The Role of Pectin Utilization in Root Colonization and Plant Growth-Promotion by *Bacillus amyloliquefaciens* subsp. *plantarum* (*Bap*)

by

Mohammad Kamrul Hassan

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Approved by

Joseph W. Kloepper, Chair, Professor of Entomology and Plant Pathology
Leonardo De La Fuente, Associate Professor of Entomology and Plant Pathology
Mark R. Liles, Professor of Biological Science

Abstract

Plant growth-promoting rhizobacteria (PGPR) and other beneficial bacteria are increasingly used as biofertilizers for enhancing crop production. After PGPR-treated seeds are planted, root colonization is considered to be an essential first step in plant growth-promotion. Plant roots exude diverse organic compounds, including sugars, and successful bacterial colonization hinges on nutrient uptake from the host plants through extracellular enzymatic activity. Strains of *Bacillus amyloliquefaciens* subsp. *plantarum* (*Bap*) colonize plant roots, and many have been used as PGPR during the past decades.

Pectin is a complex structural polysaccharide found in the middle lamella and primary cell wall of plant cells. Little is known about the possible role of pectin in root colonization and plant growth-promotion. It is possible that *Bap* strains obtain some carbon via production of extracellular pectate lyase enzymes that degrade pectin in plant root cells. Bacterial genes are involved in degradation and transport of the pectin-associated compounds D-glucuronate and D-galacturonate which can be used as energy and carbon sources. Pectin is broken down by a series of pectinolytic enzymes of soil and rhizosphere bacteria. Some pectinolytic bacteria are plant pathogens which over-produce pectinolytic enzymes, thereby causing plant cell death. Other nonpathogenic soil and rhizosphere bacteria can also degrade pectin in plant rhizospheres without causing damage to plants. Hence, understanding the expression of pectin-associated genes and their involvement in pectin degradation, transport, and utilization by PGPR is critical.

The overall objective of this study was to determine the potential role of pectin degradation and utilization in root colonization and plant growth-promotion of soybean by specific PGPR strains. Experiments were designed to evaluate the potential effects of pectin degradation and utilization by *Bap* strains on root colonization and plant growth-promotion using pectin amendment to soil. Objective 1 consisted of two parts: a) to identify *Bacillus amyloliquefaciens* subsp. *plantarum* strains with *gyrB* primers (UP-1 & UP-2r), and b) to identify *exuT* and *uxuB* genes with *exuT* and *uxuB* primers in the *Bap* strains. The hypotheses for objective 1 were i) *gyrB* primers (UP-1 & UP-2r) will identify *Bap* strains, and ii) *exuT* and *uxuB* primers will identify *exuT* and *uxuB* genes in *Bap* strains for utilization of sugars derived from pectin. Of 79 *Bacillus* species strains screened via *gyrB* universal primers, 59 were confirmed as *Bacillus amyloliquefaciens* subsp. *Plantarum*, and the remaining 20 were identified as *B. mojavensis, B. sonorensis, B. tequilensis, Bacillus subtilis*, and *B. pumilis* based on the *gyrB* phylogenetic tree. None of the strains was found to cluster together with *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens*. The *Bap* strains were then screened for the presence of the *exuT* and *uxuB* genes. Out of 59 *Bap* strains, 57 were found to contain *exuT* and 52 contained *uxuB*.

Objective 2 had two parts: a) to screen a large collection of *Bap* strains for pectin degradation and utilization as a sole carbon source, b) to conduct a comparative genomic analysis that includes *Bap* strains that lack the capacity for pectin utilization. The hypotheses for objective 2 were a) pectin degradation capacity of *Bap* strains is linked to utilization of D-Glucuronate of D-galacturonate as an energy source, and b) whole genome sequence analysis will reveal all the pectin-associated and defective genes by RAST and CLC genomics software. Pectin degradation and utilization activity of 59 *Bap* strains were tested *in vitro* on Pectate Agar

(PA) and Tris-Spizizen Salts (TSS) medium. The highest pectate lyase activities were observed in *Bap* strains AP193, AP203, AP299, AP80, AP102, and AP52, while the lowest activities were with *Bap* strains AP 194, AP214, AP215, and AP305. Twelve *Bap* strains (AP67, AP71, AP77, AP78, AP85, AP102, AP108, AP135, AP143, AP189, AP192, and AP193) grew vigorously on TSS medium. Six *Bap* strains (AP194, AP204, AP214, AP216, AP219, and HD73) had lower growth compared to other *Bap* strains on TSS medium. The whole genome sequence was determined for *Bap* strains AP194 and AP214 using an Illumina MiSeq sequencer based on *in vitro* test results.

Objective 3 was to evaluate the effect of pectin amendments to soil, together with selected pectinolytic Bap strains, for potential enhancement in root colonization, and the magnitude of *Bap*-induced plant growth of soybean plants. The hypothesis was that bacterial utilization of D-Glucuronate as a carbon source will improve root colonization and increase root and shoot biomass. Rifampicin-resistant mutants of AP143, AP193, and *Btk* strain HD73 were selected and used to evaluated effects of pectin supplement on root colonization and plant growth-promotion of soybean in greenhouse tests. The combination of *Bap* rif^R strains with pectin amendment resulted in enhanced nodule formation and weights of soybean shoots and roots weights, compared to the same *Bap* rif^R strains without pectin amendment. In contrast, *Btk* rif^R strain did not exhibit plant growth-promotion activity when used with or without pectin amendment. In summary, inoculation of selected pectinolytic *Bap* rif^R strains together with pectin amendments to soil enhanced root colonization and plant-growth promotion of soybean plant. In the greenhouse tests using nonsterilized field soil, pectin amendment with *Bap* rif^R strains also enhanced formation of nodules of indigenous *Bradyrhizobium japonicum* on soybean roots. In conclusion, the results demonstrate that the combination of selected pectinolytic *Bap*

strains and soil amendment with pectin enhanced plant growth and nodulation by indigenous symbiotic N-fixing bacteria.

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List of Abbreviations

ANOVA	Analysis of Variance
Ba	*Bacillus amyloliquefaciens*
Baa	*Bacillus amyloliquefaciens* subsp. *Amyloliquefaciens*
Bap	*Bacillus amyloliquefaciens* subsp. *plantarum*
Btk	*Bacillus thuringiensis* subsp. *kurstaki*
DNA	Deoxyribonucleic acid
exuT	*Hexuronate Transporter*
gyrB	*Subunit B protein of DNA gyrase*
OD	Optical Density
PCR	Polymerase Chain Reaction
PGPR	Plant Growth-Promoting Rhizobacteria
RIF	Rifampicin
TSS	Tris Spizizen Salts
uxuB	D-mannonate oxidoreductase

Chapter I Literature Review

1. Introduction

The global use of fertilizers in agriculture continues to increase each year to meet demands for crop production. Nitrogen, potash, and phosphate fertilizers enhance the fertility of the soil and widely uses for the different crops. The global nitrogen fertilizer market in 2011 and 2012 was 108.2 and 109.9 million tons, at a growth rate of 1.6%; it would be highest in Asia (60%) than America (19%), and Africa (7%) in 2016 (Anonymous, 2016a). The world phosphoric acid ((P2O5) fertilizer market was 38.1 million metric tons in 2015, and the demand will be 39.0 million metric tons in 2016 (Prud'homme, 2013). In the U.S., consumption of fertilizers containing N, P, and K (nitrogenous, potash and phosphate fertilizers) in 2013 was 131.9 kilograms per hectare (Anonymous, 2016b).

Even with proper fertilization, crop plants often do not receive sufficient nutrients from the soil due to nutrient run-off and to the formation of insoluble forms of some nutrients in fertilizers. Also, rainwater on slopes of agricultural lands washes the fertilizer-containing soils into nearby ponds, lakes, and rivers. Losses of phosphorous in water run-off are higher from agricultural lands than from turfgrass, pasture, and grassland systems (Soldat, 2008). Run-off of fertilizers causes pollution in which can lead to dead zones in oceans.rivers, and seas when the excessive concentrations of phosphorous and nitrogen lead to oxygen depletion (Paine, 2012).

Biofertilizers are active microorganisms that when applied to seeds, plant parts, or soil, stimulate growth by supplying nutrients, enhancing nutrient uptake capacity of the plants, and increasing root biomass (Vessey, 2003). Compared to traditional chemical fertilizers, biofertilizers are more efficient, cost effective, and could colonize in the rhizosphere to promote plant growth as well as to control plant diseases. The biofertilizers

market has been flourishing and has received substantial attention in the last decade as more biofertilizers products have been marketed and used in agriculture.

Biostimulants act as biofertilizers, and microbial inoculants that promote plant growth and yield are one category of biofertilizers (Calvo, 2014).The European Commission defined Plant Biostimulants as " microorganisms or material which contains substances whose functions on plants or in the rhizosphere to advantage nutrient uptake, nutrients efficiency, abiotic stress tolerance, crop quality, and freely of its nutrient content" (Anonymous, 2013b). The value of the biostimulant market in North America was estimated at $270 million in 2013, with projections to increase to $490 million by 2018, representing an annual growth rate of 12.4% (Anonymous, 2013a).

Ba and *Bap* are PGPR that has been using widely for the biofertilizers in multiple crops. *Bacillus amyloliquefaciens* (*Ba*) is a species of rod-shaped, Gram-positive, endospore-forming bacteria that is commonly isolated from soils. There are two subspecies of *Ba*; *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* (*Baa*) and *Bacillus amyloliquefaciens* subsp. *plantarum* (*Bap*). The *Bap* rhizobacteria with pectin supplement can be used as biofertilizers to promote plant growth. *Bap* has specific pectin-utilizing genes that can catabolize pectin present in plant roots and the rhizosphere, thereby using pectin for bacterial growth through the secretion of enzymes. (Wu et al., 2015) reported that pectin increased biocontrol effectiveness of *Bap* against *Ralstonia solanacearum* in tobacco plants by secreting the secondary metabolite surfactin. However, this study overlooked the involvement of pectin specific genes. Catabolism and utilization of pectin depend on different genes that can degrade, transport, and metabolize in order to support the rapid growth of bacteria. Expression of the *exuT* and *uxuB* genes is essential for pectin degradation, and it is possible expression hinges on the availability of D-glucuronate or D-galacturonate as carbon sources. *ExuT* is a pectin-associated gene that transport pectin compounds into the bacterial cell

encoded by Hexuronate permease enzyme (Mekjian et al., 1999a). *uxuB* is a metabolic gene that metabolize pectin compounds by bacteria as carbon and energy sources encoded by D-mannonate oxidoreductase enzyme (Nemoz et al., 1976). Elucidating the mechanism of pectin utilization for bacterial growth could aid in promoting plant growth through enhanced root colonization.

The objectives of this research are to understand better the pectin degradation mechanism through pectinolytic enzymes of *Bap* PGPR strains and how enzymes interact with bacterial colonization of plant roots to promote plant growth. This study also aims to explore the phenotypic effects of particular genes associated with degradation and utilization of pectin on plants.

1.1. PGPR background

Plant growth-promoting rhizobacteria (PGPR) are root-colonizing bacteria that enhance plant growth when applied to seed surfaces (Kloepper, 1978). “Rhiza” is a Greek word that means root, and “rhizosphere” refers to the area around the root where microbial growth is influenced by root exudates (Hiltner, 1904). The rhizosphere can be further subdivided into ectorhizosphere (outer rhizosphere) and endorhizosphere (inner rhizosphere) (Balandreau, 1978). Rhizobacterial species such as *Azospirillum, Azotobacter, Bacillus, Enterobacter, Pseudomonas, Serratia, Klebsiella,* and *Xanthomonas* contain strains which function as PGPR based on their capacity to promote plant growth (Khalid et al., 2004). Among them, *Bacillus* species are the most widely used for plant growth-promotion and biocontrol activity because of their endospore- forming capacity, which allows them to be formulated into seed treatments with a long shelf life (Li and Alexander, 1988). *Bacillus* species are also non-pathogenic and do not have any deleterious effects on tubers, plants, or seeds (Niazi et al., 2014a).

1.2. Effects of PGPR: biological control and growth promotion

Nonpathogenic, plant-associated bacteria are categorized as beneficial, neutral, or deleterious based on their impact on plants (Dobbelaere et al., 2003). PGPR is those beneficial bacteria that promote plant growth or exhibit biological control of various plant pathogens such as pathogenic bacteria, viruses, fungi, and nematodes. The use of PGPR for biocontrol has been extensively studied in *Pseudomonas stutzeri*, which lyses the mycelia of *Fusarium solani* (Sivasakthi, 2014), and in *Bacillus subtilis* isolates Sb4-23, which reduces *Meloidogyne incognita* nematode egg populations (Adam et al., 2014).

Some PGPR strains mainly exhibit biocontrol. Others mainly elicit plant growth promotion. However, the two effects can also be elicited by a specific PGPR strain. For example, *Bacillus subtilis* strain FZB24 (Kilian, 2010) and *Bacillus* sp. BPR7 (Kumar et al., 2012) have shown growth promotion, induced resistance, and antagonist activity simultaneously. These effects could be explained by the secretion of the same or different secondary metabolites of rhizobacteria that exert growth promotion and biological control. Some metabolites produced by PGPR can promote plant growth directly without communication with existing soil microflora, a phenomenon referred to as direct growth promotion (Kloepper et al., 1989). However, studies have shown that fluorescent Pseudomonad PGPR did not promote plant growth in gnotobiotic conditions (Kloepper, 1981a). Growth promotion in field soils was related to the elimination of fungi and the reduction of 23-93% gram-positive bacterial populations (Kloepper, 1981b). (Weyens, 2011) found that *Pseudomonas putida* wild-type strain W19 demonstrated root colonization and growth promotion, but gfp labeled W19 had an adverse effect on Poplar plants (*Populus deltoides*). These studies suggest that growth promotion by PGPR strains is often related to biological control.

1.3. Concept of root colonization

Hiltner (1904) coined the term “Rhizosphere” to describe the region of soil around the plant roots. Plants and microorganisms interact with each other in the rhizosphere where colonization takes place (Bolton, 1992). Microbial interaction also occurs in two other regions: the endorhiza (Egamberdieva, 2015) and the phyllosphere. However, regarding root colonization, the endosphere and phyllosphere are less important regions than the rhizosphere. The rhizosphere plays important roles in the production of root exudates, the proliferation of rhizobacteria, and promotion of root growth (Ahmad, 2011).

The bacterial community in soil is shaped by many factors such as soil particle size (Ranjard et al., 2000), soil structure (Ranjard et al., 2000), mineral composition (Carson et al., 2009), and agricultural practices (Rooney, 2009). Soil microorganisms compete with other microbiota for nutrients, and the type of this competition is influenced by plant roots (Bais, 2006). Rhizodeposition is a process in which high and low molecular weight chemical compounds, such as amino acids, sugars, and organic acids, are exuded into the rhizosphere from the various regions of the root (Bais, 2006). The rhizosphere is a vital niche in which bacterial populations colonize roots and increase nutrient uptake.

Various microbial populations, such as bacteria, fungi, yeasts, and protozoa, are available in the rhizosphere (Ahmad, 2011). Bacteria and fungi predominate in the soil rhizosphere. Cyanobacteria, Actinobacteria, Bacteroides, Firmicutes, and Proteobacteria are the main groups of phyla in which PGPR can be found (Egamberdieva, 2015). *Bacillus sp.*, *Pseudomonas sp.*, and *Azospirillum sp.* are the bacterial genera that are most often involved in growth promotion and biocontrol.

1.4. Importance of root colonization

Root colonization is the dynamic process whereby bacteria inoculated into the soil or on the seed surface and proliferate in the spermosphere in response to root exudates (Kloepper et al., 1985) in which root-colonizing, rhizobacterial species promote plant growth and biocontrol. Plant variety and species have different responses to root colonization. For example, root tips are the initial colonizing regions in faba beans (*Vicia* faba)plants for the formation of l root nodules by N-fixing *Rhizobium leguminosarum* (Desbrosses, 2011). In Poaceae plants, root hairs, and lateral roots are two regions where root colonization are efficiently higher (Combes-Meynet, 2011).

Successful growth enhancement depends on proper root colonization in the rhizosphere of plant roots. Sometimes more successful growth promotion of plants indicates the better root-colonizing capacity of rhizobacteria. Different rhizobacteria secrete various secondary metabolites in the rhizosphere which can enhance plant growth promotion. Multiple genes are involved in the formation of secondary metabolites, and these genes can be activated by nutrients from the root exudates. Root colonizing parameters are associated with different abiotic factors such as temperature, soil moisture, pH, and soil type (Ahmad, 2011). Root exudates release various organic compounds such as sugars, amino acids, and organic acids (Curl, 1986) that interact with microbial inoculants to enhance plant growth. In this fashion, rhizobacteria compete with microbes that previously existed in the soil for successful root colonization and plant growth promotion (Waard, 1993).

1.5. Need to increase root colonization

Inoculation of rhizobacteria on the seed coat initiates root colonization in the rhizosphere and rhizoplane (F. O'Gara, 2007). Colonization varies according to seed coats, temperatures, soil texture, and bacterial genera. Root colonization is the gateway for the interaction between the host plant and beneficial bacteria (Ahmad, 2011). Some plant seeds have a rigid and rough surface, and some are smooth and thin. Rhizobacteria have an attaching capacity that can easily attach to the seed coats, leaf surfaces, or any other plant parts. Colonization of the absorbing seed leads to future root colonization (Sylvia, 1999). Successful colonization on the seed surface leads to improves germination through the release of secondary metabolites in the rhizosphere.

Screening of PGPR strains based on their capacity to uptake different biochemical nutrients uptake capacity of bacterial species is another potential approach to improving root colonization. Specific genes and multiple bacterial traits are involved in root colonization. For example, the *Put* genes of *P. putida*, which are involved in proline uptake and utilization are influenced by root exudates of maize (Chabot et al., 1996).

1.6. Root colonization and growth promotion activity of *Bap* strains

The world-wide use of plant growth-promoting rhizobacteria (PGPR) has been increasing in an effort to increase crop production. *Bacillus amyloliquefaciens* subsp. *plantarum* is a gram positive endospore forming rod shaped bacteria that can colonize plant roots, promote plant growth, and enhance biocontrol activity by indole -3-acetic acid (IAA) (Idris et al., 2007), volatile compounds (Borriss, 2011), and secretion of secondary metabolites (macrolactin and difficidin) (Hossain et al., 2015). The growth-promoting potential of some PGPR is highly specific due to plant species, race, cultivar, and genotypic variability (Bashan, 1998; Lucy et al., 2004).

Various strains of *Bap* such as FZB42 (Qiao et al., 2014), YAU B9601-Y2 (Hao, 2012), and UCMB 5033 (Niazi et al., 2014b) have been reported to promote plant growth in diverse crops. *Bap* is a plant-associated bacterial species that has shown potential against soil borne pathogens and has stimulated growth on oilseed rape (*Brassica napus*) (Danielsson et al., 2007). *Bap* UCMB 5033 strains have 3,912 protein coding genes that promote growth promotion activity through stimulating compounds and biocontrol activity (Niazi et al., 2014b). *Bap* can promote plant growth by secreting phytohormones, volatile compounds (Borriss, 2011), and secondary metabolites (Koumoutsi et al., 2004; Schneider et al., 2007; Scholz et al., 2011). Some secondary metabolites can also control soil pathogens, so many *Bap* strains also function as biocontrol agent in agriculture (Borriss, 2011).

Hexuronate transporter (*exuT*) and D-mannonate oxidoreductase (*uxuB*) degradative pathway have been reported in Bap B9601-Y2 strains (He et al., 2012). It transports carbon via *exuT* gene from rhizosphere D-glucuronate or D-galacturonate to bacterial cell and moves forward through *uxuB* gene. Fructuronate changes into mannonate via *uxuB* gene in the bacterial cell to assist in the metabolic process. These bacteria have PGPR properties and can uptake glucose, fructose, and mannose as carbon sources (Danielsson et al., 2007).

2. Source of pectin

Pectin discovered by Henri Braconnot in 1825, it is a major heteropolysaccharide of primary cell walls such as cellulose and hemicellulose in plants. The primary cell wall is involved in cell to cell interactions, cell growth, and regulation of metabolic rates. In Sycamore, the primary cell wall is composed of 34% pectin, 24% hemicellulose, 23% cellulose, and 19% hydroxyproline-rich glycoprotein (Darvill et al., 1980). Pectin is also found in the middle lamella between cells in which it binds cells together. Further, availability of pectin compounds varies from plants to plants due to structure, amount, and composition (Srivastava and Malviya, 2011).

Pectin also occurs in other plant parts such as fruits, leaves, and roots apical region. No research has been done to compare the pectin concentration among the various parts of plants. The pectin levels are highest in the middle lamella of the cell wall and decrease in concentration gradually towards the plasma membrane (Kertesz, 1951). Galacturonic acid and glucuronic acid groups of pectin may be free or combined with sodium, potassium, calcium, and ammonium salts (Anonymous, 2001). Pectic materials are more commonly found in root hairs, and somewhat thicker layers found in loamy soils than in sandy soils (Howe, 1921). The duration of pectic components in roots is unknown, but it is possible that their availability depends on bacterial enzymatic activity in the soil rhizosphere.

3. Pectin degradation capacity in microorganisms

Pectin degradation occurs through pectinolytic, or pectate lyase enzymes referred to as pectinases, which are available in plant pathogenic bacteria, fungi, and higher plants (Namasivayam et al., 2011). Pectate lyase enzymatic activity was first reported in the plant pathogenic nematode *Globodera rostochiensis* (Popeijus et al., 2000). Plant pathogenic and non- pathogenic bacteria that secrete pectate lyase to degrade pectin and form a clear zone around the colony *in vitro*. Clear zones have been reported in *Bacillus cereus* isolated from the market solid waste in India (Namasivayam et al., 2011). Bacteria release pectate lyase enzymes when needed to degrade pectic materials and uptake D-glucuronate or D-galacturonate as a carbon source for nutrient supplements. Raw agricultural products and soils are related to pectin-degrading microorganisms, and up to 10% microorganisms in soils have been studied for their pectinolytic activity (Hankin, 1974).

(Hugouvieux-Cotte-Pattat et al., 2014) described eight families of polysaccharide lyase: PL1, PL2, PL3, PL4, PL9, PL10, PL11, and PL22. Three extra-cellular pectate lyases of *Dickeya dadantii* are included in PL1 (Lietzke et al., 1994; Yoder et al., 1993). PL2 and PL3 families have widely available in *γ-Proteobacteria* and plant-associated bacteria (Hugouvieux-Cotte-

Pattat et al., 2014). Pectinolytic activity has been shown in the bacterial genera of *Achromobacter, Arthrobacter, Agrobacterium, Bacillus, Clostridium, Erwinia, Pseudomonas, and Xanthomonas* (Rombouts, 1972; Voragen, 1972). *Erwinia chrysanthemi, Bacillus subtilis,* and many other bacteria convert pectin into oligogalacturonate by extracellular pectinase enzymes (Hugouvieux-Cotte-Pattat, 1987). Oligogalacturonate can be converted to D-galacturonate in *Erwinia sp.* through the enzyme oligogalacturonate lyase (Collmer, 1981). Free glucuronate and galacturonate enter *Escherichia coli* and *Erwinia sp*. bacteria by the hexuronate transporter (*exuT*) system that encodes the enzyme hexuronate permease. A *uxuB* gene encodes D-fructuronate oxidoreductase enzyme, which degrades intracellular glucuronate and galacturonate into 2-keto 3-deoxygluconate (KDG) in *E. coli* and *Erwinia chrysanthemi* bacteria (Hugouvieux-Cotte-Pattat et al., 1994). *exuT* and *uxuB* gene expressions occur when bacteria can uptake pectin derived from carbon sugar through pectinase enzymes from pectin, deemed as pectin-derived, carbon- induced genes. 2-keto 3-deoxygluconate (KDG) later metabolize into pyruvate and 3-phosphoglyceraldehyde.

4. Importance of pectate lyases

Bacteria release pectate lyase enzymes to macerate pectin components in plant cell walls. Pectin components are complexly interwoven to make plant cell walls rigid. Plant-pathogenic bacteria invade in the cell wall, degrade, and disrupt the biomolecular activity of plant cells inside the region in contrast to pathogens beneficial plant-associated bacteria do not invade in the plant cell wall. They degrade pectin compounds to help the cell wall expansion and to maintain the nutrients uptake from the rhizosphere. Hence, in nonpathogenic bacteria like *Bap* pectate lyases contribute to promoting plant growth and root colonization.

Necrotrophic plant pathogens are pathogens that attack the host cells and feed on the nutrient contents to survive in the environments (Li, 2013). Pectinolytic bacterial pathogens are

necrotrophs that degrade plant cell wall components leading to cell death. These pathogens can also produce other enzymes such as cellulases, hemicellulases, xylanases, and proteases (Kotoujansky, 1987). Various enzymes are involved in attacking α- l ,4-glycosidic linkages in pectate (Collmer, 1981). Pectate lyases (Pels) split glycosidic bonds using β-elimination to produce unsaturated products and polygalacturonase (Peh) split using hydrolysis to produce saturated product (Barras et al., 1994). Anaerobic conditions on tubers are suitable for breaking the host resistance systems such as phytoalexins, phenolics, and free radicals. Also anaerobic conditions prevents cell wall lignification and suberization that can safeguard against pectic enzyme degradation (Pérombelon, 2002). Pathogenic bacteria that cause soft rot of potato grow more profusely than any other soil-borne pectobacteria that produce pectic enzymes under field conditions (Pérombelon, 1979). *Dickeya* and *Pectobacterium* genera (Czajkowski et al., 2011) are the most important plant pathogenic agents of soft-rot disease that use endo-pectate lyase as their foremost virulence determinants (Hugouvieux-Cotte-Pattat et al., 2014). Pectic enzymes disrupt cell wall integration and the resulting leakage helps to grow bacteria profusely with tuber turgidity (Pérombelon, 2002).

5. Carbon uptake into bacterial cell

Carbon is the major component of the bacterial cellular material and contains 50% dry weight from the organic compounds or carbon dioxide sources are required for energy generation and biosynthesis (Kenneth). Bacteria need carbon to grow and survive in adverse conditions. They express different genes based on carbon availability. Bacterial cells receive nutrients from outside sources, transport, and breaks into smaller forms from complex molecules as if bacteria could grow and induce electrons from a carbon source to NADH (Bochner et al., 2001).

D-glucuronate, D-galacturonate, and D-mannose are produced from polymethyl galacturonate or pectin that are components of plant cell walls and are also found in soil

(Mekjian et al., 1999a). These three monosaccharides are hexose sugars that consist of six carbon atoms and the aldehyde is present at position one. Glucuronate, galacturonate, and mannose can be used as a primary carbon and energy source by *Bacillus subtilis, E.coli*, and *Erwinia chrysanthemi* (Hugouvieux-Cotte-Pattat, 1987; Mekjian et al., 1999a).

Plant roots discharge multiple organic components such as sugars, amino acids, vitamins, organic acids, thereby create a congenial environment for bacteria colonization (Badri et al., 2009; Bais et al., 2006; Shukla et al., 2013). Roots release enormous amounts of carbon in the rhizosphere, and it can be 10^{10}/g of soil (Gans et al., 2005; Roesch et al., 2008). *Bap* uses these sole carbon sources for growth of a bacterial cell, and it can contribute to crop yield to ameliorate soil environment around the roots of plants. In this way, it can be concluded that degradation of pectin, transport, and utilization by *Bap* strains pectin-associated enzymes could enhance root colonization, plant growth-promotion, and biocontrol activity. Pectin utilizing activity is only conserved in Bap strains, but absent in *Bacillus* group (Hossain et al., 2015). Due to this reason, understanding the carbon utilizing activity in Bap strains in crucial to study root colonization, growth-promotion, and disease control.

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Chapter II Screening of bacterial strains with gyrase subunit B (*gyrB*), *exuT and uxuB* primers

Abstract

Universal primers for *gyrB* were used to screen 79 strains of *Bacillus* species. The genomic DNA from each strain was extracted, and the PCR product of *gyrB* was amplified and purified for gene sequencing. Gene sequences were edited, and aligned, and *Bap* strains were identified based on the *gyrB* phylogenetic tree. Two primers (*exuT* and *uxuB*) were designed to detect pectin-associated transporter gene *exuT* and D-mannonate oxidoreductase gene *uxuB*. The Bap strains were then screened by PCR amplification for the presence of the two pectin-utilizing genes *exuT* and *uxuB.* Results showed that *exuT* gene was detected in 57 *Bap* strains and *uxuB* gene was detected in 54 *Bap strains.* In addition, *in vitro* tests were conducted on 59 *Bap* strains using pectin as a sole carbon source to confirm the pectate lyase enzymatic activity of *Bap* strains on pectate agar (PA) and pectin utilization on Tris-Spizizen Salts (TSS) medium.

1. Introduction

Determining the differences of *Bacillus* species among closely related groups using 16S rRNA gene sequence is challenging because of their high sequence similarity (Wang L, 2007)(Wang L, 2007)(Wang L, 2007)(Wang L, 2007)(Wang L, 2007)(Wang L, 2007)(Wang L, 2007)(Wang L, 2007)(Wang L, 2007)(Wang L, 2007)(Wang L, 2007)(Wang L, 2007) *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* and *Bacillus amyloliquefaciens* subsp. *plantarum* are two subspecies of Ba that cannot be distinguished by 16S rRNA gene sequence for their less DNA sequence dissimilarity. DNA gyrase subunit B and type II topoisomerase are referred to as *gyrB* that exerts a vital role in DNA replication is widely distributed in bacterial species and subspecies level (Huang, 1996). The *gyrB* gene provides accurate and faster results than 16S rRNA gene sequence to identify bacterial species and subspecies (Yamamoto et al., 1999). The *gyrB* gene sequences from different bacterial groups such as *Pseudomonas* (Yamamoto et al., 1999), *Acinetobacter* (Yamamoto et al., 1999), *Mycobacterium* (Kasai et al., 2000), *Bacillus thuringiesis* (La Duc et al., 2004), and *E.coli* (Fukushima et al., 2002) have been used for phylogenetic tree analysis. However, 16S rRNA gene sequence is also helpful for the identification of bacterial species (Joung, 2002). Screening of *Bacillus* species using *gyrB* gene sequence is an initial process to identify bacteria at the subspecies level.

Bacillus species utilize diverse carbon compounds from the soil rhizosphere for the survival, energy, and growth in adverse environmental conditions. *Bacillus subtilis* (Mekjian et al., 1999b) and *B. amyloliquefaciens* subsp. *plantarum* (Hossain et al., 2015) uses galacturonate and glucuronate from the pectin compounds as a sole carbon source. *ExuT* and *UxuB* are a transporter and metabolic protein that helps in carbon transport and utilization in bacterial cell wall. These two sugars were first discovered in the bacteria (Ashwell, 1962) and reported as an aldohexuronate transport system in *E. coli* (Nemoz et al., 1976). Galacturonate

and glucuronate carbon compounds enter into the bacterial cell wall and metabolize by *ExuT* and *UxuB* enzymes. *ExuT* is the only transporter system that transports carbon into the bacterial cell. In this way, pectin degrading and utilizing *ExuT* and *UxuB* enzymes detectin by PCR are very crucial for the carbon source utilization by *Bap* PGPR strains.

The overall goal of this part of the research was to screen 79 *Bacillus* species for identification of Bap strains and for the presence of pectin- utilizing genes (*exuT* and *uxuB*) identification in 59 *Bap* strains. The objectives of this study were i) to identify *Bacillus amyloliquefaciens* subsp. *plantarum* with *gyrB* primers (UP-1 & UP-2r), and ii) to identify *exuT* and *uxuB* genes with *exuT* and *uxuB* primers in the *Bap* strains.

2. Materials and methods

2.1. DNA extraction of *Bacillus sp.*

A total of 79 *Bacillus* strains were collected and subcultured on TSA plates at 28°C for 24 hr. Profusely-grown bacteria were harvested for genomic DNA extraction using E.Z.N.A. ® DNA Isolation Kit and DNA concentration measured by NanoDrop UV-Vis Spectrophotometer. *Bacillus* strains were screened with primers for *gyrB*, *exuT*, and *uxuB*.

2.2. PCR amplification, gel electrophoresis, and purification of PCR product of *gyrB* gene

Master Mix (2X) was prepared by several components such as *Tag* DNA polymerase, dNTPS, and $Mgcl_2$. Other components such as forward primers, reverse primers, DNA template and nuclease-free water were also added for Master Mix preparation. Touchdown PCR was used to avoid nonspecific sequence amplification, and UP-1 and UP-2r *gyrB* universal primers were used (Yamada et al., 1999).The first step of Touchdown PCR was a separation of DNA double strand through the heating process at 95^0C called denaturation for 30 seconds. The second step was lowering the temperature at 65^0C to allow primers to anneal to complementary sequences for 30 seconds, and the third step was to synthesize of DNA strand through DNA polymerase enzymes to create double strand called primer extension at 72^0C for 40 seconds. These steps were repeated 15 times. In the second step, the annealing temperature was changed to 50^0C. The remaining temperatures were the same and were repeated 30 times. The final stage was a primer extension at 72^0C for 5 minutes. Eppendorf Thermal Mastercycler (Eppendorf AG, Hamburg) was used for Touchdown PCR cycles. Agarose gel (1%) and 1X Tris Acetate EDTA (TAE) buffer were used for gel electrophoresis. The voltage was 110V for 35 minutes. The Agarose gel was stained with Ethidium bromide for 10 minutes. And was then properly de-stained for 5 minutes to remove ethidium bromide. The gel images were photographed using the AlphaImager® HP high-performance imaging System. PCR product was purified by E.Z.N.A. Cycle Pure Kit. The cleaned PCR reactions

(5 µl) were taken from 40 µl PCR reaction. One µl of the forward and the reverse primers from 20 µM primers were separately added to 5 µl of the cleaned PCR reaction, The total PCR reactions were 6 µl for forward and 6 µl for reverse primers. The cleaned PCR reaction samples were sent to Lucigen Corporation (Middleton, WI) for sequencing. Genomic sequences were edited and analyzed by Chromas Pro software in FASTA format and aligned by CLC Genomic Workbench software.

2.3. *gyrB* phylogenetic tree construction

MEGA (v6.1) software was used for construction of the *gyrB* phylogenetic tree. ClustalW alignment and Maximum likelihood statistical method were used for gene sequence alignment and phylogenetic tree. The *Bap* and other Bacillus sp. sequences were collected from Genbank and aligned with 76 strains to compare among the closely related species and subspecies. Bap sequences of gene bank and collected strains were compared for their similarities and dissimilarities. 2.4. exuT and uxuB primer design

2.4. *exuT* and *uxuB* primer design

Hexuronate transporter (*exuT*) primers 211F and 1070R were supplied by Dr. Mark Liles. D-mannonate/D-fructuronate oxidoreductase (*uxuB*) Primers 60F and 696R were designed for identification of Bap strains. Two-gene sequences (*exuT* and *uxuB)* of various Bap were collected from NCBI Genbank for primer design. Primer designer V. 2.0 software was used for *uxuB* primers. After designing the *uxuB* primers, the primers'parameters were checked by the online-based prediction tools sequence manipulation suite software interface (Stothard, 2000).

2.5. PCR amplification and gel electrophoresis of *exuT* and *uxuB* genes

The touchdown PCR method was used to avoid nonspecific sequence amplification. The parameters for touchdown PCR conditions used were the same as used for a *gyrB* gene sequence.

3. Results

3.1. DNA extraction of *Bacillus sp.*

The highest DNA concentration was 194.5 ng/µl in *Bap* strain AP218, and the lowest DNA concentration was 4.3 ng/µl in Bap strain AP52. The average DNA concentration was 44.21 ng/µl.

3.2. PCR, Gel electrophoresis, and Purification of PCR product of gyrB gene

The PCR DNA product size was ~1.2kb, and *gyrB* gene amplified successfully using UP-1 and UP-2r universal primers.

3.3. gyrB phylogenetic tree construction

Out of 79 *Bacillus* strains, 59 strains were identified as *Bap* strains. The remaining of the 19 bacterial strains were identified *B.mojavensis*, *B.sonorensis*, *B.tequilensis*, *Bacillus subtilis*, and *B.pumilis*. None of the strains were found to cluster together with *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens*.

3.4. *exuT* and *uxuB* primer design

The best-predicted primers were GTTCTCTGTTCAGCAATG (60F), GAAAAGGCAAAAGACGAG (222F), CTTATTGGATCGGTCTGT (671R), and GTTTCATCGGTGTATGTG (696R). The 60F and 696R primers were selected for Bap strains screening. The sequence length of forward and reverse primers was 18. GC content (%) was 44.44, molecular weight (Daltons) was 5480.63, and nearest neighbor Tm (degrees

C) was 56.15 in 60F *uxuB* primers. GC content (%) was 44.44, Molecular weight (Daltons) was 5445.62, and nearest neighbor Tm (degrees C) was 56.21 in 696R *uxuB* primers.

3.5. PCR amplification and gel electrophoresis of *exuT* and *uxuB* genes

The PCR DNA product size was approximately 900 base pair (bp) and 600bp. The two (*exuT* and *uxuB)* gene were amplified successfully by using *exuT* and *uxuB* primers.

6. Discussion

The results from the *Bacillus* species genomic DNA extraction indicated that some of the strains have higher DNA concentration than other strains. Out of 79 *Bacillus* species, 16 had high DNA concentrations, and 30 had moderate DNA concentrations. The remaining strains showed low DNA concentrations. The length of the amplified *gyrB* gene had detected ~1.2kb, and previous studies have reported the same size in *Bacillus* species (Wang et al., 2007).

The length of *gyrB* PCR product results suggests that all the strains were members of eight species in the genus *Bacillus.* species. The *exuT* gene was identified in 56 of the 59 tested Bap strains, and the *uxuB* gene was detected in 54 of the strains. Three *Bap* strains (AP102, AP204, and AP214) lacked the *exuT* and *uxuB* genes.

The *gyrB* phylogenetic tree results revealed that *Ba* DSM7, Ba LL3, TA208, and *Ba* XH7 *gyrB* gene sequences collected from NCBI database are clustered as a single clade separated from the *Bap* strains. Hence, the four strains were a distinct subspecies of *Ba*, and none of the *Bacillus* species strains were associated with *Ba*. A total of 59 strains were grouped together with the reference strains indicating that they were *Bap* strains with high bootstrap values. A bootstrap replication value was used for constructing the *gyrB* phylogenetic tree. Bootstrap uses for making inferences and robustness, similar to the use of p values in statistical analysis (Holmes, 2003). Bootstrap value 95% or higher for a given branch indicates topology correctness at the branch (Nei, 2000). AP 82 and *B. cereus* were sister groups that indicate closest relatives in 99% bootstrap replications. *B. pumilus* and AP 70 were sister groups and AP 100 was the outgroup in it. This type of relationship indicates monophyletic

clade. Monophyletic clade consists of ancestor and descendants. Similarly, strong bootstrap support was found for *B. subtilus, B. mojavensis,* and *B. tequilensis*. *B. mojavensis* and AP242 were sister group with 99% bootstrap value. In this way, 79 *Bacillus* species were compared among reference strains collected from NCBI database.

ExuT is a hexuronate transporter protein coding gene that can transport free extracellular galacturonate and glucuronate (Eichenberger et al., 2003; Feucht et al., 2003; Mekjian et al., 1999b) from the rhizosphere. This transport system is a vital part of the microorganism using exuT gene to receive carbon from the soils. Detection of *exuT* gene in *Bap* strains was important by PCR amplification methods. In the absence of *exuT* gene, *E.coli* and *Erwinia sp*. bacteria cannot degrade pectin compounds galacturonate and glucuronate (Mekjian et al., 1999b). *ExuT* gene is also known as *yjmA* and it is the prior steps of *uxaC* gene. On the contrary, exuT gene was absent in *Bap* strains AP102 and AP204. *ExuT* primers did not amplify *exuT* gene these two Bap strains by PCR methods. It might be false negative results of Bap strains AP102 and AP204. In this way, *Bap* strains may secrete hexuronate permease enzyme encoded by *exuT* gene and degrades pectin compounds from the rhizosphere in such way that it can transport in the bacterial cell wall.

Uxu is a symbol functions only as glucuronate metabolism (Nemoz et al., 1976) and uxuB involves in intracellular metabolic functions in *E.coli* and *Erwinia chrysanthemi* bacteria (Mekjian et al., 1999b). *UxuB* gene was detected in 54 Bap strains by PCR methods. The presence of uxuB gene in Bap strains indicate that it is capable to metabolize galacturonate and glucuronate carbon compounds for their energy and carbon source.

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Figure 1: *gyrB* phylogenetic tree of 79 *Bacillus sp.*

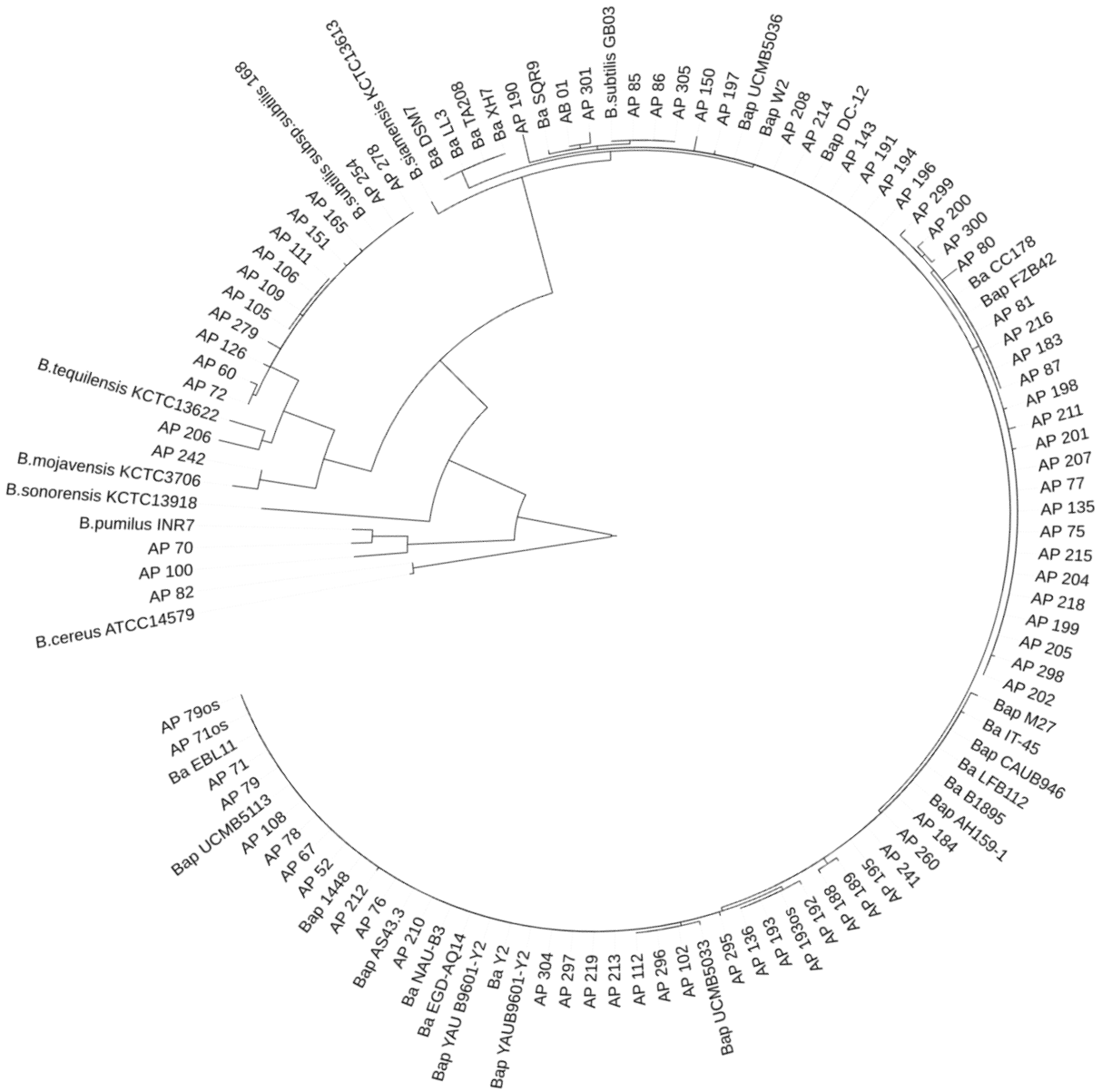

Table 1: List of PCR detected *exuT* and *uxuB* genes in *Bap* strains (+ = Present, - = Absent).

***Bap* strains**	***exuT* gene**	***uxuB* gene**
AP52	+	+
AP67	+	+
AP71	+	+
AP75	+	+
AP76	+	+
AP77	+	+
AP78	+	+
AP79	+	+
AP80	+	+
AP81	+	+
AP85	+	+
AP86	+	+
AP87	+	+
AP102	-	-
AP108	+	+
AP112	+	+
AP135	+	+
AP136	+	+
AP143	+	+
AP150	+	+
AP183	+	+
AP184	+	+
AP188	+	+
AP189	+	+
AP190	+	+
AP191	+	+
AP192	+	+
AP193	+	+
AP194	+	+
AP195	+	+
AP196	+	+
AP197	+	+
AP198	+	+
AP199	+	-
AP200	+	+

Table 2: List of PCR detected *exuT* and *uxuB* genes in *Bap* strains (+ = Present, - = Absent).

***Bap* strains**	***exuT* gene**	***uxuB* gene**
AP201	+	+
AP202	+	-
AP203	+	+
AP205	+	+
AP207	+	+
AP208	+	+
AP210	+	+
AP211	+	-
AP212	+	+
AP213	+	+
AP214	-	-
AP215	+	+
AP216	+	+
AP218	+	-
AP219	+	-
AP241	+	+
AP260	+	+
AP295	+	+
AP296	+	+
AP297	+	+
AP298	+	+
AP299	+	+
AP300	+	+
AP301	+	-
AP304	+	+
AP305	+	+

Chapter III Pectate lyase activity and pectin carbon uptake test *in vitro*; whole genome sequencing of *Bap* strains for a pectin defective pathways

Abstract

The pectin lyase activity of 59 *Bap* strains was tested *in vitro* on Pectate Agar (PA) and Tris-Spizizen Salts (TSS) medium. *Bap* strains were cultured on TSA medium and washed three times with sterile water before the inoculation on PA media. Higher and lower pectate lyase activity were observed in six (AP193, AP203, AP299, AP80, AP102, and AP52) and four (AP 194, AP214, AP215, and AP305) *Bap* strains compared to other Bap strains. A total of 12 *Bap* strains (AP67, AP71, AP77, AP78, AP85, AP102, AP108, AP135, AP143, AP189, AP192, and AP193) grew vigorously on TSS medium. A total of six *Bap* strains (AP194, AP204, AP214, AP216, AP219, and HD73) had lower growth compared to other *Bap* strains. Pectin (1%) were used for *in vitro* PA and TSS medium. Pectate lyase and utilization activity were not found in *Bacillus thuringiensis* subsp. *kurstaki* strain HD73 compared to *Bap* strains. A draft genome sequence for strains AP194 and AP214 that were lower for pectin utilization were generated using an Illumina MiSeq. Based on the results, it can be concluded that most of the *Bap* strains were capable of degrading and utilizing pectin as a sole carbon source. In addition, amino acid differences were found in *Bap* strains AP194 and AP214 that does not indicate they have a lack of function to degrade and utilize pectin.

1. Introduction

Pectate lyase enzyme was first discovered in *Erwinia carotovora* and *Bacillus polymyxa* in 1962 (Starr, 1962) and has since been reported in many plant pathogenic and non-pathogenic bacteria such as *E. aroideae* (Kamimiya et al., 1977); *E. chrysanthemi* (Starr, 1972); *Clostridium multifermentas* (Macmillan, 1964); *Fusarium solani* (Crawford, 1987), *B. amyloliquefaciens* (Fan X., 2008); and *B. subtilis* (Soriano et al., 2006). Mechanism of this enzyme activity in pathogenic and non-pathogenic bacteria could have a subtle difference in the mode of action. Pectate lyase breaks down polygalacturonate into D-galacturonate and D-glucuronate, making it available form for bacteria to use as a carbon source. Previous studies have reported that *B. subtilis* (Mekjian et al., 1999), *E. coli* K-12 (Nemoz et al., 1976), *E. carotovora* (Abbott, 2008), and *E. chrysanthemi* (Abbott, 2008) are capable of utilizing pectin as a sole carbon source and energy. Pectate lyase involves in plant cell wall degradation using β-elimination

mechanism and produced by plant pathogens, plant-associated bacteria, and hardly animals (Hugouvieux-Cotte-Pattat et al., 2014). Recent studies have shown that pectin enzymes involve in border cell separation (Hawes et al., 1998), reduction of bacterial wilt of tobacco (Wu et al., 2015), and Arabidopsis root colonization (Beauregard et al., 2013). Pectin utilizing activity is correlated to pectin degradation activity. Plant pathogenic bacteria uses pectate lyase to disintegrates cell wall and hamper plant cell walls metabolic activity. But, plant-associated bacteria separate the plant cell wall in order to receive pectin compounds for their survival and energy production. In this way, pectate lyase enzymes are vital for the root colonization, plant growth-promotion, and biocontrol.

Genome sequencing is a useful method to find out the high resolution, base by base view, gene expression, and regulation of the strains (Anonymous, 2016). Rapid Annotation using

Subsystem Technology (RAST) is designed to annotate the gene of complete prokaryotic genomes, and it uses the highest confidence first assignment that guarantees a high degree of genome consistency (Anonymous, undated). This study was designed to screen 59 *Bap* strains for pectate lyase and utilization of pectin as a sole carbon source. In addition, this study also included genome sequence analysis of two *Bap* strains AP194 and AP214 that were lower in the utilization of pectin as a carbon source. The purpose of this research was i) to screen a large collection of *Bap* strains for pectin degradation and utilization as a sole carbon source, ii) to conduct a comparative genomic analysis that includes *Bap* strains that have a low capacity for pectin utilization.

2. Material and methods

2.1. *Bap* strains preparation

A total of 59 *Bap* strains were streaked onto Tryptic Soy Agar (TSA) plates from the cryo stocks stored in the -80ºC and incubated at 28°C for 24 hours. One loopful of bacteria was inoculated into 10ml TSB in a glass test tube and placed into a shaking incubator at 28°C overnight using 220 Revolutions Per Minute (RPM) for 24-48 hours. Three replicates were used for each of the *Bap* strains.

2.2. Pectate lyase activity test

The bacteria grew from cryo stocks in the -80ºC on Tryptic Soy Broth (TSB) at 28°C overnight using 220 rpm for 5 ml culture. A one ml aliquot was pipetted into the 1.5 ml microcentrifuge tube, and centrifugation was done for 5 minutes at 10,000 x g speed. The supernatant was discarded, and the process was repeated three times using sterile water. In the final bacterial pellet, 1 ml of the sterile water was added to a microcentrifuge tube and vortexed

thoroughly to uniform the bacterial suspension. Then, it was transferred to 1 ml of the sterile water containing test tube to measure the turbidity of a bacterial suspension of all strains until the optical density at 600 nm was approximately 0.5. Twenty µl of this standardized bacterial suspension was used in triplicate onto pectate-agar (Pa) media (Kobayashi et al., 1999) to determine the pectin lyase activity. The pH 8.0 of 0.1M Tris-HCl buffer was adjusted for the medium and sterilized using 0.45 µm Nalgene syringe filter (Thermo Scientific, USA) separately. The pectate-agar media plates were incubated at 28°C for 24-48 hours and then 1% Cetyltrimethylammonium bromide (CTAB) was poured over the surface of the plate at room temperature.

2.3. Pectin carbon uptake test

The capacity of the 59 *Bap* strains to utilize as a sole carbon source were assessed using a minimal Tris-Spizizen salt (TSS) (Shingaki et al., 2003) as the base media supplemented with 1% citrus pectin (citrus source) (Tokyo Chemical Industry Co., Ltd). The TSS media was filter-sterilized using a 0.2 µm polyethersulfone (PES) vacuum filter unit (VWR, USA) and adjusted the medium p^H 7.0 using 10N NaOH. Each of the bacterial cultures was prepared for the pectin lyase assays with bacterial suspensions washed three times in sterile water, normalized to an OD_{600} = 0.5, and then 100 microliters (µl) of a 1:100 dilution were used to inoculate 1.9 ml TSS + 1% pectin cultures to adjust the OD_{600} = 0.030, in triplicate. Broth cultures were incubated at 28°C with 220 rpm, and OD_{600} readings were recorded over a 40 hr period. *Bap* strains AP 193 was used as the positive control and *Bacillus thuringiensis* subsp. *kirstaki* HD 73 was used as the negative control. *Bacillus thuringiensis* subsp. *kirstaki* HD 73 was obtained from the USDA-ARS culture collection (Ames, Iowa) that was identified as a non-pectin utilizing strain based on

its genome sequence. This strain was not observed to growth using pectin as a sole carbon source.

2.4. Genome sequencing of *Bap* strains

The genomic DNA of AP194 and AP214 was extracted by E.Z.N.A. ® DNA Isolation Kit and the DNA concentration was measured by Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA). Nextera DNA Library Preparation Kit (Illumina, Inc.) was used for Illumina MiSeq® sequencing. A total of 50 ul reaction (3.75 µl genomic DNA, 25 µl TD buffer, 5 µl enzyme, and 16.25 µl nuclease free water) was prepared using Nextera sample prep kit. The prepared reaction was vortexed and centrifuged at 10,000x rpm before the incubation for 5 minutes at 55°C. Nextera PCR program cycle was used for PCR amplification using Eppendorf Thermal Mastercycler (Eppendorf AG, Hamburg). The holding temperature was 10°C. Zymo Research DNA Clean & Concentrator™-5 was used for purifying the tagmented DNA. A total of 250 µl DNA binding buffer was used for the 50 ul sample (5:1) and centrifuged for 30 seconds using 10,000 x g. A total of 200 µl DNA wash buffer was added and centrifuged it for 30 seconds. This process was repeated twice. The empty column was used for drying the sample and centrifuged for 2 minutes. A total of 25 µl DNA wash buffer was added and kept room temperature for one minute. Then, 50 µl reactions (20 µl tagmented DNA, 5 µl indexes 1, 5 µl indexes 2, 15 µl PCR master mix, and 5 µl PCR primer cocktail) was prepared using Nextera prep kit. The temperatures for the PCR cycles were 12°C (3 min), 98°C (30 sec) for one cycle, 98°C (10 sec), 63°C (30 sec), and 72°C (3 min) for five cycles. The size-select purification kit was used for the cleaning of the 50 µl PCR products. The last step was a measurement of the DNA library concentration. DNA concentration was followed 16ng/µl, and 3.75µl genomic DNA were used for 60 ng concentration. Before running the MiSeq system, all instruments were cleaned using

0.1% Tween buffer. Then, all the samples were loaded to run the MiSeq® system. After successful running the system, fastq.gz files were generated and saved as output files. Then, fastq.gz output files were imported, trimmed, and de novo assembled using CLC Genomics Workbench.

2.5. RAST analysis

Rapid Annotation using Subsystem Technology (RAST) version 2.0 was used for annotation of the whole genome sequences for strains AP194 and AP214. FASTA-formatted sequence files of AP194 and AP214 were uploaded in RAST server. The annotated genome was viewed in a seed viewer link for AP194 and AP214.

2.6. tBLASTn and tBLASTx analysis

Pectin-associated gene sequences of *Bap* strains AP193, AP194, and AP214 were analyzed using CLC Genomics Workbench 4.9 (CLC bio, Cambridge, MA). Pectin-associated gene sequences of *Bap* strains AP193, AP194, and AP214 were aligned and compared to reference strain FZB42 on NCBI database.

2.7. Multiple alignment of *exuT* and *uxuB* protein gene sequences

Two pectin-associated genes (*exuT* and *uxuB*) sequences of 15 *Bap* reference strains were collected from the NCBI database. Each gene sequence was translated into an amino acid sequence using CLC Genomics Workbench 4.9 (CLC bio, Cambridge, MA, USA) software. Reference strains gene sequences were aligned with *exuT* and *uxuB* gene sequences of AP193, AP194, and AP214 strains.

3. Results

3.1. *Bap* strains preparation

A total of 59 *Bap* strains grew profusely within 24 hours in TSB and TSA plates. Most strains exhibited similar growth rate, but strains AP194, AP214, and AP52 grew slower than the other strain (Table 1).

3.2. Pectate lyase, utilization, and clear zone diameter activity of *Bap* strains

A clear zone appeared around bacterial colonies after 30 minutes. The magnitude of the zone of clearing was measured in milliliters (mm) and recorded in an Excel spreadsheet, with average zones of clearing determined for each of the *Bap* strains (Table 1). The clear zone images of each plate were photographed using the AlphaImager® HP high-performance imaging System.

The highest apparent pectate lyase activity was observed in AP193, AP203, AP299, AP80, AP102, and AP52. The lowest pectate lyase activity was observed in AP 194, AP214, AP215, and AP305. No pectate lyase activity was observed for strain HD73. The average pectate lyase activity was 35.45 mm.

The highest pectin utilization OD value at 600 nm was observed in *Bap* strain AP188. The lowest pectin utilization OD value at 600 nm was observed in *Bap* strain AP300.

Table 1. Pectate lyase, utilization, and clear zone diameter activity of *Bap* strains

***Bap* strain**	**CZD[1] (mm)**	**OD value**	**PUA[2]**	**PLA[3]**	***Bap* strain**	**CZD[1] (mm)**	**OD value**	**PUA[2]**	**PLA[3]**
AP52	52	0.36	++	+++	AP197	43	0.38	++	++
AP67	41	0.51	+++	++	AP198	40	0.35	++	++
AP71	32	0.51	+++	++	AP199	41	0.29	++	++
AP75	42	0.49	+++	++	AP200	44	0.24	++	++
AP76	40	0.39	++	++	AP201	40	0.33	+	++
AP77	38	0.55	+++	++	AP203	52	0.46	++	+++
AP78	39	0.49	+++	++	AP205	40	0.34	+++	++
AP79	52	0.4	++	++	AP207	35	0.24	+	+++
AP80	50	0.32	++	+++	AP208	56	0.38	++	++
AP81	39	0.35	++	+++	AP210	39	0.15	+	++
AP85	38	0.57	+++	++	AP211	38	0.2	+	++
AP86	35	0.4	+++	++	AP212	44	0.22	++	++
AP87	50	0.45	++	++	AP213	35	0.29	+	+++
AP108	39	0.66	+++	+++	AP214	34	0.2	+	++
AP112	42	0.52	+++	++	AP215	32	0.09	+	++
AP135	37	0.52	+++	++	AP216	25	0.38	++	+
AP136	39	0.44	+++	++	AP218	37	0.1	+	+
AP143	42	0.49	++	++	AP219	36	0.21	++	++
AP150	40	0.35	+	++	AP241	40	0.1	++	++
AP183	41	0.54	+++	++	AP260	33	0.17	+	++
AP184	35	0.6	+	++	AP295	40	0.18	++	++
AP188	55	0.72	+	++	AP296	45	0.11	+	++
AP189	40	0.37	+++	++	AP297	38	0.22	++	++
AP190	45	0.27	+++	+++	AP298	42	0.22	+	++
AP191	41	0.67	++	++	AP299	50	0.19	++	++
AP192	42	0.66	+	++	AP300	30	0.05	+	++
AP193	39	0.68	+	++	AP301	35	0.09	+	+++
AP194	36	0.33	+++	+++	AP304	30	0.2	+	++
AP195	38	0.36	+	+	AP305	22	0.11	++	++
AP196	43	0.34	++	++					

CZD[1] – Clear Zone Diameter, PUA[2] – Pectin Utilization Activity, PLA[3] – Pectate Lyase Activity

3.3. Genome sequencing of *Bap* strains

For *Bap* strains AP194 and AP214 a draft genome sequence was generated with 1,179,872 and 1,056,594 sequence reads resulting in 28.77x and 28.12x estimated genome coverage, respectively. Analysis of the genome sequences indicated a percent G+C content of 46.3% and 46.2%, an estimated genome size of 3.988 Mbp and 4.039 Mbp, the largest contigs were 688,671 bp and 486,108 bp, and the mean contig lengths were 86,333 and 65,067, respectively.

3.4. RAST analysis

For *Bap* strains AP194 and AP214 a RAST analysis were done with 59 and 51 protein-coding genes. RAST analysis indicated a number of subsystems were 462 and 462, the number of coding sequences were 4014 and 4060, the number of RNAs were 98 and 75, pectin-associated subsystems were 14 and 13, respectively.

3.5. tBLASTn and tBLASTx analysis

Table 2: tBLASTn (% identity) and tBLASTx (% identity) result for comparison of *Bap* strain AP193 against the genome sequence for *Bap* strains AP194 and AP214. The % identity for the top BLAST hit is indicated.

***Bap* Strains**	**Pectin associated genes**	**Tblastn (% Identity)**	**Tblastx (% Identity)**
AP194	*exuT*	94	96
AP214	*exuT*	94	96
AP194	*kdgA*	96	99
AP214	*kdgA*	96	99
AP194	*kdgK*	98	98
AP214	*kdgK*	98	98
AP194	*uxaA*	92	95
AP214	*uxaA*	93	97
AP194	*UxaB*	98	98
AP214	*UxaB*	98	98
AP194	*uxaC*	93	100
AP214	*uxaC*	94	99
AP194	*uxuA*	94	98
AP214	*uxuA*	94	98
AP194	*uxuB*	99	97
AP214	*uxuB*	98	98
AP194	*uxuR*	98	100
AP214	*uxuR*	98	99

3.6. ExuT and UxuB amino acid sequence alignment

In translation frame three of ExuT amino acid sequence alignment, glycine (G) amino acid was found in AP194 and AP214 *Bap* strains. However, cysteine (C) amino acid was found in the same translation frame of reference strains CC178, FZB42, Trigocor1448, CAU-B946, IT-45, and AS43.3. cysteine (C) also was identified in *Bap* strainAP193. Isoleucine (I) was found only in *Bap* strain AP214. Phenylalanine (F) was found in AP193, AP194, and other reference strains.

In translation frame one and two of ExuT amino acid sequence alignment, arginine (R) amino acid was found in *Bap* strains AP194 and AP214. On the contrary, glycine (G), and leucine (L) amino acid were found in the same translation frame of reference strains CC178, FZB42, Trigocor1448, CAU-B946, IT-45, and AS43.3. Arginine (R) also was found in *Bap* strain AP193.

In translation frame two and three of UxuB amino acid sequence alignment, isoleucine (I), tryptophan (W), methionine (M), tyrosine (Y), and histidine (H) were found in *Bap* strains AP 194, and AP214. However, threonine (T), cysteine (C), valine (V), leucine (L), and arginine (R) amino acid were found in the same translation frame of reference strains UCMB5113, UCMB5033, CC178, FZB42, Trigocor1448, CAU-B946, IT-45, NAU-B3, Y2, and AS43.3.

3. Discussion

The presented results indicate that all the *Bap* strains pectate lyase activity were not the same. It is possible that their enzyme secretion involves many different environmental factors in bacteria. The remaining 56 *Bap* strains growth increased noticeably after 6 hr of incubation and attained a moderate cell density. The pectate lyase test results demonstrate that all the *Bap* strains were capable of degrading pectin in the presence of 1% cetyltrimethylammonium bromide. Two *Bap* strains (AP194 and AP214), and one *Btk* strain (HD73) had the lowest and zero clear zones around the colony. Based on the results, it can be concluded that the highest clear zone showing strains have the highest pectate lyase activity. In contrast, the lowest clear zone showing strains had the lowest pectate lyase activity, and zero clear zone strain has no activity. Previous studies found that the clear zone formed in *Bacillus sp.* Strain KSM-P15 (Kobayashi et al., 1999) in 10 minutes. Another study has reported that hexadecetyltrimethylammonium bromide was used in the extracellular pectinolytic activity in freshwater ascomycetes to detect the clear zone around the colonies (Abdel-Raheem, 2002). However, the clear zone around the Bap strains colony were observed after 30 minutes.

Based on the pectin carbon uptake test, 12 *Bap* strains grew in 1% pectin within a minimal TSS medium. The remaining strains have shown lowest and average growth. *In vitro* pectin degradation and utilization results of *Bap* strains AP194 and AP214 indicates that their pectin degrading and utilizing activity were lower in PA and TSS media. Although, exuT and uxuB genes were detected in *Bap* strain AP194. Catabolite gene activator protein (CAP) in E. coli and catabolite control protein (CcpA) functions as an autoregulatory device that keeps sugar utilization at a certain level (Brückner, 2002). These two proteins may vary in vitro and in vivo

environment for the degradation and utilization of sugar by bacteria. It might be possible that these or other regulatory functions were responsible for the lower rate of pectin utilization in *Bap* strains AP194 and AP214 when grown in PA or TSS media. Previous studies have studies the complex genetic regulation of the lac operon that is required for lactose metabolism in E. coli (Ralston, 2008), and it is expected that the genetic regulation of pectin-utilization pathways in *Bap* strains is similarly complex. Based on the results, it can be hypothesized that pectin-associated functions necessary for degradation, uptake, and/or utilization were not expressed highly in the pectin media for the growth of *Bap* strains AP194 and AP214. The degree of expression of the pectin-utilization pathways in *Bap* AP194 and AP214 within the rhizosphere is unknown, and it is possible that these strains are fully capable of utilizing pectin as a carbon and energy source in plants.

Bioinformatics analysis of the genome sequences from *Bap* strains AP193, AP194, and AP214 have revealed that they have differences among them. Pectin pathway-associated subsystem features identified in three *Bap* strains AP193, AP194, and AP214 are 16, 14, and 13. However, pairwise BLASTx with different reference genome sequences has indicated that exuT and uxuB genes are present in three *Bap* gene sequences. Pectin-associated other genes were identified in three *Bap* strains AP193, AP194, and AP214.

tBLASTn and tBLASTx results have revealed that maximum identities (%) of pectin-associated genes in *Bap* strains AP194 and AP214 were almost similar compared to reference strain FZB42 and *Bap* positive control strain AP193. In the pectin-associated gene study, nine genes were found in *Bap* strains AP194 and AP214. This genomic comparison analysis showed that all the genes are available for the pectin degradation, transport, and utilization.

Based on the ExuT amino acid sequence alignment results (figure 1), it can be concluded that Glycine (G), and Arginine (R) amino acid differences exist in *Bap* strains AP194 and AP214 in comparison with reference strain FZB42 (Chen et al., 2007). This type of differences might affect the hexuronate transport of D-glucuronate and D-galacturonate chemical compounds into the bacterial cell. Lower pectin degradation and growth in PA and TSS media may be implied amino acid changes in *Bap* strains AP194 and AP214.

The UxuB amino acid sequence alignment results (figure 2) indicate that Isoleucine (I), Tryptophan (W), Methionine (M), Tyrosine (Y), and Histidine (H) amino acid changes occurred in AP194 and AP214 in comparison with reference strain FZB42 (Chen et al., 2007). This type of amino acid changes could hamper the overall metabolic activity of strains AP194 and AP214 in such way that it can reduce *Bap* strains growth in carbon source utilizing media. Amino acid variations in each protein on three different translation frames does not indicate that they are incapable of use pectin since that was not tested *in vivo*. Although *Bap* strains AP194 and AP214 had lower pectin degradation and utilization activity than other Bap strains, none of the genetic analyses indicated any defect in degradation, uptake or utilization. It may be that there is a regulatory change in these strains that results in lower expression of these functions, at least when studies *in vitro*, and that it is not known whether these two strains could use pectin with greater efficacy in the rhizosphere. Previous studies have reported that amino acid changes did not affect the cellular abundance of CodY gene in *B. subtilis* that controls dozens gene (Brinsmade et al., 2014). However, another study concluded that single amino acid changes could affect endotoxicity and expression level of protein in *B. thuringiensis* var. *Israelis* (Ward et al., 1988). In summary, it can be concluded that all of the *Bap* strains studied could use pectin as

a sole carbon source, which shows the importance of this for the metabolic activity of the *Bap* strains through multiple pectate lyase enzymes, and potentially for plant-bacterial interactions.

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Figure 1: Multiple amino acid alignment of *exuT* gene (AP193, AP194, and AP214) with reference strains.

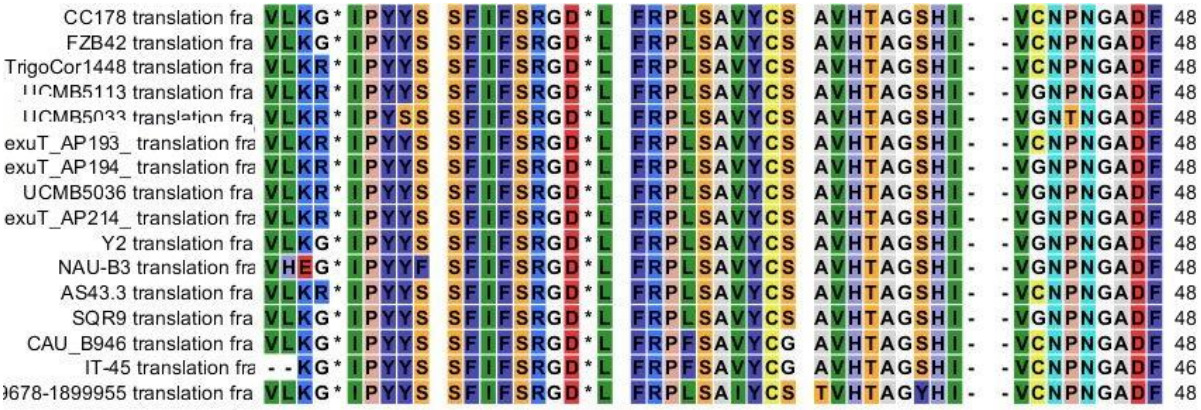
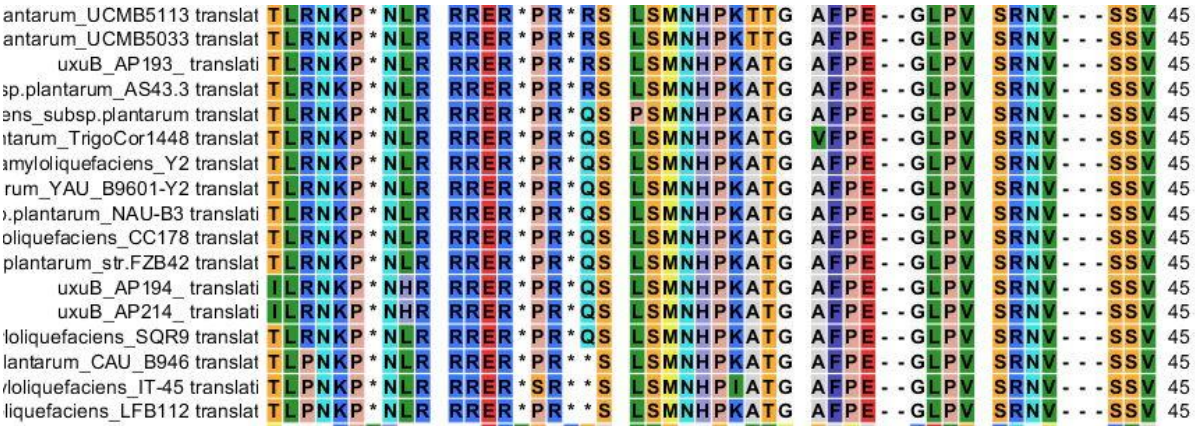


Figure 2: Multiple amino acid alignment of *uxuB* gene (AP193, AP194, and AP214) with reference strains.



Chapter IV Evaluating pectin amendments of soil for enhancing root colonization and growth-promotion of soybean by selected *Bap* strains

Abstract

Pectin amendments of soil were evaluated in the greenhouse for enhancement of root colonization and plant growth promotion of soybean by *Bacillus amyloliquefaciens* subsp. *plantarum* strains AP193 and AP143 and *B. thuringiensis* subsp. *kurstaki* strain HD73. Pectin supplement from the citrus peel source was mixed thoroughly into non-autoclaved field soil at a rate of 0.1 g pectin to 100 g soil. Rifampicin-resistant *Bap* strains were applied as seed treatments at log 6.0 rif^R mutants per seed at the time of planting. At four weeks after planting, soybean plants were removed, and effects treatments on root colonization, plant growth, and nodulation by indigenous *Bradyrhizobium* were recorded. Results indicated that root and shoot dry weights, and nodule formation were increased significantly by supplementing soil with pectin. Bap rif^R colonies were enumerated 5.98 and 6.04 log cfu/g in pectin treated AP193 and AP143 strains. *Bap* rif^R colonies were enumerated 5.80 and 5.78 log CFU/g in non-pectin treated AP193 and AP143 strains. The results support the hypothesis that pectin amendments to soil with selected pectinolytic *Bap* strains have plant growth-promotion activity.

1. Introduction

Root exudates include various low-molecular-weight organic compounds, including sugars, amino compounds, organic acids, fatty acids, and growth factors (Bolton, 1992), into the rhizosphere that are used as carbon and energy sources by PGPR inoculated onto seeds or into soils. *Bacillus* sp. secrete extracellular enzymes to degrade these organic compounds into available forms of nutrients for use as carbon and energy source for the plant growth. Carbohydrates are one of the key carbon and energy sources for microbial growth in the rhizosphere (Foster, 2012). Secretion of specific bacterial enzymes depends on plant-derived compounds that are actively released from the root caps, root tips, and sloughed-off cells. For example, rhizobacteria secrete different pectate lyase enzymes in the presence of pectin sugar compounds around plant roots. (Stephenson, 1994; Wen, 1999) reported that the pectin-degrading enzymes methyl esterases and polygalacturonases are responsible for the separation of border cells from the root tip. Border cells are sloughed-off from the root-cap cells into the rhizosphere during root growth (Driouich, 2010; Hamamoto, 2006). Khammas (1992) reported that co-inoculation of *Azospirillum sp.* with *Bacillus subtilis* or *B. polymyxa* enhanced nitrogen fixation as a result of nitrogenase activity (Khammas, 1992). Although the capacity of *Azospirillum sp.* to both degrade and utilize pectin would seem to be antithetical, (Plazinski, 1985) reported pectin degradation by *Azospirillusm sp.*, but Khammas (1991) and Myers (9187) did not find any evidence of pectin degradation by *A. brasilense*, *A. lipoferum*, *A. amazonense*, and *A. halopraeferens*.

Protopectin is the term for water-insoluble pectic substances in plant parts and consists of pectose and pectinogen (Lowe, 1938). Enzymes that convert protopetin to water-soluble pectic substances are called protopectinases (Sakai, 1987). Protopectinases have been reported in the yeast *Trichosporon penicillatum* strain SNO-3 and in several species of the genus *Bacillus*, including *B. subtilis* IFO 3108, 3134, 3336, 3513, 12112, 12113, 12210, 13719, 13721, 14117 and 14140, *B. amyloliquefaciens* IFO 14141, *B. cereus* IFO 3002 and 3132, *B. circulans* IFO 13632, *B. coagulans* IFO 12583, *B. firmus* IFO 3330, *B. lichenifomis* IFO 14206, *B. pumilus* IFO 12087, and *B. macerans* IFO 3490 (Sakai, 1989; Sakai, 1978). Protopectinases enzymatic activity has not previously been reported in *Bacillus amyloliquefaciens* subsp. *plantarum.*

Cells that are sloughed-off from the root caps as separate individual blocks or sheets of cells are termed border cells (Driouich, 2010; Hamamoto, 2006) and can serve as a nutrient source for rhizospheric bacteria. Rhizobacteria secrete different enzymes when different nutrient sources are present in the rhizosphere. For example, a rcpme1 gene encoded by pectinmethylesterase (PME) is involved in border cell separation (Hawes, 1998). Pectinmethylesterase released by rhizobacteria allows the bacteria to use pectic substances from the root caps as a carbon and energy source. (Hawes, 1992) reported that border cells in soybean roots are tightly attached with root mucilage in the absence of water, but in the presence of water, the border cells detached from the mucilage and enter the rhizosphere.

The rhizosphere region consists of plant roots and the surrounding soil that is affected by root exudates (Kenedy, 1999). In the rhizosphere, the plant root provides energy for growth of the soil microbiota, with bacterial population densities being 2-3 log units greater in the rhizosphere than in root-free soil (Arora, 2013) (Benizri, 2001). When a seed is planted, it

imbibes water and gives off many nutrients, called the spermosphere (Lynch, 1978; Verona, 1963; Kloepper et al., 1985). Soil microbes that become metabolically active in the spermosphere can continue growing in the rhizosphere.

The purpose of this greenhouse study was to evaluate pectin amendments to soil, together with selected pectinolytic *Bap* strains, for potential enhancement in root colonization, and the magnitude of *Bap*-induced plant growth of soybean plants.

2. Materials and methods

2.1. Selection of Bap and Btk rifampicin-resistant mutants

Bap strains AP193 and AP143, and *Btk* strain HD73 were streaked onto TSA plates for 24 hr to ensure the purity of each strain. One colony of each strain was then 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, USA) antibiotic was used for the selection of *Bap* and *Btk* mutants. To prepare the stock solution of 50 mg/ml rifampicin (Sigma-Aldrich, Product code 101594249, USA), 500 mg rifampicin was added to 10 ml dimethyl sulfoxide (DMSO). The stock solution was sterilized using 0.45 µm Nalgene syringe filter (Thermo Scientific, USA). After 24 hr, 50µg/ml rifampicin working concentration was added in 50 ml TSB bacterial culture media. The rif^R-TSB culture tube was wrapped with aluminum foil to prevent degradation of rifampicin by light and placed in a shaking incubator at 28˚C. After 48 hr, one loop from each rif^R-TSB culture tube was streaked onto a TSA+rif^R plate of each strain and placed into the incubator at 28˚C. Single colonies that grew on TSA+rif^R plates were removed, labeled as AP193 rif^R, AP143 rif^R, and HD73rif^R, and placed into the -80 freezer.

2.2. Determination of sporulation activity of *Bap* and *Btk* mutants

AP193 rif^R, AP143 rif^R, and HD73 rif^R –cultures were removed from the 80˚C freezer and streaked onto TSA plates. After incubation at 28˚C for 24 hours, one colony of each rif^R mutant was transferred into 20ml TSB containing VWR® Super Clear™ ultra-high performance centrifuge tubes separately for each of bacterial strain. Bacterial cultures were incubated for 48 hr at 28˚C using a shaking incubator (220 rpm) and were then centrifuged at 3600 x g for 5 minutes using Sorvall Legend RT Refrigerated Centrifuge (Thermo Scientific, USA). Supernatants of each bacterial culture were discarded, added to 5 ml sterile water, and vortexed to re-suspend the pellets in each culture tube. Bacterial cultures were transferred into test tubes and placed in an oven (Thermo Fisher Scientific, USA) at 80˚C for 20 minutes. Bacterial cultures were categorized as vegetative cells and water bath heated cultures. Vegetative cells and water bath heated cultures were serially diluted 10^{-1} to 10^{-5} onto standard TSA plates (BD BBL™).

2.3. Determination of doubling times for the rif^R mutants

The three rif^R mutants were removed from the -80˚C freezer, streaked onto TSA plates, and incubated at 28˚C for 24 hr. One colony of each strain was transferred into individual tubes containing 5ml TSB. After incubation at 28˚C for 24 hr, 50µl of each strain were added to individual tubes containing 2 ml TSB, and the optical density (OD) of each strain was adjusted to 0.030 at 600 nm using a spectrophotometer. Three replicate tubes of each strain were prepared and placed in a shaking incubator at 220 rpm and 28°C. After incubation for 12 hr, the OD of each bacterial strain was recorded using GENESYS™ 10S UV-Vis Spectrophotometer (Thermo Scientific, USA). Bacterial doubling times and growth rates were calculated using doubling time software Doubling Time (V., 2006).

2.4. Root colonization and growth promotion on soybean

The rif^R mutants of the two *Bap* strains (AP193 and AP143) and one *Btk* strain (HD73) were tested in the greenhouse experiments. The rif^R mutants were grown in TSB + rifampicin as described above. TSB centrifuge tubes were wrapped in aluminum foil and placed in a shaking incubator (220 rpm) at 28°C for 48 hr. Bap and Btk mutants were centrifuged at 10,000 x g for 10 minutes to collect bacterial pellets and were then washed three times with sterile water. Vegetative cells at 10^{-6} CFU/ml of each bacterial strains were used directly on the soybean seed surface.

Lightweight large Deepots D40L cone-tainer were used, and each D40 pots had a cell diameter of 2.5", a depth of 10", and are made with post-industry, pre-consumer recycled polypropylene resin (Stuewe & Sons, Danville, IL, USA). A clay load field soil from E.V. Smith field station was used for the greenhouse experiments. Soil analysis by Waters Agricultural Labs Camilla, GA) and that soil concentrations of P, K, Mg, Ca, B, S, Zn, Mn, Fe, and Cu were sufficient. Pectin

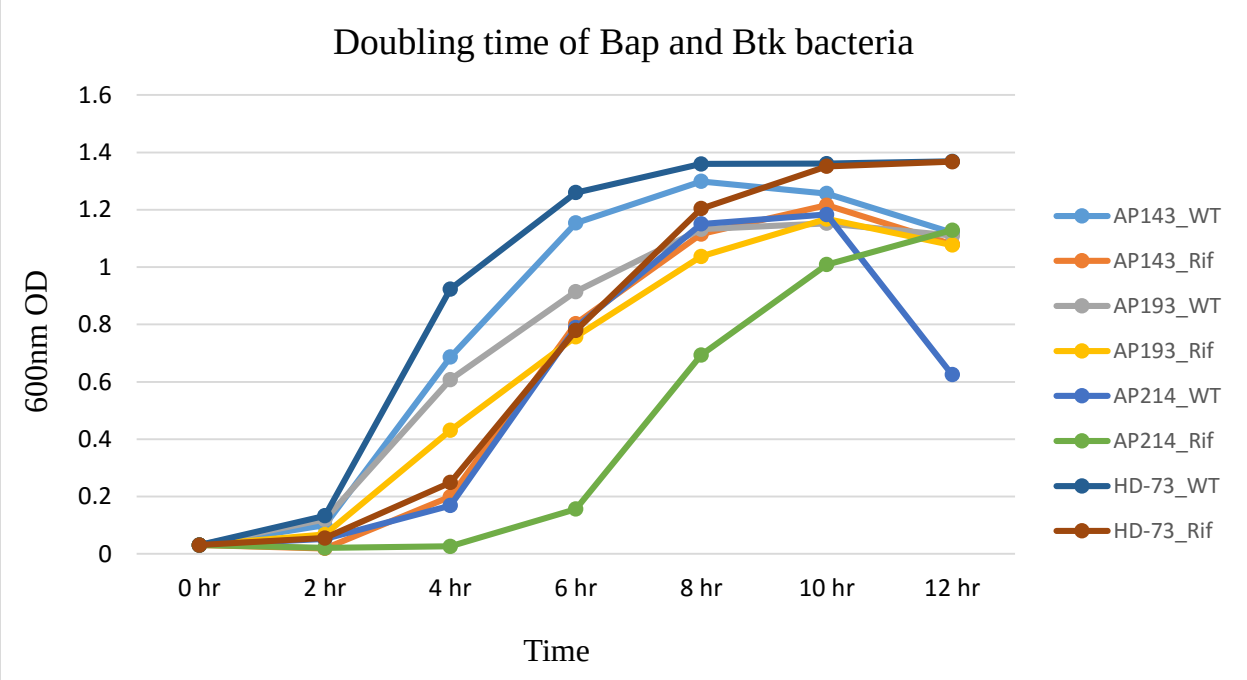
powder (0.1%) (EC No. 232-553-0, Tokyo chemical industry co., Toshima, Kita-Ku, Tokyo, Japan) was mixed thoroughly with field soil at a rate of 1 g per 1,000 g soil (0,1%) before filling the cone-tainers. Three cotton balls were placed at the bottom of each container to prevent soil loss through the drainage holes. Soil was watered to near field capacity, and one soybean seed ('Asgrow 6702 RR') was sown in each container. Five g of soil was place over eachseed, and racks of cone-tainers were covered with a plastic sheet to prevent the need for watering during seed imbibition. After 48 hr, the plastic sheet was removed, and the cone-tainers were transferred into the greenhouse chamber. Cone-tainers were watered twice daily twice with 5 ml water per cone-tainer. Plants were sampled at 28 days after inoculation (DAI) by gently removing from cone-tainers. Roots were gently shaken to remove loosely adhering soil. Root samples with attached rhizosphere soils were serially diluted and allowed to incubate at 28°C for 48 hr to count the bacterial populations in each plate. The data were analyzed with SAS 9.4 software (SAS Institute, Carry, NC) using the GLIMMIX procedure, and treatment means were compared using LSMEANS at p =0.05 level.

3. Results

3.1. Selection and characterization of *Bap* and *Btk* rifampicin-resistant mutants

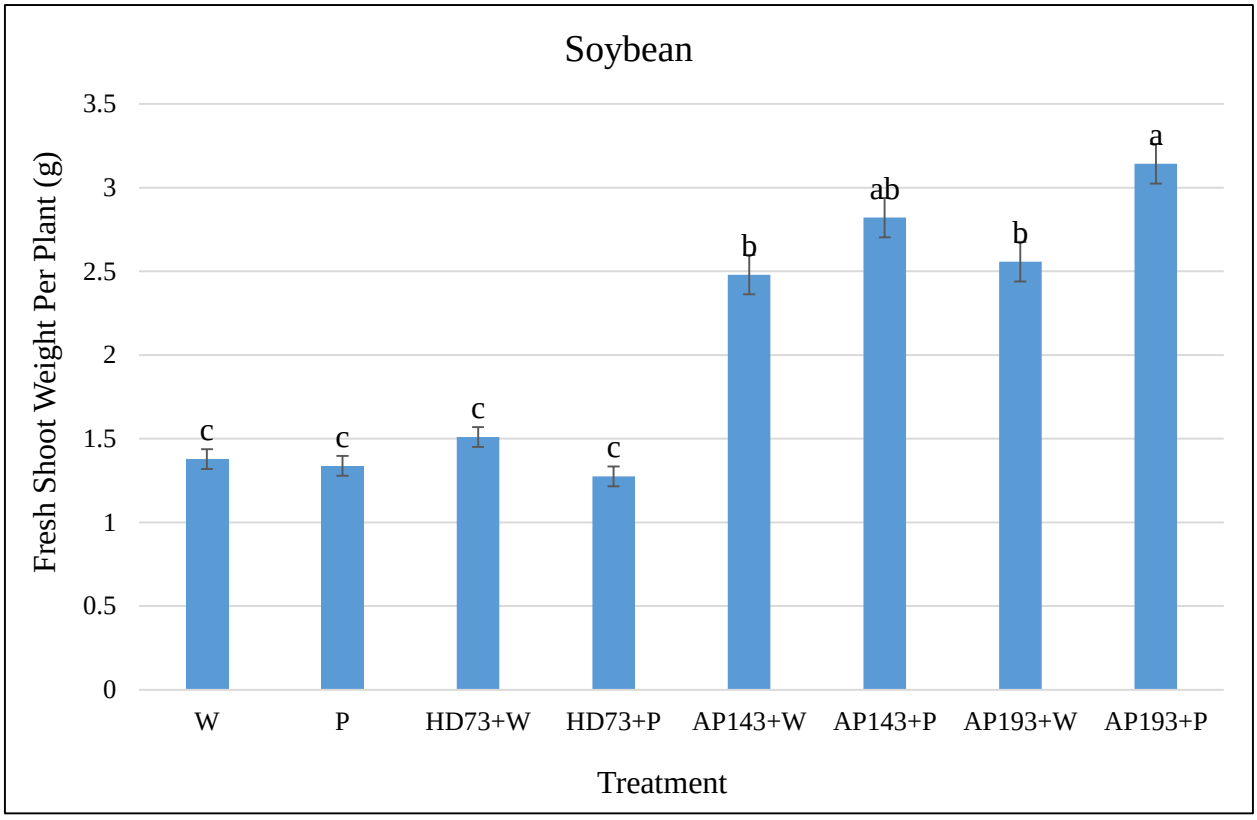
Rifampicin-resistant mutants were selected for *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) strain HD 73 and for Bap strains AP193 and AP143. The rif^R mutants for Btk stain HD73 grew within 24 hr on TSA rif^R plate, while the rif^R Bap strains grew within 36 hr. The colony morphologies and growth rates of the rif^R mutants were similar to those of the three wild-type strains. The selected rif^R mutants of all three strains formed spores when cultured in tryptic soy broth with 50 ppm rif^R for 48 hours at 28˚C. The calculated spore concentration of each of the rif^R strains was log 8.53 spores/ml for AP193 rif^R, log 8.80 spores/ml, forAP143 rif^R, and log 6.68 spores/ml for HD 73.

Figure 1. The doubling time of *Bap* and *Btk* strains (wild type and rif^R mutants).



3.2. Effects of pectin amendment on soybean plant growth promotion by *Bap* and *Btk* rif^R strains.

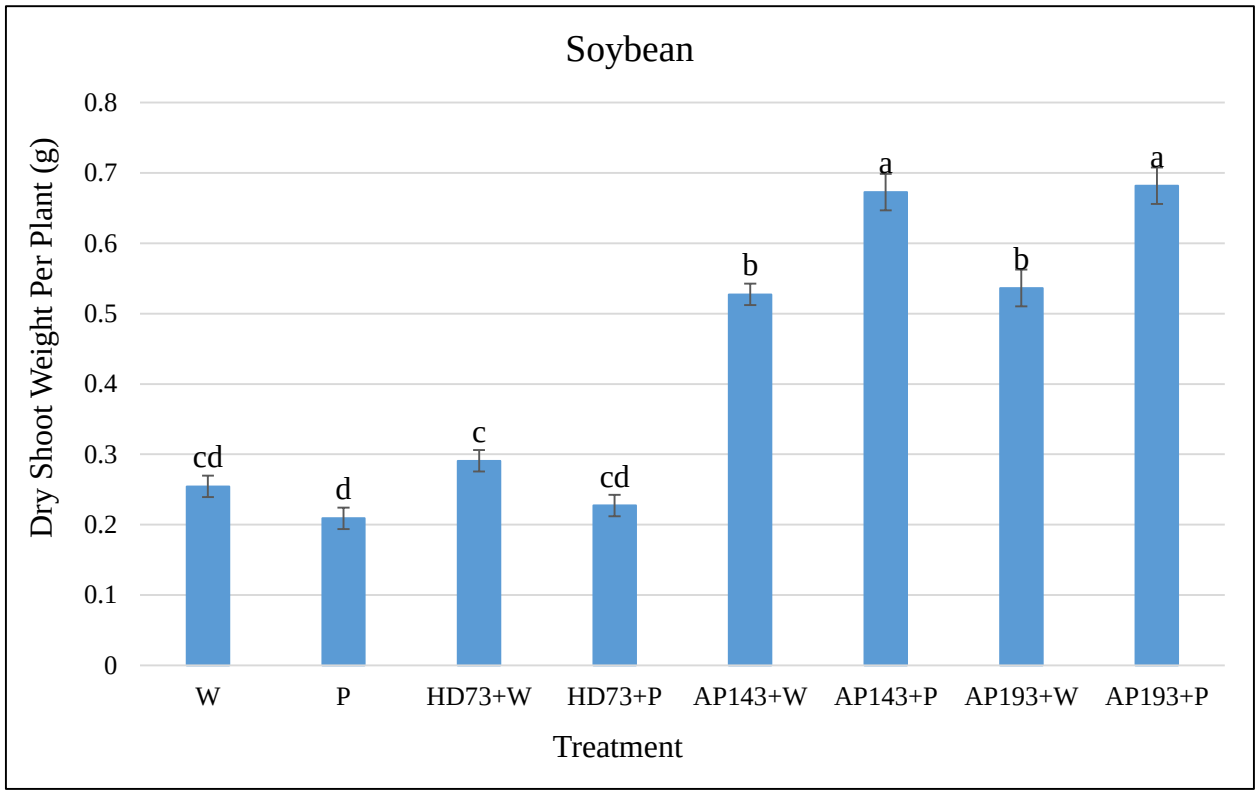
Figure 2A. Effect of pectin amendment on fresh weight of soybean shoots



Mean and standard error values of fresh shoot weight on the bar followed by the same letter are not significantly different at P ≤0.05 with Tukey-Kramer's multiple comparison tests.

Treatment with rif^R mutants AP193 and AP143 resulted in increased fresh shoot weight of soybean plants with and without pectin amendment. In contrast, rif^R mutant HD73 did not increase fresh shoot weight with or without pectin amendment. The greatest shoot weight occurred with rif^R mutant AP193 plus pectin amendment which was significantly greater than the weight with AP193 without pectin amendment.

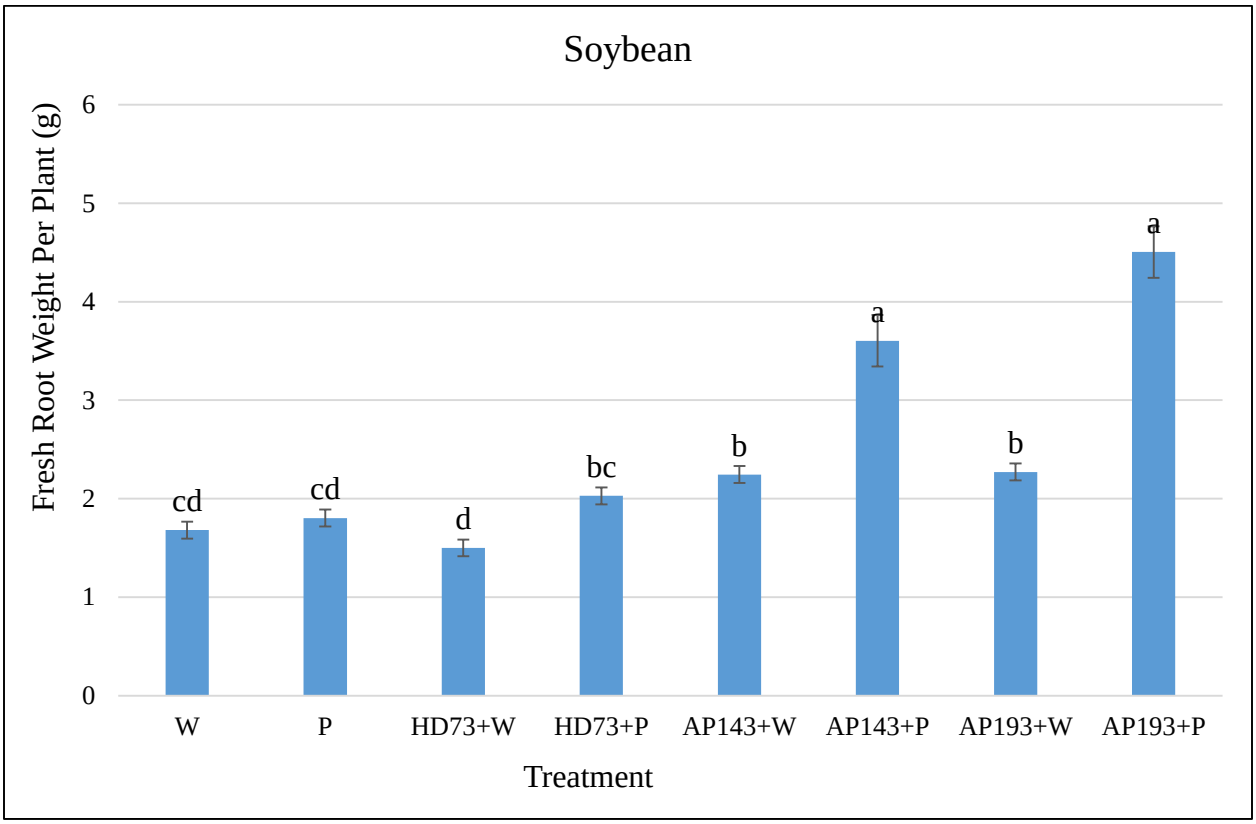
Figure 2B. Effect of pectin amendment on dry weight of soybean shoots.



Mean and standard error values of dry shoot weight on the bar followed by the same letter are not significantly different at P ≤0.05 withTukey-Kramer's multiple comparison tests.

Treatment with rif^R mutants AP193 and AP143 resulted in increased dry shoot weight of soybean plants with and without pectin amendment. In contrast, rif^R mutant HD73 did not increase dry shoot weight with or without pectin amendment. The greatest shoot weight occurred with rif^R mutant AP193 plus pectin amendment which was significantly greater than the weight with AP193 without pectin amendment.

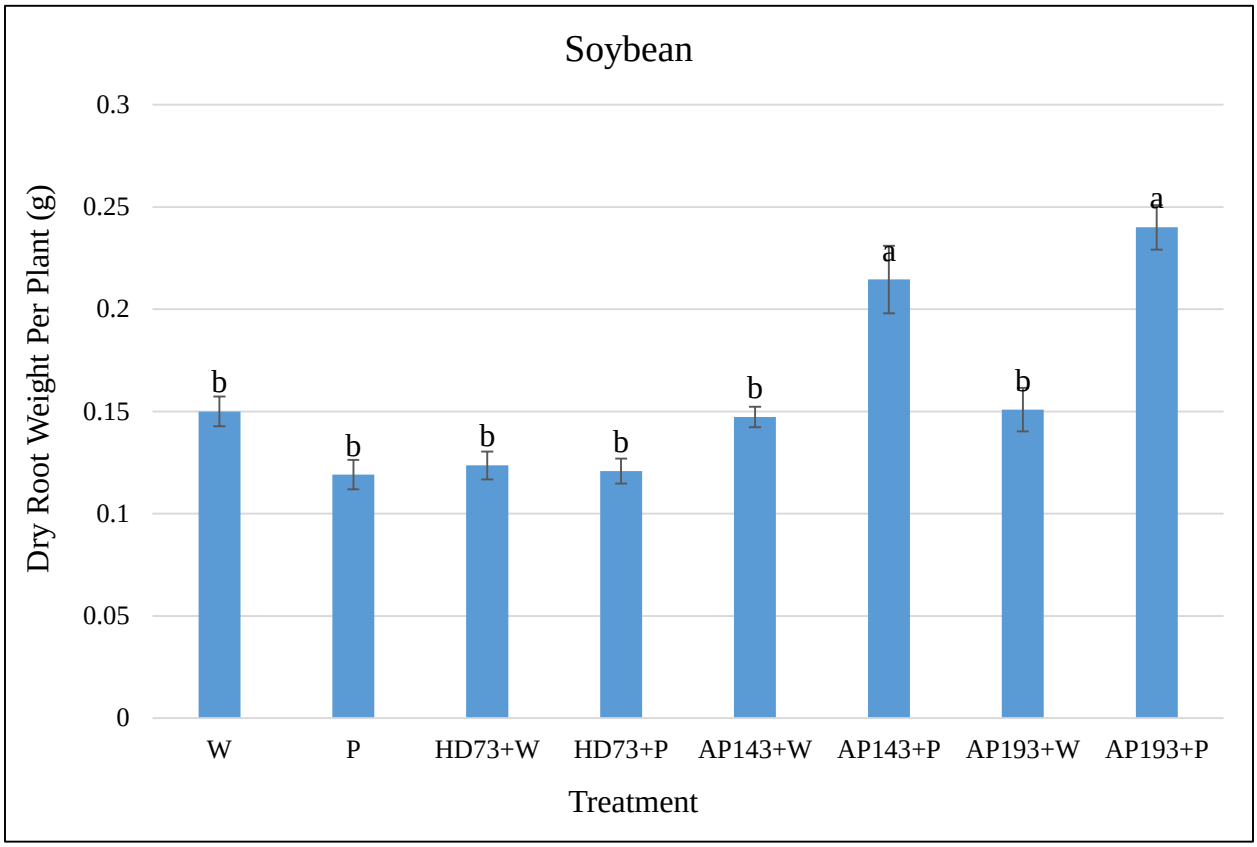
Figure 2C. Effect of pectin amendment on fresh root weight of soybean



Mean and standard error values of fresh root weight on the bar followed by the same letter are not significantly different at P ≤0.05 with Tukey-Kramer's multiple comparison tests.

Fresh root weights of pectin amendments plus treatment of AP143 and AP 193 were significantly greater than the corresponding weights for each strain without pectin amendment. In contrast, root weights resulting from treatment of HD73 with or without pectin were not significantly different from the water and the pectin controls.

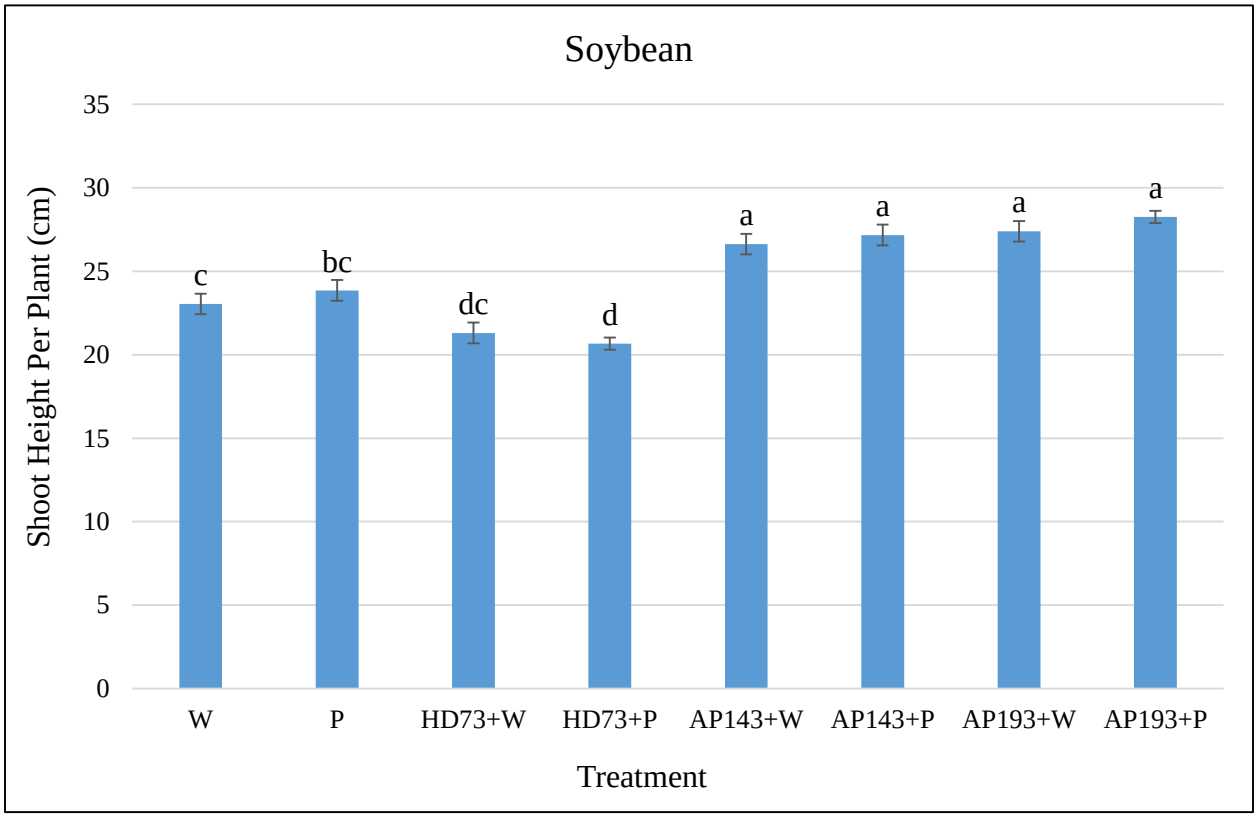
Figure 2D. Effect of pectin amendment on dry root weight of soybean.



Mean and standard error values of dry root weight on the bar followed by the same letter are not significantly different at P ≤0.05 to Tukey-Kramer's multiple comparison tests.

Dry root weights of pectin amendments plus treatment of AP143 and AP 193 were significantly greater than the corresponding weights for each strain without pectin amendment. In contrast, dry root weights resulting from treatment of HD73 with and without pectin were not significantly different from the water and the pectin controls. Pectin treated dry root weight of soybean plant had increased compared to non-pectin treated plant. The enhancement of dry root weight was similar to fresh root weight of soybean plant.

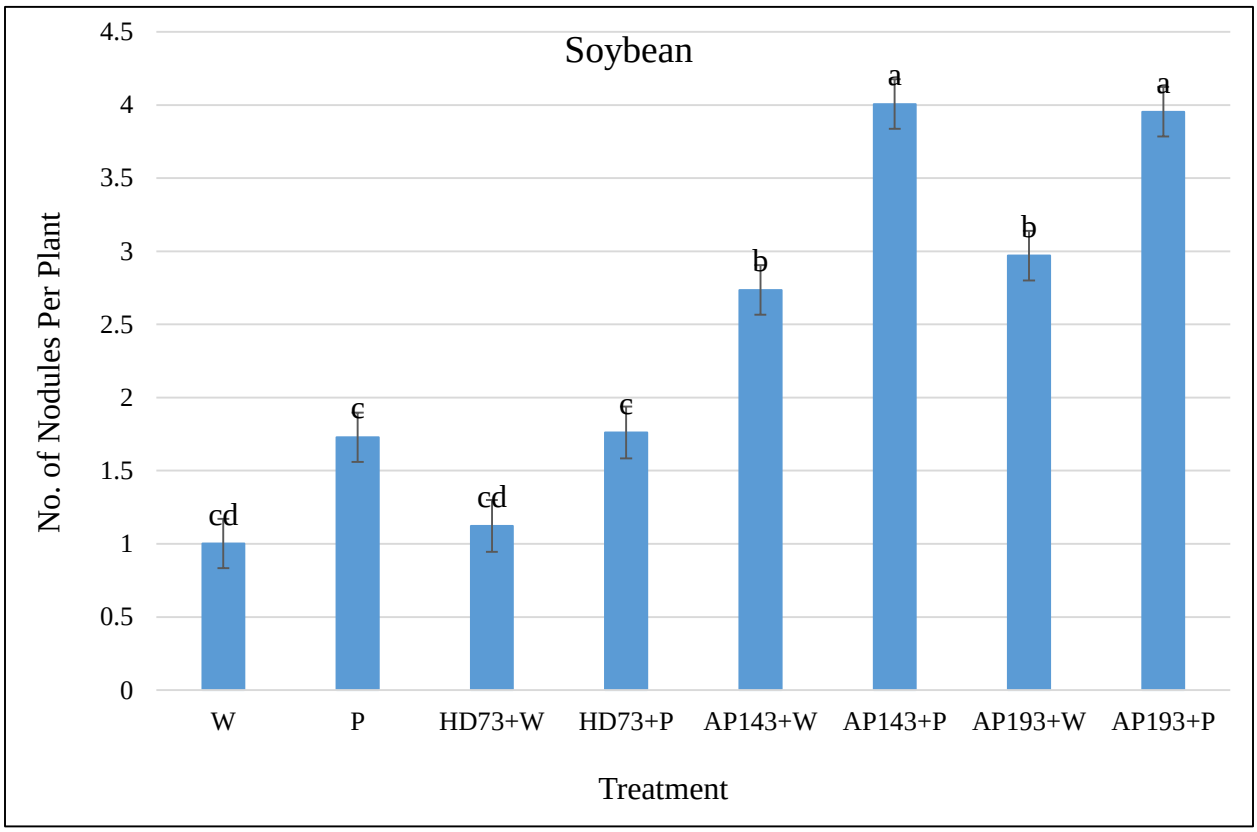
Figure 2E. Effect of pectin amendment on height of soybean plants.



Mean and standard error values of plant shoot height on the bar followed by the same letter are not significantly different at P ≤0.05 with Tukey-Kramer's multiple comparison tests.

Plant height was significantly greater with rif^{R} AP193 and AP143 with and without pectin amendments than both the water control and the pectin control. Rif^{R} HD73 did not increase shoot height with or without pectin amendment.

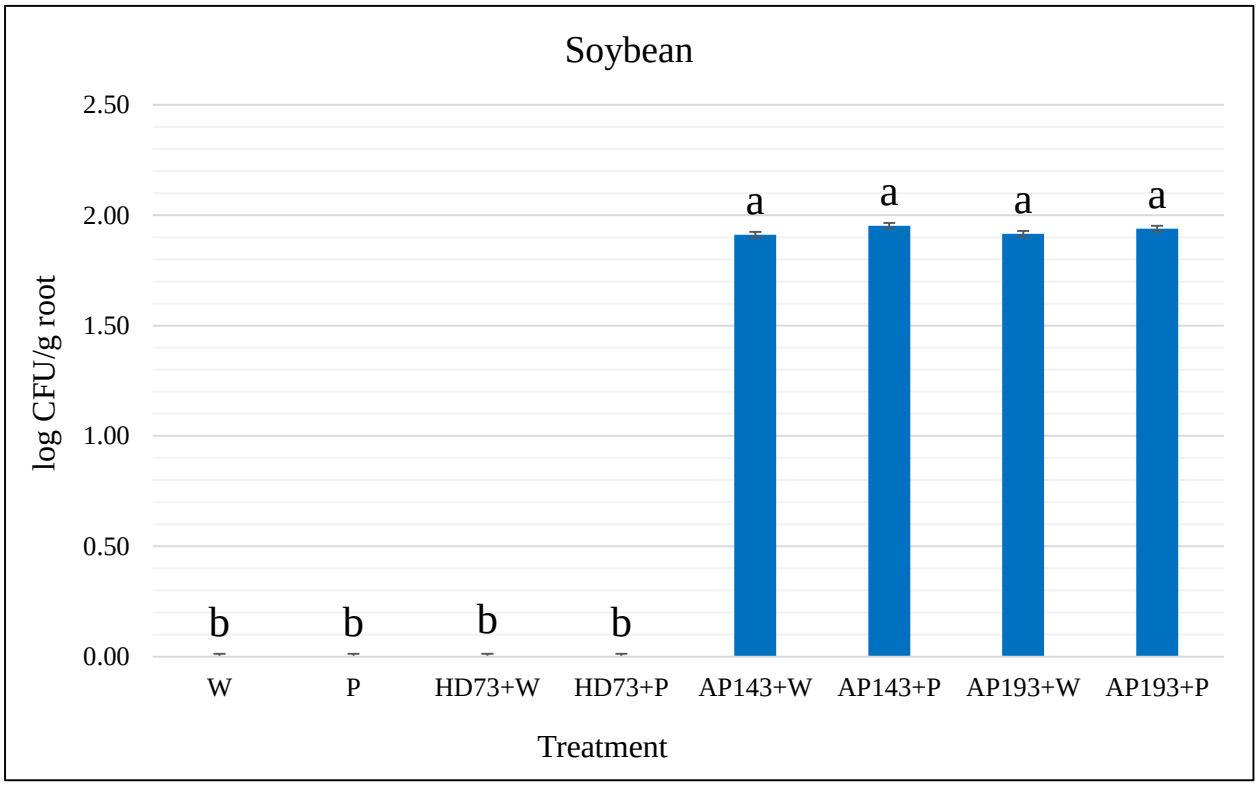
Figure 3. Effect of pectin amendment on numbers of root nodules of soybean plants.



Mean and standard error values of root nodules per treatment on the bar followed by the same letter are not significantly different at P ≤0.05 with Tukey-Kramer's multiple comparison tests.

The numbers of rhizobial nodules resulting from pectin amendments plus treatment of AP143 and AP 193 were significantly greater than the corresponding weights for each strain without pectin amendment. In addition, nodule numbers with both strains without pectin amendment were significantly greater than all other treatments.

Figure 4. Effect of pectin amendment on root colonization by AP143 and AP193 at 28 days after inoculation (DAI).



Mean and standard error values of CFU log unit in the same column followed by the same letter are not significantly different at P ≤0.05 with Tukey-Kramer's multiple comparison tests.

The application of pectin amendments to soil did not affect root colonization of Bap rif^{R} AP193 and AP143.

4. Discussion

The overall results of this study support the novel hypothesis that the combination of a pectin amendment to soil and the inoculation with pectinolytic *Bap* strains enhances plant growth-promotion activity. (Beauregard et al., 2013) previously investigated the effects of amendment on root colonization by *B. subtilis*, but did not determine effects on plant growth. An extensive review of the scientific literature did not find any reports investigating the effects of PGPR with pectin amendments on plant growth-promotion. In the current study, *Bap* rif^R strains together with pectin amendment substantially increased plant growth-promotion on soybean. More specifically, the two *Bap* rif^R strains AP143 and AP193, when inoculated into soil with pectin amendment, caused increased shoot and root weight (Figure 2A-2E). In contrast pectin amendment with *Btk* rif^R strain HD73 did not increase plant growth-promotion, most likely because the pectin-associated transporter and metabolic genes are not present in *Btk* strain HD73.

One recent study evaluated effects of pectin on biocontrol activity (Wu et al., 2015). In this study, pectin amendment was added into the pot with *B. amyloliquefaciens SQY* 162 for biocontrol of bacterial wilt in tobacco. In the greenhouse study, bacterial and fungal disease symptoms were not observed in soybean roots, shoots, and leaves. Fungal colonies were not also found on *Bap* rif^R TSA plates suggesting that antifungal potentiality of *Bap* strains. *Bap* with pectin amendments could enhance biocontrol activity, but further studies should be needed to ensure biocontrol potentiality inoculating different plant pathogens.

In the current study, bacterial populations of *Bap* rif^{R} strains with pectin amendment were not significantly different than *Bap* rif^{R} strains suggesting that pectin had no effect on bacterial colonization of roots (Table 8). However, in the greenhouse experiments, bacterial populations were assessed only in the rhizosphere and not inside roots. Therefore, it is possible that endophytic populations of the *Bap* strains were increased with pectin amendments. Alternatively, if neither the rhizosphere or endophytic populations of Bap strains are increased with pectin amendment, then the resulting plant growth promotion cannot be accounted for by increased bacterial production of metabolites reported to be related to growth-promoting capacity of some PGPR strains. In this case the increased growth promotion could result from changes in the transcriptional regulation of soybean genes related to plant growth promotion. caused by Future studies will be conducted to elucidate these possible mechanisms.

Examination of soybean roots after washing the plants revealed that the combination of pectin amendment plus *Bap* strains AP193 rif^{R,} and AP143 rif^{R} resulted in a more dense root system than non-pectin treated soils (data not shown). In addition, the primary and lateral roots were longer and more highly branched in pectin-treated soils. Based on the root morphology, it can be summarized that *Bap* strains using pectin amendment substantially enhanced root biomass of soybean. Future studies will determine if this effect is highly reproducible on soybean and on other crops, including maize.

In the current study, the effects of pectin amendment on PGPR was assessed only using vegetative cells of the *Bap* strains. Approximately 10^{-6} cfu/ml vegetative cells were applied directly on the soybean seed surface to ensure that cells are metabolically active to receive carbon nutrients from the pectin amendment. PGPR bacilli spores remain inert for an extended period and potential inoculants in agriculture due to their survivability in soil environment (Nelson, 2004). In addition, bacilli PGPR are routinely applied in agriculture as

seed treatments in which spores are applied together with various chemicals onto seeds months prior to planting. Therefore, future studies are needed to determine whether spores of select pectin-ulilizing Bap srains can enhance root colonization and plant growth-promotion in the presence of pectin amendments.

The selection of the optimum pectin concentration in an amendment is critical for crop health. High pectin levels could lure pectinolytic pathogenic bacteria to cause disease in plants. In our study, we used 0.1g pectin amendment to soil and found that this concentration increased the rate of seedling emergence. However, adding 0.5 g pectin in soil delayed germination time of soybean seed (data not shown), a result which could make seedlings more susceptible to some soilborne pathogens that cause seedling damping-off diseases..

The results of the current study also indicated that amendment of soil with pectin in the presence of pectinolytic *Bap* strains has the potential to influence root nodulation of soybean by *Bradyrhizobium japonicum* (Table 7). Recent studies have reported that *Bacillus amyloliquefaciens* strain LL2012 with *Bradyrhizobium japonicum* substantially increased soybean nodulation and plant growth-promotion by degradation of complex macromolecules (Masciarelli, 2014). Treatment with *Bap* rif^R strain AP193 plus pectin amendment resulted in 132% root nodulation compared to 75% root nodulation by the same strain without pectin amendment. Similarly, *Bap* rif^R strain AP143 plus pectin amendment increased 146% root nodulation compared to 68% root nodulation for the same strain without pectin amendment. Further studies are needed to determine how root nodulation of soybean is affected by pectin amendments. Khammas (1991) reported that mixed suspensions of *Azospirillum* spp. and *Bacillus* spp. with pectin amendment (0.05%) increased nitrogen fixation. Pectin amendment supplied carbon compounds for the *Bap* rif^R strain that interacts with soil rhizobia and increased nodule on soybean roots. In the current study, greenhouse experiments, soybean root nodulation enhanced by *Bap* rif^R strain with pectin amendment without co-inoculation

with *Rhizobia* species. Field soils from fields with a history of growing soybean were used for the greenhouse experiments and therefore, *Bradyrhizobium japonicum* was likely present in the soil.

Several suggestions for future work arise from this study. First, because some soil borne pathogens use pectinolytic enzymes to invade and macerate plant cell walls, the safety of pectin amendments together with pectinolytic *Bap* strains needs to be investigated to determine if adding pectin poses any risk to crops. Second, the robustness of pectin amendments should be determined using various concentrations on different crops, including potato which is susceptible to pectinolytic soft rot bacterial pathogens. Finally, pectin amendments should be evaluated for their effects on the magnitude of biocontrol elicited by specific *Bap* strains.

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Greenhouse experiment results of root colonization and growth-promotion on soybean seeds using pectin supplements as a sole carbon source

Table 1. Sporulation activity of *Bap* and *Btk* rif mutants and wild type strains.

Name	Time (hr)	Strain	Log spore/ml Wild type	Selected rif mutants
B. amyloliquefaciens subsp. *plantarum*	24-48	AP193	8.62	8.53
B. amyloliquefaciens subsp. *plantarum*	24-49	AP143	8.77	8.8
B. thuringiensis subsp. *kurstaki*	24-50	HD73	8.6	6.68

Table 2. The doubling time of *Bap* and *Btk* rif mutants and wild type strains.

Name	Strain	Time (hr)	Doubling time (hr) Rif type	Wild type
B. amyloliquefaciens subsp. *plantarum*	AP193	8	1.93	1.69
B. amyloliquefaciens subsp. *plantarum*	AP143	8	1.7	1.55
B. thuringiensis subsp. *kurstaki*	HD73	8	1.62	1.4

Table 3. Fresh shoot weight results of soybean plants at 28 days after inoculation (DAI).

Treatment	**Fresh Shoot Weight**
Water	1.37±0.06c
Pectin (0.1%)	1.34±0.06c
HD73+Water	1.51±0.06c
HD73+Pectin (0.1%)	1.28±0.06c
AP143+Water	2.48±0.12b
AP143+Pectin (0.1%)	2.82±0.12ab
AP193+Water	2.56±0.12b
AP193+Pectin (0.1%)	3.14±0.12a

Mean and standard error (mean ± SE) values of fresh shoot weight in the same column followed by the same letter do not significantly different at $P \leq 0.05$. Statistics were run using Tukey's multiple comparison tests ($F_{7, 35.42}=67.21$, $P<0.0001$).

Table 4. Dry shoot weight results of soybean plant at 28 days after inoculation (DAI).

Treatment	**Dry Shoot Weight**
Water	0.25±0.02cd
Pectin (0.1%)	0.21±0.02d
HD73+Water	0.29±0.02c
HD73+Pectin (0.1%)	0.23±0.02cd
AP143+Water	0.53±0.02b
AP143+Pectin (0.1%)	0.67±0.03a
AP193+Water	0.54±0.03b
AP193+Pectin (0.1%)	0.68±0.03a

Mean and standard error (mean ± SE) values of dry shoot weight in the same column followed by the same letter do not significantly different at $P \leq 0.05$. Statistics were run using Tukey's multiple comparison tests ($F_{7, 41.16}=100.28$, $P<0.0001$).

Table 5. Fresh root weight results of soybean plant at 28 days after inoculation (DAI).

Treatment	**Fresh Root Weight**
Water	1.68± 0.09cd
Pectin (0.1%)	1.8± 0.09cd
HD73+Water	1.5± 0.09d
HD73+Pectin (0.1%)	2.03± 0.09bc
AP143+Water	2.25± 0.09b
AP143+Pectin (0.1%)	3.6± 0.26a
AP193+Water	2.27± 0.09b
AP193+Pectin (0.1%)	4.51± 0.26a

Mean and standard error (mean ± SE) values of fresh root weight in the same column followed by the same letter do not significantly different at $P \leq 0.05$. Statistics were run using Tukey's multiple comparison tests ($F_{7, 35.91} = 28.77$, $P < 0.0001$).

Table 6. Dry root weight results of soybean plants at 28 days after inoculation (DAI).

Treatment	Dry Root Weight
Water	0.24±0.01b
Pectin (0.1%)	0.15±0.01b
HD73+Water	0.21±0.02b
HD73+Pectin (0.1%)	0.15±0.01b
AP143+Water	0.12±0.01b
AP143+Pectin (0.1%)	0.12±0.01a
AP193+Water	0.12±0.01b
AP193+Pectin (0.1%)	0.15±0.01a

Mean and standard error (mean ± SE) values of dry root weight in the same column followed by the same letter do not significantly different at $P \leq 0.05$. Statistics were run using Tukey's multiple comparison tests ($F_{7, 70}=18.82$, $P<0.0001$).

Table 7. Root nodules results of soybean plants at 28 days after inoculation (DAI).

Treatment	Mean Root Nodules Per Treatment at 28 DAI
Water	1.00±0.17d
Pectin (0.1%)	1.73±0.17c
HD73+Water	1.12±0.18cd
HD73+Pectin (0.1%)	1.76±0.18c
AP143+Water	2.74±0.17b
AP143+Pectin (0.1%)	4.01±0.17a
AP193+Water	2.97±0.17b
AP193+Pectin (0.1%)	3.95±0.17a

Mean and standard error (mean ± SE) values of root nodules at 28 DAI in the same column followed by the same letter do not significantly different at $P \leq 0.05$. Statistics were run using Tukey's multiple comparison tests ($F_{7, 68}=51.26$, $P<.0001$).

Table 8. Average bacterial log cfu/g population results of soybean plants at 28 days after inoculation (DAI).

Treatment	**CFU Log Unit**
Water	0±0.01b
Pectin (0.1%)	0±0.01b
HD73+Water	0±0.01b
HD73+Pectin (0.1%)	0±0.01b
AP143+Water	1.91±0.01a
AP143+Pectin (0.1%)	1.95±0.01a
AP193+Water	1.92±0.01a
AP193+Pectin (0.1%)	1.94±0.01a

Mean and standard error (mean ± SE) values of cfu log unit in the same column followed by the same letter do not significantly different at $P \leq 0.05$. Statistics were run using Tukey's multiple comparison tests ($F_{7, 70}=6511.99$, $P<.0001$).

Table 9. Shoot height results of soybean plants at 28 days after inoculation (DAI).

Treatment	**Plant Shoot Height**
Water	23.05±0.62c
Pectin (0.1%)	23.85±0.62bc
HD73+Water	21.31±0.62dc
HD73+Pectin (0.1%)	20.67±0.36d
AP143+Water	26.63±0.62a
AP143+Pectin (0.1%)	27.17±0.62a
AP193+Water	27.40±0.62a
AP193+Pectin (0.1%)	28.25±0.36a

Mean and standard error (mean ± SE) values of plant shoot height in the same column followed by the same letter do not significantly different at $P \leq 0.05$. Statistics were run using Tukey's multiple comparison tests ($F_{7, 48.97}=42.9$, $P<0.0001$).