Soybean growth response to inoculation with plant growth-promoting rhizobacteria supplemented with orange peel amendment

by

Maria Leticia Pacheco da Silva

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 7, 2022

Keywords: plant growth-promoting rhizobacteria, orange peel, soybean, cultivar variation, strain selection, seed inoculation

Approved by

Alvaro Sanz-Saez, Chair, Assistant Professor Crop, Soil and Environmental Sciences
Mark R. Liles, Professor Biological Sciences
Yucheng Feng, Professor Crop, Soil and Environmental Sciences

Abstract

In order to meet the projected global food demand from population by 2050, current crop production will need to double. In this context, researchers are studying sustainable strategies to improve nutrient absorption by the plants that enhances crop yield such as the use of biofertilizers in agriculture. Plant growth-promoting rhizobacteria (PGPR) have been developed as biofertilizers to promote plant growth. In legume plants, such as soybean, PGPR are associated with more nodulation and therefore higher N_2-fixation rate. To exert plant growth promotion, the PGPR must survive and colonize the plant root surface under soil conditions. To persist in the field soils, the inoculated PGPR must compete for carbon source with native microbiota present in the rhizosphere of that specific soil. Researchers have demonstrated that pectin-rich amendments, for instance orange peel, can be degraded by some *Bacillus velezensis* PGPR strains and used as a sole carbon source, enhancing the PGPR activity and promoting soybean growth and nodulation. Hence, understanding the physiological response of inoculation with PGPR and orange peel on plants is critical. The overall objective of this thesis was to study the effect of inoculation with PGPR supplemented with orange peel amendment on soybean growth parameters. To accomplish this objective, several greenhouse and field experiments were conducted. A preliminary screening with 20 soybean cultivars in the greenhouse showed that the PGPR plus OP treatment produced a positive increase in all plant growth parameters. Further experiments revealed that the environment, soybean cultivar, inoculation method, or the capacity of the PGPR to use orange peel may be also playing an important role in the plant responsiveness.

Acknowledgements

I would like to specially thank my advisor Dr. Alvaro Sanz-Saez for believing in my work. His patience, assistance, and guidance have always given me encouragement during these years. He is an excellent professor and an incredible human being.

I am thankful for the Crop Physiology lab assistance with all the greenhouse and field experiments. The help of this team members was crucial to complete this thesis.

I would like to tank Dr. Mark Liles, Dr. Yucheng Feng, and all their lab team members, especially Frank Moen, for the support while conducting these experiments.

I am grateful to my all family: Maria Ignes, Geni (in memory), Maurilio (in memory), Márcio, Lizete, Maria Lígia, Tiago, João Mathias, Maria Laura, Marcelo, and Aline; for their unconditional love and caring for me. Without them, it would be hard to accomplish this dream.

This thesis is dedicated in memory of my grandfather João Berto. He taught me that education is the key to success in life.

I am thankful to all my friends in Brazil for being there for me and always loving me. Also, I would like to thank my Auburn's friends that that become my "family". When friends become family**,** you have a support system.

Finally, I would like to thank God, my beloved Father, for giving me the strength to keep going even when I thought that I was incapable to.

Table of Contents

CHAPTER I: Literature Review

The world population, crop production, and the need for sustainable yield increases

The world population is expected to grow to almost 9.73 billion by 2050 (FAO, 2017) in an annual rate of 77 million people per year (Carvalho, 2006). According to Cleland (2013) population increase has a direct link to the need for increased food production. In order to meet the projected global food and fiber demands from population in 2050, current crop production will need to double (Tilman et al., 2011) requiring a ~2.4% grow rate per year (Ray et al., 2013). There are two strategies to increase food production: 1) increasing agricultural land, which is very limited due to the lack of suitable land for agriculture (Brown, 1997) or 2) producing more food from the same amount of agricultural land, therefore closing yield gap between actual and potential yield (Godfray et al., 2010). However, the biggest challenge for modern agriculture is to increase yield in an environmentally sustainable manner (Morrissey et al., 2004).

Nowadays, soil infertility is the major crop yield constraint in developing nations (Mohammadi & Sohrabi, 2012). Presently, chemical fertilizers are the major input used to increase soil fertility and crop yield. However, excessive use of chemical fertilizers leads to environmental pollution and soil structure degradation (Savci, 2012). In this context, researchers are studying sustainable strategies to improve nutrient absorption by plants as part of a strategy to reduce chemical fertilization (Pilbean, 2015). Soil degradation, characterized by decline in soil quality, can be reversed trough adoption of recommended management practices, such as integrated nutrient management, involving the combined use of chemical fertilizers and biofertilizers (Lal, 2015).

Biological fertilizers (biofertilizers) or microbial inoculants are substances that contains viable microorganisms capable to enhance nutrient uptake and transportation by plants when

applied to the seeds, drenched in the soil, or sprayed in the leaves (Azizoglu, 2019). Biofertilizers are ecofriendly and cost-effective source of plant nutrients and could be an important component in sustainability of soils (Mohammadi & Sohrabi, 2012). For example, nitrogen fixing bacteria are beneficial microorganisms that are commonly used as biofertilizers components in legume plants. Another important biofertilizer broadly used is the arbuscular mycorrhizal fungi (AMF) or simply mycorrhiza. They are oligotropic microorganisms that can colonize root cells forming a mutualistic association with the plants (Igiehon & Babalola, 2017) which can benefit the plants host with water, nutrients, and pathogen protection (Berruti et al., 2016) while the plant supply photoassimilates to the microorganism.

Plant growth-promoting rhizobacteria (PGPR) have been developed as biofertilizers to promote plant growth (Kloepper et al., 1994) and currently several PGPR strains are available as commercial biofertilizers products. Biofertilization is the process of raising the abundance of microorganisms into the rhizosphere (Igiehon & Babalola, 2017) suppling the plant nutrient requirements, which has been a beneficial alternative to chemical fertilization and reduced environmental pollution (Singh et al., 2016). Therefore, a greater understanding of how plants and soil microorganisms interact and benefit each other can provide new strategies to increase yield, at the same time helping to protect the environment (Morrissey et al., 2004).

According to a new report by Market Research (2021), the global biofertilizers market is promising and it is expected to reach $3.5 billion by 2025 with projected 8.7% annual growth rate over the next five years. Examples of commercial biofertilizer products used in the United States are listed on Table 1.

Table 1. Examples of commercial biofertilizers products used in the US

Product	Organisms	Manufacturer	Crop	Reference
Nodulator Duo SCG	*B. subtilis* and *Rhizobium leguminosarum biovar viceae*	BASF	Pea and Lentil	agriculture.basf.us
Vault IP Plus	*Bradyrhizobium japonicum, Bacillus amyloliquefaciens, Bacillus subtilis*	BASF	Soybean	agriculture.basf.us
Quickroots	*Bacillus amyloliquefancies* and *Trichoderma virens*	NexusBioAg	Canola, corn, field pea, lentil, small grains, and soybean	nexusbioag.com
Cell-Tech	*Bradyrhizobium japonicum*	NexusBioAg	Soybean	nexusbioag.com

Nitrogen (N) is one of the major nutrients required for grain production. Nitrogen is a mineral required for nucleic acids, enzymes, proteins, and chlorophyll synthesis being an essential mineral for plant growth (Gopalakrishnan et al., 2015). Nitrogen constitutes 16% of protein, and the protein content of cereal grain averages 10%. Thus, the N content of cereal grain is approximately 1.6% (Gilland, 2014). As nitrogen is one of the most important elements for agriculture, N fertilizer use has increased with the years and is expected to increase in the future. According to Sutton et al. (2013) a “high” projection of the global nitrogen fertilizer requirement in 2050 is 190 Mt, a “mid” projection 140 Mt, and a “low” projection 80 Mt. Approximately, the annual global demand for nitrogen requires an increase of 1.6 Mt per year until 2050 (Gilland, 2002). For productivity to be improved in the future, the nitrogen removed from the soil by grains or lost from the system through runoff, erosion, leaching, and denitrification must be replaced by

nitrogen derived both from nitrogen fertilizers and biological nitrogen fixation (Peoples et al., 1995).

In 1908, Fritz Haber synthesized ammonia (NH3) from a combination between the nitrogen derived from the air with hydrogen gas, and, and subsequently in 1914, Karl Bosch completed the first large scale manufacturing plant (Frink et al., 1999). Globally, the N fertilizer is produced using Haber-Bosch synthesis which requires a minimum energy of 23 MJ per kg N (Gilland, 2014) and another 9-10 GJ per ton N to convert ammonia to urea (more easily available form of fertilizer for the plants) (Smil, 2008). Nowadays a variety of industrial N fertilizers are used for enhancing agricultural productivity. However, the N fertilizer production is costly for the economy and environment. Currently, N fertilizer production consumes 4% of the world's natural gas production (Gilland, 2014). On the other hand, biological inoculants do not require high levels of energy to be produced (Yadegari & Rahmani, 2010). Due to energy expended in manufacturing N fertilizers (Pilbeam, 2015), their cost and their environmental impacts, the appeal for the use of biological alternatives has grown in the last 20 years (Gopalakrishnan et al., 2015).

Biological nitrogen fixation (BNF) is the process of converting atmospheric N_2 into plant usable forms (ammonia) through complex mechanisms (Wilson & Burris, 1947). BNF can occur in legume plants via association with nitrogen fixing bacteria (NFB) that are able to fix the atmospheric nitrogen. Legume crops can reduce the N requirement derived from chemical fertilizers (Bhattacharyya & Jha, 2012), contributing for a sustainable agriculture. Nitrogen fixing species in cropping systems can meet their own N needs increasing their yield, improve soil quality by increasing the soil nitrogen content, and benefit following crops (Kannaiyan, 2002) therefore reducing the need for N fertilizers.

Soybean and nitrogen fixation

Legume crops are an important component in crop production due the ability to build and restore soil fertility (Gopalakrishnan et al., 2015). Soybean (*Glycine max* [L.] Merrill) is one of the most important crops in the world. In the United States, nearly 4.44 billion bushels were produced in 2021 on approximately 86.3 million acres attaining a yield average of 51.4 bu/acre (National Agricultural Statistics Service (NASS), 2022). Globally, soybean is an important source of protein (approximately 40%) for human and livestock nutrition, as well as an important source of oil (20% of seed weight) that is used for cooking and production of biodiesel (Kumar et al., 2010). Soybean, as a legume, is an important component on crop rotations due the ability to promote nitrogen fertility via N_2 fixation (Grossman et al., 2011).

The discovery of BNF in legumes is attributed to the scientists Hellriegel and Wilfarth, who in 1888 reported that soil bacteria present in legumes root nodules could convert atmospheric N_2 into ammonia, a N form usable by the plant (Hellriegel & Wilfarth, 1888). Nitrogenase, the enzyme responsible for BNF, is only found in prokaryotes (Postgate, 1982). The nitrogenase complex is highly conserved in free-living (e.g. *Azotobacter*, *Azosporilum*) and symbiotic diazotrophs such as *Rhizobium* and *Frankia* (Franche et al., 2009).

Free-living bacteria have been found responsible of occasional nitrogen fixation in non-legume plants such as corn. They associate with adventitious roots that excrete mucilage which provides an oxygen free media that is rich in sugars and organic acids that promotes BNF (Van Deynze et al., 2018).

In leguminous plants, the bacteria infect the plant root, and the plant modifies its structure to enclose the bacteria in a specialized structure, the nodule, where the atmospheric N_2 is fixed (Oldroyd & Downie, 2008). In exchange of the atmospheric nitrogen fixed by the bacteria, the plant transmits carbohydrates produced during the photosynthesis to fuel the bacteria (Oldroyd

& Downie, 2008). In soybean, the main bacteria species that associates with the plant is *Bradyrhizobium japonicum* (Schubert et al., 1978).

Nitrogen fixation contribution of legume-rhizobia symbiosis ranges from 126 to 319 kg N ha^{-1} in groundnut, 18 to 183 kg N ha^{-1} in common bean, 25 to 100 kg N ha^{-1} in cowpea, and 33 to 643 kg N ha^{-1} in soybean; being soybean the species that fix more atmospheric nitrogen of all cultivated legumes (Peoples & Craswell, 1992). Biological nitrogen fixation is responsible for about 65% of the nitrogen currently used in agriculture in developing countries, where the price of synthetic fertilizers is prohibitive, and ensures adequate supply of N when chemical fertilizers cannot be applied (Gopalakrishnan et al., 2015).

In the past decades there has been a lot of concern about environmental pollution caused by the excessive use of chemical pesticides, such as ground water and drinking water pollution, and how to reduce chemical inputs in a sustainable way (de Weger, 1995). In this context, research on BNF have a lot to contribute for a sustainable agriculture since it eliminates the risk of groundwater contamination by nitrate, provides vegetative cover of the soils reducing the risk of erosion, and a is a "free" source of nitrogen for the plants (Peoples & Craswell, 1992). The use of a grain legume plant can also benefit the following cereal crops as the N fixed by the legume remains in the soil. For instance, soybean followed by maize increased 0.49 t ha^{-1} the following corn crop due to the rotation and to the BNF capacity of the soybean (Peoples & Craswell, 1992). For these reasons legume nitrogen fixation has been identified as a useful tool to reduce the need for nitrogen fertilizers while helps crops to yield more (Sibponkrung et al., 2020).

According to Koester et al. (2014), on 84 years of soybean breeding, yield increases have been achieved due to increases in total aboveground biomass and particularly in seed biomass,

mainly driven by improvement in seed partitioning efficiency or harvest index. However, the increase in seed yield over the years, has been associated with larger nitrogen demand that may not be met exclusively by BNF (Balboa et al., 2018). Therefore, improving soybean BNF at the same time that yield improves is important to increase food supply in a sustainable way without having to depend on only synthetic fertilizers.

Many factors modulate the efficiency of BNF, including the bacterial strain (Schubert et al., 1995), the plant genotype (Dhanapal et al., 2015), the interaction between plant and bacterial genotype (Heath, 2010), and the physiological status of the host plant, which is highly dependent upon environmental conditions (Montanez et al., 1995; King & Purcell, 2001). Environmental stresses such as drought and high temperature can reduce plant photosynthesis and growth, decreasing the amount of sugars that the plant sends to fuel the bacteria resulting in a drop in BNF (Montanez et al., 1995; King & Purcell, 2001). However, the specificity of the plant and bacterial genotype relationship can be leveraged to maintain higher N_2 fixations. Schubert et al., (1978) was able to select *Bradyrhizobium japonicum* strains that were more effective fixing nitrogen and therefore resulted in higher biomass production. In addition, the selection of rhizobium strains can improve crops response to environmental stresses such as low ambient temperatures (Montanez et al., 1995) and elevated CO_2 (Bertrand et al., 2007; Sanz-Saez et al., 2012) by maintaining a higher N_2 fixation under these stressful conditions. Optimizing the numbers and effectiveness of rhizobia through strain selection and inoculation techniques is a strategy to increase the amount of N_2 fixed by legumes (Peoples & Craswell, 1992).

The nitrogen fixation can also be improved by plant selection and breeding since nitrogen fixation is also controlled by the plant's genes (Dhanapal et al., 2015). For example, cultivars with bigger nodules are able to fix more nitrogen than cultivars with smaller nodules (Schubert

et al., 1978; King & Purcell, 2001). Therefore, exploit cultivar host vs. strain specificity is a strategy to enhance nitrogen fixation by legumes and obtain consistent yield responses. Efficient nitrogen fixation also requires adequate numbers of effective rhizobia species in the rhizosphere. Rhizobia occupying root nodules can come from either commercial inoculant applied to seed at sowing or from indigenous populations in the soil (Grossman et al., 2011). Treatments that increase the most efficient *Bradyrhizobium* populations in the soil and therefore in the nodules tend to increase BNF (Sanz-Saez et al., 2015).

Effect of PGPR on plant growth

Lorenz Hiltner, more than a century ago, first coined the term "rhizosphere" referring to the area around the plant roots that is colonized by microorganisms influenced by exudates released from the roots (Hiltner, 1904; Hartmann et al., 2008). Root exudates include low molecular weight compounds such as organic acids, amino acids, proteins, sugar, phenolics, and other secondary metabolites (McNear, 2013) that serve as a rich nutrient and energy source for microorganisms, increasing the number of bacteria within the rhizosphere (Gray & Smith, 2005). For example, root mucilage, a high molecular weight material in root exudates, purified from pea can be used as sole carbon source for growth of *Rhizobium leguminosarum* 8401, a pea rhizosphere bacteria (Knee et al., 2001). Living roots can release anywhere from 1 to 10 g of exudates per 100 g root dry weight (Newman, 1985), which represents 5 to 21% of the carbon fixed by the plant in the photosynthesis (Govindasamy et al., 2010). While these soil microorganisms utilize the nutrients present in the root exudates released by the plant host, in return they also secrete metabolites into the rhizosphere that can influence the plant growth (Van Loon, 2007).

Recently, plant growth-promoting rhizobacteria (PGPR) emerged as an important and promising tool for sustainable agriculture (Bishnoi, 2015). The term PGPR was first defined by Kloepper and Schroth in 1978 as a part of the rhizosphere microbiota that competitively colonize plant roots living from the root exudates and resulting in plant growth promotion (Kloepper & Schroth, 1978). PGPRs can form symbiotic, associative, or neutral associations with plants affecting the host growth and development when applied to seed surfaces through inoculation or directly to the soil (Swarnalakshmi et al., 2020).

Although the concentration of bacteria in the rhizosphere is high, microbial colonization is not uniform on the root surface, where only 15- 40% of the total plant root surface is generally occupied by microbial cells, and the density of microorganisms is influenced by nutrient availability in the rhizosphere (McNear, 2013). Root colonization refers to the bacterial attachment to the plant roots (Chandra et al., 2015). PGPR strains must be able to survive and grow in the rhizosphere in free living form until they find a root (Lugtenberg & Kamilova, 2009). After the bacteria encounter the root, the PGRR will attach to the root surface and exert its plant growth promoting beneficial mechanisms, such as the production of phytostimulators (e.g. hormone auxin) that promotes plant growth (Lugtenberg & Kamilova, 2009). Many factors, such as the plant genotype, and even different cultivars of the same plant species, can select for or against certain microbial populations within the rhizosphere (Danhorn & Fuqua, 2007; Morrissey et al., 2004).

The production of plant growth-promoting substances has been reported for many bacterial species. For example, bacterial strains from the genera *Pseudomonas, Azospirillum, Azotobacter, Bacillus, Klebsiella, Enterobacter, Xanthomonas, Arthrobacter, Burkhoolderia, Paenibacillus* and *Serratia* have been shown to have PGPR activity (Khalid et al., 2004; Spaepen

et al., 2009). Among these genera, *Pseudomonas* and *Bacillus* are the most predominant bacteria reported with PGPR activity (Podile & Kishore, 2007). The genera *Bacillus* have been closely studied due to their advantageous physiological traits such as spore-forming ability that contributes to their survival in the soil or in laboratory conditions for prolonged periods of time (Kumar et al., 2011). The spore-forming activity is a natural advantage of certain Gram-positive bacteria which can be formulated effectively into commercial products (Emmert & Handelsman, 1999). Many *Bacillus* strains have been identified as PGPR with commercial potential use as biofertilizers and biocontrol agents (Govindasamy et al., 2010). Within this genera, strains of *Bacillus velezensis* (Bv), previously known as *Bacillus amyloliquefaciens* subsp. *plantarum*, BAC03 (Meng et al., 2016) and FZB42 exert plant growth promoting activities through efficient colonization of plant roots (Idris et al., 2007).

PGPR can affect plant growth in two ways: 1) directly and 2) indirectly. The direct mechanisms of plant growth promotion by PGPR consist of providing plants with compounds synthesized by the bacteria that promote a specific reaction such as stomatal closure that increases water use efficiency, promotes root growth that increase water uptake and facilitates the uptake of specific nutrients from the soil by helping in the solubilization (Glick, 1995). PGPRs can directly alter root architecture (Kloepper et al., 2007) and promote plant development through the production of phytohormones such as indole-3-acetic acid (IAA), gibberellic acid, and cytokines (Goswami et al., 2016). The IAA accelerates plant growth because it stimulates cell division, and it is also an essential hormone regulating nodule formation and therefore it could increase BNF (Vessey, 2003, Gopalakrishnan et al., 2015). It has been documented that some PGPRs produce phytosiderophores that helps in the absorption of minerals such as iron (Kloepper et al., 1980), and produce some organic acids that can help in the solubilization of phosphorus

(Ashrafuzzaman, et al., 2009). In a greenhouse experiment performed in soybean, the application of a PGPR strain (*Bacillus cereus* GS6) promoted root growth, phosphorus solubilization, improved nodulation, and increased nodule N_2-fixation efficiency resulting in plant growth stimulation and higher yield (Arif et al., 2017). Indirectly, PGPRs can promote plant growth and health by suppression of plant pathogens or by induction of resistance against specific pathogens (Gopalakrishnan et al., 2015). Therefore, PGPRs are beneficial microbes which can act as environmentally friendly alternatives for agrochemicals increasing the sustainability of agriculture (Lugtenberg et al., 2013). The use of microbial agents for improving agricultural production and plant health had been practiced for centuries as an alternative for the use of chemical fertilizers and agrochemicals (Gopalakrishnan et al., 2015). It is known that PGPR can promote plant growth, but the impact on nutrient uptake such as BNF and P absorption is a newer theme that requires further investigation (Adesemoye & Kloepper, 2009).

Most of the experiments with PGPRs have been performed under controlled environmental conditions, such as greenhouses. In addition, these studies are usually performed in small pots where the soil can be easily sterilized to ensure that the introduced PGPR gets established into the rhizosphere. However, when PGPRs are applied into agricultural soils under field conditions, their efficacy is more variable (Hassan, 2016). The lack of consistent success suggests that maybe the introduced microbial population declines rapidly after the introduction of the bacterial population (Di Cello et al., 1997, van Veen et al., 1997). The decline in the PGPR population it is thought to be caused by the inability of the inoculated PGPR to compete with the native rhizosphere microbiota of that specific soil (Herschkovitz et al., 2005) and by environmental factors that can limit the PGPR population size and activity (Martínez-Viveros et al., 2010). PGPRs that are adapted to agricultural soils usually show their adaptability by a faster

growth rate, and high versatility to metabolize various natural and xenobiotic compounds (Bhattacharyya & Jha, 2012). For example, to be successful in the rhizosphere, some PGPRs are able to degrade organic compounds from the root exudates to use as a carbon source (Hassan, 2016) and maintain a critical population density for a longer period of time (Bhattacharyya & Jha, 2012). It has been tested that to observe a clear effect on plant growth promotion, it is necessary that the PGPR maintains a population of 10^3-10^4 cells per gram of root tissue (Spaepen et al., 2009). If we intend to increase the sustainability of agricultural systems using PGPRs as biofertilizers to increase plant growth and nutrient efficiency; we need to find new strategies to increase the survival and competitiveness of the PGPRs in agronomical soils in order to unleash their full potential.

Pectin and Orange Peel as an amendment to improve PGPR response in filed conditions

Researchers at Auburn University have demonstrated that some PGPR strains of the Bv species can use purified pectin as their sole carbon source, increasing their survival in the soil and promoting soybean growth and nodulation (Hassan et al., 2019). Pectin was first isolated and described by Henri Braconnot in 1825 (Braconnot, 1825) and consists of complex polysaccharides present in the primary cell walls in plants, such as cellulose and hemicellulose, and middle lamella between cells binding them together (Willats et al., 2001; Hassan, 2016). Pectin is present in many lignocellulosic plant materials such as leaves, stems, fruits, and seeds; however, the availability of pectin varies between plant species and organs (Srivastava & Malviya, 2011).

Pectinase, the pectinolytic enzyme that breakdown pectin, is produced by many bacteria, fungi, and higher plants (Namasivayam et al., 2011). Some bacteria are pectate-lyase-producing species, they can release pectinase, degrade pectic substances, and absorb pectin-derived sugars

such as glucuronate or galacturonate as primary carbon source (Mekjian et al., 1999). Studies performed at Auburn University showed that purified pectin can enhance PGPR benefits in soybean because some PGPR strains have the ability to degrade pectin or a pectin-rich amendment and use it as a sole carbon source (Hassan et al., 2019). Pectin used as C source could contribute for an efficient root colonization producing a major effect in the plant (Hossain et al., 2015). Selected *Bacillus* strains have the capacity to produce and secrete large quantities (20-25 g/L) of extracellular enzymes (Schallmey et al., 2004). It has been demonstrated that some *Bacillus velezensis* strains metabolize D-galacturonate and D-glucuronate from the pectin compounds present in the soil rhizosphere and use them as a sole carbon source (Hossain et al., 2015). The results support the hypothesis that pectin rich amendments inoculated to seed with selected pectinolytic Bv strains have plant growth-promotion activity. From a total 59 Bv strains, Hassan (2016) observed that Bv AP193 strain has the highest apparent pectate lyase activity, being capable to degrade pure pectin and use it as a sole carbon source. They also found that soybean root and shoot dry weights, and nodule formation increased significantly when the Bv strain AP193 was supplemented with pure pectin.

Although purified pectin has been demonstrated to increase the growth of some Bv strains and promote soybean growth, it is a very expensive chemical to be used as a seed amendment by soybean farmers. Therefore, other cheap pectin rich amendments need to be investigated so they can be applied in combination with PGPRs to increase soybean growth. Pectin is present in most of the plant tissues, but apple pomace and orange peel are the two most important sources of industrial pectin (Thakur et al., 1997). Citrus crops are among the most widely cultivated fruits and more than 80% of the citrus is manufactured to obtain juice, jams, jellies, etc (Sharma et al., 2017). After the industrial extraction of orange juice, large amounts of orange peel remain as by-

product (Yeoh et al., 2008). Citrus peel waste accounts for almost 50% of the wet fruit mass (Sharma et al., 2017). Therefore, orange peel is a good source of pectin as it contains up to 52,9% of pectin yield (Tiwari et al., 2017) and has been identified as a potential candidate to be consumed by PGPR strains with pectinase activity. We hypothesize that increasing the availability of a pectin-rich amendments like orange peel can be a source of carbon and promote the survival and persistence of PGPR within root surface. Moreover, orange peel amendment could be used as a cost-effective inoculant supplement to improve the PGPR efficacy on promoting plant growth in soybean.

Variable soybean cultivar response to inoculation with PGPR and *Bradyrhizobium* strains

Many factors regulate the efficacy of rhizobium response in plants when in symbiosis, including the rhizobium strain (Sanz-Saez et al., 2019), host genotype, and the host genotype × bacterial genotype (G x G) interactions (Heath, 2010). The plant host genotype directly influences nodulation efficacy, which indirectly affects the biological N_2-fixation (Sprent, 1982). Each host cultivar has a variable potential for nitrogen fixation and response toward rhizobial inoculation (Imran et al., 2015). Different soybeans cultivars differ in their tendency to remobilize nitrogen. Cultivars that maintain higher N_2-fixation and dry matter accumulation was associated with higher leaf N concentrations and lower rates of vegetative tissue N remobilization during the reproductive stage (Israel, 1981). The best performance in beans cultivars and rhizobia strains was correlated with the highest %N as ureides in the xylem sap (Hungria & Neves, 1987). Ureides represent the greatest proportion of the N transported in the xylem of soybeans and the synthesis of ureides is largely dependent upon nodulation and N_2-fixation. (McClure & Israel, 1979). Another important factor is that the rhizobia need to survive in the rhizosphere in order to perform their beneficial effects in the plant. It was demonstrated that the compatibility between

the composition of the host plant root exudates, and ability of the PGPR to utilize those compounds has a high influence on the PGPR subsistence (Strigul & Kravchenko, 2006). Therefore, it is important to select the right cultivar and strain to obtain the best symbiotic performance.

Co-inoculation of PGPR with *Rhizobium* species to increase BNF in soybean

There is inconsistence response to microbial introduction in field conditions because sometimes the introduced microorganism have difficult to survive (Van Elsas & Heijnen, 1990). The inconsistent positive results of individual PGPR treatments when applied to field studies can be caused by more competitive soil microbiota that out-competes the selected PGPR (Bashan & de-Bashan, 2005). We believe that this can be solved with the pectin rich amendments. However, preliminary studies performed in Auburn University shows that inoculation with PGPR plus orange peel in agricultural soils with previous history of soybean plantation thus with native *Bradyrhizobium* strains available in the soil, does not result in positive responses of some cultivars. We hypothesize that the lack of response of some cultivars is due either to the lack of synergy between the soybean cultivar and PGPR strain, or the lack of synergy between soybean cultivar and native *Bradyrhizobium* strains. To avoid this problem several authors have tested the concept of synergic co-inoculation. Synergic co-inoculation causes one microorganism to improve the performance of the other one, bringing benefits to the host crop. For instance, the co-inoculation of PGPR with selected rhizobia that are more active than the rhizobia, usually found in agricultural soils, can improve legume growth and yield (Gopalakrishnan et al., 2015). *Azospirillum* (Remans et al., 2008; Hungria et al., 2013), *Azotobacter* (Akhtar et al., 2012; Dashadi et al., 2011), and *Bacillus* (Rajendran et al., 2008; Stajkovic et al., 2011) are examples of PGPR genera that have been successfully used as co-inoculants with rhizobia in legume crops.

Some PGPR strains can also improve nodulation and nitrogen fixation when combined with selected rhizobia in legume crops (Sibponkrung et al., 2020). For example, Sibponkrung et al. (2020) observed that the co-inoculatation of *Bradyrhizobium diazoefficiens* USDA 110 (previously named *Bradyrhizobium japonicum* USDA 110) with *Bacillus velezensis* strain S141 increased nodulation (29.4%) and N_2 fixation efficiency (55.8%) in soybean when compared to single inoculation of USDA110. The helpful effect of PGPR co-inoculated with rhizobia on legumes nodulation has been applied due to the PGPR ability to synthesize different phytohormones (Drogue et al., 2013). The nod genes of *Bradyrhizobium* are the responsible for nodule formation in legume plants and these genes are induced by metabolites such as flavonoids or isoflavonoids (Lugtenberg et al., 2013). Prakamhang et al. (2015) also observed significant increase in nifH gene expression level, the gene encoded the structure of dinitrogenase reductase (NifH) which is the enzyme responsible to fix N_2, in soybean root nodules after co-inoculation of *Bradyrhizobium* and PGPR. In nodule formation, phytohormones such as auxins and cytokinins are required for the initiation and elongation of the infection thread (Sibponkrung et al., 2020). According to Prakamhang et al. (2015), the strain S141 produced amounts of indole-3-acetic acid (IAA) into 19.33 $\mu g\ mL^{-1}$ but they did not detect IAA production in USDA110. Therefore, it seems that IAA produced by S141 affect cell elongation of nodules, resulting in larger root nodules. Moreover, not only the IAA biosynthesis pathway was found in S141 but also the cytokinin biosynthesis pathway. Both auxin and cytokinin regulate cortical cell differentiation and proliferation, being crucial for nodule development (Sibponkrung et al., 2020). All of these results seem to point that co-inoculation of PGPR with efficient strain of *Bradyrhizobium* strains in soybean seems to improve plant performance and BNF. However,

these experiments have not been tested under field conditions and in combination with pectin rich amendments that should promote the growth of PGPR.

With the information exposed above, the objectives of the present research are:

1. Test if PGPR plus orange peel amendments inoculated to soybean seeds can improve plant growth and yield in both greenhouse and field experiments.
2. Select the best PGPR strains for their capability to grow in OP and test their performance on soybean growth promotion when inoculated with OP.

References

Adesemoye, A. O., & Kloepper, J. W. (2009). Plant–microbes interactions in enhanced fertilizer-use efficiency. *Applied microbiology and biotechnology*, *85*(1), 1-12.

Akhtar, N., Qureshi, M. A., Iqbal, A., Ahmad, M. J., & Khan, K. H. (2012). Influence of Azotobacter and IAA on symbiotic performance of Rhizobium and yield parameters of lentil. *J Agric Res*, *50*, 361-372.

Arif, M. S., Muhammad, R. I. A. Z., Shahzad, S. M., Yasmeen, T., Shafaqat, A. L. I., & Akhtar, M. J. (2017). Phosphorus-mobilizing rhizobacterial strain Bacillus cereus GS6 improves symbiotic efficiency of soybean on an Aridisol amended with phosphorus-enriched compost. *Pedosphere*, *27*(6), 1049-1061.

Ashrafuzzaman, M., Hossen, F. A., Ismail, M. R., Hoque, A., Islam, M. Z., Shahidullah, S. M., & Meon, S. (2009). Efficiency of plant growth-promoting rhizobacteria (PGPR) for the enhancement of rice growth. *African Journal of Biotechnology*, *8*(7), 1247-1252.

Azizoglu, U. (2019). Bacillus thuringiensis as a biofertilizer and biostimulator: a mini-review of the little-known plant growth-promoting properties of Bt. *Current microbiology*, *76*(11), 1379-1385.

Balboa, G. R., Sadras, V. O., & Ciampitti, I. A. (2018). Shifts in soybean yield, nutrient uptake, and nutrient stoichiometry: A historical synthesis-analysis. *Crop Science*, *58*(1), 43-54.

Bashan, Y., & de-Bashan, L. E. (2005). Plant growth-promoting. *Encyclopedia of soils in the environment*, *1*, 103-115.

Berruti, A., Lumini, E., Balestrini, R., & Bianciotto, V. (2016). Arbuscular mycorrhizal fungi as natural biofertilizers: let's benefit from past successes. *Frontiers in microbiology*, *6*, 1559.

Bertrand, A., Prévost, D., Bigras, F. J., & Castonguay, Y. (2007). Elevated atmospheric CO 2 and strain of rhizobium alter freezing tolerance and cold-induced molecular changes in alfalfa (Medicago sativa). *Annals of Botany*, *99*(2), 275-284.

Bhattacharyya, P. N., & Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*, *28*(4), 1327-1350.

Bishnoi, U. (2015). PGPR interaction: an ecofriendly approach promoting the sustainable agriculture system. *Advances in botanical research*, *75*, 81-113.

Braconnot, H. (1825). Nouvelles observations sur l'acide pectique. In *Annales de Chimie et de Physique* (Vol. 30, pp. 96-102).

Brown, L. (1997). *The Agricultural Link: How Environmental Deterioration Could Disrupt Economic Progress*. Worldwatch Institute, Washington.

Carvalho, F. P. (2006). Agriculture, pesticides, food security and food safety. *Environmental Science and Policy, 9*(7–8), 685–692.

Chandra, D., Srivastava, R., & Sharma, A. K. (2015). Environment-friendly Phosphorus Biofertilizer as an Alternative to Chemical Fertilizers. In Pati B.R. & Mandal S.M. (Eds.) *Recent Trends in Biofertilizers* (pp. 43-71). IK International Publisher, New Delhi.

Cleland, J. (2013). World population growth; past, present and future. *Environmental and Resource Economics, 55*(4), 543-554.

Danhorn, T., & Fuqua, C. (2007). Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol., 61*, 401-422.

Dashadi, M., Khosravi, H., Moezzi, A., Nadian, H., Heidari, M., & Radjabi, R. (2011). Co-inoculation of Rhizobium and Azotobacter on growth indices of faba bean under water stress in the green house condition. *Adv Stud Biol, 3*, 373-385.

de Weger, L. A., van der Bij, A. J., Dekkers, L. C., Simons, M., Wijffelman, C. A., & Lugtenberg, B. J. (1995). Colonization of the rhizosphere of crop plants by plant-beneficial pseudomonads. *FEMS Microbiology Ecology*, *17*(4), 221-227.

Dhanapal, A. P., Ray, J. D., Singh, S. K., Hoyos-Villegas, V., Smith, J. R., Purcell, L. C., ... & Fritschi, F. B. (2015). Genome-wide association analysis of diverse soybean genotypes reveals novel markers for nitrogen traits. *The Plant Genome*, *8*(3), 1-15.

Di Cello, F, Bevivino, A, Chiarini, L, Fani, R, Paffetti, D, Tabacchioni, S, Dalmastri, C (1997) Biodiversity of a Burkholderia cepecia population isolated from the maize rhizosphere at different plant growth stages. *Appl Environ Microbiol 63*(11), 4485–4493.

Drogue, B., Combes-Meynet, E., Moënne-Loccoz, Y., Wisniewski-Dyé, F., & Prigent-Combaret, C. (2013). Control of the cooperation between plant growth-promoting rhizobacteria and crops by rhizosphere signals. *Molecular microbial ecology of the rhizosphere, 1*, 279-293.

Emmert, E. A., & Handelsman, J. (1999). Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiology letters*, *171*(1), 1-9.

FAO, F. (2017). The future of food and agriculture–Trends and challenges. *Annual Report, 296*, 1-180.

Franche, C., Lindström, K., & Elmerich, C. (2009). Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant and soil*, *321*(1), 35-59.

Frink, C. R., Waggoner, P. E., & Ausubel, J. H. (1999). Nitrogen fertilizer: retrospect and prospect. *Proceedings of the National Academy of Sciences*, *96*(4), 1175-1180.

Gilland, B. (2002). World population and food supply: can food production keep pace with population growth in the next half-century?. *Food policy*, *27*(1), 47-63.

Gilland, B. (2014). Is a haber-bosch world Sustainable? Population, nutrition, cereals, nitrogen and environment. *J Soc Polit Econ Stud*, *39*(2), 166-185.

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. *Canadian journal of microbiology*, *41*(2), 109-117.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., ... & Toulmin, C. (2010). Food security: the challenge of feeding 9 billion people. *Science*, *327*(5967), 812-818.

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R. K., Gowda, C. L., & Krishnamurthy, L. (2015). Plant growth promoting rhizobia: challenges and opportunities. *3 Biotech*, *5*(4), 355-377.

Goswami, D., Thakker, J. N., & Dhandhukia, P. C. (2016). Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. *Cogent Food & Agriculture*, *2*(1), 1-19.

Govindasamy, V., Senthilkumar, M., Magheshwaran, V., Kumar, U., Bose, P., Sharma, V., & Annapurna, K. (2010). Bacillus and Paenibacillus spp.: potential PGPR for sustainable agriculture. In Maheshwari D. K. (Ed.), *Plant growth and health promoting bacteria* (Vol. 18, pp. 333-364). Springer, Berlin, Heidelberg.

Gray, E. J., & Smith, D. L. (2005). Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. *Soil biology and biochemistry*, *37*(3), 395-412.

Grossman, J. M., Schipanski, M. E., Sooksanguan, T., Seehaver, S., & Drinkwater, L. E. (2011). Diversity of rhizobia in soybean [Glycine max (Vinton)] nodules varies under organic and conventional management. *Applied Soil Ecology*, *50*, 14-20.

Hartmann, A., Rothballer, M., & Schmid, M. (2008). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant and Soil*, *312*(1), 7-14.

Hassan, M. (2016). *The Role of Pectin Utilization in Root Colonization and Plant Growth-Promotion by Bacillus amyloliquefaciens subsp. plantarum (Bap).* [Master's thesis, Auburn University]

Hassan, M. K., McInroy, J. A., Jones, J., Shantharaj, D., Liles, M. R., & Kloepper, J. W. (2019). Pectin-rich amendment enhances soybean growth promotion and nodulation mediated by Bacillus Velezensis strains. *Plants*, *8*(5), 120.

Heath, K. D. (2010). Intergenomic epistasis and coevolutionary constraint in plants and rhizobia. *Evolution: International Journal of Organic Evolution*, *64*(5), 1446-1458.

Hellriegel, H., & Wilfarth, H. (1888). *Untersuchungen uber die Stickstoffnahrung der Gramineen und Leguminosen.* Buchdruckerei der "Post" Kayssler & Co.

Herschkovitz, Y., Lerner, A., Davidov, Y., Rothballer, M., Hartmann, A., Okon, Y., & Jurkevitch, E. (2005). Inoculation with the plant-growth-promoting rhizobacterium Azospirillum brasilense causes little disturbance in the rhizosphere and rhizoplane of maize (Zea mays). *Microbial Ecology*, *50*(2), 277-288.

Hiltner, L. (1904). Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie und unter Berücksichtigung der Gründüngung und Brache. *Arbeiten der Deutschen Landwirtschaftlichen Gesellschaf*, *98*, 59-78.

Hossain, M.J., Ran, C., Liu, K., Ryu, C.M., Rasmussen-Ivey, C.R., Williams, M.A., Hassan, M.K., Choi, S.K., Jeong, H., Newman, M., Kloepper, J.W., & Liles, M.R. (2015). Deciphering the conserved genetic loci implicated in plant disease control through comparative genomics of Bacillus amyloliquefaciens subsp. plantarum. *Frontiers in plant science*, *6*, 631.

Hungria, M., & Neves, M. C. (1987). Cultivar and Rhizobium strain effect on nitrogen fixation and transport in Phaseolus vulgaris L. *Plant and Soil*, *103*(1), 111-121.

Hungria, M., Nogueira, M. A., & Araujo, R. S. (2013). Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability. *Biology and Fertility of Soils*, *49*(7), 791-801.

Idris, E. E., Iglesias, D. J., Talon, M., & Borriss, R. (2007). Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by Bacillus amyloliquefaciens FZB42. *Molecular plant-microbe interactions*, *20*(6), 619-626.

Igiehon, N. O., & Babalola, O. O. (2017). Biofertilizers and sustainable agriculture: exploring arbuscular mycorrhizal fungi. *Applied microbiology and biotechnology*, *101*(12), 4871-4881.

Imran, A., Mirza, M. S., Shah, T. M., Malik, K. A., & Hafeez, F. Y. (2015). Differential response of kabuli and desi chickpea genotypes toward inoculation with PGPR in different soils. *Frontiers in microbiology*, *6*, 859.

Israel, D. W. (1981). Cultivar and Rhizobium Strain Effects on Nitrogen Fixation and Remobilization by Soybeans 1. *Agronomy Journal*, *73*(3), 509-516.

Kannaiyan, S. (2002) Biofertilizers for sustainable crop production. In Kannaiyan S. (Ed.) *Biotechnology of biofertilizers* (pp. 9-49). Nerosa Publishing House, Delhi.

Khalid, A., Arshad, M., & Zahir, Z. A. (2004). Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. *Journal of Applied Microbiology*, *96*(3), 473-480.

King, C. A., & Purcell, L. C. (2001). Soybean nodule size and relationship to nitrogen fixation response to water deficit. *Crop Science*, *41*(4), 1099-1107.

Kloepper, J. W., Schroth M.N (1978). Plant growth-promoting rhizobacteria on radishes. In Station de Pathologie Vegetale et Phytobacteriologie (Ed.) *Proc. of the 4th Internet. Conf. on Plant Pathogenic Bacter* (Vol. 2, pp. 879-882). Gilbert-Clarey.

Kloepper, J. W. (1994). Plant growth-promoting rhizobacteria (other systems). *Azospirillum/plant associations*, *187*, 137-166.

Kloepper, J. W., Gutierrez-Estrada, A., & McInroy, J. A. (2007). Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria. *Canadian Journal of Microbiology*, *53*(2), 159-167.

Kloepper, J. W., Leong, J., Teintze, M., & Schroth, M. N. (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature*, *286*(5776), 885-886.

Knee, E. M., Gong, F. C., Gao, M., Teplitski, M., Jones, A. R., Foxworthy, A., ... & Bauer, W. D. (2001). Root mucilage from pea and its utilization by rhizosphere bacteria as a sole carbon source. *Molecular Plant-Microbe Interactions*, *14*(6), 775-784.

Koester, R. P., Skoneczka, J. A., Cary, T. R., Diers, B. W., & Ainsworth, E. A. (2014). Historical gains in soybean (Glycine max Merr.) seed yield are driven by linear increases in light interception, energy conversion, and partitioning efficiencies. *Journal of experimental botany*, *65*(12), 3311-3321.

Kumar, A., Prakash, A., & Johri, B. N. (2011). Bacillus as PGPR in crop ecosystem. In Maheshwari, D. K. (Ed.) *Bacteria in agrobiology: crop ecosystems* (pp. 37-59). Springer, Berlin, Heidelberg.

Kumar, V., Rani, A., & Chauhan, G. S. (2010). Nutritional Value of Soybean. In Singh, G. (Ed.) *The soybean: botany, production and uses* (pp. 375-403). CAB International.

Lal, R. (2015). Restoring soil quality to mitigate soil degradation. *Sustainability*, *7*(5), 5875-5895.

Lugtenberg, B. J., Malfanova, N., Kamilova, F., & Berg, G. (2013). Plant growth promotion by microbes. *Molecular microbial ecology of the rhizosphere*, *2*, 561-573.

Lugtenberg, B., & Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual review of microbiology*, *63*, 541-556.

Market Research (2021). *Global Biofertilizer Market Growth 2021-2026*. LP Information, Inc. https://www.marketresearch.com/LP-Information-Inc-v4134/Global-Biofertilizer-Growth-14129963/

Martínez-Viveros, O., Jorquera, M. A., Crowley, D. E., Gajardo, G. M. L. M., & Mora, M. L. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of soil science and plant nutrition*, *10*(3), 293-319.

McClure, P. R., & Israel, D. W. (1979). Transport of nitrogen in the xylem of soybean plants. *Plant Physiology*, *64*(3), 411-416.

McNear Jr, D. H. (2013). The rhizosphere-roots, soil and everything in between. *Nature Education Knowledge*, *4*(3), 1.

Mekjian, K. R., Bryan, E. M., Beall, B. W., & Moran, C. P. (1999). Regulation of Hexuronate Utilization in Bacillus subtilis. *Journal of bacteriology*, *181*(2), 426-433.

Meng, Q., Jiang, H., & Hao, J. J. (2016). Effects of Bacillus velezensis strain BAC03 in promoting plant growth. *Biological Control*, *98*, 18-26.

Mohammadi, K., & Sohrabi, Y. (2012). Bacterial biofertilizers for sustainable crop production: a review. *ARPN J Agric Biol Sci*, *7*(5), 307-316.

Montanez, A., Danso, S. K. A., & Hardarson, G. (1995). The effect of temperature on nodulation and nitrogen fixation by five Bradyrhizobium japonicum strains. *Applied Soil Ecology*, *2*(3), 165-174.

Morrissey, J. P., Dow, J. M., Mark, G. L., & O'Gara, F. (2004). Are microbes at the root of a solution to world food production? Rational exploitation of interactions between microbes and plants can help to transform agriculture. *EMBO reports*, *5*(10), 922-926.

Namasivayam, E., Ravindar, J. D., Mariappan, K., Akhil, J., Mukesh, K., & Jayaraj, R. (2011). Production of extracellular pectinase by Bacillus cereus isolated from market solid waste. *J Bioanal Biomed*, *3*(3), 70-75.

National Agricultural Statistics Service (2022). *NASS – Quick Stats*. US Department of Agriculture. https://quickstats.nass.usda.gov/

Newman, E. I. (1985). The rhizosphere: carbon sources and microbial populations. *Ecological interactions in soil: plants, microbes and animals*, 107-121.

Oldroyd, G. E., & Downie, J. A. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annu. Rev. Plant Biol.*, *59*, 519-546.

Peoples, M. B., & Craswell, E. T. (1992). Biological nitrogen fixation: investments, expectations and actual contributions to agriculture. *Plant and soil*, *141*(1-2), 13-39.

Peoples, M. B., Herridge, D. F., & Ladha, J. K. (1995). Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production?. In *Management of Biological Nitrogen Fixation for the Development of More Productive and Sustainable Agricultural Systems* (pp. 3-28). Springer, Dordrecht.

Pilbeam, D. J. (2015). Breeding crops for improved mineral nutrition under climate change conditions. *Journal of experimental botany*, *66*(12), 3511-3521.

Podile, A. R., & Kishore, G. K. (2007). Plant growth-promoting rhizobacteria. In *Plant-associated bacteria* (pp. 195-230). Springer, Dordrecht.

Postgate, J. R. (1982). Biological nitrogen fixation: fundamentals. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *296*(1082), 375-385.

Prakamhang, J., Tittabutr, P., Boonkerd, N., Teamtisong, K., Uchiumi, T., Abe, M., & Teaumroong, N. (2015). Proposed some interactions at molecular level of PGPR coinoculated with Bradyrhizobium diazoefficiens USDA110 and B. japonicum THA6 on soybean symbiosis and its potential of field application. *Applied Soil Ecology*, *85*, 38-49.

Rajendran, G., Sing, F., Desai, A. J., & Archana, G. (2008). Enhanced growth and nodulation of pigeon pea by co-inoculation of Bacillus strains with Rhizobium spp. *Bioresource technology*, *99*(11), 4544-4550.

Ray, D. K., Mueller, N. D., West, P. C., & Foley, J. A. (2013). Yield trends are insufficient to double global crop production by 2050. *PloS one*, *8*(6), e66428.

Remans, R., Ramaekers, L., Schelkens, S., Hernandez, G., Garcia, A., Reyes, J. L., ... & Vanderleyden, J. (2008). Effect of Rhizobium–Azospirillum coinoculation on nitrogen fixation and yield of two contrasting Phaseolus vulgaris L. genotypes cultivated across different environments in Cuba. *Plant and soil*, *312*(1-2), 25-37.

Sanz-Sáez, Á., Erice, G., Aguirreolea, J., Irigoyen, J. J., & Sánchez-Díaz, M. (2012). Alfalfa yield under elevated CO2 and temperature depends on the Sinorhizobium strain and growth season. *Environmental and experimental botany*, *77*, 267-273.

Sanz-sáez, Á., Heath, K. D., Burke, P. V., & Ainsworth, E. A. (2015). Inoculation with an enhanced N 2-fixing Bradyrhizobium japonicum strain (USDA 110) does not alter soybean (G lycine max M err.) response to elevated [CO2]. *Plant, cell & environment*, *38*(12), 2589-2602.

Sanz-Saez, A., Pérez-López, U., Del-Canto, A., Ortiz-Barredo, A., Mena-Petite, A., Aranjuelo, I., ... & Lacuesta, M. (2019). Changes in environmental CO2 concentration can modify Rhizobium-soybean specificity and condition plant fitness and productivity. *Environmental and Experimental Botany*, *162*, 133-143.

Savci, S. (2012). An agricultural pollutant: chemical fertilizer. *International Journal of Environmental Science and Development*, *3*(1), 77-80.

Schallmey, M., Singh, A., & Ward, O. (2004). Developments in the use of Bacillus species for industrial production. *Canadian Journal of Microbiology*, *50*(1), 1-17.

Schubert, K. R., Jennings, N. T., & Evans, H. J. (1978). Hydrogen reactions of nodulated leguminous plants: II. Effects on dry matter accumulation and nitrogen fixation. *Plant physiology*, *61*(3), 398-401.

Schubert, S. (1995). Nitrogen assimilation by legumes-processes and ecological limitations. *Fertilizer Research*, *42*(1), 99-107.

Sharma, K., Mahato, N., Cho, M. H., & Lee, Y. R. (2017). Converting citrus wastes into value-added products: Economic and environmently friendly approaches. *Nutrition*, *34*, 29-46.

Sibponkrung, S., Kondo, T., Tanaka, K., Tittabutr, P., Boonkerd, N., Yoshida, K. I., & Teaumroong, N. (2020). Co-Inoculation of Bacillus velezensis Strain S141 and Bradyrhizobium Strains Promotes Nodule Growth and Nitrogen Fixation. *Microorganisms*, *8*(5), 678.

Singh, M., Dotaniya, M. L., Mishra, A., Dotaniya, C. K., Regar, K. L., & Lata, M. (2016). Role of biofertilizers in conservation agriculture. In Bisht, J., Meena, V., Mishra, P., & Pattanayak, A. (Eds.) *Conservation Agriculture* (pp. 113-134). Springer, Singapore.

Smil, V. (2008). *Energy in nature and society: general energetics of complex systems*. MIT press.

Spaepen, S., Vanderleyden, J., & Okon, Y. (2009). Plant growth-promoting actions of rhizobacteria. *Advances in botanical research*, *51*, 283-320.

Sprent, J. I. (1982). Nitrogen fixation by grain legumes in the U. K. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *296*(1082), 387-395.

Srivastava, P., & Malviya, R. (2011). Sources of pectin, extraction and its applications in pharmaceutical industry− An overview. *Indian Journal of Natural Products and Resources*, *2*(1), 10-18.

Stajkovic, O., Delic, D., Josic, D., Kuzmanovic, D., Rasulic, N., & Knezevic-Vukcevic, J. (2011). Improvement of common bean growth by co-inoculation with Rhizobium and plant growth-promoting bacteria. *Romanian Biotechnological Letters*, *16*(1), 5919-5926.

Strigul, N. S., & Kravchenko, L. V. (2006). Mathematical modeling of PGPR inoculation into the rhizosphere. *Environmental Modelling & Software*, *21*(8), 1158-1171.

Sutton, M. A., Bleeker, A., Howard, C. M., Erisman, J. W., Abrol, Y. P., Bekunda, M., ... & Zhang, F. S. (2013). *Our nutrient world: The challenge to produce more food & energy with less pollution*. Centre for Ecology & Hydrology, Edinburgh.

Swarnalakshmi, K., Yadav, V., Tyagi, D., Dhar, D. W., Kannepalli, A., & Kumar, S. (2020). Significance of plant growth promoting rhizobacteria in grain legumes: Growth promotion and crop production. *Plants*, *9*(11), 1596.

Thakur, B. R., Singh, R. K., Handa, A. K., & Rao, M. A. (1997). Chemistry and uses of pectin—a review. *Critical Reviews in Food Science & Nutrition*, *37*(1), 47-73.

Tilman, D., Balzer, C., Hill, J., & Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the national academy of sciences*, *108*(50), 20260-20264.

Tiwari, A. K., Saha, S. N., Yadav, V. P., Upadhyay, U. K., Katiyar, D., & Mishra, T. (2017). Extraction and characterization of pectin from orange peels. *International Journal of Biotechnology and Biochemistry*, *13*(1), 39-47.

Van Deynze, A., Zamora, P., Delaux, P. M., Heitmann, C., Jayaraman, D., Rajasekar, S., ... & Bennett, A. B. (2018). Nitrogen fixation in a landrace of maize is supported by a mucilage-associated diazotrophic microbiota. *PLoS biology*, *16*(8), e2006352.

Van Elsas, J. D., & Heijnen, C. E. (1990). Methods for the introduction of bacteria into soil: a review. *Biology and Fertility of Soils*, *10*(2), 127-133.

Van Loon, L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. In *New perspectives and approaches in plant growth-promoting Rhizobacteria research* (pp. 243-254). Springer, Dordrecht.

van Veen, J. A., van Overbeek, L. S., & van Elsas, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews*, *61*(2), 121-135.

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant and soil*, *255*(2), 571-586.

Willats, W. G., McCartney, L., Mackie, W., & Knox, J. P. (2001). Pectin: cell biology and prospects for functional analysis. *Plant molecular biology*, *47*(1-2), 9-27.

Wilson, P. W., & Burris, R. H. (1947). The mechanism of biological nitrogen fixation. *Bacteriological reviews*, *11*(1), 41-73.

Yadegari, M., & Rahmani, H. A. (2010). Evaluation of bean (Phaseolus vulgaris) seeds inoculation with Rhizobium phaseoli and plant growth promoting Rhizobacteria (PGPR) on yield and yield components. *African Journal of Agricultural Research*, *5(9)*, 792-799.

Yeoh, S., Shi, J. T. A. G., & Langrish, T. A. G. (2008). Comparisons between different techniques for water-based extraction of pectin from orange peels. *Desalination*, *218*(1-3), 229-237.

CHAPTER II: The Response of Inoculation with PGPR plus Orange Peel Amendment on Soybean is Cultivar and Environmental Dependent

Abstract

Plant growth-promoting rhizobacteria (PGPR) effects on plant yield are highly variable under field conditions due to competition with soil microbiota. Previous research determined that many *Bacillus velezensis* PGPR strains can use pectin as a sole carbon source and that seed inoculation with a PGPR probiotic and a pectin-rich orange peel (OP) prebiotic can enhance PGPR-mediated increases in plant responses. Because the previous studies used a single soybean cultivar, the objective of this research was to test the effect of PGPR plus OP inoculation on the plant response in a wide range of soybean cultivars. To accomplish this objective, several greenhouse and field experiments were conducted to determine the efficacy of inoculation with *B. velezensis* (Bv) strain AP193 plus OP on several soybean cultivars. A preliminary screening with 20 soybean cultivars in the greenhouse showed that the PGPR plus OP treatment produced a positive increase in all plant growth parameters when all cultivar data was averaged. However, when the inoculation response was examined cultivar by cultivar, there was a range of cultivar response from a 60% increase in growth parameters to a 12% decrease pointing to the presence of a cultivar-PGPR specificity that may influence this prebiotic and probiotic (i.e. synbiotic) response. Further greenhouse and field experiments, studying cultivars that contrast in their response to synbiotic inoculation, revealed that the environment or the capacity of the PGPR to catabolize orange peel may be also playing an important role in the plant synbiotic responsiveness. Future research investigating ways to enhance synbiotic efficacy in seed treatment formulations need to be carried out in greenhouse and field trials.

Abbreviations: **A**, photosynthetic rate; **ANOVA**, analysis of variance; **Bv**, *Bacillus velezensis*; **CFU**, colony forming unit; $\mathbf{g_s}$, stomatal conductance; $\mathbf{J_{max}}$, RuBP regeneration rate; **Ndfa**, Nitrogen derived from the air (%); **OP**, orange peel; **PGPR**, Plant growth promotion rhizobacteria; $\mathbf{Vc_{max}}$, Maximum rubisco carboxylation activity; $\mathbf{WUE_i}$, intrinsic water-use efficiency; $\mathbf{\delta^{15}N}$, Nitrogen isotope discrimination.

Key Words: plant growth promotion rhizobacteria, root growth, nitrogen fixation, yield, photosynthesis, cultivar variation, orange peel.

Introduction

The world population is expected to grow to almost 9.73 billion by 2050 (FAO, 2017) at an annual rate of 77 million people per year (Carvalho, 2006). According to Cleland (2013), the population increase has a direct link to the need for increased food production. To meet the projected global food and fiber demands for a growing population in 2050, current crop production will need to double (Tilman et al., 2011) requiring a ~2.4% growth rate per year (Ray et al., 2013). However, the biggest challenge for modern agriculture is to increase plant productivity in an environmentally sustainable manner (Morrissey et al., 2004). Soil infertility is a major crop yield constraint in developing nations (Mohammadi & Sohrabi, 2012). Chemical fertilizers are the principal input used to increase soil fertility and crop yield. However, excessive use of chemical fertilizers leads to environmental pollution and soil structure degradation (Savci, 2012). In this context, there is a need for technologies to sustainably improve nutrient absorption by plants and reduce the use of chemical fertilizers (Pilbeam, 2015).

Soybean, as a legume, is an important component of crop rotations due the ability to promote nitrogen fertility via biological nitrogen fixation (BNF) via diazotrophic symbionts such as *Bradyrhizobium japonicum* (Grossman et al., 2011) that provide an abundant source of biologically available nitrogen for plants (Peoples & Craswell, 1992). In addition, biofertilizers, substances that contains viable microorganisms capable to enhance nutrient uptake and transportation by plants when applied to the seeds/soil (Azizoglu, 2019), are an environmentally friendly and cost-effective source of plant nutrients that can promote sustainable crop production (Mohammadi & Sohrabi, 2012). PGPR have been used as biofertilizers either by helping to provide nutrients to the plants or by influencing plant growth (Vessey, 2003).

PGPRs are microorganisms that evolved plant associations (Kloepper & Schroth, 1978) and consume root exudates released by the plant host and in return secrete metabolites (e.g. root

hormones such as Indole-3-acetic acid) that can promote plant growth (Van Loon, 2007). For example, bacterial strains from the genera *Pseudomonas, Azospirillum, Azotobacter, Bacillus, Klebsiella, Enterobacter, Xanthomonas, Arthrobacter, Burkhoolderia, Paenibacillus* and *Serratia* have been shown to have PGPR activity (Khalid et al., 2004; Spaepen et al., 2009). The genus *Bacillus* in particular have been studied as PGPR due to their advantageous physiological traits, such as spore-forming ability, that contributes to their survival in soils and a long shelf life that is conducive to field applications (Kumar et al., 2011). Many *Bacillus* strains have been identified as PGPR with commercial potential use as biofertilizers and biocontrol agents (Govindasamy et al., 2010). Within this genus, strains of *B. velezensis* (Bv), previously known as *Bacillus amyloliquefaciens* subsp. *plantarum*, such as Bv BAC03 (Meng et al., 2016) and Bv FZB42 exert plant growth-promoting activities through efficient colonization of plant roots (Idris et al., 2007).

Most of the experiments with PGPRs have been performed under controlled environmental conditions, such as greenhouses. In addition, these studies are usually performed in small pots where the soil can be easily sterilized to ensure that the introduced PGPR gets established into the rhizosphere. However, when PGPRs are used in agricultural soils under field conditions their efficacy is more variable and even inexistent (Hassan, 2016). The lack of consistent success suggests that the introduced microbial population declines rapidly after inoculation (Di Cello et al., 1997, van Veen et al., 1997). The decline in the PGPR population it is thought to be caused by the inability of the inoculated PGPR to compete with the native microbiota of that specific soil (Herschkovitz et al., 2005) and/or by the decrease in the PGPR population and activity due to environmental factors (Martínez-Viveros et al., 2010). To observe significant plant growth promotion, it is necessary that the PGPR maintains a population of 10^3-10^4 cells per gram of root

tissue (Spaepen et al., 2009). PGPRs that are adapted to agricultural soils typically are observed to have a faster growth rate and an ability to metabolize various natural and xenobiotic compounds (Bhattacharyya & Jha, 2012). Furthermore, there are plant cultivar-specific differences that can select for or against rhizosphere microbial populations (Danhorn & Fuqua, 2007; Morrissey et al., 2004).

Previous research determined that PGPR strains of Bv commonly can use pectin as a sole carbon source, and that soybean seed co-inoculation with Bv and purified pectin or pectin-rich orange peel can promote soybean growth and nodulation (Hassan et al., 2019). Pectin was first isolated and described by Henri Braconnot in 1825 (Braconnot, 1825) and consists of complex polysaccharides present in the primary cell walls in plants, such as cellulose and hemicellulose, and middle lamella between cells binding them together (Willats et al., 2001; Hassan, 2016). Although purified pectin has been demonstrated to increase the survival of some Bv strains and promote soybean growth, it is not a cost-effective source of pectin for use as a seed amendment. Therefore, other less expensive, pectin-rich amendments were investigated for compatibility with PGPRs to increase soybean growth. Pectin is present in most of the plant tissues, but apple pomace and orange peel are the two most important sources of industrial pectin (Thakur et al., 1997). Citrus crops are among the most widely cultivated fruits and more than 80% of the citrus is manufactured to obtain juice, jams, jellies, etc. (Sharma et al., 2017). After the industrial extraction of orange juice, large amounts of orange peel remain as by-product (Yeoh et al., 2008). Orange peel is a good source of pectin as it contains up to 52.9% pectin content (Tiwari et al., 2017) and has been identified to be used by PGPR strains such as pectinolytic Bv AP193 (Hassan et al., 2019). Therefore, to increase the sustainability of agricultural systems by using PGPRs as biofertilizers, it is important to research new strategies to improve PGPR efficacy in agricultural soils in

combination with a cost-effective pectin-rich amendment such as orange peel. The primary objective of this study was to test the responsiveness of different soybean cultivars to seed treatment consisting of PGPR and orange peel.

Material and Methods

Preliminary experiment to test soybean cultivar variation to orange peel amendments

In order to test if all soybean cultivars respond the same to inoculation with Bv AP193 and orange peel (OP), a greenhouse experiment with 20 soybean cultivars was performed in December of 2019.

PGPR strains, soybean cultivars and growing conditions

As a substrate for the experiment, Sandy Loam field soil texture was collected from E.V. Smith Research Center in Shorter, AL, USA, specifically from fields with a history of soybean cultivation to ensure viable populations of *B. japonicum*. Standard Classic 400 pots (3.8 liters) were prepared with fabric mesh in the bottom and filled with 4.7 kg of moist soil. Twenty commercially available cultivars were used for this experiment (Table S1). Seeds were surface sterilized in a 2% sodium hypochlorite solution and then washed several times in sterile water to remove chlorine residues as described in Sanz-Saez et al. (2019).

Two treatments were evaluated in this experiment: uninoculated and inoculated seeds with Bv AP193 plus OP, with four replications in total for each treatment. Bv AP193 spores were prepared following the methods of Hassan et al. (2019) and added to each seed at a final concentration of $1x10^6$ spore colony forming units (CFUs) in 50 µL of sterile water. OP powder (Citrus Extracts LLC, Fort Pierce, FL USA) was used to prepare the OP suspension at a final concentration of 10 mg/200 µL per seed.

Treatments were applied on seeds at sowing time. Five seeds were evenly placed 2.5 cm below the soil surface of each pot to ensure germination. Each seed, in the inoculated treatment group, was inoculated first with 200 µL of OP powder solution and then with 50 µL of Bv AP193. The seeds in the uninoculated treatment group received 250 µL of sterile water. Soil was moist at

the moment of planting and no water was added to either treatment group for at least 24-48 h to allow the seeds in the inoculated treatment group to remain in contact with the Bv and OP suspension. After emergence (approximately one week after sowing), only one seedling was kept per pot.

Pots were aligned in rows of four with four pots per row (16 per table) and rearranged in a randomized complete block design within each repetition. The pots were rotated around the tables on the greenhouse each week, preventing any biases based on pot location and light intensity among pots. Artificial LED light was used to maintain a photoperiod of 14 h of light and 8 h of darkness. Temperatures in the greenhouse ranged from 18 to 25 ºC during the day and 10 to 20 ºC at night. Each pot received 500 mL of water every 2 days. Each week plants were sprayed with pesticides to prevent insect infestation.

Physiological and growth parameters measurements

When the plants reached the R2 growth stage (flowering, ~30 days after planting, Fehr et al.,1971), SPAD values, a proxy for chlorophyll concentration, was measured using a SPAD-502 (Minolta, Tokyo, Japan). After that, plants were harvested, and total above ground biomass (g $plant^{-1}$) was calculated by separating leaves and stems and drying them at 60 ºC for at least 72 h and then weighing them on a precision scale. Before drying, total leaf area (cm^2 $plant^{-1}$) was calculated by passing each trifoliated leaf through a LI-3000 Leaf Area Meter (LI-COR Biosciences, Lincoln, Nebraska, USA).

Roots were cleaned after harvesting using tap water and the nodules were separated. Fresh nodules were cleaned and placed over a clean white paper and were imaged with a digital camera. The pictures were analyzed for quantitative nodule characteristics using the ImageJ software, the same way as used in Riedell et al. (2009), and nodule number and size (total cm^2 $plant^{-1}$and

individual nodule cm^2 $plant^{-1}$) were calculated. The imaged nodules were dried at 60 ºC for at least 72 h to determine total nodule dry weight (g $plant^{-1}$). Cleaned roots were scanned in a Winrhizo desk top scanner (Regent Instruments Inc., Sainte-Foye, Quebec, Canada) to calculate total root area (cm^2 $plant^{-1}$), root width (cm $plant^{-1}$), root height (cm $plant^{-1}$) and total root length (cm $plant^{-1}$). After scanning the roots, they were dried at 60 ºC for at least 72 h to calculate total root dry weight (g $plant^{-1}$).

Statistical analysis

A two-way ANOVA was performed for each parameter to test the effect of inoculation (Non-inoculated control and Bv AP193 plus OP), cultivars (Table 1) and their interaction. A two-way ANOVA, with inoculation and cultivars as main factors and replication as random variable, was performed using PROC GLIMMIX in SAS (SAS 9.4, SAS Institute, Cary, NC, USA). When the main effect of inoculation and/or cultivar, or their interaction was significant, the least square means post-hoc test was performed to compare means (LSMEANS, SAS 9.4, SAS Institute, Cary, NC, USA).

Green House Experiment to Test the Response of Inoculation with PGPR plus Orange Peel Amendment on Contrasting Soybean Cultivars

PGPR strains, soybean cultivars and growing conditions

A greenhouse experiment was established at Auburn University from March to May 2020 with three commercial soybean cultivars (S49XT39, AG53X0, and S52XT08) that showed contrasting response to inoculation with Bv AP193 and OP and a non-nodulating soybean cultivar (Lee) as a check to measure nitrogen fixation. Sandy Loam field soil was obtained from E.V. Smith Research Center (Shorter, AL) and used to fill 3.9 gallon pots.

Four treatments per cultivar with five replications were prepared and applied to the seeds: (1) non-inoculated control (NI) prepared by adding water, (2) Bv AP193 alone, (3) OP alone, and (4) Bv AP193 plus OP. Bv AP193 spores were prepared at a final concertation of $1x10^6$ CFU/50 µL per seed. Orange peel powder solution was prepared at a final concentration of 10 mg/200 µL per seed. At sowing, five seeds were evenly placed 2.5 cm below the soil surface. Each seed received the follow inoculations according with the treatment group: (1) 250 µL of sterile water, (2) 50 µL of Bv AP193 spores and 200 µL of distilled water, (3) 200 µL of orange peel powder solution and 50 µL of water, and (4) first 200 µL of orange peel powder solution and then 50 µL of Bv AP193 spores. The planting method was performed as in the preliminary experiment explained above.

Physiological and growth measurements

When the plants reached the R5 developmental growth stage (~60 days after planting), SPAD values, a proxy for chlorophyll concentration, was measured using a SPAD-502 (Konica Minolta Inc. Tokyo, Japan). Mid-day leaf photosynthesis and stomatal conductance was also measured at R5 developmental growth stage (beginning of pod filling, ~60 days after planting, Fehr et al., 1971) on the youngest fully expanded trifoliate leaf in the top of the main stem during 10:30 am to 2 pm using two or three sets of LI-6400XT Portable Photosynthesis System (LI-COR Biosciences, Lincoln NE, USA). Leaf chamber environmental conditions were adapted to meet outside environmental conditions of that day such as light intensity (1500 µmol mol^{-1} PAR), temperature (28 ºC) and relative humidity (65%).

Maximum rates of Rubisco carboxylation (Vc_{max}) and Ribulose 1,5-bisphosphate (RuBP) regeneration rate (J_{max}) were estimated from the response of photosynthesis to intercellular [CO_2] (C_i) as previously described (Sanz-Saez et al., 2017). Briefly, A-c_i curves were measured when

plants were at the beginning of seed filling (R5) according to growth stages defined by Fehr et al. (1971). Photosynthesis was initially measured at the growth [CO_2] (ambient, 410 ppm), and then [CO_2] was reduced stepwise to the lowest concentration of 50 ppm, followed by a stepwise increase to the highest concentration of 1,500 ppm. A total of 11 measurements per curve were recorded. During measurements, the block temperature was set at 28 °C and PPFD was set at saturated light conditions (1,750 μmol m^{-2} s^{-1}). Variables Vc_{max} and J_{max} were calculated using equations developed by Sharkey et al. (2007).

To measure total canopy photosynthesis, a modular transparent custom chamber was designed as a closed system according to Soba et al. (2020). Summarizing, the chamber consisted of a base module to hold the container and seal the chamber, an intermediate transparent module to adjust chamber height, and a top module with ceiling and all sensors and tube fittings. Both the middle and top modules had four fans to ensure air mixing. The top module contains a temperature sensor (LI-1000-8, LI-COR Biosciences, Lincoln, NE, USA) placed under the side frame, a PAR sensor (LI-190, LI-COR Bioscience, Lincoln, NE, USA) on top of the frame, and 5 m of polytetra-fluoroethylene (PTFE) tubing that connects the custom chamber inlet and outlet fittings to the LI-8100 (LI-COR Bioscience, Lincoln, NE, USA) that serves as CO_2 analyser. For purposes of this study, CO_2 fluxes were calculated as temporal changes in CO_2 concentration of air passing through a closed loop in the canopy chamber. Measurements were performed within 90 sec to avoid chamber over-heating. Temperatures were not observed to increase more than 1 ºC during measurements. The CO_2 evolution data were analysed using Soil-Flux-Pro software (LI-COR Biosciences, Lincoln, NE, USA) by fitting a linear regression line to the CO_2 evolution in the chamber, which provides normalized sum of square residuals of the fits and R^2 values.

After the physiological measurements were done, above ground plant organs were separated and total above ground biomass (g plant^{-1}) was calculated by separating leaves, stems, and pods and drying them at 60 ºC for at least 72 h to afterwards weighting them in a precision scale. Before drying, total leaf area (cm^2 plant^{-1}) was calculated by passing each trifoliated leaf through a LI-3000 Leaf Area Meter (LI-COR Biosciences, Lincoln, Nebraska, USA).

The total aboveground biomass including leaves stems and pods was ground to pass a 1 mm screen, weighed into tin capsules, and shipped to the UC-Davis Stable Isotopes Facility (Davis, California, USA) for ^{15}N isotope analysis. Samples were analyzed using an isotope ratio mass spectrometer (IsoPrime, Elementar France, Villeurbanne) coupled to an elemental analyzer (EA3000, EuroVector, Milan, Italy). The natural ^{15}N isotopic ratio (δ^{15}N) in the aboveground biomass was calculated using the formula described by Shearer and Kohl (1986):

$$\delta^{15}N = \frac{R_{sample}}{(R_{air} - 1)} * 1000$$

where, R_{sample} and R_{air} are the isotope ratios (^{15}N/^{14}N) of the sample and air, respectively. The proportion of N derived from the atmosphere (%Ndfa), estimating of the biological nitrogen fixation, was determined by the ^{15}N natural abundance method (Shearer & Kohl, 1986) following the formula:

$$\text{Ndfa (\%)} = \frac{\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{soy}}}{\delta^{15}\text{N}_{\text{soy}} - \text{B}} \times 100,$$

where Ndfa (%) is the percentage of N_2 coming from the atmosphere through BNF, $\delta^{15}N_{ref}$ is the δ^{15}N signature of the non-fixing soybean reference (cultivar Lee) aboveground biomass, $\delta^{15}N_{soy}$: δ^{15}N signature of the above ground biomass for each treatment, and B is the δ^{15}N value of a soybean plant growing in a N free media relying only on BNF as source of N. The B-value used

in our study were obtained as the $\delta^{15}N$ average value (-1.86‰) from previous reports for soybean growing in greenhouse condition (Supplementary Table 1).

Roots were cleaned after harvesting using tap water and the nodules were separated. Nodule and root characteristics were measured as described in the section above.

Statistical analysis

A two-way ANOVA was performed for each parameter to test the effect of inoculation (Control, Bv AP193 alone, OP alone, AP193+OP), cultivar (S49XT39, AG53X0, and S52XT08) and their interaction. A two-way ANOVA, with inoculation and cultivars as main factors and replication as random variable, was performed using PROC GLIMMIX in SAS (SAS 9.4, SAS Institute, Cary, NC, USA). When the main effect of inoculation and/or genotype, or their interaction was significant, least square means post-hoc tests were performed to compare means (LSMEANS, SAS 9.4, SAS Institute, Cary, NC, USA).

Field Experiment to Test the Response of Inoculation with PGPR plus Orange Peel Amendment on Contrasting Soybean Cultivars

Field experimental design and inoculation treatments

During the Summer 2020, field trials were established at two different locations: E.V. Smith Research Center (EVS; Shorter, AL) and Tennessee Valley Research Center (TV; Madison, AL) in a no-tillage system, with rye as winter cover crop. E.V. Smith Research Center has a Piedmont Plateau soil with Compass Loamy Sand texture and the mean, maximum and minimum temperature during the growing season was 22.9, 33.4, and 11ºC, respectively, with a rainfall accumulation of 887.73 mm during the growing season. Tennessee Valley Research Center has a Limestone Valleys and Uplands soil with Decatur Silt Loam soil texture and the mean, maximum and minimum temperature during the growing season was 20.9, 32.1, and 8.8 ºC, respectively,

with a rainfall accumulation of 809.24 mm during the growing season. Soil tests were performed two weeks before planting and fertilizers applied according to the best cultural practices recommended by the Auburn University Extension Soil Fertility Team. Pre-emergence and post emergence herbicides and pesticides were applied following the recommendations of the Alabama Cooperative Extension System.

A randomized complete block design was used for these experiments. Totally, four commercial soybean cultivars (S49XT39, AG53X0, S52XT08, and AG69X0) and a non-nodulating soybean cultivar (Williams 82 NN), as a check for the nitrogen fixation, were evaluated. Four different inoculations were applied at sowing time: 1) non-inoculated (NI), (2) Bv AP193, (3) OP, or (4) Bv plus OP. At sowing, a Bv spore suspension at $1x10^6$ spore CFU/mL and orange peel liquid suspension (1%) was applied in-furrow in the two middle rows to avoid cross plot contamination at the rate of 37.85 liters per hectare, according to sprayer specifications and following the protocol of Hassan et al. (2019). The experimental design had four replicates, with a total of 80 plots at each location. Plots were 20 foot long and consisted of four rows with 36 inches spacing between rows. For all the treatments, a seeding rate of eight seeds per foot was used.

Physiological measurements

When the plants had reached the R2 developmental growth stage (Flowering, Fehr et al., 1971), SPAD values, a proxy for chlorophyll concentration, was measured using a SPAD-502. Mid-day leaf photosynthesis and stomatal conductance was measured at R3 developmental growth stage (First pod, Fehr et al., 1971) in two plants per plot on the youngest fully expanded trifoliate leaf in the top of the main stem during 10:30 am to 2 pm using two sets of LI-6400XT Portable Photosynthesis System (LI-COR Biosciences, Lincoln NE, USA). Leaf chamber environmental

conditions were adapted to meet outside environmental conditions of that day and location such as light intensity, temperature, and relative humidity.

Growth Parameters and %Ndfa calculation

Emergence fifteen days after planting was counted twice per plot as number of seedlings per meter to estimate the percentage of germination. Plant Height (cm) at R2, R5, and R7 was measured in three plants per plot from the soil surface to the apical meristem of the main stem. At pod filling (R3, Fehr et al., 1971) aboveground biomass accumulation was measured by harvesting a total of 0.5 meters where the stems emerge from the soil and dried for 72 h in an industrial forced air heating oven at 60 ºC and later weighted on a precision scale.

The total aboveground biomass including leaves, stems, and pods was ground to pass a 1 mm screen, weighed into tin capsules, and shipped to the UC-Davis Stable Isotopes Facility (Davis, California, USA) for ^{15}N isotope analysis. The nitrogen derived from the atmosphere (Ndfa %) was calculated as described above using the cultivar Williams 82 NN as non nodulating control and a B value of $\delta^{15}N$ =-2.78‰ from previous reports for soybean sampled around R1-R2 developmental stage (Supplementary Table 2).

Root Surface Area (cm^2) and Root Volume (cm^3) at beginning of pod developmental stage (R3, Fehr et al., 1971), was measured by collecting two roots per plot using the shovelomic method (Seethepalli et al., 2020) and stored into plastic bag in a container with ice. The roots were photographed and then analyzed for root parameters using RhizoVisionExplorer (version 2.0.3) software and set up (Seethepalli et al., 2020).

Statistical analysis

A two-way ANOVA was performed for each parameter to test the effect of inoculation (Control, Bv AP193 alone, OP alone, AP193+OP), cultivar (S49XT39, AG53X0, and S52XT08)

and their interaction independently in each location. The two-way ANOVA, with inoculation and cultivars as main factors and replication as a random variable, was performed using PROC GLIMMIX in SAS (SAS 9.4, SAS Institute, Cary, NC, USA). When the main effect of inoculation treatment and/or genotype, or their interaction was significant, least square means post-hoc tests were performed to compare means (LSMEANS, SAS 9.4, SAS Institute, Cary, NC, USA).

Results

Preliminary experiment to test soybean cultivar variation to orange peel amendments

Among the 20 soybeans cultivars tested, the inoculation with Bv plus OP significantly increased plant height (14.3%), leaf area (11.4%), and total aboveground dry weight (13.2%) compared with the non-inoculated treatment (Table 1). Additionally, there was a significant effect of the cultivar variable for those parameters but there was no significant interaction between Cultivar*Inoculation (Table 1). Despite of the lack of Cultivar*Inoculation interaction, the Bv+OP inoculation had a negative impact on plant growth parameters for the cultivar S54XT17, reducing plant height (3%), leaf area (16.5%), and aboveground biomass (15.2%) in contrast with the control treatment. For cultivars AG53X0, LS5588X, and REV4940X the inoculation with Bv+OP also reduced the leaf area and aboveground dry weight (Table 1).

On the other hand, the cultivars G4190RX and S49XT39 had the highest increase on plant height due to the inoculation (50.9% and 38.7%, respectively). For leaf area, cultivars AG69X0, G4190RX, S49XT39, and S52XT08 showed more than 25% increase in inoculated treatment. The cultivar S49XT39 can be highlighted with an 87.3% increase on leaf area with Bv+OP treatment compared with the non-inoculated control. Cultivars S49XT39 and S52XT08 showed a 69.8% and 31.6% increase in dry weight respectively with the inoculation treatment (Table 1).

The inoculation with Bv+OP significantly increased nodule number (22.9%), nodule area (26.4%), nodule dry weight (40.5%), root length (16.5%), and root dry weight (12.5%) (Table 2, 3). For all the nodulation and root growth parameters measured, there was a significant cultivar effect. Additionally, only for the nodule area parameter, there was a significant effect of the Cultivar*Inoculation interaction (Table 2).

For the effect of inoculation on cultivar, as in the aboveground parameters, the cultivar S49XT39 stands out with a 163.1% increase in nodule numbers, 166.3% in nodule area, 275.4% in nodule dry weight, 45.0% in root length, and 73.2% in root dry weight when compared with the NI treatment. The cultivar AG69X0 also showed a 67.5, 101.8, 176.5, 18.7 and 31.3% increase in nodule number, nodule area, nodule dry weight, root area and root dry weight, respectively, with the inoculation. On the other hand, as shown for the aboveground parameters, the inoculation with Bv+OP had a negative impact on the cultivar S54XT17, reducing the nodule number (30.8%), nodule area (34.4%), nodule dry weight (22.9%), and root area (3.9%) relative to non-inoculated plants. Also, there was a decrease on the root growth for AG53X0 and REV4940X when inoculated with Bv plus OP (Table 3). These contrasting results showed that although there was no significant interaction between cultivar and inoculation treatment, the response to inoculation seems to be dependent of the cultivar as it was observed that some cultivars responded positively while others had a negative response to the synbiotic treatment.

Based on these data, we selected three cultivars considered responsive (S49XT39, S52XT08, AG69X0) and one non-responsive (AG53X0) to the synbiotic treatment to study the physiological response of soybean genotype to Bv plus OP inoculation to better understand cultivar variations to inoculation and the factors that can influence this response.

Green House Experiment to Test the Response of Inoculation with PGPR plus Orange Peel Amendment on Contrasting Soybean Cultivars

Growth Parameters

No significant inoculation effect was observed for any of the aboveground plant parameters analyzed in this experiment (Table 4). However, the effect of the Cultivar*Inoculation interaction

was significant for leaf area and aboveground dry weight parameters (Table 4). For the cultivar itself, significant effects were observed for plant height, leaf area, and pod dry weight (Table 4).

There was an increase of leaf area with the inoculation AP193+OP compared with the non-inoculated control (NI) for cultivars AG53X0 (+20.7%) and S49XT39 (+17.2%). In contrast, the inoculation with AP193 plus OP had a negative impact for S52XT08, reducing leaf area by 44.5%, which resulted in a 10.2% decrease of the total aboveground dry weight compared to the non-inoculated control treatment (Table 4).

The S49XT39 cultivar inoculated with AP193 resulted in higher pod (+42.8%) and aboveground biomass (+17.1%; Table 4). The supplement of OP to the inoculation with AP193 did not improve the pod and aboveground dry weights for this cultivar. For cultivar AG53X0, there was no significant effect of the Bv AP193 and OP inoculation on pod dry weight; however, the AP193+OP treatment significantly increased the total aboveground biomass in 5.50 g (+31.8%) in comparison with the non-inoculated treatment (Table 4).

There was a significant effect of the cultivar on nodule number, area, and dry weight (Table 6). However, there was no effect of inoculation or the interaction of Cultivar*Inoculation on the nodulation and nitrogen fixation. In general, AP193 supplemented with OP reduced the nodule number, nodule area, and dry weight when compared with the control (non-inoculated) treatment (Table 6). On the contrary, this treatment increased nodule size (+5.2%) and the nitrogen derived from the air (+8.7%) compared with the control (Table 5). In cultivar AG53X0, the inoculation with AP193+OP showed no positive response on nodulation and nitrogen fixation parameters. The S52XT08 cultivar showed a negative response on nodulation but a no significant increase in nitrogen derived from the air (4.7%) compared with the non-inoculated treatment. In contrast, there was a strong positive response of the AP193+OP inoculation on cultivar S49XT39 with an increase

of 71.2% in nodule number, 65.4% in nodule area, 60.7% in nodule dry weight, and 32.3% in nitrogen derived from the air in comparison with the control treatment (Table 5).

There was no significant effect of the inoculation and the interaction Cultivar*Inoculation on the root growth parameters and there was cultivar effect only for root dry weight (Table 6). The cultivar AG53X0 had the highest root area (1302.86 cm^2), length (7673.44 cm), and dry weight (3.3172 g) when inoculated with the OP treatment alone. The cultivar S49XT39 showed a 34.2% increase in the root and a 15.1% root dry weight when inoculated with AP193. There was a negative effect of the inoculation with AP193+OP on S52XT08 root growth, with a reduction of 19.1% in root area, 22.7% in root length, and 24.1% in root dry weight (Table 6).

Photosynthesis parameters

There was a significant cultivar effect on stomatal conductance (g_s), canopy photosynthesis, and intrinsic water-use efficiency (WUE_i; Fig. 1). The effect of the inoculation and the interaction between factors was significant for both g_s and WUE_i, while it was not significant for photosynthetic rate (A). The g_s was significantly higher (87.3% increase) for the non-inoculated treatment in comparison with plants inoculated with AP193+OP. This resulted in superior WUE_i (A/g_s) for the inoculated plants with AP193+OP in comparison with the control (38.2%). In cultivar S52XT08 the treatment with AP193+OP increased the WUE_i by 113.5% in comparison to the NI treatment. For canopy photosynthesis, the control treatment had higher flux (29.1%) compared with the AP193 plus OP inoculation.

Field Experiment to Test the Response of Inoculation with PGPR plus Orange Peel Amendment on Contrasting Soybean Cultivars

A cultivar effect was observed for plant height at both E.V. Smith (EVS) and Tennessee Valley (TV) locations (Fig. 2). However, there was no effect of inoculation or the interaction

between variables for the plant growth parameters measured. The inoculation with AP193 resulted in higher biomass accumulation (6.1%) at EVS, while at TV the OP was responsible for the highest value (5.1%) compared with the non-inoculated treatment. For plant height, plants maintained the same range on the treatments within cultivars. Cultivar AG53X0 had the total aboveground biomass (+4.2% at EVS and +20.9% at TV) and plant height (+1.6% at EVS and +3.8% at TV) increased with inoculation AP193+OP compared with the non-inoculated treatment at both locations. Therefore, this cultivar was observed to have more consistent positive results in comparison with the other cultivars as some increased growth in one location and decreased it in another.

There was a slight inoculation effect only for nitrogen derived from the air (Ndfa) at EVS (Fig. 3). However, no significant response of the interaction between variables on yield and nitrogen fixation was observed at either location. Individually, cultivar S52XT08 showed the highest Ndfa on the inoculation with AP193+OP (49.73% at EVS and 62.63% at TV), which represents a 24% (EVS) and 14% (TV) increase in comparison with the non-inoculated treatment although this difference was not significant (Fig. 3). The other cultivars showed a reduction on the nitrogen fixation with the inoculation (AP193+OP) in both locations in relation to the control treatment (non-inoculated).

The cultivar AG69X0 showed the greatest yield on the inoculated treatment with AP193+OP (3.2 ton/ha at EVS and 4.1 ton/haat TV), which was a 14.9% (EVS) and 4.1% (TV) yield increase in comparison with the non-inoculated treatment (Fig. 3). For both locations, the inoculations resulted in reduction of yield for S49XT39. For AG53X0 and S52XT08, there was a decrease in yield at EVS and an increase at TV for the inoculated treatments (Fig. 3). Therefore,

for yield gain, AG69X0 was the cultivar with more consistent positive responses to the inoculation with AP193+OP at different environmental conditions.

Discussion

The effect of soybean seed inoculation with Bv AP193 plus OP was previously tested with positive results in greenhouse and field experiments but only for one year experiment and with one soybean cultivar (Hassan et al., 2019). For that reason, this study explored the response of 20 soybean cultivars to inoculation with Bv AP193 supplemented with OP as a seed treatment to assess the consistency of the synbiotic inoculant in promoting plant growth. Our results demonstrate that there was a statistically significant positive effect of the inoculation with PGPR plus OP on plant growth promotion of 13.2% in the above ground biomass (Table 2), 40.5% in the nodule dry weight (Table 3), and 12.5% in root dry weight (Table 4) when the response is averaged across all cultivars. In addition, this significant inoculation effect occurred in the absence of inoculation by cultivar interaction. If these positive results are translated to an improvement in seedling vigor and later yield in the field, this PGPR plus OP inoculation treatment could have a very significant impact on soybean crop production and contribute to improvements of yields across the world.

However, when the effect of inoculation is analyzed by percentage of change for each cultivar, we observed that the response to the synbiotic inoculation was highly cultivar-specific. Cultivars S49XT39, S52XT08, G4190RX, and AG69XT0 were found to have a positive response to the inoculation with several plant growth parameters observed to increase; in contrast, cultivars AG53X0, REV4940X, LS5588X and S54XT17 showed a negative response to the inoculation (Tables 2-4). For example, the inoculation with AP193 plus OP increased root dry weight by 73 and 28% in S49XT39 and S52XT08 cultivars, respectively, while decreasing 12% in cultivar AG53X0 (Table 4). This phenomenon could be caused by different compatibility between cultivars and PGPR strain that is derived from the capability of the PGPR strain to metabolize and use specific root exudates that can vary between the cultivar within each crop species (Strigul &

Kravchenko, 2006), as well as the presence of plant pathogens that could metabolize pectin and the relative susceptibility of the soybean cultivars to those pathogens. It has been found that rhizosphere populations change depending on the soybean cultivar planted (Liu et al., 2019); therefore, it is possible that PGPR compatibility and effectiveness in promoting growth can change with the cultivar of soybean tested. Similarly to our study, Kuzmicheva et al. (2017) found that inoculation with *Pseudomonas oryzihabitans* (strain Ep4) stimulated root growth of the soybean cultivars Nice-Mecha and Svapa which produced more organic acids, meanwhile the cultivar Bara that secreted less organic acids did not show root growth promotion. Although our preliminary results showed a positive trend of inoculation when all the cultivars response was averaged, we have found that some cultivars did not respond to the inoculations like it has been previously demonstrated in other studies (Saubidet et al., 1998; Strigul & Kravchenko, 2006; Remans et al., 2008; Walker et al., 2011; Kuzmicheva et al. 2017). These cultivar by PGPR differential responses could be a constraint for inoculant manufacturers since there is a need for consistently effective biologic inoculants that can broadly be used in agriculture. For that reason, understanding the factors that can affect the genotype by PGPR strain responses need to be further studied.

The different response of the cultivars to the inoculation could also been explained by the response of each cultivar to the OP amendment used in this preliminary study. Although carrier materials can increase plant growth in combination with PGPR, the inoculation method can also cause stress to the introduced bacteria (van Elsas & Heijnen, 1990). Furthermore, OP has a complex chemical composition including phenolic fractions that could affect the PGPR and/or each cultivar performance (Hassan et al., 2019). For that reason, the greenhouse experiment investigated the combined and separate effects of PGPR and OP on a set of cultivars selected for their high responsiveness (S49XT39 and S52XT08) or lack of response (AG53X0) to inoculation.

As in the preliminary greenhouse experiment, the cultivar S49XT39 showed positive plant growth promotion after inoculation with AP193 plus OP with a 9, 60, and 32% increase on above ground biomass, nodule dry weight and nitrogen fixation respectively in comparison with the non-inoculated treatment (Table 5, 6). In contrast, the cultivar AG53X0 that did not show an increase in aboveground biomass with the inoculation in the preliminary experiment, showed a 31% increase in biomass in this experiment. Additionally, one of the other responsive cultivars during the preliminary experiment, S52XT08, showed a negative response to the inoculation. This lack of consistency between experiments could be due to a strong influence of the environment and/or soil microbiota on the plant response to PGPR and OP inoculation. According to Nadeem et al. (2014), the effectiveness of inoculation with PGPRs on plant growth promotion might vary depending on microbial populations and their interactions with environmental factors such as soil nutrition, moisture, and temperature. Since our preliminary experiment was conducted during the winter of 2020 and the second experiment during spring 2020 in a greenhouse, the environmental conditions such as light intensity, quality and temperature may have affected plant responses to synbiotic inoculation. Light intensity and quality can affect photosynthesis (Shafiq et al., 2021), which ultimately affect the amount of root exudates produced by the plant and therefore might interfere in the plant-PGPR crosstalk (Strigul & Kravchenko, 2006).

The detailed greenhouse experiment also showed that inoculated plants with PGPR plus OP had lower stomatal conductance (g_s) without any negative effect on plant photosynthesis, which lead to superior intrinsic water-use efficiency (WUE_i). WUE_i is an instantaneous measurement of the efficiency of carbon gain per water loss. The WUE_i tended to be higher in all the cultivars inoculated with PGPR plus OP but was significantly increased in S52XT08 (Fig. 1). Changes in WUE_i are the result of decreases in transpiration rate or increases in photosynthesis

activity (Fernandes et al., 2016). In maize, soil inoculation with *Burkholderia* sp. LD-$_{11}$ also improved WUE_i, through reduction of stomatal aperture provoked by small increases in abscisic acid (ABA) concentration in the leaves, which also promoted biomass accumulation (Fan et al., 2015). Although in our current experiment we did not measure ABA concentrations, we hypothesize that inoculation with Bv AP193 may produce an increase in WUE_i due to ABA production. These results are supported by peanut experiments performed also at Auburn University where the inoculation with Bv AP203 with OP amendment resulted in increased WUE_i under well-watered and drought stress conditions (Hassan, 2020). These results indicate that inoculation with some PGPR strains plus OP may be an important tool to alleviate water stress and benefit plant survival under water shortage environments.

In the field trials, there was no significant effect of inoculation and the interaction between variables for plant growth, nitrogen fixation, and yield (Fig. 2, 3). Although non-significant, cultivar AG69X0 presented the greatest yield on the PGPR plus OP treatment with 14.9% (EVS) and 4.1% (TV) yield increases in comparison with the non-inoculated control. Although not significant, these increases are considered "acceptable" by the farmers and by inoculant manufacturers (García de Salamone & Di Salvo, 2021) and therefore it should be further studied for commercial application. On the other hand, the other two cultivars showed no effects or even decreases in yield at both locations (Fig. 3). As significant positive effects have been observed in some of the greenhouse experiments, the lack of a significant effect in the field experiment could be attributed to: (1) effects of the environment (Strigul & Kravchenko, 2006; Shafiq et al., 2021); (2) competition with soil indigenous microbiota (Jagnow, 1987; Sanz-Saez et al., 2015; Björklöf et al., 2003, van Veen et al., 1997), (3) influence of parasites and pathogens (Keel et al., 2002; van Veen et al., 1997), or (4) leaching of the inoculum and amendment due to the spray application

and the occurrence of extreme precipitation events. Although Bv AP193 was selected for its capability to grow and consume pectin (Hassan et al., 2019), if the pectin washed away or was diluted in the soil, this may explain the lack of significant effect observed in the field. It could also be that there may be other *Bacillus* strains or pathogenic microorganisms that were able to grow more rapidly on a pectin-rich substrate and therefore may be able to survive better under field conditions. Future experiments should focus on isolating new *Bacillus* strains that catabolize pectin-rich substrates rapidly to produce a better plant response. In addition, it needs to be tested whether seed coating or in-furrow seed treatment will be more effective in producing a stable growth promotion response in multiple cultivars under different environments.

Conclusion

A preliminary experiment showed a significant positive effect of the inoculation with AP193 plus orange peel on plant growth promotion, when analyzed for cultivar-specific responses. However, we observed cultivars with a very positive response and some with no response to inoculation. The cultivar-specific responses may be explained by cultivar-strain crosstalk, where the PGPR is able or not to degrade and use the root exudates as C and energy source, inoculation method used, and/or orange peel composition. Furthermore, we noticed a lack of consistency of results when analyzing the cultivars selected from the preliminary experiment. This can be due to a strong influence of the environment on the plant response to the PGPR inoculation, such as soil nutrient status, moisture, temperature, and light intensity. Also, the competition of the introduced PGPR strain with the soil native bacteria can influence PGPR survival in the rhizosphere, thereby reducing their beneficial effect on plant growth promotion. Further studies are needed to assess the factors that can affect the communication between soybean cultivar and PGPR and ways to enhance the efficacy of a synbiotic treatment in promoting plant growth.

References

Ainsworth, E. A., & Rogers, A. (2007). The response of photosynthesis and stomatal conductance to rising [CO_2]: mechanisms and environmental interactions. *Plant, cell & environment*, *30*(3), 258-270.

Amarger, N., Mariotti, A., Mariotti, F., Durr, J. C., Bourguignon, C., & Lagacherie, B. (1979). Estimate of symbiotically fixed nitrogen in field grown soybeans using variations in 15 N natural abundance. *Plant and Soil*, *52*(2), 269-280.

Araujo, K. E., Vergara, C., Guimarães, A. P., Rouws, J. R., Jantalia, C. P., Urquiaga, S., ... & Boddey, R. M. (2018). Changes in 15 N natural abundance of biologically fixed N 2 in soybean due to shading, rhizobium strain and plant growth stage. *Plant and Soil*, *426*(1), 413-428.

Azizoglu, U. (2019). Bacillus thuringiensis as a biofertilizer and biostimulator: a mini-review of the little-known plant growth-promoting properties of Bt. *Current microbiology*, *76*(11), 1379-1385.

Balboa, G. R., & Ciampitti, I. A. (2020). Estimating biological nitrogen fixation in field-grown soybeans: impact of B value. *Plant and Soil*, *446*(1), 195-210.

Bergersen, F. J., Turner, G. L., Gault, R. R., Chase, D. L., & Brockwell, J. (1985). The natural abundance of 15N in an irrigated soybean crop and its use for the calculation of nitrogen fixation. *Australian Journal of Agricultural Research*, *36*(3), 411-423.

Bhattacharyya, P. N., & Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*, *28*(4), 1327-1350.

Björklöf, K., Sen, R., & Jørgensen, K. S. (2003). Maintenance and impacts of an inoculated mer/luc-tagged Pseudomonas fluorescens on microbial communities in birch rhizospheres developed on humus and peat. *Microbial ecology*, *45*(1), 39-52.

Braconnot, H. (1825). Nouvelles observations sur l'acide pectique. In *Annales de Chimie et de Physique* (Vol. 30, pp. 96-102).

Carvalho, F. P. (2006). Agriculture, pesticides, food security and food safety. *Environmental Science and Policy*, *9*(7–8), 685–692.

Cleland, J. (2013). World population growth; past, present and future. Environmental and *Resource Economics*, *55*(4), 543-554.

Danhorn, T., & Fuqua, C. (2007). Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.*, *61*, 401-422.

Di Cello, F, Bevivino, A, Chiarini, L, Fani, R, Paffetti, D, Tabacchioni, S, Dalmastri, C (1997) Biodiversity of a Burkholderia cepecia population isolated from the maize rhizosphere at different plant growth stages. *Appl Environ Microbiol 63*(11), 4485–4493.

Fan, X., Hu, H., Huang, G., Huang, F., Li, Y., & Palta, J. (2015). Soil inoculation with Burkholderia sp. LD-11 has positive effect on water-use efficiency in inbred lines of maize. *Plant and Soil*, *390*(1), 337-349.

FAO, F. (2017). The future of food and agriculture–Trends and challenges. *Annual Report, 296*.

Fehr, W. R., Caviness, C. E., Burmood, D. T., & Pennington, J. S. (1971). Stage of development descriptions for soybeans, Glycine Max (L.) Merrill 1. *Crop science*, *11*(6), 929-931.

Fernandes, T. J., Del Campo, A. D., Herrera, R., & Molina, A. J. (2016). Simultaneous assessment, through sap flow and stable isotopes, of water use efficiency (WUE) in thinned pines shows improvement in growth, tree-climate sensitivity and WUE, but not in WUEi. *Forest Ecology and Management*, *361*, 298-308.

García de Salamone, I. E., & Di Salvo, L. P. (2021). Interactions Between Plant Genotypes and PGPR are a Challenge for Crop Breeding and Improvement Inoculation Responses. In *Microbiological Activity for Soil and Plant Health Management* (pp. 331-349). Springer, Singapore.

Govindasamy, V., Senthilkumar, M., Magheshwaran, V., Kumar, U., Bose, P., Sharma, V., & Annapurna, K. (2010). Bacillus and Paenibacillus spp.: potential PGPR for sustainable agriculture. In *Plant growth and health promoting bacteria* (pp. 333-364). Springer, Berlin, Heidelberg.

Gray, E. J., & Smith, D. L. (2005). Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. *Soil biology and biochemistry*, *37*(3), 395-412.

Grossman, J. M., Schipanski, M. E., Sooksanguan, T., Seehaver, S., & Drinkwater, L. E. (2011). Diversity of rhizobia in soybean [Glycine max (Vinton)] nodules varies under organic and conventional management. *Applied Soil Ecology*, *50*, 14-20.

Hassan, M. (2016). *The Role of Pectin Utilization in Root Colonization and Plant Growth-Promotion by Bacillus amyloliquefaciens subsp. plantarum (Bap).* [Master's thesis, Auburn University]

Hassan, M. (2020). Enhancing plant growth, drought stress tolerance, and biological control capacities of PGPR strains with exogenous pectin-rich amendments. [Doctoral dissertation, Auburn University]

Hassan, M. K., McInroy, J. A., Jones, J., Shantharaj, D., Liles, M. R., & Kloepper, J. W. (2019). Pectin-rich amendment enhances soybean growth promotion and nodulation mediated by Bacillus Velezensis strains. *Plants*, *8*(5), 120.

Herschkovitz, Y., Lerner, A., Davidov, Y., Rothballer, M., Hartmann, A., Okon, Y., & Jurkevitch, E. (2005). Inoculation with the plant-growth-promoting rhizobacterium Azospirillum brasilense causes little disturbance in the rhizosphere and rhizoplane of maize (Zea mays). *Microbial Ecology, 50*(2), 277-288.

Idris, E. E., Iglesias, D. J., Talon, M., & Borriss, R. (2007). Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by Bacillus amyloliquefaciens FZB42. *Molecular plant-microbe interactions, 20*(6), 619-626.

Jagnow, G. (1987). Inoculation of cereal crops and forage grasses with nitrogen-fixing rhizosphere bacteria: Possible causes of success and failure with regard to yield response–a review. *Zeitschrift für Pflanzenernährung und Bodenkunde, 150*(6), 361-368.

Keel, C., Ucurum, Z., Michaux, P., Adrian, M., & Haas, D. (2002). Deleterious impact of a virulent bacteriophage on survival and biocontrol activity of Pseudomonas fluorescens strain CHA0 in natural soil. *Molecular plant-microbe interactions, 15*(6), 567-576.

Khalid, A., Arshad, M., & Zahir, Z. A. (2004). Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. *Journal of Applied Microbiology, 96*(3), 473-480.

Kloepper, J. W., Schroth M.N (1978). Plant growth-promoting rhizobacteria on radishes. In Station de Pathologie Vegetale et Phytobacteriologie (Ed.) *Proc. of the 4th Internet. Conf. on Plant Pathogenic Bacter* (Vol. 2, pp. 879-882). Gilbert-Clarey.

Kumar, A., Prakash, A., & Johri, B. N. (2011). Bacillus as PGPR in crop ecosystem. In Maheshwari, D. K. (Ed.) *Bacteria in agrobiology: crop ecosystems* (pp. 37-59). Springer, Berlin, Heidelberg.

Kuzmicheva, Y. V., Shaposhnikov, A. I., Petrova, S. N., Makarova, N. M., Tychinskaya, I. L., Puhalsky, J. V., ... & Belimov, A. A. (2017). Variety specific relationships between effects of rhizobacteria on root exudation, growth and nutrient uptake of soybean. *Plant and Soil, 419*(1), 83-96.

Liu, F., Hewezi, T., Lebeis, S. L., Pantalone, V., Grewal, P. S., & Staton, M. E. (2019). Soil indigenous microbiome and plant genotypes cooperatively modify soybean rhizosphere microbiome assembly. *BMC microbiology, 19*(1), 1-19.

Martínez-Viveros, O., Jorquera, M. A., Crowley, D. E., Gajardo, G. M. L. M., & Mora, M. L. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of soil science and plant nutrition, 10*(3), 293-319.

Meng, Q., Jiang, H., & Hao, J. J. (2016). Effects of Bacillus velezensis strain BAC03 in promoting plant growth. *Biological Control, 98*, 18-26.

Mohammadi, K., & Sohrabi, Y. (2012). Bacterial biofertilizers for sustainable crop production: a review. *ARPN J Agric Biol Sci, 7*(5), 307-316.

Morrissey, J. P., Dow, J. M., Mark, G. L., & O'Gara, F. (2004). Are microbes at the root of a solution to world food production? Rational exploitation of interactions between microbes and plants can help to transform agriculture. *EMBO reports*, *5*(10), 922-926.

Nadeem, S. M., Ahmad, M., Zahir, Z. A., Javaid, A., & Ashraf, M. (2014). The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. *Biotechnology advances*, *32*(2), 429-448.

Peoples, M. B., & Craswell, E. T. (1992). Biological nitrogen fixation: investments, expectations and actual contributions to agriculture. *Plant and soil*, *141*(1-2), 13-39.

Pilbeam, D. J. (2015). Breeding crops for improved mineral nutrition under climate change conditions. *Journal of experimental botany*, *66*(12), 3511-3521.

Ray, D. K., Mueller, N. D., West, P. C., & Foley, J. A. (2013). Yield trends are insufficient to double global crop production by 2050. *PloS one*, *8*(6), e66428.

Remans, R., Beebe, S., Blair, M., Manrique, G., Tovar, E., Rao, I., ... & Vanderleyden, J. (2008). Physiological and genetic analysis of root responsiveness to auxin-producing plant growth-promoting bacteria in common bean (Phaseolus vulgaris L.). *Plant and soil*, *302*(1), 149-161.

Riedell, W. E., Catangui, M. A., & Beckendorf, E. A. (2009). Nitrogen fixation, ureide, and nitrate accumulation responses to soybean aphid injury in Glycine max. *Journal of plant nutrition*, *32*(10), 1674-1686.

Sanz-Saez A, Perez-Lopez U, del Canto A, Ortiz-Barredo A, Mena-Petite A, Aranjuelo I, Munoz-Rueda A, Lacuesta M. (2019) Changes in environmental CO_2 concentration can modify *Rhizobium*-soybean specificity and condition plant fitness and productivity. *Environmental Experimental Botany, 162*, 133-143.

Sanz-Sáez, Á., Heath, K. D., Burke, P. V., & Ainsworth, E. A. (2015). Inoculation with an enhanced N 2-fixing B radyrhizobium japonicum strain (USDA 110) does not alter soybean (G lycine max M err.) response to elevated [CO2]. *Plant, Cell & Environment*, *38*(12), 2589-2602.

Sanz-Sáez, Á., Koester, R.P., Rosenthal, D.M., Montes, C.M., Ort, D.R., Ainsworth E.A. (2017). Leaf and canopy scale drivers of genotypic variation in soybean response to elevated carbon dioxide concentration. *Global Change Biology, 23*(9), 3908-3920.

Saubidet, M. I., & Barneix, A. J. (1998). Growth stimulation and nitrogen supply to wheat plants inoculated with Azospirillum brasilense. *Journal of plant nutrition*, *21*(12), 2565-2577.

Savci, S. (2012). An agricultural pollutant: chemical fertilizer. *International Journal of Environmental Science and Development*, *3*(1), 73.

Schipanski, M. E., Drinkwater, L. E., & Russelle, M. P. (2010). Understanding the variability in soybean nitrogen fixation across agroecosystems. *Plant and Soil*, *329*(1), 379-397.

Seethepalli, A., Guo, H., Liu, X., Griffiths, M., Almtarfi, H., Li, Z., ... & York, L. M. (2020). Rhizovision crown: an integrated hardware and software platform for root crown phenotyping. *Plant Phenomics, 2020*, 3074916.

Shafiq, I., Hussain, S., Raza, M. A., Iqbal, N., ASGHAR, M. A., Ali, R. A. Z. A., ... & Feng, Y. A. N. G. (2021). Crop photosynthetic response to light quality and light intensity. *Journal of Integrative Agriculture*, *20*(1), 4-23.

Sharkey, T. D., Bernacchi, C. J., Farquhar, G. D., & Singsaas, E. L. (2007). Fitting photosynthetic carbon dioxide response curves for C3 leaves. *Plant, cell & environment*, *30*(9), 1035-1040.

Sharma, K., Mahato, N., Cho, M. H., & Lee, Y. R. (2017). Converting citrus wastes into value-added products: Economic and environmently friendly approaches. *Nutrition*, *34*, 29-46.

Shearer, G., & Kohl, D. H. (1986). N2-fixation in field settings: estimations based on natural 15N abundance. *Functional Plant Biology*, *13*(6), 699-756.

Soba, D., Shu, T., Runion, G. B., Prior, S. A., Fritschi, F. B., Aranjuelo, I., & Sanz-Saez, A. (2020). Effects of elevated [CO2] on photosynthesis and seed yield parameters in two soybean genotypes with contrasting water use efficiency. *Environmental and Experimental Botany*, *178*, 104154.

Spaepen, S., Vanderleyden, J., & Okon, Y. (2009). Plant growth-promoting actions of rhizobacteria. *Advances in botanical research*, *51*, 283-320.

Strigul, N. S., & Kravchenko, L. V. (2006). Mathematical modeling of PGPR inoculation into the rhizosphere. *Environmental Modelling & Software*, *21*(8), 1158-1171.

Thakur, B. R., Singh, R. K., Handa, A. K., & Rao, M. A. (1997). Chemistry and uses of pectin—a review. *Critical Reviews in Food Science & Nutrition*, *37*(1), 47-73.

Tilman, D., Balzer, C., Hill, J., & Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the national academy of sciences*, *108*(50), 20260-20264.

Tiwari, A. K., Saha, S. N., Yadav, V. P., Upadhyay, U. K., Katiyar, D., & Mishra, T. (2017). Extraction and characterization of pectin from orange peels. *International Journal of Biotechnology and Biochemistry*, *13*(1), 39-47.

Van Elsas, J. D., & Heijnen, C. E. (1990). Methods for the introduction of bacteria into soil: a review. *Biology and Fertility of Soils*, *10*(2), 127-133.

Van Loon, L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. In *New perspectives and approaches in plant growth-promoting Rhizobacteria research* (pp. 243-254). Springer, Dordrecht.

van Veen, J. A., van Overbeek, L. S., & van Elsas, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews*, *61*(2), 121-135.

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant and soil*, *255*(2), 571-586.

Walker, V., Bertrand, C., Bellvert, F., Moënne-Loccoz, Y., Bally, R., & Comte, G. (2011). Host plant secondary metabolite profiling shows a complex, strain-dependent response of maize to plant growth-promoting rhizobacteria of the genus Azospirillum. *New Phytologist*, *189*(2), 494-506.

Willats, W. G., McCartney, L., Mackie, W., & Knox, J. P. (2001). Pectin: cell biology and prospects for functional analysis. *Plant molecular biology*, *47*(1-2), 9-27.

Yeoh, S., Shi, J. T. A. G., & Langrish, T. A. G. (2008). Comparisons between different techniques for water-based extraction of pectin from orange peels. *Desalination*, *218*(1-3), 229-237.

List of Tables

Table 6. Root area, root length and root dry weight of three soybean cultivars (AG53X0, S49XT39, S52XT08) inoculated with Bv AP193, OP, the combination of Bv AP193 plus OP, or the NI control and grown under greenhouse conditions. The bottom section of the table shows the ANOVA results (p-value) for the effect of Cultivar, Inoculation, and the interaction Cultivar*Inoculation.

List of Figures

Table 1.

Cultivar	Inoculation	Plant Height (cm)		Leaf area (cm^2)		Total Aboveground Dry Weight (g)	
		Mean	% Change	Mean	% Change	Mean	% Change
AG44X0	**NI**	16.3	14.0	142.6	10.9	1.5	22.7
	Bv+OP	18.6		158.2		1.8	
AG53X0	**NI**	19.8	3.5	192.6	-5.5	2.0	-12.9
	Bv+OP	20.5		181.9		1.8	
AG69X0	**NI**	37.4	10.9	172.3	33.1	1.7	22.3
	Bv+OP	41.5		229.3		2.1	
CZ 4539GTLL	**NI**	14.1	14.0	116.6	7.0	1.2	12.7
	Bv+OP	16.1		124.7		1.4	
CZ 5859LL	**NI**	30.1	7.4	236.5	9.5	1.9	10.0
	Bv+OP	32.3		258.9		2.1	
CZ 6515LL	**NI**	22.6	18.9	149.5	22.0	1.6	27.0
	Bv+OP	26.8		182.3		2.1	
G4190RX	**NI**	14.5	50.8	134.1	26.7	1.5	25.4
	Bv+OP	21.9		170.0		1.9	
G5000RX	**NI**	16.6	9.9	150.5	13.4	1.5	7.8
	Bv+OP	18.3		170.6		1.6	
GoSoy 512E18	**NI**	25.9	25.1	161.5	20.1	1.6	20.1
	Bv+OP	32.5		194.0		1.9	
LS4798X	**NI**	19.2	14.2	160.4	4.2	1.6	13.4
	Bv+OP	22.0		167.1		1.9	
LS5087X	**NI**	16.7	26.2	155.5	18.7	1.4	22.4
	Bv+OP	21.1		184.6		1.7	
LS5588X	**NI**	29.6	5.7	283.7	-7.3	1.9	-3.5
	Bv+OP	31.3		262.9		1.9	
LSX6501XS	**NI**	20.4	8.7	183.6	16.9	1.6	18.5
	Bv+OP	22.2		214.6		1.8	
NKS49-F5X	**NI**	18.0	9.9	164.0	8.8	1.7	9.6
	Bv+OP	19.8		178.5		1.9	
REV 4940X	**NI**	17.4	14.7	148.6	-8.1	1.3	-3.3
	Bv+OP	20.0		136.5		1.3	
REV 5659X	**NI**	28.1	22.8	259.6	14.2	1.9	14.5
	Bv+OP	34.5		296.6		2.1	
S49XT39	**NI**	17.1	38.7	108.2	87.3	1.0	69.8
	Bv+OP	23.7		202.6		1.7	
S52XT08	**NI**	19.5	22.8	192.9	26.6	1.7	31.6
	Bv+OP	23.9		244.3		2.2	
S54XT17	**NI**	33.9	-3.0	280.6	-16.5	2.0	-15.2
	Bv+OP	32.9		234.2		1.7	
S56XT99	**NI**	32.9	6.0	240.4	6.1	1.8	8.1
	Bv+OP	34.9		255.0		2.0	
NI Mean		22.5 b	14.3	181.7 b	11.4	1.6 b	13.2
Bv+OP Mean		25.7 a		202.4 a		1.8 a	
2-WAY ANOVA RESULTS							
Cultivar (P-value)		<.0001		<.0001		0.0361	
Inoculation (P-value)		0.0002		0.0107		0.0032	
Cultivar*Inoculation		0.994		0.7378		0.8728	

[1]NI – non-inoculated; [2]Bv+OP – *Bacillus velezensis* plus orange peel

Table 2.

Cultivar	Inoculation	Nodule Number		Nodule Area (cm²)		Nodule Dry Weight (g)	
		Mean	% Change	Mean	% Change	Mean	% Change
AG44X0	**NI**	36.2	-3.4	2.2	1.9	0.045	18.3
	Bv+OP	35.0		2.2		0.053	
AG53X0	**NI**	24.2	12.4	1.5	15.9	0.043	25.6
	Bv+OP	27.2		1.8		0.053	
AG69X0	**NI**	30.0	67.5	1.7	101.8	0.041	176.5
	Bv+OP	50.2		3.5		0.114	
CZ 4539GTLL	**NI**	18.7	49.3	1.2	43.2	0.025	82.1
	Bv+OP	28.0		1.7		0.045	
CZ 5859LL	**NI**	31.2	-2.4	2.1	15.3	0.057	30.7
	Bv+OP	30.5		2.4		0.074	
CZ 6515LL	**NI**	23.9	49.8	1.8	42.1	0.051	40.3
	Bv+OP	35.8		2.6		0.071	
G4190RX	**NI**	17.6	68.7	1.3	63.1	0.032	89.8
	Bv+OP	29.7		2.2		0.062	
G5000RX	**NI**	25.7	36.9	1.8	9.5	0.052	0.0
	Bv+OP	35.2		2.0		0.052	
GoSoy 512E18	**NI**	29.7	-10.9	2.3	-5.7	0.064	7.8
	Bv+OP	26.5		2.1		0.069	
LS4798X	**NI**	32.2	11.6	1.8	17.3	0.054	-0.8
	Bv+OP	36.0		2.1		0.054	
LS5087X	**NI**	22.2	52.8	1.1	121.3	0.024	218.0
	Bv+OP	34.0		2.5		0.076	
LS5588X	**NI**	23.2	41.9	1.5	21.9	0.042	25.3
	Bv+OP	33.0		1.8		0.052	
LSX6501XS	**NI**	32.2	35.7	2.1	28.7	0.054	45.2
	Bv+OP	43.7		2.8		0.079	
NKS49-F5X	**NI**	37.0	26.3	2.3	11.1	0.061	8.8
	Bv+OP	46.7		2.6		0.067	
REV 4940X	**NI**	40.2	-32.9	2.2	-19.7	0.047	4.7
	Bv+OP	27.0		1.7		0.050	
REV 5659X	**NI**	40.2	6.2	2.3	37.8	0.049	71.2
	Bv+OP	42.7		3.2		0.084	
S49XT39	**NI**	16.2	163.1	0.9	166.3	0.016	275.4
	Bv+OP	42.7		2.5		0.061	
S52XT08	**NI**	30.0	55.0	1.6	88.5	0.040	108.5
	Bv+OP	46.5		3.1		0.083	
S54XT17	**NI**	45.5	-30.8	3.7	-34.4	0.101	-22.9
	Bv+OP	31.5		2.4		0.078	
S56XT99	**NI**	35.0	27.9	2.5	18.9	0.062	17.8
	Bv+OP	44.7		2.9		0.072	
NI Mean		29.6 b	22.9	1.9 b	26.4	0.048 b	40.5
Bv+OP Mean		36.3 a		2.4 a		0.067 a	
2-WAY ANOVA RESULTS							
Cultivar (P-value)		0.0007		0.0003		0.0017	
Inoculation (P-value)		<.0001		<.0001		<.0001	
Cultivar*Inoculation		0.0323		0.0171		0.0443	

[1]NI – non-inoculated; [2]Bv+OP – *Bacillus velezensis* plus orange peel

Table 3.

Cultivar	Inoculation	Root Area (cm^2)		Root Length (cm)		Root Dry Weight (g)	
		Mean	% Change	Mean	% Change	Mean	% Change
AG44X0	**NI**	361.6	5.8	2935.6	29.8	0.36	11.1
	Bv+OP	382.7		3810.7		0.40	
AG53X0	**NI**	417.5	-3.9	4175.2	-11.9	0.49	-12.7
	Bv+OP	401.1		3679.1		0.43	
AG69X0	**NI**	330.5	18.7	3584.6	30.4	0.40	31.3
	Bv+OP	392.3		4673.4		0.52	
CZ 4539GTLL	**NI**	326.0	9.5	2188.8	19.5	0.32	-13.1
	Bv+OP	356.8		2616.0		0.28	
CZ 5859LL	**NI**	383.3	-3.4	3896.8	3.7	0.48	1.4
	Bv+OP	370.3		4042.4		0.49	
CZ 6515LL	**NI**	374.4	6.3	3038.3	38.0	0.35	32.5
	Bv+OP	397.9		4192.4		0.47	
G4190RX	**NI**	392.3	0.0	2951.2	18.0	0.39	11.7
	Bv+OP	392.5		3483.8		0.44	
G5000RX	**NI**	351.3	7.4	2702.1	31.8	0.37	9.3
	Bv+OP	377.4		3562.1		0.41	
GoSoy 512E18	**NI**	413.6	-4.2	3078.5	19.4	0.38	16.9
	Bv+OP	396.1		3676.9		0.44	
LS4798X	**NI**	376.3	10.5	3614.3	11.0	0.37	-6.3
	Bv+OP	415.7		4010.9		0.34	
LS5087X	**NI**	416.1	-1.9	2779.3	16.9	0.30	31.2
	Bv+OP	408.0		3249.2		0.39	
LS5588X	**NI**	395.2	2.4	3958.7	11.4	0.49	2.5
	Bv+OP	404.6		4408.4		0.51	
LSX6501XS	**NI**	399.9	-2.8	3638.0	13.3	0.42	32.2
	Bv+OP	388.5		4122.7		0.55	
NKS49-F5X	**NI**	378.4	3.4	3350.6	9.7	0.42	4.8
	Bv+OP	391.5		3675.9		0.44	
REV 4940X	**NI**	362.9	-13.2	3327.2	-7.5	0.35	-4.5
	Bv+OP	315.0		3077.4		0.34	
REV 5659X	**NI**	364.1	5.2	3489.3	20.4	0.57	-4.0
	Bv+OP	383.1		4200.9		0.55	
S49XT39	**NI**	320.4	21.8	2567.2	45.0	0.24	73.2
	Bv+OP	390.3		3722.4		0.41	
S52XT08	**NI**	362.9	8.3	3535.3	24.5	0.43	28.3
	Bv+OP	393.2		4402.1		0.55	
S54XT17	**NI**	379.4	-3.8	3601.5	4.7	0.35	12.9
	Bv+OP	364.9		3770.3		0.39	
S56XT99	**NI**	375.3	12.4	4033.0	25.6	0.46	27.5
	Bv+OP	421.9		5064.8		0.59	
NI Mean		374.1	3.5	3322.3 b	16.5	0.40 b	12.5
Bv+OP Mean		387.2		3872.1 a		0.45 a	
2-WAY ANOVA RESULTS							
Cultivar (P-value)		0.0146		<.0001		<.0001	
Inoculation (P-value)		0.0529		<.0001		0.0088	
Cultivar*Inoculation		0.5024		0.9111		0.8248	

[1]NI – non-inoculated; [2]Bv+OP – *Bacillus velezensis* plus orange peel

Table 4.

Cultivar	Inoculation	Plant Height (cm)	Leaf area (cm^2)	Pod Dry Weight (g)	Aboveground Dry Weight (g)
AG53X0	NI	55.5	1394.2 b	7.7	17.3 bcd
	AP193	58.5	1451.8 b	4.7	15.0 d
	OP	56.4	1479.8 b	6.5	17.2 bcd
	AP193+OP	51.2	1682.6 b	7.6	22.8 a
S49XT39	NI	59.7	1409.1 b	5.8	16.5 bcd
	AP193	59.6	1498.3 b	8.3	19.3 abc
	OP	58.5	1270.5 b	6.3	16.0 bcd
	AP193+OP	58.2	1652.0 b	6.3	18.1 bcd
S52XT08	NI	54.1	2553.1 a	5.2	19.9 a
	AP193	45.5	1632.1 b	5.4	15.9 cd
	OP	48.1	2928.5 a	4.3	18.3 bcd
	AP193+OP	43.0	1415.8 b	5.4	17.9 bcd
NI Mean		56.4	1785.5	6.2	17.9
AP193 Mean		54.5	1527.4	6.1	16.7
OP Mean		54.4	1892.9	5.7	17.2
AP193+OP Mean		50.8	1583.5	6.4	19.6
2-WAY ANOVA RESULTS					
Cultivar (P-value)		<.0001	0.0003	0.0354	0.8293
Inoculation (P-value)		0.2382	0.1839	0.8263	0.1502
Cultivar*Inoculation		0.6569	0.0019	0.142	0.0343

[1]NI – non-inoculated; [2]AP193 – *Bacillus velezensis* strain AP193; [3]OP – orange peel

Table 5.

Cultivar	Inoculation	Nodule Number	Nodule Area (cm^2)	Nodule Dry Weight (g)	Nodule size (mm^2)	Ndfa (%)
AG53X0	**NI**	219.2	12.1	0.51	5.2	48.6
	AP193	164.2	4.8	0.15	3.0	26.9
	OP	248.4	11.4	0.47	4.8	49.8
	AP193+OP	178.0	8.8	0.37	5.7	45.3
S49XT39	**NI**	166.6	7.8	0.27	4.3	41.3
	AP193	281.0	13.2	0.44	4.6	42.8
	OP	280.6	11.2	0.38	4.1	42.2
	AP193+OP	285.2	13.0	0.43	4.6	54.6
S52XT08	**NI**	367.0	17.8	0.70	4.9	54.4
	AP193	324.6	16.8	0.69	5.1	48.0
	OP	298.4	13.5	0.57	4.6	56.2
	AP193+OP	237.8	10.7	0.47	4.8	57.0
NI Mean		251.0	12.6	0.49	4.8	48.1
AP193 Mean		256.6	11.6	0.43	4.2	39.3
OP Mean		275.8	12.0	0.47	4.5	49.4
AP193+OP Mean		233.7	10.9	0.42	5.0	52.3
Cultivar (P-value)		0.0279	0.0214	0.007	0.5937	0.0677
Inoculation (P-value)		0.8095	0.8763	0.8456	0.4745	0.1083
Cultivar*Inoculation		0.3136	0.1213	0.1633	0.2784	0.5526

[1]NI – non-inoculated; [2]AP193 – *Bacillus velezensis* strain AP193; [3]OP – orange peel

Table 6.

Cultivar	Inoculation	Root Area (cm^2)	Root Length (cm)	Root Dry Weight (g)
AG53X0	NI	1146.4	6609.3	2.86
	AP193	969.6	5543.3	2.56
	OP	1302.9	7673.4	3.32
	AP193+OP	1136.4	5635.3	3.06
S49XT39	NI	1051.1	4993.0	2.80
	AP193	1411.0	6979.5	3.22
	OP	1153.3	7559.8	2.70
	AP193+OP	1210.7	7305.4	2.84
S52XT08	NI	1138.9	6078.3	3.87
	AP193	1139.1	4635.3	3.99
	OP	1052.2	5308.9	3.61
	AP193+OP	921.7	4699.1	2.94
NI Mean		1112.1	5893.6	3.18
AP193 Mean		1173.2	5719.4	3.26
OP Mean		1169.5	6847.4	3.21
AP193+OP Mean		1089.6	5879.9	2.94
2-WAY ANOVA RESULTS				
Cultivar (P-value)		0.4616	0.0944	0.0097
Inoculation (P-value)		0.8963	0.5448	0.6931
Cultivar*Inoculation		0.5281	0.4514	0.2593

[1]NI – non-inoculated; [2]AP193 – *Bacillus velezensis* strain AP193; [3]OP – orange peel

Figure 1.

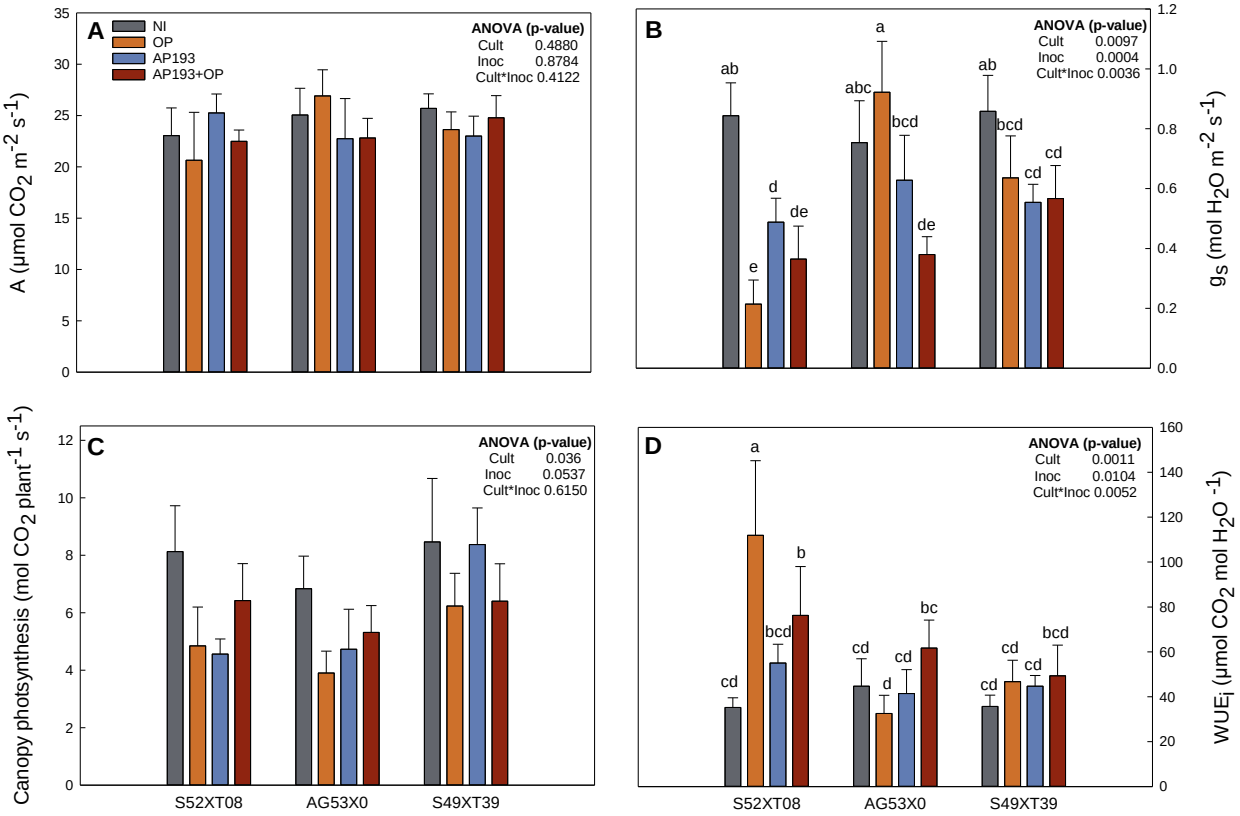

A
NI
OP
AP193
AP193+OP
ANOVA (p-value)
Cult 0.4880
Inoc 0.8784
Cult*Inoc 0.4122
A (µmol CO2 m-2 s-1)
B
ANOVA (p-value)
Cult 0.0097
Inoc 0.0004
Cult*Inoc 0.0036
gs (mol H2O m-2 s-1)
ab
e
d
de
a
abc
bcd
de
ab
bcd
cd
cd
C
ANOVA (p-value)
Cult 0.036
Inoc 0.0537
Cult*Inoc 0.6150
Canopy photsynthesis (mol CO2 plant-1 s-1)
D
ANOVA (p-value)
Cult 0.0011
Inoc 0.0104
Cult*Inoc 0.0052
WUEi (µmol CO2 mol H2O -1)
cd
a
bcd
b
cd
d
cd
bc
cd
cd
cd
bcd
S52XT08
AG53X0
S49XT39

Figure 2.

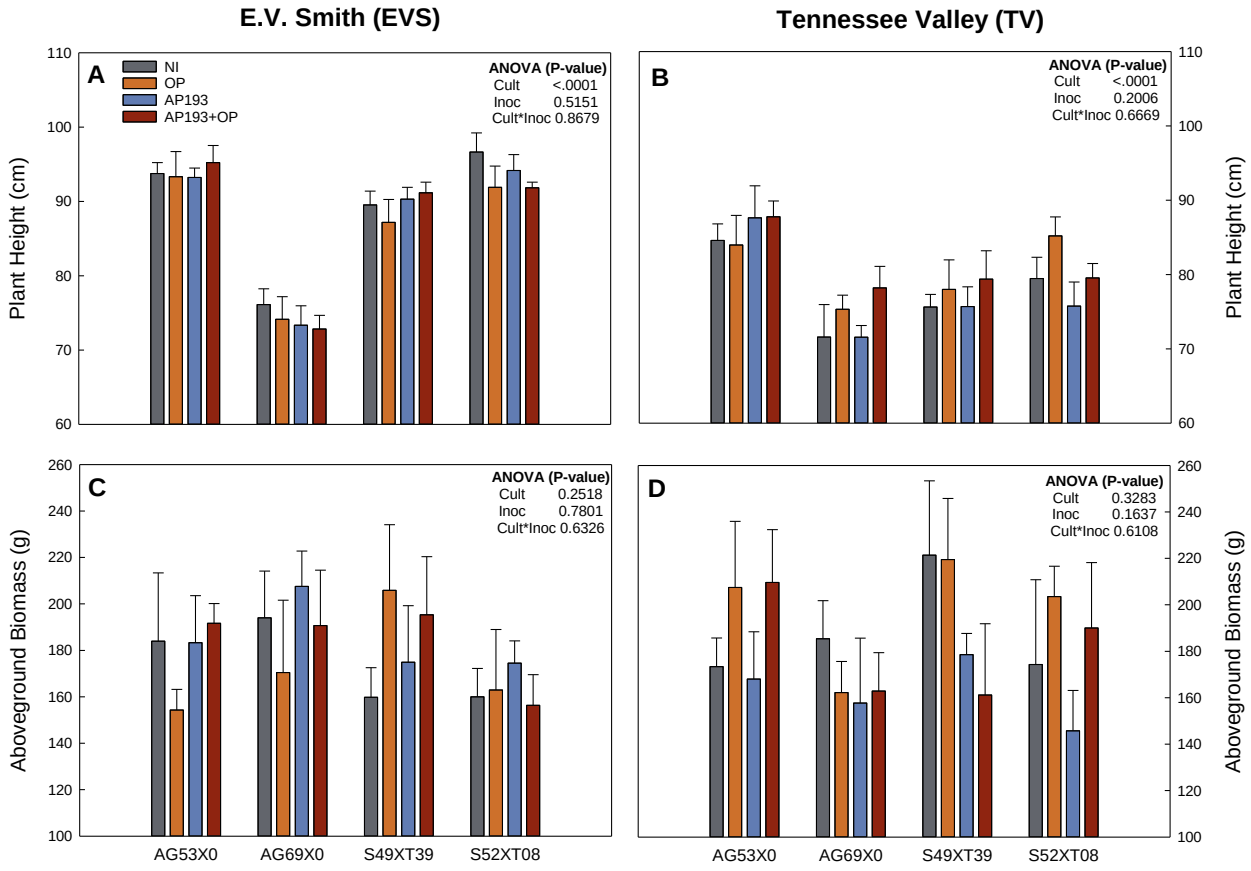
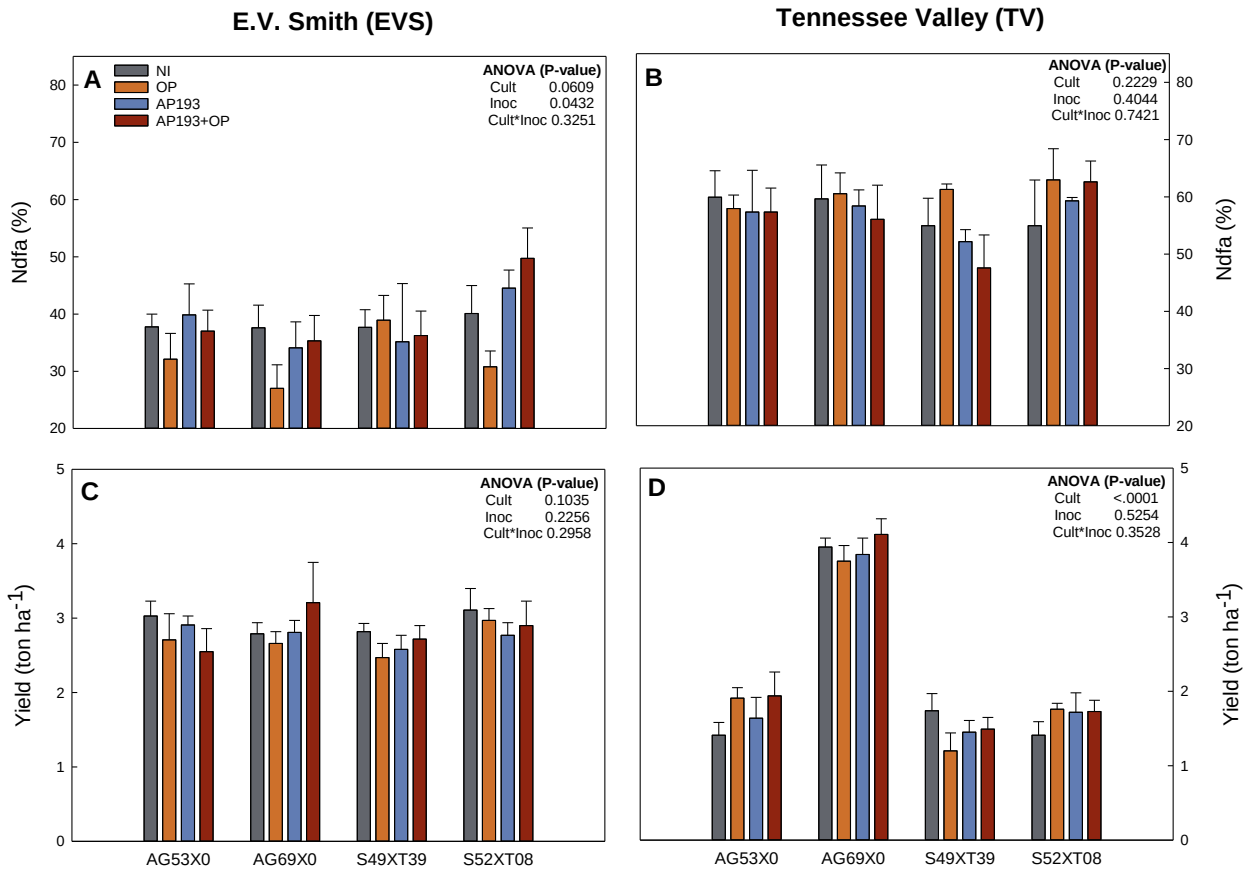

E.V. Smith (EVS)
Tennessee Valley (TV)
A
B
C
D
NI
OP
AP193
AP193+OP
ANOVA (P-value)
Cult <.0001
Inoc 0.5151
Cult*Inoc 0.8679
ANOVA (P-value)
Cult <.0001
Inoc 0.2006
Cult*Inoc 0.6669
ANOVA (P-value)
Cult 0.2518
Inoc 0.7801
Cult*Inoc 0.6326
ANOVA (P-value)
Cult 0.3283
Inoc 0.1637
Cult*Inoc 0.6108
Plant Height (cm)
Aboveground Biomass (g)
AG53X0
AG69X0
S49XT39
S52XT08

Figure 3.


E.V. Smith (EVS)
Tennessee Valley (TV)
A
NI
OP
AP193
AP193+OP
ANOVA (P-value)
Cult 0.0609
Inoc 0.0432
Cult*Inoc 0.3251
B
ANOVA (P-value)
Cult 0.2229
Inoc 0.4044
Cult*Inoc 0.7421
C
ANOVA (P-value)
Cult 0.1035
Inoc 0.2256
Cult*Inoc 0.2958
D
ANOVA (P-value)
Cult <.0001
Inoc 0.5254
Cult*Inoc 0.3528
Ndfa (%)
Yield (ton ha-1)
AG53X0
AG69X0
S49XT39
S52XT08

Supplementary Table 1. Characteristics of the soybean cultivars tested in the preliminary greenhouse experiment.

Cultivar	Brand/Company	Maturity Group	Trait
AG44X0	Asgrow	4.4	R2X[1]
AG53X0	Asgrow	5.3	R2X
AG69X0	Asgrow	6.9	R2X
CZ 4539GTLL	Credenz	4.5	GTLL[2]
CZ 5859LL	Credenz	5.8	LL[3]
CZ 6515LL	Credenz	6.5	LL
G4190RX	Agrigold	4.1	R2X, STS[4]
G5000RX	Agrigold	5.0	R2X, STS
GoSoy 512E18	Stratton Seed Co.	5.1	Enlist[5]
LS4798X	Local Seed Co.	4.7	R2X
LS5087X	Local Seed Co.	5.0	R2X
LS5588X	Local Seed Co.	5.5	R2X
LSX6501XS	Local Seed Co.	6.5	R2X, STS
S49-F5X	NK Seeds	4.9	R2X
REV 4940X	REV Brand Seeds	4.9	R2X
REV 5659X	REV Brand Seeds	5.6	R2X
S49XT39	Dyna-Gro	4.9	R2X
S52XT08	Dyna-Gro	5.2	R2X
S54XT17	Dyna-Gro	5.4	R2X
S56XT99	Dyna-Gro	5.6	R2X

[1]Roundup Ready 2 Xtend® (R2X)
[2]LibertLink® GT27® (GTLL)
[3]LibertLink® (LL)
[4]Sulfonylurea Tolerant Soybean (STS®)
[5]Enlist E3® (Enlist)

Supplementary Table 2. Author, publication year, soybean shoot B values (used to calculate the percentage of nitrogen derived from the air), and maturity stage retrieved from literature for soybean grown in greenhouse experiments.

Author	Publication year	Shoot B value %	Stage
Amarger	1979	-1.2	R4-R5
Amarger	1979	-1.5	R4-R5
Amarger	1979	-1.6	R4-R5
Amarger	1979	-1.3	R4-R5
Bergersen et al.	1985	-1.36	R3-R5
Bergersen et al.	1985	-1.3	R4-R5
Bergersen et al.	1985	-1.4	R4-R5
Schipanski et al.	2010	-2.28	R4
Schipanski et al.	2010	-2.7	R4
Balboa & Ciampitti	2020	-2.37	R3
Balboa & Ciampitti	2020	-2.32	R5
Balboa & Ciampitti	2020	-2.05	R3
Balboa & Ciampitti	2020	-1.97	R5
Balboa & Ciampitti	2020	-1.96	R3
Balboa & Ciampitti	2020	-2	R5
Balboa & Ciampitti	2020	-2.18	R3
Balboa & Ciampitti	2020	-2.2	R5
Average		**-1.86**	

Supplementary Table 3. Author, publication year, soybean shoot B values (used to calculate the percentage of nitrogen derived from the air), and maturity stage retrieved from literature for soybean cultivated under field conditions.

Author	Publication year	Shoot B value %	Stage
Araujo et al.	2018	-2.85	R1
Araujo et al.	2018	-3.17	R1
Araujo et al.	2018	-2.76	R1
Araujo et al.	2018	-2.35	R2
Average		**-2.78**	

CHAPTER III: The effect of PGPR strains selected for their capacity to grow in orange peel on soybean growth and yield

Abstract

Researchers are implementing plant growth-promoting rhizobacteria (PGPR) as biofertilizers to enhance plant growth and crop yield in an environmentally sustainable manner. However, when PGPRs are introduced into agricultural soils, their survival is restricted due to limited nutrient and energy source available in the rhizosphere. It has been demonstrated that selected *Bacillus velezensis* (Bv) strains can degrade orange peel (OP) pectin and use the derived sugars as their sole carbon source increasing their survival and promoting soybean growth and nodulation. Therefore, it is important to select the best Bv strains for their capability to grow in OP and test their performance on soybean growth promotion when inoculated with OP. Among the 6 Bv strains tested in our greenhouse experiment AP191, AP215, and AP215 showed the best results in plant growth promotion when supplemented with OP, where only AP191+OP significantly enhanced pod dry weight by 15.8%. Additionally, the effectiveness of the introduced PGPR was affected by the inoculation method. With that in mind, we tested the effect of selected PGPR strains plus OP co-inoculated with *Bradyrhizobium japonicum* in different soybean cultivars with in-furrow or seed coating inoculation at two different locations. Inoculation with AP191+OP showed the greatest yield being the only treatment significantly different from the control in the seed inoculated treatment (+0.5 ton ha^{-1}, 9.4%). In the in-furrow inoculation treatment, the commercial product, Vault, showed the highest yield. We conclude that seed coating inoculation is probably a better method to introduce the selected PGPR plus OP into agricultural soils.

Abbreviations: ANOVA, analysis of variance**; Brad**, *Bradyrhizobium japonicum*; **Bv**, *Bacillus velezensis*; **C**, Carbon; **CFU**, colony forming unit; **Ndfa**, Nitrogen derived from the air (%); **OP**, orange peel; **PGPR**, Plant growth promotion rhizobacteria; **$\delta^{15}N$**, Nitrogen isotope discrimination.

Key Words: plant growth promotion rhizobacteria, root growth, yield, strain selection, orange peel, pectin, co-inoculation, seed inoculation

Introduction

In order to meet the projected global food and fiber demands from an increasing population in 2050, current crop production will need to double (Tilman et al., 2011). However, the biggest challenge for modern agriculture is to increase yield in an environmentally sustainable manner (Morrissey et al., 2004). In this context, researchers are adopting strategies to improve yield gains by implementing beneficial microorganisms in agriculture. For instance, plant growth-promoting rhizobacteria (PGPR) have been used as biofertilizers (Kloepper, 1994) and biostimulants (Calvo et al., 2014) to promote plant growth. Currently, several PGPR strains are available as commercial biofertilizer products, for example, Vault IP Plus (*Bradyrhizobium japonicum, Bacillus amyloliquefaciens*, and *Bacillus subtilis;* BASF) and Cell-Tech (*Bradyrhizobium japonicum;* NexusBioAg).

PGPRs are a part of the rhizosphere biota that associates with the plant host roots (Bhattacharyya & Jha, 2012). Rhizosphere consist of the area around the plant roots that is colonized by microorganisms influenced by the exudates released from them (Hiltner, 1904; Hartmann et al., 2008). Root exudates include low molecular weight compounds such as organic acids, amino acids, proteins, sugar, phenolics, and other secondary metabolites (McNear, 2013) and serve as a nutrient and energy source for microorganisms present in the rhizosphere (Gray & Smith, 2005). In return for the root exudates, PGPR secrete metabolites into the rhizosphere that can promote the host growth (Van Loon, 2007). For example, PGPRs can directly alter root architecture (Kloepper et al. 2007) and promote plant development through the production of phytohormones such as indole-3-acetic acid, gibberellic acid, and cytokines (Goswami et al., 2016).

Many *Bacillus* strains have been identified as PGPR with commercial potential use as biofertilizers (Govindasamy et al., 2010) due to their advantageous physiological traits, such as

spore-forming ability, that contributes to their survival in the soil for prolonged periods of time (Kumar et al., 2011). However, when PGPRs are applied into agricultural soils under field conditions, their efficacy is variable (Hassan, 2016), suggesting that maybe the introduced microbial population declines rapidly after the introduction of the bacterial population (Di Cello et al., 1997, van Veen et al., 1997). These can be due to limited carbon source for the bacteria in the rhizosphere. Having that in mind, researchers at Auburn University have demonstrated that selected PGPR strains of *Bacillus velezensis* (Bv) specie can use purified pectin as their sole carbon source, increasing their survival in the soil and promoting soybean growth and nodulation (Hassan et al., 2019). These bacteria are pectate-lyase-producing (pectinolytic) species; they can release pectinase, degrade pectic substances, and use pectin-derived sugars such as glucuronate or galacturonate as primary carbon source (Mekjian et al., 1999). Pectin is present in most of the plant tissues, but orange peel (OP) is one of the most important sources of industrial pectin (Thakur et al., 1997). After the industrial extraction of orange juice, large amounts of OP remain as by-product (Yeoh et al., 2008). Orange peel is a good source of pectin as it contains up to 52.9% of pectin yield (Tiwari et al., 2017) and has been identified as a potential candidate to be consumed by PGPR strains with pectinolytic activity.

Preliminary studies performed in Auburn University shows that inoculation with pectinolytic strains plus OP in agricultural soils does not result in statistically significant positive responses of some soybean cultivars (Pacheco da Silva et al., under review). We hypothesize that the lack of response of the plant to the inoculation is due to the lack of synergy between the soybean cultivar and PGPR strains (Pacheco da Silva et al., under review) and/or the capacity of the PGPR strain to adequately use OP as carbon sole source(Hassan et al., 2019; Pacheco da Silva et al., under review). This problem could be solved by screening different PGPR strains for their growth

in the OP, select the ones that grow better and faster, to later in the greenhouse, select the best performer before bringing them to the field tests.

Other problem that could be causing the absence of positive effects in the inoculation with PGPR plus OP and the variability of results (Pacheco da Silva et al., under preparation) is the lack of synergy between soybean cultivars and native *Bradyrhizobium* strains (Kang et al., 1991; Saeki et al., 2000); Pacheco da Silva et al., under preparation). Positive soybean yield response to inoculation can be attributed to the use of more effective *Bradyrhizobium* strains than those indigenous bradyrhizobia living in the soil (Hungria & Mendes, 2015). To avoid this problem, several authors have tested the concept of synergic co-inoculation. Synergic co-inoculation causes one microorganism to improve the performance of the other one, bringing benefits to the host crop. For instance, the co-inoculation of PGPR with selected rhizobia can improve legume growth and yield (Gopalakrishnan et al., 2015) as is the example of the commercial biofertilizer Vault IP Plus. Some PGPR strains can also improve nodulation and nitrogen fixation when combined with selected rhizobia in soybean (Sibponkrung et al., 2020). For this reason, the field experiment in our research included treatments with commercial *Bradyrhizobium japonicum* in combination with PGPR and orange peel in order to test if the lack of effective *Bradyrhizobium* strains could cause the lack of effect of the inoculation.

There are several methods of inoculant application, but according to Gault et al. (1982), the main methods used for introduction of rhizobia into the agricultural ecosystem can be either via seed inoculation or by direct application into the soil via liquid furrow inoculation. Testing these two inoculation methods on peanut, Bogino et al. (2011) found that in-furrow inoculation increased the competitiveness of the introduced bacteria, nitrogen-fixing efficiency, and plant biomass, compared to on-seed inoculation. Usually, the in-furrow inoculation provides larger

amount of viable rhizobacteria than seed inoculation; however, for bacterial strains that do not survive well in the soils, this inoculation method may be disadvantageous since they do not provide a protecting environment for the introduced bacteria (Bashan, 1998). In consideration of that, testing the different inoculation methods on soybean is important in order to access if the effectiveness of the introduced PGPR is affect by the inoculation method.

Therefore, to increase the sustainability of soybean production systems by using PGPRs as biofertilizers, it is important to research new strategies to make PGPR strains to survive and thrive in agricultural soils, such as strain selection, use of orange peel amendments, co-inoculation with *Bradyrhizobium japonicum*, and the method of inoculation. Therefore, the objectives of this research were to: 1) Select the PGPR strains that grow better in OP powder and test the plant response in the greenhouse; 2) Test the effect of greenhouse selected PGPR strains plus OP co-inoculated with *Bradyrhizobium japonicum* under field condition; and 3) Test if in furrow seed or seed coating treatment is more effective in promoting plant growth under field conditions.

Material and Methods

Effect of orange peel on PGPR strain growth

To assess PGPR's ability to utilize OP powder as a sole carbon source, an *in vitro* assay was developed to evaluate the growth of each *Bacillus* spp strain (Fig. 1) in a M9 minimal medium (Difco, Detroid, MI). The M9 Minimal Medium was prepared according to manufacturer's instructions, including addition of 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$, along with the respective carbon source (OP powder), to a final concentration in 1X M9 Base Medium (Mageshwaran et al., 2014). A 10% (w/v) OP stock suspension was prepared and diluted to a final concentration of 0.5% in the 1X M9 medium (pH 7.0). Overnight cultures of each PGPR strain were prepared in 10 mL of tryptic soy broth (TSB, 24 hours, 30ºC, 175rpm). The 0.5% OP + 1X M9 medium was distributed in 3 mL per culture tube while mixing. PGPR overnight cultures were subjected to centrifugation at 10,000 x *g* for 8 min at 25ºC to pellet cells, which were washed in 1 mL 1X M9 medium, and then pelleted again before a final resuspension in 1 mL 1X M9 medium in a sterile 1.5 mL tube. PGPR inocula were normalized to an OD_{600} ~ 0.1 in 3 mL cultures of 0.5% OP + 1X M9 media. Once all PGPR were inoculated an OD_{600} was recorded, and tubes were incubated while shaking at 200 rpm at 30ºC. OD_{600} readings were recorded every 24 hours for 72 hours. Each strain culture was replicated three times. The ΔOD_{600} values were calculated by the OD_{600} at time 72 hours subtracted from the OD_{600} at time 0 hours, and a one-way ANOVA was performed using PROC GLIMMIX in SAS (SAS 9.4, SAS Institute, Cary, NC, USA) using strains as main factor and replications as random factor.

Greenhouse experiment to select PGPR strains

In order to select the most effective PGPR strains on soybean growth, inoculation with 6 different PGPR strains (known for their pectinolytic activity) and orange peel (OP) were tested in a greenhouse experiment in September of 2020.

PGPR strains, soybean cultivar and growing conditions

As a substrate for the experiment, Sandy Loam field soil texture was collected from E.V. Smith Research Center (Shorter, AL). The soil was mixed with sand (1:1 proportion) and the mixture was used to fill 3.9-gallon pots. Six different PGPR strains, previously selected from the *in-vitro* experiment and with known pectate-lyase-producing activity, were used in this experiment (AP191, AP193, AP215, AP216, AP218, and MB315) with the soybean cultivar S49XT39 (Dyna-Gro).

Four treatments per strain were evaluated: (1) non-inoculated control (NI), (2) inoculation with orange peel suspension (OP), (3) inoculation with the PGPR strains alone (AP191, AP193, AP215, AP216, AP218, or MB315) and (4) inoculation with each PGPR strain plus orange peel (AP191+OP, AP193+OP, AP215+OP, AP216+OP, AP218+OP, and MB315+OP, all strains individually), with seven replications in total for each treatment. PGPR strains spores were prepared following the methods of Hassan et al., (2019) and added to each seed at a final concentration of $1x10^6$ spore colony forming unit (CFU) in 50 µL of sterile water. Orange peel powder (Citrus Extracts LLC, Fort Pierce, FL 34982, USA) was used to prepare the OP suspension at a final concentration of 10 mg/200 µL per seed.

Treatments were inoculated on seeds at the sowing time. Five seeds were evenly placed 2.5 cm below the soil surface of each pot to ensure proper germination. Each seed received the following inoculations according with the treatment group: (Non-Inoculated) 250 µL of distilled

water, (OP) 200 µL of orange peel powder solution and 50 µL of water, (PGPR) 50 µL of respective PGPR spores and 200 µL of distilled water, (PGPR+OP) first 20 µL of orange peel powder solution and then 50 µL of respective PGPR spores. No water was added to either treatment group for at least 24-48 hours after planting to allow the seeds in the inoculated treatment group to remain in contact with the PGPR and OP solution. After emergence (approximately one week after sowing), one seedling was kept per pot and the other ones were removed.

Pots were aligned by replication and rearranged in a randomized complete block design. The pots were rotated around the tables in the greenhouse each week, preventing any biases based on plant location and light intensity. Artificial LED light was used to maintain a photoperiod of 16 h of light and 8 h of night. Temperatures in the greenhouse oscillated between 18-30ºC during the day and 15-20ºC at night. Each pot received 500 mL of water every 2 days. Each week plants were sprayed with pesticides to prevent insect infestation.

Physiological and growth parameters measurements

When the plants had reached the R4 growth stages (Fehr et al., 1971), SPAD values, a proxy for chlorophyll concentration, was measured using a SPAD-502 (Minolta, Tokyo, Japan). At R5, plants were harvested, and above ground plant organs were separated and total above ground biomass (g $plant^{-1}$) was calculated by separating leaves, stems, and pods and drying them at 60ºC for at least 72 h to afterwards weighting them in a precision scale. Before drying, total leaf area (cm^2 $plant^{-1}$) was calculated by passing each trifoliated leaf by a LI-3000 Leaf Area Meter (LI-COR Biosciences, Lincoln, Nebraska, USA).

Roots were cleaned after harvesting using tap water and the nodules were separated. Fresh nodules were cleaned and placed over a clean white paper and were imaged with a digital camera. The pictures were analyzed for quantitative nodule characteristics using ImageJ according to

Riedell et al. (2009), and nodule number and size (total cm^2 $plant^{-1}$ and individual nodule cm^2 $plant^{-1}$) were calculated. The imaged nodules were dried at 60ºC for at least 72 h to calculate total nodule dry weight (g $plant^{-1}$). Cleaned roots were scanned in a Winrhizo desk top scanner (Regent Instruments Inc., Sainte-Foye, Quebec, Canada) to calculate total root area (cm^2 $plant^{-1}$), root volume (cm^3 $plant^{-1}$), total root length (cm $plant^{-1}$), and total root average diameter (mm $plant^{-1}$). After scanning, the roots were dried at 60ºC for at least 72 h to calculate total root dry weight (g $plant^{-1}$).

Statistical analysis

A two-way ANOVA was performed for each parameter to test the effect of PGPR strain (AP191, AP193, AP215, AP216, AP218, and MB315), inoculation (NI, OP, PGPR, PGPR+OP) and their interaction. A two-way ANOVA, with PGPR and inoculation as main factors and replication as random variable, was performed using PROC GLIMMIX in SAS (SAS 9.4, SAS Institute, Cary, NC, USA). When the main effect of PGPR and/or inoculation, or their interaction was significant, least square means post-hoc tests were performed to compare means (LSMEANS, SAS 9.4, SAS Institute, Cary, NC, USA).

Field Experiment to Test the Response of Co-inoculation of PGPR plus Orange Peel Amendment with *Bradyrhizobium japonicum* on Soybean Cultivars

Field experimental design and inoculation treatments

During the Summer 2021, field trials were established at two different locations: E.V. Smith Research Center (EVS; Shorter, AL) and Tennessee Valley Research Center (TV; Madison, AL) in a no-tillage system, with rye as winter cover crop. E.V. Smith Research Center has a Piedmont Plateau soil with a Sandy Loam soil texture and the mean, maximum and minimum temperature during the growing season was 22.6, 32.8, 11.1ºC respectively, with a rainfall

accumulation of 1,040.7 mm during the growing season. Tennessee Valley Research Center has a Limestone Valleys and Uplands soil with Dewey Silt Loam soil texture and the mean, maximum and minimum temperature during the growing season was 21, 31.8, 8.3ºC respectively, with a rainfall accumulation of 1,115.9 mm during the growing season. Soil tests were performed two weeks before planting and fertilization was added in agreement with best cultural practices recommended by the Auburn University Extension Soil Fertility Team. Pre-emergence and post emergence herbicides and pesticides were applied following the recommendations of the Auburn University Extension Practices.

A complete randomized block experimental design was used for these experiments. Totally, three commercial soybean cultivars (S49XT39, S52XT08, and AG69X0) known for their good responsiveness to PGPR plus OP inoculation (Pacheco da Silva et al., under preparation) and a non-nodulating soybean cultivar (Lee), as a check for the nitrogen fixation, were evaluated. Fourteen different inoculation treatments were applied at sowing time: (1) non-inoculated (NI), (2) orange peel amendment (OP), (3) *Bradyrhizobium japonicum* (Brad), (4) Brad plus OP, (5) *Bacillus velezensis* (Bv) strain AP191, (6) AP191 plus OP, (7) AP191 plus Brad, (8) AP191 plus Brad and OP, (9) Bv strain AP193, (10) AP193 plus OP, (11) AP193 plus Brad, (12) AP193 plus Brad and OP, (13) Cell-Tech® (*Bradyrhizobium japonicum;* NexusBioAg) inoculant, and (14) Vault® (*Bradyrhizobium japonicum, Bacillus amyloliquefaciens*, and *Bacillus subtilis;* BASF) inoculant.

The different PGPR strains spores were prepared following the methods of Hassan et al., (2019) and the spore suspension was prepared at a final concentration of $1x10^6$ spore CFU/mL. Orange peel powder (Citrus Extracts LLC, Fort Pierce, FL 34982, USA) was used to prepare the orange peel suspension at a final concentration of 1% (w/v). Cell-Tech® and Vault® commercial

inoculants suspensions were prepared following the respective label recommendations. *Bradyrhizobium japonicum* powder (Exceed® Peat for Soybean, Visjon Biologics) was applied to the seeds previously to sowing according with the label recommendation.

For each location, the seed inoculation was applied differently onto the seeds. For Tennessee Valley Research Center, at sowing, the PGPR suspensions at $1x10^6$ spore CFU/mL, orange peel liquid suspension (1%), and the commercial inoculant solutions were applied in-furrow in the two middle rows to avoid cross plot contamination at the rate of 37.85 liters per hectare, according to sprayer specifications and following the protocol of Hassan et al., (2019). For E.V. Smith Research Center, the treatments were applied to the seeds before sowing. Each batch of untreated seed, containing 425 g of seeds, received the respective inoculant and/or PGPR suspension (2 mL for each PGPR at a 10^9 CFU mL^{-1}) and 18 mL of sterile water. Seeds were mixed to ensure that all the liquid was evenly spread on the seeds surface. Then 25.75 grams of orange peel powder was added to the seed batch. Seeds were mixed again to ensure that all the powder added was equally distributed into the seeds surface. Seeds were left to dry during 24 h at room temperature and later packaged for seed planting. Seed packages were kept in a refrigerator at 4ºC.

The experimental design had four replications, with 14 treatments per cultivar, with 3 cultivars and non-nodulating controls totaling 180 plots at each location. Plots were 20 foot long and consisted of four rows with 36 inches spacing between rows. For all the treatments, a seeding rate of 8 seed per foot was used.

Physiological and growth measurements

Emergence fifteen days after planting was counted twice per plot as number of seedlings per meter to estimate the percentage of germination. At V2 vegetative growth stage (Fehr et al., 1971) plant vigor scores were assigned for each plot ranging from 1 to 5, where the lowest value

represents significantly weaker and slower growth plants and the highest value significantly stronger and faster growth plants compared to control treatment.

When the plants had reached the R5 developmental growth stage (Flowering, Fehr et al., 1971), SPAD values, a proxy for chlorophyll concentration, was measured using a SPAD-502 (Minolta, Tokyo, Japan) in two plants per plot. Plant Height (cm) at R7 was measured in three plants per plot from the soil surface to the apical meristem of the main stem.

At beginning of pod formation (R3, Fehr et al. (1971)) aboveground biomass accumulation was measured by harvesting a total of 0.5-meter row. Collected plants were dried for 72 h in a forced air oven at 60ºC and later weighted in a precision scale. The total aboveground biomass including leaves, stems, and pods was ground to pass a 1 mm screen, weighed into tin capsules, and shipped to the UC-Davis Stable Isotopes Facility (Davis, California, USA) for ^{15}N isotope, N content, and carbon isotopeanalysis. Samples were analyzed using an isotope ratio mass spectrometer (IsoPrime, Elementar France, Villeurbanne) coupled to an elemental analyzer (EA3000, EuroVector, Milan, Italy).

The natural ^{15}N isotopic ratio ($\delta^{15}N$) in the aboveground biomass was calculated using the formula described by Shearer and Kohl (1986):

$$\delta^{15}N = \frac{R_{sample}}{(R_{air} - 1)} * 1000$$

where, R_{sample} and R_{air} are the isotope ratios ($^{15}N/^{14}N$) of the sample and air respectively. The proportion of N derived from the atmosphere (%Ndfa), an estimation of the biological nitrogen fixation, was determined by the ^{15}N natural abundance method (Shearer & Kohl, 1986) following the formula:

$$\text{Ndfa } (\%) = \frac{\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{soy}}}{\delta^{15}\text{N}_{\text{soy}} - \text{B}} \times 100,$$

where Ndfa (%) is the percentage of N_2 coming from the atmosphere through BNF, $\delta^{15}N_{ref}$ is the $\delta^{15}N$ signature of the non-fixing soybean reference (cultivar Lee) aboveground biomass, $\delta^{15}N_{soy}$ is the $\delta^{15}N$ signature of the above ground biomass for each treatment, and B is the $\delta^{15}N$ value of a soybean plant growing in a N free media relying only on BNF as source of N. The B-value used in our study were obtained as the $\delta^{15}N$ average value (-2.78‰) from previous reports for soybean sampled around R1-R2 developmental stage (Supplementary Table 1).

The ratio (R) of $^{13}C/^{12}C$ was showing as $\delta^{13}C$ (‰), indicating the C isotope composition relative to Vienna Pee Dee Belemnite calcium carbonate (V-PDB):

$\delta^{13}C = (R_{samples}/R_{standard})-1$

$\delta^{13}C$ (‰) values were standardized to C isotope discrimination ($\Delta^{13}C$, ‰) data calculated as:

$$\Delta^{13}\mathrm{C}\ (‰) = \left(\frac{\partial^{13}\mathrm{C}_{\mathrm{atm}} - \partial^{13}\mathrm{C}_{\mathrm{sample}}}{1 + (\frac{\partial^{13}\mathrm{C}_{\mathrm{sample}}}{1000})} \right)$$

where $\delta^{13}C_{atm}$ is the C isotope composition of atmospheric CO_2 (-8‰; Farquhar et al., 1989), and $\delta^{13}C_{sample}$ is the C isotope composition of the aboveground biomass sample.

Root Surface Area (cm^2) and Root Volume (cm^3) at the beginning of pod developmental stage (R3, Fehr et al., 1971), was measured by collecting two roots per plot using the shovelomic method (Seethepalli et al., 2020) and stored into plastic bags in a container with ice, to keep them fresh. The roots were photographed and then analyzed for root parameters using RhizoVisionExplorer (version 2.0.3) software and set up (Seethepalli et al., 2020). At harvest, each plot was assigned with a lodging score ranging from 0 to 5, according with Supplementary Table 2.

Statistical analysis

A two-way ANOVA was performed for each parameter to test the effect of inoculation, cultivar, and their interaction independently in each location. The two-way ANOVA, with inoculation and cultivars as main factors and replication as random variable, was performed using PROC GLIMMIX in SAS (SAS 9.4, SAS Institute, Cary, NC, USA). When the main effect of inoculation treatment and/or genotype, or their interaction was significant, least square means post-hoc tests were performed to compare means (LSMEANS, SAS 9.4, SAS Institute, Cary, NC, USA).

Results

Effect of orange peel on PGPR strain growth

All of the 18 Bv PGPR strains grew well in M9 minimal medium containing OP as a sole carbon source (Fig. 1). The highest Δ OD values after 72 h were observed for Bv PGPR strains AP193, AP218, JJ1144, JJ1368, and JJ523. Based on their observed ability to grow in vitro using OP as a sole carbon source, the strains from the AP family (AP191, AP193, AP215, AP216, and AP218) were selected for the greenhouse trial experiment as Bv AP193 showed the highest growth in OP (Fig. 1). Also, the Bv MB315 strain was selected because it showed the lowest growth in OP and therefore less effect of the inoculation on plant growth was expected.

Greenhouse experiment to select PGPR strains

Among the 6 Bv strains tested in the greenhouse AP191, AP215, and AP216 showed the best results in plant growth promotion when supplemented with OP (Table 1, Fig. 2, Fig. 3). There were significant effects of the inoculation on plant height, leaf area, and SPAD measurements (Table 1). Additionally, for the leaf area parameter, there was an effect of the interaction between Strain and Inoculation. Strains AP216 and AP191 when supplemented with OP, resulted in taller plants with 13.4% and 12.5%, increase in plant height, respectively. The general mean of plant height, among all inoculation treatment, was increased in 4.09 cm when inoculated with PGPR+OP. In addition, the inoculation of PGPR+OP significantly increased leaf area for strains AP215 (+39.8%) and AP216 (+33.6%) when compared with the control treatment. In contrast, leaf area was reduced for strains AP193 (12.6%), AP218 (10.1%), and MB315 (2.2%) supplemented with OP. In general, the inoculation with PGPR+OP increased leaf area in 12.2% (35.3 cm^2) in comparison with the non-inoculated treatment. For SPAD, all the three inoculations showed greater value than the non-inoculated treatment. Strain AP215 can be highlighted with an

improvement of +11.7% in SPAD when inoculated with PGPR+OP (Table 1). As expected, based on the OP growth data, the strain MB315 was one of the worst performers in combination with OP.

There was significant effect of the inoculation and the interaction between Inoculation and Strain for pod, root, aboveground, and total biomass dry weight (Fig. 2). Aboveground dry weight (+18%, +17.9%, and +17.6%), root dry weight (+51.2%, +42.5%, and +31.7%), and total biomass (+22.1%, +21.1%, and +19.7%) were significantly improved with the inoculations of AP191+OP, AP215+OP, and AP216+OP, respectively. However, only AP191+OP significantly enhanced pod dry weight increasing it in 15.8%. The inoculation with OP alone had a negative impact on plant dry biomass, reducing pod dry weight in 23.2% and aboveground in 15.7% (Fig. 2).

For the nodule parameters, there was a significant effect of the inoculation on nodule number, nodule dry weight, nodule area, and nodule number by root length (Table 2). However, there was no effect of the interaction between Strain and Inoculation for any nodulation parameter which mean that none of the strains was more advantageous than other promoting nodule growth. The inoculation with PGPR+OP significantly increased nodule number (~22 more nodules) for all the strains in comparison with the NI control. The inoculation with OP alone had a negative impact on nodulation, reducing nodule number (-29.8%), nodule dry weight (-39.2%), nodule size (-5.5%), nodule area (-32.8%), and nodule number by root length (-21.8%). Although the interaction was not significant, strains AP191 and AP215 showed an increase in nodule number of 22.2 and 24.7%, respectively. There was not statistically difference for the inoculation with PGPR+OP and NI treatments for nodule dry weight, nodule area, and nodule number by root length parameters. For nodule size, there was no effect of the factors and their interaction (Table 2). When analyzing the nodule diameter individually, the inoculation with PGPR and PGPR+OP significantly

increased small nodules in comparison with the non-inoculated treatment (Table 3). Also, PGPR+OP increased median (+21.8%) and large nodules (+6.8%) in in comparison with the NI treatment. Strain AP191 when supplement with OP improved small (+90%) and median (+45.5%) nodule number in comparison with the non-inoculated plants (Table 3).

The bellow ground parameters were significantly improved by the inoculation with PGPR+OP (Fig. 3). There was a significant effect of the inoculation treatment and the Strain by Inoculation interaction for root length, diameter, surface area, and volume. The strain AP191 showed the highest root improvement when supplemented with OP increasing root length in 3,099.64 cm (+51.7%), surface area in 488.42 cm^2 (+70.2%), root volume in 5.95 cm^3 (+92.4%), and average diameter in 0.1095 mm (+29%) in comparison with the non-inoculated treatment. Additionally, strains AP215 and AP216 significantly increased root length, surface area, and volume but in smaller measure than AP191 (Fig. 3).

Having in mind all the greenhouse results, we selected the strain AP191 and AP193 (standard strain used in previous experiments and with higher growth in OP) to test the effect of the co-inoculation with *Bradyrhizobium* on soybean growth, yield, and nitrogen fixation.

Field Experiment to Test the Response of Co-inoculation of PGPR plus Orange Peel Amendment with *Bradyrhizobium japonicum* on Soybean Cultivars

Among the 3 cultivars and 14 treatments tested, there was effect of cultivar, inoculation, and the interaction Cultivar by Inoculation in EVS (seed inoculation) for emergence and plant vigor (Table 4). In TV (in-furrow inoculation) there was only effect of cultivar and inoculation for emergence (Table 4). In both locations, the inoculation with AP193+Brad+OP and AP191+Brad+OP increased the plant emergence in 5.99% and 2.07%, respectively for EVS and

16.72% and 11.82% for TV compared with the control treatment. Additionally, the inoculation with AP191+Brad+OP and AP193+Brad+OP significantly increased the plant vigor (+30.56% and +27.78%, respectively) in EVS (Table 4).

There was effect of cultivar and inoculation variables for SPAD and plant height at EVS (seed inoculation), but the interaction was not significant (Table 5). Inoculation with AP191+Brad+OP and AP193+Brad+OP significantly improved the chlorophyll content on leaves by 4.2% and 4.1% respectively for EVS (seed inoculation). Cultivar S49XT39 inoculated with AP193+Brad+OP showed the highest percentage of change for SPAD value, increasing the chlorophyll content in 7.5% in comparison with the control (Supplementary Table 4). The standard inoculation with Cell-Tech had the highest plant height in EVS (seed inoculation), increasing plants in 14.6 cm compared with the non-inoculated treatment. In addition, the inoculation with AP193+Brad+OP improved plant height in 16.6% (Table 5). When looking for the cultivars individually, inoculation with AP193 in cultivar S49XT39 showed the greater improvement on plant height, increasing plants in 31% at EVS (Supplementary Table 4). For TV (in-furrow inoculation), there was no effect of inoculation and the interaction between Cultivar and Inoculation for SPAD and plant height parameters (Table 5).

At EVS (seed inoculation) from all the root parameters, only root surface area had a slight effect of the inoculation and none of the parameters were affected by the Inoculation by Cultivar interaction (Table 6). The root surface area was improved in 29.2% with the OP treatment at EVS. Additionally, the inoculation with AP193+OP and AP191+OP increased root surface area by 19 and 7% respectively, although the differences was not statistically different from the NI control. At TV (in-furrow inoculation), inoculation was significant for total root tips and total root length, being slightly significant for root surface area (P= 0.072). Average root diameter was not affected

by the inoculation (Table 6). In TV (in-furrow inoculation), the treatment with AP193+OP significantly increased root tips (+20.6) and root length (+26.4%) in comparison with the NI control. Also, this treatment improved the root surface area (+24.5%) when compared with the non-inoculated treatment although the inoculation treatment was not significant at 0.05 (Table 6). Inoculation with AP191+OP increased total root tips (+8.6%) and total root length (+14.8%), although this difference was not statistically significant. When analyzing each cultivar individually at TV (in-furrow inoculation) we observed no significant changes in total root tips between inoculations on AG69X0 and S52XT08 (Supplementary Table 6). In the other hand, cultivar S49XT39 inoculated with AP193+Brad significantly improved the total root tips, increasing it by 37.80% compared to the non-inoculated treatment on that cultivar. For the total root length parameter, there was no significant difference between inoculation for cultivar S49XT39. However, the cultivars AG69X0 and S52XT08 inoculated with AP193+OP significantly improved the root length (+31.4% and +53.8%, respectively) when compared to the respective non-inoculated treatment (Supplementary Table 6).

There was a significant effect of the inoculation on $\Delta^{13}C$, Ndfa, and N uptake only at EVS (seed inoculation), while the interaction between Inoculation and Cultivar effects were not significant for any parameter or location (Table 7). Treatment with AP193+Brad+OP decreased the $\Delta^{13}C$ in 1.52%. Only the inoculation with OP alone improved the nitrogen fixation in soybeans at EVS (seed inoculation) with an 8.9% increase in Ndfa in comparison with the no-inoculated control. In contrast, inoculation with AP191+Brad+OP significantly reduced the Ndfa in 10.8%. Also, almost all the other inoculation treatments reduced the nitrogen derived from the air compared to the non-inoculated treatment in EVS (Table 7). The commercial inoculant Cell-Tech had the highest N uptake at EVS (103 kg N ha^{-1}), non-differing statistically from inoculation with

Brad+OP, Vault, AP191, AP191+Brad, AP191+Brad+OP, AP193, AP193+Brad, AP193+OP, and AP193+Brad+OP (Table 7). In TV (in-furrow inoculation), inoculation affected slightly $\Delta^{13}C$, with plants inoculated with AP191 showing the lowest values and the ones inoculated with AP193 with the highest value (21.49 ‰). For Ndfa and N uptake, the inoculation treatment did not affect these parameters. However, plants inoculated with Vault, AP193, AP193+Brad, and AP193+Brad+OP increased the percentage of change Ndfa (+6.7, 8, 6.3, and 7.1%, respectively) and AP191+Brad+OP increased the N uptake in 25.7% in comparison with the non-inoculated treatment (Table 7).

There was effect of inoculation on aboveground dry weight and yield at EVS (seed inoculation) (Fig. 4). Inoculations with AP193+Brad+OP and AP191+Brad+OP showed the highest values for aboveground biomass significantly increasing this parameter by 33.9% and 33% in comparison with the non-inoculated control. For yield, inoculation with AP191+OP showed the greatest yield (5.84 tons ha^{-1}) which resulted in the only inoculation treatment significantly different from the NI control (+0.5 ton ha^{-1}, 9.4%). When looking at each cultivar individually, S49XT39, S52XT08, and AG69X0, had the highest yield response when inoculated with AP191+OP, Brad+OP, and AP191+Brad respectively (Supplementary Table 4). At TV, where the inoculation was provided by in furrow, there was effect of the inoculation variable for yield with the commercial product Vault showed the highest value (4.75 tons ha^{-1}) being the only inoculation treatment significantly different from the non-inoculated treatment with a 6.6% of change (Fig. 4). Although not statistically different from the NI, inoculation with AP193+OP increased yield in 3.7% in TV (in-furrow inoculation). There was no significant effect of the inoculation for the aboveground dry weight, but the inoculation with AP191+Brad+OP increased it in 22.4% in comparison with the control (Fig. 4).

Discussion

Different *Bacillus velezensis* strains from the Auburn University collection were tested for their capacity to grow in M9 minimal medium with OP as their sole C source, with the idea that the strains that are able to grow in OP would be able to better survive in the soil in presence of OP as an amendment until the "symbiosis" between PGPR and plant is well stablished. In general, the results of the *in vitro* growth experiment indicated that all Bv strains can degrade and use pectin derived from OP powder, although some strains grew better in the OP media than others (Fig. 1). The strains from the AP family were selected for the greenhouse experiment as Bv AP193 showed the highest growth in OP (Fig. 1) and the others showed acceptable growth (Δ OD after 72 h higher than 0.4) and have been researched widely in the literature (Hassan et al., 2019; Hossain et al., 2015; Liu et al., 2017). The Bv MB315 strain was also selected for this experiment because it showed the lowest growth in OP and therefore less effect of the inoculation on plant growth was expected.

The plant response to pectin amendment has been demonstrated to be PGPR strain (Hassan et al., 2019) and soybean cultivar dependent (Pacheco da Silva et al., under review). For that reason, in our greenhouse experiment, we tested the growth response of 6 different Bv strains supplemented with OP on one soybean cultivar to select the most responsive PGPR strains; and later, in our field experiments, we tested the combined and separate effects of the selected PGPR strains, OP, and *Bradyrhizobium* on a set of three soybean cultivars. Our results demonstrate that in the greenhouse experiment, the inoculation with any PGPR plus OP tended to increase soybean growth parameters (Table 1, Fig. 2, Fig. 3). Specifically, the inoculations with AP191, AP215, and AP216 supplemented with OP significantly improved root and aboveground biomass growth with the plants inoculated with AP191 plus OP showing the greatest response at shoot, pod and root

level. For that reason, the strain AP191 was selected for field experiments as well as AP193 as this strain showed the highest growth in OP and has demonstrated growth promotion (Hassan et al., 2019) and disease resistance characteristics (Hossain et al., 2015; Shantharaj et al., 2021).

Inoculation with Bv strains AP191, AP215, and AP216 plus OP resulted in above and below ground organs stimulation showing a more significant effect in root parameters (Fig. 2 and 3). Other authors have found that inoculation with other Bv strains such as KPS46 (Buensanteai et al., 2008) and other PGPR species tend to increase aboveground growth, but more significantly root growth due to the production of Indol-3-Acetic Acid (IAA, Barea & Brown, 1971; Shahab et al., 2009). This plant hormone produced by PGPR has been demonstrated to increase total biomass but more significantly primary and secondary roots as well as root hairs (Barea & Brown, 1971; Buensanteai et al., 2008, Shahab et al., 2009; Grover et al., 2021). In our greenhouse experiment, we found that inoculation with AP191, AP 215, and AP2016 increased root characteristics such as total root length, root surface area, and root volume only when OP was added in the inoculation (Fig. 3). This result suggests that in presence of OP, these strains may be able to metabolize the OP and produce more IAA or some other precursors of it that would be responsible of the increased root growth observed in these strains but this hypothesis needs to be further tested using in-vitro and greenhouse experiments. In the field experiment, the increase in root growth parameters with AP191 and AP193 plus OP was not always significant although noticeable with the inoculation with AP191 and AP193 plus OP increasing parameters such as number of root tips, root length, and root surface area (Table 6). This less significant response in the field experiment could be related with the fact that in the field, only the "root crown" was collected according to the shovelomic method, therefore a lot of roots remained in the soil. By the contrary, in the greenhouse experiment, all the roots were collected as the plants were grown in pots and carefully cleaned.

The increase in root growth due to PGPR inoculation, sometimes result in an increase nutrient and water uptake (Vacheron et al., 2013; Cassan et al., 2020). In our experiment, the aboveground N concentration (data not shown) was not increased with any inoculation treatment, however, N uptake was stimulated when the plants were inoculated with AP193 and/or AP191 plus OP (Table 7) demonstrating that the inoculation with PGPR plus OP can sustain a major growth promotion through higher nitrogen uptake probably due to a bigger root system or to higher N_2-fixation.

When looking at root nodule traits, there was a positive significant effect of PGPR+OP inoculation on nodule number (Table 2), where strain AP191 plus OP inoculation resulted in a 90 and 45% increase in small and medium size nodule number respectively (Table 3). However, the nodule number per root length parameter was not higher in the plants inoculated with PGPR plus OP. These results together with the increase in root length and root surface area may indicate that PGPR+OP inoculation could induce soybean nodulation but only thanks to an increased root growth. In the literature it has been reported that inoculation with PGPR may increase the number of secondary roots (Barea & Brown, 1971; Buensanteai et al., 2008, Shahab et al., 2009; Grover et al., 2021) which could increase the infection area for rhizobium, promoting more nodulation. Other authors have reported increased nodule numbers and dry weight with the inoculation of PGPR (Kumar & Chandra, 2008; Arif et al. 2017), however, ours is the first report that points that increase in nodule number may be caused by an overall root growth.

The stimulation of small nodule numbers could increase the contact with external area facilitating the O_2 entry in nodules which could lead to a higher N_2-fixation (Mohammadi et al., 2012). In our field experiment, the inoculation with PGPR plus OP did not result in an increase in N_2-fixation as it can be observed by the Ndfa value (Table 7). However, plants inoculated with AP191 plus OP exhibited more growth than control plants (see more discussion about aboveground

and yield below) maintaining the same levels of aboveground nitrogen concentration and higher N uptake. As it seems that the extra N attained by the PGPR plus OP inoculation was not achieved through a greater N_2-fixation, the stimulation of root traits by the inoculation may be responsible of the higher N-uptake observed in this experiment as it has been observed before (Vacheron et al., 2013; Cassan et al., 2020).

In the current research we tested if seed or in-furrow inoculation was more effective for the deliverance of the PGPR plus OP inoculation by measuring growth promotion in the field. In our EVS location we performed the seed inoculation experiment while in TV we tested the in-furrow inoculation. Although we recognize that the results can be affected by different soil types and environmental conditions, we observed that the seed inoculation (EVS) using AP191+OP resulted in significant higher yield with a 9.4% increase with respect to the control (Fig. 4). In the other hand, in the in-furrow inoculation treatment (TV), we did not observe significant increase with any of the PGPR plus OP treatments, although the inoculation with AP193 plus OP resulted in a not significant 3.7% yield increase. In the in-furrow treatment in TV, inoculation with Vault, which is a commercial product formulated specifically for in-furrow inoculation, resulted in a significant increase in yield (Fig.4). The lack of effect of the PGPR plus OP and the high efficiency of Vault in TV may indicate that the seed treatment could be more adequate than the in-furrow inoculation. Bogino et al. (2011) found that in furrow inoculation was more effective than seed treatment; however, they only tested the effect of PGPR inoculation and not the effect of a seed amendment such OP as in our experiment. This is important because in the seed inoculation the PGPR and OP were in close contact between them and the growing root than in an in-furrow treatment where the OP would be suspended in water as it is an insoluble product and therefore would be susceptible to be washed away easily or being diluted in the soil water. .

The inoculation with AP191 plus OP in the greenhouse and in the field in the case of the seed treatment, resulted in very significant increases in biomass, and what is more relevant for the farmers, yield (Fig. 4). This strain was not the one that grew the best in the OP *in vitro* media, as it was AP193, however, AP191 was the one that produced higher yields. This could suggest that the capacity of growing more in OP media is not the only predictor of good plant growth promotion. It also could be that the strain AP193 effects in plant are more related with disease control than with growth promotion as it has been demonstrated before (Hossain et al., 2015; Shantharaj et al., 2021). Our results also show that there is not interaction between the inoculation treatment and the cultivar used which could be of great importance for the bio-stimulant industry, as it would mean that no matter the cultivar that a farmer uses the effects are going to be always positive and noticeable. However, when the effect of the inoculation with AP191 plus OP is analyzed by cultivar, we can find that some cultivars like AG69X0 show a 7.3% increase in yield while S52XT08 show a 11.5% increase (Supplementary Table 4). Although both positive responses, this variability between cultivars needs to be investigated and homogenized to make bio-fertilizer products more attractive for companies and farmers worldwide.

Previous research have found that co-inoculation of PGPR strains plus highly efficient *Bradyrhizobium* results in better growth and yield stimulation than native *Bradyrhizobium* strains that usually are less efficient in the N_2-fixation (Hungria & Mendes, 2015). Our results showed no extra effect of the co-inoculation with *Bradyrhizobium* plus PGPR and OP on soybean yield. However, when looking at the initial plant phenological stages, inoculation with PGPR plus OP and *Bradyrhizobium* resulted in higher emergence and plant vigor (Table 4). In a meta-analysis study about the effect of PGPR co-inoculated with *Bradyrhizobium* between 1987 and 2018, Zeffa et al. (2020) found that co-inoculation significantly increases soybean nodulation, root and shoot

biomass without an increase in yield. Our results are similar to this probably because the positive effect of very efficient *B. japonicum* species is more evident in the vegetative stages than in the reproductive, due to the change of carbohydrate sinks from the nodules to the developing pods (Sanz-Saez et al., 2015).

Conclusion

Different PGPR strains have variable capability to grown in OP. Although it seems that being able to use OP as a sole C source is an advantage for the PGPR, the level of growth does not seem to predict plant performance. In the greenhouse experiment, inoculation of strains AP191, AP215, and AP216 only in presence of OP resulted in a major root, nodule and aboveground biomass growth, with AP191 being the only one that showed pod stimulation. For that reason, AP191 was selected for a field experiment in addition to AP193 as it was the strain that showed higher growth in OP. In the field experiment seed inoculation demonstrated to be more successful promoting plant growth than in-furrow irrigation probably because it can maintain a close contact between the seed and OP/PGPR. In the field, plants inoculated with AP191 plus OP showed a 9.4% increase in yield in comparison with the no inoculated control when the three cultivars were tested. There was not a cultivar by inoculation interaction for yield with a cultivar variation between 7-11% in comparison with the control. These results are very encouraging for the future of the biofertilizer industry and demonstrate that field performance can be improved with the use of synbiotic treatments such as PGPR plus OP treatments.

References

Arif, M. S., Muhammad, R. I. A. Z., Shahzad, S. M., Yasmeen, T., Shafaqat, A. L. I., & Akhtar, M. J. (2017). Phosphorus-mobilizing rhizobacterial strain Bacillus cereus GS6 improves symbiotic efficiency of soybean on an Aridisol amended with phosphorus-enriched compost. *Pedosphere*, *27*(6), 1049-1061.

Barea, J. M., & Brown, M. E. (1974). Effects on plant growth produced by Azotobacter paspali related to synthesis of plant growth regulating substances. *Journal of Applied Bacteriology*, *37*(4), 583-593.

Bashan, Y. (1998). Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnology advances*, *16*(4), 729-770.

Bhattacharyya, P. N., & Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*, *28*(4), 1327-1350.

Bogino, P., Nievas, F., Banchio, E., & Giordano, W. (2011). Increased competitiveness and efficiency of biological nitrogen fixation in peanut via in-furrow inoculation of rhizobia. *European Journal of Soil Biology*, *47*(3), 188-193.

Buensanteai, N., Yuen, G. Y., & Prathuangwong, S. (2008). The biocontrol bacterium Bacillus amyloliquefaciens KPS46 produces auxin, surfactin and extracellular proteins for enhanced growth of soybean plant. *Thai J Agric Sci*, *41*(3–4), 101-116.

Calvo, P., Nelson, L., & Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant and soil*, *383*(1), 3-41.

Cassán, F., Perrig, D., Sgroy, V., Masciarelli, O., Penna, C., and Luna, V. (2009). Azospirillum brasilense Az39 and Bradyrhizobium japonicum E109 inoculated singly or in combination, promote seed germination and early seedling growth in corn (Zea mays L.) and soybean (Glycine max L.). *European Journal of Soil Biology, 45*(1), 28-35.. 45, 28–35.

Di Cello, F, Bevivino, A, Chiarini, L, Fani, R, Paffetti, D, Tabacchioni, S, Dalmastri, C (1997) Biodiversity of a Burkholderia cepecia population isolated from the maize rhizosphere at different plant growth stages. *Appl* Environ Microbiol 63(11), 4485–4493.

Farquhar, G. D., Ehleringer, J. R., & Hubick, K. T. (1989). Carbon isotope discrimination and photosynthesis. *Annual review of plant biology*, *40*(1), 503-537.

Fehr, W. R., Caviness, C. E., Burmood, D. T., & Pennington, J. S. (1971). Stage of development descriptions for soybeans, Glycine Max (L.) Merrill 1. *Crop science*, *11*(6), 929-931.

Gault, R. R., Chase, D. L., & Brockwell, J. (1982). Effects of spray inoculation equipment on the viability of Rhizobium spp. in liquid inoculants for legumes. *Australian Journal of Experimental Agriculture*, *22*(117), 299-309.

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R. K., Gowda, C. L., & Krishnamurthy, L. (2015). Plant growth promoting rhizobia: challenges and opportunities. *3 Biotech, 5*(4), 355-377.

Goswami, D., Thakker, J. N., & Dhandhukia, P. C. (2016). Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. *Cogent Food & Agriculture, 2*(1), 1127500.

Govindasamy, V., Senthilkumar, M., Magheshwaran, V., Kumar, U., Bose, P., Sharma, V., & Annapurna, K. (2010). Bacillus and Paenibacillus spp.: potential PGPR for sustainable agriculture. In *Plant growth and health promoting bacteria* (pp. 333-364). Springer, Berlin, Heidelberg.

Gray, E. J., & Smith, D. L. (2005). Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. *Soil biology and biochemistry, 37*(3), 395-412.

Grover, M., Bodhankar, S., Sharma, A., Sharma, P., Singh, J., & Nain, L. (2021). PGPR mediated alterations in root traits: Way toward sustainable crop production. *Frontiers in Sustainable Food Systems*, *4*, 618230.

Hartmann, A., Rothballer, M., & Schmid, M. (2008). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant and Soil, 312*(1), 7-14.

Hassan, M. (2016). *The Role of Pectin Utilization in Root Colonization and Plant Growth-Promotion by Bacillus amyloliquefaciens subsp. plantarum (Bap).* [Master's thesis, Auburn University]

Hassan, M. K., McInroy, J. A., Jones, J., Shantharaj, D., Liles, M. R., & Kloepper, J. W. (2019). Pectin-rich amendment enhances soybean growth promotion and nodulation mediated by Bacillus velezensis strains. *Plants, 8*(5), 120.

Hiltner, L. (1904). Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie und unter Berücksichtigung der Gründüngung und Brache. *Arbeiten der Deutschen Landwirtschaftlichen Gesellschaf, 98*, 59-78.

Hossain, M. J., Ran, C., Liu, K., Ryu, C. M., Rasmussen-Ivey, C. R., Williams, M. A., ... & Liles, M. R. (2015). Deciphering the conserved genetic loci implicated in plant disease control through comparative genomics of Bacillus amyloliquefaciens subsp. plantarum. *Frontiers in plant science, 6*, 631.

Hungria, M., & Mendes, I. C. (2015). Nitrogen fixation with soybean: the perfect symbiosis? In de Bruijn, F. J. (Ed.), *Biological nitrogen fixation* (Vol. 2, pp. 1009–1024). John Wiley & Sons, Inc, Hoboken.

Kang, U. G., Somasegaran, P., Hoben, H. J., & Bohlool, B. B. (1991). Symbiotic potential, competitiveness, and serological properties of Bradyrhizobium japonicum indigenous to Korean soils. *Applied and environmental microbiology*, *57*(4), 1038-1045.

Kloepper, J. W., Schroth M.N (1978). Plant growth-promoting rhizobacteria on radishes. In Station de Pathologie Vegetale et Phytobacteriologie (Ed.) *Proc. of the 4th Internet. Conf. on Plant Pathogenic Bacter* (Vol. 2, pp. 879-882). Gilbert-Clarey.

Kloepper, J. W. (1994). Plant growth-promoting rhizobacteria (other systems). *Azospirillum/plant associations, 187*, 137-166.

Kloepper, J. W., Gutierrez-Estrada, A., & McInroy, J. A. (2007). Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria. *Canadian Journal of Microbiology*, *53*(2), 159-167.

Kumar, A., Prakash, A., & Johri, B. N. (2011). Bacillus as PGPR in crop ecosystem. In *Bacteria in agrobiology: crop ecosystems* (pp. 37-59). Springer, Berlin, Heidelberg.

Kumar, R., & Chandra, R. (2008). Influence of PGPR and PSB on Rhizobium leguminosarum bv. viciae strain competition and symbiotic performance in lentil. World Journal of *Agricultural Sciences*, *4*(3), 297-301.

Liu, K., Newman, M., McInroy, J. A., Hu, C. H., & Kloepper, J. W. (2017). Selection and assessment of plant growth-promoting rhizobacteria for biological control of multiple plant diseases. *Phytopathology*, *107*(8), 928-936.

Mageshwaran V., Inmann F.III., and Holmes L.D. (2014). Growth Kinetics of Bacillus subtilis in Lignocellulosic Carbon Sources. *Intl. J. Microbiol. Research. 6*, 579-574.

McNear Jr, D. H. (2013). The rhizosphere - roots, soil and everything in between. *Nature Education Knowledge*, *4*(3), 1.

Mekjian, K. R., Bryan, E. M., Beall, B. W., & Moran, C. P. (1999). Regulation of Hexuronate Utilization in Bacillus subtilis. *Journal of bacteriology*, *181*(2), 426-433.

Mohammadi, K., Sohrabi, Y., Heidari, G., Khalesro, S., & Majidi, M. (2012). Effective factors on biological nitrogen fixation. *African Journal of Agricultural Research*, *7*(12), 1782-1788.

Morrissey, J. P., Dow, J. M., Mark, G. L., & O'Gara, F. (2004). Are microbes at the root of a solution to world food production? Rational exploitation of interactions between microbes and plants can help to transform agriculture. *EMBO reports*, *5*(10), 922-926.

Riedell, W. E., Catangui, M. A., & Beckendorf, E. A. (2009). Nitrogen fixation, ureide, and nitrate accumulation responses to soybean aphid injury in Glycine max. *Journal of plant nutrition*, *32*(10), 1674-1686.

Saeki, Y., Akagi, I., Takaki, H., & Nagatomo, Y. (2000). Diversity of indigenous Bradyrhizobium strains isolated from three different Rj-soybean cultivars in terms of randomly amplified polymorphic DNA and intrinsic antibiotic resistance. *Soil science and plant nutrition*, *46*(4), 917-926.

Sanz-Sáez, Á., Heath, K. D., Burke, P. V., & Ainsworth, E. A. (2015). Inoculation with an enhanced N 2-fixing B radyrhizobium japonicum strain (USDA 110) does not alter soybean (Glycine max Merr.) response to elevated [CO2]. *Plant, Cell & Environment, 38*(12), 2589-2602.

Seethepalli, A., Guo, H., Liu, X., Griffiths, M., Almtarfi, H., Li, Z., ... & York, L. M. (2020). Rhizovision crown: an integrated hardware and software platform for root crown phenotyping. *Plant Phenomics, 2020*, 3074916.

Shahab, S., Ahmed, N., & Khan, N. S. (2009). Indole acetic acid production and enhanced plant growth promotion by indigenous PSBs. *African Journal of Agricultural Research, 4*(11), 1312-1316.

Shantharaj, D., Williams, M. A., Potnis, N. S., & Liles, M. R. (2021). Burkholderia gladioli C101 metabolites protect tomato plants against Xanthomonas perforans infection. *Journal of Plant Diseases and Protection, 128*(2), 379-390.

Shearer, G., & Kohl, D. H. (1986). N2-fixation in field settings: estimations based on natural 15N abundance. *Functional Plant Biology, 13*(6), 699-756.

Sibponkrung, S., Kondo, T., Tanaka, K., Tittabutr, P., Boonkerd, N., Yoshida, K. I., & Teaumroong, N. (2020). Co-Inoculation of Bacillus velezensis Strain S141 and Bradyrhizobium Strains Promotes Nodule Growth and Nitrogen Fixation. *Microorganisms, 8*(5), 678.

Thakur, B. R., Singh, R. K., Handa, A. K., & Rao, M. A. (1997). Chemistry and uses of pectin—a review. *Critical Reviews in Food Science & Nutrition, 37*(1), 47-73.

Tilman, D., Balzer, C., Hill, J., & Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the national academy of sciences, 108*(50), 20260-20264.

Tiwari, A. K., Saha, S. N., Yadav, V. P., Upadhyay, U. K., Katiyar, D., & Mishra, T. (2017). Extraction and characterization of pectin from orange peels. *International Journal of Biotechnology and Biochemistry, 13*(1), 39-47.

Vacheron, J., Desbrosses, G., Bouffaud, M. L., Touraine, B., Moënne-Loccoz, Y., Muller, D., et al. (2013). Plant growth-promoting rhizobacteria and root system functioning. *Frontiers in Plant Science, 4*, 356.

Van Loon, L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. In *New perspectives and approaches in plant growth-promoting Rhizobacteria research* (pp. 243-254). Springer, Dordrecht.

van Veen, J. A., van Overbeek, L. S., & van Elsas, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews, 61*(2), 121-135.

Yeoh, S., Shi, J. T. A. G., & Langrish, T. A. G. (2008). Comparisons between different techniques for water-based extraction of pectin from orange peels. *Desalination, 218*(1-3), 229-237.

List of Tables

Table 3. Response of inoculation with different *Bacillus velezensis* (Bv) strains (AP191, AP193, AP215, AP216, AP218, and MB315) supplemented or not with orange peel (OP) on soybean nodulation in a greenhouse experiment testing 6 different PGPR strains. The table shows the nodule number separated by small (< 2 mm), median (2-3 mm), large (3-4 mm), and very large (> 4 mm) diameter. Means are grouped to show the combined effect of inoculation and strain. The bottom section of the table shows the ANOVA results (p-value) for the effect of Cultivar, Inoculation, and the interaction Cultivar*Inoculation. Means followed by different letters were statistically different.

Table 4. Emergence and plant vigor of soybean treated with different inoculation treatments and grown at field conditions during Summer 2021 at two different Alabama locations (E.V. Smith and Tennessee Valley Research and Extension Centers). The data shown in here for each inoculation treatment is the mean of 3 soybean cultivar. Data for each cultivar can be found in supplemental material. The bottom section of the table shows the ANOVA results (p-value) for the effect of Cultivar, Inoculation, and the interaction Cultivar*Inoculation. Means followed by different letters were statistically different. The percentage of change of each treatment in comparison with their respective control treatment was showed to better show the effect of each treatment.

Table 5. Plant height and SPAD of soybean treated with 14 different inoculation treatments and grown at field conditions during Summer 2021 at two different Alabama locations (E.V. Smith and Tennessee Valley Research and Extension Centers). The data shown in here for each inoculation treatment is the mean of 3 soybean cultivar. Data for each cultivar can be found in supplemental material. The bottom section of the table shows the ANOVA results (p-value) for the effect of Cultivar, Inoculation, and the interaction Cultivar*Inoculation. Means followed by different letters were statistically different. The percentage of change of each treatment in comparison with their respective control treatment was showed to better show the effect of each treatment.

Table 6. Total root tips, root length, average root diameter, and root surface area of soybean treated with 14 different inoculation treatments and grown at field conditions during Summer 2021 at two different Alabama locations (E.V. Smith and Tennessee Valley Research and Extension Centers). The data shown in here for each inoculation treatment is the mean of 3 soybean cultivar. Data for each cultivar can be found in supplemental material. The bottom section of the table shows the ANOVA results (p-value) for the effect of Cultivar, Inoculation, and the interaction Cultivar*Inoculation. Means followed by different letters were statistically different. The percentage of change of each treatment in comparison with their respective control treatment was showed to better show the effect of each treatment.

Table 7. Carbon isotope discrimination and Nitrogen derived from the air of soybean treated with 14 different inoculation treatments and grown at field conditions during Summer 2021 at two different Alabama locations (E.V. Smith and Tennessee Valley Research and Extension Centers). The data shown in here for each inoculation treatment is the mean of 3 soybean cultivar. Data for each cultivar can be found in supplemental material. The bottom section of the table shows the ANOVA results (p-value) for the effect of Cultivar, Inoculation, and the interaction Cultivar*Inoculation. Means followed by different letters were statistically different. The percentage of change of each treatment in comparison with their respective control treatment was showed to better show the effect of each treatment.

List of Figures

Table 1.

Strain	Inoculation	Plant Height (cm)		Leaf area (cm^2)		SPAD	
		Mean	% Change	Mean	% Change	Mean	% Change
AP191	NI	46.5	-	290.4 de	-	36.9	-
	PGPR	43.7	-6.0	304.8 cde	4.9	40.6	9.9
	OP	48.3	3.9	242.4 e	-16.5	40.1	8.6
	PGPR+OP	52.3	12.5	361.4 bcd	24.5	39.6	7.2
AP193	NI	46.5	-	290.4 de	-	36.9	-
	PGPR	51.3	10.3	449.2 a	54.7	41.7	13.0
	OP	48.3	3.9	242.4 e	-16.5	40.1	8.6
	PGPR+OP	49.2	5.8	253.8 e	-12.6	40.6	9.9
AP215	NI	46.5	-	290.4 de	-	36.9	-
	PGPR	46.1	-0.7	261.1 e	-10.1	40.2	8.9
	OP	48.3	3.9	242.4 e	-16.5	40.1	8.6
	PGPR+OP	49.3	6.0	406.1 ab	39.8	41.2	11.7
AP216	NI	46.5	-	290.4 de	-	36.9	-
	PGPR	42.1	-9.5	319.6 cde	10.1	41.0	11.2
	OP	48.3	3.9	242.4 e	-16.5	40.1	8.6
	PGPR+OP	52.7	13.4	388.2 abc	33.7	41.2	11.6
AP218	NI	46.5	-	290.4 de	-	36.9	-
	PGPR	46.0	-1.0	289.6 de	-0.3	41.0	11.2
	OP	48.3	3.9	242.4 e	-16.5	40.1	8.6
	PGPR+OP	51.2	10.2	261.0 e	-10.1	40.3	9.2
MB315	NI	46.5	-	290.4 de	-	36.9	-
	PGPR	47.6	2.5	293.4 de	1.0	40.0	8.5
	OP	48.3	3.9	242.4 e	-16.5	40.1	8.6
	PGPR+OP	48.7	4.9	283.9 de	-2.2	40.3	9.2
NI Mean		46.5 b	-	290.4 b	-	36.9 b	-
PGPR Mean		46.1 b	-0.8	319.6 ab	10.1	40.8 a	10.5
OP Mean		48.3 ab	3.9	242.4 c	-16.5	40.1 a	8.6
PGPR+OP Mean		50.6 a	8.8	325.7 a	12.2	40.5 a	9.8
2-WAY ANOVA RESULTS							
Strain (P-value)		0.9677		0.2716		0.9977	
Inoculation (P-value)		0.0048		<.0001		0.0012	
Strain*Inoculation		0.7389		0.0002		1	

[1]NI – non-inoculated; [2]PGPR+OP – Plant growth-promoting rhizobacteria strain plus orange peel

Table 2.

Strain	Inoculation	Nodule Number		Nodule Dry Weight (g)		Nodule size (mg)		Nodule Area (cm^2)		Nodule Number/Root Length (m)	
		Mean	% Change	Mean	% Change	Mean	% Change	Mean	% Change	Mean	% Change
AP191	NI	137.9	-	0.445	-	2.9	-	11.2	-	25.8	-
	PGPR	137.8	-0.04	0.383	-14.0	2.8	-1.5	10.0	-10.4	28.8	11.5
	OP	96.7	-29.9	0.270	-39.3	2.7	-5.5	7.5	-32.9	20.2	-21.9
	PGPR+OP	168.6	22.2	0.346	-22.4	2.3	-17.7	11.8	5.3	26.1	1.1
AP193	NI	137.9	-	0.445	-	2.9	-	11.2	-	25.8	-
	PGPR	132.6	-3.9	0.325	-27.0	2.4	-14.2	9.6	-14.1	25.7	-0.3
	OP	96.7	-29.9	0.270	-39.3	2.7	-5.5	7.5	-32.9	20.2	-21.9
	PGPR+OP	143.2	3.9	0.364	-18.3	2.7	-5.3	11.3	0.9	30.1	16.7
AP215	NI	137.9	-	0.445	-	2.9	-	11.2	-	25.8	-
	PGPR	141.3	2.5	0.350	-21.3	2.5	-12.2	9.0	-19.1	30.8	19.2
	OP	96.7	-29.9	0.270	-39.3	2.7	-5.5	7.5	-32.9	20.2	-21.9
	PGPR+OP	172.1	24.8	0.343	-22.9	2.2	-24.2	11.8	5.6	20.5	-20.6
AP216	NI	137.9	-	0.445	-	2.9	-	11.2	-	25.8	-
	PGPR	147.6	7.0	0.335	-24.8	2.4	-16.8	9.4	-15.8	34.0	31.6
	OP	96.7	-29.9	0.270	-39.3	2.7	-5.5	7.5	-32.9	20.2	-21.9
	PGPR+OP	160.6	16.5	0.402	-9.7	2.5	-13.1	12.0	7.5	20.4	-21.1
AP218	NI	137.9	-	0.445	-	2.9	-	11.2	-	25.8	-
	PGPR	157.6	14.3	0.442	-0.7	2.6	-8.3	11.0	-1.8	25.5	-1.1
	OP	96.7	-29.9	0.270	-39.3	2.7	-5.5	7.5	-32.9	20.2	-21.9
	PGPR+OP	152.3	10.5	0.473	6.1	3.4	19.4	12.0	7.6	30.4	17.8
MB315	NI	137.9	-	0.445	-	2.9	-	11.2	-	25.8	-
	PGPR	173.0	25.4	0.517	16.2	3.0	4.3	13.2	17.9	21.1	-18.3
	OP	96.7	-29.9	0.270	-39.3	2.7	-5.5	7.5	-32.9	20.2	-21.9
	PGPR+OP	165.3	19.9	0.491	10.2	3.0	5.8	12.7	13.4	31.6	22.3
NI Mean		137.9 b	-	0.445 a	-	2.9	-	11.2 ab	-	25.8 a	-
PGPR Mean		148.3 ab	7.5	0.392 a	-11.9	2.6	-10.3	10.4 b	-7.1	27.6 a	7.0
OP Mean		96.7 c	-29.9	0.270 b	-39.3	2.7	-6.9	7.5 c	-33.0	20.2 b	-21.7
PGPR+OP Mean		160.3 a	16.2	0.403 a	-9.4	2.6	-10.3	11.8 a	5.3	26.5 a	2.7
2-WAY ANOVA RESULTS											
Strain (P-value)		0.816		0.0733		0.2117		0.5491		0.9943	
Inoculation (P-value)		<.0001		<.0001		0.4271		<.0001		0.0002	
Strain*Inoculation		0.9876		0.627		0.3941		0.9511		0.1075	

[1]NI – non-inoculated; [2]PGPR+OP – Plant growth-promoting rhizobacteria strain plus orange peel

Table 3.

Strain	Inoculation	Small Nodules	Median Nodules	Large Nodules	Very Large Nodules
AP191	**NI**	22.1	42.8	48.5	25.3
	PGPR	36.6	40.6	37.1	22.6
	OP	20.0	31.1	28.4	17.1
	PGPR+OP	42.0	62.3	46.1	18.1
AP193	**NI**	22.1	42.8	48.5	25.3
	PGPR	19.1	52.9	46.4	14.1
	OP	20.0	31.1	28.4	17.1
	PGPR+OP	27.4	42.4	49.5	23.8
AP215	**NI**	22.1	42.8	48.5	25.3
	PGPR	40.1	49.6	37.1	14.4
	OP	20.0	31.1	28.4	17.1
	PGPR+OP	37.1	59.1	59.6	16.1
AP216	**NI**	22.1	42.8	48.5	25.3
	PGPR	47.9	44.0	39.7	16.8
	OP	20.0	31.1	28.4	17.1
	PGPR+OP	29.0	55.3	55.5	21.3
AP218	**NI**	22.1	42.8	48.5	25.3
	PGPR	34.6	56.1	49.3	17.9
	OP	20.0	31.1	28.4	17.1
	PGPR+OP	30.7	44.7	48.9	26.9
MB315	**NI**	22.1	42.8	48.5	25.3
	PGPR	44.3	45.3	52.7	30.7
	OP	20.0	31.1	28.4	17.1
	PGPR+OP	37.0	49.1	51.3	27.9
NI Mean		22.1 b	42.8 a	48.5 ab	25.3 a
PGPR Mean		37.1 a	48.1 a	43.7 b	19.4 bc
OP Mean		20.0 b	31.1 b	28.4 c	17.1 c
PGPR+OP Mean		33.9 a	52.1 a	51.8 a	22.3 ab
2-WAY ANOVA RESULTS					
Strain (P-value)		0.3914	0.9898	0.8894	0.339
Inoculation (P-value)		<.0001	<.0001	<.0001	0.0137
Strain*Inoculation		0.7416	0.9509	0.9101	0.8036

[1]NI – non-inoculated; [2]PGPR+OP – Plant growth-promoting rhizobacteria strain plus orange peel

Table 4.

Inoculation Mean	EVS				TV			
	Emergence (%)		Vigor		Emergence (%)		Vigor	
	Mean	% Change	Mean	% Change	Mean	% Change	Mean	% Change
NI	87.7 bc	-	3.0 cd	-	62.9 c	-	3.0	-
OP	85.0 cd	-3.1	3.6 abc	19.4	62.9 c	0.0	3.5	16.7
Brad	86.4 bcd	-1.4	2.9 d	-2.8	64.7 bc	2.9	3.3	11.1
Brad+OP	86.8 bcd	-1.0	3.5 abcd	16.7	60.3 c	-4.0	3.3	11.1
Cell Tech	87.9 abc	0.2	3.4 abcd	13.9	61.8 c	-1.7	3.4	13.9
Vault	86.0 bcd	-1.9	3.8 ab	27.8	59.0 c	-5.2	4.0	33.3
AP191	90.2 ab	2.9	3.1 cd	2.8	63.2 c	0.6	3.4	13.9
AP191+Brad	82.6 d	-5.8	3.4 abcd	13.9	62.4 bc	-0.7	3.7	22.2
AP191+OP	85.5 bcd	-2.5	3.2 cd	5.6	63.8 bc	1.4	3.4	13.9
AP191+Brad+OP	89.5 abc	2.1	3.9 a	30.6	70.3 ab	11.8	3.3	11.1
AP193	86.8 bcd	-1.0	3.2 bcd	8.3	59.6 c	-5.2	3.2	5.6
AP193+Brad	87.0 bcd	-0.8	3.2 bcd	8.3	65.4 bc	4.0	3.2	8.3
AP193+OP	85.7 bcd	-2.3	3.3 abcd	11.1	63.6 bc	1.2	3.6	19.4
AP193+Brad+OP	92.9 a	6.0	3.8 ab	27.8	73.4 a	16.7	3.6	19.4
ANOVA								
Cultivar (P-value)	<.0001		<.0001		<.0001		0.099	
Inoculation (P-value)	0.0401		0.0368		0.0052		0.3783	
Cultivar*Inoculation	<.0001		0.0139		0.6418		0.3543	

[1]NI – non-inoculated; [2]OP – orange peel; [3]Brad – *Bradyrhizobium japonicum*

Table 5.

Inoculation Mean	EVS				TV			
	SPAD		Plant Height (cm)		SPAD		Plant Height (cm)	
	Mean	% Change	Mean	% Change	Mean	% Change	Mean	% Change
NI	46.8 c	-	85.6 e	-	46.2	-	90.7	-
OP	47.5 bc	1.5	90.3 de	5.5	49.1	6.30	90.5	-0.3
Brad	47.8 abc	2.1	89.6 de	4.6	47.3	2.3	95.4	5.2
Brad+OP	47.0 c	0.4	96.1 abcd	12.3	48.9	5.8	90.9	0.3
Cell Tech	47.8 abc	1.9	100.2 a	17.0	48.3	4.5	92.0	1.4
Vault	47.3 c	1.0	97.5 abc	14.0	48.7	5.3	94.5	4.2
AP191	47.6 abc	1.6	94.3 abcd	10.2	48.5	5.0	92.5	2.0
AP191+Brad	47.8 abc	2.1	98.2 abc	14.7	48.7	5.3	92.2	1.7
AP191+OP	47.0 c	0.3	92.2 cde	7.8	47.4	2.7	91.5	0.9
AP191+Brad+OP	48.8 a	4.2	98.0 abc	14.5	48.4	4.8	93.8	3.4
AP193	48.5 ab	3.6	98.3 abc	14.8	48.6	5.2	89.7	-1.1
AP193+Brad	48.7 ab	3.9	96.5 abcd	12.8	47.5	2.7	91.7	1.1
AP193+OP	47.8 abc	2.1	93.0 bcd	8.6	48.2	4.3	93.1	2.6
AP193+Brad+OP	48.8 a	4.1	99.8 ab	16.6	48.2	4.3	93.7	3.3
ANOVA								
Cultivar (P-value)	<.0001		<.0001		<.0001		<.0001	
Inoculation (P-value)	0.0026		0.0008		0.4519		0.6997	
Cultivar*Inoculation	0.2957		0.3817		0.7192		0.9753	

[1]NI – non-inoculated; [2]OP – orange peel; [3]Brad – *Bradyrhizobium japonicum*

Table 6.

E.V. SMITH				
Inoculation Mean	**Total Root Tips**	**Total Root Length (cm)**	**Average Root Diameter (mm)**	**Root Surface Area (cm^2)**
NI	315.2	509.6	1.93	303.7 bcde
OP	396.7	669.3	1.95	392.2 a
Brad	346.5	553.1	1.90	321.0 abcde
Brad+OP	362.7	583.1	1.77	316.0 abcde
Cell Tech	375.0	594.5	1.88	338.8 abcd
Vault	347.2	546.0	1.87	313.8 bcde
AP191	325.4	488.7	1.83	274.2 de
AP191+Brad	322.7	501.8	1.97	295.8 bcde
AP191+OP	367.3	614.8	1.95	362.2 ab
AP191+Brad+OP	307.3	456.4	1.88	262.3 e
AP193	350.3	567.6	2.06	357.0 abc
AP193+Brad	330.5	504.6	1.87	284.4 cde
AP193+OP	356.6	586.3	1.79	326.2 abcde
AP193+Brad+OP	328.2	516.0	1.93	304.6 bcde
ANOVA				
Cultivar (P-value)	<.0001	<.0001	<.0001	<.0001
Inoculation (P-value)	0.6550	0.1963	0.2109	0.0530
Cultivar*Inoculation	0.9079	0.9670	0.2914	0.8825
TENNESSEE VALLEY				
Inoculation Mean	**Total Root Tips**	**Total Root Length (cm)**	**Average Root Diameter (mm)**	**Root Surface Area (cm^2)**
NI	257.0 bcdef	470.0 bcd	2.19	316.3
OP	284.3 abcd	512.8 abc	2.22	342.0
Brad	294.4 abc	541.4 ab	2.17	358.4
Brad+OP	273.0 abcde	490.2 bc	2.27	336.1
Cell Tech	249.0 cdef	466.0 bcd	2.35	340.2
Vault	259.1 bcdef	452.3 bcd	2.24	304.6
AP191	269.8 abcde	500.3 bc	2.23	347.2
AP191+Brad	243.9 def	440.7 cd	2.27	306.5
AP191+OP	279.0 abcde	539.7 ab	2.18	362.5
AP191+Brad+OP	231.4 ef	446.8 cd	2.32	314.3
AP193	274.5 abcde	517.2 abc	2.24	360.1
AP193+Brad	302.1 ab	528.3 abc	2.13	347.1
AP193+OP	309.8 a	594.0 a	2.16	393.9
AP193+Brad+OP	219.5 f	400.1 d	2.36	284.8
ANOVA				
Cultivar (P-value)	<.0001	<.0001	<.0001	<.0001
Inoculation (P-value)	0.012	0.0049	0.3867	0.0729
Cultivar*Inoculation	0.0095	0.0110	0.0921	0.0904

[1]NI – non-inoculated; [2]OP – orange peel; [3]Brad – *Bradyrhizobium japonicum*

Table 7.

Inoculation Mean	EVS						TV					
	Carbon Isotope Discrimination (‰)		Nitrogen Derived from the Air (%)		Nitrogen Uptake (kg N ha^{-1})		Carbon Isotope Discrimination (‰)		Nitrogen Derived from the Air (%)		Nitrogen Uptake (kg N ha^{-1})	
	Mean	PC (%)	Mean	PC (%)	Mean	PC (%)	Mean	PC (%)	Mean	PC (%)	Mean	PC (%)
NI	21.67 ab	-	71.2 ab	-	76.8 c	-	21.37	-	41.2	-	40.7	-
OP	21.65 ab	-0.09	77.5 a	8.9	77.7 c	1.1	21.36	-0.05	38.4	-6.9	39.4	-3
Brad	21.71 a	0.18	67.0 bc	-5.9	82.1 bc	6.9	21.29	-0.37	33.4	-18.9	39.3	-3.4
Brad+OP	21.38 de	-1.34	68.7 bc	-3.4	99.7 ab	29.8	21.34	-0.14	37.2	-9.9	43.9	7.9
Cell Tech	21.38 de	-1.34	67.7 bc	-4.9	103.0 a	34.0	21.39	0.09	29.8	-27.7	45.7	12.4
Vault	21.37 de	-1.38	64.5 bc	-9.4	95.8 ab	24.6	21.24	-0.61	44.0	6.7	46.2	13.8
AP191	21.63 ab	-0.18	70.8 ab	-0.5	87.0 abc	13.2	21.14	-1.08	30.0	-27.2	40.9	0.6
AP191+Brad	21.42 cde	-1.15	69.5 bc	-2.3	93.5 abc	21.6	21.29	-0.37	35.5	-14.0	42.5	4.6
AP191+OP	21.58 abc	-0.42	68.0 bc	-4.5	82.6 bc	7.5	21.21	-0.75	38.9	-5.7	36.1	-11.1
AP191+Brad+OP	21.39 cde	-1.29	63.5 c	-10.8	96.7 ab	25.8	21.40	0.14	35.9	-13.1	51.1	25.7
AP193	21.51 abcde	-0.74	65.5 bc	-8.0	90.5 abc	17.8	21.49	0.56	44.5	8.0	32.7	-19.5
AP193+Brad	21.57 abcd	-0.46	69.8 bc	-2.0	98.0 ab	27.5	21.41	0.19	43.8	6.3	41.4	1.9
AP193+OP	21.49 cde	-0.83	69.0 bc	-3.1	85.5 abc	11.2	21.30	-0.33	31.8	-22.9	45.9	12.9
AP193+Brad+OP	21.34 e	-1.52	65.3 bc	-8.3	97.9 ab	27.4	21.36	-0.05	44.2	7.1	46.6	14.6
ANOVA												
Cultivar (P-value)	0.0353		0.5347		0.7333		0.0100		0.1104		0.0348	
Inoculation (P-value)	0.0005		0.0316		0.0336		0.0775		0.1053		0.3149	
Cultivar*Inoculation	0.5839		0.9243		0.3735		0.8074		0.1024		0.4812	

[1]NI – non-inoculated; [2]OP – orange peel; [3]Brad – *Bradyrhizobium japonicum*

Figure 1.

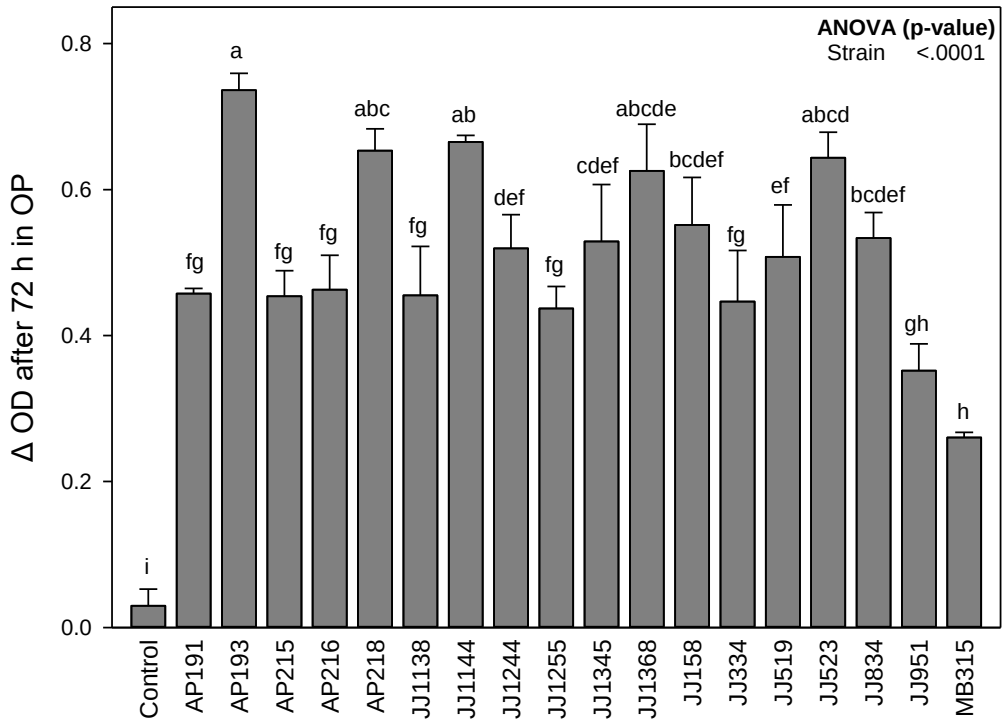

ANOVA (p-value)
Strain <.0001
Δ OD after 72 h in OP
0.0
0.2
0.4
0.6
0.8
i
fg
a
fg
fg
abc
fg
ab
def
fg
cdef
abcde
bcdef
fg
ef
abcd
bcdef
gh
h
Control
AP191
AP193
AP215
AP216
AP218
JJ1138
JJ1144
JJ1244
JJ1255
JJ1345
JJ1368
JJ158
JJ334
JJ519
JJ523
JJ834
JJ951
MB315

Figure 2.

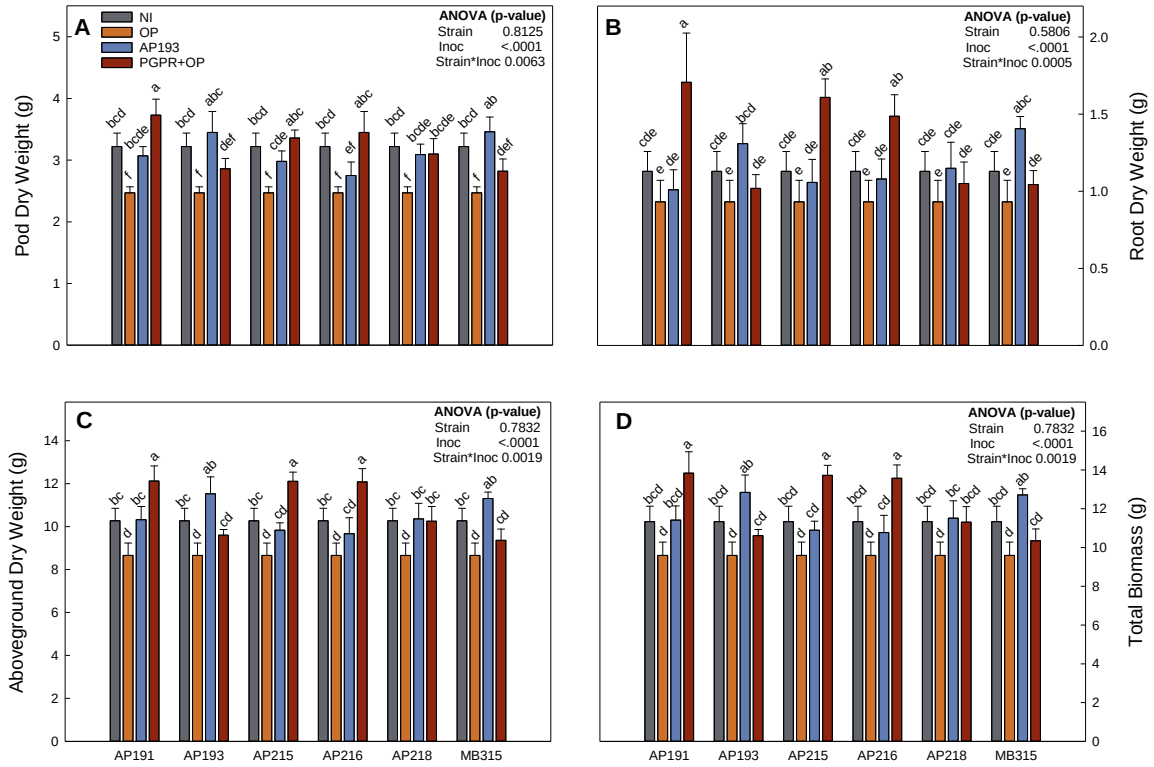

A
NI
OP
AP193
PGPR+OP
ANOVA (p-value)
Strain 0.8125
Inoc <.0001
Strain*Inoc 0.0063
Pod Dry Weight (g)
B
ANOVA (p-value)
Strain 0.5806
Inoc <.0001
Strain*Inoc 0.0005
Root Dry Weight (g)
C
ANOVA (p-value)
Strain 0.7832
Inoc <.0001
Strain*Inoc 0.0019
Aboveground Dry Weight (g)
D
ANOVA (p-value)
Strain 0.7832
Inoc <.0001
Strain*Inoc 0.0019
Total Biomass (g)
AP191
AP193
AP215
AP216
AP218
MB315

Figure 3.

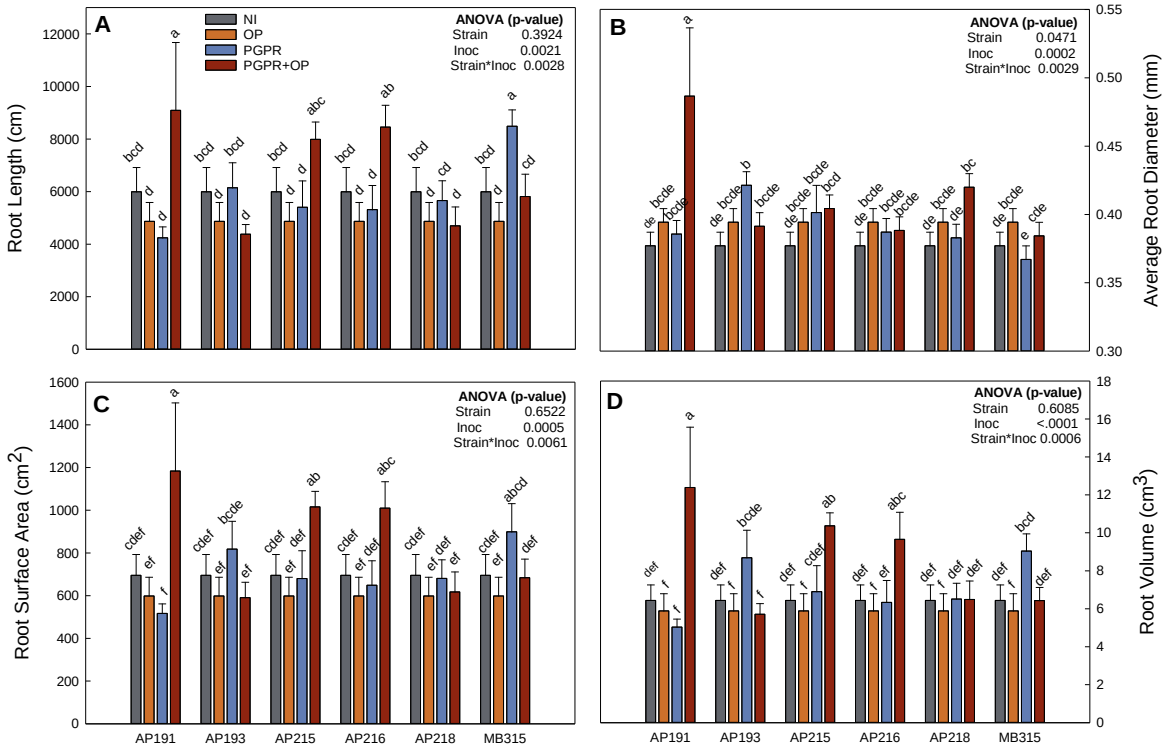
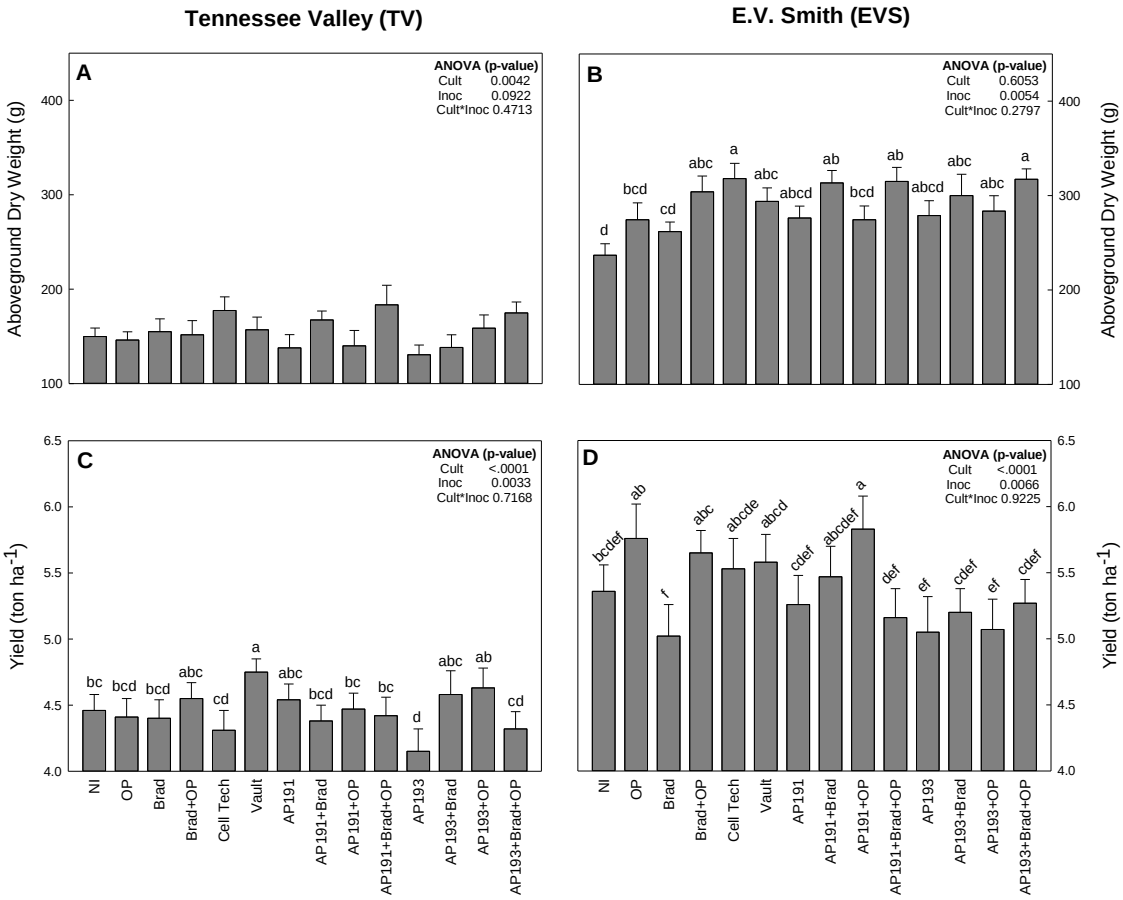

A
NI
OP
PGPR
PGPR+OP
ANOVA (p-value)
Strain 0.3924
Inoc 0.0021
Strain*Inoc 0.0028
Root Length (cm)
B
ANOVA (p-value)
Strain 0.0471
Inoc 0.0002
Strain*Inoc 0.0029
Average Root Diameter (mm)
C
ANOVA (p-value)
Strain 0.6522
Inoc 0.0005
Strain*Inoc 0.0061
Root Surface Area (cm2)
D
ANOVA (p-value)
Strain 0.6085
Inoc <.0001
Strain*Inoc 0.0006
Root Volume (cm3)
AP191
AP193
AP215
AP216
AP218
MB315

Figure 4.


Tennessee Valley (TV)
E.V. Smith (EVS)
A
B
C
D
ANOVA (p-value)
Cult 0.0042
Inoc 0.0922
Cult*Inoc 0.4713
ANOVA (p-value)
Cult 0.6053
Inoc 0.0054
Cult*Inoc 0.2797
ANOVA (p-value)
Cult <.0001
Inoc 0.0033
Cult*Inoc 0.7168
ANOVA (p-value)
Cult <.0001
Inoc 0.0066
Cult*Inoc 0.9225
Aboveground Dry Weight (g)
Yield (ton ha-1)
NI
OP
Brad
Brad+OP
Cell Tech
Vault
AP191
AP191+Brad
AP191+OP
AP191+Brad+OP
AP193
AP193+Brad
AP193+OP
AP193+Brad+OP

Supplementary Table 1. Characteristics and source of the soybean commercial cultivars tested in the field experiments.

Cultivar	Brand/Company	Maturity Group	Trait
AG69X0	Asgrow	6.9	R2X
S49XT39	Dyna-Gro	4.9	R2X
S52XT08	Dyna-Gro	5.2	R2X

Supplementary Table 2. B-Values retrieved from the literature that were used to calculate the N derived from the atmosphere in the field study.

Author	Publication year	Shoot B value %	Stage
Araujo et al.	2018	-2.85	R1
Araujo et al.	2018	-3.17	R1
Araujo et al.	2018	-2.76	R1
Araujo et al.	2018	-2.35	R2
Average		**-2.78**	

Supplementary Table 3. Lodging scores and definition used to evaluate lodging before harvest soybeans plants in the field experiment.

Lodging Score	Definition
0	All plants erect
1	Almost all plants erect
2	Either all plants leaning slightly or a few plants down
3	Either all plants leaning moderately or 25-50% down
4	Either all plants leaning considerably or 50-80% down
5	All plants down

Supplementary Table 4. Yield, aboveground dry weight, SPAD, and plant height for three different soybean cultivars grown at field conditions during Summer 2021 at E.V. Smith Research and Extension Center. Fourteen inoculation treatments were tested, and the table shows the mean of inoculations for each cultivar tested. The table also shows the ANOVA results (p-value) for the effect of Cultivar, Inoculation, and the interaction Cultivar*Inoculation.

Cultivar	Inoculation	Yield (ton ha^{-1})		Aboveground Dry Weight (g)		SPAD		Plant Height (cm)	
		Mean	PC (%)	Mean	PC (%)	Mean	PC (%)	Mean	PC (%)
AG69X0	NI	5.98	-	234.8	-	48.6	-	81.9	-
	OP	6.42	7.4	271.5	15.6	50.0	2.8	88.9	8.5
	Brad	5.87	-1.9	287.7	22.5	49.1	1.0	90.1	10.0
	Brad+OP	6.14	2.6	331.4	41.2	49.4	1.7	94.0	14.7
	Cell Tech	6.29	5.2	323.6	37.8	50.0	3.0	88.8	8.4
	Vault	6.29	5.2	297.5	26.7	49.3	1.4	90.0	9.9
	AP191	5.58	-6.7	304.8	29.8	49.1	1.0	85.6	4.5
	AP191+Brad	6.44	7.7	309.0	31.6	49.0	0.8	95.5	16.6
	AP191+OP	6.42	7.3	281.6	19.9	47.3	-2.8	89.1	8.7
	AP191+Brad+OP	5.86	-2.0	310.9	32.4	50.5	3.9	95.6	16.7
	AP193	6.05	1.2	251.3	7.0	50.0	2.8	97.4	18.9
	AP193+Brad	5.84	-2.3	314.5	34.0	51.2	5.3	88.3	7.8
	AP193+OP	5.76	-3.8	305.2	20.0	48.6	-0.1	85.8	4.8
	AP193+Brad+OP	5.84	-2.4	306.7	30.6	50.0	2.8	95.7	16.9
S52XT08	NI	5.04	-	252.5	-	46.6	-	97.2	-
	OP	5.41	7.4	311.8	23.5	46.1	-1.1	91.7	-5.6
	Brad	4.61	-8.6	254.2	0.7	47.5	1.9	91.3	-6.1
	Brad+OP	5.70	13.1	284.5	12.6	45.8	-1.7	99.4	2.2
	Cell Tech	5.34	6.1	279.9	10.8	46.2	-0.9	110.1	13.2
	Vault	5.41	7.5	291.2	15.3	46.2	-0.9	109.3	12.4
	AP191	5.16	2.4	253.2	0.2	47.4	1.7	102.7	5.7
	AP191+Brad	5.27	4.5	299.4	18.6	47.2	1.2	101.3	4.2
	AP191+OP	5.62	11.6	277.5	9.9	47.1	1.0	99.2	2.0
	AP191+Brad+OP	4.57	-9.4	322.7	27.8	47.3	1.5	106.9	9.9
	AP193	4.26	-15.5	299.9	18.8	47.3	1.4	95.7	-1.5
	AP193+Brad	4.72	-6.3	233.8	-7.4	46.4	-0.5	104.7	7.7
	AP193+OP	4.82	-4.3	295.6	17.1	46.8	0.3	98.4	1.2
	AP193+Brad+OP	4.91	-2.5	344.8	36.5	47.6	2.1	104.1	7.0
S49XT39	NI	4.99	-	223.5	-	45.3	-	77.6	-
	OP	5.46	9.4	240.3	7.5	46.6	2.8	90.2	16.2
	Brad	4.61	-7.7	243.7	9.0	46.9	3.6	87.2	12.4
	Brad+OP	5.13	2.7	296.2	32.5	45.9	1.2	94.9	22.3
	Cell Tech	4.97	-0.4	350.5	56.8	47.0	3.8	101.6	30.9
	Vault	5.05	1.2	293.0	31.1	46.4	2.5	93.2	20.2
	AP191	5.04	1.0	271.2	21.3	46.3	2.1	94.6	21.9
	AP191+Brad	4.72	-5.3	332.0	48.5	47.4	4.6	88.5	14.1
	AP191+OP	5.48	9.8	265.6	18.8	46.6	2.8	94.7	22.0
	AP191+Brad+OP	5.07	1.5	311.7	39.4	48.6	7.2	94.5	21.8
	AP193	4.86	-2.6	285.7	27.8	48.4	6.7	101.7	31.0
	AP193+Brad	5.06	1.3	351.8	57.4	48.4	6.8	96.5	24.4
	AP193+OP	4.65	-6.8	250.3	12.0	48.1	6.1	94.7	22.0
	AP193+Brad+OP	5.07	1.6	300.6	34.5	48.7	7.5	99.5	28.2
ANOVA									
Cultivar (P-value)		<.0001		0.6053		<.0001		<.0001	
Inoculation (P-value)		0.0066		0.0054		0.0026		0.0008	
Cultivar*Inoculation		0.9225		0.2797		0.2957		0.3817	

Supplementary Table 5. Yield, aboveground dry weight, SPAD, and plant height for three different soybean cultivars grown at field conditions during Summer 2021 at and Tennessee Valley Research and Extension Center. Fourteen inoculation treatments were tested, and the table shows the mean of inoculations for each cultivar tested. The table also shows the ANOVA results (p-value) for the effect of Cultivar, Inoculation, and the interaction Cultivar*Inoculation.

Cultivar	Inoculation	Yield (ton ha^{-1})		Aboveground Dry Weight (g)		SPAD		Plant Height (cm)	
		Mean	PC (%)	Mean	PC (%)	Mean	PC (%)	Mean	PC (%)
AG69X0	NI	4.82	-	157.7	-	45.5	-	89.8	-
	OP	4.95	2.7	141.7	-10.2	50.6	11.3	88.9	-1.0
	Brad	4.93	2.3	157.8	0.1	45.3	-0.4	95.1	5.8
	Brad+OP	4.95	2.6	168.8	7.0	48.7	7.0	87.7	-2.3
	Cell Tech	4.79	-0.7	216.4	37.2	49.6	9.0	92.2	2.6
	Vault	5.03	4.4	129.4	-18.0	50.4	10.8	88.5	-1.5
	AP191	4.92	2.1	146.3	-7.2	49.7	9.3	91.7	2.1
	AP191+Brad	4.81	-0.1	189.0	19.8	49.7	9.3	92.1	2.5
	AP191+OP	4.80	-0.5	184.4	16.9	49.8	9.4	90.0	0.2
	AP191+Brad+OP	4.60	-4.6	226.7	43.7	49.7	9.1	90.1	0.3
	AP193	4.77	-1.0	153.6	-2.6	49.0	7.7	89.3	-0.6
	AP193+Brad	5.17	7.3	172.4	9.3	48.1	5.8	90.7	1.0
	AP193+OP	4.93	2.3	147.6	-6.4	49.7	9.3	87.5	-2.6
	AP193+Brad+OP	4.81	-0.2	177.2	12.4	49.4	8.6	91.1	1.4
S52XT08	NI	4.23	-	146.5	-	47.2	-	99.4	-
	OP	4.12	-2.6	138.6	-5.4	47.3	0.3	96.4	-3.0
	Brad	4.14	-2.0	148.9	1.6	47.4	0.5	100.2	0.8
	Brad+OP	4.28	1.0	147.7	0.8	46.9	-0.7	98.3	-1.0
	Cell Tech	3.92	-7.3	194.6	32.8	47.0	-0.5	96.7	-2.6
	Vault	4.49	6.1	162.4	10.8	46.1	-2.4	104.2	4.9
	AP191	4.36	3.0	152.3	3.9	47.5	0.5	100.6	1.2
	AP191+Brad	4.13	-2.3	152.0	3.8	47.1	-0.2	99.0	-0.4
	AP191+OP	4.21	-0.4	114.8	-21.6	43.7	-7.5	100.2	0.9
	AP191+Brad+OP	4.11	-2.8	161.1	10.0	46.6	-1.3	102.5	3.1
	AP193	3.78	-10.6	135.2	-7.7	47.3	0.2	95.1	-4.3
	AP193+Brad	4.38	3.6	116.3	-20.6	46.3	-2.0	95.2	-4.2
	AP193+OP	4.26	0.7	145.4	-0.7	46.2	-2.1	101.7	2.4
	AP193+Brad+OP	4.07	-3.7	161.4	10.2	46.8	-0.9	102.3	3.0
S49XT39	NI	4.33	-	145.7	-	45.5	-	82.9	-
	OP	4.14	-4.4	158.4	8.7	49.4	8.5	86.1	3.8
	Brad	4.12	-4.8	158.4	8.7	49.1	7.9	90.9	9.6
	Brad+OP	4.43	2.2	138.8	-4.7	48.1	5.7	86.3	4.1
	Cell Tech	4.23	-2.5	121.3	-16.8	48.3	6.0	87.0	4.9
	Vault	4.75	9.5	179.5	23.2	49.5	8.7	90.8	9.5
	AP191	4.34	0.1	115.1	-21.00	48.4	6.4	85.2	2.8
	AP191+Brad	4.19	-3.4	161.5	10.8	49.1	8.0	85.6	3.2
	AP191+OP	4.40	1.5	120.8	-17.1	48.9	7.4	84.2	1.6
	AP191+Brad+OP	4.55	4.9	162.8	11.7	49.0	7.7	88.9	7.2
	AP193	3.90	-10.1	103.1	-29.2	49.5	8.8	84.7	2.1
	AP193+Brad	4.14	-4.5	126.5	-13.2	48.0	5.4	89.2	7.5
	AP193+OP	4.69	8.2	156.3	7.2	48.6	6.8	90.0	8.5
	AP193+Brad+OP	4.09	-5.7	186.1	27.7	48.3	6.2	87.7	5.8
ANOVA									
Cultivar (P-value)		<.0001		0.0042		<.0001		<.0001	
Inoculation (P-value)		0.0033		0.0922		0.4519		0.6997	
Cultivar*Inoculation		0.7168		0.4713		0.7192		0.9753	

Supplementary Table 6. Total root tips and root length for three different soybean cultivars grown at field conditions during Summer 2021 at and Tennessee Valley Research and Extension Center. Fourteen inoculation treatments were tested, and the table shows the mean of inoculations for each cultivar tested. The table also shows the ANOVA results (p-value) for the effect of Cultivar, Inoculation, and the interaction Cultivar*Inoculation.

Cult.	Inoculation	Total Root Tips			Total Root Length (cm)		
		Mean		PC (%)	Mean		PC (%)
AG69X0	NI	329.6	abcde	-	633.9	bcd	-
	OP	393.6	ab	19.4	701.2	abc	10.6
	Brad	344.8	abc	4.6	614.6	bcd	-3.0
	Brad+OP	282.8	cdefghijk	-14.2	508.2	defghijkl	-19.8
	Cell Tech	263.5	cdefghijkl	-20.0	541.0	defghij	-14.6
	Vault	343.3	abc	4.2	615.3	bcdef	-2.9
	AP191	268.4	cdefghijkl	-18.6	524.4	defghijk	-17.3
	AP191+Brad	287.7	cdefghi	-12.7	548.2	cdefghij	-13.5
	AP191+OP	346.8	abc	5.2	734.5	ab	15.9
	AP191+Brad+OP	291.7	cdefghi	-11.5	570.9	cdefghi	-9.9
	AP193	334.6	abcd	1.5	613.4	bcdef	-3.2
	AP193+Brad	322.4	bcdef	-2.2	588.5	bcdefgh	-7.2
	AP193+OP	410.2	a	24.4	833.0	a	31.4
	AP193+Brad+OP	286.3	cdefghij	-13.1	545.1	defghij	-14.0
S52XT08	NI	213.7	hijklm	-	329.6	mno	-
	OP	162.9	m	-23.8	298.0	o	-9.6
	Brad	283.2	cdefghijk	32.5	506.8	defghijkl	53.8
	Brad+OP	296.1	cdefgh	38.5	505.2	efghijklm	53.3
	Cell Tech	214.3	hijklm	0.3	398.0	jklmno	20.8
	Vault	187.5	lm	-12.3	307.8	no	-6.6
	AP191	244.3	fghijklm	14.3	455.4	ghijklmn	38.2
	AP191+Brad	188.6	lm	-11.7	319.2	no	-3.1
	AP191+OP	167.9	m	-21.4	292.6	o	-11.2
	AP191+Brad+OP	202.0	jklm	-5.5	367.0	lmno	11.3
	AP193	212.9	hijklm	-0.4	373.1	klmno	13.2
	AP193+Brad	270.3	cdefghijkl	26.5	429.4	jklmno	30.3
	AP193+OP	235.2	ghijklm	10.0	398.0	jklmno	20.8
	AP193+Brad+OP	210.5	ijklm	-1.5	348.1	mno	5.6
S49XT39	NI	227.6	hijklm	-	433.9	ghijklmno	-
	OP	296.3	cdefgh	30.2	539.2	defghij	24.2
	Brad	255.2	defghijkl	12.1	475.8	efghijklm	9.6
	Brad+OP	240.2	fghijklm	5.5	457.1	ghijklmn	5.3
	Cell Tech	269.1	cdefghijkl	18.2	459.1	fghijklmn	5.8
	Vault	246.5	efghijklm	8.3	433.9	hijklmno	0.0
	AP191	296.7	cdefgh	30.3	521.2	defghijkl	20.1
	AP191+Brad	255.3	defghijkl	12.2	454.8	ghijklmn	4.8
	AP191+OP	322.4	bcdef	41.6	592.1	bcdefg	36.4
	AP191+Brad+OP	200.4	klm	-12.0	402.6	jklmno	-7.2
	AP193	275.9	cdefghijk	21.2	565.1	cdefghi	30.2
	AP193+Brad	313.6	bcdefg	37.8	567.1	cdefghi	30.7
	AP193+OP	284.1	cdefghijk	24.8	550.9	cdefghi	27.0
	AP193+Brad+OP	161.6	m	-29.0	307.0	no	-29.3
ANOVA							
Cultivar (P-value)		<.0001			<.0001		
Inoculation (P-value)		0.0120			0.0049		
Cultivar*Inoculation		0.0095			0.0110		