Enhancing plant growth, drought stress tolerance, and biological control capacities of PGPR strains with exogenous pectin-rich amendments

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 8th, 2020

Keywords: Pectin, orange peel, PGPR, plant growth-promotion, drought stress tolerance, biological control

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Abstract

Bacillus velezensis is a plant growth-promoting rhizobacterium (PGPR) that promotes plant growth, enhances drought stress tolerance, and suppresses plant pathogens. However, little is known about the effects of exogenous pectin or orange peel amendments on plants by *B. velezensis* PGPR strains. The objectives of the present studies were: (i) to evaluate the combined effects of *B. velezensis* PGPR strains with exogenous pectin or orange peel amendments in soybean growth promotion and nodulation, (ii) to determine the capacity of *B. velezensis* PGPR strain with orange peel amendment to enhance drought stress tolerance in peanut (iii) to evaluate *B. velezensis* PGPR strain with orange peel amendment for their potential biological control of the southern root-knot nematode, *Meloidogyne incognita* in soybean and cotton. The treatments for objective one included soybean seeds planted in field soil that inoculated *B. velezensis* PGPR strains amended with or without exogenous pectin or orange peel and untreated control. The treatments for objective two included untreated peanut genotypes planted in field soil mixed with a potting mix that inoculated with *B. velezensis* PGPR strain amended with or without the orange peel. The treatments for objective three included soybean and cotton seeds planted in field soil that inoculated with cell pellet suspension, culture broth, and cell-free supernatant of *B. velezensis* PGPR strain grown in orange peel amended media and untreated control. In the greenhouse test, soybean seeds inoculated with *B. velezensis* PGPR strain AP193 and pectin had significantly increased shoot length, dry weight, and nodulation compared to the same strain without pectin amendment. Orange peel amendment in greenhouse test, with AP193 at 10 mg significantly increased the dry weight of shoots and roots compared to the same strain without pectin amendment. In the field test, pectin with *B. velezensis* PGPR strain AP193 significantly increased shoot length, dry weight, and nodulation compared to the same strain without pectin amendment. The biological control test results indicate that cell pellet suspension (CPS), culture broth (CB),

and cell-free supernatant (CFS) of *B. velezensis* PGPR strain AP203 significantly reduced *M. incognita* population compared to *M. incognita* inoculated positive control at 45 days after planting (DAP) compared to *M. incognita* inoculated positive control in soybean and cotton. However, there were no significant differences between CPS, CB or CFS of *B. velezensis* strains grown in orange peel amended media. The specific leaf area (SLA), SPAD chlorophyll meter readings (SCMR), chlorophyll density (ChlD), and root length of *B. velezensis* strain AP203 with orange peel amendment were statistically significant compared to *B. velezensis* strain AP203 without orange peel amendment under drought stress tolerance conditions. Significant genotype x drought stress interactions were observed on most of the investigated agronomic traits. Genotype AU 18-33 of *B. velezensis* strain AP203 with orange peel amendment showed significant plant dry weight compared to *B. velezensis* strain AP203 without orange peel amendment under both water regimes. These results are significant as they illustrate the potential of *B. velezensis* PGPR strains to enhance soybean plant growth, peanut drought stress tolerance, and biological control of *M. incognita* by exogenous pectin or orange peel amendments.

Acknowledgments

I would first like to thank my beloved mother, Jahanara Nargis; you are indeed an inspiration to me. Without your unconditional love, I would not complete this arduous journey. Whenever I experienced tough times during this journey, you encouraged and showed me a path to fulfill my cherished dream.

This dissertation is dedicated to my father, Mohammad Altaf Hossain. His courageous and positive attitude made me a motivated and confident person to overcome the hurdle under any circumstances.

I am grateful to my brothers and sister-in-laws, Mohammad Jahangir Hossain, Mohammad Jahid Hossain, Shamima Nasrin, and Tahmina Ripa, for their constant support and guidance. I am also thankful to my dearest nieces, Sarina, Samrina, and Jarin. They always encourage me to fulfill my cherished dream.

I am delighted to express my gratefulness to my beloved wife, Madhabi Rahman, for her caring and love. I would like to extend my appreciation for her valuable guidance, constant support, and encouragement during this journey.

I wish to express my sincere gratitude to my major professor Dr. Joseph W. Kloepper. His mentorship, advice, and support encouraged and inspired me to be a good researcher. It is always the utmost pleasure working with him. I feel privileged to have had the opportunity of working under the tutelage of a pioneer Plant Pathologist like him.

I am grateful to my co-advisors, Dr. Kathy S. Lawrence and Dr. Mark R. Liles. It was unthinkable to complete nematode and molecular works without their constant support and advice during my Ph.D. study.

I express my sincerest thanks to my committee member, Dr. Edward J. Sikora, for always supporting me. Your guidance and advice helped me a lot to complete the project on time.

I would like to thank my dissertation reader, Dr. Charles Chen, for his valuable suggestions in revising this dissertation, and Dr. Alvaro Sanz-Saez, for his advice during the greenhouse drought stress tolerance test.

I also thank my father and mother-in-laws for their tremendous support during my Ph.D. study.

Finally, I would like to thank God for giving me the strength to complete this journey.

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List of Abbreviations

Bv	*Bacillus velezensis*
PGPR	Plant growth-promoting rhizobacteria
LC-MS	Liquid chromatography-mass spectrometry
VOCs	Volatile organic compounds
DAP	Days after planting
DAE	Days after emergence
RW	Regular water
DT	Drought stress tolerance
ChlD	Chlorophyll density
SPAD	Soil plant analytical development
SCMR	SPAD chlorophyll meter readings
SLA	Specific leaf area
RWC	Relative water content
WUEi	Instantaneous water use efficiency

Chapter I Literature Review

1. Introduction

The world's population is projected to increase more than 25% by 2050 (207) which will require increased crop production worldwide. To produce more crops, more fertilizers are needed for arable lands to maintain soil health and quality. In 2016, worldwide fertilizer (N, P_2O_5, and K_2O) consumption was 292,429 thousand tons and was projected to reach 318,652 thousand tons by 2022 (8).

Although fertilizers enhance plant growth and crop production, plants cannot utilize all the nutrients in fertilizers due to the formation of insoluble forms such as nitrogen and phosphate. Nutrients also often are washed off agricultural fields into waterways. Run-off reduces crop growth and harms aquatic ecosystems through depletion of oxygen level in rivers, lakes, ponds, and seas. Excessive concentrations of nitrogen and phosphorous are responsible for the oxygen depletion in the water (165). This depletion causes dead zones that have developed in many coastal areas, including the Gulf of Mexico, the Black Sea, the Baltic Sea, and the East China Sea (52). The depletion of oxygen in oceans, called anoxia, leads to hypoxia of fish, resulting in changes in diversity and abundance of fish species, decreases in growth rates, and altered spatial distribution of fish (27).

Plant diseases often reduce crop production in agricultural fields. Plant pathogenic bacteria, fungi, and nematodes attack plant parts through natural openings, wounds, and in some cases, through the help of insect vectors. Once inside the plant, pathogens can multiply, reduce the plants physiological activity, and sometimes produce toxins. In this way, plants either die before maturation or produce less yield. For example, In 2017, soybean yield loss due to bacterial, fungal, viral, and nematode associated diseases was estimated at a total of 92.43 million bushels in the Southern United States (4). In 2017, soybean yield loss estimated 4.19 % due to diseases in Alabama (4). Managing plant diseases is a continual challenge because

pathogens routinely develop resistance to many bactericides, nematicides, and fungicides. This resistance often results from the overuse of these chemicals, requiring the continual introduction of new fungicides. Hence, alternative plant disease control approaches, such as the use of biocontrol agents, including PGPR, can help delay development of resistance to bactericides and fungicides.

Biofertilizers are bacterial or fungal inoculants that enhance the bioavailability of nutrients in the rhizosphere and nutrient uptake capacity by plants (29; 57). Biofertilizers do not directly influence plant pathogens. PGPR biofertilizers use at least one mechanism to enhance plant growth, such as increased nutrient accumulation or plant growth hormone elicitation (5). Biofertilizers can be applied as a seed treatment, as foliar applications, or directly into soil (220). In the past decade, biofertilizers products have been developed based on the various rhizobacterial genera that are now available in crop production.

PGPR are plant growth-promoting rhizobacteria that colonize roots in the rhizosphere and stimulate plant growth by the production and secretion of chemical compounds such as secondary metabolites, phytohormones, and volatile organic compounds (2). Microbial synthesis of the phytohormone auxin has been known (2) and reported that 80% microbes isolated from the rhizosphere of multiple crops have the capacity to synthesize and release auxin as a secondary metabolite (170). Volatile organic compounds (VOCs) emitted by PGPR contribute to plant growth-promotion and induced systemic resistance (ISR) to pathogens (190; 191). Park *et al.* (2015) have shown that VOCs (13-Tetradecadien-1-ol, 2-butanone, and 2 Methyl-n-1-tridecene) produced by *P. fluorescens* SS101 enhanced tobacco seedling growth *in vitro* and *in planta* (166). The species *B. velezensis* (previously known as *B. amyloliquefaciens* subsp. *plantarum*) is a spore-forming, gram-positive, rod-shaped bacterium. Many strains of *B. velezensis* have been reported to promote plant growth and exhibit biological control against pathogens on several crops and vegetables, including

potato, pepper, tomato, cucumber (146), and cabbage (127). Strains of *B. velezensis* have significantly increased plant growth of tea plants (137), maize (102), soybean (28), and *Arabidopsis thaliana* (69). Biological control activity of *B. velezensis* strains have been reported against strawberry Fusarium wilt (*F. oxysporum* f. sp. *fragariae*) (154), black rot of cabbage (*Xanthomonas campestris* pv. *campestris*) (128), fire blight of apple (*Erwinia amylovora*) (36), root rot of hydroponically-grown vegetables (*Pythium spp.*) (107), peanut bacterial wilt (*Ralstonia solanacearum*) (230), anthracnose crown rot of strawberry (*Colletotrichum gloeosporioides*) (153), and both gray mold (*Botrytis cinerea*) and powdery mildew (*Sphaerotheca aphanis*) fungal pathogen of strawberry (197). Recent research also reported that *B. velezensis* strains Bve2 and Bve12 enhanced cotton yield in both field trials and microplot conditions and reduced numbers of *Meloidogyne incognita* eggs in the microplot conditions at 45 days after planting (239). Gao *et al.* (2017) described 29 volatile compounds (VOCs) of *B. velezensis* that inhibited several fungal pathogens, including *Alternaria solani, Botrytis cinerea, Valsa mali, Monilinia fructicola, Fusarium oxysporum f. sp. capsicum,* and *Colletotrichum lindemuthianum* (75).

It has been estimated that pectin makes up 35% of the dry cell wall weight of dicotyledonous plants (147). However, little is known about the interactions of exogenous pectin and PGPR on plant growth-promotion, drought stress tolerance, and biological control. Root border cells contributed up to 98% on the carbon-rich material that have impacts on plant-microbe interactions under controlled conditions (80). Border cell separation is influenced by the activity of pectin-degrading methylesterases and polygalacturonases enzymes (208; 233). Pectin is a complex polysaccharide present in the primary cell wall and middle lamella of higher plants (235). It plays an important role in cell elongation during early plant growth stages and plant cell wall formation in higher plants that give plant structural strength and support (224). Pectin is present around growing roots and PGPR colonizing roots may be able

to utilize pectin as a carbon source. Recent studies have found that oligogalacturonides oligomers of alpha-1, 4-linked galacturonic acid hydrolyzed from pectin are able to act as damage-associated molecular patterns (DAMPs) against pathogen infections (17; 71).

The composition of pectin differs from plant to plant based on the cell wall structure. The main currently-available commercial sources of pectin extracts are sugar beet (6), apple pomace, and citrus peel (229). A recent study found that the pectin content in citrus genera ranged from 29.4% in *Citrus lemon* to 36.7% in *C. limetta* (109). To date, only one study has reported interactions of pectin and PGPR strains to promote plant-growth and provide biological control (87). In this study, the pectin amendment (0.1%) with two *B. velezensis* PGPR strains significantly increased plant growth-promotion and nodulation of soybean plants. Hence, further studies are needed to evaluate the effects of pectin-rich amendments on the plant growth-promotion, drought stress tolerance, and biological control by *B. velezensis* PGPR strains.

Accordingly, the objectives of this research were i) to investigate the effects of pectin or orange peel amendments on plant growth-promotion, nodulation, and drought stress tolerance of legume plants by *B. velezensis* PGPR strains, and ii) determine if pectin amendment from an orange peel source increases the biological control against root-knot nematode, *Meloidogyne incognita* in soybean and cotton by selected *B. velezensis* strain.

2. Plant growth-promoting rhizobacteria (PGPR)

2.1. Background of PGPR

PGPR are plant growth-promoting rhizobacteria that colonize plant roots and the rhizosphere and suppress other microorganisms through the competition (2; 111). Hiltner (1904) first coined the term “rhizosphere,” which is obtained from the word “rhiza,” meaning root, and “sphere,” meaning an area of activity. Pinton *et al.* (2001) later defined the rhizosphere as the volume of soil affected by exudates from the root and root tissues and colonized by

rhizobacteria (174). In the rhizosphere, the rhizobacterial interactions with plant roots can be positive, negative, or neutral. Positive interactions can result in enhanced plant growth/or suppression of plant pathogens (150). Lynch (134) proposed three zones of the rhizosphere: the endorhizosphere (the endodermis and cortical layers inside the root), the rhizoplane (the root surface including the mucilaginous polysaccharide layer), and the ectorhizosphere (soil particles past the root surface that are impacted by root exudates). Currently, the zone that Lynch termed "endorhizosphere" is referred to as "internal root colonization" because it is a location and not a "sphere " (135).

2.2. Root colonization and nutrient uptake capacities of PGPR

The concept of root colonization in the broadest sense refers to the multiplication of rhizobacteria ectophytically in the rhizosphere, on the root surface, and endophytically inside roots (168). Root colonization by PGPR is considered a prerequisite for plant growth-promotion. Rhizobacteria disseminate from a source of inoculum to the actively growing root region and multiply or grow in the rhizosphere due to root colonization (167). The dispersal of rhizobacteria from the inoculation site to the growing region of roots supervised by both active and passive movement of bacteria (19). One root colonization model proposed by Newman (1977) predicts that an abundance of rhizobacteria occur near the root tip region of the growing plant (156). The migration of rhizobacteria from the seed (point of inoculation) to the growing region of roots is governed by both active and passive movement mechanisms (19). Howie *et al*. (1987) suggested that root colonization by *Pseudomonas fluorescens* strain 2-79 takes place by passive transport on the root tip (99). Bacterial lipopolysaccharides (LPS), especially the O-antigen can play an important role in root colonization (50), but this activity is strain-dependent. For example, the O-antigenic side chain of *Pseudomonas fluorescens* WCS374 does not help root adhesion of potato (47). Studies showed that synthesis of B_1 vitamin and secretion of NADH dehydrogenases contribute to root

colonization by PGPR (203). Another study revealed that type IV pili of bacteria involve in plant root colonization by endophytic bacteria *Azospirillum* species (206). In certain bacteria, successful root colonization is associated with secretion of a site-specific recombinase gene (49), and transfer of this gene from competent to incompetent *Pseudomonas* strain increased colonization of root tips (48).

2.3. The significance of root colonization by PGPR

Plants uptake nutrients through roots as an essential part of their growth and survival. Active root colonization in the rhizosphere is crucial for plant growth and hinges on the interactions between soil, plant, and rhizobacteria. Successful plant growth-promotion suggests that rhizobacteria colonized and communicated with roots properly in the rhizosphere. During root colonization, some PGPR enhance the availability of nutrients in the rhizosphere, thereby enabling increased plant growth (76). The process by which this enhancement occurs is the solubilization of unavailable forms of nutrients (phosphate and nitrogen) and siderophore production which chelate and release iron for the plant in iron-limited conditions (220). Various rhizobacteria produce one or more types of phytohormones in the rhizosphere. Different phytohormones-producing genes are involved and can be activated by organic compounds that are abundant in the root cap and the elongation zone (87). For example, Lakshmanan *et al.* (2013) reported that *Arabidopsis thaliana* roots treated with *B. subtilis* FB17 strain expressed multiple genes, including auxin-regulated genes involved in metabolism, stress response, and plant defense during the root colonization (122).

2.4. PGPR diversity in the rhizosphere

The root rhizosphere contains diverse bacterial species that have the potential to enhance plant growth and biocontrol activity when they colonize the roots. Molecular based studies estimated that more than 4,000 microbial species are present per gram of soil (149). PGPR present in the rhizosphere includes *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*,

Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcus, Pseudomonas, Serratia, and *Cellulomonas spp.* (61; 79). Endophytic bacterial genera such as *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium,* and *Rhizobium* of the family Rhizobiaceae that colonize plant roots to form nodules and increase plant growth directly or indirectly (228). Other genera of PGPR such as *Pantoea, Methylobacterium, Exiguobacterium, Paenibacillus,* and *Azoarcus,* etc. also colonize roots and exhibit many beneficial effects on plants (35). Two novel proteobacterial strains (WRB 10 *Alkaligenes* sp. and WRB 4 *Providencia* sp.) significantly enhanced root colonization and growth-promotion of wheat (141). Actinomycetes are another group of gram-positive bacteria that stimulate plant growth and suppress plant pathogens through the root colonization. The actinomycetes genera *Streptomyces, Streptosporangium, Thermobifida,* and *Micromonospora,* have shown biocontrol activity against root fungal pathogens (72). For example, (Lee and Hwang, 2002) reported that actinomycetes isolates (*Streptomyces* sp., *Micromonospora,* sp., *Dactylosporangium,* sp., *Actinomadura* sp., *Nocardiform* sp., and *Streptosporangium* sp. (50% of 1510 strains) showed strong antifungal activity against some fungal pathogens, such as *Alternaria mali, Colletotrichum gloeosporioides, Fusarium oxysporum* f.sp. *cucumerinum,* and *Rhizoctonia solani* (124).

2.5. Root exudation process

The quantity and type of root exudates produced from the growing roots vary with plant species, age, and stress-associated factors (215). Root exudates are the niches and the central source of nutrients for the rhizosphere microorganisms (219). Bolton *et al.* (1992) defined root exudates as “low molecular weight organic compounds leaks from intact root cells” (24). The process by which root secretes or releases organic compounds are known as root exudation. This root exudation process can be divided into active root secretion with unknown functions and secretion of compounds from roots with known functions such as

lubrication and defense of plant roots (12; 215). Root exudates are divided into low and high molecular weight organic compounds (13). Low molecular weight organic compounds such as amino acids, organic acids, sugars, and phenolics are highly diverse compounds in root exudates that are released from intact root cells (23). High molecular weight organic compounds such as mucilage (polysaccharides) and proteins secreted by root cap cells and epidermal cells are involved in the enhancement of root-soil interactions and facilitate root movement through the soil (20). Plant root exudates play an essential role in resource competition and chemical interference (13). A recent study conducted by Tsuno *et al.* (2017) revealed that soybean roots secrete huge amounts of soyasaponins (a new type of root exudates) during vegetative emergence (VE) stage of growth (214). Liu *et al.* (2015) reported that soybean root exudates play an important role in triggering early symbiotic associations between *B. diazoefficiens* and soybean (130). Hence, root exudates serve as nutrient contents for the interactions with microorganisms to enhance plant growth and repeal plant pathogens by the induced defense mechanisms.

2.6. Rhizodeposition process

The release or loss of carbon compounds from plant roots into the surrounding soil environment is referred to as rhizodeposition (40), a process that results in the rhizosphere effect (133). The release of carbon during rhizodeposition drives the interactions between plant, soil, and microbial populations (231). Many biotic and abiotic factors affect rhizodeposition process in the growing roots. These include biotic factors such as plant species, photosynthesis, the supply of carbon from shoot to root, root architecture, mycorrhizas, and nodulation (106). The abiotic factors are temperature, moisture, humidity, rooting depth, soil texture, atmospheric nitrogen deposition, and available space factors that affect rhizodeposition (106). Rhizodeposits released near the apical root region or root cap and secreted polysaccharide mucilages make up to 2-12% of the total rhizodeposition (157;

201). Organic compounds released by the plant roots through the process of rhizodeposition are sugars, amino acids, organic acids, enzymes, fatty acids, growth factors, and vitamins (51; 241). These substances originate from sloughed-off root cells, mucilages, volatiles, soluble lysates, and exudates that are secreted from damaged and intact root cells (40; 42). Root exudation is a component of the rhizodeposition activity, which is a crucial source of soil organic carbon compounds released by plant roots (101; 157). Rhizodeposition controls multiple ecological soil functions such as nutrient availability and mobilization (101), the formation of aggregates (205), carbon sequestration (115), structuring of microbial communities (115), and regulating their activities at a high level (120). Organic compounds such as sugars, amino acids, and carboxylic acids exuded by roots constitute up to 10% of rhizodeposition (106). Kuzyakov (2013) reported that rhizodeposits supply energy to soil microbes for the solubilization of organic nitrogen and other nutrients from the soil organic matter (121). Laura *et al.* (2017) showed that isoflavonoids are a vital rhizodeposit elements that help in plant defense and facilitate symbiotic events with *Rhizobia* in soybean (234).

2.7. Root border cells

Root cells that are sloughed-off from the root tips are called root border cells (54; 86). Separation of these root border cells depends on the species, genotype, and environmental conditions (41; 67). Hawes *et al.* (1998) first proposed the term “root border cell”(92). Since then the role of root border cells in the rhizosphere have been extensively studied, and multiple functions of root border cells have been reported. Pectin-degrading enzymes, such as methylesterases and polygalacturonases play important roles in separating root border cells from the root tip region (208; 233). The cell walls of root border cells contain pectin polysaccharides that are also available in the secreted mucilage of root cap (32; 59). Although root border cells are separated from the root cap regions as an individual cells, they can be remained viable, and the viable percentages are greater than 90% (91). Root border cells have

multiple effects on plant pathogenic bacteria and fungi, such as chemoattraction, repulsion, and suppression of pathogenic infection (88-90). In addition, root border cells can stimulate plant growth-promotion elicited by root-colonizing PGPR (88). A recent study conducted by Canellas and Olivares (2017) reported that humic acid enhanced the production of border cells and colonization of maize root tips by *Herbaspirillum seropedicae* (31). Root border cells secrete antimicrobial compounds such as phenolics and arabinogalactan proteins that suppress pathogen attacks in the rhizosphere of plant roots (55; 232). For example, the secretion of arabinogalactan proteins inhibited the zoospores germination of *Aphanomyces euteiches* causes root-rot of legumes (32).

2.8. Rhizodeposits, exudates, and border cells in the growing plant root

The root rhizosphere is so diverse that not all the functions of the rhizosphere environment in the soil are yet entirely studied. Rhizodeposition and root exudates are largely discussed in the literature of root colonization by PGPR rather than in relation to root border cells. Root border cells contribution during growing plant roots are immense for the plant growth-promotion and development. Rhizodeposition takes place in different root zones (mature, elongation, and division zones), with the release of organic compounds by growing plant roots (119; 157). Rhizodeposits can occur in root cap cells, root tissues, mucilage, and root exudates (157; 189). Exudation occurs in the elongation zone, where root exudates and bacterial abundance are high (143). In addition, root exudates are a major part of the rhizodeposition processes (157) and are released near the apical meristem of tap and lateral roots (51). Root exudates can be active in functioning in defense and lubrication and passive based on their function and mode of secretion from the roots (13; 106). Root exudation, also known as rhizodeposition, influences plant growth and soil ecology in the rhizosphere (97). Border cells are released in the root cap and the cell division zone. Root exudates, nutrient availability, and bacterial growth are high in detached root cap cells also known as root

border cells (119; 143). Border cells secrete antimicrobial proteins, phytoalexins, and arabinogalactan proteins (32; 44). Driouich *et al*. (56) concluded that border cells can influence root-rhizosphere interactions at the root tip. Root exudates and border cells release different organic compounds and proteins, but their functions are to attract beneficial microbes and repel deleterious microbes (92). Dennis *et al.* (2010) reported that border cells are part of rhizodeposits like exudates that are released from root cap cells (51). Thus, rhizodeposits, root exudates, and root border cells shape microbial communities in the rhizosphere thereby effecting plant uptake of various nutrients for the growth (193).

2.9. Interactions of PGPR with *rhizobium* species in root colonization

Inoculation of PGPR with *Rhizobium* enhances root colonization, plant growth, and nodulation in legume plants. For example, inoculation of root-colonizing *P. putida* and *P. fluorescens* with *Bradyrhizobium japonicum* inoculant did not reduce the formation of nodules on soybean in the field (176). In the same study, Polonenko *et al.* (1987) reported that 17 of 18 *Pseudomonas* strains reduced the growth of *B. japonicum* on agar (176). However, one study showed that *Pseudomonas putida* inhibited the multiplication of *Rhizobium phaseoli* on agar media (81). In another study conducted by Siddiqui (2005) reported that the combination of PGPR with *rhizobium* constituted an alternative to promote nitrogen fixation (200). Co-inoculation of *Azospirillum* with *Rhizobium* significantly enhanced white clover plant growth (175). Camacho *et al.* (2001) reported that *Bacillus* sp. CEST 450 increased nodulation on bean when inoculated with *R. tropici* CIAT 899 in a greenhouse and under field conditions (30). PGPR also increase the root colonization capacity of *Rhizobium* species. For instance, the co-inoculation of *B. polymyxa* with *R. etli* increased *R. etli* populations in the rhizosphere of *Phaseolus vulgaris* compared to *R. etli* without *B. polymyxa* inoculation (172).

2.10. Communication between PGPR and roots in the rhizosphere

Communication between microbes and plant roots is interdependent for the mutual benefit and organic compounds. This communication can be root-root and root-microbe in which both exchange nutrients for their survival. Various types of relationships such as associative, symbiotic, neutral, or parasitic can be developed based on the nutrients of the soil, soil environment, plant defense mechanism, and multiplication of microbes itself (169). The secretion of organic compounds from plant roots helps to aggregate microbes in the rhizosphere. Root exudates are phytochemicals released by plant roots that actively regulate symbiotic interactions with rhizobacteria in the active soil zone of the rhizosphere (96; 105). Plant-microbe interactions governed by root exudates via the chemotactic response of the microbes toward root-secreted organic compounds play an important role in root colonization (247). For example, the endophytic bacteria *Corynebacterium flavescens* and *Bacillus pumilus* showed a fivefold increase of chemotaxis activity over other bacterial strains in the rice rhizosphere in the presence of amino acids and carbohydrate root exudates (10). The release of root border cells can also influence plant-microbe interactions. Hawes *et al.* (1998) reported that root border cells influence the stimulation of growth and chemoattraction of bacteria and fungi. In plant-microbe interactions, the secretion of antimicrobial compounds from the root exudates and border cells in the rhizosphere enhance plant growth and suppress various bacterial and fungal pathogens. For instance, a complex mixture of extracellular proteins released from the root cap of pea plant as root border cells in the rhizosphere inhibited the soil-borne pea pathogen *Nectria haematococca* (232). Bais *et al.* (2005) suggested that the secretion of antimicrobial metabolites by root exudation reduced multiple bacterial pathogens such as *Erwinia carotovora*, *E. amaylovora*, *Xanthomonas campestris* pv. *vesicatoria*, and *P. fluorescens* (226). In this way, assumptions can be made that root border cells are important to stimulate plant growth and inhibit a wide range of plant pathogens.

2.11. Growth promotion activity of *Bacillus velezensis* strains

Growth promotion by some bacteria is highly specific due to plant species, race, cultivar, and genotypic variability (16; 131). *B. velezensis* is a plant-associated bacterial species that has the potential to control soil-borne pathogens and to stimulate the growth of oilseed rape (*Brassica napus*) (43). *B. velezensis* strain UCMB 5033 has 3,912 protein-coding genes and exhibits both plant growth-promotion activity and biological control (159). This strain has PGPR properties and can uptake glucose, fructose, and mannose as a carbon source (43). *B. velezensis* strains typically promote plant growth by secreting phytohormones, volatile organic compounds (26), and secondary metabolites (116; 194; 195) and often control soil-borne pathogens (26). The hexuronate transporter (exuT) and the D-mannonate oxidoreductase (uxuB) degradative pathways have been reported in Bap B9601-Y2 strains (94) that transport carbon via exuT gene from rhizosphere D-glucuronate or D-galacturonate to bacterial cells. Fructuronate changes into mannonate via the uxuB gene in the bacterial cell. Genome analysis of *B. velezensis* strain UCMB5113 indicated that it can increase root colonization, plant growth-promotion, and suppress plant pathogen through the production of IAA and siderophore (158). PGPR can promote plant growth by different mechanisms such as fixation of atmospheric nitrogen, production of siderophores that chelate iron, solubilization of minerals, and biosynthesis of phytohormones (112). Phosphate solubilization and production of siderophores and IAA production have been reported in *B. velezensis* strains that enhanced the growth of tea plants (34).

Kanjanamaneesathian *et al.* (2014) reported that *B. velezensis* strains enhanced plant growth and suppressed root rot of lettuce (*Lactuca sativa)* caused by *Pythium* species (108). One study reported that a pure culture of strain AH2 of *B. velezensis* stimulated plant growth and suppressed phytopathogenic fungi *Botrytis cinerea* (144).

2.12. Mechanisms of plant growth-promotion by PGPR

2.12.1. Atmospheric nitrogen fixation

Nitrogen is the most abundant compound in the atmosphere. It is also an essential nutrient for plants. However, plants are unable to uptake atmospheric nitrogen. Soil microorganisms, cyanobacteria, and lightning can fix atmospheric nitrogen making it available for plant roots in the soil. Nitrogenase enzymes are involved in these conversion processes. Nitrogen (N_2)-fixing bacteria can fix atmospheric nitrogen by symbiotic N_2 fixation and asymbiotic N_2 fixation (22). Bacteria that are associated with symbiotic N_2 fixation are referred to as symbiotic N_2 fixing bacteria. In symbiotic N_2 fixation, bacteria colonize and make symbiotic interactions with leguminous host plants and bacteria to form nodules (25). *Sinorhizobia, Bradyrhizobia,* and *Mesorhizobia* are soil bacteria that have been studied vastly for symbiotic nitrogen fixation in leguminous plants. In asymbiotic nitrogen fixation, bacteria do not form symbiotic interactions with host plants. Asymbiotic nitrogen-fixing bacterial genera include *Azotobacter spp.* (151), *Azospirillum spp*. (46), *Burkholderia spp*. and *Bacillus polymyxa* (162). Of these bacterial genera, *Azospirillum spp*. (15) are the most extensively studied.

2.12.2. Phosphate solubilization

Phosphorous is applied in soils via fertilizers, but plants often cannot uptake it due to unavailable forms. Microorganisms can help convert some types of phosphorous to forms that are available for plant uptake. Organic acids (78) and phosphatases enzymes (185) are two ways whereby microorganisms can be taken up by plants. Several bacterial genera known as phosphate solubilizing bacteria produce organic acids and phosphatases, including *Azospirillum spp., Bacillus licheniformis* (186), and *B. amyloliquefaciens* (34; 186). Bacteria secrete phosphatases enzymes that release phosphate, which can then be taken up by plants. In addition, phosphate solubilizing bacteria lower the rhizospheric pH and dissolve soil phosphate through the production of low molecular weight organic acids such as gluconic

and ketogluconic acids (78). The most efficient phosphate solubilizing bacterial genera are *Bacillus, Rhizobium*, and *Pseudomonas* (118). Two species of *Rhizobia* such as *Mesorhizobium ciceri* and *M. mediterraneum* are good phosphate solubilizers that nodulate chickpea (184).

2.12.3. Siderophore production

Plants require iron but cannot take it up due to unavailable forms such as ferric ions, which have very low solubility (118). Microorganisms can help transport iron into plant cells through the production of low molecular weight siderophores that chelate iron in the rhizosphere. Most of the siderophores are water-soluble and can be classified as extracellular and intracellular siderophores (2). Pectin degrading and utilizing *B. velezensis* bacteria can help to boost plant growth via siderophores production. *B. amyloliquefaciens* bacteria were used in tea plants that increased plant growth through the production of siderophores (34). Siderophores are low molecular weight compounds and can be various types such as hydroxamates, phenolcatercholates, and carboxylates (118). Siderophore-associated iron sequestration is better studied in gram-negative PGPR than gram-positive PGPR (82) and 270 siderophores chemical structure have been identified (95).

2.12.4. Phytohormone production

Phytohormones are produced in the plant and control different physiological functions that increase plant growth. Gibberellins, auxins, cytokinins, and ethylene are plant hormones produced by microbial inoculants. PGPR secrete these phytohormones, which may function directly or indirectly through plant roots to promote plant growth (164). *B. velezensis* bacterium can produce auxins such as Indole-3-acetic acid that can help to increase the growth of tea plants (34). *B. subtilis* strain IB-22 involved in cytokinin production that increased lettuce shoot and root weight approximately 30% (9).

2.12.5. Volatile organic compounds (VOCs)

Volatile organic compounds (VOCs) are low molecular weight compounds produced by PGPR and plant growth-promoting fungi (PGPF) (155) that can trigger plant growth-promotion and induced systemic resistance (ISR) against pathogens (190; 191). For example, Francisca *et al.* (2010) concluded that VOCs (aldehydes, ketones, alcohols, i-octen-3-ol, and butyrolactone) produced by *B. cereus*, *B. simplex*, and *Bacillus* spp. PGPR enhanced the growth of *Arabidopsis thaliana* roots and shoots (84). Another study conducted by Santoro *et al.* (2011) provided evidence that VOCs (pulegone, menthone, menthol, and menthofuran) emitted by *P. fluorescens*, *B. subtilis*, and *Azospirillum brasilense* PGPR increased peppermint crop productivity and yield of essential oil (192). Two VOCs mixture (2-methyl-propanol and 3-methyl-butanol) produced by PGPF *Phoma* sp. GS8-3 at lower concentrations significantly enhanced tobacco plant growth-promotion *in vitro* (155).

2.12.6. Enhancement of drought stress tolerance by PGPR

Drought is one of the major abiotic stresses that affect crop health and production in the U.S. and worldwide. PGPR play a vital role in crop protection and alleviation of drought stress. These beneficial microbes colonize the plant roots and enhance drought stress tolerance by secreting phytohormones, exopolysaccharides, and volatile compounds (225). Eke *et al.* (63) reported that *B. amyloliquefaciens* strain CBa_RA37 enhanced drought stress tolerance and growth promotion in tomato.

3. Source and function of pectin

Pectin is the most complex galacturonic acid-rich polysaccharide in nature (3), constituting up to 35% of the primary walls in dicots and non-graminaceous monocots, 2-10% in grasses, and 5% in woody tissue (161; 183). Braconnot discovered pectin in 1825 and reported that it is abundant in the middle lamella and primary plant cell wall. Pectin has multiple functions in plant growth, morphology, development, cell expansion, seed hydration, and plant defense

(183; 236) and is found in the peel of several fruits, including apple (221), passion fruit (202), dragon fruit (209), and orange (117; 142). Pectin is also found in different plant parts, such as the root cap region, leaf, and fruit. However, fruit contains the highest percentage of pectin during the ripening period. For instance, orange peel is an excellent source for the extraction of pectin (64) and because it contains about 30 % of pectin (188). Agricultural waste materials such as orange peels can be used as nutrient sources for PGPR growth *in vitro* and *in vivo*. Previous studies reported that microorganisms are capable of utilizing nutrients in waste material as an energy and carbon source for the synthesis of cell biomass (21; 237). Chemical composition of orange waste materials are pectin, cellulose, starch, crude protein, and reducing sugars that were used for the growth and extracellular hydrolytic enzyme production of *B. subtilis* strain 11089 (139). Another study concluded that orange peel was used as energy and carbon source for the production of 1,4-β mannanase by *B. amyloliquefaciens* 10A1 (138).

3.1. The pectinolytic capacity of microorganisms

Pectin degradation activity has been reported in different beneficial and deleterious bacteria. Beneficial bacteria used pectin derived carbon sugar to promote plant growth and control of plant diseases. For instance, the secretion of pectinolytic enzymes by *Paenibacillus* sp. strain B2 in the presence of *Glomus mossease* arbuscular mycorrhizal fungi significantly enhanced mycorrhizal colonization of sorghum roots (73). Wu *et al.* (2015) reported that the pectin amendment of soil by *B. amyloliquefaciens* strain SQY 162 increased surfactin and iturin A secondary metabolites production that significantly reduced bacterial wilt of tobacco caused by *Ralstonia solanacearum* (238). Pectinolytic bacterial genera are *Achromobacter, Arthrobacter, Agrobacterium, Bacillus*, *Clostridium, Erwinia*, *Pseudomonas*, and *Xanthomonas* (187; 223).

3.2. Role of pectin in plant growth and drought stress tolerance

Pectin from agricultural waste orange peel has the potential to enhance plant growth and provide long-term beneficial effects in soil without causing damage to plants. The tropical forest vegetation in Costa Rica has been restored and increased by agricultural waste orange peel application in the soil (213). The Cleopetra mandarin orange (*Citrus reshni*) has the potential to reduce the adverse effects of drought stress on yield and fruit quality during period of drought (171). The low-cost biodegradable polymer from orange peel absorbs greater than 75% of water and can be a better solution for fighting drought in fruit production (7).

4. Biological control of plant pathogens

Biological control is a mechanism or strategy in which one organism suppresses, kills or reduces the inoculum density of a pathogen or the population of insect pests (62). Biological control can also be defined as the interactions among the host, the pathogen, and the biological control agent in which pathogenic damage is substantially reduced by both host and biological control agents (2). Some PGPR induce biological disease control through mechanisms of antibiosis, competition for nutrients, niche exclusion, production of antimicrobial metabolites (132). Induced systemic resistance is another indirect mechanism in which PGPR elicit systemic resistance of host against the pathogen (38).

4.1. The antagonistic mechanisms of PGPR

The antagonistic mechanisms of PGPR that have been reported to contribute to biological control include antibiotics, cell wall degrading enzymes, toxic compounds, and biosurfactants, as well as competition for nutrients and minerals (38; 85). Antagonism is an active opposition or hostility between two organisms, including antibiosis, competition, and parasitism (39). Antibiosis is the principal mechanism among other direct mechanisms in which PGPR act as an antagonistic agent against plant pathogens (65).

4.1.1. Antibiotic production

Antibiotics are a heterogeneous group of low molecular weight organic compounds secreted by microorganisms that suppress or reduce the growth of the pathogen (58). Biocontrol potentiality of PGPR often depends on the production of one or more antibiotics (77) and one of the most efficient mechanisms to reduce plant pathogens (200). Antibiotics reported to be produced by PGPR include azomycin (199), antitumor antibiotics FR901463 (152), bacillomycin (222), butyrolactones (211), cepaciamide A (100), cepafungicins (198), 2,4 diacetyl phloroglucinol (196), ecomycins (104), kanosamine (148), oomycinA (110), pyrrolnitrin (210), pyoluteorin (98), and karalicin (123). Haas *et al.* (85) stated that six classes of antibiotics phenazines, phloroglucinol, pyoluteorin, pyrrolnitrin, cyclic lipopeptides, and hydrogen cyanide are related to biocontrol of root diseases. *Pseudomonas* species are widely reported to function as biocontrol agents by the production of such antibiotics (126). For instance, lipopeptide biosurfactants (massetolide A, fengycins, and surfactins) produced from *Pseudomonas* species are prominent due to their suppressive influences to control plant pathogenic bacteria (*Xanthomonas campestris; P. syringae*) (11; 68), fungi (*R. solani; Botrytis cinerea*) (163), oomycetes (*Phytophthora infestans*) (212), and nematode (*Caenorhabditis elegans* as a model for phytopathogenic nematodes) (45; 177). Antibiotics, such as circulin, colistin, and polymyxin produced by different *Bacillus* species are active against plant pathogenic fungi (*Fusarium oxysporum, Rhizoctonia* sp., *and Pythium* sp.), gram-positive (*Streptomyces scabies*), and gram-negative (*Erwinia carotovora* var. *atroseptica*) bacteria (140). Zwittermicin A (aminopolyol) and kanosamine (aminoglycoside) are produced by *B. cereus* UW85 strain that suppresses alfalfa damping off caused by *Phytophthora medicaginis* (93).

4.1.2. Lytic enzyme production

Extracellular lytic enzymatic activity is another mechanism used by some PGPR strains to kill fungal pathogens (240) and to promote plant growth (83). The enzymes chitinases, β-1, 3-glucanases, and proteases produced by *Lysobacter enzymogenes* strain C3 actively inhibited damping-off of sugar beet caused by *P. ultimum* and leaf spot of tall fescue caused by *Bipolaris sorokiniana* (114). *P. cepacia* secreted β-1, 3-glucanases that significantly suppressed *R. solani* in cotton, *Sclerotium rolfsii* in bean, and *P. ultimum* in cucumber through fungal cell wall degradation (74). The β-1, 3-glucanases produced by *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 reduced the pathogenic activity of Fusarium wilt of cucumber (*Fusarium oxysporum* f. sp. *cucumerinum*) (204). In the same way, β-1, 3-glucanases synthesized by *B. subtilis* EPCO 16 inhibited *F. oxysporum* f. sp. *lycopersici* in tomato plants (180).

4.1.3. Volatile organic compounds (VOCs)

VOCs are low molecular weight organic compounds produced by PGPR that have gained much attention due to their strong antagonistic activity against plant pathogens (37; 53). The benzothiazoles phenol and 2,3,6-trimethyl phenol VOCs secreted by *B. velezensis* strain NJN-6 suppressed growth and spore germination of *F. oxysporum* f. sp. *cubense* (245). Four species of *Pseudomonas* (*P. fluorescens, P. corrugate, P. chlororaphis,* and *P. aurantiaca*) produced the VOCs benzothiazole, cyclohexanol, *n*-decanal, dimethyl trisulfide, 2-ethyl 1-hexanol, or nonanal that significantly inhibited mycelial growth or formation of sclerotia by *Sclerotinia sclerotiorum* (70).

4.1.4. Siderophore production

Siderophore production by some PGPR strains has been reported to play a significant role in biological control of plant pathogens by sequestering ferric ions (Fe^{3+}) near plant roots, resulting in iron unavailability for the pathogen (145). For instance, the production of

hydroxamate type siderophores by *Bacillus* species SC1 and *B. firmus* D 4.1 significantly reduced the severity of rice blast caused by *Pyricularia oryzae* (33). The siderophore 2, 3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin secreted by *B. subtilis* CAS15 suppressed Fusarium wilt (*F. oxysporum* f. sp. *capsici*) of pepper (244).

4.1.5. Induced systemic resistance (ISR)

PGPR triggered host defense that reduces the incidence or severity of plant disease produced in plants, which are spatially segregated from the pathogen is known as induced systemic resistance (ISR) (113). ISR expression occurs locally and systemically by increasing levels of defense against a broad spectrum of pathogens (227).

4.1.6. The mechanisms of ISR-elicited by PGPR

Some PGPR strains elicit ISR via alterations in biochemical and physiological processes of the host plant that result in less infection of the pathogen (179). PGPR-mediated ISR has been related to the accumulation of pathogenesis-related proteins (PR) (217), the synthesis of phytoalexins (243), and secondary metabolites in host plants (246). For example, PGPR isolate *Pseudomonas fluorescens* Pf1 induced PR proteins such as chitinase and *β*-1,3 glucanase that successfully reduced the incidence of finger millet blast fungus (*Pyricularia grisea*) (178). Inoculation of bean root by fluorescent pseudomonads (*P. putida* isolate Corvalis, *P. tolaasii* P9A, and *P. aureofaciens* REW1-I-1) was associated with systemic resistance and induction of PR proteins against *Botrytis cinerea* (246). Bargagus *et al.* (14) reported that ISR by *B. mycoides* isolate Bac J against the Cercospora leaf spot pathogen (*Cercospora beticola*) in sugar beet was correlated with increased production of the PR proteins chitinase, *β*-1,3 glucanase, and peroxidase. In pepper, ISR against *X. axonopodis* pv. *vesicatoria* elicited by *B. cereus* strain BS107 was associated with expression of PR proteins 4 and CaPR1 (242).

ISR elicited by PGPR enhances defense against a broad-spectrum of diseases through structural modifications of the cell wall, such as the accumulation of phenolic compounds and deposition of callose in the host plant (18). For example, the formation of callose and deposition of phenolic compounds in tomato plants inhibited the growth of *F. oxysporum* f. sp. *radicis-lycopersici* inoculated with the *P. fluorescens* strains 63-28 (136). ISR elicited by *B. cereus* strain AR156 was associated with callose deposition and expression of PR1, PR2, and PR5 genes in the leaves of *Arabidopsis* ecotype Col-0 plants (160).

PGPR-mediated resistance is referred to as ISR (218) and is reliant on the jasmonic acid (JA) and ethylene (ET) signaling pathways (173). On the other hand, pathogen-mediated resistance is referred to as systemic acquired resistance (SAR) and is reliant on the salicylic acid (SA) pathway (60; 216). Despite the different pathways used by ISR and SAR, both types of resistance are effective against a broad-spectrum of pathogens (218). Both ISR and SAR activate dormant resistant mechanisms that are expressed during or after pathogens infect host plants (217).

4.1.7. Broad-spectrum protection

PGPR-elicited ISR typically exerts a broad range of protection against multiple phytopathogens (66; 113). Liu *et al.* (129) reported that *B. velezensis* strains AP197, AP199, AP200, AP298, and *B. altitudinis* strain AP69 exhibited biocontrol activity against diverse diseases, such as damping-off of pepper (*R. solani*), damping-off of cucumber (*Pythium ultimum*), the bacterial spot of tomato (*X. axonopodis* pv. *vesicatoria*), and bacterial speck of tomato (*P. syringae* pv. *tomato*).

4.1.8. Synergistic roles of PGPR strains

Mixtures of PGPR strains can sometimes exhibit more effective biocontrol than single PGPR strains due to synergistic effects on plant pathogens (103; 181). For example, a combination of four *B. velezensis* PGPR strains (AP136, AP209, AP282, and AP305) had a

synergistic activity to inhibit black rot of cabbage (*X. campestris* pv. *campestris*) and increased marketable yield in field conditions compared to single strain (128). Wichitra *et al.* (125) showed that the extracellular enzyme β-1,3-glucanase in mixture with antibiotics produced by *B. subtilis* NSRS 89-24 synergistically inhibited the hyphal growth of rice blast (*Pyricularia oryzae*) and rice sheath blight (*R. solani*).

5. Role of pectin or orange peel amendments for inhibiting plant pathogens

PGPR strains amended with pectin or orange peel inhibit multiple plant pathogens through the secretion of secondary metabolites. The production of surfactin and iturin A by *B. amyloliquefaciens* SQY 162 plus pectin amendments strongly inhibited bacterial wilt of tobacco caused by *Ralstonia solanacearum* (238). Separated cow manure amended with orange peels significantly reduced root galls and *Meloidogyne javanica* egg populations in tomato (182). Another study conducted by Abolusoro *et al.* (2010) reported that sweet orange peel aqueous extract is more effective and significantly suppressed *M. incognita* populations and root galls in tomato root (1).

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Chapter II Pectin-rich amendment enhances soybean growth promotion and nodulation mediated by *Bacillus Velezensis* strains

Abstract

Plant growth-promoting rhizobacteria (PGPR) are increasingly used in crops worldwide. While selected PGPR strains can reproducibly promote plant growth under controlled greenhouse conditions, their efficacy in the field is often more variable. Our overall aim was to determine if pectin or orange peel (OP) amendments to *Bacillus velezensis* (Bv) PGPR strains could increase soybean growth and nodulation by *Bradyrhizobium japonicum* in greenhouse and field experiments to reduce variability. The treatments included untreated soybean seeds planted in field soil that contained Bv PGPR strains and non-inoculated controls with and without 0.1% (*w/v*) pectin or (1 or 10 mg/200 μL) orange peel (OP) amendment. In greenhouse and field tests, 35 and 55 days after planting (DAP), the plants were removed from pots, washed, and analyzed for treatment effects. In greenhouse trials, the rhizobial inoculant was not added with Bv strains and pectin or OP amendment, but in the field trial, a commercial *B. japonicum* inoculant was used with Bv strains and pectin amendment. In the greenhouse tests, soybean seeds inoculated with Bv AP193 and pectin had significantly increased soybean shoot length, dry weight, and nodulation by indigenous *Bradyrhizobium* compared to AP193 without pectin. In the field trial, pectin with Bv AP193 significantly increased the shoot length, dry weight, and nodulation of a commercial *B. japonicum* compared to Bv AP193 without pectin. In greenhouse tests, OP amendment with AP193 at 10 mg significantly increased the dry weight of shoots and roots compared to AP193 without OP amendment. The results demonstrate that pectin-rich amendments can enhance Bv-mediated soybean growth promotion and nodulation by indigenous and inoculated *B. japonicum*.

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) colonize the plant rhizosphere and stimulate plant growth through diverse mechanisms such as nitrogen fixation [1], phosphate-solubilization [2], siderophore production [3], phytohormone production [4], and the secretion of volatile organic compounds (VOCs) [5]. *B. velezensis* (Bv) (previously known as *B. amyloliquefaciens* subsp. *plantarum*) is a gram-positive, rod-shaped PGPR species that includes strains reported to enhance the plant growth of several plants, including maize [6], soybean [7], oilseed rape (*Brassica napus*) [8], and *Arabidopsis thaliana* [9].

In addition to promoting plant growth, many Bv strains inhibit plant pathogens through the secretion of bioactive secondary metabolites and volatile organic compounds (VOCs). Palazzini et al. [10] reported that iturin and fengycin secreted from Bv RC 2018 suppressed *Fusarium* head blight caused by *Fusarium graminearum*. Three volatile organic compounds (pyrazine, benzothiazole, and phenol-2,4-bis) of Bv strain ZSY-1 exhibited antifungal activity against *Alternaria solani* and *Botrytis cinerea* [11]. Our previous comparative genomic study of *B. amyloliquefaciens* and Bv strains [12] predicted 73 genes that were exclusively identified among Bv PGPR strains, including genes involved in carbon source utilization and secondary metabolite production. Interestingly, this previous study predicted that all the Bv PGPR strains for which genome sequences were available (n = 28) could degrade pectin and utilize it as a sole carbon source. Hence, in the current study, we screened a collection of 59 Bv PGPR strains for the capacity to use purified pectin as a sole carbon source to determine if this is a conserved trait among plant growth-promoting Bv strains.

Pectin has multiple functions in plant growth, morphology, plant development, cell expansion, seed hydration, and plant defense [13,14]. Pectin is present in the peel of several fruits, including apple [15], passion fruit [16], and orange [17,18]. The separation of root border cells from the root cap is induced by pectin methylesterase activity [19], resulting in

the release of soluble, de-esterified pectin that can have multiple impacts on bacterial-mediated plant growth-promotion and plant health [20]. The soluble pectin produced by root border cells could be used as a nutrient source by rhizobacteria [19,21]. While pectin is found in various plant tissues such as the root tip, leaves, and fruits, the highest percentage of pectin occurs in fruit. For example, orange peel contains 30% pectin [22], making it the preferred source of pectin [23]. There are currently only three commercially available sources of pectin extracts in the United States: sugar beet [24], apple pomace [25], and citrus peel [26]. A recent study conducted by Wu et al. [27] demonstrated that exogenous pectin or other carbohydrate amendments induced *B. amyloliquefaciens* SQY 162 to increase biofilm formation and secretion of the secondary metabolite surfactin, resulting in enhanced biocontrol activity against *Ralstonia solanacearum* in tobacco. However, this study did not investigate the potential synergy between pectin and PGPR-mediated plant growth-promotion. Hence, the overall objective of this study was to test the hypothesis that pectin-rich amendments enhance the plant growth-promoting effects of Bv PGPR strains on soybean.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Fifty-nine Bv strains were previously isolated and identified as being affiliated with Bv based on 16S rRNA and *gyrB* gene sequences, and each strain had been previously shown to have PGPR activity [12,28–30]. Bv strains were transferred from cryostocks at −80 °C onto tryptic soy agar (TSA) and were incubated at 28 °C for 24 h. A single colony of each strain was streaked onto spore preparation medium [31] and incubated at 28 °C for seven days. Sterilized distilled water (15 mL) was added to each plate, and the cellular mass was transferred to a 50 mL centrifuge tube. Bv suspensions were heat-treated at 80 °C for 20 min, serially diluted, and adjusted to 1.0×10^6 spore colony-forming units (CFU)/mL.

2.2. Pectate lyase activity test

Bacteria were cultured from cryostocks at −80 °C into tryptic soy broth (TSB) at 28 °C overnight using 220 rpm for 5 mL culture. A one-ml aliquot was pipetted into a 1.5 mL microcentrifuge tube, and tubes were centrifuged for 5 min at 10,000 × *g* speed. The supernatant was discarded, and the process was repeated three times using sterile water. To the final bacterial pellet, 1.0 mL of sterile water was added to each microcentrifuge tube and vortexed thoroughly to produce a uniform bacterial suspension. A 1.0 mL aliquot of each strain was transferred to a cuvette to measure turbidity, adding sterilized water until the optical density at 600 nm (OD_{600}) was approximately 0.5. Twenty µL of this standardized bacterial suspension was transferred in triplicate onto pectate-agar (Pa) medium [32] to determine the pectate lyase activity. Tris-HCl buffer was adjusted (0.1M, pH 8.0) for the Pa medium separately and sterilized using a 0.45 µm Nalgene syringe filter (Thermo Scientific, Waltham, MA, USA). The Pa medium plates were incubated at 28 °C for 24 to 48 h, and then 1% cetyltrimethyl ammonium bromide (CTAB) was poured over the surface of each plate at room temperature. The resulting pectin clear zones were measured in millimeters (mm), and pectate lyase activity (PLA) was rated on a scale of low (OD_{600} 0.1–0.2) (+), medium (OD_{600} 0.2–0.4) (++), and high (OD_{600} 0.4 – 0.6) (+++).

2.3. Growth of Bv PGPR strains using pectin as a sole carbon source

Each Bv strain was assessed for its ability to utilize pectin as a sole carbon source using a Tris-Spizizen Salts (TSS) [33] minimal medium supplemented with 0.1% pectin powder (EC No. 232-553-0, Tokyo Chemical Industry Co., Toshima, Kita-Ku, Tokyo, Japan). The TSS minimal medium was filter sterilized using a 0.45-µm polyethersulfone (PES) vacuum filter unit (VWR, Radnor, PA, USA). Each of the bacterial cultures was grown overnight in TSB medium, and the cell pellets were washed three times in sterilized water, normalized to OD_{600} = 0.5, and then 100 µL of a 1:100 dilution was used to inoculate 1.9 mL TSS+0.1%

pectin cultures to adjust the OD_{600} = 0.030, in triplicate. Bacterial cells were grown at 28 °C with 200 rpm continuous shaking for 72 h in a shaking incubator, and readings at OD_{600} were recorded.

2.4. Greenhouse trials of pectin and PGPR amendments on soybean to assess root colonization, growth promotion, and nodulation

2.4.1. Preparation of pectin powder and liquid suspensions

Pectin powder (from the citrus peel source, described above) was mixed thoroughly with field soil using a soil mixer at a rate of 1.0 g per 1000 g of field soil. In addition, pectin powder (0.1 g) was suspended with sterilized water at a rate of 1.0 g per 1000 mL water for application as an aqueous pectin suspension.

2.4.2. Field soil preparation

Sandy loam field soil collected from the E.V. Smith Research Center (Shorter, AL), and sieved to remove root debris, was used for the greenhouse experiment. Soil (450 g) was placed in each cone-tainer tube (lightweight large Deepots D40L, Stuewe & Sons, Danville, IL, USA) that contained three cotton balls in the bottom to retain soil.

2.4.3. Soybean seed inoculation

Soybean seeds of variety (‘Progeny P5333 RY’) without chemical treatments were used for all greenhouse experiments. One mL of Bv PGPR strains (1.0×10^6 spore CFUs/mL) was pipetted over each seed. Two seeds were placed in each cone-tainer to ensure germination, and one seedling was removed one week after planting. Then, 5.0 g of soil was placed over each seed. Each cone-tainer rack was covered by a plastic sheet for 48 h to prevent soil desiccation. Afterward, racks were transferred to the greenhouse and tubes were watered twice daily.

2.4.4. Soybean plant growth measurement

At 35 days after planting (DAP), all the plants were harvested for plant morphometric measurements. Shoot length was measured from the growing apical region to the basal region connected to the root. Root length was measured from the root tip to the basal region connected to the root. For dry weight measurements, shoots and roots were oven dried at 70 °C for 48 h.

2.4.5. Selection of Bv rifampicin-resistant mutants and evaluation of Bv PGPR strains root colonizing capacity

Three strains (AP136, AP143, and AP193) were streaked onto TSA plates for 24 h to ensure the purity of the bacterial colony. From each strain, one colony was transferred into 30 mL TSB in a sterile 50 mL centrifuge tube and placed in a shaking incubator (220 rpm) at 28 °C. Rifampicin (Sigma, St. Louis, MO, USA) antibiotic was used for the selection of Bv mutants. To prepare the stock solution of 50 mg/mL of rifampicin (Sigma-Aldrich, Product code 101594249, St. Louis, MO, USA), 500 mg of rifampicin (rif) was added to 10 mL of dimethyl sulfoxide (DMSO). The stock solution was sterilized using a 0.45 μm Nalgene syringe filter (Thermo Scientific, Waltham, MA, USA). After 24 h, 50 μg/mL of rifampicin working concentration was added to 50 mL of TSB bacterial culture media. The rif–TSB culture tube was wrapped with aluminum foil to prevent the degradation of rifampicin by light and placed in a shaking incubator at 28 °C. After 48 h, one loop from each rif–TSB culture tube was streaked onto a TSA+rif plate of each strain and placed into the incubator at 28 °C. Single colonies that grew on TSA+rif plates were removed, labeled as AP136-rif, AP143-rif, and AP193-rif, and placed into the −80 °C freezer. At 35 DAP, the populations of Bv PGPR populations in the soybean rhizosphere were evaluated. The adherent soil was removed gently from the roots of each plant and placed in 15 mL screw-cap tubes. Nine ml of sterile water was added to each tube, and the tubes were vortexed thoroughly. Then, serial

dilutions were made from 1:10 to 1:1000 in sterile Milli-Q water in microcentrifuge tubes, and 100 µL was plated on TSA+rif plates for each dilution and incubated at 28 °C for 24 to 48 h. Colonies that grew on the TSA+rif plates that had the same colony morphology as the inoculated strain were counted and expressed in log CFU/mL.

2.4.6. Evaluation of soybean nodulation

At 35 DAP, soybean nodules per plant were removed from the roots, counted, and oven dried in a mechanical convection oven at 70 °C for 48 h. Then, the nodule dry weight was recorded.

2.5. Field trial of pectin and PGPR amendments on soybean growth promotion and nodulation

2.5.1. Soil type and application of *Bradyrhizobium Japonicum* inoculant

The field trial was conducted in the Alabama Agricultural Experiment Station's (AAES) Gulf Coast Research and Extension Center (GCREC) located in Fairhope, Alabama (AL). GCREC Soil was a Malbis fine sandy loam type. A commercial *B. japonicum* inoculant powder (HiStick N/T, BASF, North Carolina, NC, USA) was directly applied to the seed at planting via a hopper-box treatment into the furrow. According to the label, the population of *B. japonicum* inoculant was (2.0×10^9 cells/g).

2.5.2. Soybean seed inoculation

Soybean seeds of variety "Asgrow 75×6" was used for the field trial. The soybean seeds were planted directly from a hopper box into each plot. Each plot had four rows. A Bv spore suspension at 1.0×10^6 spore CFU/mL was applied in-furrow at the rate of 37.85 liters per hectare, and pectin liquid suspension (0.1%) was sprayed over seeds at the time of planting.

2.5.3. Soybean plant growth measurement

Soybean shoot length was assessed at 35 and 55 days after planting (DAP). Soybean dry shoot weight was measured after drying in an oven at 70 °C for 48 h at 35 and 55 DAP. Soybean yield was assessed by harvesting at 140 DAP, and soybean seed weights were recorded from the two center rows of each four-row plot.

2.5.4. Evaluation of soybean nodulation

Treatment effects on soybean nodulation were determined by counting the number of nodules per plant and by measuring the total dry weight of nodules per plant at 35 DAP. To assess nodule dry weight, nodules were removed from roots and dried in a mechanical convection oven at 70 °C for 48 h.

2.6. Greenhouse trials of orange peel liquid suspension and PGPR amendments on soybean growth promotion and nodulation in field soil

2.6.1. Growth of Bv PGPR strains using orange peel as a sole carbon source

The same methods were followed for the *in vitro* Bv strain growth experiments using orange peel powder as a sole carbon source. Organic orange peel powder was collected from Citrus Extracts (Fort Pierce, FL 34982, USA).

2.6.2. Preparation of orange peel liquid suspensions

Orange peel powder (500 mg) was used for the greenhouse tests (Citrus Extracts, Fort Pierce, FL 34982, USA), which was added into 10 mL of sterilized water until thoroughly dispersed and applied (1 or 10 mg/200 μL) onto the soybean seed surface after inoculation with the Bv strains as described above.

2.6.3. Field soil preparation

A sandy loam field soil was collected and prepared for orange peel liquid suspensions test by the same methods described above. The same amount of soil was placed in each cone-tainer tube that contained three cotton balls in the bottom to retain soil.

2.6.4. Soybean seed inoculation

A soybean seeds of variety (‘Progeny P5333 RY’) without chemical seed treatments was used for the greenhouse experiment. Two seeds were placed in each cone-tainer to ensure germination, and one seedling was removed one week after planting. Orange peel suspensions (1 or 10 mg/200 µL) and 50 µL of Bv strains (1.0×10^6 spore CFUs) were pipetted separately over each seed. Then, 5.0 g of soil was placed over each seed. Each cone-tainer rack was covered by a plastic sheet to prevent soil desiccation for 48 h. Afterward, cone-tainer racks were transferred to the greenhouse and were watered twice daily.

2.6.5. Soybean plant growth measurement and nodule evaluation

The soybean plant growth parameters and the numbers of nodules per plant were assessed by the same methods that were followed for the pectin experiments in the greenhouse tests.

2.7. Statistical analyses and experimental design

In the greenhouse tests, cone-tainers were arranged in a randomized complete block design (RCBD) with eight treatments and 12 replications, with each replication being a single plant in a single cone-tainer. The data of mean shoot height, mean root length, mean dry shoot weight, mean dry root weight, a mean number of root nodules per plant, and rhizobacterial CFUs were analyzed with SAS 9.4 software (SAS Institute, Cary, NC, USA) using the PROC GLIMMIX. Treatment means were compared using LSMEANS at the $p < 0.05$ level of significance.

In the field test, the experiment design was a 6 × 6 Latin square design with 36 total plots. Each plot consisted of four 9.1 m long rows. The planting rate was 120,000 seeds per acre or eight seeds per 0.3 m of row. The data of plant mean shoot height, mean dry shoot weight, mean nodule numbers per root, and mean nodule dry weight were analyzed with SAS

9.4 software (SAS Institute, Cary, NC, USA) using Duncan's multiple range test at the 5% level of significance.

3. Results

3.1. Pectate lyase activity

Pectinase clear zones appeared around the colonies of all the tested Bv strains after 30 min (Table 1). Very strong pectate lyase activities were observed for strains AP52, AP80, AP81, AP87, AP112, AP143, AP183, AP188, AP190, AP191, AP192, AP199, AP200, AP203, AP207, AP208, AP212, AP296, AP298, and AP299, while all other Bv strains exhibited pectate lyase activity albeit to a lesser degree (Table 1).

3.2. Growth of Bv PGPR strains using pectin as a sole carbon source

All the 59 Bv PGPR strains grew well in TSS minimal medium containing purified pectin as a sole carbon source (Table 1 and Figure 1). The highest OD_{600} values were observed for Bv PGPR strains AP67, AP71, AP75, AP77, AP78, AP85, AP87, AP108, AP112, AP135, AP135, AP143, AP183, AP184, AP188, AP191, AP192, AP193, and AP203. Based on their observed ability to grow *in vitro* using pectin as a sole carbon source, the four best-growing Bv PGPR strains AP136, AP143, AP193, and AP203 were selected for greenhouse and field trials.

3.3. Greenhouse trials of pectin and PGPR amendments on soybean to assess root colonization, growth promotion, and nodulation

Soybean shoot length was significantly enhanced by the combination of purified pectin powder amendment and inoculation with Bv AP193 relative to the shoot length observed when Bv AP193 was applied in the absence of pectin (Figure 2); however, the same enhanced shoot length was not observed with the combination of pectin liquid and Bv AP193 (Figure 2). The best result was observed when pectin was applied as a liquid suspension, with the combination of Bv AP136 and pectin showing a significant increase in shoot length

compared to Bv AP136 alone (Figure 2). Mean root lengths were not significantly different when comparing Bv strains AP193 and AP143 with and without pectin powder amendment (Table 2). Pectin powder amendment with Bv PGPR strain AP193 significantly increased dry shoot weight compared to Bv PGPR strain AP193 without pectin powder amendment (Table 2).

Interestingly, a significant increase in soybean nodulation was observed when seeds were inoculated with both purified pectin and Bv spores (Figure 3). When pectin and Bv strains were applied together, there was a significant increase in nodules, of 331% and 388%, when pectin was applied as a powder together with Bv AP143 or Bv AP193, respectively (Figure 3). Application of purified pectin as a powder or liquid suspension together with Bv AP193 similarly induced a significant increase in the number of nodules per plant (Figure 3). In each of these experiments, the nodules were formed either by infection with indigenous rhizobia in the field soils or by commercial *B. japonicum* inoculant. While significant effects on nodulation due to *B. japonicum* were observed, the populations of the inoculated Bv PGPR strains did not significantly change as a result of pectin amendment (Table 2).

3.4. Field trial of pectin and PGPR amendments on soybean growth promotion and nodulation

In the field trial, the treatment of soybean seeds with Bv AP193 and a pectin liquid suspension resulted in a significant increase in shoot length and dry shoot weight at 55 DAP (Table 3). The inoculation of seeds with Bv AP193 resulted in a mean shoot length of 57.2 cm and a mean dry shoot weight of 14.4 g, whereas an inoculation with both pectin and Bv AP193 resulted in an average shoot length of 78.7 cm (37.6% increase) and an average dry shoot weight of 16.93 g (17.6% increase). The mean number of nodules on plants treated with Bv AP136 or AP193 and a pectin amendment were significantly more than on plants seeds treated with these Bv strains alone (Figure 3). The nodule dry weight of plants treated with

Bv AP136 and pectin was significantly greater than the Bv AP136 control alone (Figure 4). The effect of pectin amendment on soybean yield was also assessed in the field trial, with no significant differences observed among the different treatment groups for plot or test weights (Table 4). The combination of Bv strain and pectin amendment did not increase soybean yield over the Bv strains alone, pectin, or water control, nor did soybean yield change in response to pectin amendment compared to the water control.

3.5. Greenhouse trials of orange peel liquid suspension and PGPR amendments on soybean growth promotion and nodulation in field soil

The lack of consistent plant growth-promoting effects using purified pectin as an amendment in greenhouse and field trials led to the evaluation of orange peel powder as a pectin-rich organic amendment. To first evaluate Bv strains *in vitro* growth using orange peel powder as a growth substrate, Bv strains were inoculated into the TSS minimal medium. A rapid growth of each Bv strain was observed compared to growth using purified pectin as a sole carbon source (Figure 1).

Given the much greater growth rate observed for Bv strains *in vitro* on a pectin powder growth substrate, it was of interest to evaluate the combination of Bv spores and orange peel powder as a seed amendment. A greenhouse trial using field soil was conducted as before, but in this case, the amendment consisted of orange peel powder as a liquid suspension with two different doses (1 or 10 mg) applied with or without Bv inoculum (Table 5). At 35 DAP, the mean dry shoot weights of Bv AP193 with orange peel amendment at 1 mg or 10 mg were significantly increased compared to that of Bv AP193 alone (Figure 5). Similarly, the dry root weights were significantly greater when seeds were amended with Bv AP193 with either rate of orange peel amendment compared to Bv AP193 alone (Figure 5). The dry root weights and the numbers of nodules per plant also increased significantly when Bv AP203

was applied together with orange peel (10 mg) compared to plants that received Bv AP203 without orange peel amendment (Figure 5).

4. Discussion

The results of the *in vitro* growth assays indicated that Bv strains could degrade and utilize exogenous pectin or pectin-rich citrus peel as a sole carbon and energy source. While a slow rate of growth was observed when purified pectin was added to a minimal medium, the *B. velezensis* growth observed in the presence of orange peel powder was significantly greater, suggesting that additional nutritional requirements for Bv growth were supplied from the orange peel powder. A previous study [27] assessed the effects of adding different carbohydrates to the complex medium Lysogeny broth (LB) on the growth of *B. amyloliquefaciens* SQY 162 (a strain that may now be affiliated with *B. velezensis*), and did not observe any significant differences in growth for any added carbohydrate; however, due to the use of the nutrient-rich LB medium in these experiments, this precluded any assessment of *Bacillus* growth due to the use of any of these carbohydrates as a sole carbon and energy source. Hence, our study is therefore the first demonstration of the growth of *B. velezensis* strains using pectin or a pectin-rich organic source such as orange peel powder as a substrate. Given the ubiquity of *B. velezensis* strain growth using a pectin substrate, this suggests that pectin utilization is an important function among these rhizobacteria that may be important in their root colonization and plant growth-promoting activities. The overall results of this study support the hypothesis that pectin amendment can enhance plant growth promotion mediated by selected Bv strains. Plant responses to pectin amendment depended on the specific PGPR strain, and how pectin was inoculated onto a soybean seed. We observed differences in our results depending on whether the purified pectin was applied as a powder or a liquid suspension, and we suspect that Bv strains may have utilized the pectin applied as a liquid suspension more efficiently because of increased bioavailability. There

were also differences observed concerning Bv strain performance. For example, Bv strain AP193 was one of the best-performing strains in this study, causing significant increases in the shoot length of soybean plants observed after treatment together with pectin and in a pectin-rich orange peel powder. While Bv strain AP193 was one of the strains observed to grow well in a minimal medium containing either pectin or orange peel powder, other Bv strains showed comparable *in vitro* growth results and yet did not perform as well in plant trials, suggesting that other bacterial functions such as secondary metabolite biosynthesis might explain these strain differences. A previous study [27] demonstrated that pectin increased biofilm formation, chemotactic activity, and extracellular polymeric substance (EPS) production by *B. amyloliquefaciens* SQY 162, resulting in the enhanced root colonization of tobacco. In our study, there were no significant increases in Bv populations in plant rhizospheres despite significant effects observed on plant physiology. This suggests that pectin-rich amendments are used by Bv strains in the rhizosphere for production of bacterial products such as EPS or secondary metabolites that can have effects on plant growth promotion, rather than simply being used to increase bacterial populations as was observed *in vitro.*

Soybean growth parameters varied with the pectin source, the doses of application, and individual Bv strains' performance in field soils. For example, while Bv strain AP193 combined with either 1 or 10 mg of orange peel amendment significantly increased the dry shoot and root weights, the synergy between Bv strain AP203 and orange peel powder only occurred at the 10-mg dose. These results suggested that different Bv strains degrade and utilize pectin differently based on pectin sources. In addition, the composition of the pectin-rich amendment, such as phenolic compounds from orange peel [40,41], may also influence the PGPR-mediated induction of soybean plant growth. Citrus fruit peel contains a large amount of pectin [42], and Treuer et al. [43] demonstrated that the application of pectin-rich

orange peel to soils provided a long-term benefit for the soil and increased forest vegetation in Costa Rica. Given that different citrus peel sources have different compositions of phenolic, carbohydrate, and other chemical moieties, this could affect the PGPR strain response to these organic amendments; therefore, in a preliminary study, we evaluated different citrus peel powders for their synergy in promoting soybean growth and observed comparable results between orange, grapefruit, lemon, and tangerine peel powders (data not shown). Due to the relatively low cost of orange peel powder compared to other pectin-rich amendments, we selected orange peel powder as the most practical and sustainable amendment for these studies. Interestingly, we consistently observed enhanced nodulation in soybean amended with Bv strains and either purified pectin or orange peel powder. Soybean root nodulation was significantly greater when both pectin and Bv spores were applied, compared to spores alone. These results were observed in both greenhouse and field trials and with pectin applied as a powder or in liquid suspension. These results indicate that pectin mixed with Bv strains can induce soybean nodulation by indigenous and by inoculated rhizobia. Masciarelli et al. [44] reported that a mixed inoculation of *B. japonicum* with *B. amyloliquefaciens* strain LL2012 enhanced soybean nodulation. Another study indicated that *Bacillus cereus* UW85 increased soybean nodulation in a growth chamber and under field conditions without the inoculation of *Bradyrhizobium* spp. [45]. These reports collectively support the conclusion that there are synergistic interactions between *Bradyrhizobium* spp. and some *Bacillus* spp. that either directly or indirectly (e.g. by Bv interactions with plant root cells) result in enhanced soybean root nodulation. The results of our study support these previous conclusions and indicate the role of complex carbohydrates such as pectin in enhancing these rhizobacteria–plant interactions. Further research should explore the molecular interactions between Bv PGPR strains and rhizobia in promoting legume infection and nodulation processes.

In conclusion, the results of this study indicate the importance of pectin as a complex carbohydrate that can be utilized by Bv PGPR strains, and that the exogenous application of pectin-rich amendments can enhance soybean growth and *Bradyrhizobium* nodulation. Future studies are required to extend our understanding of the use of pectin-rich amendments in synergy with select Bv PGPR strains to enhance plant growth, legume nodulation, and/or disease control under field conditions.

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Table 1. *In vitro* degradation and utilization activities of pectin as a sole carbon source for growth for each Bv strain.

Bv strain	*Pectate Lyase Activity	OD_{600}	Reference
AP52	+++	0.36	Kumar et al., 2011 [34]
AP67	++	0.51	This study
AP71	++	0.51	Hossain et al., 2015 [12]
AP75	++	0.49	This study
AP76	++	0.39	This study
AP77	++	0.55	This study
AP78	++	0.49	This study
AP79	++	0.4	Hossain et al., 2015 [12]
AP80	+++	0.32	This study
AP81	+++	0.35	This study
AP85	++	0.57	This study
AP86	++	0.4	This study
AP87	+++	0.45	This study
AP108	++	0.66	This study
AP112	+++	0.52	This study
AP135	++	0.52	This study
AP136	++	0.44	Liu et al., 2016 [29]
AP143	+++	0.49	Coy et al., 2017 [28]
AP150	++	0.35	This study
AP183	+++	0.54	Nasrin et al., 2015 [35]
AP184	++	0.6	This study
AP188	+++	0.72	Zebelo et al., 2016 [36]
AP189	++	0.37	This study
AP190	+++	0.27	This study
AP191	+++	0.67	This study
AP192	+++	0.66	This study
AP193	++	0.68	Ran, 2013 [37]
AP194	++	0.33	Liu et al., 2016 [29]
AP195	++	0.36	Liu et al., 2016 [29]
AP196	++	0.34	This study
AP197	++	0.38	Liu et al., 2016 [29]
AP198	++	0.35	This study
AP199	+++	0.29	Liu et al., 2016 [29]
AP200	+++	0.24	Liu et al., 2016 [29]
AP201	++	0.33	Liu et al., 2016 [29]
AP203	+++	0.46	Liu et al., 2016 [29]
AP205	++	0.34	This study
AP207	+++	0.24	This study
AP208	+++	0.38	Liu et al., 2016 [29]
AP210	++	0.15	Liu et al., 2016 [29]
AP211	++	0.2	This study
AP212	+++	0.22	Liu et al., 2016 [29]
AP213	++	0.29	Liu et al., 2016 [29]
AP214	++	0.2	Liu et al., 2016 [29]
AP215	++	0.09	This study
AP216	++	0.38	This study
AP218	+	0.1	Coy et al., 2017 [28]
AP219	++	0.21	Kumar et al., 2011 [34]
AP241	++	0.1	This study
AP260	++	0.17	This study
AP295	++	0.18	Liu et al., 2016 [29]
AP296	+++	0.11	This study
AP297	++	0.22	Liu et al., 2018 [38]
AP298	+++	0.22	Liu et al., 2018 [38]
AP299	+++	0.19	This study
AP300	++	0.05	This study
AP301	++	0.09	Yellareddygari et al., 2014 [39]
AP304	++	0.2	Kumar et al., 2011 [34]
AP305	++	0.11	Liu et al., 2016 [29]

* Pectate lyase activity were rated on a scale of low (OD_{600} 0.1–0.2) (+), medium (OD_{600} 0.2–0.4) (++), and high (OD_{600} 0.4–0.6) (+++).

Table 2. Effect of pectin powder or liquid amendments on soybean growth, nodulation by indigenous soil rhizobia, and root colonization by Bv PGPR strains in greenhouse tests at 35 days after planting (DAP[#]). The mean values in the column followed by the same letter are not significantly different at $p \leq 0.05$ using Tukey's multiple comparison tests.

Treatment	Dry Shoot Weight (g)	Root Length (cm)	Dry Root Weight (g)	Root Colonization[#] (log CFU/g)
Control	0.4bc	24.2a	0.07cd	1.1c
Pectin Powder (0.1%)	0.3d	18.6b	0.07cd	2.4c
AP143	0.4b	22.9a	0.16a	3.7b
AP143+ Pectin Powder (0.1%)	0.6ab	22.8ab	0.15ab	4.1ab
AP193	0.4bc	25.3a	0.10bc	4.5a
AP193+ Pectin Powder (0.1%)	0.6a	26.4a	0.15ab	4.9a
Control	0.9a	20.7b	0.23b	1.2c
Pectin Liquid (0.1%)	0.1a	23.1ab	0.22b	6.5b
AP136	1.3a	28.3ab	0.21a	7.7a
AP136+ Pectin Liquid (0.1%)	1.1a	30.8a	0.27a	7.8a
AP193	1.7a	28.8ab	0.34a	7.4a
AP193+ Pectin Liquid (0.1%)	1.8a	32.1a	0.40a	7.4a

Table 3. Effect of pectin amendment when applied as a liquid suspension on soybean plant growth and nodulation by *B. velezensis* (Bv) PGPR strains AP136, AP193, and commercial *B. japonicum* inoculant in the field trial. Note that for all the treatment groups, pectin was applied as a liquid suspension at 1% (*w/v*). The mean values in the columns followed by the same letter are not significantly different at $p \leq 0.05$ using Duncan's multiple range tests (DAP[#] – days after planting).

Treatment	DAP[#]	Shoot length (cm)	Dry shoot weight (g)	Root length (cm)	Dry root weight (g)
Control	35 DAP	37.4b	2.6ab	14.5c	0.7ab
Pectin		41.9b	3.6ab	16.6bc	0.7ab
AP136		53.0a	4.3a	22.0ab	0.9a
AP136 + Pectin		53.6a	4.9a	25.1a	0.9a
AP193		32.7b	2.5b	13.4c	0.5b
AP193 + Pectin		38.4b	3.6ab	18.1bc	0.6ab
Control	55 DAP	70.6c	13.8b	12.1b	1.7a
Pectin liquid		74.5bc	17.0a	13.5b	1.9a
AP136		92.7a	19.8a	19.4a	2.1a
AP136 + Pectin		95.6a	20.6a	24.7a	2.3a
AP193		57.1c	14.4b	11.5b	1.8a
AP193 + Pectin		78.7ab	16.9a	19.8a	1.8a

Table 4. Effect of pectin amendment as a 1% (w/v) liquid suspension on soybean yield by *B. velezensis* PGPR strains AP136, AP193 and commercial *B. japonicum* inoculant in the field trial. The mean value in the column followed by the same letter are not significantly different at $P \leq 0.05$ using Duncan's multiple range tests. *Plot weights indicated the total pounds harvested from the two center rows of the four-row plot. **Test weights indicated the number of pounds in one bushel of soybeans.

Treatment	Plot weight (kg)*	Test weight (kg)**
Control	4.0ab	16.6ab
Pectin liquid (PL)	4.5ab	16.6ab
AP136 + Pectin liquid (PL)	5.6a	20.4ab
AP136	5.6a	20.8a
AP193 + Pectin liquid (PL)	3.9ab	16.5ab
AP193	3.5b	12.5b

Table 5. Effect of orange peel (OP) amendment on soybean growth promotion and nodulation by *B. velezensis* (Bv) PGPR strains with *B. japonicum* inoculant in the greenhouse trial (OP – orange peel). The mean value in the column followed by the same letter are not significantly different at $p \leq 0.05$ using Tukey's multiple comparison tests (mg – milligram).

Treatment	Shoot Length (cm)	Root Length (cm)	Mean Nodule Numbers	Dry Nodule Weight (g)
Control	46.8b	22.4b	11.6d	0.02b
OP 1 mg	55.1ab	25.8ab	17.2cd	0.03b
OP 10 mg	64.6a	26.0ab	15.1cd	0.03b
AP193	58.1a	27.7ab	27.2abc	0.05ab
AP203	65.6a	27.1ab	20.9bcd	0.04ab
AP193 + OP 1 mg	53.7ab	33.7a	39.7a	0.06a
AP193 + OP 10 mg	59.9ab	29.9ab	35.0ab	0.06a
AP203 + OP 1 mg	56.0ab	34.7a	36.5a	0.05ab
AP203 + OP 10 mg	53.6ab	32.6ab	37.7a	0.06a

Figure 1. In vitro growth assay of *B. velezensis* (Bv) plant growth-promoting rhizobacteria (PGPR) strains in Tris-Spizizen Salts (TSS) minimal medium including 0.1% (*w/v*) pectin powder (P) or 0.5% (*w/v*) orange peel (OP) amendments.

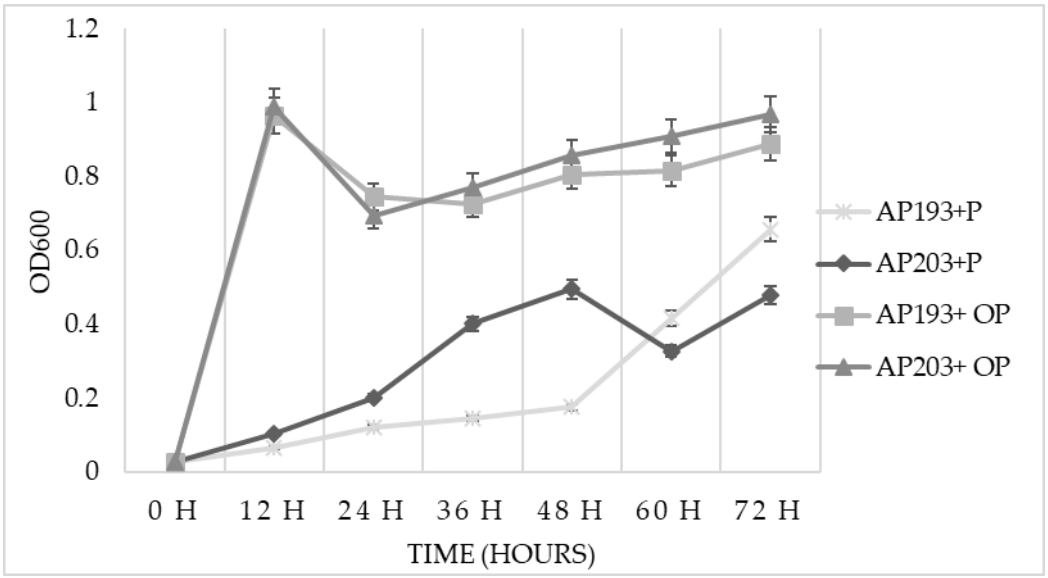


Figure 2. Effect of pectin powder or liquid suspension amendments on soybean shoot length by Bv PGPR strains at 35 DAP[#] in the greenhouse trials. The gray bar indicates amendment with 0.1% (*w/v*) pectin liquid suspension (L), while the white bar indicates amendment with the comparable amount of pectin powder (P) (* indicates significance at the 5% level relative to the Bv PGPR strains alone) (DAP[#] – days after planting).

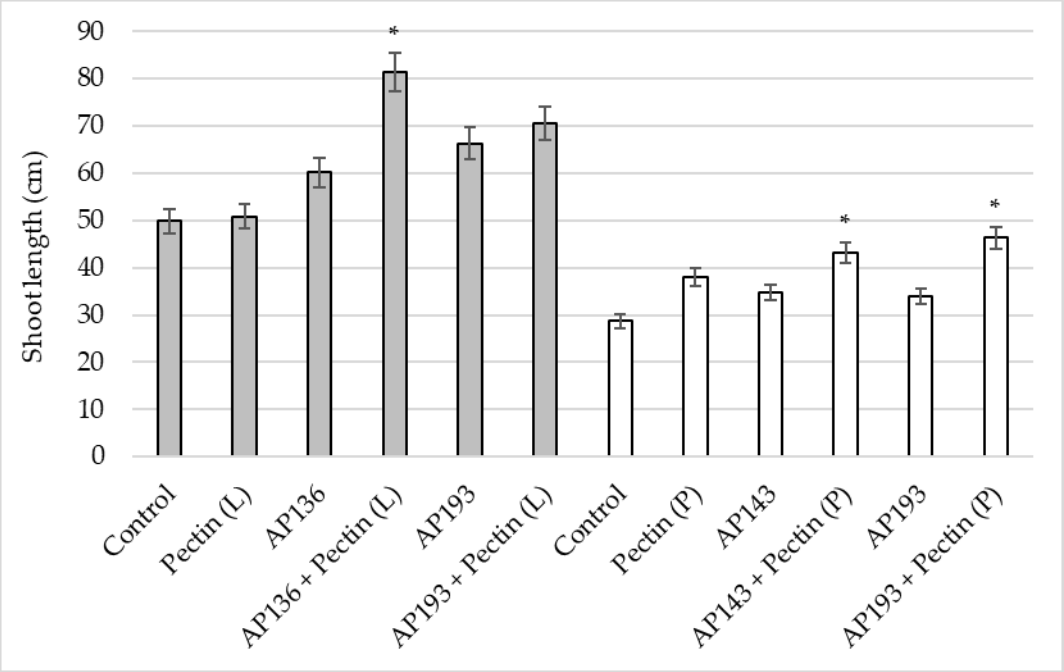


Figure 3. Effect of pectin powder or liquid suspension amendments on soybean nodulation by Bv PGPR strains at 35 DAP[#] in the greenhouse and field trials. The gray-colored bar indicates the results of the field trial with a 0.1% (*w/v*) pectin liquid suspension, and the white-colored bar indicates greenhouse trials in which pectin was applied as a powder (P) or as a liquid suspension (L) amendment (* indicates significance at the 5% level relative to the Bv PGPR strains alone) (DAP[#] – days after planting).

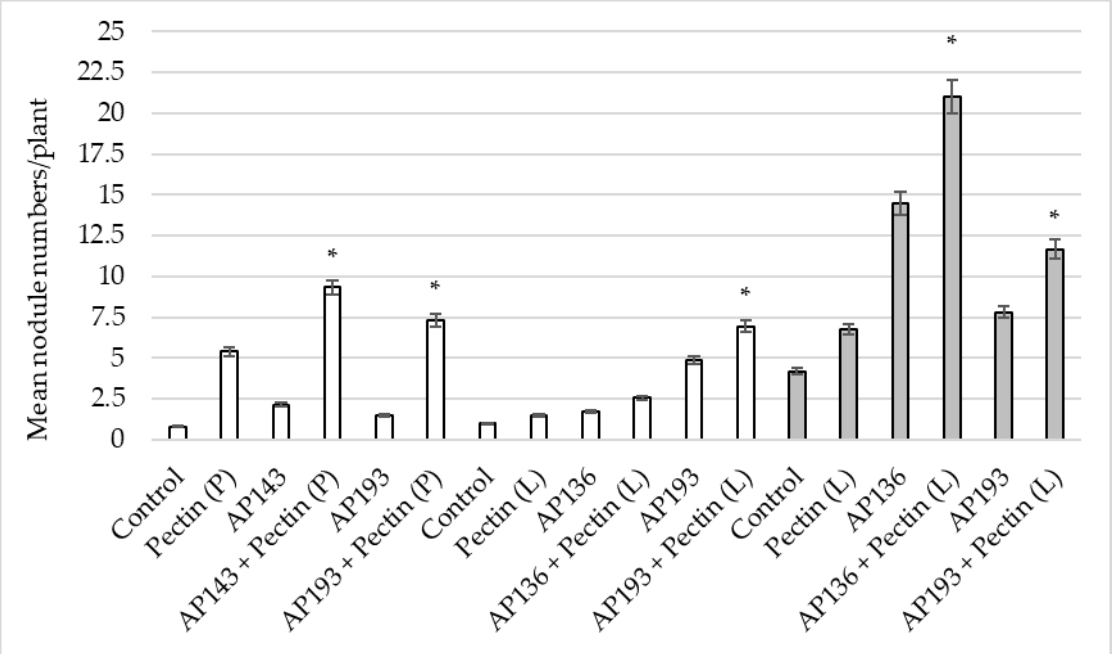


Figure 4. Effect of pectin powder or liquid suspension amendments on soybean dry nodule weight by Bv PGPR strains at 35 DAP[#] in the greenhouse and field trials. The gray-colored bar indicates the results of the field trial with a 0.1% (*w/v*) pectin liquid suspension, and the white-colored bar indicates greenhouse trials in which pectin was applied as a powder (P) or as a liquid suspension (L) amendment (* indicates significance at the 5% level relative to the Bv PGPR strains alone) (DAP[#] – days after planting).

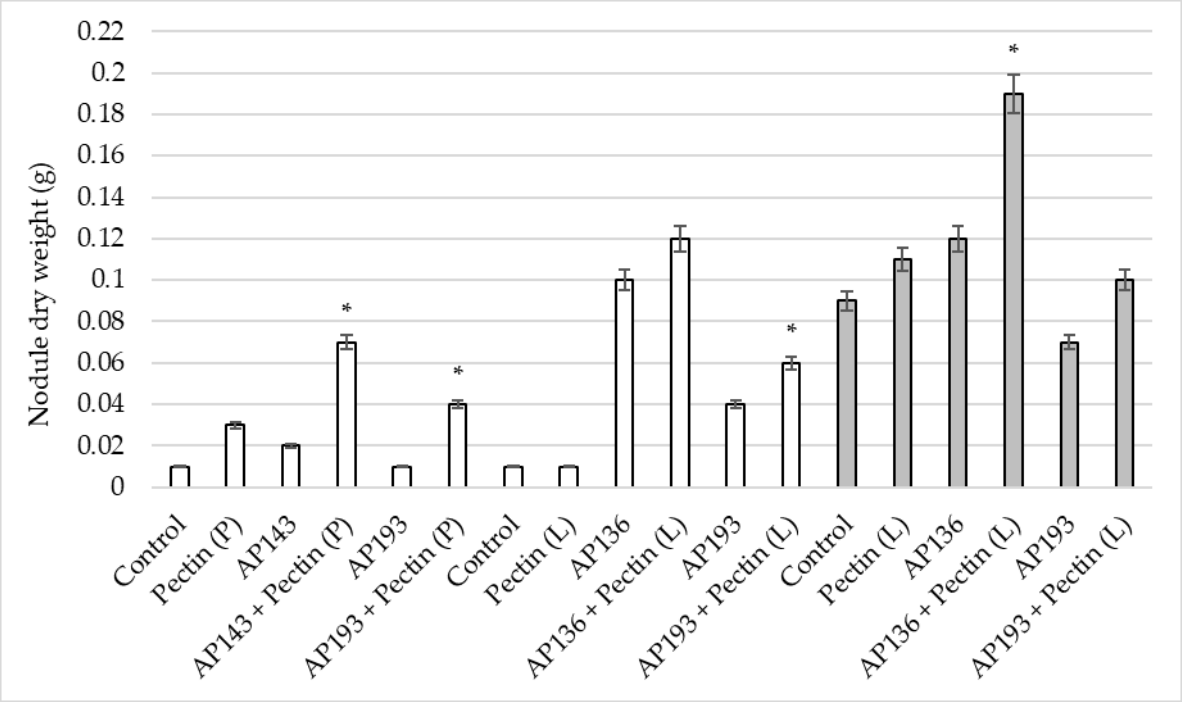
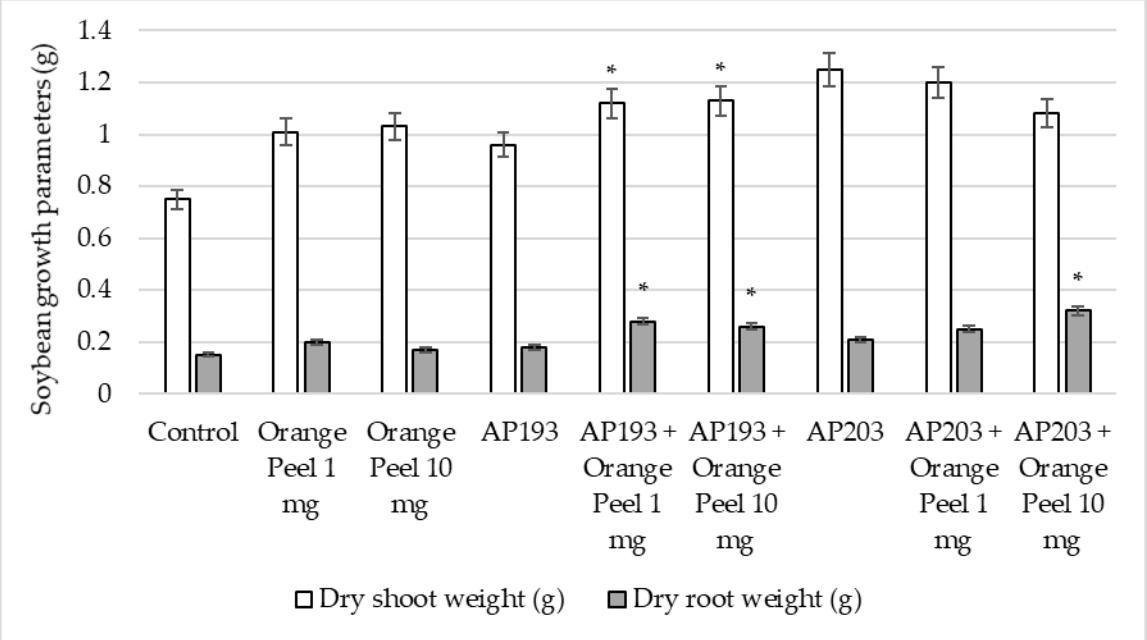


Figure 5. Effect of orange peel (OP) amendments on soybean dry shoot (white bar graph) and root weights (gray bar graph) by Bv PGPR strains at 35 DAP in the greenhouse tests (* indicates significance at the 5% level relative to the Bv PGPR strains alone) (DAP[#] – days after planting).



Chapter III Biological control of root-knot nematode, *Meloidogyne incognita* by the agricultural waste orange-peel and plant growth-promoting rhizobacteria

Abstract

Experiments were conducted to evaluate *Bacillus velezensis* plant growth-promoting rhizobacterial (PGPR) strain AP203 with orange peel amendment for their potential biological control of the root-knot nematode, *Meloidogyne incognita* population on soybean and cotton. LC-MS tests were performed to evaluate the secondary metabolites produced by *B. velezensis* strain, grown in media amended with orange peel. The overall hypothesis was that the model *B. velezensis* strain, grown in orange peel amended media will express different secondary metabolites and thereby will inhibit *M. incognita* population *in vitro* and *in planta* compared to *B. velezensis* PGPR strain without orange peel amendment. The treatments for *in vitro* tests included inoculation of second-stage juveniles (J2) with *B. velezensis* spores plus orange peel suspension, second-stage juveniles (J2) with orange peel suspension, and non-inoculated control. The treatments for greenhouse tests included soybean and cotton seeds planted in field soil mixed with sand (2:1) that contained cell pellet suspension, culture broth, and cell-free supernatant of *B. velezensis* strain grown in orange peel amended media and non-inoculated controls. *In vitro* test results suggest that spores of *B. velezensis* strain AP203 with orange peel suspension significantly increased second-stage juveniles (J2) mortality percentage compared to other treatments ($P \leq 0.05$). The second-stage juveniles (J2) mortality rate increased with *B. velezensis* strain AP203 with orange peel suspension (94%) compared to the same strain without orange peel suspension (53%), orange peel suspension (59%), and the non-inoculated control (7%). The greenhouse test results indicate that cell pellet suspension, culture broth, and cell-free supernatant of *B. velezensis* strain AP203 amended with orange peel significantly reduced *M. incognita* population at 45 days after planting (DAP) compared to *M. incognita* inoculated positive control in soybean

and cotton. However, there were no significant differences between cell pellet suspension, culture broth, or cell-free supernatant. The cell pellet suspension, culture broth, and cell-free supernatant of *B. velezensis* strain AP203 amended with orange peel did not significantly increase shoot length and fresh weight of soybean (shoot and root) compared to other treatments (*B. velezensis* strain AP203 with glucose amendment, *B. velezensis* without orange peel or glucose amendment, glucose alone, orange peel alone, positive, and untreated control). The root length of soybean significantly increased by culture broth of *B. velezensis* strain AP203 amended with orange peel compared to other treatments (*B. velezensis* strain AP203 with glucose amendment, glucose alone, positive, and untreated control). The fresh root weight of soybean significantly increased by cell pellet suspension of *B. velezensis* strain AP203 amended with orange peel compared to other treatments (glucose alone, and positive control). The shoot and root length of cotton significantly increased by cell pellet suspension of *B. velezensis* strain AP203 amended with orange peel compared to the *M. incognita* inoculated positive control. However, fresh shoot and root weight of cotton did not significantly increase by cell pellet suspension, culture broth, and cell-free supernatant of *B. velezensis* strain AP203 amended with orange peel compared to other treatments. LC-MS/MS test results showed that the combination of *B. velezensis* strain AP203 and orange peel suspension produced numerous secondary bioactive metabolites.

1. Introduction

Cotton (*Gossypium hirsutum* L.) and soybean (*Glycine max* L.) are economically important crop in the United States and worldwide. In 2018, cotton yield in the U.S. was 18.4 million bales, and soybean yield was 4.54 billion bushels [1]. *Meloidogyne incognita* (Kofoid and White) Chitwood, the southern root-knot nematode, is broadly distributed in soils cultivated with crops [2] and vegetables [3], and causes yield losses annually. In 2016, cotton yield losses in the U.S. due to *Meloidogyne* spp. were estimated at 414,700 bales [4]. In 2018,

soybean yield losses due to *M. incognita* in the southern U.S. were estimated at 11.92 million bushels in total with 70,000 bushels loss in Alabama [5]. Multiple methods are used for reducing *M. incognita* populations in the field, including cultural practices, chemical nematicides, and resistant varieties [6]. However, environmental and health concerns have limited the use of chemical nematicides for controlling *M. incognita* populations, and there is a need for developing environmentally friendly methods to manage the pathogen such as the use of biological control agents [7].

Plant growth-promoting rhizobacteria (PGPR), beneficial root-colonizing bacteria that enhance plant growth, and biological control of plant pathogens [8]. *Bacillus velezensis* is a Gram-positive rod-shaped PGPR. Strains of *B. velezensis* have been reported to enhance the biological control of *M. incognita* on cotton [2]. These PGPR strains have pectin-associated genes related to degradation and utilization of pectin compounds as a sole carbon and energy sources [9]. The agricultural waste orange peel contains pectin, limonene, and phenolic compounds [10] that can be used as an amendment for biological control by PGPR strains. In the presence of different growth substrates such as carbohydrates (pectin, sucrose, xylan, and galactose), *Bacillus* spp. produce multiple secondary metabolites that inhibit multiple plant pathogens. For example, *B. amyloliquefaciens* PGPR strain SQY 162 grown on pectin amended media increased production of the secondary metabolite surfactin and inhibited bacterial wilt of tobacco caused by *Ralstonia solanacearum* [11]. Another study concluded that cell-free supernatant, cell pellet suspension, and culture broth of *B. subtilis* strains significantly reduced eggs and second-stage juveniles (J2) of *M. incognita* under laboratory and greenhouse conditions [6]. Previous studies have reported that the combination of separated cow manure and orange peels (SCM-OP) reduced the number of eggs of *M. javanica* in tomato roots [12]. However, the effect of exogenous orange peel amendments on

PGPR-mediated biological control activity against plant pathogenic nematode such as *M. incognita* has not been investigated.

The overall goal of this study was to evaluate selected *B. velezensis* PGPR strain with orange peel amendment for their potential biological control of *M. incognita* population on soybean and cotton under greenhouse conditions. The first objective was to investigate the antagonistic effects of *B. velezensis* PGPR strain with orange peel amendment on the mortality of second-stage juveniles (J2) of *M. incognita in vitro*. The second objective was to determine the effects of orange peel amendment by *B. velezensis* PGPR strain on the expression of secondary bioactive metabolite(s) responsible for the reduction of *M. incognita* population. Objective three was to evaluate the efficacy of orange peel amendment by *B. velezensis* PGPR strain to reduce the number of *M. incognita* egg populations in the roots of soybean and cotton under greenhouse conditions.

2. Materials and Methods

2.1. *In vitro* experiment

2.1.1. Preparation of *B. velezensis* PGPR strains and orange-peel suspensions

B. velezensis PGPR strain AP203 was streaked onto tryptic soy agar (TSA) from the stock culture maintained at -80 °C and incubated at 28 °C for 24 h. A single colony of PGPR strain was transferred into a spore preparation medium [13], and incubated for seven days at 28 °C. The 20 mL of sterilized distilled water was added to each Petri plate, and the bacterial mass was transferred to a 50 mL centrifuge tube. *B. velezensis* strain was heat-treated for 20 min at 80 °C in the unstirred water bath (VWR, Radnor, PA, USA), serially diluted, and adjusted to 1.0 X 10^7 spore colony-forming units (CFU/mL). The orange peel powder was suspended in sterilized distilled water by a magnetic stirrer at a rate of 1.0 g per 100 mL (1.0% w/v) water and was applied as an aqueous suspension (Citrus Extracts, Fort Pierce, FL, USA).

2.1.2. Preparation of *M. incognita* inoculum and enumeration of mortality percentage

M. incognita egg was isolated and extracted from corn plant roots Mycogen 2H273 (Dow AgroScience, Indianapolis, IN) at the Plant Science Research Center (PSRC) (Auburn University, Auburn, AL) using sucrose centrifugation-flotation method [14]. The eggs were enumerated using an inverted TS100 Nikon microscope at 40X magnification. All the eggs of *M. incognita* were hatched for seven days at 30 °C in an incubator. Then 10 µL J2 of *M. incognita* (30-40) counted and transferred in a 96-well plate for the J2 mortality test. The 96-well plate was sealed by a parafilm and incubated at room temperature for 48 h. The number of live second-stage juveniles (J2) were counted at the beginning (0 h) and at the end (48 h) of this experiment. The viability of second-stage juveniles (J2) were determined by the sodium hydroxide [15] and the mortality percentage was calculated by the equation: [(live J2 at 0 h – live J2 at 48 h) / live J2 at 0 h] X 100 [2].

2.2. LC-MS experiment:

2.2.1. Preparation of *B. velezensis* PGPR strains

B. velezensis strain was prepared as previously described. A single colony of each PGPR strain was transferred into TSA, TSS (Tris-Spizizen Salts), and TSS + OPP (0.5% w/v) media and grown for 72 h in a shaking incubator at 28 °C. *B. velezensis* strain was then centrifuged in a Sorvall Legend RT centrifuge (Thermo Scientific, USA) at 10,000 x g for 10 min. The supernatant was collected and passed through a 0.2 µm syringe filter (VWR, Radnor, PA, USA) and was then transferred into a 1 mL microcentrifuge tube for LC-MS tests.

2.2.2. LC-MS analysis

LC-MS analysis was performed at the Auburn University Chemistry, and Biochemistry Mass Spectrometry Center on an ultra-performance LC system (ACQUITY, Waters Corp., USA) coupled with a quadrupole time-of-flight mass spectrometer (Q-Tof Premier, Waters) with electrospray ionization (ESI) in positive and negative mode using Masslynx software (V4.1).

Injection of 10 µl of the solution was made onto a C4 column (Aeris Widepore C4, 3.6 µm, 2.1 × 50 mm, Phenomenex) with a 300 µL/min flow rate of the mobile phase. In positive mode, the mobile phase was solution A (0.1% formic acid in water) and solution B (95% acetonitrile, 5% H_2O, and 0.1% formic acid) beginning at 0% B, held for 2 min, then linear ramp to 50% B in 18 min, followed by ramp to 100% B in 8 min and held at 100% B for 2.5 min, and back to 0%B in 0.5 min with 4 min of re-equilibration at 0% B. In negative mode, the mobile phase was solution A (2mM ammonium formate in water) and solution B (100% acetonitrile) beginning at 2% B, held for 2 min, then linear ramp to 50% B in 18 min, followed by ramp to 95% B in 8 min, held at 95% B for 2.5 min, and back to 2% B in 0.5 min with 4 min of re-equilibration at 2% B. The capillary voltage was set at 3.1 kV in positive mode and 2.8 kV in negative mode, the sample cone voltage was 30 V, and the extraction cone was 4.3 V. The source and desolvation temperature were maintained at 105 and 300 °C, respectively, with the desolvation gas flow at 600 L/h. The Time of Flight Mass Spectrometry (TOF/MS) scan was 1 s long from 80 to 1400 m/z with a 0.02 s inter-scan delay using the centroid data format. The lock mass was used to correct instrument accuracy with a 2.5 µg/mL solution of leucine encephalin (Bachem H-2740). The data was converted to mzXML and analyzed with XCMS Online [16].

2.3. Serial dilutions and colony enumeration of *B. velezensis* strain from cell-free supernatants

B. velezensis strain, orange peel, and glucose amended TSS media were prepared as mentioned previously. *B. velezensis* strain was then centrifuged in a Sorvall Legend RT centrifuge (Thermo Scientific, USA) at 10,000 x g for 10 mins and the supernatant was collected and passed through a 0.2 µm syringe filter (VWR, Radnor, PA, USA) and was then transferred into a 1 mL microcentrifuge tube for seven-fold serial dilutions. 50 µl samples were taken from serially diluted supernatants (10^{-5} – 10^{-7} CFU/mL) and were plated on TSA

plates. The plates were incubated at 28 °C for 24 h and triplicate samples were used for each dilution.

2.4. Greenhouse experiment

2.4.1. Preparation of *B. velezensis* PGPR strain and orange-peel suspension

B. velezensis strain and orange peel suspension were prepared as mentioned previously. *B. velezensis* strain AP203 were streaked onto Tryptic Soy Agar (TSA) from the stock culture maintained at -80 °C and grown at 28 °C for 24 h. A single colony of each PGPR strain was transferred into TSA, TSS (Tris-Spizizen Salts) + glucose (0.5% w/v), and TSS + orange peel (0.5% w/v) media and grown for 48 h in a shaking incubator at 28 °C. *B. velezensis* strain was then centrifuged in a Sorvall Legend RT centrifuge (Thermo Scientific, USA) at 10,000 x g for 10 mins and were then adjusted to 1.0 X 10^7 CFU/mL. The TSA grown strain was suspended directly in TSS broth and normalized to approximately 1.0 X 10^7 CFU/mL based on OD_{600} readings. 1 mL of this sample (TSA-GROWN CELLS) was applied to each seed. For the PGPR strains grown in broth media, 35 mL of these cultures were subjected to centrifugation at 10,000 x *g* for 10 mins. The supernatant was saved and passed through a 0.2 µm syringe filter (VWR, Radnor, PA, USA) and 1 mL of this sample (CELL-FREE SUPERNATANT) was applied to each seed. For the cell pellet suspension, the pellet was suspended in TSS and then subjected to centrifugation again to remove spent media, and then resuspended in 35 mL of TSS. 1 mL of this sample (TSS-GROWN CELLS) was applied on each seed. 35 mL of the broth culture were prepared (TSS-GROWN CELLS and SUPERNATANT) and 1 mL of the broth culture will be applied to each seed. Eleven treatments of biological control of *M. incognita* in the greenhouse as follows: 1. *M. incognita* as a positive control (RKN); 2. untreated control (UC); 3. Tryptic soy agar grown cells + *M. incognita* (TR); 4. cell-free supernatant (TSS+OPP) + *M. incognita* (COR); 5. cell-free supernatant (TSS+glucose) + *M. incognita* (CGR); 6. cell pellet (TSS+OPP) + *M. incognita*

(CPOR); 7. cell pellet (TSS+glucose) + *M. incognita* (CPGR); 8. culture broth (TSS-grown cells and supernatant) (TSS+OPP) + *M. incognita* (CBOR); 9. culture broth (TSS-grown cells and supernatant) (TSS+glucose) + *M. incognita* (CBGR); 10. TSS + OPP + *M. incognita* (TSOR); and 11. TSS + glucose + *M. incognita* (TSGR).

2.4.2. Preparation of *Meloidogyne incognita* inoculum

M. incognita eggs were isolated and extracted from corn roots as described previously. 2,000 *M. incognita* eggs/mL were inoculated into a 2 cm depth of soil in each cone-tainer during seed planting and were then covered with field soil. *M. incognita* inoculated soybean and cotton seeds were incubated at room temperature in the greenhouse for 24 h before transferring to a growth chamber at 25 – 35 °C.

2.4.3. Soil preparation and seed inoculation

Field soil was collected from the E.V. Smith Research Center of Auburn University (Shorter, AL) was mixed with sand at a ratio (2:1) of two parts soil to one part sand. In the greenhouse experiments, I70 g of the field soil/sand mix was placed into each 150 cm^3 cone-tainers (Stuewe & Sons, Tangent, OR, USA). Two soybeans (DD VSG 75140) and cotton seeds (DPL-1558 NRB2RF) were placed into 2 cm depth of each cone-tainer to ensure seed germination. Cell pellet suspension, culture broth, and cell-free supernatant of *B. velezensis* strain were applied on the soybean and cotton seed surface. The seeds were then covered with 5 g of soil/sand, incubated at room temperature for 24 h, and then transferred to a greenhouse chamber (25 – 35 °C).

2.5. Statistical analysis

All data collected from the *in vitro* bioassay and greenhouse tests were analyzed with SAS 9.4 software (SAS Institute, Cary, NC, USA) using the PROC GLIMMIX procedure at the $P \leq 0.05$ level of significance. In the *in vitro* experiments, the mortality percentages of second-stage juveniles (J2) of *M. incognita* were analyzed with nine treatments and eight replicates.

In the greenhouse experiment, plant height, root length, root and shoot fresh weight, and *M. incognita* eggs/plant data were collected and analyzed. The greenhouse experiment was arranged in a randomized complete block design (RCBD) with eleven treatments and eight replicates.

3. Results

3.1. *In vitro* antagonistic effects of *B. velezensis* strain AP203 with orange peel amendment

A spore preparation of *B. velezensis* strain AP203 with orange peel amendment was tested *in vitro* for the potential to increase mortality of *M. incognita* second-stage juveniles (J2). The mortality percentage of *M. incognita* J2 ranged from 0 – 100%, and there was a significant reduction with *B. velezensis* strain AP203 with 1.0% (w/v) orange peel amended treatment compared to the other three treatments (*B. velezensis* strain AP203 alone, 1.0% orange peel suspension, and the control) (Figure 1). The highest mortality percentage of *M. incognita* J2 was 94% with the *B. velezensis* strain AP203 and 1.0% orange peel amended treatment. The mortality percentages of *M. incognita* J2 in *B. velezensis* strain AP203 alone, 1.0% orange peel, and the control treatments were recorded as 53%, 59%, and 7% respectively.

3.2. Antagonistic effect of cell pellet suspension, culture broth, and cell-free supernatant of *B. velezensis* strain AP203 with orange peel amendment in soybean and cotton under greenhouse conditions

Cell pellet suspension, culture broth, and cell-free supernatant of *B. velezensis* strain AP203 with orange peel amendment were tested for the potential to reduce *M. incognita* population in the soil under greenhouse conditions. The soybean root lengths of culture broth (CBOR) of *B. velezensis* strain AP203 amended with orange peel treatment was significantly greater compared to the *B. velezensis* strain AP203 amended with glucose (CGR, CPGR, and CBGR) and *M. incognita* inoculated positive control treatments. The soybean root lengths of cell-free supernatant (COR), cell pellet suspension (CPOR), and culture broth (CBOR) of *B.*

velezensis strain AP203 with orange peel amended treatments were 21.87, 22.0, and 22.62 centimeters (Table 1). The soybean shoot lengths and fresh weight of cell-free supernatant (COR), cell pellet suspension (CPOR), and CBOR of *B. velezensis* strain AP203 with orange peel amended treatments were not significantly increased compared to the *B. velezensis* strain AP203 amended with glucose, *B. velezensis* strain AP203 alone, glucose alone, orange peel alone, and *M. incognita* inoculated positive control treatment. The soybean fresh root weight of the CPOR of *B. velezensis* strain AP203 with orange peel amended treatment was significantly increased compared to compared to glucose alone, and *M. incognita* as a positive control treatment. The soybean root fresh weight of the CPOR of *B. velezensis* strain AP203 with orange peel amended treatment was 4.92 grams (Table 1). COR, CPOR, and CBOR of *B. velezensis* strain AP203 with orange peel amended treatments had a maximum antagonistic activity against *M. incognita* eggs in soybean roots at 45 DAP (Figure 2). The cotton shoot and root lengths of CPOR of *B. velezensis* strain AP203 with orange peel amended treatments were significantly increased compared to the *M. incognita* inoculated positive control treatment. However, there were no significant differences between cell-free supernatant, culture broth or cell pellet suspension of *B. velezensis* strain AP203 amended with orange peel. The cotton shoot and root lengths of CPOR of *B. velezensis* strain AP203 with orange peel amended treatments were 30.75 and 21.25 centimeters (Table 2). The cotton root length of COR and CBOR of *B. velezensis* strain AP203 with orange peel amended treatments were significantly greater compared to the *M. incognita* inoculated positive control treatment. The cotton root lengths of COR and CBOR of *B. velezensis* strain AP203 with orange peel amended treatments were 20.25 and 20.12 centimeters (Table 2). The shoot and root fresh weight did not significantly increase by COR, CPOR, and CBOR of *B. velezensis* strain AP203 with orange peel amended treatments compared to the other treatments. COR, CPOR, and CBOR of *B. velezensis* strain AP203 with orange peel amended treatments had a

maximum antagonistic activity against *M. incognita* eggs in cotton roots at 45 DAP (Figure 3). *B. velezensis* strain AP203 with glucose amended treatments (CGR, CPGR, CBGR, and TSGR) did not significantly reduce *M. incognita* eggs compared to the *M. incognita* positive control treatment in the roots of cotton and soybean at 45 DAP under the greenhouse conditions (Figure 2 and 3).

3.3. Secretion of secondary bioactive metabolites by *B. velezensis* strain AP203 with orange peel amendment

Numerous secondary bioactive metabolites were found in cell-free supernatant of *B. velezensis* strain AP203 amended with orange peel (Table 4-8). However, four secondary bioactive metabolites [1,3-Diphenyl-2-propanone, p-(3,4-Dihydro-6-methoxy-2-naphthyl) phenol, (E)-1,1'-(1,2-Diethyl-1,2-ethenediyl) bis (4-methoxybenzene), 3-(Dimethylamino) propyl benzoate] were reported in this study because of their biological control capacities reported previously (Table 3) [25]. The retention times (RT) of these secondary metabolites were 6.69, 6.63, 3.39, and 2.40 mins (Table 3). The product mass to ions charge ratio (m/z) of these secondary metabolites were 211.11, 253.12, 295.17, and 206.12 (Table 3). The relative abundances (RA) per colony forming units (CFU) (10^{-5}, 10^{-6}, and 10^{-7}) of secondary metabolite 1,3-Diphenyl-2-propanone were 5.27, 21.11, and 26.38 (Table 3). The relative abundances (RA) per colony forming units (CFU) (10^{-5}, 10^{-6}, and 10^{-7}) of secondary metabolite p-(3,4-Dihydro-6-methoxy-2-naphthyl) phenol were 6.32, 25.31, and 31.64 (Table 3). The relative abundances (RA) per colony forming units (CFU) (10^{-5}, 10^{-6}, and 10^{-7}) of secondary metabolite (E)-1,1'-(1,2-Diethyl-1,2-ethenediyl) bis (4-methoxybenzene) were 7.37, 29.51, and 36.89 (Table 3). The relative abundances (RA) per colony forming units (CFU) (10^{-5}, 10^{-6}, and 10^{-7}) of secondary metabolite 3-(Dimethylamino) propyl benzoate were 5.15, 20.61, and 25.76 (Table 3).

3.4. Serial dilutions and colony enumeration of *B. velezensis* strain from cell-free supernatants

The calculated average number of *B. velezensis* colonies (10^{-5}, 10^{-6}, and 10^{-7}) on TSA plates were 2.0 X 10^{8} CFU/mL, 5.0 X 10^{8} CFU/mL, and 4.0 X 10^{9} CFU/mL that were plated from supernatant of *B. velezensis* plus orange peel amended TSS media (figures 4 & 5). The calculated average number of *B. velezensis* colonies (10^{-5}, 10^{-6}, and 10^{-7}) on TSA plates were 1.25 X 10^{8} CFU/mL, 4.5 X 10^{8} CFU/mL, and 3.5 X 10^{9} CFU/mL that were plated from the supernatant of *B. velezensis* plus glucose amended TSS media.

4. Discussion

This study demonstrated that the PGPR *B. velezensis* strain AP203 with 1.0% orange peel amendment significantly enhanced mortality of *M. incognita* J2 compared to the *B. velezensis* strain alone, and untreated control, suggesting that there are nematicidal secondary bioactive metabolites produced by *B. velezensis* strain in the presence of orange peel growth substrate. In the presence of different carbohydrate substrates, the production of secondary metabolites can vary at different levels by PGPR [17]. Because the production of the secondary metabolite surfactin from *B. velezensis* strain SQY 162 (previously *B. amyloliquefaciens*) with exogenous pectin amendment significantly increased biological control efficacy against bacterial wilt of tobacco caused by *Ralstonia solanacearum* [11]. A previous study reported that *B. velezensis* strains have pectinolytic activity and can utilize pectin as a sole carbon and energy source [9]. Hence, the exogenous pectin amendment enhanced plant growth-promotion and biofilm formation by PGPR [18, 19]. Previous study reported that chitinolytic bacteria with chitin substrate significantly reduced soybean cyst nematode (*Heterodera glycines*) in a greenhouse test [20]. The combined application of *B. velezensis* strain AP203 and 1.0% orange peel amendment showed significant *in vitro* antagonistic activity against *M. incognita* second-stage juveniles (J2) after 48 h treatment (Figure 1).

The results from the greenhouse tests suggested that cell pellet suspension (CPOR), culture broth (CBOR), and cell-free supernatant (COR) of *B. velezensis* strain AP203 with 1.0 % (w/v) orange peel amended media significantly increased soybean and cotton plant growth (root length) compared to the *M. incognita* inoculated positive control. In addition, the numbers of *M. incognita* eggs of COR, CPOR, and CBOR were reduced in the roots of cotton and soybean compared to the *M. incognita* inoculated positive control. However, there were no significant differences between COR, CPOR or CBOR of *B. velezensis* strain AP203 amended with orange peel. Previous studies have reported that extracts of fresh orange peel significantly reduced *M. incognita* eggs and second-stage juveniles (J2) *in planta* and *in vitro* [12, 21, 22]. Recent studies showed that *B. velezensis* strain enhanced cotton and soybean yields, and reduced *M. incognita* eggs in a greenhouse, microplots, and field experiments [2, 23]. To date, the combination of *B. velezensis* strains and orange peel amendment has not been investigated against the southern root-knot nematode, *M. incognita.* Agricultural waste can be an environmental problem and waste management is an enormous challenge worldwide. The use of the agricultural waste orange peel for plant pathogenic nematode control not only reduces the use of chemical nematicides but also improves plant and soil health. The present findings indicated that *B. velezensis* strain with orange peel amendment can be used to reduce *M. incognita* population density and increase yield in the field, thereby providing an alternative option to chemical nematicides. In addition to improving plant health and suppressing plant-parasitic nematodes, orange peel amendment with *B. velezensis* strain can enhance soil nutrients levels. A recent study showed that agricultural waste orange peel significantly enhanced soil nutrients level and regenerated tropical forest vegetation in Costa Rica [24].

As showed in Table 3, the four secondary bioactive metabolites [1,3-Diphenyl-2-propanone, p-(3,4-Dihydro-6-methoxy-2-naphthyl) phenol, (E)-1,1'-(1,2-Diethyl-1,2-ethenediyl) bis (4-

methoxybenzene), 3-(Dimethylamino) propyl benzoate] produced by *B. velezensis* strain AP203 in orange peel amended TSS media were reported in this study, suggesting that these metabolites could have reduced *M. incognita* eggs and second-stage juveniles (J2) *in vitro* and *in planta*. Previous study reported that the same volatile organic compounds (VOCs) (phenol, propyl benzene, propanone, and 1-ethenyl-4-methoxy benzene) produced by *B. megaterium* YFM3.25 showed nematicidal effects and significantly reduced *M. incognita* eggs in pot experiment [25]. Orange peel contains pectin, limonene, and phenolic compounds that have antioxidant properties and exerts beneficial effects in plant health [10, 26]. *B. velezensis*-mediated secondary metabolites production *in vitro* by adding exogenous orange peel amended medium as a sole carbon and energy source not studied yet. Our study is the first report that a *B. velezensis* strain can use orange peel as a sole carbon and energy source and can reduce numbers of *M. incognita* eggs and second-stage juveniles (J2) through the production of secondary metabolites. *In vitro* test results showed that *B. velezensis* strain grows faster in orange peel amended TSS media (within 6 to 8 h) compared to *B. velezensis* strain grows glucose amended TSS media (within 12-14 h), suggesting that *B. velezensis* strain have the potential to degrade and utilize pectin because of their pectin-associated genes. Thus, *B. velezensis* strain with orange peel amendment might contribute to reduce plant-pathogenic nematode population density *in vitro* and *in planta*.

In conclusion, *B. velezensis* strain AP203 with orange peel amendment significantly reduced *M. incognita* populations *in vitro* and *in planta.* Hence, the combined use of *B. velezensis* strain and orange peel may represent a promising and sustainable biological control technique for plant-parasitic nematodes. Further studies are needed to evaluate four secondary metabolites for potential use against plant-parasitic *M. incognita* nematode produced by *B. velezensis* strain AP203 amended with orange peel.

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Table 1. Effects of culture broth, cell pellet suspension, and cell-free supernatant of *B. velezensis* strain AP203[1] on soybean plant growth at 45 DAP[2] in greenhouse trials.

Treatment	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Root fresh weight (g)
TR	58.00a	22.50ab	6.51ab	3.91ab
COR	67.37a	21.87abc	8.12ab	4.20ab
CGR	66.75a	19.87cde	7.55ab	3.98ab
CPOR	72.25a	22.00abc	9.06ab	4.92a
CPGR	62.50a	20.12bcde	8.43ab	3.42ab
CBOR	72.00a	22.62a	10.20ab	4.11ab
CBGR	60.75a	19.00e	8.25ab	4.00ab
TSOR	62.50a	21.37abce	8.12ab	3.65ab
TSGR	67.87a	20.00cde	5.95ab	2.53b
RKN	57.50a	19.37de	3.81b	2.53b
UC	67.25a	22.62a	6.65ab	3.31ab

The greenhouse test was repeated twice, and data were analyzed in SAS 9.4 using PROC GLIMMIX procedure. Means with the same letter are not significantly different at $P \leq 0.05$ level of significance. 1- *B. velezensis* strain AP203 grew in 1.0% (w/v) orange peel powder (OPP) amended Tris Spizizen Salts (TSS) media and 2 – days after planting.

Table 2. Effects of culture broth, cell pellet suspension, and cell-free supernatant of *B. velezensis* strain AP203[1] on cotton plant growth at 45 DAP[2] in greenhouse trials.

Treatment	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Root fresh weight (g)
TR	26.00ab	18.50abc	2.65a	1.75a
COR	27.37ab	20.25ab	2.54a	1.70a
CGR	26.87ab	18.25abc	2.53a	1.63a
CPOR	30.75a	21.25ab	2.27a	1.97a
CPGR	27.00ab	16.12bc	2.72a	1.66a
CBOR	30.62ab	20.12ab	2.41a	1.48a
CBGR	28.87ab	18.12abc	2.57a	1.62a
TSOR	23.25ab	17.75abc	2.10a	1.75a
TSGR	22.62b	16.12bc	2.30a	1.17a
RKN	20.00b	14.37c	1.70a	0.96a
UC	26.25ab	21.87a	2.73a	1.37a

The greenhouse test was repeated twice, and data were analyzed in SAS 9.4 using PROC GLIMMIX procedure. Means with the same letter are not significantly different at $P \leq 0.05$ level of significance. 1- *B. velezensis* strain AP203 grew in 1.0% (w/v) orange peel powder (OPP) amended Tris Spizizen Salts (TSS) media and 2 – days after planting.

Table 3: Secondary metabolites found in cell-free supernatants of *B. velezensis* strain AP203 after 48 h growth in 0.5% (w/v) orange peel powder (OPP) amended Tris Spizizen Salts (TSS) media.

Treatment	RT[1] (min)	Product ions (m/z)	RA/CFU[2] (10^-5)	RA/CFU (10^-6)	RA/CFU (10^-7)	Secondary metabolites
OPAP203 1	6.69min	211.11	5.27	21.11	26.38	1,3-Diphenyl-2-propanone
OPAP203 2	6.63min	253.12	6.32	25.31	31.64	p-(3,4-Dihydro-6-methoxy-2-naphthyl) phenol
OPAP203 3	3.39min	295.17	7.37	29.51	36.89	(E)-1,1'-(1,2-Diethyl-1,2-ethenediyl) bis (4-methoxybenzene)
OPAP203 4	2.40min	206.12	5.15	20.61	25.76	3-(Dimethylamino) propyl benzoate
GluAP203	0	0	0	0	0	0
OP	0	0	0	0	0	0
Glu	0	0	0	0	0	0

The *in vitro B. velezensis* strain AP203 growth test was repeated twice. 1 - retention time, and 2- relative abundance / colony forming units.

Table 4: List of putative secondary bioactive metabolites found in the cell-free supernatant of *B. velezensis* strain AP203 grown on orange peel amended TSS minimal media *in vitro*.

Query ID	Query m/z	Name	Formula	Exact Mass
1	101.0711	Gyromitrin;Acetaldehyde methylformylhydrazone	C4H8N2O	100.0636629
2	103.0559	Indoleacetic acid	C10H9NO2	175.0633285
3	103.0559	5-Hydroxyindoleacetaldehyde	C10H9NO2	175.0633285
4	104.0549	2-Ethyl-1-hexanol, 9CI; (Â±)-form, O-Sulfate	C8H18O4S	210.0925798
5	104.0568	2-Ethyl-1-hexanol, 9CI; (Â±)-form, O-Sulfate	C8H18O4S	210.0925798
7	104.0585	Histidinyl-Glycine	C8H12N4O3	212.0909403
8	104.0585	D-Glycero-D-galacto-heptitol	C7H16O7	212.0896029
9	104.0585	Glycyl-Histidine	C8H12N4O3	212.0909403
10	104.0707	N-methyl-beta-alanine	C4H9NO2	103.0633285
11	104.0707	(2S)-2-Nitrobutane	C4H9NO2	103.0633285
13	104.0707	Ethyl carbamic acid methyl ester	C4H9NO2	103.0633285
14	104.0707	N-Methyl-L-alanine	C4H9NO2	103.0633285
15	104.0707	HBA	C4H9NO2	103.0633285
16	104.0707	DL-3-aminobutyrate	C4H9NO2	103.0633285
17	104.0707	Mefenamic acid	C15H15NO2	241.1102787
18	104.0707	N-[2-(4-Hydroxyphenyl)ethyl]benzamide	C15H15NO2	241.1102787
19	104.0707	2-Amino-2-methylpropanoate;2-Aminoisobutyric acid	C4H9NO2	103.0633285
20	104.0707	(R,S)-3-Amino-2-methylpropanoate	C4H9NO2	103.0633285
21	104.0707	beta-alanine-methyl-ester	C4H9NO2	103.0633285
22	104.0707	N,N-Dimethylglycine;Dimethylglycine	C4H9NO2	103.0633285
23	104.0707	N-Ethylglycine	C4H9NO2	103.0633285
24	104.0707	(R)-2-Aminobutanoic acid;(S)-2-Aminobutanoate	C4H9NO2	103.0633285
25	104.0707	1-nitrobutane	C4H9NO2	103.0633285
26	104.0707	4-Aminobutanoate;4-Aminobutanoic acid	C4H9NO2	103.0633285
27	105.0366	3-methylthiopropanal	C4H8OS	104.0295856
28	105.0366	Acutifolane A	C16H22O3	262.1569
29	105.0366	tetrahydrothiophene 1-oxide	C4H8OS	104.0295856
30	105.0367	3-methylthiopropanal	C4H8OS	104.0295856
31	105.0367	tetrahydrothiophene 1-oxide	C4H8OS	104.0295856
32	105.0372	3-methylthiopropanal	C4H8OS	104.0295856
33	105.0372	tetrahydrothiophene 1-oxide	C4H8OS	104.0295856
34	105.0376	3-methylthiopropanal	C4H8OS	104.0295856
35	105.0376	tetrahydrothiophene 1-oxide	C4H8OS	104.0295856
37	105.044	3-cyanopyridine	C6H4N2	104.0374481
38	105.044	2-Cyanopyridine	C6H4N2	104.0374481
39	105.044	4-Cyanopyridine	C6H4N2	104.0374481
40	105.0441	(+)-18-Hydroxy-7,16-sacculatadiene-11,12-dial	C20H30O3	318.2195
41	105.0441	ent-7alpha-hydroxykaur-16-en-19-oic acid	C20H30O3	318.2195
42	105.0441	2-Cyanopyridine	C6H4N2	104.0374481
43	105.0441	Oxymesterone	C20H30O3	318.2194948

Table 5: List of putative secondary bioactive metabolites found in the cell-free supernatant of *B. velezensis* strain AP203 grown on orange peel amended TSS minimal media *in vitro*.

Query ID	Query m/z	Name	Formula	Exact Mass
44	105.0441	4-Cyanopyridine	C6H4N2	104.0374481
45	105.0441	3-cyanopyridine	C6H4N2	104.0374481
46	105.0441	8-oxo-5E,9Z,11Z,14Z-eicosatetraenoic acid	C20H30O3	318.2195
47	105.0441	9-oxo-5E,7Z,11Z,14Z-eicosatetraenoic acid	C20H30O3	318.2195
48	105.0441	11-oxo-5E,8Z,12Z,14Z-Eicosatetraenoic acid	C20H30O3	318.2195
49	105.0441	(+)-7beta-Hydroxy-15-beyeren-19-oic acid	C20H30O3	318.2195
50	105.0557	Tyrosyl-Tyrosine	C18H20N2O5	344.1372218
51	105.0651	Aminoserine	C3H8N2O2	104.0585775
52	105.0651	L-2,3-Diaminopropionate	C3H8N2O2	104.0585775
53	105.0651	Hydroxyaminoalanine	C3H8N2O2	104.0585775
54	105.0662	2,3-Diaminopropanoic acid	C3H8N2O2	104.0585775
55	105.0672	4'-O-Methylbavachalcone	C22H24O4	352.1675
56	105.0672	Ovalichalcone	C22H24O4	352.1675
57	105.0672	Pongagallone A	C22H24O4	352.1675
58	105.0672	Candidone	C22H24O4	352.1675
59	105.0672	Methylhildgardtol A	C22H24O4	352.1675
60	105.0672	Methylhildgardtol B	C22H24O4	352.1675
61	105.0672	Xuulanin	C22H24O4	352.1675
62	105.0715	Valganciclovir	C14H22N6O5	354.1651678
63	105.0733	12-hydroxyjasmonic acid 12-O-beta-D-glucoside	C19H30O8	386.1941
64	105.0733	Citroside A	C19H30O8	386.1940679
65	105.0733	6,9-Dihydroxy-4,7-megastigmadien-3-one	C19H30O8	386.1940679
66	107.0845	p-Xylene;1,4-Dimethylbenzene;p-Methyltoluene	C8H10	106.0782503
67	107.0845	Ethylbenzene;Phenylethane;Ethylbenzol;Ethylenzene	C8H10	106.0782503
68	107.0845	o-Xylene;o-Dimethylbenzene;o-Methyltoluene	C8H10	106.0782503
69	107.0845	m-Xylene;1,3-Dimethylbenzene;1,3-Xylene	C8H10	106.0782503
70	107.0848	o-Xylene;o-Dimethylbenzene;o-Methyltoluene	C8H10	106.0782503
71	107.0848	m-Xylene;1,3-Dimethylbenzene;1,3-Xylene	C8H10	106.0782503
72	107.0848	p-Xylene;1,4-Dimethylbenzene;p-Methyltoluene	C8H10	106.0782503
73	107.0848	Ethylbenzene;Phenylethane;Ethylbenzol;Ethylenzene	C8H10	106.0782503
74	107.0848	O-6-deoxy-a-L-galactopyranosyl	C20H33NO14	511.1901048
75	107.0851	o-Xylene;o-Dimethylbenzene;o-Methyltoluene	C8H10	106.0782503
76	107.0851	m-Xylene;1,3-Dimethylbenzene;1,3-Xylene	C8H10	106.0782503
77	107.0851	p-Xylene;1,4-Dimethylbenzene;p-Methyltoluene	C8H10	106.0782503
78	107.0851	Ethylbenzene;Phenylethane;Ethylbenzol;Ethylenzene	C8H10	106.0782503
79	107.0858	p-Xylene;1,4-Dimethylbenzene;p-Methyltoluene	C8H10	106.0782503
80	107.0858	Ethylbenzene;Phenylethane;Ethylbenzol;Ethylenzene	C8H10	106.0782503
81	107.0858	o-Xylene;o-Dimethylbenzene;o-Methyltoluene	C8H10	106.0782503
82	107.0858	m-Xylene;1,3-Dimethylbenzene;1,3-Xylene	C8H10	106.0782503
83	107.0858	Daunorubicin	C27H29NO10	527.1791462
84	107.0859	m-Xylene;1,3-Dimethylbenzene;1,3-Xylene	C8H10	106.0782503
85	107.0859	p-Xylene;1,4-Dimethylbenzene;p-Methyltoluene	C8H10	106.0782503
86	107.0859	Ethylbenzene;Phenylethane;Ethylbenzol;Ethylenzene	C8H10	106.0782503
87	107.0859	o-Xylene;o-Dimethylbenzene;o-Methyltoluene	C8H10	106.0782503
88	109.0287	1,2-Benzoquinone	C6H4O2	108.0211294
89	109.0287	Quinone;p-Benzoquinone;Chinone	C6H4O2	108.0211294

Table 6: List of putative secondary bioactive metabolites found in the cell-free supernatant of *B. velezensis* strain AP203 grown on orange peel amended TSS minimal media *in vitro*.

Query ID	Query m/z	Name	Formula	Exact Mass
90	109.0306	Hordatine B glucoside	C35H50N8O10	742.3649899
91	109.0306	Hydroxymethylmethylsilanediol	C2H8O3Si	108.0242707
92	109.0309	Hydroxymethylmethylsilanediol	C2H8O3Si	108.0242707
93	109.0309	Monothioglycerol	C3H8O2S	108.0245002
94	109.0315	Hydroxymethylmethylsilanediol	C2H8O3Si	108.0242707
95	109.0316	Hydroxymethylmethylsilanediol	C2H8O3Si	108.0242707
96	109.0316	Hydroxymethylmethylsilanediol	C2H8O3Si	108.0242707
97	109.0321	Hydroxymethylmethylsilanediol	C2H8O3Si	108.0242707
98	109.0642	Benzenemethanol;Phenylmethanol;Phenylcarbinol	C7H8O	108.0575149
99	109.0642	o-Cresol;2-Hydroxytoluene;o-Methylphenol	C7H8O	108.0575149
100	109.0642	3-Cresol;m-Cresol;3-Hydroxytoluene	C7H8O	108.0575149
101	109.0642	4-Cresol;p-Cresol;4-Hydroxytoluene	C7H8O	108.0575149
102	109.0642	Anisole;Methoxybenzene;Methyl phenyl ether	C7H8O	108.0575149
103	111.0434	Resorcinol;Resorcin;1,3-Benzenediol	C6H6O2	110.0367794
104	111.0434	Hydroquinone;p-Benzenediol;1,4-Benzenediol	C6H6O2	110.0367794
105	111.0434	5-Methyl-2-furaldehyde;5-Methyl-2-furfural	C6H6O2	110.0367794
106	111.0434	o-Benzosemiquinone	C6H6O2	110.0367794
107	111.0434	Catechol;1,2-Benzenediol;o-Benzenediol	C6H6O2	110.0367794
108	111.0434	Benzosemiquinone;p-Benzosemiquinone	C6H6O2	110.0367794
109	111.0437	Resorcinol;Resorcin;1,3-Benzenediol	C6H6O2	110.0367794
110	111.0437	Hydroquinone;p-Benzenediol;1,4-Benzenediol	C6H6O2	110.0367794
111	111.0437	5-Methyl-2-furaldehyde;5-Methyl-2-furfural	C6H6O2	110.0367794
112	111.0437	Catechol;1,2-Benzenediol;o-Benzenedio	C6H6O2	110.0367794
113	111.0437	Benzosemiquinone;p-Benzosemiquinone	C6H6O2	110.0367794
114	111.0438	2-Furanmethanol	C5H6O2	98.03677944
115	111.0438	Benzosemiquinone;p-Benzosemiquinone	C6H6O2	110.0367794
116	111.0438	Resorcinol;Resorcin;1,3-Benzenediol	C6H6O2	110.0367794
117	111.0438	Hydroquinone;p-Benzenediol;1,4-Benzenediol	C6H6O2	110.0367794
118	111.0438	5-Methyl-2-furaldehyde;5-Methyl-2-furfural	C6H6O2	110.0367794
119	111.0438	o-Benzosemiquinone	C6H6O2	110.0367794
120	111.0438	penta-2,4-dienoic acid;beta-vinyl acrylic acid	C5H6O2	98.0368
121	111.0438	Catechol;1,2-Benzenediol;o-Benzenediol	C6H6O2	110.0367794
122	111.0438	5-Methyl-2(3H)-furanone	C5H6O2	98.03677944
123	111.0447	o-Benzosemiquinone	C6H6O2	110.0367794
124	111.0447	Catechol;1,2-Benzenediol;o-Benzenediol	C6H6O2	110.0367794
125	111.0447	Benzosemiquinone;p-Benzosemiquinone	C6H6O2	110.0367794
126	111.0447	Resorcinol;Resorcin;1,3-Benzenediol;1,3-Dihydroxybenzene	C6H6O2	110.0367794
127	111.0447	Hydroquinone;p-Benzenediol;1,4-Benzenediol	C6H6O2	110.0367794
128	111.0447	5-Methyl-2-furaldehyde;5-Methyl-2-furfural	C6H6O2	110.0367794
129	111.0448	o-Benzosemiquinone	C6H6O2	110.0367794
130	111.0448	Catechol;1,2-Benzenediol;o-Benzenediol	C6H6O2	110.0367794
131	111.0448	Benzosemiquinone;p-Benzosemiquinone	C6H6O2	110.0367794
132	111.0448	Resorcinol;Resorcin;1,3-Benzenediol;1,3-Dihydroxybenzene	C6H6O2	110.0367794
133	111.0448	Hydroquinone;p-Benzenediol;1,4-Benzenediol	C6H6O2	110.0367794

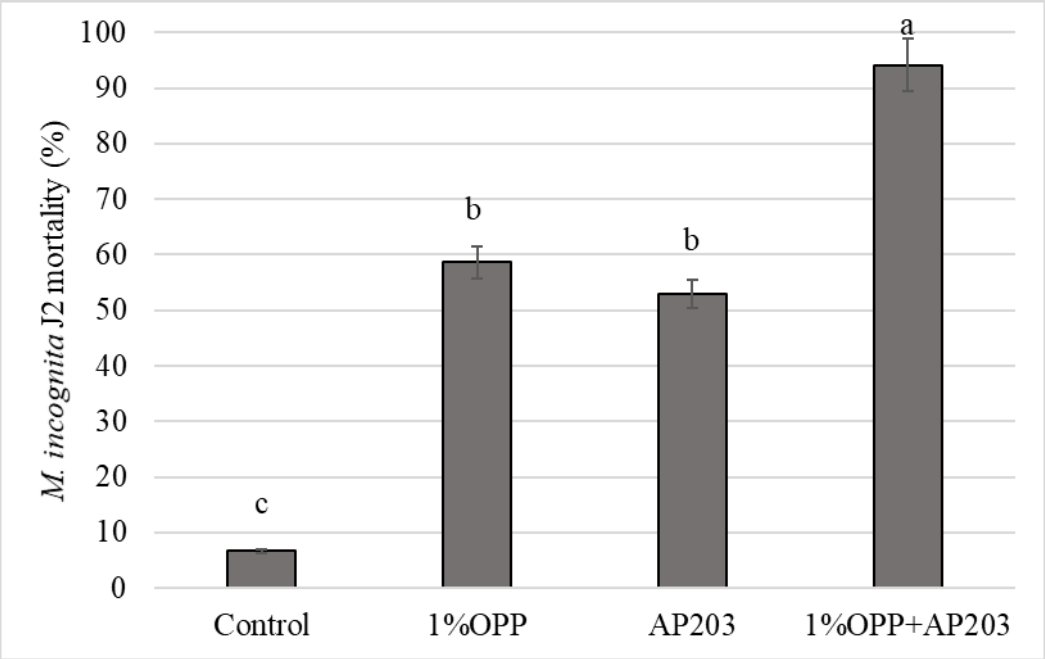
Table 7: List of putative secondary bioactive metabolites found in the cell-free supernatant of *B. velezensis* strain AP203 grown on orange peel amended TSS minimal media *in vitro*.

Query ID	Query m/z	Name	Formula	Exact Mass
134	111.0451	Catechol;1,2-Benzenediol;o-Benzenediol	C6H6O2	110.0367794
135	111.0451	Benzosemiquinone;p-Benzosemiquinone	C6H6O2	110.0367794
136	111.0451	Resorcinol;Resorcin;1,3-Benzenediol;1,3-Dihydroxybenzene	C6H6O2	110.0367794
137	111.0451	Hydroquinone;p-Benzenediol;1,4-Benzenediol	C6H6O2	110.0367794
138	111.0451	5-Methyl-2-furaldehyde;5-Methyl-2-furfural	C6H6O2	110.0367794
139	121.0316	Dimethylsulfonioacetate	C4H8O2S	120.0245002
140	121.0316	3-(Methylthio)propionic acid;3-Methylthiopropionate	C4H8O2S	120.0245002
141	121.0316	sulfolane	C4H8O2S	120.0245002
142	121.032	Dimethylsulfonioacetate	C4H8O2S	120.0245002
143	121.032	3-(Methylthio)propionic acid;3-Methylthiopropionate	C4H8O2S	120.0245002
144	121.032	sulfolane	C4H8O2S	120.0245002
145	121.0324	Dimethylsulfonioacetate	C4H8O2S	120.0245002
146	121.0324	3-(Methylthio)propionic acid;3-Methylthiopropionate	C4H8O2S	120.0245002
147	121.0324	sulfolane	C4H8O2S	120.0245002
148	121.0325	Dimethylsulfonioacetate	C4H8O2S	120.0245002
149	121.0325	3-(Methylthio)propionic acid;3-Methylthiopropionate	C4H8O2S	120.0245002
150	121.0325	sulfolane	C4H8O2S	120.0245002
151	121.0325	Dimethylsulfonioacetate	C4H8O2S	120.0245002
152	121.0325	3-(Methylthio)propionic acid;3-Methylthiopropionate	C4H8O2S	120.0245002
153	121.0325	sulfolane	C4H8O2S	120.0245002
154	121.0325	Dimethylsulfonioacetate	C4H8O2S	120.0245002
155	121.0325	3-(Methylthio)propionic acid;3-Methylthiopropionate	C4H8O2S	120.0245002
156	121.0325	sulfolane	C4H8O2S	120.0245002
157	121.037	3-nitro-1-propionate	C3H6NO4	120.0296827
158	121.0379	3-nitro-1-propionate	C3H6NO4	120.0296827
159	121.05	2,3-dihydroxy-2-methyl-propanoic acid	C4H8O4	120.0422587
160	121.05	L-(+)-Erythrose;D-threo-Aldose;D-Erythrulose	C4H8O4	120.0422587
161	121.05	3-Deoxytetronic acid	C4H8O4	120.0422587
162	121.05	4-Deoxyerythronic acid	C4H8O4	120.0422587
163	121.05	L-Erythrulose;L-glycero-Tetrulose	C4H8O4	120.0422587
164	121.05	3,4-Dihydroxybutyric acid	C4H8O4	120.0422587
165	121.05	D-Threose;D-threo-Tetrose;D-Erythrose	C4H8O4	120.0422587
166	121.0516	Purine	C5H4N4	120.0435961
167	122.0962	3,4-DIMETHYLANILINE	C8H11N	121.0891494
168	122.0962	1-Phenylethylamine;alpha-Phenylethylamine	C8H11N	121.0891494
169	122.0962	N-Ethylaniline;N-Ethylbenzenamine	C8H11N	121.0891494
170	122.0962	Phenethylamine;2-Phenylethylamine;beta-Phenylethylamine	C8H11N	121.0891494
171	122.0962	2,4-Dimethylaniline;2,4-DMA	C8H11N	121.0891494
172	122.0962	N,N-Dimethylaniline; N,N-Dimethylbenzenamine	C8H11N	121.0891494
173	122.0965	1-Phenylethylamine;alpha-Phenylethylamine	C8H11N	121.0891494
174	122.0965	2,6-Dimethylaniline	C8H11N	121.0891494
175	122.0965	N-Ethylaniline;N-Ethylbenzenamine	C8H11N	121.0891494
176	122.0965	2,5-Dimethylalanine	C8H11N	121.0891494
177	122.0965	2-Phenylethylamine;beta-Phenylethylamine	C8H11N	121.0891494

Table 8: List of putative secondary bioactive metabolites found in the cell-free supernatant of *B. velezensis* strain AP203 grown on orange peel amended TSS minimal media *in vitro*.

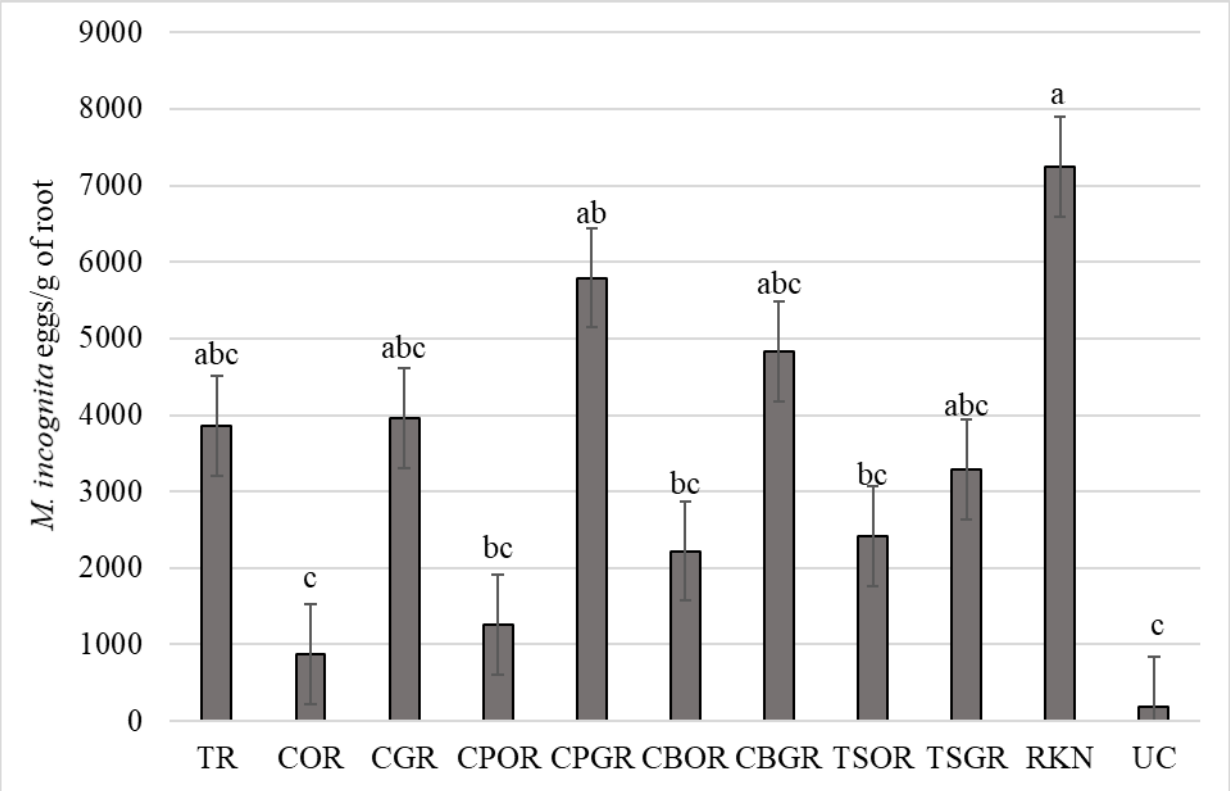
Query ID	Query m/z	Name	Formula	Exact Mass
178	181.0694	Sorbose;xylo-Hexulose;D-Fructose	C6H12O6	180.0633881
179	181.0694	2-Deoxy-D-gluconate	C6H12O6	180.0633881
180	181.0694	Ketose	C6H12O6	180.0633881
181	181.0996	Methylphophonic acid diisopropyl ester	C7H17O3P	180.0915309
182	229.1235	1,1-Bis(4-hydroxyphenyl)propane	C15H16O2	228.1150298
183	229.1235	Mansonone C	C15H16O2	228.1150298
184	229.1235	Bisphenol A;2,2-Bis(4-Hydroxyphenyl)propane	C15H16O2	228.1150298
185	229.1235	dihydropinosylvin monomethylether	C15H16O2	228.1150298
186	229.1285	Deoxyguanidinoproclavaminic acid	C9H16N4O3	228.1222404
187	229.1298	Deoxyamidinoproclavaminate	C9H16N4O3	228.1222404
188	230.058	Lamivudine;3TC;2',3'-Dideoxy-3'-thiacytidine	C8H11N3O3S	229.0521119
189	230.058	Carbonylphophonic acid	C9H12NO4P	229.0503944
190	230.058	2,3-Dihydroxy-2'-carboxybiphenyl	C13H9O4-	229.0500838
191	357.1718	Rutamarin	C21H24O5	356.1623739
191	357.1718	Gingerenone A	C21H24O5	356.1623739
192	357.1718	alpha,beta-dihydroxanthohumol	C21H24O5	356.1623739
193	357.1718	Kadsurenone;Denudatin B	C21H24O5	356.1623739
194	371.0795	Rebamipide	C19H15ClN2O4	370.0720347
195	371.0795	Digalacturonate;Digalacturonic acid	C12H18O13	370.0747407
196	371.0795	1,2-beta-D-Glucuronosyl-D-glucuronate	C12H18O13	370.0747407
197	371.145	Napththalene-2-sulfonamide	C20H22N2O3S	370.1351133
198	371.1501	iso-dehydrocycloxanthohumol hydrate	C21H22O6	370.1416384
199	371.1501	xanthohumol D	C21H22O6	370.1416384
200	371.1501	5'-Prenylhomoeriodictyol;Sigmoidin B 3'-methyl ether	C21H22O6	370.1416384
201	371.1501	xanthohumol B	C21H22O6	370.1416384
202	371.1501	curcumin	C21H22O6	370.1416384
203	371.1501	Alkannin beta,beta-dimethylacrylate	C21H22O6	370.1416384
204	371.1501	Sophoraisoflavanone A	C21H22O6	370.1416384
205	371.1509	iso-dehydrocycloxanthohumol hydrate	C21H22O6	370.1416384
206	371.1509	xanthohumol D	C21H22O6	370.1416384
207	371.1509	5'-Prenylhomoeriodictyol;Sigmoidin B 3'-methyl ether	C21H22O6	370.1416384
208	371.1509	xanthohumol B	C21H22O6	370.1416384
209	371.1509	curcumin	C21H22O6	370.1416384
210	371.1509	Alkannin beta,beta-dimethylacrylate	C21H22O6	370.1416384
211	371.1509	Sophoraisoflavanone A	C21H22O6	370.1416384
212	371.1528	Galactan;Amylose	C14H26O11	370.1475117
213	371.1528	Galactan;Amylose	C14H26O11	370.1475117
214	371.1541	Galactan;Amylose	C14H26O11	370.1475117
215	404.1365	trifluoperazine	C21H20F3N3S	403.1330029
216	404.1484	Ampicillin trihydrate	C16H25N3O7S	403.1413209
217	405.1191	Spectinomycin dihydrochloride	C14H26Cl2N2O7	404.1117066
218	405.1267	Sulfinpyrazone;Sulfoxyphenylpyrazolidine	C23H20N2O3S	404.1194632
219	405.1269	Sulfinpyrazone;Sulfoxyphenylpyrazolidine	C23H20N2O3S	404.1194632

Figure 1. Effects of orange peel powder (OPP) amendment on the mortality of second-stage juveniles (J2) of *Meloidogyne incognita in vitro* by *Bacillus velezensis* strain AP203.



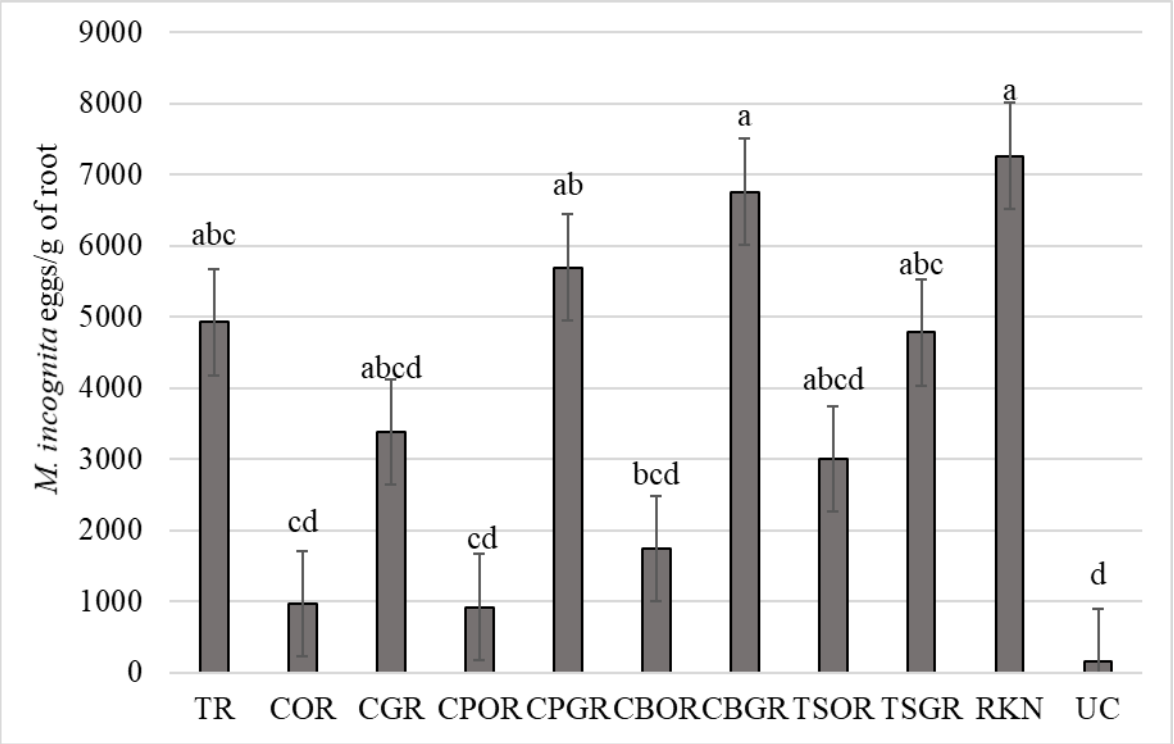
The *in vitro* test was repeated twice, and data were analyzed in SAS 9.4 using PROC GLIMMIX procedure. Means with the same letter are not significantly different at $P \leq 0.05$ level of significance.

Figure 2: Effects of culture broth, cell pellet suspension, and cell-free supernatant of *B. velezensis* strain AP203[1] on the number of *M. incognita* eggs in the roots of soybean at 45 DAP[2] in greenhouse trials.



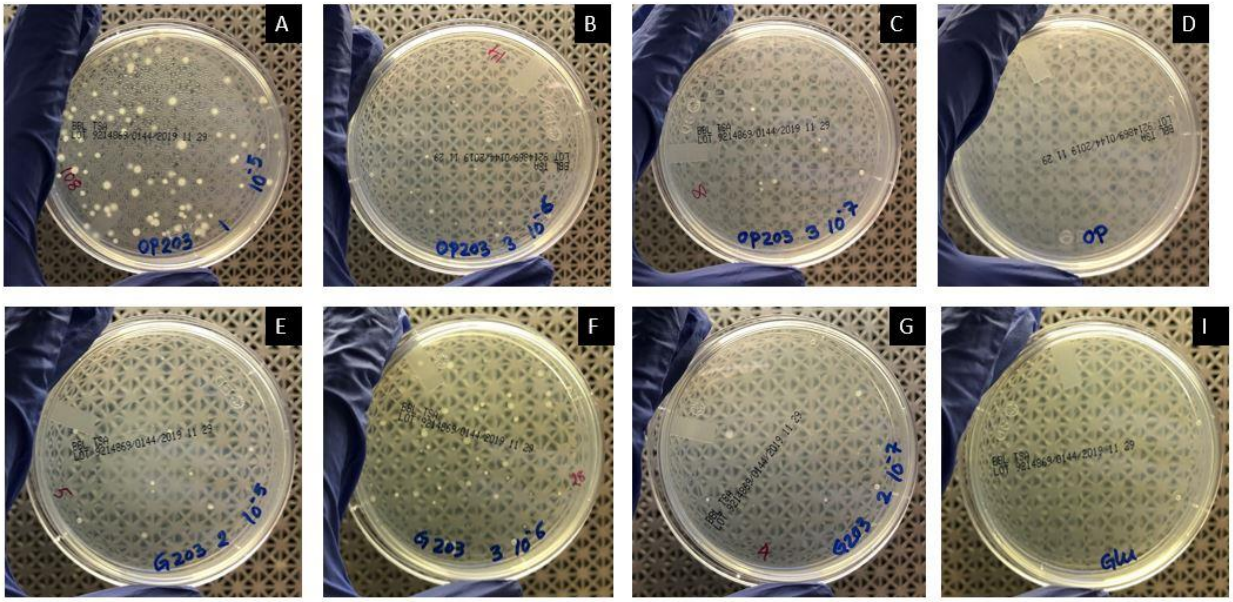
The greenhouse test was repeated twice, and data were analyzed in SAS 9.4 using PROC GLIMMIX procedure. Means with the same letter are not significantly different at $P \leq 0.05$ level of significance. 1- *B. velezensis* strain AP203 grew in 1.0% (w/v) orange peel powder (OPP) amended Tris Spizizen Salts (TSS) media and 2 – days after planting.

Figure 3. Effects of culture broth, cell pellet suspension, and cell-free supernatant of *B. velezensis* strain AP203[1] on the number of *M. incognita* eggs in the roots of cotton at 45 DAP[2] in greenhouse trials.



The greenhouse test was repeated twice, and data were analyzed in SAS 9.4 using PROC GLIMMIX procedure. Means with the same letter are not significantly different at $P \leq 0.05$ level of significance. 1- *B. velezensis* strain AP203 grew in 1.0% (w/v) orange peel powder (OPP) amended Tris Spizizen Salts (TSS) media and 2 – days after planting.

Figure 4. *B. velezensis* strains on TSA plates after diluting and transferring from supernatant of *B. velezensis* strain growing on orange peel and glucose amended TSS media.



A-C; *B. velezensis* strain AP203 grown on orange peel amended TSS minimal media (10^{-5} – 10^{-7} CFU/mL), D; orange peel, E-G; B. velezensis strain AP203 grown on glucose amended TSS minimal media (10^{-5} – 10^{-7} CFU/mL), I; Glucose.

Figure 5. The amount of *B. velezensis* colonies present on TSA plates after 24 h of growth under three different dilutions. The supernatants were diluted and transferred from *B. velezensis* strain growing on orange peel and glucose amended TSS media.

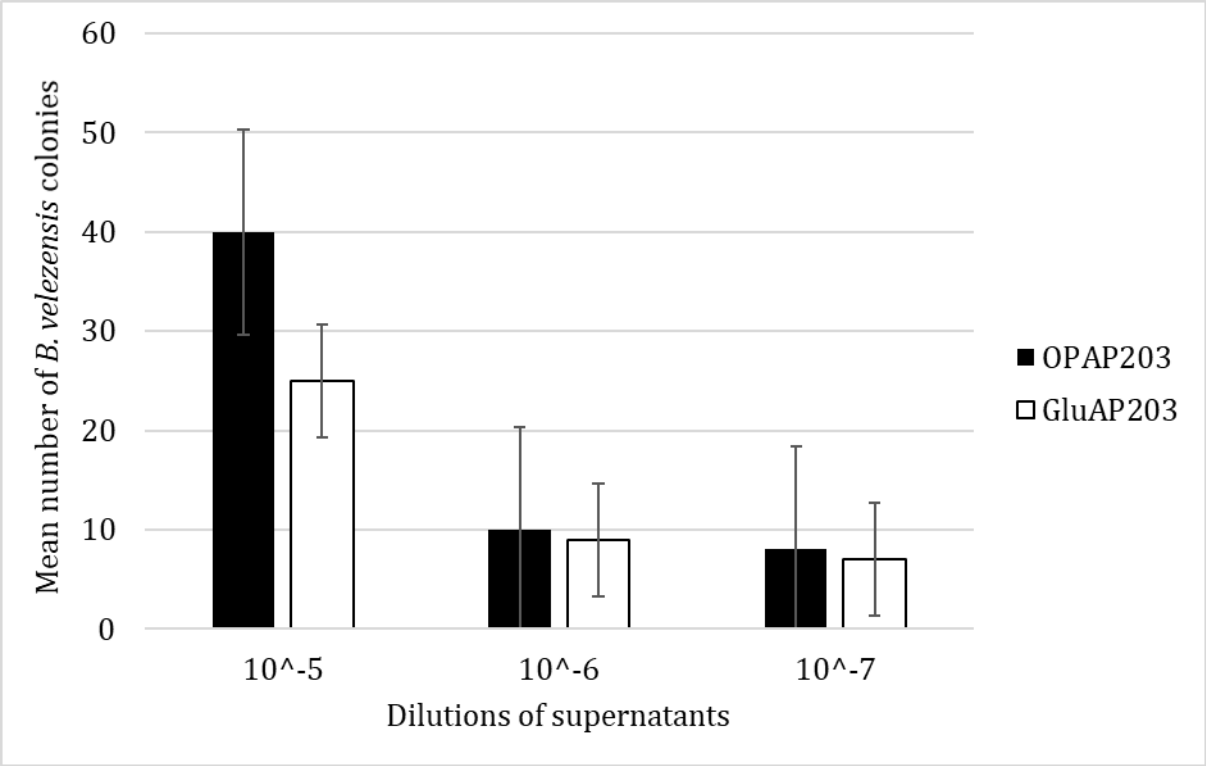


Figure 6. LC-MS/MS spectra of the peaks eluted at 6.69 min (m/z of 211.11).

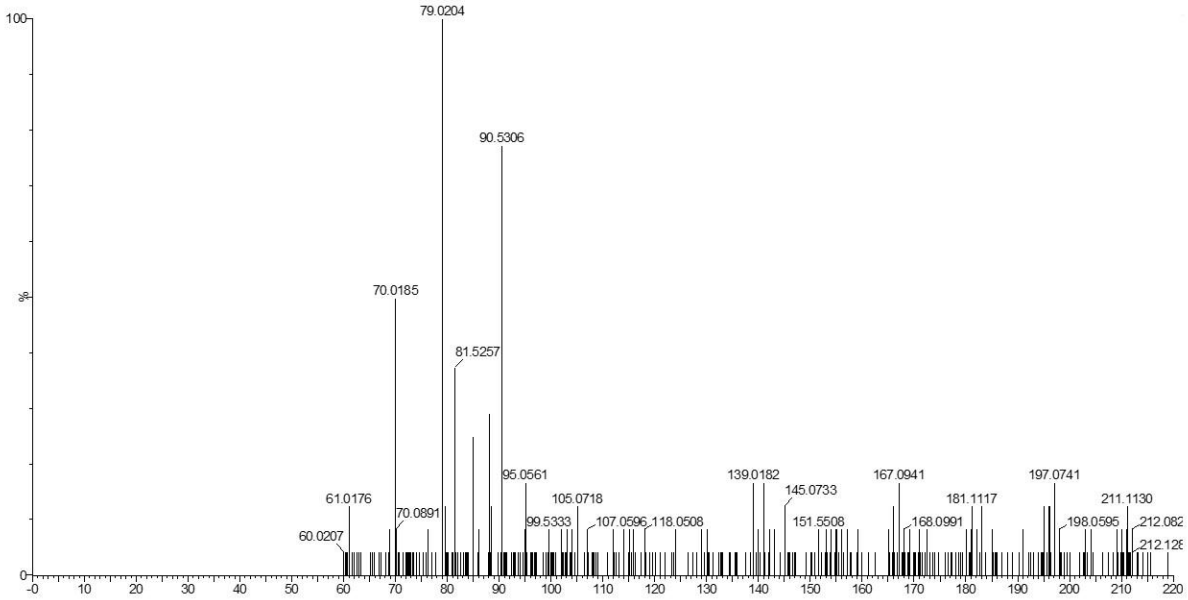


Figure 7. LC-MS/MS spectra of the peaks eluted at 2.40 min (m/z of 206.12).

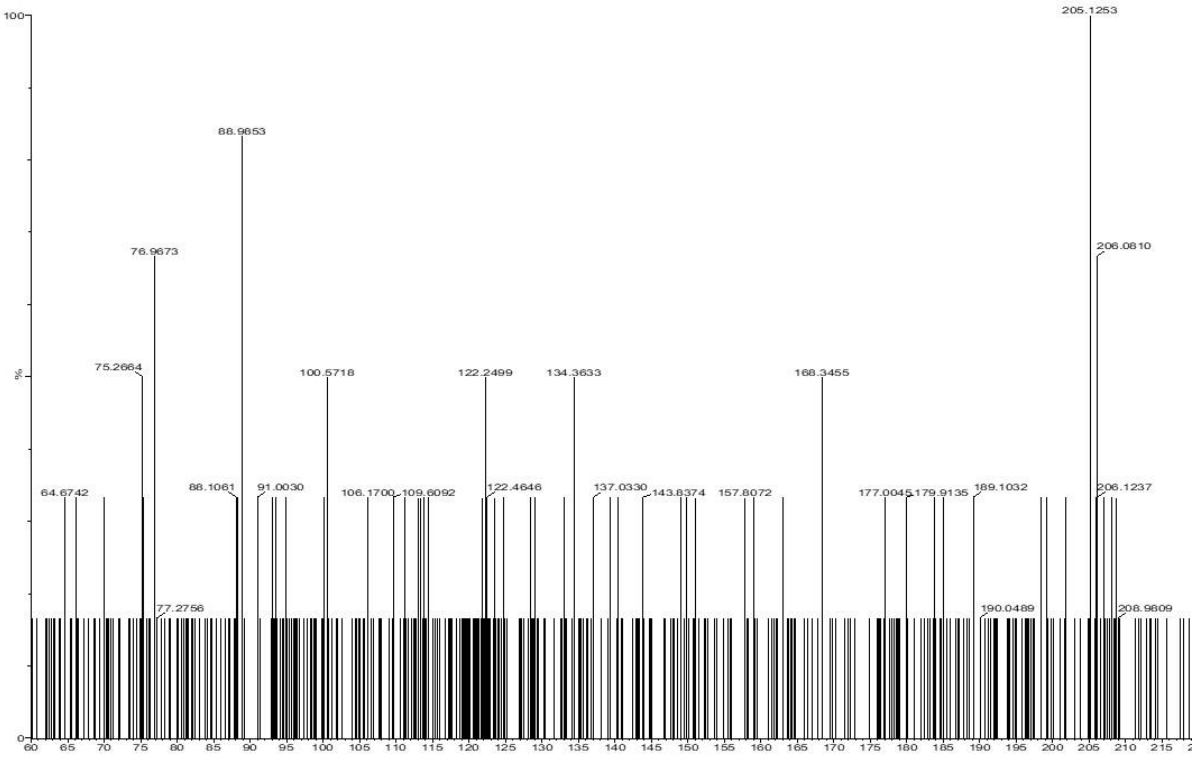
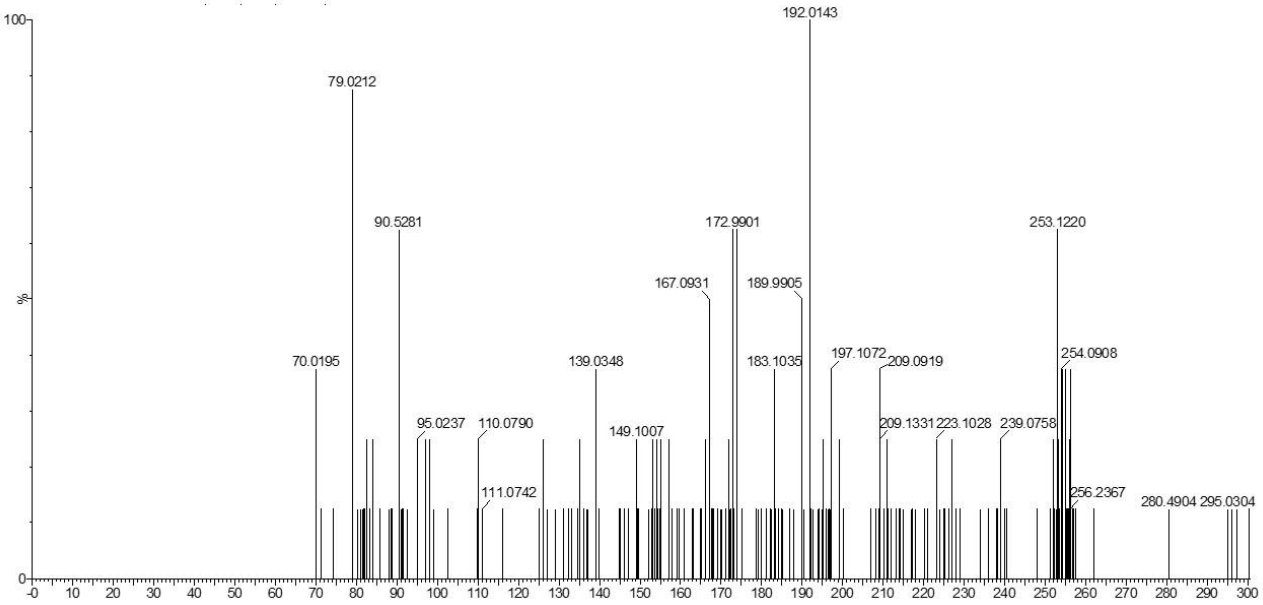


Figure 8. LC-MS/MS spectra of the peaks eluted at 6.63 min (m/z of 253.12) and 3.39 min (m/z of 295.17).



Chapter IV The effect of orange peel amendment on peanut drought stress tolerance by PGPR *Bacillus velezensis*

Abstract

Drought stress is a significant abiotic environmental factor that negatively affects crop production in the U.S. and worldwide. An investigation of the *Bacillus velezensis* plant growth-promoting rhizobacterial (PGPR) strain with orange peel amendment on the peanut genotypes x drought stress interactions could contribute to enhancing drought stress tolerance under greenhouse conditions. The objective of this study was to evaluate orange peel amendment for its capacity to increase drought stress tolerance of peanut genotypes with inoculation of *B. velezensis* PGPR strain AP203 compared to *B. velezensis* PGPR strain alone. The split plot experimental design included with four treatments (*B. velezensis* strain AP203 with or without orange peel amendment, orange peel suspension alone, and nontreated control) and ten replications. The nontreated peanut genotypes (AU 18-33, AU 18-53, GA06-G, and AU-NPL 17) planted in sandy loam field soil mixed with the potting mix at a mix ratio of 4:3 (field soil: potting mix). *B. velezensis* PGPR spores (1.0 X 10^6 CFU spores/100 µL) and orange peel suspension doses were applied separately on the peanut seeds and incubated for 24 h at room temperature. The water regimes were regular watering (RW) and reduced watering to stimulate drought (DW). At 135 days after emergence (DAE), the peanut plants with pods were removed from pots, washed, and analyzed for significant treatment effects. The morphophysiological parameters (specific leaf area, SPAD chlorophyll meter reading, chlorophyll density, and root length) of genotypes were statistically significant under drought stress tolerance conditions. Significant genotype x drought stress interactions were observed on most of the investigated parameters. Genotype AU 18-33 of *B. velezensis* strain AP203 with orange peel amendment showed significant plant dry weight under both water regimes compared to *B. velezensis* strain AP203 without orange peel amendment. This study

indicates that the co-application of orange peel with *B. velezensis* PGPR strain can enhance peanut growth and drought stress tolerance.

1. Introduction

Peanut (*Arachis hypogaea* L.) is an economically important oil-seed and food crops in the United States and many other regions around the world. It is cultivated on approximately 35.5 million ha worldwide (27). More than half of the peanut cultivation areas are located in arid and semi-arid regions, where drought is a key limiting factor for peanut production (18). Peanut production in the U.S. was 5.6 billion pounds (lbs.) in 2018 and 2019, which that was 1.5 billion pounds (lbs.) less than in 2017 (3). Drought substantially affected and reduced peanut growth and production based on the severity or duration (10). If drought persists for an extended period, significant yield losses can be incurred annually in the U.S. The selection of superior drought-tolerant plants and breeding are important approaches to ensure good production in drought conditions (29). However, drought-adaptive traits and plant selection are complicated due to trait-associated gene arrangements and extreme variability depending on year and location (4; 11). The extensive root systems (root length and distribution) in soil may enhance the plants capacity to withstand drought in the field (7). A combination of orange peel amendment and plant growth-promoting rhizobacteria (PGPR) may provide better uptake of nutrients in the soil through the root colonizing approach to enhance peanut yield and overcome drought stress conditions.

Bacillus velezensis is a root colonizing PGPR species that promotes plant growth and suppresses phytopathogens by a wide variety of mechanisms such as nitrogen fixation, phosphate solubilization, sequestration of iron by siderophores, and phytohormone production (1; 22; 34). Islam *et al.* (12) reported that *B. velezensis* strain 5113 enhanced drought stress tolerance capacity in the plant by accumulating stress metabolites during drought conditions. Da-Yeon *et al.* (19) reported that *B. velezensis* strain YP2 increased plant

growth and reduced drought stress in kale as it colonized roots. Strains of *B. velezensis* have pectin-related genes that can encode for degradation and utilization of exogenous pectin as a sole carbon and energy source (16). Wu *et al.* (35) and Solecka *et al.* (28) reported that two pectin-associated enzymes, pectin methylesterase and polygalacturonase involved in temperature-dependent modifications and increased drought stress tolerance capacity in plants.

Treuer *et al.* (31) reported that the agricultural waste orange peel enhanced forest vegetation and soil nutrients in Costa Rica. Manneh *et al.* (21) conducted a greenhouse test and concluded that orange peel amendments significantly reduced root-knot nematodes in tomato roots. A recent study also found that orange peel-derived pectin and carbohydrate increased water holding capacity of plant roots *in vitro* during drought conditions (9). While orange peel amendments have been examined as biofertilizers (17) and biological control of plant pathogens (32), there has been no report on the drought tolerance activity of orange peel. The overall goal of this greenhouse experiment was to test orange peel powder amendment for its potential to increase drought stress tolerance of peanut with inoculation of *B. velezensis* PGPR strain AP203. The two specific objectives were i. to evaluate chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) under drought stress conditions in peanut genotype by *B. velezensis* strain AP203 with orange peel amendment and ii. to evaluate the effect of orange peel amendment together with *B. velezensis* strain AP203 on growth of peanut genotypes under drought stress conditions.

2. Materials and methods

2.1. *Bacillus velezensis* PGPR strain preparation

B. velezensis PGPR strain AP203 was maintained in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) as a stock culture supplemented with 30% glycerol at -80 °C. The PGPR strain was streaked onto tryptic soy agar (TSA) from cryostocks and incubated

at 28 °C for 24 h. A single colony was transferred onto the spore preparation medium (37) and incubated for seven days at 28 °C. The 20 mL of sterilized distilled water was added to the Petri plate, and the PGPR strain was transferred to a 50 mL centrifuge tube. The PGPR strain was heat-treated for 20 mins at 80 °C in the unstirred water bath (VWR, Radnor, PA, USA) and adjusted to 1.0 X 10^7 spore colony-forming units (CFU/mL).

2.2. Orange peel suspension preparation and peanut seed inoculation

The orange peel powder was suspended in sterilized distilled water using a magnetic stirrer at a rate of 0.5 g per 10 mL water (10 mg of orange peel powder/200 µl of water) and was applied as an aqueous suspension (Citrus Extracts, Fort Pierce, FL, USA). The PGPR spore (1.0 X 10^6 CFU spores / 100 µL) and orange peel suspension were applied on the peanut seed surface separately, and seeds were then covered with 100 g of field soil. Four peanut seeds were planted into 2 cm soil depth to ensure proper germination. Two weeks after emergence, plants were thinned to two plants per pot.

2.3. Field soil preparation

Sandy loam field soil collected from the E.V. Smith Research Center (Shorter, AL, USA) and potting substrate (Sun Gro Horticulture, Agawam, MA, USA) were used for the greenhouse experiment. The pots (C 2000 3.9 gallons) (Northcoast Horticulture Supply, Inc 513 K St. Arcata, CA, USA) were filled with field soil and potting substrate (4:3; 4 buckets full of soil and three buckets full of the potting substrate).

2.4. Plant growth conditions and peanut genotypes

Four peanut genotypes (AU 18-33, AU 18-53, GA06-G, & AU-NPL 17) and one non-nodulation peanut (NNP) genotype AG55X9 were tested to evaluate the drought tolerance capacity in greenhouse conditions. The NNP was used to assess N_2 fixation in the pots with the ^{15}N nature abundance method (2). All plants were watered equally before starting the drought stress tolerance test. Four treatments (water control, orange peel, PGPR, orange peel

+ PGPR) per peanut cultivar was used for the greenhouse test. Two water regimes (well water and drought stress tolerance water) were used after starting the drought tolerance test. 70% and 30% of soil water content were maintained for well water and drought stress tolerance water treatments and repeated until the maturity of peanut pods.

2.5. SPAD chlorophyll meter readings (SCMR) and chlorophyll measurement

The SCMR and ChlD (chlorophyll content per unit leaf area) data were recorded at 30, 60, 90 days after emergence (DAE) to study the chlorophyll status of the leaf before and after starting the drought stress tolerance water application. Six plants from each treatment of peanut cultivar were randomly sampled, and the second fully expanded leaves were selected for SCMRs in the morning (08:30 – 09.30 hours) (6). Six leaflets from each leaf of the peanut plant were selected for the SCMR data collection using a Minolta SPAD-502 meter (Konica Minolta, Tokyo, Japan). An average SCMR for each treatment was obtained from 36 single observations (6 leaflets X 6 plants treatment^{-1}). Six leaflets from each plant were used for chlorophyll assessment. One leaf disc from each leaf was taken with a cork borer (1 cm^2 in diameter) and was soaked in 5 mL of N, N-dimethylformamide, and kept in the dark for 24 h before determining chlorophyll (a+b) using a light absorption with a spectrophotometer (6; 24). A 3 mL of aliquot was used to analyze the sample spectrophotometrically at 645 and 663 nanometers (nm). The concentration of chlorophyll extract (mg/cm^2) was calculated using a formula as follows (5): [a x (OD 645 nm) + b x (OD 663 nm)], where a = leaf disc weight, and b = leaf disc weight. The SCMR and ChlD data were used to analyze correlation coefficients for each peanut cultivar at 30, 60, 90 DAE.

2.6. Specific leaf area (SLA) measurement

Six fresh leaves from each treatment were collected once a week for four consecutive weeks during the drought period. The second fully expanded leaves from the top of each plant were sampled in the morning, placed into a plastic bag, and put on ice in coolers.

Afterward, the leaf area (LA) of each leaf was immediately measured using a LI-3100 area meter (LI-COR Biosciences, Lincoln, NE, USA). The leaf was then placed into a mechanical convection oven at 70 °C for 48 h to obtain the dry leaf mass (DW). SLA was calculated using the ratio of leaf area to dry leaf mass (LA/DW) (10).

2.7. Relative water content (RWC) and soil moisture measurement

Six fully expanded leaves from the top of the main stem of six plants were sampled to determine the RWC at 30, 60, 90 DAE. The leaf sample was immersed in distilled water at room temperature under the dark condition for 8 h. After imbibition, the leaf surface was dried with absorbent paper and measured turgid weight. The leaf sample was then oven-dried at 70°C for 48 h. The formula was used to calculate RWC as follows (6; 30): (RWC) (%) = (FW-DW)/ (TW-DW) X 100, where FW – fresh leaf weight, TW – turgid leaf weight, and DW – dry leaf weight. The pots were regularly watered to maintain their weight for their required water treatment standards, and the amount of water used for each pot was documented in a Microsoft Excel spreadsheet. The pots were weighed with a scale (OHAUS Corporation, Parsippany, NJ, USA) to ensure they were properly watered. Before and after watering weights were obtained, which were used to obtain a difference between the 2 numbers for transpiration efficiency data analysis.

2.8. Photosynthetic measurement

Li-Cor 6400 portable photosynthetic system (Li-Cor Biosciences, Lincoln, NE, USA) was used for the midday photosynthetic measurements (net photosynthetic rate and stomatal conductance) at 15 and 30 days after drought stress tolerance experiment initiated. Three fully expanded young leaves from each treatment were sampled randomly to measure net photosynthetic rate (Pn, $\mu molCO_2 \cdot m^{-2} \cdot s^{-1}$) and stomatal conductance (Gs, $molH_2O \cdot m^{-2} \cdot s^{-1}$). Instantaneous water use efficiency (WUEi) ($\mu molCO_2/\mu molH_2O$) was calculated as follows (15): net photosynthetic rate/transpiration rate (stomatal conductance).

2.9. Transpiration efficiency

The amount of water used per pot was documented to account for transpiration efficiency (TE), also known as water use efficiency (WUE). The transpiration efficiency (TE) was calculated based on the formula suggested by Vadez *et al*., (2014) as follows (33): total biomass/evapotranspiration (water received).

2.10. Nitrogen concentration analysis

The isotopes of each plant were analyzed to measure N_2 fixation in the plants. First, all the biomass, including stems, roots, leaves, and pods, was ground together. Then the samples were ground using a UDY Corporation Model 3010-030 grinder (UDY Corporation, Fort Collins, CO, USA) to reduce particles each to 1 mm in size. The samples were then taken to a Sartorius Microbalance (Southern Balance Calibrations, Inc., Braselton, GA, USA) to weight out 2.9-3.4 mg samples into 5X9 mm tin capsules (Costech Analytical Technologies Inc., Valencia, CA, USA) and sent to the University of California-Davis Stable Isotope Facility to conduct the isotope analysis, specifically for dual ^{13}C and ^{15}N natural abundance.

2.11. Peanut plant growth measurement

At 135 DAE, mature peanut plants were harvested. Plants were washed, and plant growth parameters were assessed. The peanut pod numbers were recorded for each plant. To assess dry matter per treatment, the peanut plants and pods were dried in a mechanical convection oven at 70 °C for 48 h.

2.12. Statistical analysis

In the greenhouse test, the pots were arranged in a split-plot design with four treatments per peanut cultivar and ten replications per treatment. The data were analyzed by analysis of variance, and the treatment means were compared by using Tukey's multiple

range test at the 5% level of significance in R statistical package software (RStudio, Inc., Boston, MA, USA).

3. Results

3.1. Plant water status

Relative water content (RWC) of *B. velezensis* strain AP203 with orange peel amendment was >90% at 30, 60, and 90 DAE under regular water and drought stress tolerance conditions (Tables 12 and 13). There were no significant treatment differences of *B. velezensis* strain AP203 with orange peel amendment compared to *B. velezensis* strain AP203 without orange peel amendment at 30, 60, and 90 DAE under either water regime.

3.2. Specific leaf area

Among the peanut genotypes, GA06-G and AU 18-53 showed the highest specific leaf area (SLA) in the second week under both water regimes (regular water and drought stress tolerance) (Tables 3 and 4). The SLA of *B. velezensis* strain AP203 with orange peel amendment of peanut genotype AU-NPL 17 was statistically significant in the first and third week under drought tolerance conditions compared to *B. velezensis* strain AP203 without orange peel amendment, orange peel alone, and control treatments (Figures 1 and 2).

3.3. SPAD chlorophyll meter readings and chlorophyll density

SPAD chlorophyll meter reading (SCMR) of *B. velezensis* strain AP203 with orange peel amendment of peanut genotypes (AU 18-33 and AU 18-53) were statistically greater at 30 DAE compared to *B. velezensis* strain AP203 without orange peel amendment under regular water and drought stress tolerance conditions (Figures 4 and 5). The SCMR of *B. velezensis* strain AP203 with orange peel amendment of peanut genotype AU 18-53 showed a significant increase at 90 DAE under drought stress tolerance conditions (Figure 8). However, the SCMR values of the other three genotypes were not significantly different at 90 DAE under drought stress tolerance conditions (Table 9).

At 90 DAE, the chlorophyll density (ChlD) of *B. velezensis* strain AP203 with orange peel amendment treatments of the three peanut genotypes (AU 18-33, GA06-G, and AU-NPL 17) were significantly increased compared to the *B. velezensis* strain AP203 without orange peel amendment under drought tolerance conditions (Figures 11, 14, and 15). The average ChlD of peanut genotypes (AU 18-33, GA06-G, and AU-NPL 17) were 6.55, 9.97, and 8.17 µg cm^{-2} (Table 11). There was a positive correlation between SCMR and ChlD at 30, 60, and 90 DAE under regular water and drought stress tolerance conditions (Figure 15-26).

3.4. Photosynthetic measurement

The photosynthetic rate, stomatal conductance, and instantaneous water use efficiency (WUEi) of drought-stressed plants were decreased compared to the regularly watered plants at 75 DAE (Table 6 and 7). The photosynthetic rate of *B. velezensis* strain AP203 with orange peel amendment treatments of the four peanut genotypes (AU 18-33, AU 18-53, GA06-G, and AU-NPL 17) were 7.65, 8.11, 10.98, and 6.94 µmol m^{-1} s^{-1} under drought stress tolerance conditions (Table 7). The stomatal conductance of *B. velezensis* strain AP203 with orange peel amendment treatments of the four peanut genotypes (AU 18-33, AU 18-53, GA06-G, and AU-NPL 17) were 0.06, 0.07, 0.15, and 0.07 mmol m^{-1} s^{-1} (Table 7). The WUEi values of *B. velezensis* strain AP203 with orange peel amended treatments of the four peanut genotypes (AU 18-33, AU 18-53, GA06-G, and AU-NPL 17) were 99.76, 97.66, 75.66, and 96.66 µmol CO_2 mmol H_2O m^{-1} s^{-1} (Table 7).

3.5. Transpiration efficiency

There were no significant differences among peanut genotypes for transpiration efficiency (TE) in *B. velezensis* strain AP203 with orange peel amendment compared to *B. velezensis* strain AP203 without orange peel amendment, orange peel alone, and control treatments under drought stress tolerance conditions (Table 5).

3.6. Shoot and root length

The average shoot and root lengths from peanut genotypes are shown in Table 1. *B. velezensis* strain AP203 with orange peel amended treatments of the three peanut genotypes (AU 18-33, AU 18-53, and AU-NPL 17) enhanced root growth under drought stress tolerance conditions (Figures 25, 26, and 28). The shoot length of the three peanut genotypes (AU 18-33, GA06-G, and AU-NPL 17) did not significantly increase compared to the *B. velezensis* strain AP203 without orange peel amendment, orange peel alone, and control treatments under drought stress tolerance conditions (Table 2). Also, the number of pods per plant did not significantly increase by *B. velezensis* strain AP203 with orange peel amendment under regular water and drought stress tolerance conditions (Tables 1 and 2).

3.7. Total plant dry matter

In the greenhouse experiment, a significant effect of *B. velezensis* strain AP203 with an orange peel amendment was observed on the plant dry weight of the peanut genotype AU 18-33 under regular water and drought tolerance conditions (Tables 1 and 2). The plant dry weight of *B. velezensis* strain AP203 with orange peel amended treatments of the peanut genotype AU 18-33 was 27.92 g under regular water conditions and 28.42 g under drought stress tolerance conditions.

4. Discussion

Drought stress is a major abiotic environmental factor that causes significant yield loss of diverse agronomic crops, including peanut. In this study, drought stress significantly decreased the pod and plant dry weight in a greenhouse trial. Previous studies reported that pod and plant weight decreased due to intermittent drought stress conditions (8; 13). Our results showed that the number of pods per plant and pod dry weight of peanut genotypes (GA06-G and AU-NPL 17) reduced under drought stress conditions (Table 2). *B. velezensis* PGPR strain AP203 with orange peel amendment showed significant growth effects on the

peanut genotypes under both regular and drought water regimes. For example, *B. velezensis* strain AP203 with orange peel amendment of peanut genotype GA06-G significantly increased root growth under both water regimes compared to *B. velezensis* strain AP203 without orange peel amendment, although the root growth was reduced under drought stress compared to regular water (Tables 1 and 2). Previous studies showed that *B. velezensis* strain BAC03 produced 1-aminocyclopropane-1-carboxylate (ACC) deaminase and volatile organic compounds under drought stress and enhanced plant growth (14; 23). Wu *et al.* (36) reported that *B. amyloliquefaciens* strain SQY 162 increased surfactin secondary metabolite production in the presence of pectin carbohydrate and significantly reduced bacterial tobacco wilt caused by *Ralstonia solanacearum*. Thus, the degradation and utilization of orange peel-mediated pectin and other phenolic compounds by PGPR could contribute to enhancing drought stress tolerance and plant growth. The genotypic variations by *B. velezensis* PGPR strain AP203 with orange peel amendment observed in this study revealed that GA06-G was high pod yielding genotype under regular water (Table 1).

The genotypes differed significantly for SCMR, ChlD, and SLA. At 90 DAE, SCMR, ChlD, and SLA values of genotype AU 18-53 showed a significant increase under drought stress compared to the same genotype under regular water conditions (Tables 4, 9, and 11). For the first data collection at 30 DAE, SCMR and ChlD of treated drought stress plants were not different compared to the regularly watered plants. However, SCMR, ChlD, and SLA showed similar trends under both water regimes. SCMR and ChlD recorded at three different times (30, 60, and 90 DAE) showed a positive correlation with each other (Figures 15-26). From the morphophysiological trait results, it is evident that SCMR, ChlD, and SLA observations were consistent with previous reports and can be recorded at any time after 60 DAE (25). RWCs of *B. velezensis* strain AP203 with orange peel amendment of four genotypes ranged from 85% to 95% compared to the control and orange peel treatments

under drought stress conditions (Table 13). At 90 DAE, RWCs for *B. velezensis* strain AP203 with orange peel amendment showed no significant differences compared to the *B. velezensis* strain AP203 without orange peel amendment under drought stress conditions. In our study, a significant genotype x treatment interaction was found and consistent under drought stress conditions for TE at 90 DAE. TE for drought stress was significantly higher compared to the regular water conditions (Table 5). Puangbut et al. (26) reported that root architecture is responsible for retaining water for transpiration under drought conditions. Under drought stress conditions, the lowest water input justified the effects on stomatal conductance and net photosynthetic rate (Table 7). The water stress effects on the net photosynthetic rate are connected to WUEi that is further associated with stomatal conductance (20).

In conclusion, *B. velezensis* strain AP203 with orange peel amendment responded differently to the peanut genotypes under both water regimes. These various responses of the genotypes could have triggered by the production of orange peel-mediated secondary metabolites by *B. velezensis* strain AP203 in soil. Further studies are needed to evaluate best performing genotype as highly drought-tolerant using a different doses of orange peel suspension with *B. velezensis* strain AP203 in different field locations.

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Table 1. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on peanut genotype growth (shoot length, root length, and the number of pods per plant) and total dry matter (plant and pod dry weight) under regular water conditions.

Treatment	Shoot Length (cm)	Root length (cm)	pod number/plant	Plant dry weight (g)	Pod dry weight (mg)
Control 33	52.10a	23.10a	6.50a	14.94a	12.25a
OP 33	44.70a	25.40a	9.10a	23.23b	18.70bc
AP203 33	54ab	41.80b	5.60a	21.14ab	16.95ab
OPAP203 33	64b	40.70b	7.50a	27.92c	23.59c
Control 53	51.80a	20.80a	4.60a	15.85a	12.75a
OP 53	69a	27.20ab	9.50b	26.52b	20.06b
AP203 53	64.80a	32.70bc	10b	26.11b	18.98ab
OPAP203 53	63.10a	38.20c	10.50b	27.20b	20.16b
Control G	60.4a	29.50a	7.50a	15.27a	15.30a
OP G	56.10a	31.40a	10.20b	26.05b	19.38ab
AP203 G	63.20a	29.50a	11.60ab	25.55b	22.45ab
OPAP203 G	65.10a	38.50b	15b	32.18c	25.40b
Control 17	51.80a	16a	6.60a	17.14a	14.75a
OP 17	57.50a	19.10a	12.30bc	26.24b	24.42b
AP203 17	52.30a	18.50a	9.120ab	23.29ab	20.76ab
OPAP203 17	60.80a	35.60b	15.10c	29.15b	22.52b

OP: orange peel; 33, 53, G, and 17: peanut genotype; and AP203= *B. velezensis* PGPR strain. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 2. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on peanut genotype growth (shoot length, root length, and the number of pods per plant) and total dry matter (plant and pod dry weight) under drought stress tolerance conditions.

Treatment	Shoot Length (cm)	Root length (cm)	pod number/plant	Plant dry weight (g)	Pod dry weight (mg)
Control 33dt	52.30a	22.50a	6.10a	14.68a	13.14a
OP 33dt	47.72a	18.90a	6.81a	21.95b	17.88ab
AP203 33dt	51.90a	21a	8.10a	22.36b	19.66b
OPAP203 33dt	53.75a	28.87b	8.87a	28.42c	20.66b
Control 53dt	52.40a	20.80a	7.80a	16.48a	15.17a
OP 53dt	52.90a	25.90a	12.20a	26.52b	21.64a
AP203 53dt	56.80a	26.70a	11.50a	26.11b	19.66a
OPAP203 53dt	55.20a	33.60b	10.40a	27.20b	21.93a
Control Gdt	47.30a	23.40a	6.90a	15.94a	15.11a
OP Gdt	53.60b	24.70a	12.30b	25.44a	22.92b
AP203 Gdt	46.40a	34.40b	14.40b	24.07b	19.94ab
OPAP203 Gdt	53.40a	25.51a	9.60ab	23.32b	21.94b
Control 17dt	43.20a	23.60a	7a	14.06a	15.74a
OP 17dt	43.22a	20.22a	12.44b	25.44b	19.53ab
AP203 17dt	42.60a	21.30a	12.20b	23.32b	23.33b
OPAP203 17dt	46.41a	32.25b	11.25ab	24.07b	20.56ab

OP: orange peel; 33, 53, G, and 17: peanut genotype; dt=drought tolerance; and AP203= *B. velezensis.* Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 3. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on specific leaf area (SLA) of peanut genotype under regular water conditions.

Treatment	SLA – 1st week	SLA – 2nd week	SLA – 3rd week	SLA – 4th week
Control 33	176.13a	265.67a	196.11a	206.66a
OP 33	335.27ab	385.22ab	197.88a	192.94a
AP203 33	322.19ab	625.80b	344.30a	302.30a
OPAP203 33	589.88b	487.02ab	409.58a	262.47a
Control 53	187.52a	289.70a	211.63a	213.82ab
OP 53	461.97a	413.47ab	368.38a	166.77a
AP203 53	469.83a	730.83b	372.94a	295.41ab
OPAP203 53	447.61a	516.16ab	393.75a	375.63b
Control G	207.36a	251.31a	240.08a	190.05a
OP G	258.97a	206.49a	367.55a	344.20ab
AP203 G	460.16ab	662b	420.86a	461.31ab
OPAP203 G	647.80b	703.25b	388.66a	583.75b
Control 17	219.94a	262.72a	197.61a	158.13a
OP 17	310.70ab	330.04a	291.97ab	346.74a
AP203 17	327.97ab	469.58ab	421.08ab	234.15a
OPAP203 17	577.70b	600.80b	542.33b	351.05a

OP: orange peel; 33, 53, G, and 17: peanut genotype; and AP203= *B. velezensis* PGPR strain. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 4. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on specific leaf area (SLA) of peanut genotype under drought stress tolerance conditions.

Treatment	SLA – 1st week	SLA – 2nd week	SLA – 3rd week	SLA – 4th week
Control 33dt	194.02a	220.67a	262.27a	244.86a
OP 33dt	430.16ab	327.58ab	365.74ab	171.31a
AP203 33dt	507.80ab	460.49ab	385.52ab	538.88b
OPAP203 33dt	651.80b	602.19b	589.33b	551.52b
Control 53dt	235.95a	255.81a	146.69a	180.30a
OP 53dt	500.11ab	406.40ab	387.97ab	364.52ab
AP203 53dt	506.86ab	577.08ab	382.91ab	291.11ab
OPAP203 53dt	619.27b	853.55b	597.47b	478.09b
Control Gdt	226.70a	267.76a	234.99a	328.22a
OP Gdt	408.99ab	270.58a	246.77a	311.94a
AP203 Gdt	606.22ab	493.76ab	381.97ab	257.13a
OPAP203 Gdt	730.69b	712.91b	559.72b	706.63b
Control 17dt	207.36a	177.86a	143.25a	213.99a
OP 17dt	221.44a	229.41a	207.88ab	310.19ab
AP203 17dt	310.58a	601.36b	330.91b	567.27b
OPAP203 17dt	515.66b	474.08ab	509.08c	388.44ab

OP: orange peel; 33, 53, G, and 17: peanut genotype; dt=drought tolerance; and AP203= *B. velezensis*. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 5. Transpiration efficiency (TE) of peanut genotype under drought stress tolerance conditions at 90 DAE in a greenhouse trial.

Treatment	TE (g kg^{-1})	
	RW - 90 DAE	DT - 90 DAE
Control 33	3.4a	3.56a
OP 33	2.89b	2.15b
AP203 33	2.52b	2.24b
OPAP203 33	2.31bc	2.12b
Control 53	3.15a	3.35a
OP 53	2.54b	2.25b
AP203 53	2.12b	2.42b
OPAP203 53	1.49c	2.85b
Control G	3.21a	3.24a
OP G	1.25c	2.14b
AP203 G	1.02c	2.78b
OPAP203 G	2.15b	2.45b
Control 17	3.15a	3.23a
OP 17	2.15b	2.58b
AP203 17	2.11b	2.14b
OPAP203 17	1.54c	2.24b

OP: orange peel; 33, 53, G, and 17: peanut genotype; TE: transpiration efficiency, RW: regular water; DT: drought tolerance; and AP203= *B. velezensis* PGPR strain. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 6. Photosynthesis, stomatal conductance, and instantaneous water use efficiency (WUEi) of peanut genotype under regular water conditions.

Treatment	75 days after drought tolerance			90 days after drought tolerance		
	Photosynthesis (µmol m^{-1} s^{-1})	Stomatal conductance (mmol m^{-1} s^{-1})	WUEi (µmol CO_2 mmol H_2O m^{-1} s^{-1})	Photosynthesis (µmol m^{-1} s^{-1})	Stomatal conductance (mmol m^{-1} s^{-1})	WUEi (µmol CO_2 mmol H_2O m^{-1} s^{-1})
Control 33	21a	0.31a	77.46a	17.70a	0.25a	86.53a
OP 33	18.73a	0.29a	67.34a	14.67a	0.28a	95.83a
AP203 33	21.83a	0.41a	56.86a	11.92a	0.25a	96.37a
OPAP203 33	14.30a	0.17a	94.44a	20.80a	0.47a	96.89a
Control 53	13.70a	0.11a	40.22a	20.13a	0.34a	68.85a
OP 53	17.10a	0.22a	61.91b	13.55a	0.18a	66.72a
AP203 53	19.43a	0.32a	95.47b	15.40a	0.29a	94.56b
OPAP203 53	15.73a	0.16a	97.66b	18.32a	0.31a	99.65b
Control G	17.43a	0.16a	55.17a	12.44a	0.12a	69.14a
OP G	17.80a	0.24a	91.80b	12.57a	0.13a	80.03a
AP203 G	17.96a	0.13a	97.05b	13.94a	0.22a	90.25b
OPAP203 G	15.33a	0.16a	98.86b	16.20a	0.33a	91.26b
Control 17	12.55a	0.14a	56.45a	13.69a	0.22a	43.59a
OP 17	11.66a	0.19a	85.56b	15.44a	0.20a	64.67b
AP203 17	13.99a	0.17a	90.22b	11.28a	0.14a	88.01c
OPAP203 17	15.41a	0.15a	96.12b	11.75a	0.26a	78.34bc

OP: orange peel; 33, 53, G, and 17: peanut genotype; dt=drought tolerance; and AP203= *B. velezensis*. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 7. Photosynthesis, stomatal conductance, and instantaneous water use efficiency (WUEi) of peanut genotype under drought stress tolerance conditions.

Treatment	75 days after drought tolerance Photosynthesis (µmol m^{-1} s^{-1})	75 days after drought tolerance Stomatal conductance (mmol m^{-1} s^{-1})	75 days after drought tolerance WUEi (µmol CO_2 mmol H_2O m^{-1} s^{-1})	90 days after drought tolerance Photosynthesis (µmol m^{-1} s^{-1})	90 days after drought tolerance Stomatal conductance (mmol m^{-1} s^{-1})	90 days after drought tolerance WUEi (µmol CO_2 mmol H_2O m^{-1} s^{-1})
Control 33dt	5.11a	0.04a	59.64a	16.20a	0.45a	36.43a
OP 33dt	3.42a	0.05a	65.44a	15.50a	0.58a	28.44a
AP203 33dt	2.08a	0.02a	74.88b	16.03a	0.75a	22.7a
OPAP203 33dt	7.65a	0.06a	99.76b	17.63a	0.56a	33.17a
Control 53dt	6.37a	0.05a	53.12b	18.53a	0.49a	40.57a
OP 53dt	6.79a	0.10a	74c	21.50a	0.72a	31.75a
AP203 53dt	8.14a	0.09a	62.33b	15.06a	0.43a	35.18a
OPAP203 53dt	8.11a	0.07a	97.66a	19.36a	0.41a	51.5b
Control Gdt	6.90a	0.08a	60.21a	16.26a	0.34a	49.49b
OP Gdt	6.78a	0.07a	65.32a	18.43a	0.41a	57.64b
AP203 Gdt	10.15a	0.11a	91.33c	23.96a	0.65a	36.56a
OPAP203 Gdt	10.98a	0.15a	75.66b	17.80a	0.38a	51.24b
Control 17dt	8.57a	0.12a	61.33a	13.69a	0.38a	61.76b
OP 17dt	11.08a	0.12a	62.66a	15.44a	0.50a	30.03a
AP203 17dt	7.68a	0.09a	84.33b	17.46a	0.48a	75.12c
OPAP203 17dt	6.94a	0.07a	96.66c	13.69a	0.29a	81.85c

OP: orange peel; 33, 53, G, and 17: peanut genotype; dt=drought tolerance; and AP203= *B. velezensis* PGPR strain. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 8. SPAD chlorophyll meter readings (SCMR) of peanut genotype under regular water conditions.

Treatment	SCMR - 30 DAE	SCMR - 60 DAE	SCMR - 90DAE
Control 33	30.96a	34.93a	33.58a
OP 33	35.90a	39.88ab	34.16ab
AP203 33	41.58b	43.53b	37.03ab
OPAP203 33	47.66c	46.90b	39.78b
Control 53	33.93a	28.28a	30.90a
OP 53	37.25a	43.83b	31.70a
AP203 53	37.61a	44.21b	39.08b
OPAP203 53	49.81b	48.61b	43.28b
Control G	34.83a	29.85a	36.73a
OP G	33.01a	35.55ab	46.30b
AP203 G	42.41b	39.08b	41.15ab
OPAP203 G	47.53b	45.43c	39.21a
Control 17	31.38a	32.68a	26.71a
OP 17	32.38ab	30.26a	39.05b
AP203 17	38.48bc	47.33b	40.10b
OPAP203 17	40.58c	46.93b	45.53b

OP: orange peel; 33, 53, G, and 17: peanut genotype; DAE: days after emergence; and AP203= *B. velezensis* PGPR strain. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 9. SPAD chlorophyll meter readings (SCMR) of peanut genotype under drought stress tolerance conditions.

Treatment	SCMR - 30 DAE	SCMR - 60 DAE	SCMR - 90DAE
Control 33dt	30.46a	33.53a	34.11a
OP 33dt	35.96a	34.33a	39.43ab
AP203 33dt	41.98b	43.36b	40.96ab
OPAP203 33dt	47.33c	45.80b	44.96b
Control 53dt	32.91a	36.40a	32.81a
OP 53dt	36.60ab	32.46a	36.31a
AP203 53dt	39.95b	45.53b	35.73a
OPAP203 53dt	50.26c	45.63b	45.93b
Control Gdt	33.90a	38.25b	36.11a
OP Gdt	32.66a	29.86a	46.96b
AP203 Gdt	42.66b	44.68c	42.28b
OPAP203 Gdt	47.93c	46.05c	45.63b
Control 17dt	30.90a	42.08b	29.70a
OP 17dt	32.16a	28.73a	41.48b
AP203 17dt	38.55b	44.83b	44.65b
OPAP203 17dt	41.36b	47b	45.51b

OP: orange peel; 33, 53, G, and 17: peanut genotype; dt=drought tolerance; DAE: days after emergence; and AP203= *B. velezensis*. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 10. Chlorophyll density (ChlD) of peanut genotype under regular water conditions.

Treatment	ChlD - 30 DAE	ChlD - 60 DAE	ChlD - 90DAE
Control 33	11.18a	9.57a	4.04a
OP 33	13.71a	8.22a	5.11b
AP203 33	20.89b	18.09b	6.17c
OPAP203 33	22.80b	19.37b	6.60c
Control 53	9.71a	9.10a	3.87a
OP 53	14.72b	8.25a	4.62ab
AP203 53	19.31c	18.78b	5.24b
OPAP203 53	22.74c	18.64b	6.98c
Control G	7.45a	7.75a	2.53a
OP G	14.41b	9.11ab	3.58ab
AP203 G	16.93b	14.81bc	4.22b
OPAP203 G	21.80c	20.47c	7.08c
Control 17	5.98a	7.40a	2.86a
OP 17	10.47b	9.97a	4.45b
AP203 17	17.22c	14.26b	5.56c
OPAP203 17	20.21c	18.97c	7.10d

OP: orange peel; 33, 53, G, and 17: peanut genotype; DAE: days after emergence; and AP203= *B. velezensis* PGPR strain. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 11. Chlorophyll density (ChlD) of peanut genotype under drought stress tolerance conditions.

Treatment	ChlD - 30 DAE	ChlD - 60 DAE	ChlD - 90DAE
Control 33dt	9.72a	13.88b	4.16a
OP 33dt	13.71b	10.12a	4.44a
AP203 33dt	20.89c	21.68c	5.12a
OPAP203 33dt	22.80c	20.01c	6.55b
Control 53dt	8.50a	13.92a	4.97a
OP 53dt	14.72b	10.06a	4.47a
AP203 53dt	19.31c	20.94b	7.98b
OPAP203 53dt	22.74c	19.64b	7.24b
Control Gdt	6.58a	9.70a	4.78a
OP Gdt	14.41b	9.50a	5.93a
AP203 Gdt	16.93b	19.64b	6.04a
OPAP203 Gdt	21.80c	19.97b	9.97b
Control 17dt	5.20a	11.75a	4.41a
OP 17dt	10.17b	13.21ab	5.48b
AP203 17dt	17.22c	18.06bc	7.23c
OPAP203 17dt	20.21c	20.05c	8.17d

OP: orange peel; 33, 53, G, and 17: peanut genotype; dt=drought tolerance; DAE: days after emergence; and AP203= *B. velezensis*. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 12. Relative water content (RWC) of peanut genotype under regular water conditions.

Treatment	RWC - 30 DAE	RWC - 60 DAE	RWC - 90DAE
Control 33	81.92a	71.01a	74.08a
OP 33	74.93a	70.67a	81.62ab
AP203 33	85.29ab	93.40a	88.35ab
OPAP203 33	95.07b	94.95a	95.59b
Control 53	79.51a	66.48a	69.99a
OP 53	73.04a	76.19ab	87.57b
AP203 53	85.80a	91.80b	97.68b
OPAP203 53	90.99a	97.68b	97.03b
Control G	77.54a	59.21a	70.43a
OP G	84.68a	79.79ab	88.96b
AP203 G	85.29a	91.52b	95.77b
OPAP203 G	89.96a	95.08b	95.95b
Control 17	67.22a	74.25a	94.79a
OP 17	83.55b	72.72a	86.09a
AP203 17	85.83b	92.33a	90.44a
OPAP203 17	93.24b	92.42a	97.35a

OP: orange peel; 33, 53, G, and 17: peanut genotype; DAE: days after emergence; and AP203= *B. velezensis* PGPR strain. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Table 13. Relative water content (RWC) of peanut genotype under drought stress tolerance conditions.

Treatment	RWC - 30 DAE	RWC - 60 DAE	RWC - 90DAE
Control 33dt	79.86ab	53.61a	68.73a
OP 33dt	76.98a	69.60ab	87.63b
AP203 33dt	85.60ab	84.95b	91.10b
OPAP203 33dt	94.79b	92.34b	93.66b
Control 53dt	81.85a	57.80a	74.70a
OP 53dt	66.10a	75.12ab	89.86ab
AP203 53dt	83.86a	91.31b	95.76b
OPAP203 53dt	91.42a	91.53b	97.05b
Control Gdt	80.32a	68.74a	81.76a
OP Gdt	76.01a	85.72ab	88.19a
AP203 Gdt	88.59a	93.39b	95.38a
OPAP203 Gdt	92.43a	93.03b	92.84a
Control 17dt	51.11a	76.08a	77.55a
OP 17dt	82.92b	80.14a	91.19ab
AP203 17dt	92.10b	95.41a	95.95b
OPAP203 17dt	91.92b	97.91a	91.19ab

OP: orange peel; 33, 53, G, and 17: peanut genotype; dt=drought tolerance; DAE: days after emergence; and AP203= *B. velezensis*. Means with the same letter are not statistically significant by Tukey's test ($p \leq 0.05$).

Figure 1. Specific leaf area (SLA) of peanut genotype (AU-NPL 17) under regular water (RW) and drought stress tolerance (DT). The asterisk (*) indicates a significant difference compared to other treatments.

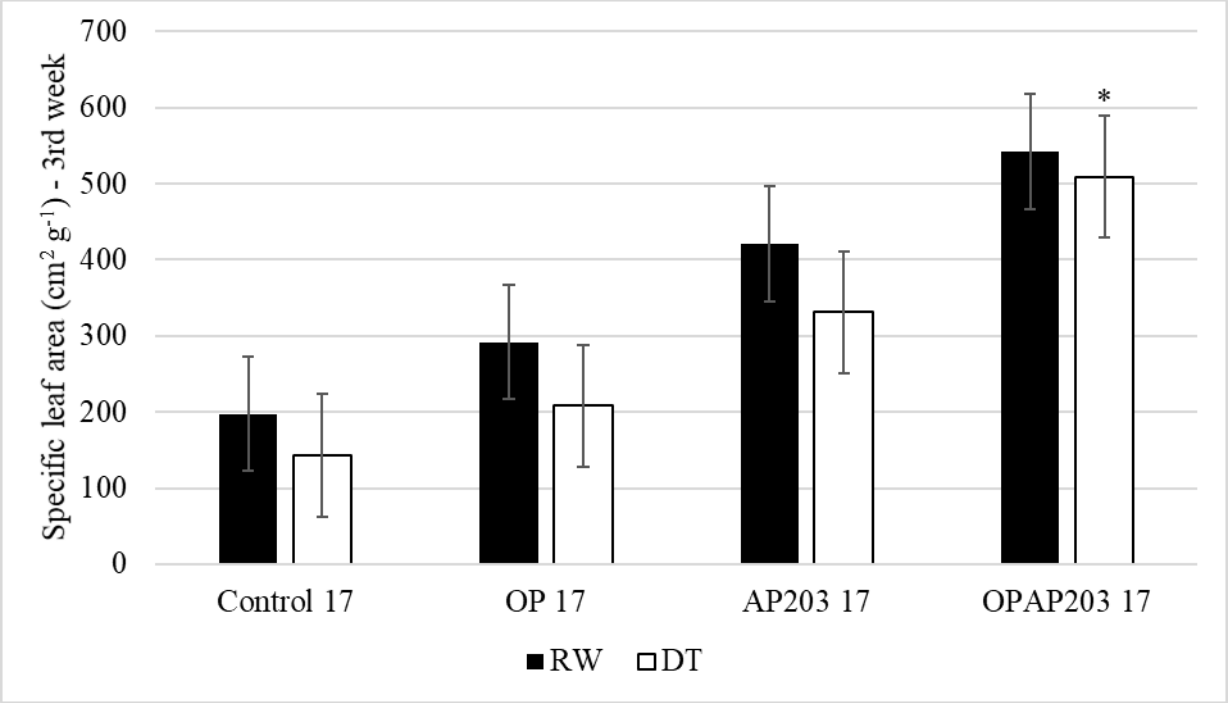


Figure 2. Specific leaf area (SLA) of peanut genotype (AU-NPL 17) under regular water (RW) and drought stress tolerance (DT). The asterisk (*) indicates a significant difference compared to other treatments.

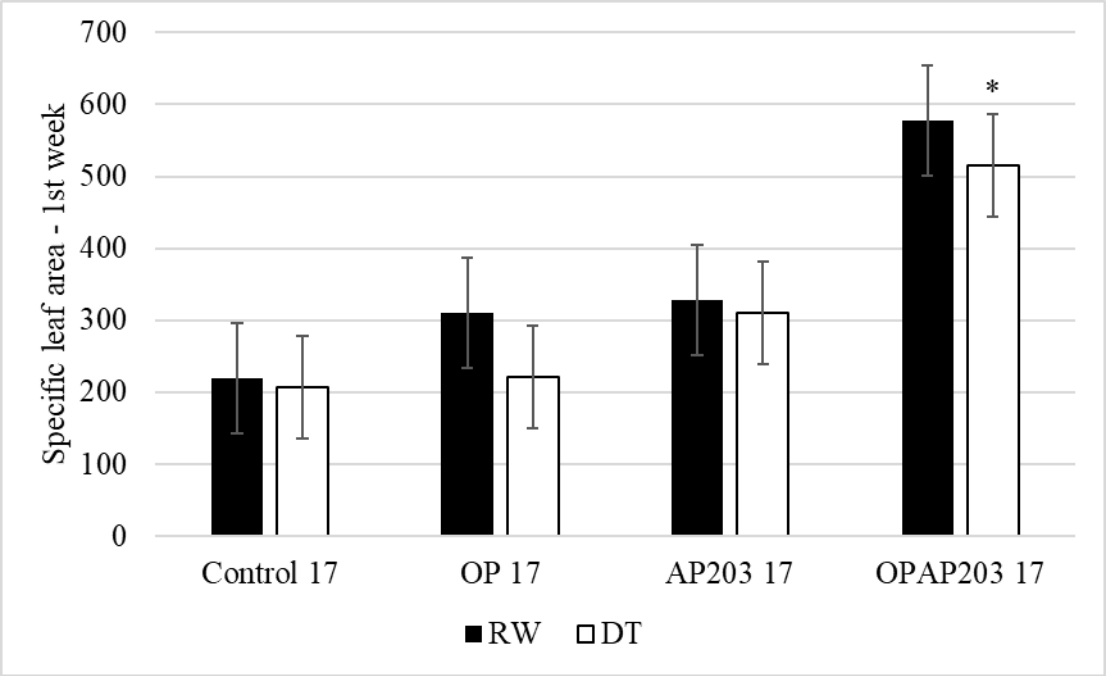


Figure 3. Specific leaf area (SLA) of peanut genotype (GA06-G) under regular water (RW) and drought stress tolerance (DT). The asterisk (*) indicates a significant difference compared to other treatments.

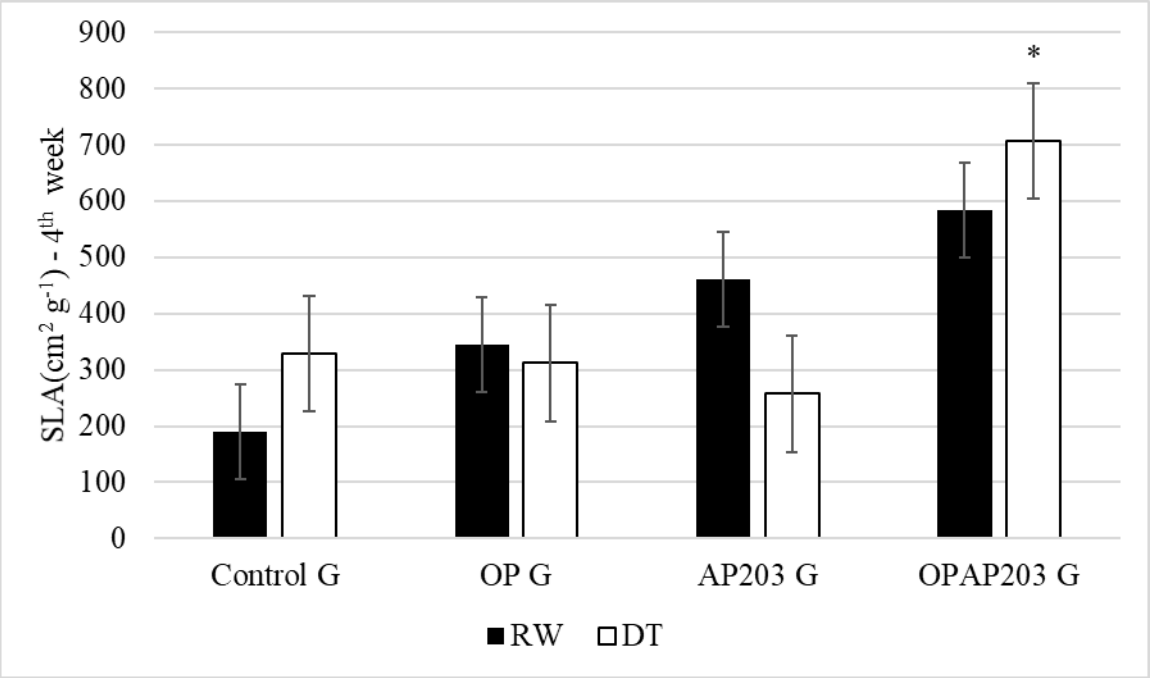


Figure 4. SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU 18-33) under regular water (RW) and drought stress tolerance (DT) at 30 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

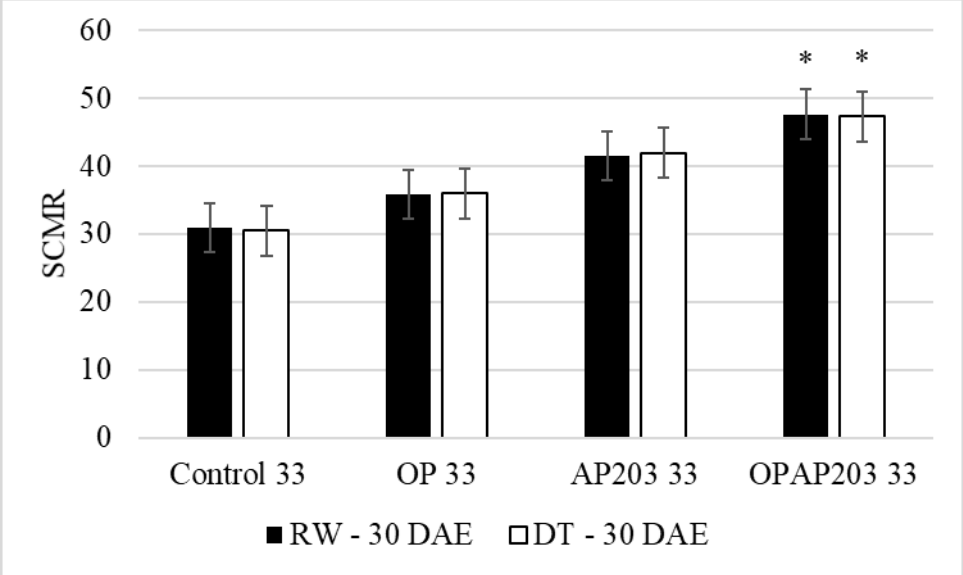


Figure 5. SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU 18-33) under regular water (RW) and drought stress tolerance (DT) at 30 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

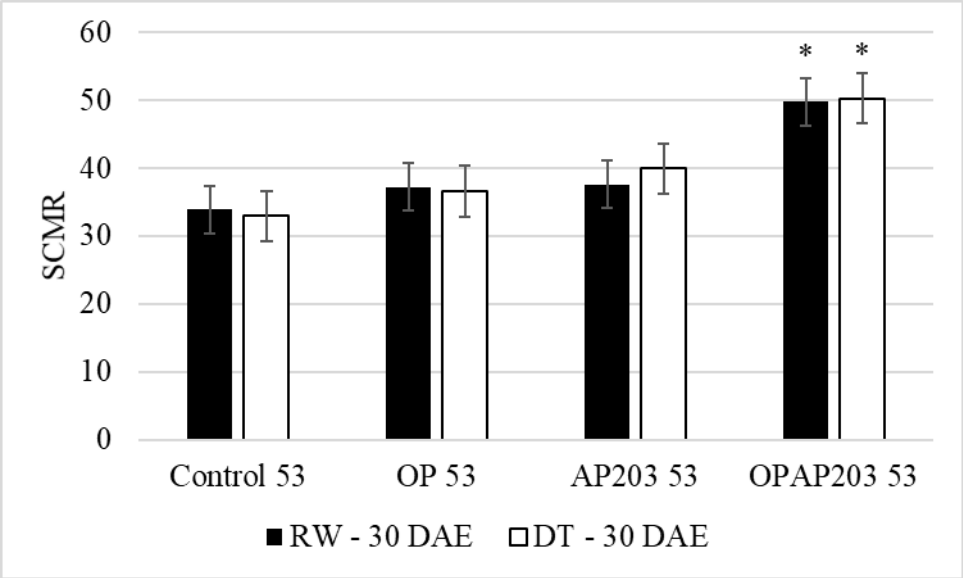


Figure 6. SPAD chlorophyll meter readings (SCMR) of peanut genotype (GA06-G) under regular water (RW) and drought stress tolerance (DT) at 30 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

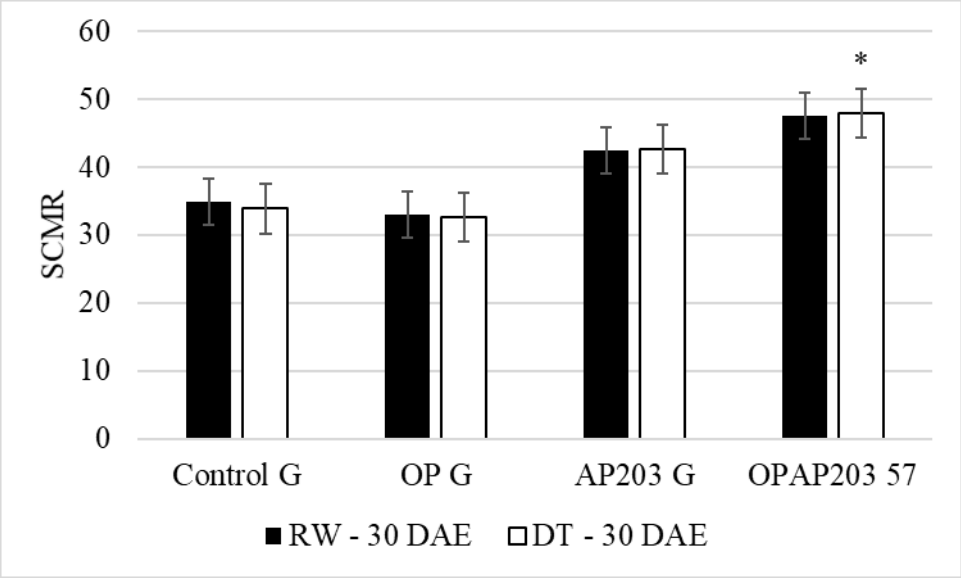


Figure 7. SPAD chlorophyll meter readings (SCMR) of peanut genotype (GA06-G) under regular water (RW) and drought stress tolerance (DT) at 60 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

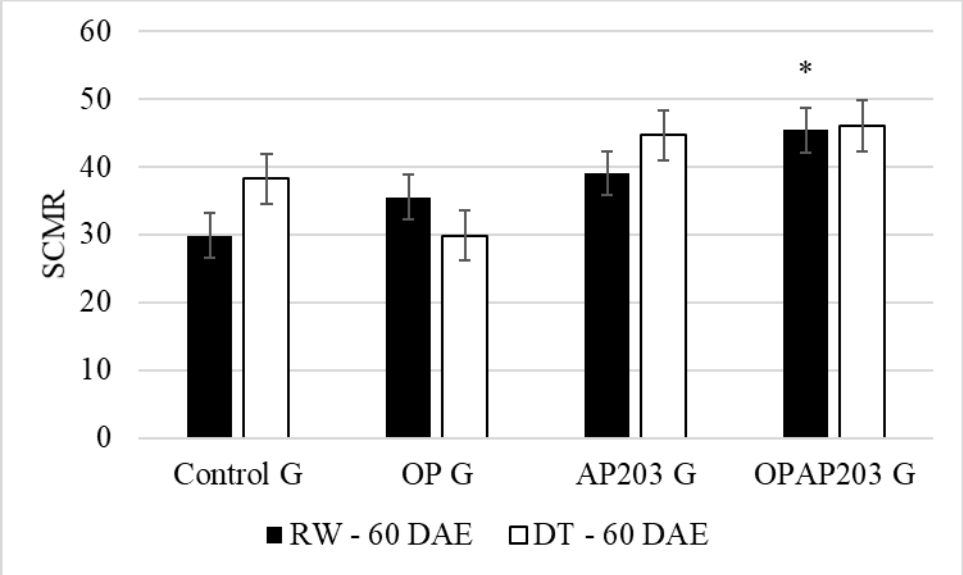


Figure 8. SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU 18-53) under regular water (RW) and drought stress tolerance (DT) at 90 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

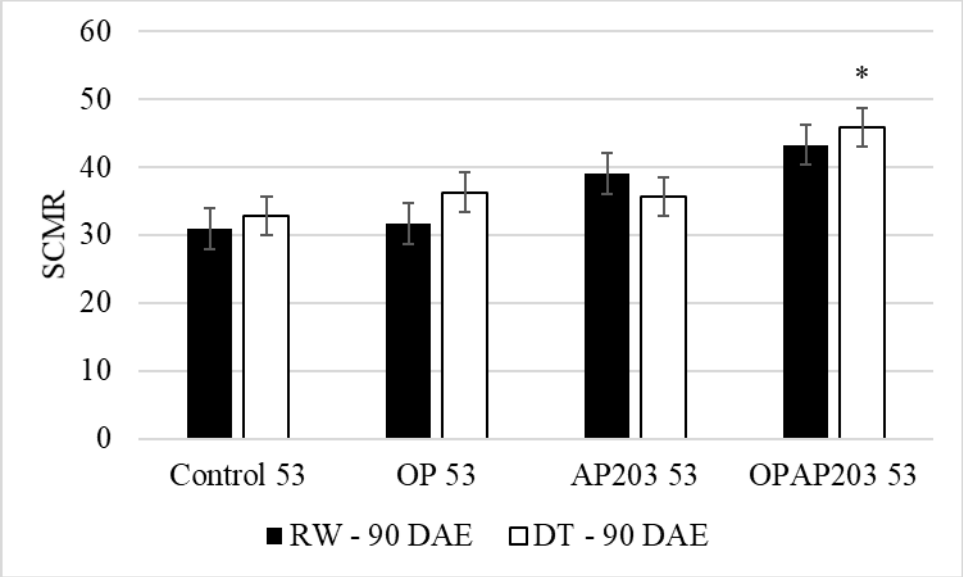


Figure 9. Chlorophyll density (ChlD) of peanut genotype (GA06-G) under regular water (RW) and drought stress tolerance (DT) at 30 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

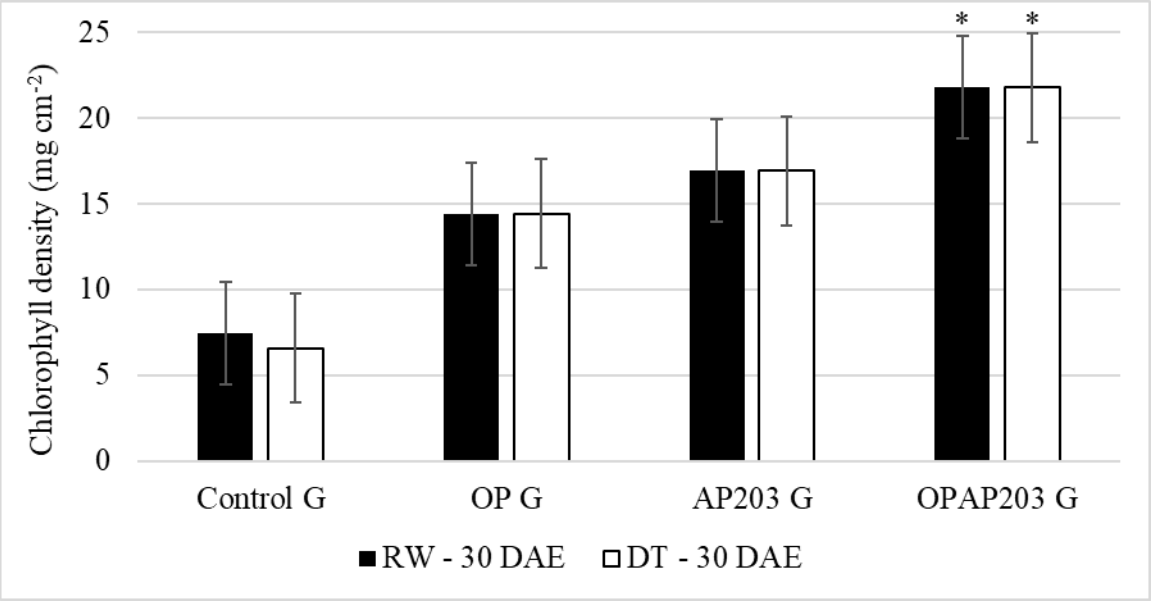


Figure 10. Chlorophyll density (ChlD) of peanut genotype (AU-NPL 17) under regular water (RW) and drought stress tolerance (DT) at 60 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

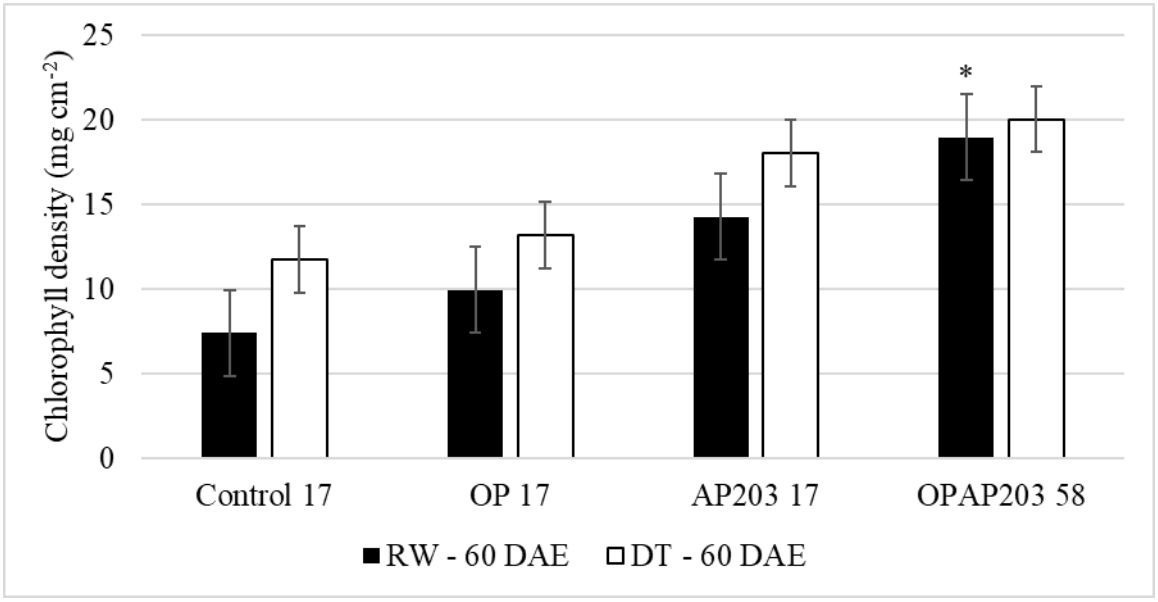


Figure 11. Chlorophyll density (ChlD) of peanut genotype (AU 18-33) under regular water (RW) and drought stress tolerance (DT) at 90 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

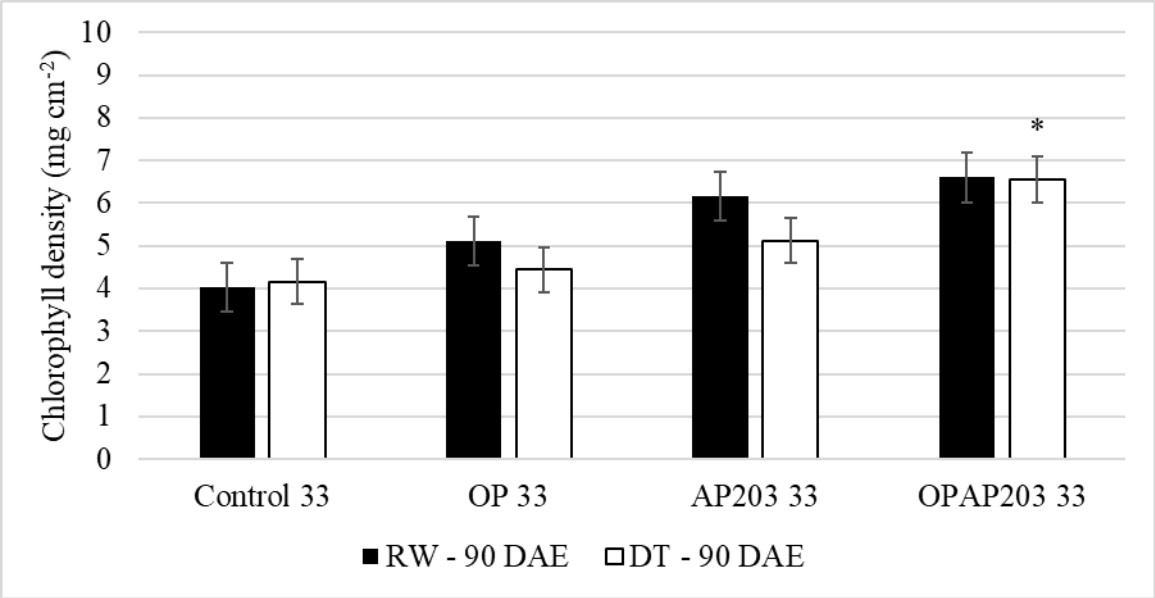


Figure 12. Chlorophyll density (ChlD) of peanut genotype (AU 18-53) under regular water (RW) and drought stress tolerance (DT) at 60 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

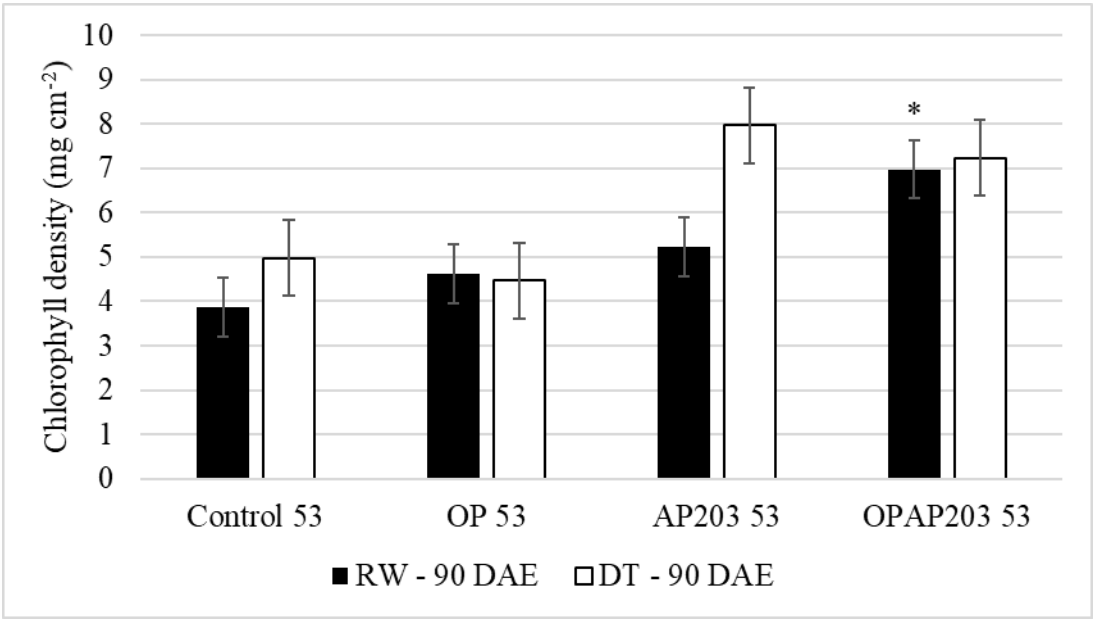


Figure 13. Chlorophyll density (ChlD) of peanut genotype (GA06-G) under regular water (RW) and drought stress tolerance (DT) at 60 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

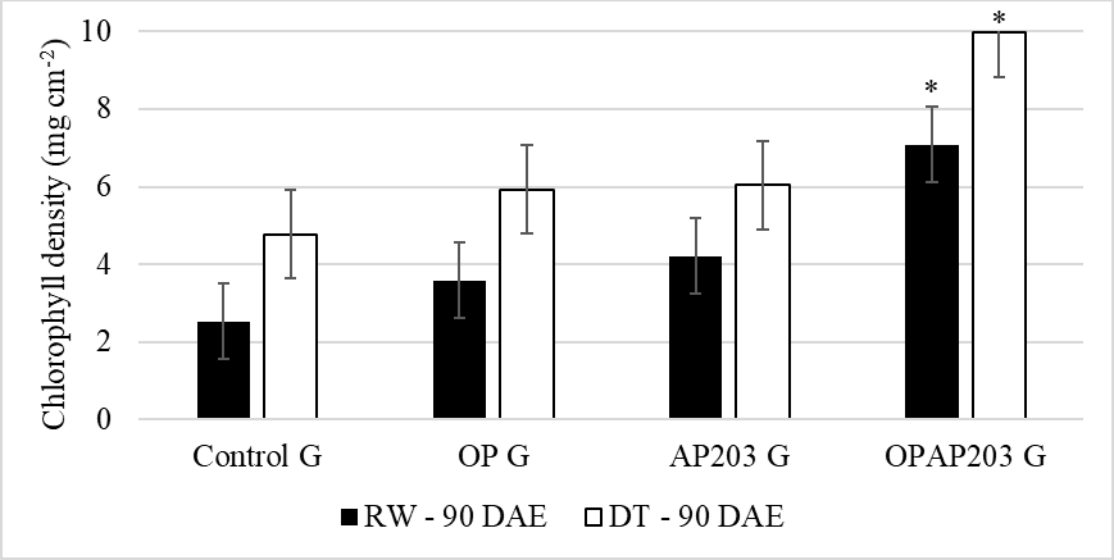


Figure 14. Chlorophyll density (ChlD) of peanut genotype (AU-NPL 17) under regular water (RW) and drought stress tolerance (DT) at 60 days after emergence (DAE). The asterisk (*) indicates a significant difference compared to other treatments.

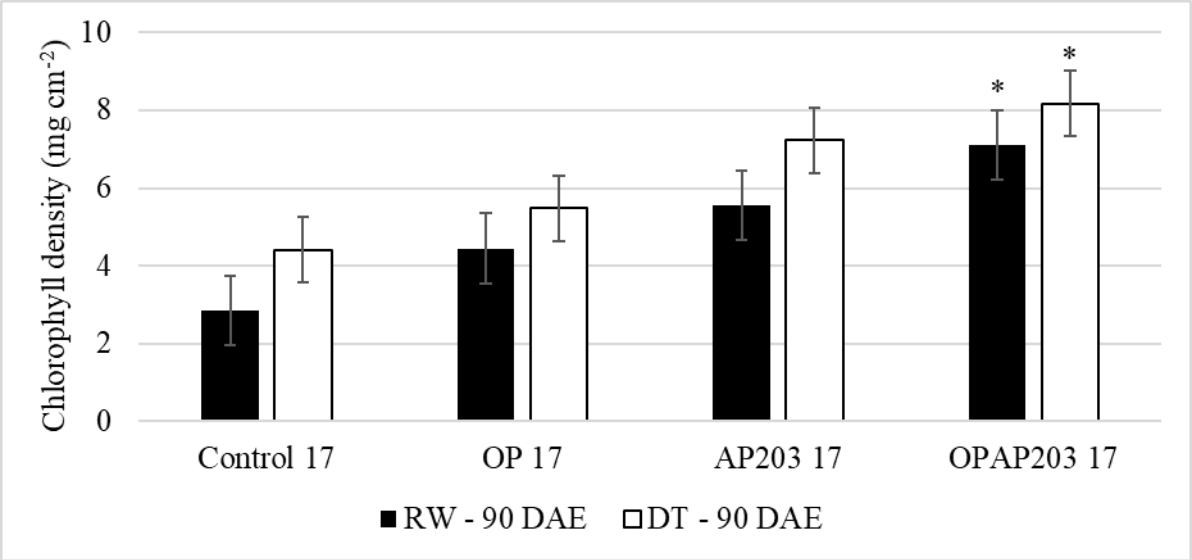


Figure 15. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU 18-33) under regular water and drought stress tolerance condition at 30 days after emergence (DAE). The r indicates correlation coefficients.

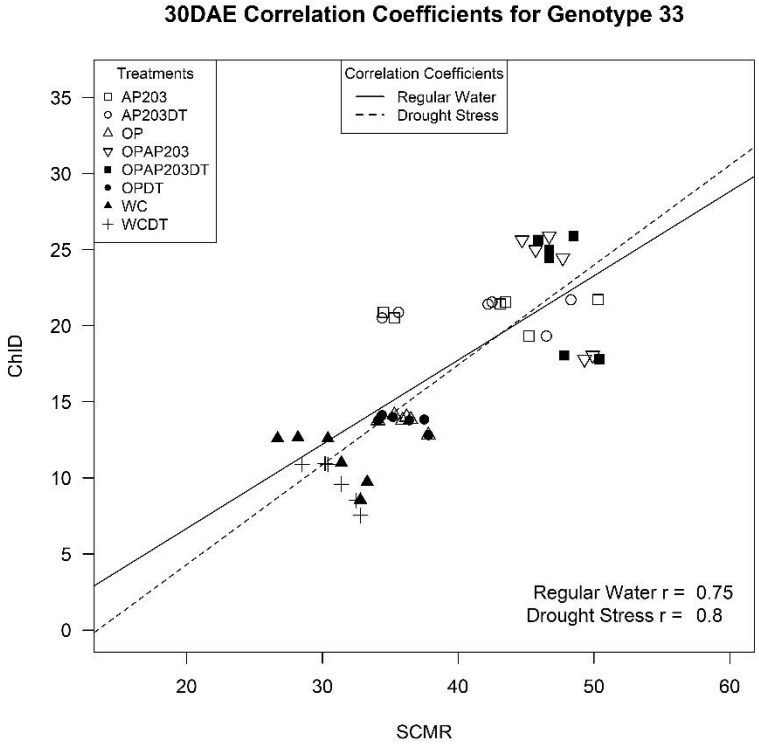


Figure 16. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU 18-53) under regular water and drought stress tolerance condition at 30 days after emergence (DAE). The r indicates correlation coefficients.

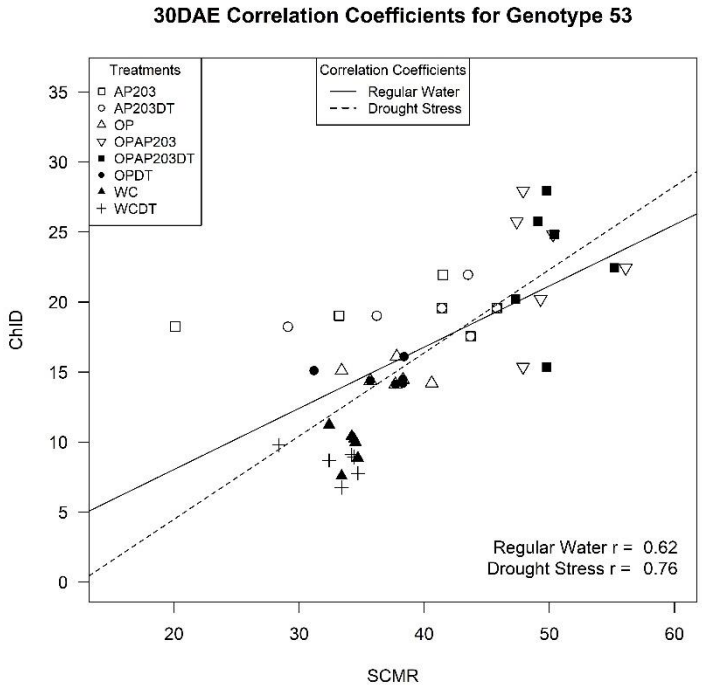


Figure 17. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (GA06-G) under regular water and drought stress tolerance condition at 30 days after emergence (DAE). The r indicates correlation coefficients.

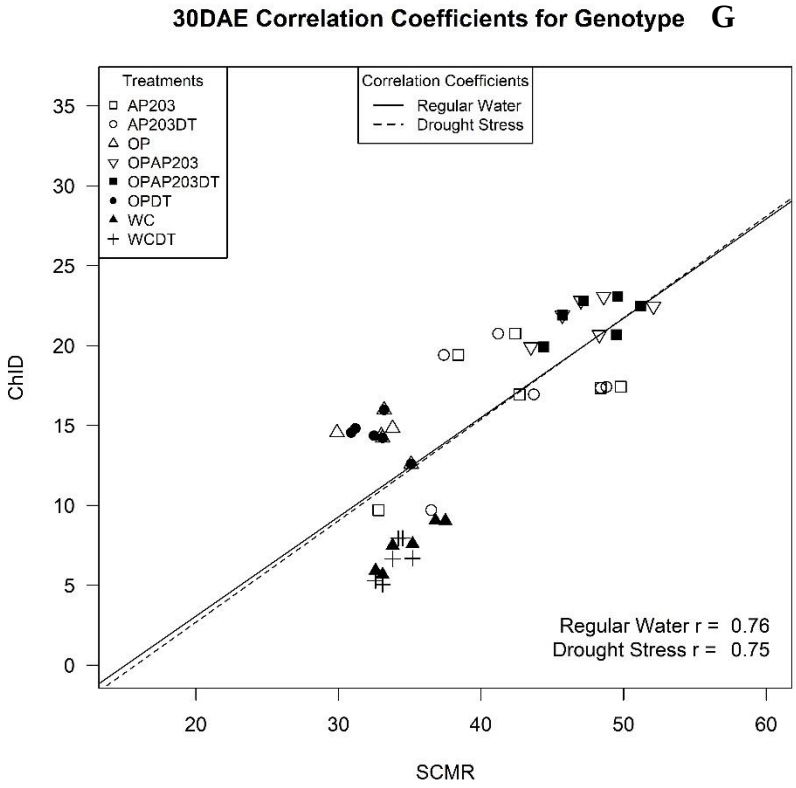


Figure 18. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU-NPL 17) under regular water and drought stress tolerance condition at 30 days after emergence (DAE). The r indicates correlation coefficients.

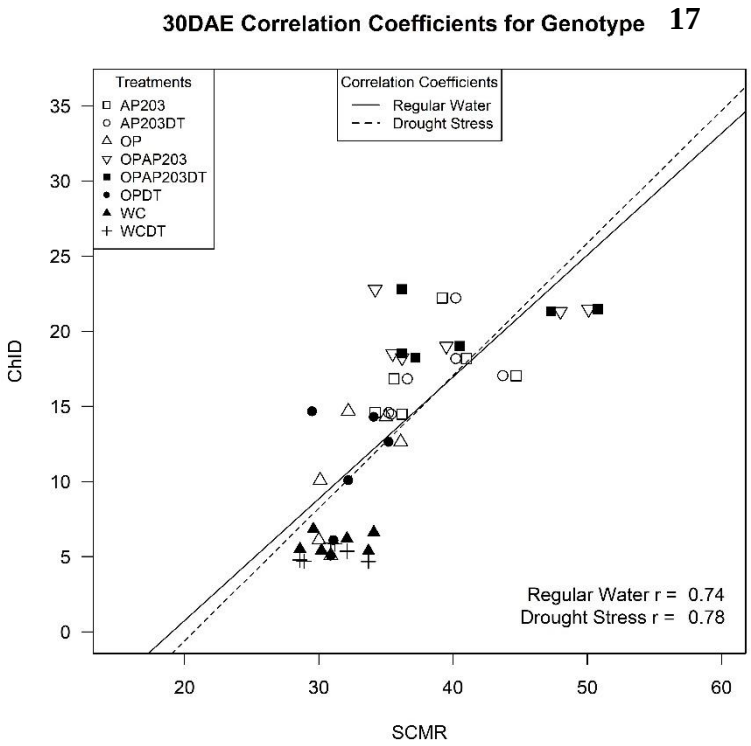


Figure 19. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU 18-33) under regular water and drought stress tolerance condition at 60 days after emergence (DAE). The r indicates correlation coefficients.

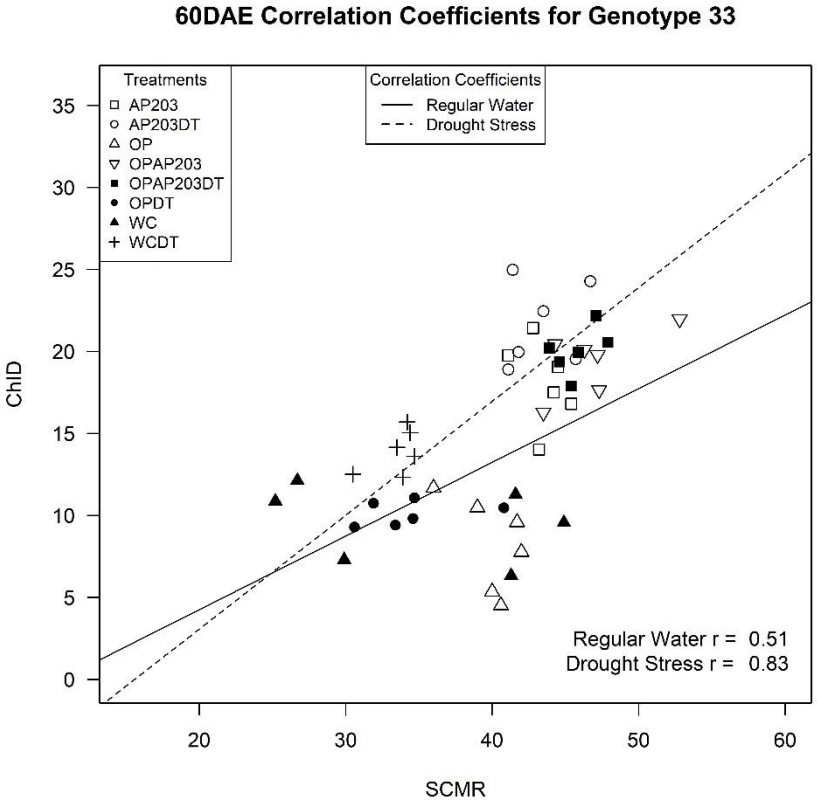


Figure 20. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU 18-53) under regular water and drought stress tolerance condition at 60 days after emergence (DAE). The r indicates correlation coefficients.

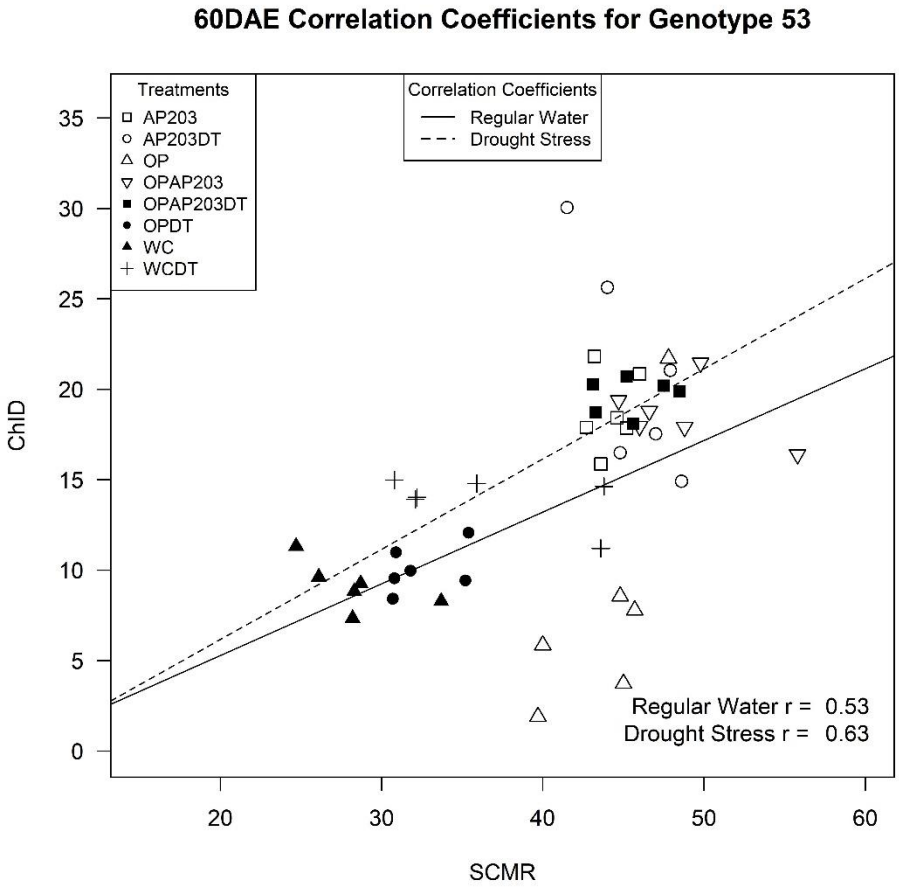


Figure 21. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (GA06-G) under regular water and drought stress tolerance condition at 60 days after emergence (DAE). The r indicates correlation coefficients.

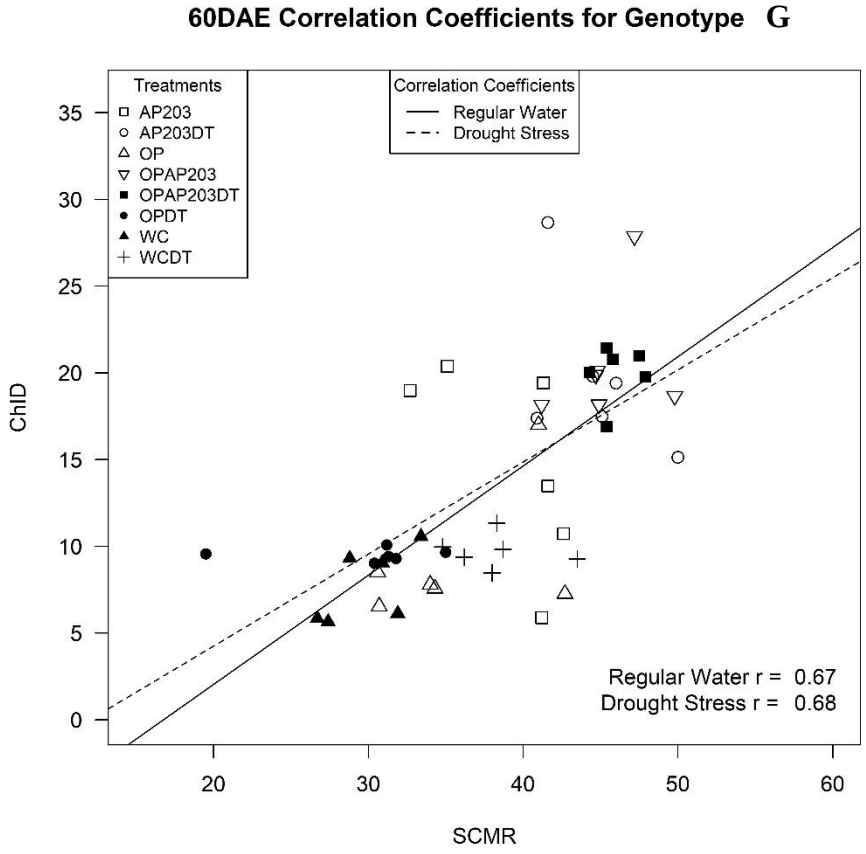


Figure 22. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU-NPL 17) under regular water and drought stress tolerance condition at 60 days after emergence (DAE). The r indicates correlation coefficients.

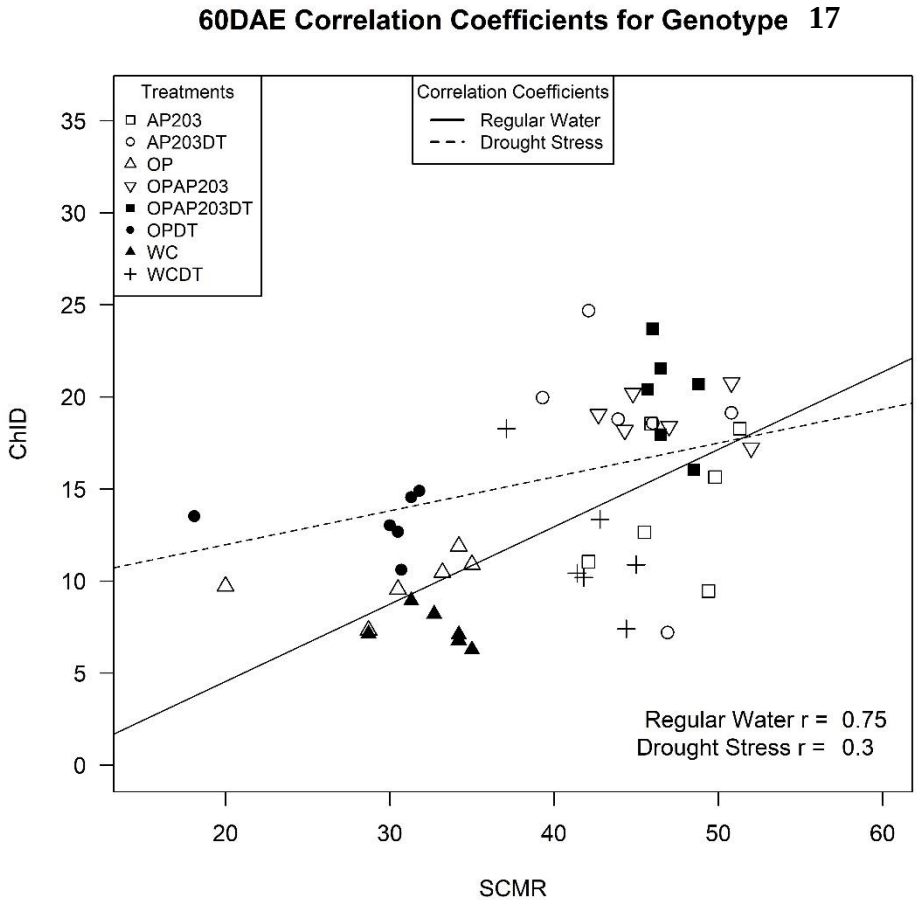


Figure 23. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU 18-33) under regular water and drought stress tolerance condition at 90 days after emergence (DAE). The r indicates correlation coefficients.

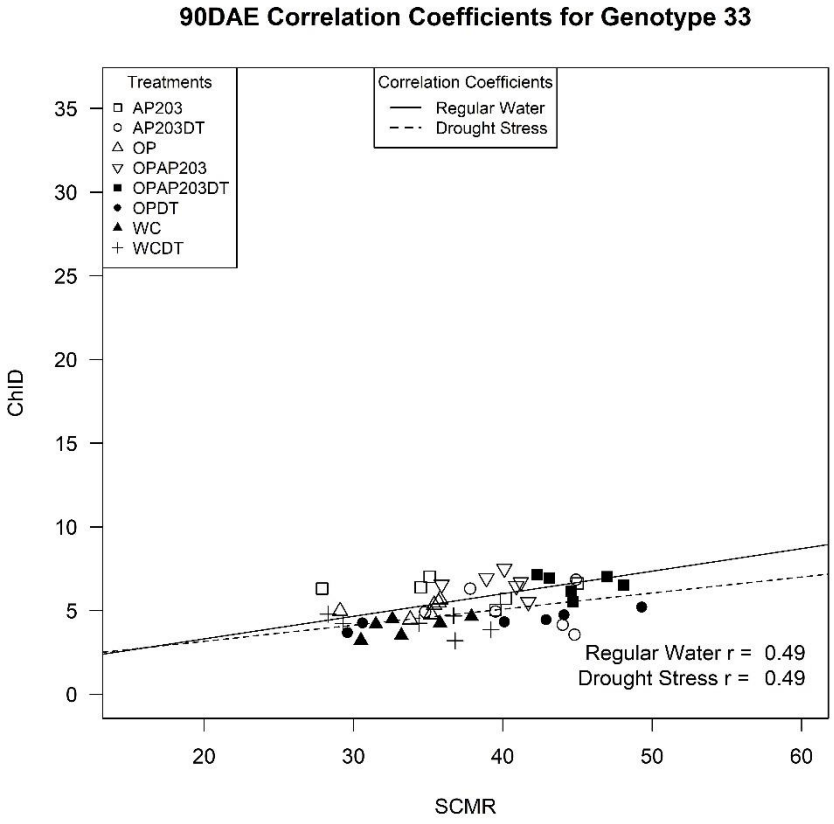


Figure 24. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU 18-53) under regular water and drought stress tolerance condition at 90 days after emergence (DAE). The r indicates correlation coefficients.

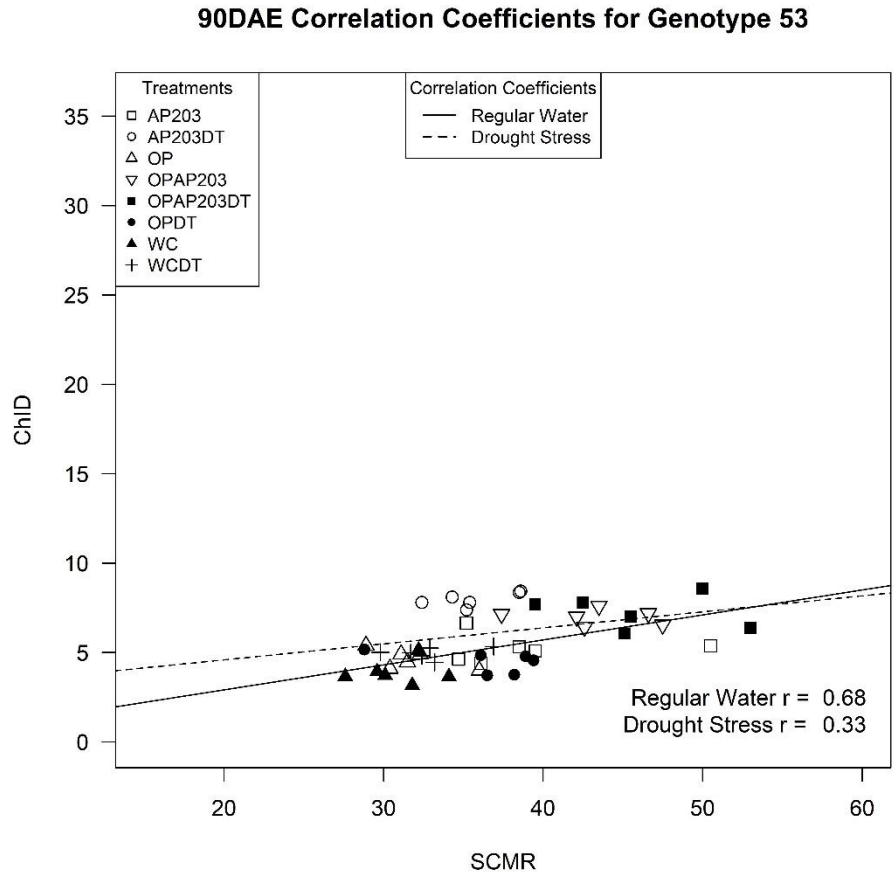


Figure 25. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (GA06-G) under regular water and drought stress tolerance condition at 90 days after emergence (DAE). The r indicates correlation coefficients.

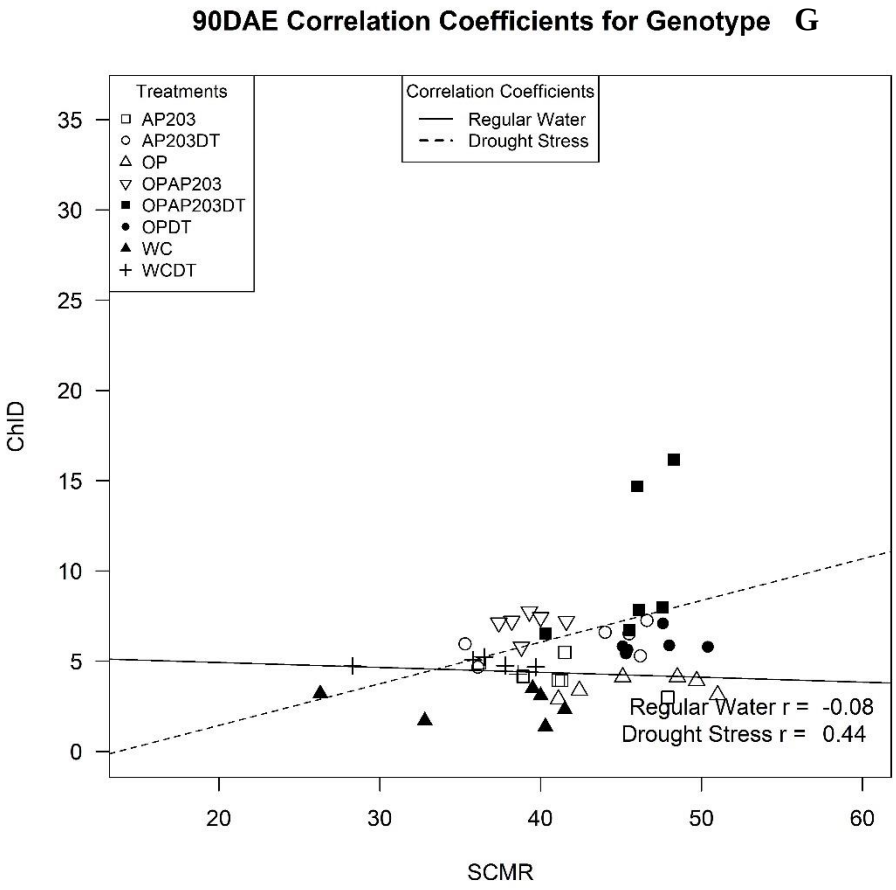


Figure 26. Relationship between chlorophyll density (ChlD) and SPAD chlorophyll meter readings (SCMR) of peanut genotype (AU-NPL 17) under regular water and drought stress tolerance condition at 90 days after emergence (DAE). The r indicates correlation coefficients.

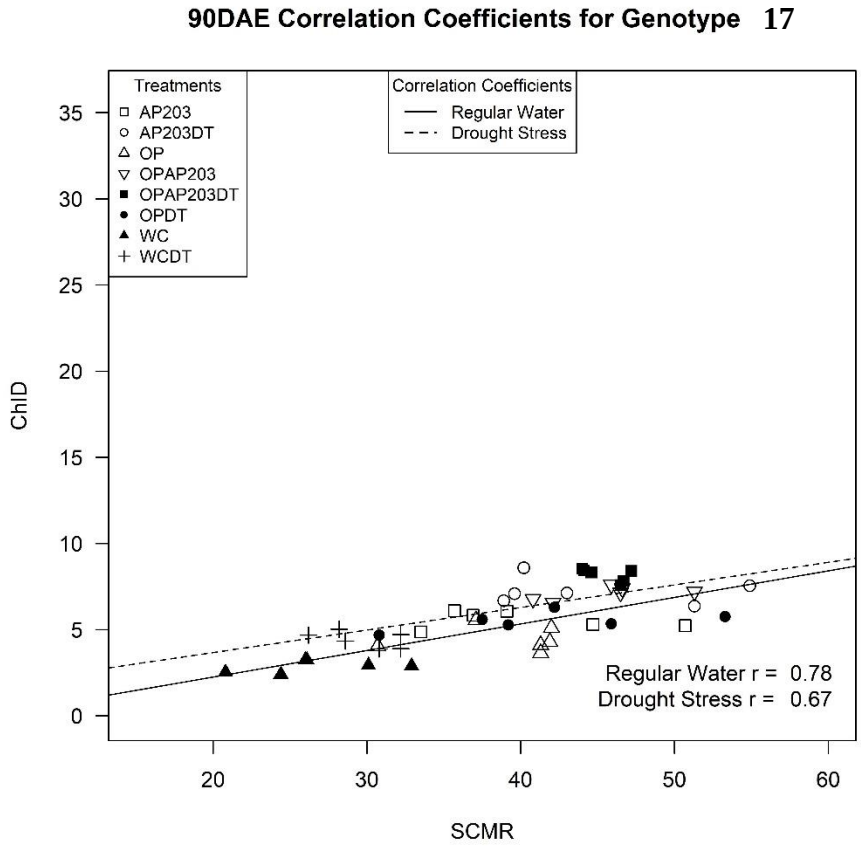


Figure 27. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on root length of peanut genotype (AU 18-33) under regular water (RW) and drought stress tolerance (DT) condition. The asterisk (*) indicates a significant difference compared to other treatments.

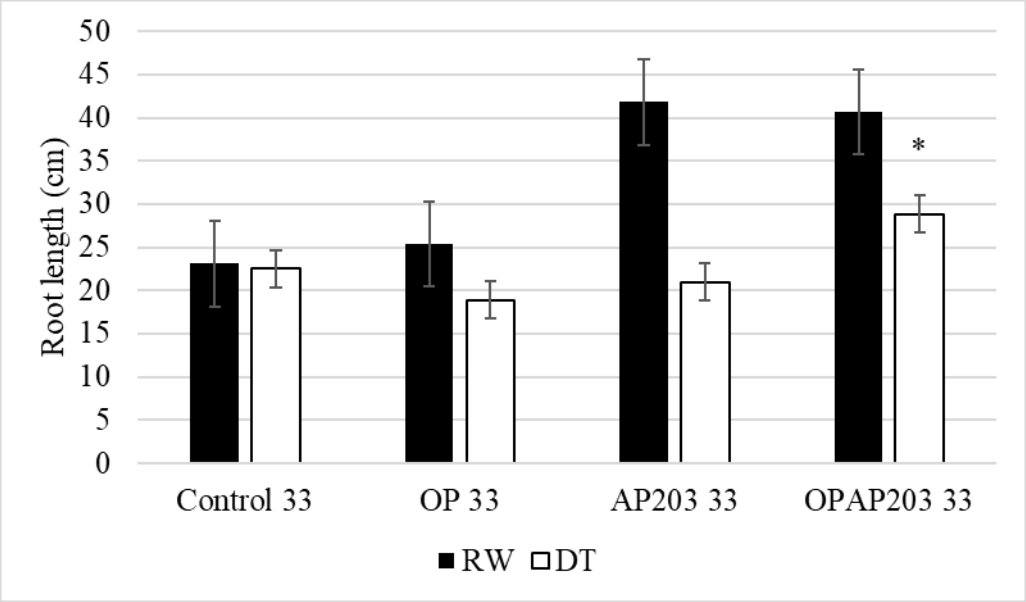


Figure 28. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on root length of peanut genotype (AU 18-53) under regular water (RW) and drought stress tolerance (DT) condition. The asterisk (*) indicates a significant difference compared to other treatments.

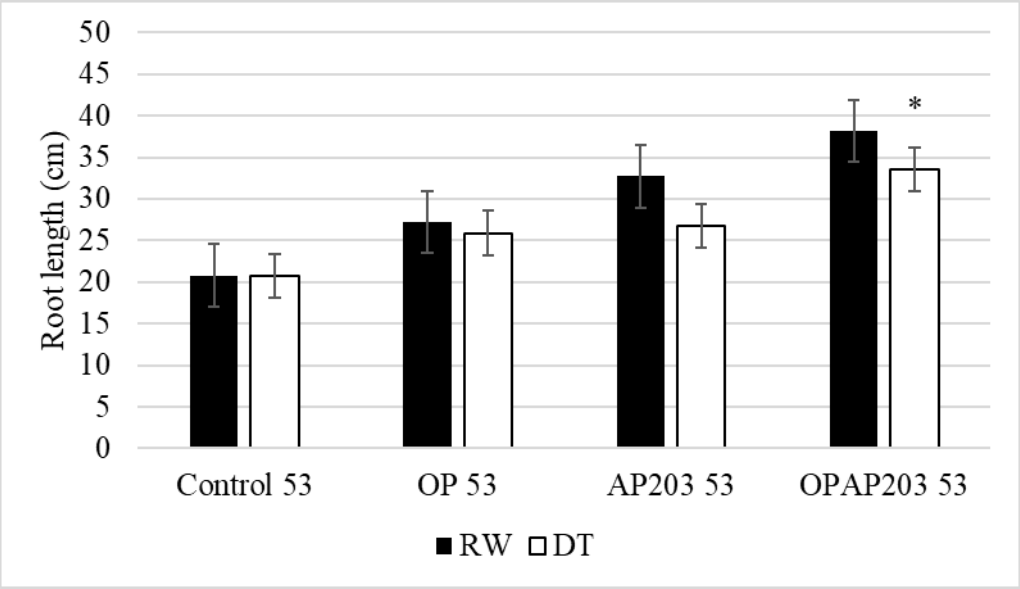


Figure 29. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on root length of peanut genotype (GA06-G) under regular water (RW) and drought tolerance (DT) condition. The asterisk (*) indicates a significant difference compared to other treatments.

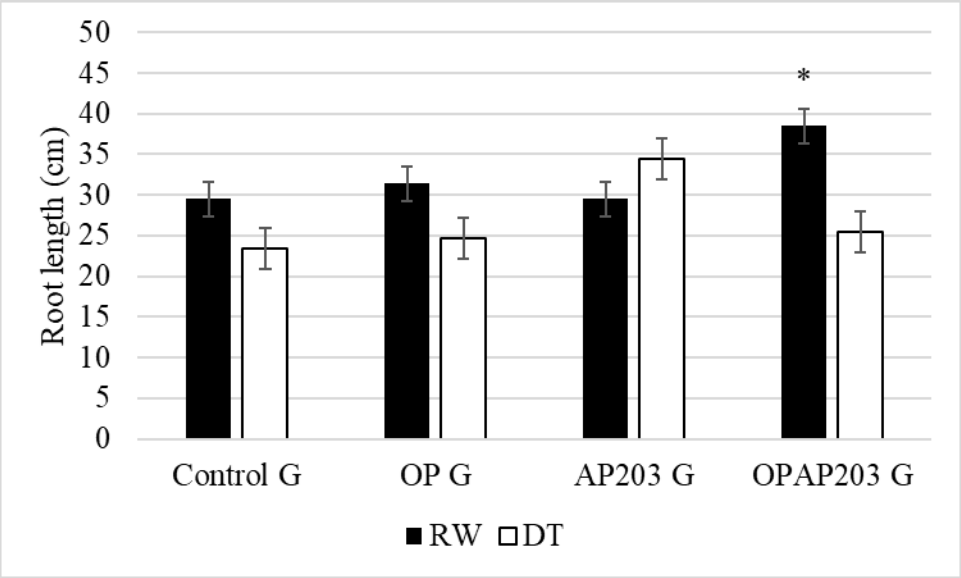


Figure 30. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on root length of peanut genotype (AU-NPL 17) under regular water (RW) and drought tolerance (DT) condition. The asterisk (*) indicates a significant difference compared to other treatments.

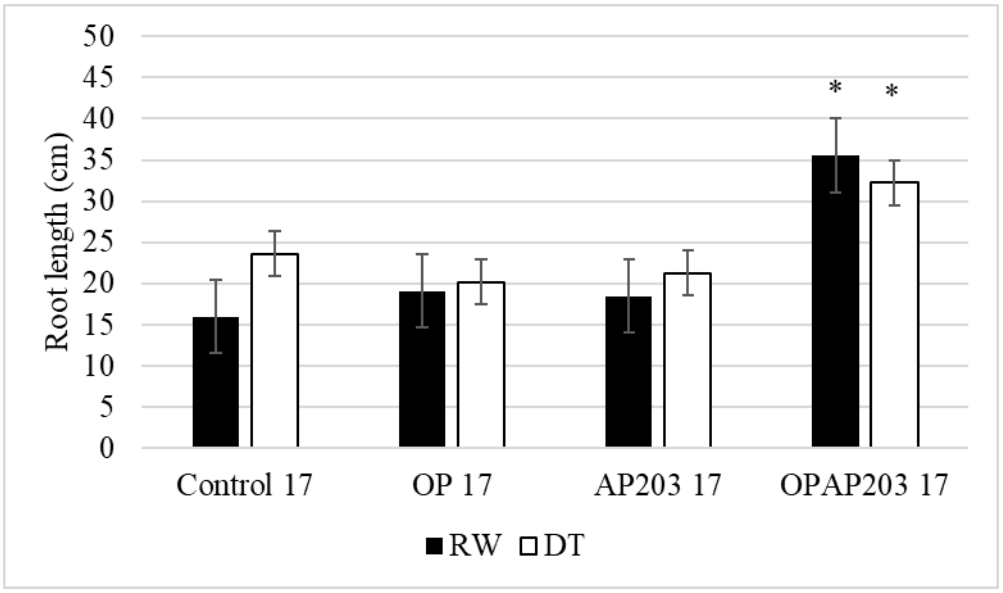


Figure 31. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on plant dry weight of peanut genotype (AU 18-33) under regular water (RW) and drought tolerance (DT) condition. The asterisk (*) indicates a significant difference compared to other treatments.

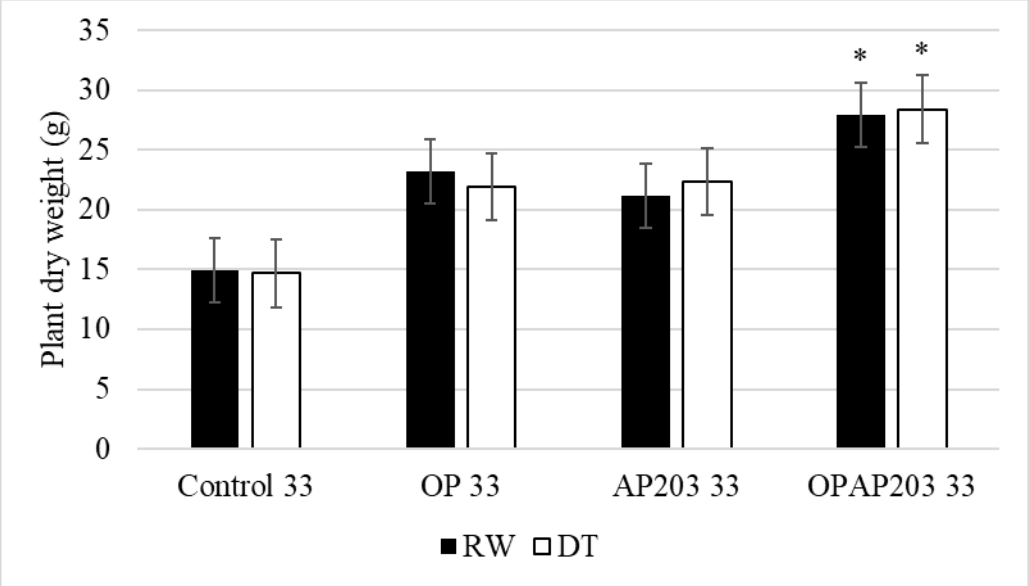
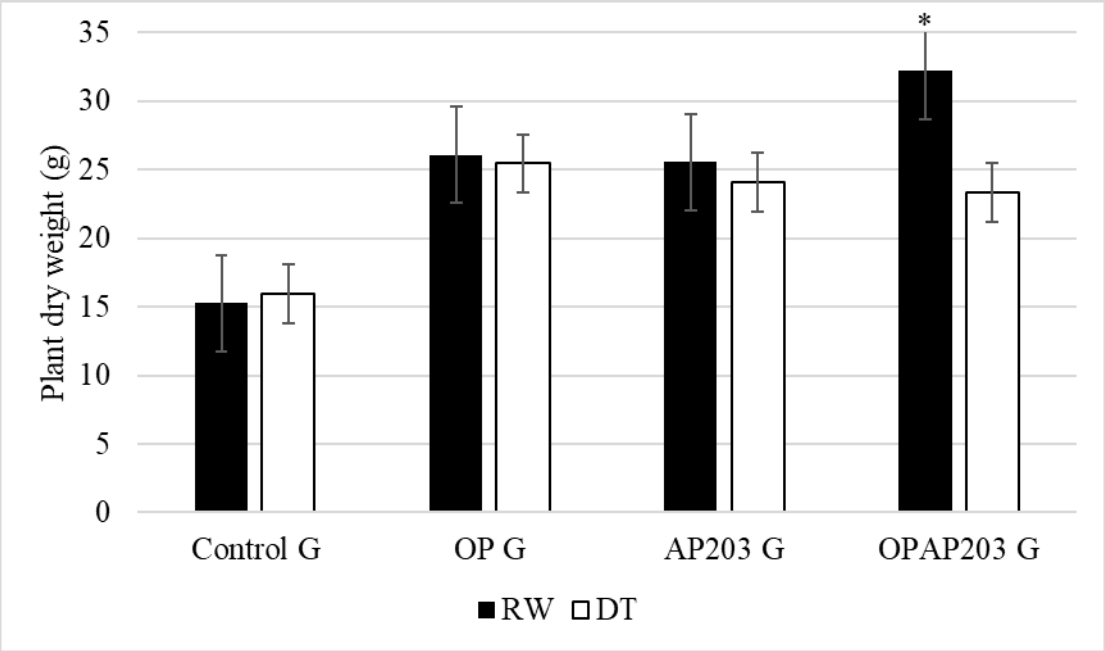


Figure 32. Effects of *B. velezensis* PGPR strain AP203 with orange peel amendment on plant dry weight of peanut genotype (GA06-G) under regular water (RW) and drought tolerance (DT) condition. The asterisk (*) indicates a significant difference compared to other treatments.



Chapter V Conclusions and future directions

An agricultural waste pectin-rich orange peel amendments with beneficial bacteria are promising and can be beneficial not only for enhancing plant growth and health but also for suppressing plant pathogens [1]. In addition to promoting plant growth and reducing multiple plant pathogens, it also increases soil macro and micronutrients [2]. However, the concentration of exogenous orange peel amendments are critical for degrading and utilizing by plant growth-promoting rhizobacterial strains. Our studies revealed that orange peel amendments range from 0.1 – 1.0% significantly increased soybean growth parameters, peanut drought stress tolerance, and *M. incognita* mortality rate. *B. velezensis* strains with pectin-rich amendment significantly increased soybean nodules, shoot, and root growth in a greenhouse and field experiments compared to *B. velezensis* strains alone. Cell-free supernatant, Cell pellet, and culture broth of *B. velezensis* strain grown on orange peel amended TSS media significantly suppressed *M. incognita* populations compared to *M. incognita* inoculated positive control. *B. velezensis* strains with pectin-rich amendment significantly increased peanut root length under drought stress conditions compared to *B. velezensis* strain alone. Future transcriptomic studies are needed to determine whether *B. velezensis* strains with orange peel amendments have direct interactions with *Bradyrhizobium* spp. in the soil or indirect interactions with soil metabolites. In addition to the transcriptomic study, field experiments can be conducted in different locations to evaluate the biological control and drought stress tolerance capacities *B. velezensis* strains with orange peel amendments.

References

1. Tsai, B.Y., *Effect of peels of lemon, orange, and grapefruit against Meloidogyne incognita.* Plant Pathology Bulletin, 2008. **17**(3): p. 195-201.
2. Treuer, T.L., et al., *Low-cost agricultural waste accelerates tropical forest regeneration.* Restoration ecology, 2018. **26**(2): p. 275-283.