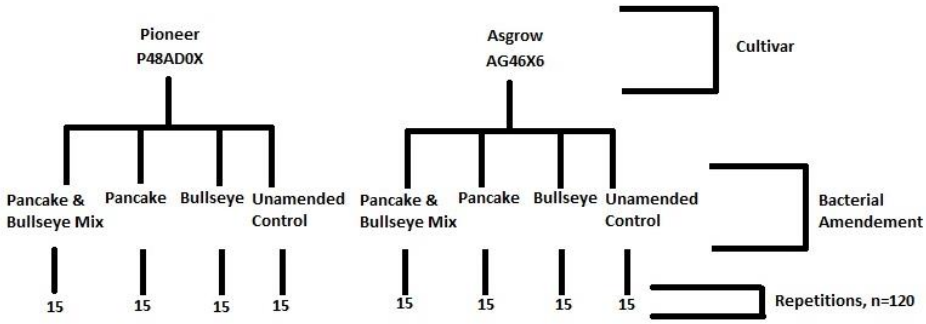

Figure 3.2.2: Experimental design showing the bacterial amendments and repetitions applied to each soybean cultivar. Pancake = PAMW1, Bullseye =BUMW2

3.3 Soybean Growth Measurements

Measurements were taken during various soybean growth stages to monitor differences in growth and productivity among sites. During the 12-week growing period, chlorophyll content, stomatal conductance, and height were measured over three time points. After harvest, the wet and dry mass of the shoots and roots were measured. The nodules were counted and massed for each sample.

A soil plant analysis development (SPAD) meter measures leaf chlorophyll concentration rapidly and non-destructively. This measurement indicates the plant's growth and productivity because SPAD measurements can be related to nitrogen status in the plant's leaf material (Ling et al., 2011). Before making measurements, the growth stages for each plant were assessed. Using this information, the most commonly new and fully matured trifoliate leaf was subjected to a SPAD (Minolta, Osaka, Japan) reading for all experimental soybeans. To clarify, experimental soybeans were approaching V3 on the day of the first SPAD reading, so the second trifoliate leaf was used for measurements across all plants. The SPAD meter was used to measure three locations on the middle leaflet of the chosen trifoliate leaf. These values were averaged to have one reading per plant for each time point.

Porometers are used to measure stomatal conductance, which can be associated with plant growth and productivity. The stomatal control of conductance limits water loss for the plant. The rate of stomatal opening is often dependent on the available water content of the soil. With this metric, the rate of photosynthesis for each plant is better understood (McDermitt, 1990). A trifoliate leaf was chosen for this measurement as described in the previous paragraph. The middle leaf of the chosen trifoliate was used to measure the real-time conductance rates over a

30-second period using a portable unit (Decagon Devices, Pullman, WA, USA); however, only the final measurement was recorded. Porometer readings can be altered by cloud coverage, so measurements were only taken during periods of full sun.

Plant height was measured throughout the growing period to generate a metric that directly relates to plant growth. A tape measure was used to measure from the base of the soybean to the top of its terminal bud. These values were recorded in centimeters for each of the three time points.

Dry mass, length, nodule count, and nodule mass were recorded for each experimental root sample. The roots were washed to remove excess soil while using a sieve to ensure that nodules were not washed away. The nodules were counted and massed, then stored at -20°C. This data was used to determine metrics including average individual nodule mass. Root lengths were measured with a ruler, then the roots were wrapped in aluminum foil and dried for three days at 37°C. Root dry mass was weighed to a hundredth of a gram after drying. Additionally, once the dry shoots were massed, total biomass and shoot-to-root ratios were calculated.

3.4 Nodule Sterilization and Bacterial Extraction

The nodule microbiome of various amended soybean plants was analyzed to determine if the applied inoculum integrated into the nodule. Since the bacterial amendments used for this study were initially extracted from soybean nodules, it was hypothesized that the *Pseudomonas* amendment would colonize the soybean root and infect through interactions with *Bradyrhizobia*, resulting in their nodule incorporation. To test this hypothesis, nodules were surface-sterilized, and the interior microbiome was extracted onto a general media to observe any growth of the applied bacterial amendments or other nodule-associated bacteria. If either PAMW1 or BUMW2 exhibit growth from their respective plant's bacterial extraction, this could address the microbes' survivability in the rhizosphere and support characterizing the isolates as nodule endophytes.

On the day of the harvest, several experimental plants were selected for interior nodule bacterial extraction. Three plants associated with each bacterial treatment were randomly selected for both cultivars. These samples were harvested and subjected to post-harvest measurements before other samples. Once data were collected, nodule samples were surface-sterilized on the day of harvest to ensure the nodules were fresh.

From prior experience, it is difficult to sterilize and extract bacteria from nodules after more extended storage periods. Perhaps -20°C storage results in the dehydration and desiccation of the soybean nodule, despite the addition of glycerol, which aids in preservation. Fresh nodules are often more spherical and lack grooves, while older nodules are often rigid from dryness. Desiccation raises problems during nodule sterilization as it is difficult for sterilizing reagents to

interact with bacteria that may be residing within these grooves. Thus, the surface-sterilization of older nodules often fails, and prioritizing fresh nodules is recommended.

Throughout the literature, methods for nodule surface-sterilization have been inconsistent and often lack citations. Many studies have consistently suggest using ethanol, hydrogen peroxide, hypochlorite, DI water, or saline solution to achieve a sterilized nodule surface; however, the use of these reagents often varies between studies. After considering numerous published methods (Muresu et al., 2008; Sturz et al., 1997; Tokgöz et al., 2020), nodules were first cleaned with 0.9% saline solution, and surface-sterilized using a detergent consisting of 12% *v/v* NaClO, 0.1% *v/v* Tween 80, and sterile DI water.

The primary saline wash removes soil that covers the surface of the nodule. This step is essential to ensure the nodule surface is sterile and clear of soil that covers the nodule. For each sample, 10 nodules of various sizes were selected and transferred into a 2-mL tube. Then, 1.5 mL of saline solution was added to each tube and the samples were vortexed (Vortex-Genie 2, Scientific Industries, Bohemia, NY, USA) for 30 s, with the first 5 s on the high setting and lower intensity for the remaining 25s. Next, the contents of the 2 mL tube were emptied onto a 250µm sieve and washed under a DI water faucet. Finally, the tube was rinsed, and the nodules were returned. The saline wash was performed three times before proceeding into sterile conditions.

The nodules were sterilized under a biosafety cabinet using the previously described detergent. First, the nodules were transferred into sterile 2-mL tubes. Each tube received 1.5 mL of detergent. The tubes were vortexed in the same manner as the saline wash. Next, the contents of each tube were dumped onto individual sterile filter paper in the biosafety cabinet. The nodules were rolled around the filter paper using sterile forceps and returned to their tube. Between samples, the forceps were placed in an incinerator until it appeared red to practice aseptic technique. After returning the nodules to their respective tubes, an additional 1.5 mL of detergent was added to repeat the process two more times. After completing the sterilization process three times, the nodules were transferred into new sterile tubes.

Finally, samples were subjected to three washes using sterile deionized water (SDW) to wash the sterilizing detergent off the nodule. Fresh sterile filter paper replaced the ones used during sterilization to begin washing away any detergent on the nodule surface. The SDW wash was performed by repeating the sterilization process under the biosafety cabinet; however, the detergent was substituted for SDW. Similar to the sterilization process, nodules received an SDW wash three times.

Rolling nodules onto yeast-mannitol agar (YMA) confirmed nodule surface-sterilization. Around 3-4 nodules, depending on their size, were aseptically transferred to a YMA plate. The plate was

closed shut with its lid and shaken by hand. The surface of the nodules rolled around the media, essentially inoculating the plate with any potential bacteria still residing on the nodule following sterilization. Then, the plates were opened, and the nodules were aseptically returned to their tube. The plates were incubated for five days at 28℃ to allow for potential long-growing microorganisms. If there was no visible growth following the incubation period, then the nodules were considered sterilized.

Then, the interior nodule microbiome was extracted from the sterilized nodules. Tubes containing sterile nodules received 1.5mL of sterile 80% glycerol and were crushed inside the tube using a sterile L-shaped rod. Afterward, the tube was lightly vortexed. After crushing and vortexing the nodules, their interior microbiome were released, allowing the microbes to interact with the glycerol. Next, a YMA plate was inoculated with 100µL of glycerol from the vortexed tube and incubated for 3-5 days at 28℃. The incubation period depended on its enumeration, as higher numbers would interfere with long-growing microbes because of the limited room on an agar plate. Additionally, a relatively shorter incubation period favors *Pseudomonas* growth.

After this incubation period, plates were analyzed for *Pseudomonas* based on morphology. Any colony that resembled PAMW1 or BUMW2 was T-streaked onto LBA. The 16s rRNA gene of the colonies were amplified by PCR and sequenced by Virginia Tech's Biocomplexity Institute (Blacksburg, VA, USA). The extract's 16s rRNA gene sequence was compared to the bacterial amendments used in the field experiment. A match to the original amendment suggests that the amendment may have been incorporated and recovered from the nodule.

3.5 Quantitative PGPR Phenotypes

Certain species of the *Pseudomonas* genus are often considered PGPR because of their ability to produce siderophores, organic acids, and phytohormones. Incorporating these compounds into the rhizosphere often increases available plant nutrients and subsequent plant growth promotion. PAMW1 and BUMW2 were grown on various indicator media to quantify the production of these compounds. A microbe with greater production of one of these compounds may promote observable soybean growth (Vasseur-Coronado et al., 2021).

Microbial siderophore production can be detected through a dye complex containing ferric chloride. Prior to adding the dye complex to nutrient-rich media, it is supplemented with hexadecyltrimethylammonium bromide (HDTMA) and chrome azurol S (CAS), which act as indicators upon interaction with siderophores. (Schwyn & Neilands, 1987). The microbial production of a strong iron chelator on this Blue CAS Assay results in iron removal from the dye complex. As a result, there will be an observable change in media color from blue to orange as siderophores are produced in liquid culture.

Numerous studies have quantified the production of siderophores by *Pseudomonas spp.* using liquid Blue CAS assay. (Bagmare et al., 2019; Hussein & Joo, 2014; R. B. Pal & Gokarn, 2010). Blue CAS dye was made by creating a 100mL solution consisting of 0.12% CAS, 0.027% $FeCl_3$-6 H_2O, and 0.18% HDTMA *w/v* (Louden et al., 2011). The dye complex was autoclaved and then added to a sterile nutrient broth. Next, 25-mL aliquots were added to conical tubes and inoculated, making three replications of each isolated strain. The assay was incubated for 24 hrs at 28°C. After incubation, color change was observed. The samples were centrifuged and aliquoted into cuvettes to quantify color change. A comparison between strains was performed through spectrophotometry at a 630 nm wavelength. Siderophore production (psu) was found by subtracting the absorbance of the sample from the absorbance of the reference (CAS solution and uninoculated broth). This value was multiplied by 100 and divided by the absorbance of the reference (Arora & Verma, 2017).

The microbial production of organic acids can be detected using the National Botanical Research Institute's phosphate growth media (NBRIP) (Nautiyal, 1999). Active microbial organic acid production results in a clear halo surrounding colonies. The halo forms as precipitated phosphate dissolves upon interacting with organic acids (Katznelson et al., 1962). First, the two isolates were T-streaked onto a modification of lysogeny broth (LB Miller), a general media containing yeast extract, NaCl, and agar. The two solid cultures were incubated for 24 hrs at 28°C. Next, the NBRIP agar plates were inoculated by transferring cells from a colony grown on LBA with a sterile pipette tip, inoculating a section of the NBRIP media. This method of inoculation results in the growth of a single colony within a quadrant of the selective media. The NBRIP agar plates were incubated for two weeks at 28°C. After observing a halo surrounding the colony, the diameter of the halo was measured. The measurement was divided by the diameter of the colony to quantify organic acid production. Multiple replications were performed for each isolate.

The production of indole-3-acetic acid (IAA) by the *Pseudomonas* strains was determined and quantified using multiple cultures of LB Miller liquid media containing concentrations of L-tryptophan between 0.5 to 2.0mg/mL (Heo et al., 2022). L-tryptophan serves as a precursor toward the synthesis of IAA through the tryptophan-dependent IAA biosynthetic pathway; therefore, manipulating the concentration of this limited resource and assessing the bacterial response can indicates differences in resource use efficiency (RUE) (B. Wang et al., 2015; Woodward & Bartel, 2005). Although RUE is an ecological term suggesting a resource is used efficiently to increase microbial biomass, it can be used loosely here in terms of the application of plant-growth promoters because microbial IAA production can lead to an increase in root biomass, which in turn can enhance bacterial proliferation and colonization (S. Gilbert et al., 2018; Hodapp et al., 2019).

Two 10-mL vials containing sterile LB Miller broth were inoculated with either strain of *Pseudomonas* and incubated for 24 hrs at 28°C to prepare the IAA production assay described by

Heo (Heo et al., 2022). After incubation, the cultures were subjected to spectrophotometry, measuring each culture's absorbance at 600 nm. Next, the cultures were diluted to have similar CFUs/mL. Separately, an aqueous solution of L- tryptophan was vacuum filter-sterilized using Seitz filters with a 0.1µm pore size. The solution was applied to vials containing 10 mL of sterile LB Miller broth, resulting in LB Miller broth ranging between 0.5-1.5% *w/v* L-tryptophan. Then, the vials were inoculated with a diluted culture and incubated for 24h in a shaking incubator (Excella E24 Incubator Shaker Series, New Brunswick Scientific) set for 200 rpm and 28°C. Afterward, 3mL aliquots were taken from the cultures and centrifuged at 13,000 rpm (Centrifuge 5430, FA-45-30-11, Eppendorf, Hamburg) for 10 min at 4°C. After centrifugation, the supernatant was mixed with Salkowski's reagent and left in the dark for 30 minutes. An OD reading at 530 nm was taken on this mixture to quantify IAA production across numerous replications.

3.6 Statistical Analysis

The statistical analyses in our study determine relationships between treatment and soybean growth using recorded response variables. JMP Pro version 16 (SAS Institute Inc., Cary, NC, USA) and PC-ORD version 6 (MJM Software, Gleneden Beach, OR, USA; McCune and Medfford 2011) were used to run univariate and multivariate analyses, respectively. Two-way factorial ANOVAs accompanied by Tukey HSD pairwise comparisons were a univariate approach to observe variance among independent variables. Non-metric multidimensional scaling (NMS) functioned as a multivariate approach. Box plots were used to visualize the results of the univariate approach, while scatter plots were used to visualize multivariate analysis results.

Two-way factorial ANOVA assumes that each response variable follows a Gaussian distribution; however, the distribution of numerous response variables in our study appeared skewed. Nevertheless, appropriately transforming response variables achieved a Gaussian distribution. For example, one dependent variable, nodule mass, had a strong right skew distribution. Therefore, the cube root for each nodule mass data point was determined, transforming the metric to a Gaussian distribution. Distributions were analyzed using a normal quantile plot while considering the position of the mean between the upper and lower 95% mean. *Table 3.6.1* organizes this interpretation, presenting any necessary transformations.

Once all the response variables were in a Gaussian distribution, outliers were assessed. An outlier box plot was created for each response variable, and data points within 1.5x of the interquartile range were flagged as potential outliers. The interquartile range is found through software, subtracting the 25th percentile value from the 75th percentile. If a sample was flagged across multiple response variables, it was considered an outlier and excluded from the data set.

A model was made for each response variable following their transformation. The two independent variables, cultivar and bacterial treatment, were added to the construct model effects. Additionally, a cross between these two independent variables was included to observe potential variation in the interaction between cultivar and bacterial treatment. Although numerous timepoint measurements were recorded throughout the study, only the final timepoints were used in the analysis to preserve statistical independence. The model was set to have an α-value of 0.1. Through this model, the p-value and F-statistic were recorded and interpreted for significant effects by treatment levels.

Upon observing significant differences among samples for a specific response variable, a Tukey HSD test was performed as a means of a hypothesis test; however, a Student T-test was used as a substitute for a Tukey HSD pairwise comparison when analyzing the effects of cultivar. Tukey HSD tests cannot be used to test cultivar because only two cultivars were used in this study. Similarly, a Student T-test was used when analyzing a significant interaction between cultivar and bacterial treatment because a Tukey HSD test was too conservative. These tests analyze the treatment levels through their least square means and standard error to indicate significant differences among samples.

A least square means table indicating that a bacterial treatment level is significantly greater than the control for a given response variable supports our study's first hypothesis. If the univariate analysis shows that samples amended with a mixed culture treatment level are significantly greater than the remaining bacterial amendments, then the model supports our second hypothesis. A least square means plot and box plots visualize these significant differences.

The final timepoints of all response variables were relativized for a multivariate approach (McCune & Grace, 2002). Relativization is suitable for multivariate analyses using a Sorensen similarity distance measure. A distance measure calculates a matrix of distance, or dissimilarity, in a multidimensional space. Sorensen distance measures the shared abundance among samples and divides by the total abundance. This common distance measure is used in non-metric multidimensional scaling, which interprets quantitative ecological data.

NMS is an ordination method suited for nonnormal or discontinuous data, which is appropriate considering missing values in the data. These missing values or discountinous data may occur for a variety of reasons. For example, a sample may have been measured for height, stomatal conductance, and chlorophyll content but experienced severe herbivory shortly afterward. In this example, the shoot mass would be omitted from the dataset, while root data could still be collected. Ultimately, NMS ordination considers all recorded response variables to rank overall soybean growth and productivity, translated by varying distances between ordinates.

It is necessary to determine the appropriate dimensionality to analyze data through NMS. First, a six-dimensional preliminary run was performed on the relativized dataset. The dimensions were then reduced to a single-dimensional solution to observe changes in stress. Stress is the departure from monotonicity in a distance matrix. The preliminary run was performed using an instability criterion of 0.005 and 200 iterations. The data were run under these conditions ten times, followed by 20 runs using randomized data to provide a basis for a randomization test. Significance at each dimension is acquired by a randomization test, which is necessary to evaluate the strength of the patterns in the dataset.

A Monte Carlo randomization test was performed simultaneously with the preliminary runs. The Monte Carlo test ensures that the number of axes or dimensions used in the final solution was not obtained by random chance. Similarly, a scree plot was used to evaluate improvement in fit as more dimensions were added to the analysis. A scree plot visualizes the final stress against the number of dimensions in the analysis. Using the scree plot, the number of dimensions that resulted in low final stress was considered the best fit and most stable model. Ultimately, the final model included the highest number of dimensions that resulted in minimal stress reduction upon adding each dimension.

A final NMS rerun was performed using the appropriate number of dimensions. The rerun is performed once without randomized runs or step-downs in dimensionality. The ordination was then visualized using a scatter plot. NMS was performed on data filtered by cultivar, as the univariate analysis indicated that the bacterial amendments behave differently based on cultivar for some response variables. As a result, two NMS ordinations were created to account for significant differences through cultivar effects and cultivar-bacterial interactions.

Response Variable	Distribution	Transformation
Chlorophyll Content	Gaussian	None
Stomatal Conductance	Gaussian	None
Height	Gaussian	None
Nodules Per Plant	Strong Right-Skew	Cube Root
Nodule Mass	Strong Right-Skew	Cube Root
Shoot Dry Mass	Weak Right-Skew	Square Root
Root Dry Mass	Strong Right-Skew	Cube Root
Total Dry Mass	Weak Right-Skew	Square Root
Shoot/Root Mass Ratio	Weak Right-Skew	Square Root

Table 3.6.1: The recorded or calculated response variable followed by its distribution and performed transformation to achieve a Gaussian distribution.

Section 4: Results

4.1 Univariate Analysis of Soybean Growth and Productivity

The univariate analysis consisted of 2-way factorial ANOVAs, utilizing the final time point for each measurement of soybean growth and productivity as dependent variables within a parametric construct. These dependent variables were individually added into each model as construct effects, including the cultivar of soybean, its applied bacterial amendment, and their interaction. The results of the ANOVA indicated significant differences among samples by the applied treatments using an α-value of 0.1 for each dependent variable. In turn, a p-value < 0.1 suggests a statistically significant difference between means was calculated for each response variable.*(Table 4.1.1)*. The least square mean values and standard errors for each independent and dependent variable are reported on *Table 8.0.3*.

Pairwise comparisons by Student T-tests indicated that the Asgrow cultivar had higher values than Pioneer across the three significant response variables. The following response variables were significantly different between cultivars: chlorophyll content (SPAD), nodules per plant, and nodule mass. Using the mean provided by the least square means table, these response variables were 4%, 21.4%, and 19.7% higher for Asgrow samples, respectively. The least square means plot agreed with the results of the Student T-test, which can be visualized through the boxplots in *Figure 4.1.1*.

The results of the 2-way factorial ANOVA suggest that there are significant differences by bacterial amendment for soybean stomatal conductance, height, shoot dry mass, and biomass (*Table 4.1.1*). These observed variances consider the bacterial effect across both cultivars. Although significant differences by cultivar have been observed in chlorophyll content, nodule count, and nodule mass, the response variables affected by bacterial amendments varied little between cultivars.

A significant cultivar-bacterial interaction for stomatal conductance indicates that the bacterial treatments affect this response variable differently depending on the cultivar it was applied to (*Table 4.1.1*). The results of the Tukey HSD pairwise comparison indicate that none of the bacterial treatments were significantly greater than the control when accounting for cultivar; nonetheless, the effects were still varied by cultivar (*Graph D Figure 4.1.*3). Asgrow samples associated with a PAMW1 amendment were significantly greater than Pioneer samples associated with the same isolate by 29.5%. The remaining bacterial treatments were relatively consistent across cultivars in terms of variance in stomatal conductance.

The pairwise comparisons by Tukey HSD testing indicate that PAMW1 offers significant promotion in soybean height, shoot mass, and biomass. The box plots in *Figure 4.1.2* show this pairwise comparisons through compact letter display. The observed growth promotion for these response variables is relatively high in PAMW1 amended samples. Relative to the control, samples associated with PAMW1 were 18.2%, 22.9%, and 22.5% greater in height, shoot mass, and biomass respectively across cultivars. This substantial promotion in biomass accounts for variance in shoot and root mass.

Although the results of the 2-way factorial ANOVA suggest that PAMW1 is the dominant soybean growth promoter in this study, the mixed culture amendment is contributed some significant variation. Similar to PAMW1, the Tukey HSD pairwise comparison indicates that this treatment significantly increased biomass across cultivars relative to the control (*Figure 4.1.2*). The mean value of biomass measurements for samples associated with the mixed culture amendment was 21.9% greater than control samples. This is consistent with the effect of a mixed culture amendment on the shoot and root mass, which was 19.5% and 14.6% greater than the

control, although insignificant. Additionally, a noteworthy yet statistically insignificant, variance in height was observed as the mixed culture amended samples were 16.8% taller than the control.

The univariate approach allowed for the pairwise comparisons between the mixed amendment and the other applied bacterial treatments for each response variable. Although this analysis did not show significant differences between mixed and isolated culture amended samples, some response variables tended to be greater in samples associated with a mixed culture. When analyzing the differences in nodule count and mass, the mixed culture amended samples were 9.9% and 14.0% greater than samples amended with PAMW1. This is rather noteworthy as samples amended with isolated cultures were not greater than the control for those two response variables. Simply, the mixed culture amendment was the only bacterial treatment associated with samples that have greater values than the control for these response variables.

The isolated BUMW2 amendment resulted in significant differences in soybean growth. Amending samples with BUMW2 resulted in significant shoot mass increses relative to the control. The pairwise comparison by Tukey HSD indicated that the isolate had similar effects on shoot mass as PAMW1, which is shown in *Graph C Figure 4.1.2*. The shoot mass of samples that received a BUMW2 amendment was 19.0% more massive than the control. Samples amended with BUMW2 tended to have the highest measurements in stomatal conductance among all bacterial treatments. This trend contributed to a significant bacterial effect for this response variable indicated by ANOVA (*Graph D Figure 4.1.2*). The box plot shows that BUMW2 amended samples had significantly greater stomatal conductance relative to samples amended with a mixed culture.

Response Variable	**Transformation**	**Cultivar Variance: *p*-values**	**Bacteria Variance: *p*-values**	**Cultivar-Bacteria Interaction: *p*-values**
Chlorophyll Content	None	0.045*	0.691	0.298
Stomatal Conductance	None	0.955	0.074*	0.049*
Height	None	0.514	0.087*	0.139
Nodules Per Plant	Cube Root	0.079*	0.481	0.866
Nodule Mass	Cube Root	0.074*	0.425	0.977
Shoot Dry Mass	Square Root	0.969	0.028*	0.737
Root Dry Mass	Cube Root	0.636	0.111	0.424
Total Dry Mass	Square Root	0.817	0.022*	0.638
Shoot/Root Mass Ratio	Square Root	0.221	0.778	0.187

Table 4.1.1: Two-way factorial ANOVA analysis showing the effects of treatments on indices of soybean growth and productivity. Columns indicate each response (dependent) variable, the transformation performed to achieve a Gaussian distribution, and the reported p-value for each treatment factor level in the model. (*) Indicates $p < 0.1$.

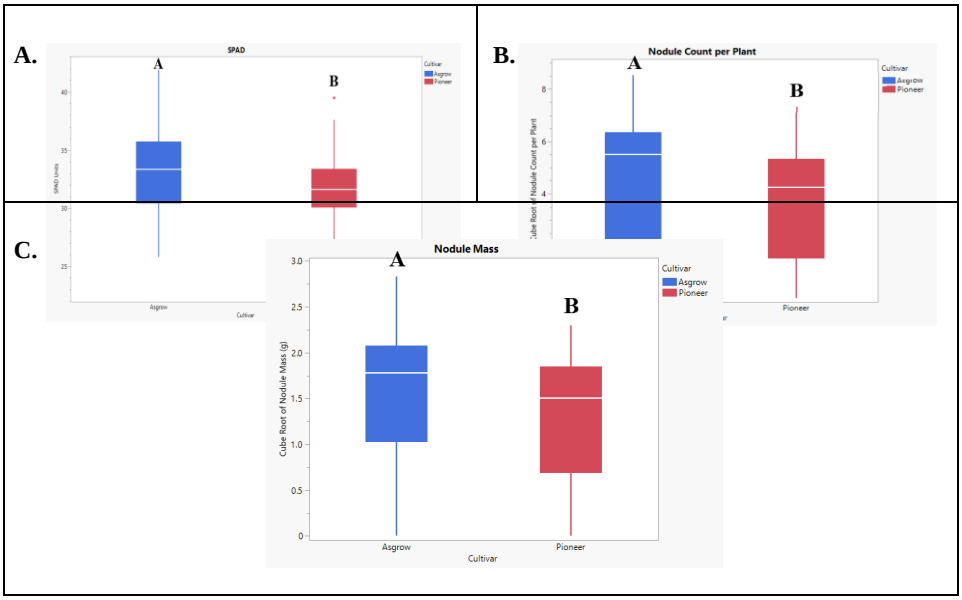

Figure 4.1.1: Boxplots visualize the significant differences in (A.) chlorophyll content (SPAD), (B.) Nodule Count per Plant, (C.) Nodule mass by cultivar effect. Significant differences between cultivars are determined by Student's T-test pairwise comparison and indicated by compact letter display above each box.

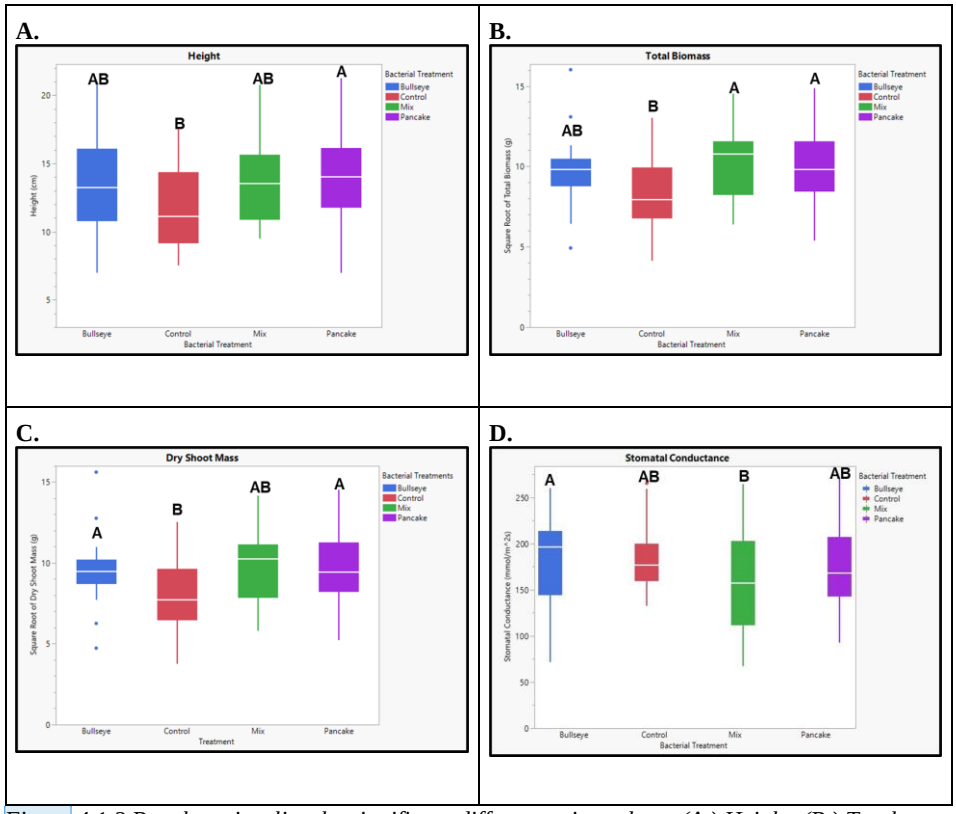

Figure 4.1.2 Boxplots visualize the significant differences in soybean (A.) Height, (B.) Total Biomass, (C.) Dry Shoot Mass, and (D.) Stomatal Conductance by bacterial treatment. Each color box represents an applied bacterial treatment: PAMW1 "Pancake" is colored violet, BUMW2 "Bullseye" is colored blue, Control is colored red, and PAMW1 and BUMW2 mix is colored green. Significant differences between bacterial treatments are determined by Tukey HSD pairwise comparison and indicated by compact letter display above each box.

Commented [1]: puzzled here.
B and C look like the same graph but w different letters over box plots. also, shoot dm and total dm do not differ.

more puzzles
in B., how is Pancake A but lower than Bullseye which is AB? in C., how is Mix AB with mean greater than Bullseye and Pancake?

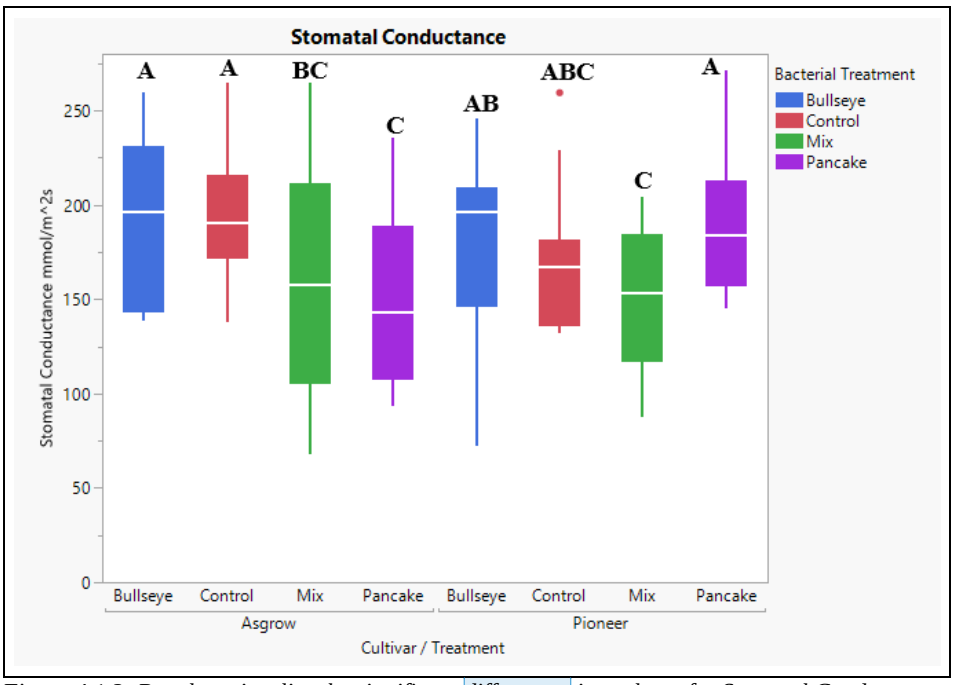

Figure 4.1.3: Boxplots visualize the significant differences in soybean for Stomatal Conductance by the interaction between cultivar and bacterial treatment. Each color box represents an applied bacterial treatment: PAMW1 "Pancake" is colored violet, BUMW2 "Bullseye" is colored blue, Control is colored red, and PAMW1 and BUMW2 mix is colored greenSignificant differences between treatments are determined by Student T-test pairwise comparison and indicated by compact letter display above each box.

Commented [2]: Pioneer Bullseye = AB but greater than Asgrow Bullseye and Control?

4.2 Multivariate Analysis of Soybean Growth and Productivity

Nonmetric multidimensional scaling was used as a multivariate approach to visualize similarities or differences among samples in terms of growth and productivity. For each sample, the final time point measurements of each response variable and its applied bacterial treatment were considered. The data were filtered by cultivar and relativized to ensure each response variable was equally impactful while emphasizing the bacterial effect on soybean growth.

This ordination method requires preliminary work to ensure the final ordination has an appropriate number of dimensions, low stress, and stability. The relationship between these basic principles and the number of iterations in the final solution help determine the best fitting model. The output results for preliminary testing are summarized in *Table 4.2.1*. This table summarizes both ordinations whereas each column corresponds to the cultivar of samples analyzed. Table 4.2.2 reports statistical differences of the multivariate ordination using multi-response permutation procedure (MRPP) and Permutational Multivariate Analysis of Variance.

Stress was analyzed using both Kruskal and Clarke's rule of thumb (Clarke, 1993; Kruskal, 1964). Software rescales these values to allow for comparison. A stress value of 9.38 for the Asgrow ordination was achieved, which can be interpreted as a "fair" ordination by Kruskal's formula and a "good ordination with no real risk of drawing false inferences" by Clarke's rule of thumb. Kruskal's formula interprets a stress value of 3.52 for the Pioneer ordination as "good," while Clarke considers this stress value to be "an excellent representation with no prospect of misinterpretation" (McCune & Grace, 2002).

The number of dimensions in the final solution was chosen after considering its final stress and Monte Carlo test results. Both ordinations had a p-value of 0.0040 from this analysis, which can be interpreted as a better-than-random solution. The solution provides the final iterations used in the ordination. The final solution of the Asgrow ordination consisted of 111 iterations, while the Pioneer ordination used 82 iterations. This was compared to the final stress of the solution, which presented an instability rating that was less than 10^{-4}. These results suggest that a 2-dimensional solution is the best fitting for this dataset for both ordinations.

The Asgrow ordination was plotted for an interpretative aid (*Figure 4.2.1*). This solution shows the placement of the mean point and standard error for each bacterial amendment. If two different bacterial treatments experience overlap in their mean point standard error, there is a similarity regarding their effect on soybean growth. This is easily visualized by color corresponding ellipses that encompass the mean point standard error.

The standard errors for PAMW1 and the Mix amendment overlapped, while the standard errors for BUMW2 and the control overlapped in a separate space. As a result, this solution had fewer

spatial differences (or relatively more similarity) between samples associated with the PAMW1 and Mix amendments. Similarly, there was less spatial difference between the BUMW2 and control amendments. Because these two different overlaps occurred in separate spaces within the matrix, this suggests thatthe two groups of bacterial treatments act differently in terms of their effect on soybean growth and productivity.

A biplot is superimposed onto the scatterplot presented in *Figure 4.2.1*. The biplot contains vectors that correspond to an individual response variable. These vectors vary in length and direction. A longer vector that is aimed toward a cluster of samples indicates that its corresponding response variable strongly influenced its position in the matrix.

The biplot shows that Asgrow samples that received either the PAMW1 or mix amendment are dissimilar from the other bacterial amendments because of their shared variance in nodule count and nodule mass. The length of these vectors suggests that nodule count had a greater influence on the position of these samples relative to the influence of nodule mass. The vectors that correspond to the height and root mass are aimed away from sample clusters, which suggests that these response variables are less influential. Vectors that correspond to chlorophyll content and stomatal conductance are lengthy and aimed toward samples that were amended with BUMW2 or the control. This suggests that their position, or dissimilarity from other samples, was strongly influenced by those response variables.

Figure 4.2.2 visualizes the NMS ordination for Pioneer samples. In this figure, the mean point corresponding to the mix amendment resides in a separate space relative to the remaining bacterial treatments. The mean points for samples that received either PAMW1, BUMW2, or a control reside in the same space within the matrix. There is a substantial overlap in standard errors belonging to their three mean points, suggesting samples associated with Mix amendment differ from the remaining samples which were similar in terms of soybean growth and productivity.

The superimposed biplot within *Figure 4.2.2* suggests that samples associated with the Mix amendment are dissimilar from the other treatments in terms of nodule mass and nodule count. This is represented by a lengthy vector running along the vertical axis. The vectors that correspond to height, root mass, and shoot mass, are short and aimed away from those samples, that those response variables have little influence on the sample distribution. A lengthy vector corresponding to stomatal conductance is aimed towards samples associated with the isolates or control, while a shorter vector corresponding to chlorophyll content is aimed in the same direction. This suggests that plants treated withPAMW1, BUMW2, or control treatment are in a similar space because of their common stomatal conductance and chlorophyll content measurements.

Cultivar	**Asgrow**	**Pioneer**
Max Iterations	250	250
Runs with Real Data	150	150
Distance Measure	Sorensen	Sorensen
Stress	9.37842	3.52333
Monte Carlo p-value	0.0040	0.0040
Final Iteration	111	82
Final, Random Solution	2-D	2-D

Table 4.2.1 Reported NMS ordination output from relativized datasets filtered by cultivar. Stress, Monte-Carlo, and the final iterations support that this ordination is not obtained by random chance and is the best fitting solution. The max iterations, runs with real data, and distance measure are fixed criteria necessary for testing, while the remaining metrics are obtained from preliminary tests.

Cultivar	**Relativized MRPP**	**Relativized PERMANOVA**
Asgrow	0.324	0.230
Pioneer	0.422	0.422

Table 4.2.2 Statistical differences of the multivariate ordination using multi-response permutation procedure (MRPP) and Permutational Multivariate Analysis of Variance.

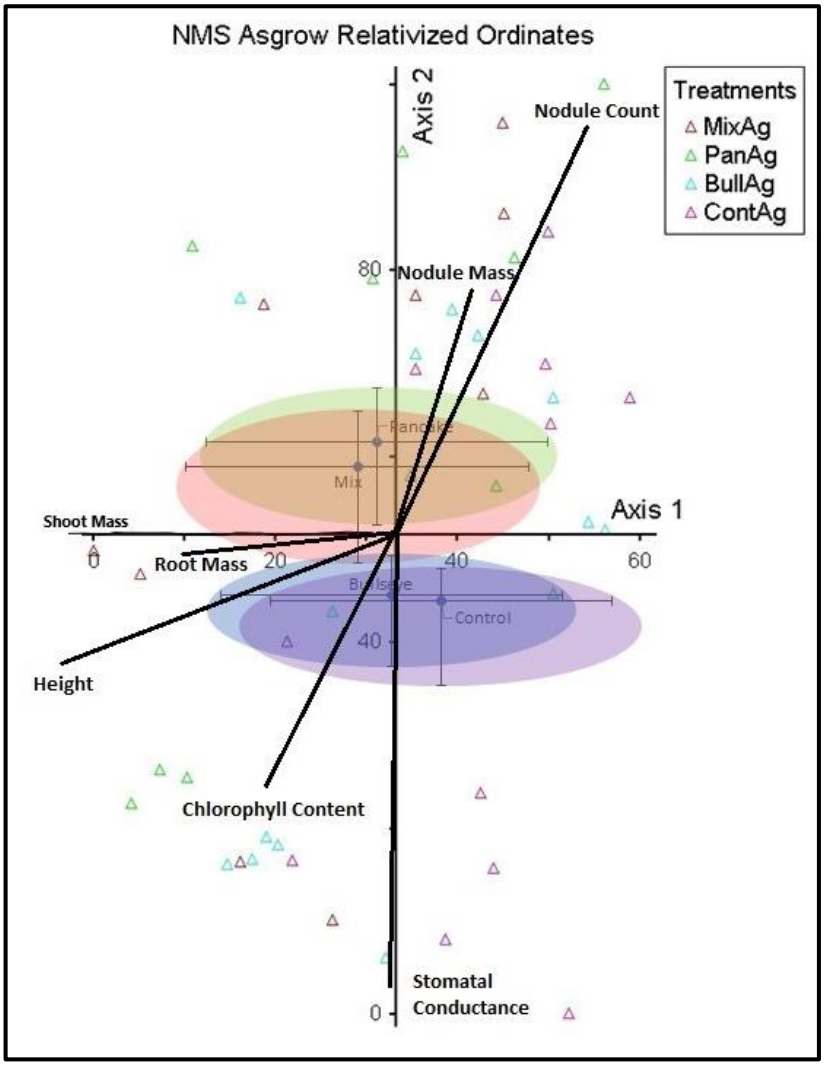

Figure 4.2.1: Nonmetric Multidimensional Scaling (NMS) of relativized Asgrow samples. This ordination organizes samples by soybean growth and productivity, while considering their applied bacterial treatment. Bacterial treatments are represented by colored triangular points (PAMW1=green, BUMW2=blue, Mix=red, Control=purple). The centroid, or center mean point, is represented by a blue, labeled point on the graph. The standard error of the centroid is encompassed by an ellipse corresponding in color to the applied bacterial amendment.

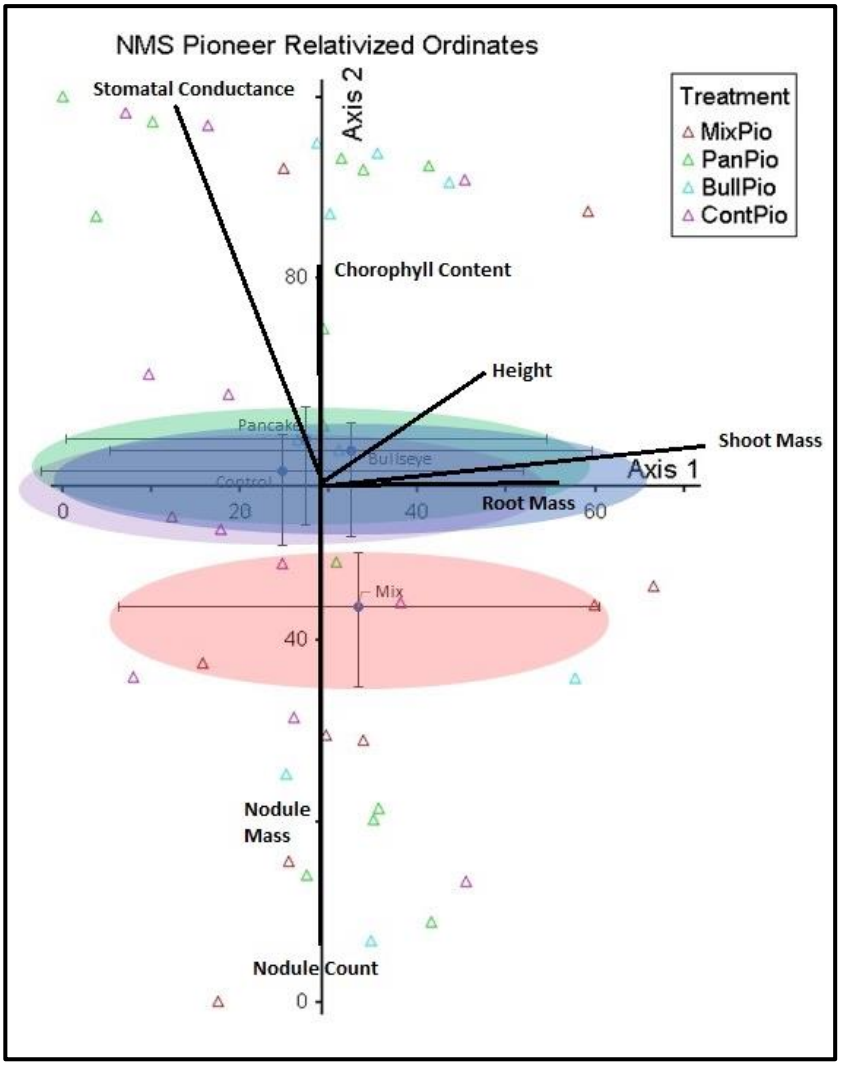

Figure 4.2.2: Nonmetric Multidimensional Scaling (NMS) of relativized Pioneer samples. This ordination organizes samples by soybean growth and productivity, while considering their applied bacterial treatment. Bacterial treatments are represented by colored triangular points (PAMW1=green, BUMW2=blue, Mix=red Control=purple). The centroid, or center mean point, is represented by a blue, labeled point on the graph. The standard error of the centroid is encompassed by an ellipse corresponding in color to the applied bacterial amendment.

4.3 Interior Nodule Extraction

The interior of sterile nodules was subjected to a bacterial extraction, which resulted in colony growth on *Pseudomonas* selective media. The colonies that resembled the morphology of PAMW1 or BUMW2 underwent PCR amplification and ribosomal RNA 16s gene sequencing. The sequences were compared to the original amendments. It was hypothesized that if PAMW1 is a dominant member in the nodule following the amendments in this study then it can be extracted from the nodule and grow abundantly.

Five of the selected bacterial extracts colonies had relatively close matches in percent identity to the original PAMW1 or BUMW2 16s rRNA gene, summarized in *Table 4.3.1*. The remaining samples are reported in *Table 8.0.2*. However, PAMW1 and BUMW2 have an identical 16s rRNA gene, where whole genome sequencing confirmed their classification as two different strains. Thus, whether these five colonies are related more closely to either isolate is inconclusive.

One extracted colony, belonging to a Pioneer sample amended with BUMW2, has a 98.54% identity with the original strains. Similarly, an Asgrow sample that served as a control had an identical percent identity to the aforementioned bacterial extract. Two colonies that belong to Pioneer samples amended with PAMW1 had a 97.81% and 97.66% identity of the original strains. Lastly, a Pioneer sample that served as a control has a 97.65% identity of the original strains.

A phylogenetic tree allows visualization of the relatedness between the original strains and the extracted colonies (*Figure 4.3.1*)based on the percent of related identity.. For example, the first node belongs to the original cultures used for the bacterial amendment to the experimental soybean, labeled as PAMW1. The two colonies with the closest percent identity to PAMW1 or BUMW2 reside within its clade. Of the two colonies in this clade, one originates from a control soybean sample, while the other originates from a soybean sample amended with BUMW2.

The diversity or richness of isolates that were found the results of this extraction do not support the hypothesis. The high richness of isolated could have made it more difficult to sample Pancake from the mixture. However, it does tend to suggest that Pancake was not a abundant member of the nodule.

Sample (Cultivar; Bacterial Treatment)	Percent Identity
Pioneer; Bullseye	98.54
Asgrow; Control	98.54
Pioneer; Pancake	97.81
Pioneer; Pancake	97.66
Pioneer; Control	97.65
Bullseye	100

Table 4.3.1 The percent identity of bacterial extract's ribosomal 16s rRNA gene sequence. The sample in which the bacterial colony was extracted from serves as its label. The first five samples have the highest percent identity of the tested samples. This is a comparison to the original PAMW1 culture that was applied to experimental soybean. Beneath the blackened columns shows the 16s rRNA gene percent identity of the original BUMW2 culture to PAMW1.

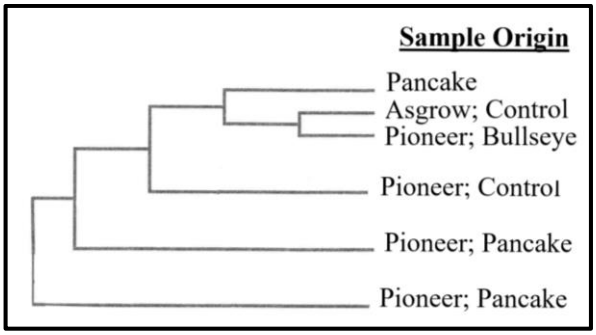

Figure 4.3.1: Phylogenetic tree with cladogram branch lengths. The sample in which the extracted bacterial colony originates from serves as the label on the phylogenetic tree.

4.4 Quantified PGPR Traits

The growth promoting potential of four novel strains of *Pseudomonas* sp. were investigated using quantitative assays for the production of siderophores, organic acids, and IAA production. The results of the three assays were positive for all four strains. The production of these secondary metabolites furthers the potential of these strains to be efficient biostimulanst.

Table 4.4.1 shows the quantification of these assays for the two strains used as amendments in this field study. The data was not subjected to statistical analysis and may only be interpreted as observations. PAMW1, or Pancake, shows the greatest potential for plant growth promotion compared to BUMW2, or Bullseye. The results of the Blue CAS assay suggests that the siderophore production of PAMW1 is 15.5% greater than BUMW2. Furthermore, the results of the liquid NBRIP media shows that all strains produce similar amounts of organic acids. 1.5% L-tryptophan results in optimal IAA production for PAMW1 , while BUMW2 produces the greatest amount of IAA in 2.0% L-Tryptophan. In terms of IAA production, the absorbance of the PAMW1 culture was 82% higher than the BUMW2 culture indicatingthat PAMW1 produces more IAA than BUMW2 in a 1.5% L-Tryptophan solution. *Figure 4.4.1* qualitatively visualizes the color change in Blue CAS and NBRIP media following incubation for all four isolated strains, while *Figure 4.4.2* shows their culture color following incubation in Salkowski reagent to measure IAA production.

Strain	**Siderophore Production** (psu: siderophore production unit)	**Organic Acid Production**	**IAA Production** **1.5% L-Tryptophan** (Absorbance at 530 nm)
PAMW1 “Pancake”	22.67	1.75	0.153
BUMW2 “Bullseye”	19.63	1.63	0.084
STMW3 “Starfish”	19.63	1.75	0.100
JEMW4 “Jellyfish”	27.03	1.66	0.663

Table 4.4.1 Quantification of PGPR traits in the four novel strains using numerous assays. Blue CAS Media was used to measure siderophore production in psu through spectrophotometry at a 630nm wavelength. Organic acid production was measured on NBRIP media by finding the diameter of the colony and halo and dividing that by the diameter of the colony. IAA production was quantified through spectrophotometry at a 530 nm wavelength to measure variance in the effect of Salkowski reagent on culture color.

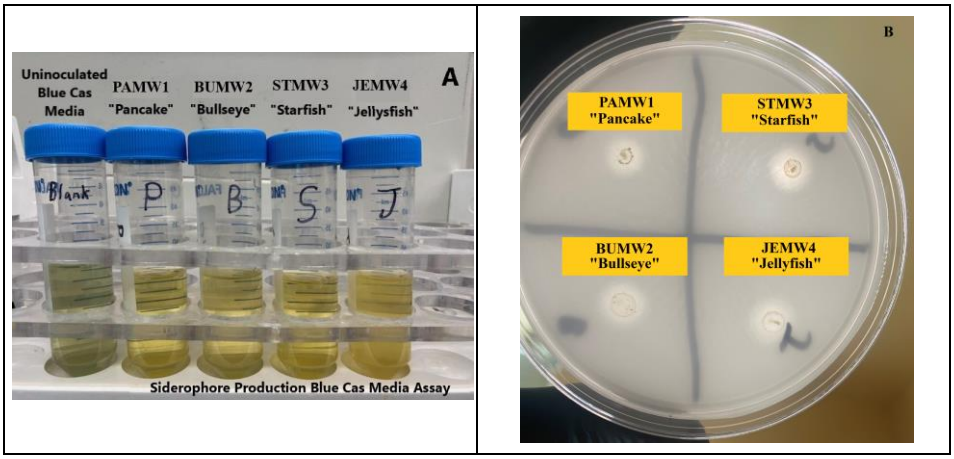

Figure 4.4.1 Assays used to quantify numerous PGPR traits: (A.) Blue CAS, (B) NBRIP. A color change from blue to yellow indicates positive results for siderophore production in Blue CAS media, while a color change from opaque to clear indicates positive results for organic acid production on the NBRIP assay.

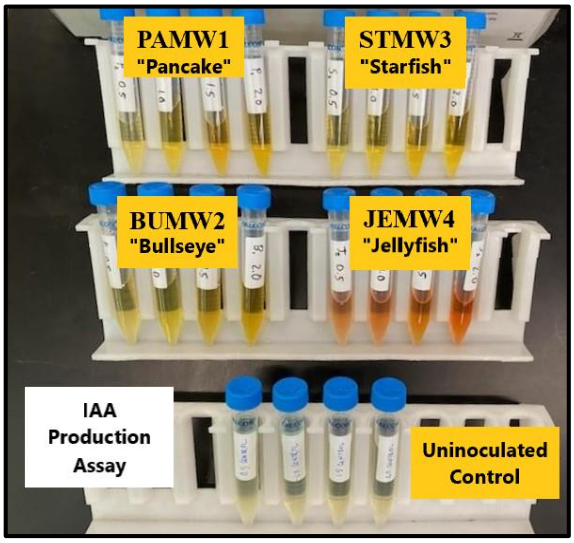

Figure 4.4.2 IAA Assays showing variance in phytohormone production by the four novel strains of *Pseudomonas*. The addition of Salkowski reagent to the cultures following incubation changes the color of the culture to red in the presence of IAA. The intensity of the color is positively correlated to the amount of IAA produced in the culture.

Section 5: Discussion

After extracting and isolating strains of a novel species of *Pseudomonas* from soybean nodules, these microbes were applied to soybean rhizospheres to investigate their effects on plant growth parameters. As hypothesized, the novel *Pseudomonas* strains positively influence the growth of soybean plants following inoculation into the rhizosphere under field conditions. Significant increases in PAMW1-amended soybean biomass, shoot mass, and height support this hypothesis; furthermore, NMS analyses suggest differences in soybean growth due to bacterial treatment (*Figure 4.1.2; 4.2.1; 4.2.2*). By demonstrating consistent soybean growth promotion in the field and those documented previously in the greenhouse, PAMW1 shows potential to be a real-world crop biostimulant (Griggs et al., 2022).

The mechanisms responsible for growth promotion are not known. I outline some of the most possible ways that growth may be increased due to bacterial inoculation.This will be done through further analysis of these bacteria's functional properties and through a review of the literature. Microbes can increase nutrient availability and produce phytohormones in the rhizosphere, which may promote plant growth (B. Lugtenberg & Kamilova, 2009; B. Singh & Nehra, 2011). PAMW1 and BUMW2 produce siderophores, organic acids, and IAA, which hypothetically contribute to their plant-growth-promoting abilities. (*Table 4.4.1*). However, *in vitro* analyses of bacterial function are not necessarily robust indicators of plant growth promotion and should be accompanied by greenhouse or field experiments that directly demonstrate those functions (Smyth et al., 2011). Indeed, it is also unknown whether distal or proximate growth drivers cause *Pseudomonas* growth promotion in soybean (Bharti et al., 2018). An example of the former, *Pseudomonas*, could directly affect the rhizosphere microbiome, which in turn could be causing the growth-promoting effects on soybean (*Figure 5.0.1*).

It is hypothesized that soybean growth promotion observed in our study, where these helper bacteria were observed, but not confirmed to be, within root nodules, may indicate a broader mutualistic relationship between the soybean nodule microbiome system (Egamberdieva et al., 2010). However, this is still somewhat of a speculative idea. Perhaps the mutualism occurs through interactions with several species of *Bradyrhizobium* (Afkhami et al., 2020). Although these bacteria reside together with *Bradyrhizobium*, it is not known the extent to which this novel species interacts with *Bradyrhizobium* to result in positive growth effects on soybean. Through NMS ordinations, the interactions between strains in a mixed culture amendment may have resulted in different soybean growth relative to isolated amendments (*Figure 4.1.2; 4.2.1; 4.2.2*). Thus, different interactions with other bacteria and the soybean host may alter the effects of MWPA1 or MWBU2 on plant growth and productivity.

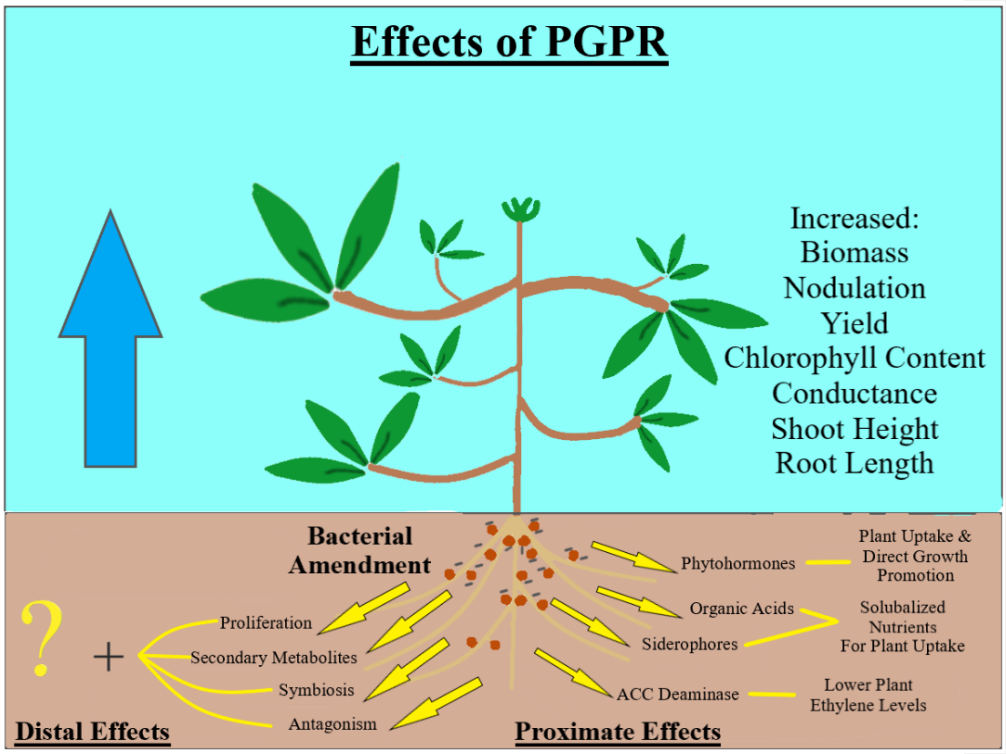

Figure 5.0.1 A few examples of microbial interactions or mechanisms that may have either a distal or proximate effect on plant growth. Microbes are enlarged relative to the scale of the illustration to emphasize their interaction with the plant.

5.1 Inoculants Affected Soybean Growth in a Field Setting

Observing consistency in microbial plant-growth promotion in both the greenhouse and field setting is a significant step toward identifying an applicable PGPR or biostimulant (Backer et al., 2018; Vasseur-Coronado et al., 2021). The amendment of PAMW1 to greenhouse soybean resulted in growth promotion; however, the observable increases in growth were similar, but not identical, to its effect in the field. Both studies suggested increases in overall productivity, but the effect was more substantial in the field. The greenhouse study observed, furthermore, that the PAMW1 amendment influenced soybean nodule mass and root length (Griggs et al., 2022). Although the univariate statistics in the field study did not observe a significant variance in nodulation, multivariate NMS analysis showed that greater nodule count and mass were associated with the mixed inoculum. Hence, there were many expected outcomes in the field study based on findings in the greenhouse.

It is common for PGPR applications to have inconsistent results depending on whether the crop was grown in the greenhouse or the field (Nelson, 2004). For example, applying *Bacillus vallismortis* EXTN-1 in tomato (*Solanum lycopersicum)*, potato (*Solanum tuberosum)*, and black pepper (*Piper nigrum)* reduced bacterial wilt, fusarium wilt, and foot rot by a mean level of 80 to 90% under greenhouse conditions. However, its effect on disease reduction was limited to 45% under field conditions (Thanh et al., 2009). Alternatively, in a separate study, two strains of *Bacillus megaterium* offered growth promotion in sugar beet (*Beta vulgaris)* comparable to the effects of a sole P fertilizer amendment in both the greenhouse and the field. Overall, there is a tendency for field and greenhouse studies to come to different conclusions, but given the consistent results herein, there is strong evidence that the bacterial treatment has potential for development as a crop-based growth promoter for soybean (Çakmakçi et al., 2006). Further studies from other geographical location should be done to further test the veracity of this conclusion.

Compared to BUMW2, PAMW1 has greater potential to develop as a reliable growth promoter. There is considerable variation in soybean growth promotion by PAMW1 compared to BUMW2. Both strains significantly improved soybean shoot mass under field conditions, but the effects of BUMW2 were often less influential than PAMW1. The production of siderophores by PAMW1 is 15.4% greater than BUMW2, while PAMW1 cultures have an 82% greater absorbance than BUMW2 cultures in regards to measures of IAA production.

Despite their variable effects on soybean growth, these two strains are very similar on a genomic level. As indicated in *Table 4.3.1*, the two strains have identical 16s rRNA genes (100% ANI). Consequently, the two isolates cannot be discerned as individual strains at a 16s rRNA gene level. Per personal communication with Dr. Hazem Sharaf regarding his work analyzing these two isolates' whole-genome DNA sequences, the isolates can be considered two unique strains. Differences in the genomic sequence result from single nucleotide polymorphisms (SNPs), where six known SNPs have been reported between the two strains, of which one is non-synonymous in the "lipopolysaccharide export system permease protein." Ultimately, SNPs help distinguish these isolates as individual strains with apparent functional differences, which are strong candidates for differences in their effects on soybean growth promotion.

Promotion in plant growth and biomass often translates to increased yield (Antoun & Prévost, 2006; Jiménez et al., 2020). The biomass of rapeseed (*Brassica napus)* and corn gromwell (*Lithospermum arvense)* significantly increased upon the amendment of *Pseudomonas fluorescens* LBUM677. The amendment significantly increased the seed mass of all studied plants while significantly increasing the seed count in soybean (Jiménez et al., 2020). Additionally, *Pseudomonas fluorescens* MKB232 significantly increased wheat head weight and yield upon its amendment in glasshouse trials (Smyth et al., 2011). With the effect of PAMW1 on soybean biomass, there is potential for an effect on soybean yield, which should be further

investigated. Significant increases in soybean yield by the novel strains could rationalize its application as a biofertilizer for use in sustainable agriculture.

The amendment of PAMW1 in Pioneer samples significantly decreased soybean stomatal conductance. Stomata co-regulate CO_2 influxes for photosynthesis and transpirational water loss, resulting in its metabolically mediated responses to leaf water status (Buckley, 2019). Therefore, stomatal conductance influences the water use efficiency (WUE) of a crop, which is a metric for carbon gains relative to water used for its assimilation (M. E. Gilbert et al., 2011). A more massive plant may require greater water requirements. A lower stomatal conductance can improve WUE in soybean if the available water does not increase. (Sinclair et al., 2008; Teare et al., 1973). Perhaps the effect of PAMW1 on the height of Pioneer samples requires greater hydraulic conductance, resulting in lower stomatal conductance to maintain WUE (D. Li et al., 2013; Mencuccini, 2003). Furthermore, the leaf area is a plastic response to environmental conditions depending on the soybean cultivar, directly affecting stomatal conductance (Franks et al., 2009). Plasticity may explain the lower stomatal conductance in PAMW1 amended plants. However, the result was inconsistent, where bacterial inoculum applied to the Pioneer cultivar had no discernable effect on stomatal conductance and thus WUE.

The conservation of water in plant tissues through decreased stomatal conductance and increased WUE logically improve crop drought tolerance (Fletcher et al., 2007; Sinclair et al., 2010). Improved drought tolerance could result from bacterial-induced hormone changes (Barnawal et al., 2017; Bashir et al., 2020). For example, the amendment of *Pseudomonas simiae* AU to soybean enhanced abscisic (ABA) and salicylic acid (SA) production, resulting in stomatal closure and reduced transpiration rates. As a result, the soybean's relative water content and WUE improved during drought conditions (Vaishnav & Choudhary, 2019). In addition, the inoculation of *P. simiae* AU resulted in greater root length and number of lateral roots. The result from this independent study was thus consistent with the findings in the greenhouse experiment (Griggs et al., 2022). With plant-phenotypic responses to bacterial amendments that consequently improve drought tolerance, including increased root length and decreased stomatal conductance, it is necessary to quantify PAMW1's effect on soybean phytohormone production further. Observing increased ABA or SA levels in PAMW1 amended soybean can indicate that PAMW1 may potentially improve soybean drought tolerance.

In summary, the observed soybean growth promotion by the applied bacterial amendments in this study supports the hypothesis that the novel strains promote soybean growth in both a greenhouse and field setting. These observations may indicate an effect on soybean yield and drought tolerance, which require further investigation. The effects of PAMW1 and BUMW2 on plant growth may vary because of differences in their ability to solubilize phosphorus, produce siderophores, or synthesize phytohormones. Numerous studies attribute these common PGPR mechanisms to plant growth and productivity increases. Therefore, to test whether the

mechanisms of growth promotion by PAMW1 or BUMW2 are related to phytohormone production, siderophore production, or other mechanisms previously shown by PGPR, it is recommended to mechanistically test if these strains function in those ways to affect plant growth.

5.2 *Pseudomonas sp.* PGPR and their Associated Mechanisms

It is not yet known, as noted, how the novel strains support soybean growth, but the data can provide clues to the possible mechanisms. First, a commonly measured mechanism, microbial phosphorus solubilization, provides soybean with an otherwise limiting nutrient. The amendment of phosphorus fertilizers often results in increased plant-available phosphorus, which translates into increased plant height, shoot and root biomass, shoot-to-root ratio, chlorophyll content, nodulation, and yield (Raby et al., 2022). A microbial PGPR amendment, in contrast, is often associated with phosphorus solubilization and mobilization, which would result in similar effects on plant growth compared to that of phosphorus fertilization (Zaidi et al., 2009). The ability of PAMW1 or BUMW2 to solubilize phosphorus is an effective mechanism for plant growth. The bacterial amendments used in this study significantly increased soybean biomass, shoot mass, and height, which is consistent with phosphorus fertilization and the effects of phosphorus solubilizing *Pseudomonads*. Below I will outline additional mechanisms of growth promotion reported in the literature with the idea of developing working hypotheses, but keeping in mind that we currently have little evidence that a specific mechanism of growth promotion is at play. Measures of plant tissue nutrient concentrations are expected to help eventually support or narrow down the likelihood of some of these proposed mechanisms.

Both PAMW1 and BUMW2 were shown to produce siderophores, and the production of these molecules, which often translates into soybean growth promotion, can occur because of micronutrient solubilization, such as iron. Alternatively, siderophores have also been shown to affect iron concentrations around the plant as a means of biocontrol. Iron availability is crucial for chlorophyll synthesis and chloroplast structure and function, and it is thus not surprising that it is a common limiting nutrient for plant growth (Rout & Sahoo, 2015; Zuo & Zhang, 2011). With iron's role in photosynthesis, changes in plant chlorophyll content and biomass are expected in plants amended with siderophore-producing microbes. Also, changes in nodulation are expected because iron is a cofactor of the nitrogen-fixing enzyme nitrogenase. Recall root nodules in soybean are a major source of nitrogen for soybean. Given that nodule function is the joint result of the mutualism between plants and nodule bacteria, the ability to sequester limiting concentrations of iron could have a major impact on nodule function and plant growth. Discerning the nutrient content of soybean tissues may help indicate if greater iron, and possibly greater siderophore production, contribute to the observed soybean growth promotion in this study.

Some notable *Pseudomonas* plant growth promoters can produce phytohormones like indole-3-acetic acid (IAA) or abscisic acid (ABA). These phytohormones stimulate cell growth and division or regulate stomatal closure, respectively. Commonly, these functions tend to be essential for improving plant tolerance under environmental stress (Glick & Pasternak, 2003; Yamaguchi-Shinozaki & Shinozaki, 1994). Often increased cell growth and division results in root elongation, which can be further stimulated by ACC deaminase production (Bharti et al., 2018; Remans et al., 2008). In addition, ACC deaminase restricts ethylene production. Moderate ethylene production restricts shoot and root elongation, so its restriction in plants under abiotic stress may promote growth despite salinity, drought, or pathogenic stress (Glick et al., 2007; S. Gupta & Pandey, 2019). It has been observed that PAMW1 produces IAA, and further investigation is required to quantify potential ABA or ACC deaminase production. These effective *Pseudomonas* growth promotion mechanisms should be considered for the novel *Pseudomonas* strains.

Here, I utilize the literature to assess if plant growth promotion is consistent among *Pseudomonas*. This could support the hypothesis that PAMW1's known PGPR functions contribute to more significant plant growth with inoculation. *Table 5.2.1* organizes the functions and potential mechanisms used by *Pseudomonas* to support plant growth promotion.

Table 5.2.1 Examples of *Pseudomonas sp.* Plant Growth Promoters

Pseudomonas Species and Strain	Amended Plant	Phosphorus Solubilization	Siderophore Production	IAA Production	ACC Deaminase Production	Pseudomonas Effect on Plant Growth	Author
P. fluorescens L321	*P. sativum L.* (Pea Plant) **Greenhouse**	1312 mg/L (Gluconic Acid)				Significant increase in fresh and dry biomass	(Otieno et al., 2015)
P. corrugata 1; 7	*Amaranthus paniculatus* (Red Amaranth) **Field**	130 mg/L at 21℃				Significant increase in plant height, biomass, and root length	(A. Pandey et al., 1999, 2002)
P. striata	*Vigna Radiata* (Mung Bean) **Greenhouse**	+				Significant increase in plant height (2.37 fold), nutrient content, chlorophyll content, and yield	(Hassan et al., 2017)
P. fluorescens PMV-8; *P. aeruginosa* PMV-14	*Oryza sativa* (Rice) **Field**	+				Significant increase in plant height, root length, biomass, and yield	(Deshwal et al., 2011)
P. simaie AU	*Vigna Radiata* (Mung Bean) **Greenhouse**	Pi-solubilization index: 2.7		38.98 µg/mL	79.0 nmol/mg/h	Significant increase in plant height, root length, biomass, RWC, and stomatal conductance	(Kumari et al., 2015)
P. lini DT6	*Ziziphus jujuba* (Red Date) **Greenhouse**	69.16 mg/L		30.82 µg/mL	234.98 µmol **α**-KA/(mg Pr h)	Significant increase in height, root/shoot mass & ratio, RWC, and phytohormone levels	(Z. Zhang et al., 2014)
P. libanensis EU-LWNA-3	*Triticum aestivum L.* (Wheat) **Greenhouse**	199.10 mg/L	+		+	Significant increase in plant height, root length, dry/fresh biomass, and yield	(Kour et al., 2020)

Continue Table 5.2.1

Pseudomonas Species and Strain	Amended Plant	Phosphorus Solubilization	Siderophore Production	IAA Production	ACC Deaminase Production	Pseudomonas Effect on Plant Growth	Author
P.cepacia GW1201	*Glycine max* (Soybean) **Lightroom**		+		+	Significant increase in root length and mass	(Cattelan et al., 1999)
Pseudomonas sp. NBRI 4014	*Glycine max* (Soybean) **Greenhouse**	277.0 µg/mL	143.87µg/mL	5.6 µg/mL		Significant increase in germination, plant height. Insignificant trend in root mass promotion	(A. Gupta et al., 2002)
Fluorescent *Pseudomonas sp.* 21	*Sorghum bicolor L.* (Sorghum) **Greenhouse**	50 ppm	58 SU	-		Significant increase in root/shoot mass and chlorophyll content	(Praveen Kumar et al., 2012)
P.aeruginosa LSE-2 (Nodule Endophyte)	*Glycine max* (Soybean) **Field**	Pi-solubilization index: 2.77	+ (4.5 cm halo)	43.6 µg/mL		Significant increase in plant height, shoot mass, chlorophyll content, nodulation, and yield	(K. C. Kumawat et al., 2019)
P. fragi CS11RH1	*Triticum aestivum L.* (Wheat) **Growth Chamber**	514.97 µg/mL	-	12.13 µg/mL (30°C)		Significant increase in plant biomass, germination, and nutrient uptake	(Selvakumar et al., 2018)
PAMW1 "Pancake"	***Glycine max* (Soybean) Field**	+	22 psu	+		**Significant increase in soybean height, shoot mass, and biomass**	

Table 5.2.1 Examples of *Pseudomonas sp.* plant growth promotion and their reported mechanisms associated with growth promotion. +/- indicates an unquantified positive/negative result. If the article did not report a mechanism then it was left blank. All IAA production measurements used media containing L-Tryptophan. Our study is listed in the last row in bolded font.

The effects of four *Pseudomonas sp.* on plant growth promotion, highlighted in *Table 5.2.1*, were attributed predominantly to phosphorus solubilization. The effects of these phosphorus solubilizers on their respective plants resembled the effects of PAMW1 on soybean height and biomass. For example, *P. fluorescens* PMV-8 and *P. aeruginosa* PMV-14 significantly increased rice height and biomass by approximately 150% and 290%, respectively, while *P. striata* significantly increased mung bean height by 2.37 fold (Deshwal et al., 2011; Hassan et al., 2017). Furthermore, *P. corrugata 1; 7* significantly increased red amaranth height and biomass (A. Pandey et al., 1999, 2002). An increase in plant height and biomass may result in significantly greater yield, exemplified by *P. fluorescens* PMV-8, *P. aeruginosa* PMV-14, and *P.corrugata* 1; 7 (Rosales-Serna et al., 2004). Perhaps, the effect of PAMW1 on soybean height and biomass may lead to greater yield, consistent with other phosphorus solubilizing *Pseudomonads*. Additionally, similar observed growth promotion attributed to phosphorus solubilization may indicate that phosphorus solubilization was an influential mechanism for the results observed in our study. Based on these outcomes, it is recommended that studies are conducted to assess if phosphorus may be an important factor in bacterial treatment-induced increases in soybean productivity.

Several studies credit gluconic acid production for more efficient phosphorus solubilization that increases plant P availability relative to that of other organic acids (Miller et al., 2010; Vyas & Gulati, 2009; Zeng et al., 2016). For example, the amendment of *P. fluorescens* L321, a predominant phosphorus solubilizer, affects pea plant shoot mass and total biomass, consistent with the effects of PAMW1. The study attributed significant pea plant growth under phosphorus deficiency to increased available phosphate, concluding that phosphate availability increased because the strain produces 33.24g/L of gluconic acid *in vitro* during a 5-day incubation period. (Otieno et al., 2015). Gluconic acid is a common secondary metabolite among the *Pseudomonas* genera, where its production temporarily lowers soil pH, which solubilizes otherwise insoluble soil mineral phosphates (K. S. N. Kumar et al., 2013; Prasad et al., 2015; Yu et al., 2019). With taxonomic similarities and consistencies regarding plant growth promotion, there is rationale to hypothesize that the effects of PAMW1 soybean growth may result from solubilized phosphorus and that gluconic acid production, in particular, may be a good target to understand the mechanisms of phosphorus solubilization better.

The above noted four phosphorus solubilizers share few inconsistencies with PAMW1 regarding their effect on plant productivity; nonetheless, inconsistencies provide a rationale for future investigation. First, the amendment of *P. striata* significantly increases chlorophyll and nutrient content in mung bean, whereas the former chlorophyll effect was not observable in our amended soybean. Furthermore, *P. corrugata* 1; 7, *P. fluorescens* PMV, and *P. aeruginosa* PMV significantly increased root length in their respective grains. While root length was not measured in our field study, it is consistent with results in the greenhouse. However, greater root biomass observed in this field study supports the idea that root length under field conditions might be

worth measuring. Finally, as previously described, the latter two strains and *P. striata* significantly increased yield. Therefore, the effects of these phosphorus solubilizers and the rationale above all call for further future investigation on the effect of PAMW1 on soybean root architecture, yield, and foliar nutrient content.

Overall, with similarities regarding the effect of PAMW1 compared to other phosphorus solubilizing *Pseudomonas* species on plant growth, there is a support that the observed soybean growth promotion in this study may be a consequence of phosphorus solubilization. To further explore this hypothesis, it is useful to evaluate PAMW1's production of organic acids, like gluconic acid. For example, HPLC analysis can determine different organic acids produced as secondary microbial metabolites (Maliha et al., 2004; Panhwar et al., 2012), and this production could be measured by sampling the nodule and rhizosphere habitats during plant growth. Additionally, previous studies have quantified the rate of phosphorus solubilization and plant uptake in a field setting (R. M. N. Kucey, 1987); however, because of much greater simplicity and control, the work is recommended to be first conducted in the greenhouse.

There is hesitation in attributing a single mechanism, like phosphorus solubilization, for plant growth promotion. For example, without rationalizing this result, Deshwal observed a similar shoot length promotion by a non-P solubilizing strain, *P. aeruginosa* PMV-19 (Deshwal et al., 2011). Furthermore, two of the aforementioned phosphorus-solubilizers significantly increased root length; however, ACC deaminase production can also result in this effect. Three *Pseudomonas* sp. that can produce ACC deaminase, *P. libanesis* EU-LWNA-3, *P. simaie* AU, and *P. cepacia* GW1201, significantly increased root length in wheat, mung bean, and soybean, respectively (Cattelan et al., 1999; Kour et al., 2020; Kumari et al., 2015) (*Table 5.2.1)*. Perhaps it is more common that multiple simultaneous mechanisms are characteristic of PGPR supporting plant growth promotion.

The majority of *Pseudomonas* exemplified in *Table 5.2.1* classify as multifarious PGPR. Like the effects of ACC deaminase and organic acid-producing *Pseudomonads*, Griggs observed significant increases in soybean root length by amending PAMW1 to the plant in a greenhouse setting (Griggs et al., 2022). These *Pseudomonas* sp. have further similarities in their effect on plant growth. For example, *P. lini* DT6, *P simaie* AU, and *P. libanesis* EU-LWNA-3 promoted plant height and biomass like that of PAMW1. This consistency supports the need for further investigation regarding the ability and specific functions that PAMW1 might utilize to increase root length. ACC deaminase production may be a more effective mechanism for root growth promotion than phosphorus solubilization, exemplified by *Pseudomonas cepacia* GW1201; the only growth promoter noted as capable of ACC deaminase production without reporting phosphorus solubilization. Its amendment affects root length and mass but does not promote soybean height (Cattelan et al., 1999) (*Table 5.2.1)*. Ultimately, it is difficult to attribute a single PGPR function to observed plant growth promotion.

The greenhouse and field study results share similarities with the effects of growth promoters applied during drought. Although phosphorus solubilizing *Pseudomonas sp.* have commonly been associated with plant growth promotion under drought conditions, it is not a predominant function. Instead, drought stress alleviation commonly results from phytohormone production, including IAA and ABA, because of their consequential effect on cell growth, division, and root elongation. Following the effects of phytohormones on root growth, phosphorus solubilizing microbes gain relevancy regarding growth promotion under drought conditions by their ability to stimulate ion transport systems in plant roots (Ramaekers et al., 2010). A common misconception is that root surface area is a predominant factor in nutrient uptake; however, the regulation of nutrient uptake is also highly dependent on stimulated root ion transporters. There is inverse coordination between root ion transporters and root surface area to maintain acquisition rates (Mahdi Dar et al., 2018; Nazoa et al., 2003; Touraine, 2004). Therefore, more multifarious PGPR are possibly more applicable for their effects of drought stress alleviation resulting from phytohormones, ACC deaminase, and solubilized phosphorus on plant growth. This conclusion rationalizes an investigation of the effect of PAMW1 on soybean productivity under drought conditions because of its ability to produce phytohormones and solubilize phosphorus.

P. lini DT6, *P. simaie* AU, and *P. libanesis* EU-LWNA-3 promoted plant growth under drought conditions (Kour et al., 2020; Kumari et al., 2015; M. Zhang et al., 2020). *P. simaie* AU and *P. lini* DT6 significantly increased relative water content in mung bean and red dates, respectively, experiencing drought stress. The latter plants had significantly increased relative water content (by 11%) through changes in stomatal conductance from ABA production. Similar to the effect of PAMW1 on Pioneer samples, *P. simaie* AU significantly decreased stomatal conductance in mung bean (*Figure 4.1.3*). The mung bean amended with *P. simaie* had significantly greater stomatal closure than the control plants. Perhaps a means of stomatal regulation occurs in PAMW1-amended soybean under drought conditions. Further investigation regarding the ability of PAMW1 to produce ABA may provide a better understanding of its potential in soybean growth promotion during drought. Assuming the increased stomatal regulation is growth supportive over the plant's life, perhaps by saving water to avoid plant death, this and other mechanisms of stomatal control will be important to understand for managing cropping systems.

With the assays chosen in this study to quantify functions characteristic of PGPR, PAMW1 can solubilize phosphorus and produce siderophores and IAA. Collectively, these three functions were also reported in *P. aeruginosa* LSE-2 and *Pseudomonas sp.* NBRI 4014 (A. Gupta et al., 2002; K. C. Kumawat et al., 2019). *P. aeruginosa* LSE-2 is exceptionally comparable to PAMW1 because of its origin as a soybean nodule endophyte. The observations made in these two studies share some consistencies with the effects of PAMW1 on soybean growth. The independent amendment of *P. aeruginosa* LSE-2 to soybean resulted in significant increases in

plant height, shoot and root mass, and chlorophyll content, while *Pseudomonas sp.* NBRI 4014 significantly increased soybean height. (A. Gupta et al., 2002; K. C. Kumawat et al., 2019). Also, the amendment of *P. aeruginosa* LSE-2 resulted in significant nodulation and nodule weight increases. Although an effect on nodulation was not observed in PAMW1 amended soybean, perhaps an influence on nodulation results from siderophore production. The promotion of chlorophyll content and nodulation by *P. aeruginosa* LSE-2 is consistent with the effects of more available iron; however, this was not observed by PAMW1 (R. N. Kumawat et al., 2006; Nasar & Shah, 2017). As multivariate statistics indicate, nodulation was an influential factor in differentiating PAMW1 amended samples from bacterial effects (*Figure 4.2.1; 4.2.2)* even though univariate statistics showed no effect of bacterial inoculation on nodulation. There is thus evidence that PAMW1 can positively affect soybeans' ability to attain nitrogen from nodule-inhabiting nitrogen-fixing bacteria.

Variance in nodulation, or nodule count, across the plots was high, with a standard deviation of 24.71, a standard error of the mean of 16.81, and a range of 0 to 600 nodules per plant. After reviewing raw data and observations made during harvest, nodulation was unusually low in a specific corner of the experimental plot. *Figure 5.2.*1 divides the plot into 6-sections of 20 plants, allowing *Figure 5.2.2* to illustrate low nodulation areas within the plot. Additionally, *Figure 5.2.2* shows less variation in total biomass within each section for comparison with nodulation. Lower nodulation was not associated with a treatment like a cultivar or bacterial amendment (*Table 4.1.1)*. Instead, it may represent a region of soil with low population levels of *Bradyrhizobium spp*. Despite this confounding factor likely masking the actual effects of *Pseudomonas*, significant effects were still observed, suggesting PAMW1 is a growth promoter. Though further tests are warranted, these results tend to agree with the hypothesis that *Bradyrhizobium* and nodulation are critical components needed for PAMW1 to play the role of a PGPR in soybean. PGPR functions or traits of PAMW1 suggest a potential influence on nodulation upon its amendment to the soybean rhizosphere (*Figure 4.4.1*). A generalized randomized block design would have alleviated its impact on this study. However, no discernible reasons to block could be distinguished during planting. It is clear, nevertheless, that field-scale variation plays an important role in modulating the effects of any organisms, including PGPR.

The variable nodulation in this study may have resulted from low *Bradyrhizobium* sp. abundancy in the soil. Although soil belonging to a previously hosted soybean field was used to cover the seeds during planting, perhaps inefficient or incompatible strains of *Bradyrhizobia* sp. were amended to the plot. Additionally, this study's sections of relatively lower nodulation may have been the consequence of high soil nutrient contents or other legacy soil conditions. For example, hemp (*Cannabis sativa L.),* which was grown in these plots in 2019, can induce high nitrate concentration in the soil (Soti et al., 2016). Relatively high nitrate content in soil inhibits nodule count, mass, and microbial nitrogen fixation (El-Shemy, 2011). As a result of hemp cultivation, higher nitrate concentrations may have limited nodulation or may have created variability in

downstream soil conditions. Alternatively, low phosphorus bioavailability can be consequential to nitrogenase inhibition and low nodule ATP (H. Li et al., 2021). Therefore, if the stress was high enough in that section of the plot, it could explain the low amount of nodulation in that part of the plot. The above explanations for variability in nodulation are intended as examples of factors that can cause variability at the field level. We have no evidence that supports that any of those examples are more likely than other possibilities.

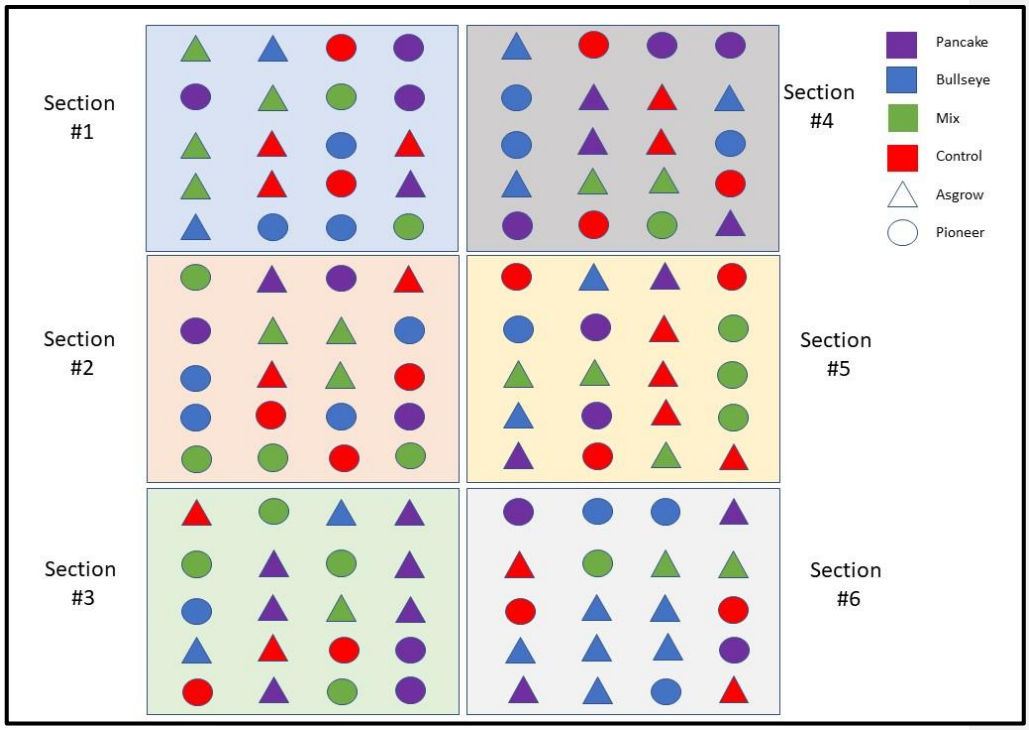

Figure 5.2.1 The plot's complete randomization design (CRD) split into 6 sections of 20 plants to measure the variance in growth parameters by location, rather than treatment.

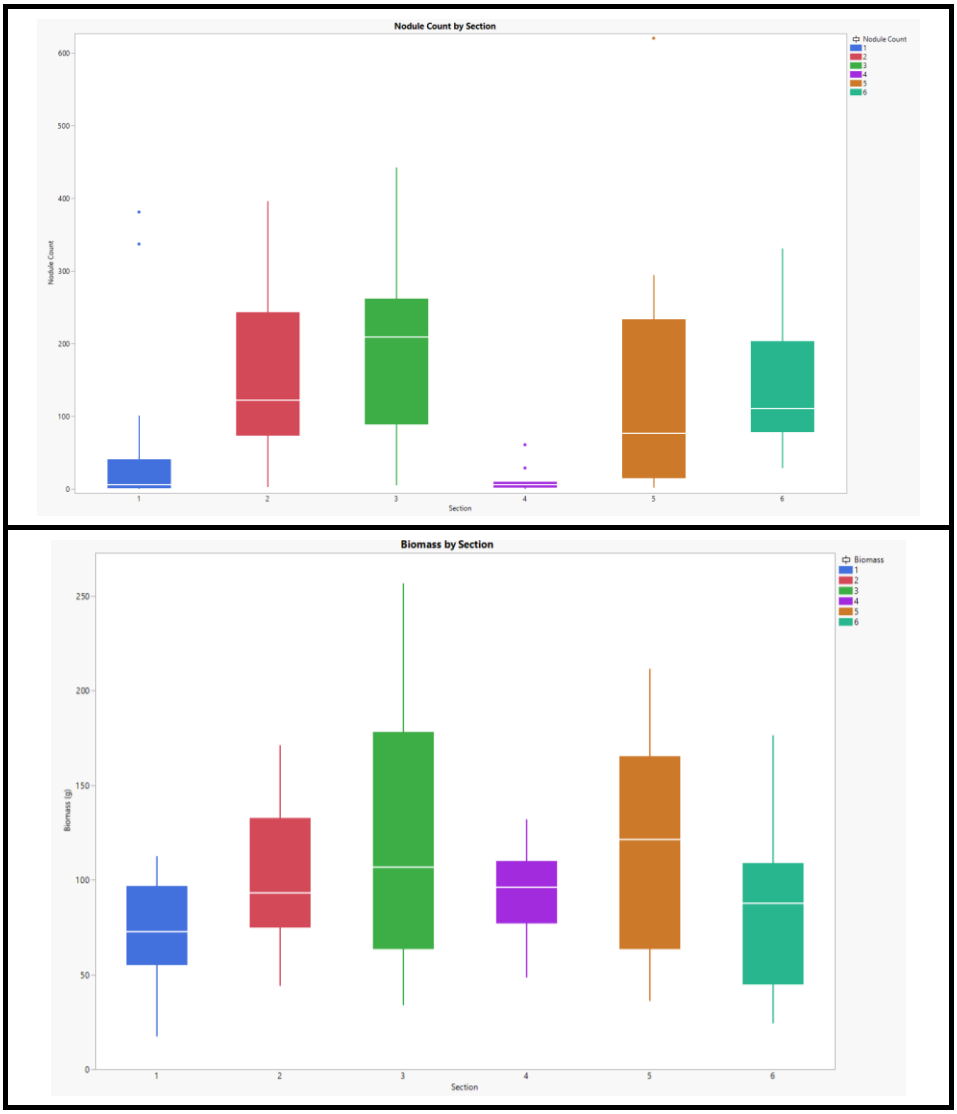

Figure 5.2.2 Variation in nodule count (top) and biomass (bottom) by section in the plot. Each section consists of 20 plants in a 4x5 formation.

5.3 Enhanced PGPR Mechanisms through *Pseudomonas* and *Bradyrhizobia* Interactions

Although the effects of PAMW1 or BUMW2 on soybean growth and productivity may be a direct result of functions that increase nutrient availability or phytohormone concentration in the rhizosphere, it is also important to consider more distal factors that may play a role in PGPR induced growth promotion. Although it is not possible to confirm the relevancy or impact of distal effects compared to that of proximate, they nevertheless deserve mention. One example of a more distal cause of our bacterial treatment-induced growth could come from the strains interacting with the indigenous rhizosphere soil microbial community. Interactions with the microbial community may influence the inoculum's survivability, colonization, or infection of soybean nodules (Egamberdieva et al., 2013; Fox et al., 2011; Santoyo et al., 2021). In turn, the bacterial inoculum could have an unknown effect on the composition of the rhizosphere soil community, which could positively impact soybean productivity. Interactions between amended inoculums and the indigenous microbial community may alter the impact of the inoculum on plant growth (K. C. Kumawat et al., 2022; Tu et al., 2021), and certainly are worthy of study to assess their potential relevance to the field of PGPR.

The origin of PAMW1 and BUMW2 as nodule endophytes suggests that these two strains interact with *Bradyrhizobium*, the microbial genus predominately responsible for soybean nodulation (Faruque et al., 2015; Limpens et al., 2003). Their interaction with *Bradyrhizobium* may directly affect the PAMW1 or BUMW2's residence within the nodule. For example, one particular species, *Pseudomonas fluorescens* IAM 12022, was observed attaching to the base of mung bean root hairs but unable to invade because of their inability to independently form an infection thread (Pandya et al., 2013). Nonetheless, *P. fluorescens* IAM 12022 was able to invade the root hair in the presence of *Ensifer adhaerens*, a *Rhizobiales* nodule endophyte capable of infection thread formation. The inability of *P. fluorescens* IAM 12022 to produce cellulase or pectinase may help explain its inability to form infection threads; however infection thread formation is a relatively complex process requiring numerous genes (Mateos et al., 1992; Pandya et al., 2013). These enzymes, nevertheless, are essential for infection thread formation because of their ability to degrade plant cell walls and pectin layers (D. P. S. Verma et al., 1978). Additionally, these enzymes promote localized microbial movement within plant cells (Carro & Menéndez, 2020; Peeters et al., 2013). PAMW1 and BUMW2 may share similar methods for root hair invasion as *P. fluorescens* IAM 12022. Perhaps they share genes and functions relevant to their taxonomic relationship.

Interactions with *Bradyrhizobium* may determine if PAMW1 or BUMW2 are capable of invasion into root nodules. Thus, other questions arise regarding how the novel species may influence the well-characterized and complex relationship that *Bradyrhizobium* has with soybean. Perhaps most important to ask is whether the novel *Pseudomonas* species has a net positive effect on *Bradrhizobium*. At first glance, the results of the greenhouse and field studies would tend to

suggest a positive interaction between these two microbes. However, this may not always be the case. The fixation of nitrogen and carbon by bacteria and plants, respectively, are the key units of exchange between these organisms that result in positive impacts on plant growth. The mutualistic theory supports the idea that relatively high nitrogen fixation levels favor *Bradyrhizobium* (Archetti et al., 2011). The plant supports and rewards high levels of nitrogen fixation by the bacteria as long as nitrogen remains in high demand. Efficient and high nitrogen-fixing nodules are supported because otherwise, the plant will limit carbon allocation to nodules (Denison, 2000). Limiting carbon allocation to nodules is based on resource sanctioning, whereby nodules that underperform in providing nitrogen to a plant do not receive photosynthetic carbon or energy (Kiers et al., 2006; Oono et al., 2009). This often results in nodule death. Hence, the working hypothesis is that the novel *Pseudomonas* species positively interacts with *Bradyrhizobium* by helping increase nitrogen fixation, perhaps by providing nutrients such as iron and phosphorus that can limit nitrogen fixation (Khosro Mohammadi, 2012).

Through siderophore production or phosphorus solubilization, PAMW1 or BUMW2 could increase nutrient availability in the nodule, resulting in more effective nitrogen fixation. The effectiveness of nitrogen fixation in a nodule may be observable through nodule mass or biomass measurements (Quigley et al., 1997; Singleton & Tavares, 1986). Thus, it may be hypothesized that the observed biomass promotion in our study may be an indicative result of the novel *Pseudomonas* species supporting the nitrogen fixation of indigenous *Bradyrhizobium*. Therefore, incorporating these bacterial treatments into the nodule would favor *Bradyrhizobium*, resulting in adequate nitrogen fixation and the prevention of sanctions.

Since PGPR application can enhance *Bradyrhizobium* nitrogen fixation efficiency, it would be favorable if their interaction also improves *Bradyrhizobium* survival (Rajendran et al., 2012). For example, the co-inoculation of *Pseudomonas* sp. 54RB and *Bradyrhizobium* sp.TAL 377 onto media results in higher CFUs for each species compared to their individual isolated growth (Afzal et al., 2010). Afzal suggests that this laboratory result may indicate if an interaction between the two strains improves their survival efficiency in the rhizosphere. After amending this mixture onto the field, soybean nodule count increases beyond the effects of either isolated microbe. Ultimately, greater survival efficacy may promote plant growth and nodulation, which is advantageous for all tripartite parties. Therefore, a laboratory test should be conducted to explore variance in survivability depending on the strain of *B. japonicum* or *B. elkanii* co-inoculated with PAMW1 (Sharaf et al., 2019). Perhaps the interaction resulting in higher enumeration could enhance the effects of PAMW1 on soybean growth promotion.

Interactions with PGPR that increase *Bradyrhizobium* efficacy of survivability may improve root colonization (F. Ahmad et al., 2011). For example, through increasing soil salinity, *P. putida* TSAU1 had more significant soybean root colonization than *B. japonicum* USDA 110; however,

B. japonicum USDA 110 had more significant root colonization in the presence of *P. putida* TSAU1 than it did as an isolated inoculation under the same conditions. Their amendment as a co-inoculation promotes their survivability in the rhizosphere. As a result, samples had significantly longer soybean roots than isolated amendments of *B. japonicum* USDA 110. Ultimately, alleviating salt stress promotes USDA 110's survival and root colonization, which may increase the number of infection threads and nodules. The potential for TSAU1 to be incorporated into soybean nodules increases (Egamberdieva et al., 2013). Since salt stress in soils is often the consequence of drought, perhaps, the potential for PAMW1 to alleviate plant drought stress begins with supporting the proliferation and colonization of a particular strain of *Bradyrhizobium*. Additionally, understanding the survivability between PAMW1 and strains of *Bradyrhizobia* may progress PAMW1 towards an applicable soybean growth promoter.

The effects of interacting PGPR and *Bradyrhizobium* may offer greater soybean growth promotion than either member's isolated amendment. For example, one study concludes that the co-inoculation of a phosphorus solubilizing *Pseudomonas sp.* and *B. japonicum* TAL-378 was beneficial over chemical fertilizers (Argaw, 2012). This interaction significantly increased soybean nodule count and mass, yield, and nutrient uptake. Furthermore, interactions between a diazotroph closely related to *B. diazoefficiens* USDA 110 and various species of *Pseudomonas* were assessed to observe differences in soybean growth promotion (Tu et al., 2021). The effects of dual inoculations on soybean growth depended on the *Pseudomonas* strain used for co-inoculations. Although most of the 7 *Pseudomonas* isolates tested significantly improved soybean growth when co-inoculated with *B. diazoefficiens* USDA 110, the greatest increases in soybean growth were observed when a species closely related to *P. punonensis* CECT 8089 was included in the dual inoculation. This amendment resulted in significantly greater soybean nodulation, biomass, and nutrient content than the effects of a single, isolated inoculation of one species of bacteria.

In conclusion, interactions between *Bradyrhizobia* and non-rhizobia PGPR may impact their individual survival, colonization, and infection. Since their co-inoculation may synergistically affect plant growth beyond the effects of an isolate, it is necessary to consider their interaction before applying PGPR to a legume rhizosphere (Basu et al., 2021; K. C. Kumawat et al., 2019). Further investigations regarding differences in survival and colonization rates of PAMW1 depending on its co-inoculated strain of *Bradyrhizobium* may further the effects of PAMW1 on soybean growth.

5.4 Additive Effects of a Mixed Culture Amendment

In contrast to results supporting our first hypothesis, that this novel species of *Pseudomonas* promotes soybean growth in a field setting, this study cannot fully support the second hypothesis. To clarify, this separate hypothesis states that the interactions between PAMW1 and BUMW2 in a mixed amendment would result in synergistic or additive effects on soybean growth compared

to an isolated amendment. Although the results from the univariate approach indicate that some growth parameters experience additive effects by the mixed culture amendment, it is inconsistent across all measured variables. Indeed, the samples amended with a mixed culture experienced an average increase in nodule count and mass by 9.9% and 13.9%, respectively, compared to samples amended with a single isolate. This result is consistent with the multivariate analysis, where nodulation strongly influenced the separation of mixed amended samples from others among both cultivars (*Figure 4.2.1; 4.2.2)*. This could tend to suggest that there is an additive effect of the two strains on nodulation. However, these results were not significantly different, and it is therefore concluded that there is insufficient evidence to conclude that the interactions within a mixed culture amendment offer additive or synergistic effects on soybean growth over that of individual strains.

There are many similarities between PAMW1 and BUMW2 regarding their growth-promoting potential. These two strains have similar growth rates at 28°C, for example. At this temperature, the production of siderophores and IAA are also similar. This trend is consistent with the ability of the strains to solubilize phosphorus. Ultimately, these data show a lack of functional differences between strains, which may have ultimately been the primary reason why a mixture of strains does not have synergistic or additive effects on soybean productivity. Veen explains that consortia consisting of bacteria with minimal diversity are often ineffective, and the use of multiple microbial consortia containing diverse functions can increase the potential for the amendment to improve plant growth in variable environments (van Veen et al., 1997).

Since these taxa are genetically very similar they may not, when mixed, increase functional richness. Perhaps the application of a consortium containing PAMW1 and a different strain (e.g. Jellyfish) might offer more additive effects toward soybean growth than a PAMW1 and BUMW2 co-inoculation. When testing the IAA production in a Jellyfish, its absorbance was 333% greater than a PAMW1 culture, while its siderophore production unit (psu) was 19.2% higher than PAMW1. Additionally, the growth rate of Jellyfish at 28°C is 26% greater than either PAMW1 or BUMW2. With a greater output of secondary metabolites associated with plant growth promotion and its higher growth rate, there is a functional variation that could help to increase the niche of a consortium consisting of both PAMW1 and Jellyfish and that of an individual strain. It would be worth testing the ability of Jellyfish as a growth-promoting inoculant and co-inoculant. Again, however, Smyth urges not to rely on *in vitro* analyses, such as siderophore production, as a robust indicator of plant growth promotion. (Smyth et al., 2011). In other words, because an organism has the ability to carry out plant growth-promoting functions does not mean they will be expressed under field conditions.

The literature shows that multiple genera, rather than multiple strain co-inocula, may maximize microbial bioactive compounds and plant-growth promotion. Impactful co-inoculations have thus far been shown to consist of two growth promoters like *Pseudomonas* sp. and *Bradyrhizobium*

sp. amendments described in Section 5.3. Applicable consortia comprising free-living rhizobacteria commonly consist of different genera also. The co-inoculation of three microbes belonging to species of either *Pseudomonas* or *Bacillus* significantly increased yield and plant growth compared to the effects of isolated amendments (He et al., 2019). The study incorporated numerous free-living PGPR into a co-inoculation based on the calculated nutrient needs of tomato through various growth stages. At an early seedling stage, *P. putida* provided phosphorus solubilization to support root and shoot growth, while *B. amyoliquefaciens* produced antifungals to suppress root diseases. Towards flowering, *B. mojavensis* and *B. pumilus* acted as diazotrophs, providing usable nitrogen for synthesizing proteins, energy, and nutrient transfer. Co-inoculations consisting of three or four selected microbes offered the greatest yield, while co-inoculations of two or three bacterial species significantly increased tomato shoot to root mass. This study justifies using multiple bacterial species and several inoculations throughout a plant's life (growth stages) to functionally service the plant's needs (He et al., 2019). The results support the idea that other nodule bacteria of soybean may be in combination with the bacterial treatments in our study, which could provideadditive growth benefits.

We applied two amendments in this study with consideration of soybean growth stages. Both amendments occurred before soybean V3 growth stage. As PAMW1, BUMW2, and *P. putida* are phosphorus solubilizers, applying the bacterial treatments used in this study to soybean seedlings may have similar effects to *P. putida* on tomato shoot and root length. However, the rationale for our application regime was to optimize the potential for incorporating PAMW1 and BUMW2 as an endophyte of soybean nodules since nodulation peaks at the R1 growth stage. Perhaps a consortium of PAMW1, BUMW2, and other PGPR genera would further maximize soybean growth if the application's timing considers the plant's growth stage requirements.

For effective bacterial consortia, it is crucial to consider the PGPR functions belonging to the *Pseudomonas* genus to select other genera most appropriate for a diverse consortium. The application of *Pseudomonas* into the rhizosphere is commonly associated with increased phosphorus and iron availability (Kalyanasundaram et al., 2021). Furthermore, its application has been shown to promote biocontrol in the rhizosphere through antibiotic production as well as the production of ISR elicitors like 2,4 diacetylphloroglucinol, pyocyanin, exopolysaccharides, or iron-regulated siderophores (Höfte & Bakker, 2007; Iavicoli et al., 2003; Kalyanasundaram et al., 2021; Schuhegger et al., 2006). Now, functions of other genera can be compared with these listed functions to find appropriate co-inoculants with the *Pseudomonas* genus.

There has been a considerable amount of research into growth-promoting bacteria, including those in the genera *Azotobacter, Azospirillum, Pancium, Enterobacter, Rhizobia, and Bacillus*. Together, any combination of these bacteria would increase the functional potential relative to any individual. These bacteria are growth promoters and may increase the diversity of PGPR functions (Kalyanasundaram et al., 2021). This may suggest that exploring how other bacterial

PGPR may interact with PAMW1 and BUMW2 to provide additive growth effects relative to their isolated effects alone is worthwhile. Together, but not individually, for example, it is known that *Azotobacter* and *Azospirillum* mediate salinity tolerance through IAA production or osmolyte accumulation (El-Esawi et al., 2019; Patil, 2011).

Furthermore, *Rhizobia* and *Bacillus* can enhance the availability of nitrogen and potassium in the rhizosphere. The latter genus is also known to increase biocontrol in the rhizosphere. *Bacillus subtilis* produces antifungal compounds against *Sclerotium rolfsii*, benzothiazole against *Monilinia fructicola*, and β-glucanase against *Aspergillus ochraceus* (Nalisha, I.* et al., 2006; Zhao et al., 2022; Zhou et al., 2019). With numerous genera and diverse functions, a mixed culture amendment may best interact positively with the plant. However, adding too many growth promoters into a co-inoculation commonly masks their effects (He et al., 2019). These results suggest that other bacteria within soybean nodules may make a suitable co-inoculum that may provide additive or synergistic benefits to plant growth compared to PAMW1.

The interior nodule of soybean has been shown to host numerous bacterial species, which could potentially interact with PAMW1 or BUMW2. There are at least ten other taxonomic units of *Pseudomonas*, and with other bacterial species, there are a diversity of possible bacterial-bacterial and plant-bacterial interactions that may alter their effect on plant growth. The literature tends to highlight bacterial mixtures of species and genera that work together additively to support plant growth over those of any individual in the mixture.

Although the results of a mixed culture amendment of PAMW1 and BUMW2 do not provide sufficient evidence to contend its co-inoculation as an efficient means of soybean growth promotion, the application of a microbial consortium have been shown to benefit plant growth. With further investigation, an efficient consortium may consist of PAMW1 or MWJE4 accompanied by microbes that are taxonomically and mechanistically unrelated to novel *Pseudomonas* species. The co-existence of these and other bacteria (e.g., *Bacillus*) in the nodules of some soybean provide a glimpse of some possible other organisms worth testing for growth-promoting features. Assuming that plant sanctions have selected nodules that are beneficial to soybean growth, it is worth characterizing these other bacteria for their functional role in the soybean life cycle.

Section 6: Conclusion

Results of our univariate analysis show *Pseudomonas* spp. PAMW1 isolated from soybean nodules and amended on a field site into the rhizosphere of soybean significantly affected the indices of plant growth and productivity. This amendment significantly promoted soybean biomass and height. Furthermore, nonmetric multidimensional scaling (NMS) indicate that there are differences in soybean growth and productivity depending on the bacterial treatment applied to the soybean. Through this multivariate analysis, it was found that nodule count, nodule mass, and overall plant biomass were influenced differently by bacterial treatments. Taken collectively, these analyses support our hypothesis that PAMW1 promotes soybean growth and productivity in a field setting.

Alternatively, the second hypothesis of this study, that the application of a mixed culture of strains belonging to the novel species of *Pseudomonas* further promotes soybean growth and productivity compared to isolated strains, was not supported. However, this finding, based on multivariate analysis, tended to suggest that the mixture might have a different effect than either isolated strain.

The findings of this study offer important insights regarding non-rhizobial occupancy of nodules and the application of PGPR. This study shows that a species of *Pseudomonas*, originating as a nodule endophyte, promotes plant growth upon inoculation to the root-zone, which helps understand the role of this species as a plant growth promoter. Furthermore, consistent soybean growth promotion in the greenhouse and field improve the potential for PAMW1 to develop into an applicable inoculumn. Its application may reduce the use of fertilizers as a biotechnology for sustainable agriculture.

Ultimately, this study raises more questions regarding the classification and application of *Psuedomonas* sp. PAMW1. This calls for further investigation regarding its ability to produce certain phytohormones or other proteins characteristic to growth promoters, like ACC deaminase production. The application of *Pseudmonas* sp. PAMW1 with other PGPR may advance sustainable agriculture by offering diverse functions that are advantageous for plant growth. Additional studies assessing the specific mechanisms that are used by the bacteria to support soybean productivity are needed. Further study of how this nodule-extracted endophyte invades the root-zone or nodules themselves to exert growth promoting effects are warranted.

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Section 8: Appendix

<u>Representative 16S rRNA gene sequence for PAMW1 (Pancake) and BUMW2 (Bullseye)</u>

GCAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGG
TGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAAC
GAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGG
CCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAA
TGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACC
TAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTC
TGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTA
CTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCC
CGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTA
GAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAG
GAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGTCAACTAGCCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCT
AACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAG
AGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACC
CTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAA
GCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAG
TCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGA
ATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC
ATGGGAGTGGGTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGT

IAA Production by L-Tryptophan Concentration

Strain	0.5% L-Tryp	1.0% L-Tryp	1.5% L-Tryp	2.0% L-Tryp
PAMW1 "Pancake"	0.081	0.082	0.153	0.102
BUMW2 "Bullseye"	<0.001	0.059	0.084	0.117
STMW3 "Starfish"	<0.001	0.066	0.1	0.104
JEMW4 "Jellyfish"	0.325	0.667	0.663	0.991

Table 8.0.1: IAA production by L-Tryptophan Concentration quantified through spectrophotmetry using a 530nm wavelength. Values are the absorbance measurements following the addition of Salkowski reagent.

Sample (Cultivar; Bacterial Treatment)	Percent Identity
Pioneer; Control	97.58
Pioneer; Bullseye	96.98
Pioneer; Bullseye	96.98
Pioneer; Control	97.66
Pioneer; Pancake	97.57

Table 8.0.2 The percent identity of bacterial extract's ribosomal 16s rRNA gene sequence. The sample in which the bacterial colony was extracted from serves as its label. The first five samples have the highest percent identity of the tested samples. This is a comparison to the original PAMW1 culture that was applied to experimental soybean.

Chlorophyll Content

Level	Least Sq Mean	Std Error	Mean
Bullseye	32.411888	0.66460743	32.4667
Control	33.170833	0.66229575	33.1708
Mix	32.032273	0.70882805	32.0619
Pancake	32.641026	0.64943441	32.6480

Level	Least Sq Mean	Std Error	Mean
Asgrow	33.247611	0.46913183	33.2563
Pioneer	31.880399	0.48067719	31.9239

Stomatal Conductance

Level	Least Sq Mean	Std Error	Mean
Bullseye	187.70769	9.1306393	188.083
Control	182.53333	9.0988804	182.533
Mix	154.58000	9.7381596	154.814
Pancake	171.32244	8.9221863	172.204

Level	Least Sq Mean	Std Error	Mean
Asgrow	173.77676	6.4451183	174.458
Pioneer	174.29497	6.6037331	175.587

Height

Level	Least Sq Mean	Std Error	Mean
Bullseye	13.532343	0.66302538	13.5104
Control	11.802083	0.66071920	11.8021
Mix	13.743182	0.70714074	13.7857
Pancake	13.992788	0.64788848	13.9500

Level	Least Sq Mean	Std Error	Mean
Asgrow	13.486815	0.46801510	13.4583
Pioneer	13.048383	0.47953298	13.0380

Nodules Per Plant (cube root)

Level	Least Sq Mean	Std Error	Mean
Bullseye	3.6503126	0.44356929	3.68714
Control	4.2211043	0.45196086	4.23873
Mix	4.6620179	0.48421570	4.66202
Pancake	4.2908600	0.44356929	4.23509

Level	Least Sq Mean	Std Error	Mean
Asgrow	4.6124775	0.32081140	4.58672
Pioneer	3.7996699	0.32425340	3.77502

Nodule Mass (cube root)

Level	Least Sq Mean	Std Error	Mean
Bullseye	1.2863984	0.13948956	1.29792
Control	1.4943559	0.14212846	1.50022
Mix	1.6307704	0.15644426	1.62658
Pancake	1.4344531	0.14212846	1.42693

Level	Least Sq Mean	Std Error	Mean
Asgrow	1.5929357	0.10246941	1.58104
Pioneer	1.3300531	0.10287519	1.32347

Shoot Dry Mass (square root)

Level	Least Sq Mean	Std Error	Mean
Bullseye	9.5482621	0.45826774	9.53082
Control	8.0239626	0.46693738	8.00987
Mix	9.5600352	0.48875925	9.57085
Pancake	9.8530962	0.44780547	9.84228

Level	Least Sq Mean	Std Error	Mean
Asgrow	9.2373879	0.32348116	9.22805
Pioneer	9.2552902	0.33499811	9.26811

Root Dry Mass (square root)

Level	Least Sq Mean	Std Error	Mean
Bullseye	1.7699500	0.07040584	1.76142
Control	1.5818453	0.06987043	1.58277
Mix	1.8116996	0.07485683	1.81170
Pancake	1.7594035	0.06700761	1.75921

Level	Least Sq Mean	Std Error	Mean
Asgrow	1.7474858	0.04905763	1.73931
Pioneer	1.7139634	0.05075879	1.71326

Table 8.0.3 The least square mean values and standard errors for each independent and dependent variable

Total Dry Biomass (square root)							
Level	**Least Sq Mean**	**Std Error**	**Mean**	**Level**	**Least Sq Mean**	**Std Error**	**Mean**
Bullseye	9.815161	0.47916365	9.7887	Asgrow	9.6345460	0.33387333	9.59537
Control	8.281415	0.47551977	8.2683	Pioneer	9.5231217	0.34545102	9.52573
Mix	10.080711	0.50945593	10.0807				
Pancake	10.138049	0.45603621	10.1275				

Shoot to Root Dry Mass Ratio (square root)							
Level	**Least Sq Mean**	**Std Error**	**Mean**	**Level**	**Least Sq Mean**	**Std Error**	**Mean**
Bullseye	4.0906277	0.14589148	4.10440	Asgrow	4.0455631	0.10165478	4.05625
Control	4.0773879	0.14478203	4.06619	Pioneer	4.2258553	0.10517985	4.22839
Mix	4.1132511	0.15511461	4.11325				
Pancake	4.2615699	0.13884985	4.26017				

Table 8.0.3 continued.