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DISSERTATION

GENETIC DIVERSITY OF ACC-DEAMINASE POSITIVE BACTERIA IN COLORADO SOIL UNDER WINTER WHEAT CULTIVARS (*Triticum aestivum* L.)

Submitted by

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ABSTRACT

GENETIC DIVERSITY OF ACC-DEAMINASE POSITIVE BACTERIA IN COLORADO SOIL UNDER WINTER WHEAT CULTIVARS (*Triticum aestivum* L.)

ACC-deaminase positive bacteria (ACC+) promote plant growth and development by lowering abiotic stress ethylene levels through deamination of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene. During drought stress, ACC+ bacteria can help plants better tolerate drought stress in arid and semi-arid areas such as Colorado. The purpose of this study was to assess the capability of ACC+ bacteria to support winter wheat cultivar growth and production under drought stress conditions.

In a field study, the relative abundance and genetic diversity of ACC+ bacteria associated with four winter wheat cultivars (Baca, Hatcher, Ripper, and RonL) were assessed under dryland, limited irrigation, and fully irrigated conditions in Colorado. The abundance of ACC deaminase positive bacteria was relatively high, with numbers ranging between 1.69×10^7 and 3.28×10^9 CFU's g^{-1} soil. At anthesis, the abundance and relative percent of ACC+ bacteria were greater under dryland and limited irrigation compared to full irrigation, and greater under RonL than other cultivars. The composition of rhizosphere ACC+ bacteria was distinct under RonL compared to Ripper. The majority of ACC+ bacteria enriched from these soils were Proteobacteria, specifically *Pseudomonas spp.* These results suggest that cultivar was a stronger driver of community composition of ACC+ bacteria than irrigation practice.

The second study was conducted using a total of 55 bacterial isolates from the original experimental soils (RonL, Ripper, and Hatcher), which were selected to assay for Plant-Growth

Promoting (PGP) traits: ACC-deaminase activity, Indole Acetic Acid (IAA) production, osmotic stress tolerance, phosphorus solubilization**,** and **s**iderophore production. Most isolates were identified as species of *Pseudomonas*, but other species such as *Arthrobacter, Variovorax, Agrobacterium, Rhizobium, Ochrobacterium, Micrococcus, Rahnella*, and *Bacillus* were represented as well. Out of 55 isolates, 47 bacterial isolates tolerated osmotic stress when tested at an osmotic stress of -0.73 MPa water potential, 42 isolates demonstrated ACC-deaminase activity (potential ACC-deaminase activity ranged from 0.012 to 4.36 nmoles mg^{-1} protein h^{-1}), 16 isolates were capable of solubilizing rock phosphate, one isolate produced IAA in the presence of 200 μg mL^{-1} tryptophan, and none of the isolates produced siderophores in vitro.

Seven bacterial isolates that exhibit multiple PGPRs traits were selected for a root elongation assay using winter wheat seeds. The isolates selected were three *Pseudomonas sp.* isolated from RonL rhizospheres in fully irrigated plots (RLF6, RLF9, and RLF12); one *Variovorax sp.* isolated from Ripper rhizosphere in a dryland plot (RD12), one *Pseudomonas sp*. from Ripper rhizosphere of a fully irrigated plot (RF12), and two *Pseudomonas sp.* isolated from Hatcher rhizospheres (HD8 and HF1). The results showed that 6 days after seeds were inoculated, Ripper seedlings had the longest roots. In addition, isolates RLF6 and RF12, both *Pseudomonas sp.*, were found to be the most effective in increasing root length as compared to sterile inoculum (control). The effect of inoculation on root length, however, was dependent on cultivar type.

A subsequent field plot study was conducted to test the effectiveness of *Pseudomonas sp.* RLF9 to improve wheat growth and productivity under drought stress. Inoculum was prepared in the form of alginate bead and distributed in trenches next to winter wheat plants (at stem elongation growth phase), in replicated field plots under irrigated and non-irrigated treatments.

Six weeks after inoculation, numbers of culturable *Pseudomonas sp.* were greater ($P \leq 0.05$) in soil inoculated with RLF9 than sterile inoculant, except for the RonL/Ripper bioculture treatment. Overall, soil under RonL accumulated the highest ACC-deaminase positive bacterial populations (1.8×10^5 CFUs g^{-1} soil) compared to other cultivar treatments. Soil ACC deaminase activity was highly variable but tended to be greatest in soil planted to monocultures of RonL and Ripper, and bicultures of RonL and Ripper. There was no significant correlation between inoculant abundance in soil and soil ACC-deaminase activity, perhaps because of ACC deaminase activity of indigenous soil bacteria. The field season was marked with abundant rainfall during the spring, followed by outbreaks of pathogens, including stripe rust, that affected all field treatments and confounded late season drought and heat stress. As a result, there were no irrigation treatment effects or inoculation effects on wheat yield indices.

The findings of this study are very attractive for further field studies, which would be helpful in extending isolate use as PGP inocula for wheat production, especially in Colorado and perhaps other semi-arid regions. Specifically, inoculation of winter wheat cultivars with PGPR's containing ACC-deaminase and other PGP traits could be an effective approach for successful crop production. Selection of PGPR inoculants and wheat cultivar combinations for obtaining optimum responses should be considered in future studies. The ability of potential inoculants to survive in the rhizosphere, compete with indigenous microbial communities, and colonize roots should also be studied. Based on laboratory studies of potential PGPR activities, *Pseudomonas sp.* RLF9 and possibly *Pseudomonas sp.* RLF6 could be good inocula selection to improve wheat growth and productivity under drought stress. Further research is needed to demonstrate the effectiveness of RLF6 and RLF9 as effective inoculants in the field.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

1. Introduction

Bacteria are the most numerous type of organisms in soil, and they are estimated in soil at populations of 10^4 -10^9 cells g^{-1} soil (Alexander, 1977). Moreover, bacteria are the most diverse organisms, and it is estimated that there are more than 10-to-100 million species on this earth (American Society for Microbiology, 1994). The number of bacterial species in soil is estimated to be over $1x10^4$ and only a few of these species have been identified and studied (about 1–10%) (Hawksworth, 1991).

One area of soil that is particularly enriched with bacteria is the rhizosphere, or the soil associated with plant roots. In the rhizosphere, bacterial populations are influenced by plant roots. The rhizosphere is the part of soil that is directly attached to the root system of plants (Curl and Truelove, 1986). This area is relatively rich in nutrients from root exudates that exude from the roots or germinating seeds; about 30% of plant photosynthates are lost from the plant root to the rhizosphere (Nelson, 2004). Theses nutrients attract microorganisms and stimulate microbial activity in rhizosphere (Rouatt and Katznelson, 1961). Microorganisms that respond and utilize theses nutrients as nutrient sources can dominate the rhizosphere. The abundance and diversity of microbial populations in the rhizosphere is affected by these exudate in addition to a wide variety of factors including: soil type, plant species, plant age, growth stage, cultural practices such as tillage and crop rotation, and other environmental factors (De Leij et al., 1994; Latour et al.,1996; Westover et al., 1997; Grayston et al., 1998; Horwath and Paul 1994; Lupwayi et al., 1998). In addition to attracting diverse and actively metabolizing soil microbes, root exudates can modify the chemical and physical properties of the soil and alter the structure of microbial

community in the rhizosphere (Dakora and Phillips, 2002). The composition of these exudates is dependent on the physiological status and species of both plants and microorganisms (Kang et al., 2010). Moreover, microbial activity in the rhizosphere makes the nutrients available to the plants and that leads to altered quality and quantity of root exudates. Furthermore, it has been reported that certain plants select and attract specific soil microorganisms, and that the change in composition and diversity of microbial communities in the rhizosphere is plant- dependent (Broeckling et al. 2008; Houlden et al. 2008).

1.1 Plant Growth Promoting Rhizobacteria (PGPR)

In the rhizosphere, some microorganisms such as pathogens can inhibit plant growth while others can promote plant growth. Those that benefit plant growth are called Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper,1994). Examples of positive effects of PGPR on plant growth are improvement of nutrient uptake such as nitrogen fixation, phosphate solubilization, and iron sequestration (Glick et al., 2007; Hirsh and Mauchline, 2012; Glick, 2012).

The ability of PGPR to promote plant growth and protect plants from various pathogens has been documented since 1950. PGPR indirectly support plant growth through the production of siderophores, which helps them to compete with other microorganisms (for colonization sites on the root & for nutrients) with other soil microorganism (including pathogens). They also compete with pathogens for iron (Crowley et al., 1991; Wang et al., 1993). PGPRs also indirectly promote plant growth as biocontrol agents. PGPRs may secrete iron-binding siderophores to obtain soluble iron from the soil and provide it to a plant, and thereby preventing fungal pathogens from using it (Neilands and Leong, 1986). Other mechanisms include the production of low molecular weight metabolites with antifungal activity such as hydrogen cyanide (Dowling

and O'Gara, 1994); production of enzymes including chitinase, β-1,3-glucanase, protease, and lipase, which can lyse some fungal cells (Chet and Inbar, 1994); out-competing phytopathogens for nutrients and niches on the root surface (Kloepper et al., 1988; O'Sullivan and O'Gara, 1992) and lowering the production of stress ethylene (Hyodo, 1991) in plants through production of ACC-deaminase enzyme (Glick et al., 1998; Penrose et al., 2001).

In the field, plant growth may be inhibited by a large number of both biotic and abiotic stresses, the latter of which includes extreme temperature, flooding, drought, toxic metals and environmental organic contaminants, and salinity (Abeles et al., 1992). Under such stress conditions, plant growth would be lower than under normal conditions. In recent years, scientists have become more interested in PGPR technologies to promote plant growth when under abiotic stress (e.g., drought, heat, salinity, and heavy metals) (Reed and Glick, 2005).

Plant Growth Promoting Rhizobacteria may increase a plant's ability to overcome environmental stress through various activities (Fig1). For example, some PGPR strains have the ability to enhance plant growth and development by altering the concentration of plant hormones, including hormones that have an important role in root system development such as auxins, abscisic acid, gibberellic acid, cytokinins, indol-3-acetic-acid (IAA), and ethylene (Mathesius et al.,1998; Yang et al., 2009; Kim et al., 2013; Dimkpa et al., 2009; Patten and Glick, 1996; Conrath, et al., 2006)). One of the most important bacterial traits of PGPR for abiotic stress is express of ACC- deaminase enzyme, which supports plant growth under wide variety of stress. ACC-deaminase cleaves of 1-aminocyclopropane-1-carboxylate (ACC) to α-ketobutyrate and ammonia. ACC-deaminase is not a secreted enzyme; however, it is localized only within the cytoplasm of the microorganism that produces it (Jacobson et al., 1994).

1.2. ACC-deaminase producing bacteria

Microorganisms that express ACC deaminase can lower the effect of different biotic and abiotic stress that plants face during their life cycle (Glick et al., 1997; Glick and Penrose, 1998). ACC-deaminase producing bacteria express the enzyme ACC-deaminase; which is responsible for cleaving ACC, the immediate precursor of ethylene in plant tissue, into (ammonia source) and α-ketobutyrate (carbon source) (Honma and Shimomura, 1978).

Ethylene (H_2C_4) is a gaseous hormone and it is essential for growth and development of plants in concentrations as low as 0.05 μL L^{-1} (Abeles et al., 1992). Under stress conditions such as drought, heat, or salinity, the plant ethylene level increases and that can inhibit plant growth. The high level of ethylene can damage the plants, leading to short roots for example, and it can inhibit whole plant growth and lead to plant death (Glick et al., 1994; Glick et al., 1995). ACC-deaminase producing microorganisms decrease the ACC level in plants and support plant growth by decreasing plant ethylene concentration (Glick et al., 1998; Glick et al., 2007; Hyodo, 1991).

Microorganisms that express ACC deaminase can lower the effect of different biotic and abiotic stress that plant face during its life cycle (Glick et al., 2007; Brotman et al., 2013).
The enzyme ACC-deaminase is found among many species of rhizosphere bacteria (Glick et al., 1994; Glick et al., 1995). ACC-deaminase was first identified in the yeast *Cyberlindnera saturnus* (previously *Hansenula satunus)* and the bacterium *Pseudomonase sp.* ACP (Honma and Shimomura, 1978). Following, ACC-deaminase activity has been reported in a number of both Gram-positive and Gram-negative bacteria with a variety of different metabolisms (Glick, 2012; Glick, 2014). In 2004, Mayak first reported that ACC-deaminase producing bacteria induced plant growth under drought stress (Mayak et al., 2004). In addition, both the

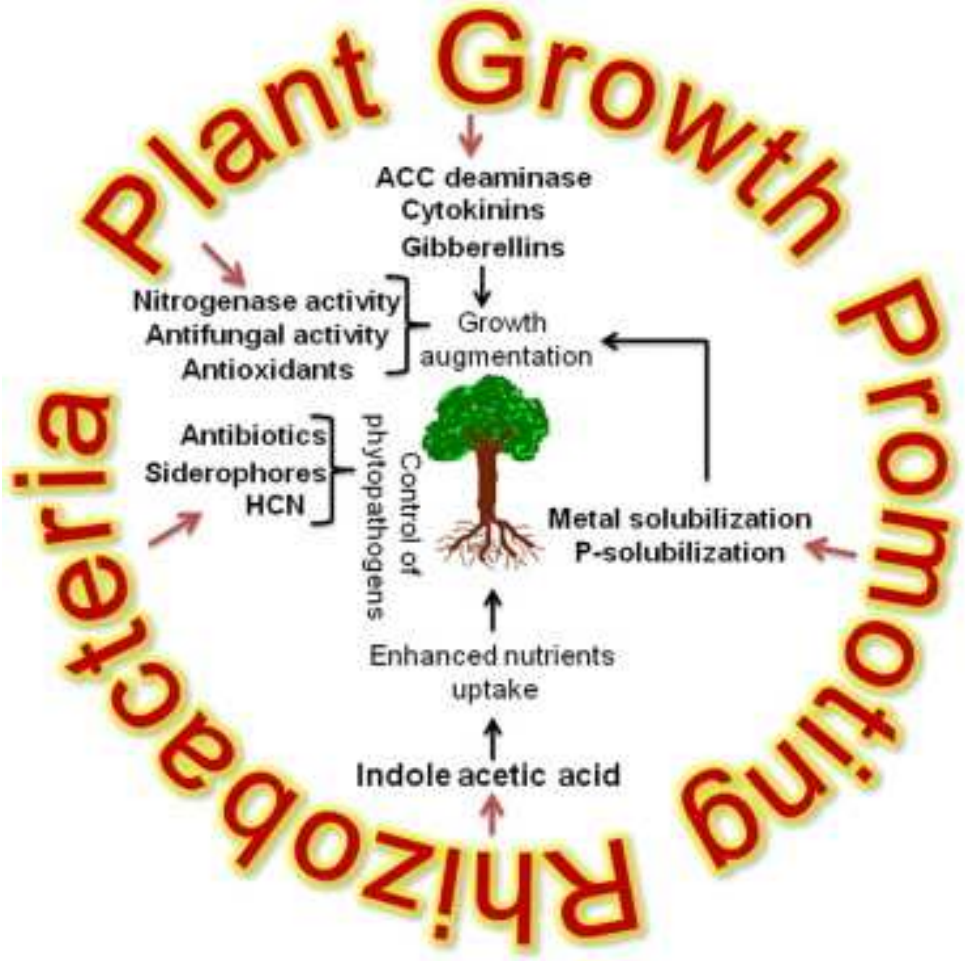

Figure 1.1 Examples of mechanisms by which Plant Growth Promoting Rhizobacteria (PGPR) support plant growth in soil (Ahmed, 2014).

ACC deaminase structure gene *acdS* and the regulator gene *acdR* have been found in many rhizhobacteria including *Pseudomonase sp., Enterobacter sp., Agrobacterium sp., Achromobacter sp.*, and *Rhizobium sp.* (Blaaha et al., 2006). However, not all strains of a particular genus or species are expected to have an *acdS* gene.

A model to explain how ACC deaminase promotes plant growth is described in Fig. 2. In this very basic model (Glick et al., 1998), ACC deaminase-producing bacteria first bind to the surface of the seed or root of the plant. In presence of the tryptophan in the root exudates, the ACC deaminase-producing bacteria synthesize and secrete indol-3-acetic-acid (IAA) (Hong et al., 1991), which is taken up by the plant and results in cell elongation. IAA can stimulate the activity of the enzyme ACC synthase to convert S-adenosylmethionine (SAM) to ACC (Kende, 1993). Through the action of ACC oxidase, ACC is then metabolized into ethylene, which, under non-stressed conditions, may be present in plant tissues in concentrations as low as 0.05 μL L^{-1} (Abeles et al., 1992). The ACC hydrolyzed by ACC deaminase enzyme into ammonia and α-ketobutyrate, and then assimilated by the bacteria (as N and C sources, respectfully). As exuded ACC is degraded, the amount of ACC outside of the plant decreases, and therefore, the plant must keep exuding ACC to maintain the balance of ACC inside and outside of plant. At the same time, the presence of ACC in root exudates induces rhizobacteria to produce more ACC deaminase enzyme. As a result of the diffusion gradient and enzyme induction, the bacteria casue the plant to synthesize greater amounts of ACC than it needs. Furthermore, under stress, production of ACC synthase is induced, and that leads to increased concentration of ACC and then ethylene. At the same time, ACC-deaminase production by bacteria is further induced by the increased amount of

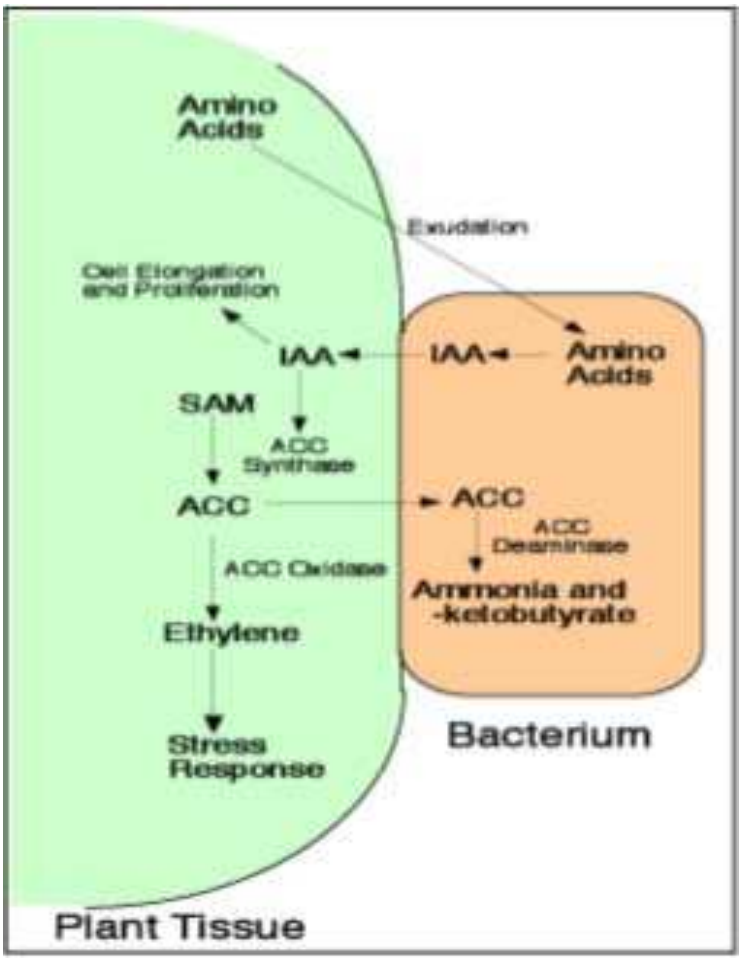

Figure 1.2 Schematic model of how Plant Growth Promoting Rhizhobacteria that both express ACC deaminase and synthesize IAA promote plant growth. First, PGPR bind to either a seed or plant root. SAM is converted to ACC by the enzyme ACC synthase; ACC is converted ether to either α-ketobutyrate or ammonia by ACC-deaminase or to ethylene by ACC- oxidase. IAA biosynthesis, both in bacteria and in plants is a complex multi-enzyme/protein process as is IAA signal transduction. Lowering the ethylene level support root elongation and plant growth.
ACC: 1-aminocyclopropane-1-carboxylate; IAA, indole-3-acetic acid; SAM, S-adenosyl methionine (Glick et al., 2008).

ACC that ensues from the induction of ACC synthase in plant. This results in increased ACC deaminase activity of ACC deaminase-positive bacteria under abiotic stress. Increased bacterial activity leads to faster degradation of ACC and lowered ethylene levels. In soil, ACC-deaminase positive bacteria can control the level of ethylene in the plant, depending on the ratio of ACC oxidase to ACC deaminase (Glick et al., 1998). In order to effectively decrease ethylene level in the plant, ACC-deaminase is required to function before ACC oxidase is induced. It was reported that when IAA-producing bacteria are associated with the plant, IAA activates the transcription of ACC synthase.

1.3. Implications for ACC-Deaminase Positive Bacteria for Improving Drought Tolerance of Winter Wheat

The Great Plains region produces about two-thirds of the wheat (*Triticum aestivum* L.) grown in the United States. Here, wheat is mainly grown under dryland conditions, and wheat yields are limited due to water deficits in most years. Based on analysis of historical yield and weather data, winter wheat yields in Colorado were mainly constrained by the amount of precipitation (Byrne, unpublished data). Moreover, the frequency and severity of drought stress is likely to increase in the Great Plains due to climate change (Karl, 2009). One mechanism by which winter wheat might cope with water stress is through interactions with ACC+ bacteria. To determine the importance of this association, more information on the ecological interactions between wheat and ACC+ bacteria are needed. For example, it is known that plant growth stage can affect the abundance and diversity of ACC deaminase positive bacteria (Mantelin and Touraine, 2004; Penrose and Glick, 2001), but not much is known regarding how ACC+ bacterial abundance and diversity are influenced by plant genotype or agronomic practices. Furthermore, in Colorado and the Great Plains, where the soil can be affected by drought, no

studies have been conducted on these bacteria or their interaction with winter wheat, or how the diversity of these bacteria are affected by agricultural management practices.

There are ten major growth stages that the wheat progresses during its life cycle shown in (Fig.3). In the present work, we will focus on the following physiological growth stages of winter wheat cultivars: emergence, green-up, anthesis, and mid-grain filling. Here we are more interested in anthesis stage (Fig.4). In this growth stage pollen is being released and the individual grains are being fertilized. We expected that wheat in this stage growth is more sensitive to drought stress. Subjecting the wheat to drought stress during flowering can reduce grain numbers, weight, or yield. It was show that applied drought stress at anthesis or late grain filling stage decreased chlorophyll content, individual grain weight, and grain yield of wheat genotypes and spring wheat cultivars (Pradhan et al., 2012).

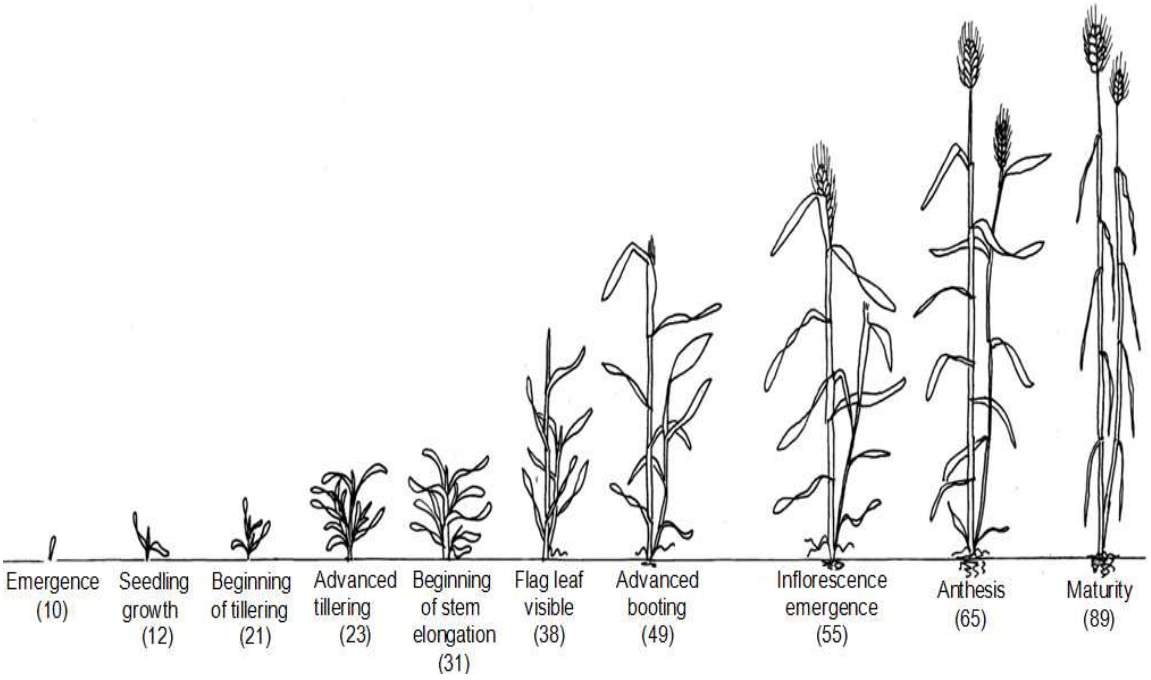

Figure 1.3 Growth stages of wheat describe by Zadoks scale (Zadoks et al., 1974).

Figure 1.4 Wheat at anthesis growth stage.

1.3. Objectives and Hypotheses

The purpose of this research was to identify ACC deaminase positive bacteria that associate with winter wheat varieties under drought stress, so that I can identify specific plant genotype-microbial species interactions that contribute to winter wheat productivity under drought stress.

My specific objectives were to:

1. Determine the diversity and ACC deaminase activity of ACC deaminase positive bacteria associated with different winter wheat genotypes and irrigation practices in Colorado (Chapter 2),
2. Characterize ACC-deaminase positive bacteria for their plant growth promoting activities, to identify potential isolates for field inoculation (Chapter 3),
3. Assess ACC deaminase bacterial inoculum survival, activity, and effects on winter wheat yields under field conditions (Chapter 4).

My hypotheses are as follows:

1. The richness and relative distribution of ACC-deaminase positive bacteria in the rhizosphere will differ among different genotypes of winter wheat and by irrigation practices that affect water availability,
2. The greatest diversity and activity of ACC deaminase positive bacteria will be associated with drought-tolerant winter wheat genotypes (eg., Ripper),
3. Microbial diversity will be greater under full irrigation compared to dryland management practice. This is based on preliminary results that found

Pseudomonas to be the only genera under dryland, whereas *Rahnella aquatilis* and *Bacilius spp.,* and *Pseudomonas spp.* were detected under full irrigation.

4. Drought tolerance in winter wheat can be improved through inoculation with ACC deaminase bacteria that possess multiple PGP traits and prepared as an alginate bead inoculum.

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CHAPTER 2: GENOTYPE-SPECIFIC ENRICHMENT OF ACC-DEAMINASE POSITIVE BACTERIA IN WINTER WHEAT RHIZOSPERES[1]

1. Introduction

Among the many ecosystem services provided by soil bacteria, perhaps none are as important within agricultural systems as supporting plant growth. Soil bacteria that influence plant growth positively are called plant growth promoting bacteria (PGPB) (Bashan and Holguin, 1998). These bacteria reside within root tissues, on the root surface, and in the rhizosphere soil, where they can improve plant growth by increasing availability of nutrients to plants, producing phytohormones that regulate plant growth, and antagonizing and outcompeting plant pathogens in the soil environment (Lugtenberg and Kamilova, 2009; Chaparro et al., 2012).

Water scarcity is among the most difficult of the challenges facing agricultural sustainability and food security. Of growing interest is the ability of certain PGPRs to reduce the effects on plants of abiotic stress in the environment, including drought. Under abiotic stress (e.g., drought, salinity, and heavy metal stress), plants produce the phytohormone ethylene, which induces defense responses such as reduced root and shoot growth and reduced productivity (Glick, 2014). ACC-deaminase positive (ACC+) bacteria degrade the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), through the action of ACC deaminase. The ACC substrate is released by plant tissues, then absorbed and degraded by

[1] A modified version was published as Stromberger, M.E., I. Abduelafez, P. Byrne, M.M. Canela, A.A. Elamari, D.K. Manter, and T. Weir. 2017. Genotype-specific enrichment of ACC-deaminase positive bacteria in winter wheat rhizospheres. Soil Science Society of America 81:796-805. Asma Elamari's contributions to the publication included performing laboratory analyses of total and ACC+ bacterial enumerations, isolating pure cultures of ACC+ bacteria, molecular analysis of LIRF rhizosphere ACC+ bacterial communities, soil ACC deaminase enzyme activity at ARDEC, and statistical analyses. Asma also contributed to the data interpretation and writing of the manuscript.

ACC+ microbes. This results in lower plant ethylene concentrations, continued root elongation and greater resistance to water stress (Mayak et al., 2004; Glick, 2005; Shaharoona et al., 2007; Naveed et al., 2008; Nadeem et al., 2010). To date, studies conducted on these bacteria have primarily focused on their isolation and subsequent testing as inocula for crops grown in the greenhouse or field (for reviews see Saleem et al., 2007 and Forni et al., 2017). Rarely have studies examined the natural abundance and diversity of indigenous ACC+ populations in rhizospheres (Timmusk et al., 2011), and none have studied the indigenous populations of the semi-arid Great Plains region of the United States, despite the potential for their positive influence on plant growth under water stress.

The Great Plains region produces about two-thirds of the wheat (*Triticum aestivum* L.) grown in the United States, mainly under dryland (rainfed) conditions. In the Colorado plains, the annual precipitation averages only 250-450 mm (semi-arid climate), and wheat yields are constrained because of water deficits in most years. Moreover, the frequency and severity of drought stress is predicted to increase in the Great Plains due to climate change (Karl, 2009; Dai 2013). One mechanism by which winter wheat might cope with water stress is through interactions with ACC+ bacteria. To determine the importance of this association, more information on the ecological interactions between wheat and ACC+ bacteria, as influenced by genotype and water stress, are needed. Therefore, the objectives of this study were to assess the abundance, diversity, species composition, and activity of ACC+ bacteria in Colorado agricultural soils, and to determine the degree to which ACC+ bacteria are influenced by winter wheat genotype, physiological growth stage of wheat, and soil water availability as determined by irrigation regime.

2. Materials and Methods

2.1. Field study 1 – Greeley

For the first study, we examined culturable ACC+ bacteria associated with four winter wheat genotypes grown under three levels of irrigation at three wheat growth stages during the course of a growing season. This study was conducted in 2010-2011 on experimental research plots at the USDA-ARS Limited Irrigation Research Farm (LIRF), located near Greeley, CO (40°26'N and 104°38'W). The average annual precipitation is 370 mm (obtained from the Colorado Agricultural Meteorological Network). The soil at LIRF is classified as an Olney fine sandy loam (mixed mesic, Ustic Haplargid). The experimental design was a split-plot design with three replicates. Main plots were irrigation regimes: 1) full irrigation (>80% field capacity); 2) limited irrigation, where soil water content was maintained above 80% field capacity until anthesis, or flowering, and 3) dryland management. Main plots were split into sub-plots of four winter wheat genotypes (Baca, Hatcher, Ripper, and RonL; Table 1). Sub-plots were 5 m long and 1.4 m wide. Each sub-plot was planted to 6 rows of wheat, and plant density was adjusted to 1,980,000 plants ha^{-1}. Row spacing was 23 cm between rows within a sub-plot, and 28 cm between sub-plots. The study was planted in October 2010 and all plots were given 43.3 mm of water to ensure even germination. Total rainfall from planting to harvesting was 185 mm. An additional 373 mm and 139 mm were given to the full irrigation and limited irrigation treatments, respectively (Fig. 1A). The number and amount of water applied was determined by weekly measurements of soil water content in the top six feet with neutron probes installed in the plots planted to Hatcher.

2.1.1. Soil sampling

Roots with rhizosphere soil (defined as soils adhering to roots) were collected at the following growth stages of wheat (Zadoks et al., 1974): seedling in November 2010 (approximate Zadoks stage 12), green-up in March 2011 (Zadoks 20-23), anthesis (flowering) in late May 2011 (Zadoks 60-65), and mid-grain filling in late June 2011 (Zadoks 80-85). Samples were collected by excavating roots and associated soil from at least three plants per plot with a trowel, to a depth of 20 cm. Samples from individual plants within each plot were composited into labeled Ziploc bags and stored on ice chests for transport back to the laboratory. Samples were homogenized by hand and stored at 4°C. A total of 36 samples were collected at each time point (4 genotypes × 3 irrigation treatments × 3 replicates).

2.1.2. Abundance of culturable ACC+ bacteria

Subsamples (10 g) of rhizosphere soil and attached roots were suspended in 90 mL of sterile phosphate buffered saline (pH 7.2) to achieve a 1:10 dilution. Suspensions were blended on high speed for one minute in a Waring blender and serial, ten-fold dilutions were made in sterile phosphate buffered saline. Total culturable bacteria were enumerated by plating diluted suspensions (10^{-4} to 10^{-6}) onto triplicate 10% tryptic soy broth (TSB) agar plates (3g TSB, 18 g agar L^{-1}). Culturable ACC+ bacteria were enumerated by plating dilutions (10^{-3} to 10^{-5}) onto Dworkin and Foster (DF) minimal salts medium containing ACC as the sole N source (Penrose and Glick, 2003). Plates were incubated at 28°C for 4-5 days, and colonies were counted on plates. The abundance of ACC+ bacteria was expressed as the number of colony forming units (CFUs) per g rhizosphere soil. This number was divided by the number of CFUs counted on the TSBA plates to determine the proportion of ACC+ bacteria relative to total culturable bacteria.

2.1.3. Species composition and diversity of culturable ACC+ bacteria

We previously experimented with published primers that target a portion of the ACC deaminase gene, acdS (Blaha et al., 2006). Unfortunately, this method employed degenerate primers that produced more than one amplicon, thereby limiting downstream molecular analyses. To assess the diversity of ACC+ bacteria at the anthesis growth stage, an alternative approach was chosen, whereby ACC+ bacteria were selectively enriched from triplicate soil samples collected from roots of Ripper and Ron L, under dryland and full irrigation treatments, in liquid medium and subsequently analyzed for bacterial community diversity by 454 pyrosequencing of 16S rDNA. Following the enrichment method outlined by Penrose and Glick (2003), 1 g of rhizosphere soil was added to 50 ml sterile medium containing (per litre) 10 g proteose peptone, 10 g casein hydrolysate, 1.5 g anhydrous $MgSO_4$, 1.5 g K_2HPO_4 and 10 ml glycerol (PAF medium) in a 250 mL flask. The flask was incubated in a shaking incubator (200 rpm at 30°C for 24 h). After 24 h, a 1 mL aliquot was removed from the growing culture, transferred to 50 mL of sterile PAF medium in a 250 mL flask and incubated a second time. A 1-mL aliquot was removed from the second culture and transferred to a 250-mL flask containing 50 mL sterile DF salts minimal medium containing $(NH_4)_2SO_4$ as a nitrogen source. After another 24-h incubation as described above, a 1-mL aliquot was removed from this culture and transferred to 50 ml sterile DF salts minimal medium containing 3.0 mM ACC as the sole source of nitrogen. After a 24-h incubation, the cultures were centrifuged in sterile 50 mL conical tubes to pellet the cells.

DNA was extracted directly from pelleted cells using the FastDNA Spin Kit for Soil (MP Biomedical, Santa Ana, CA). Extracted DNA was stored at -20°C. A 291-bp fragment of the V4 region of the 16S rDNA was amplified using fusion primers with GS Flex 454 standard chemistry compatible adaptors (forward primer 515F; 5'-GCC TTG CCA GCC CGC TCA GGT

GTG CCA GCM GCC GCG GTA A and the reverse primer 806R; 5'-GCC TCC CTC GCG CCA TCA GAN NNN NNN NNN NNG GGG ACT ACV SGG GTA TCT AAT) obtained from Invitrogen. Unique 12 nt error-correcting Golay barcodes were used to tag each PCR product (designated by NNN NNN NNN NNN). Twenty microliter reactions were prepared in 96-well plates with the following reagents: 10 µl 5-Prime Hot Master Mix (5 Prime), 30 µM each of forward and reverse primer, and 15 ng of template DNA (5 ng μl^{-1}). Thermal cycling consisted of initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 2 min, with a final extension of 10 min at 72°C. Replicate PCR reactions from each sample were combined and gel purified using the GenElute Gel Extraction kit (Sigma-Aldrich, St. Louis, MO), followed by an additional purification with AMPure beads (Beckman Coulter, Indianapolis, IN) and quantified with PicoGreen (Invitrogen, Carlsbad, CA). Amplicons were then pooled in equimolar ratios and concentrated by ethanol precipitation. The resulting amplicon library was sequenced under contract by EnGenCore, University of South Carolina (Columbia, SC).

2.1.4. Statistical analysis

Abundance of ACC+ bacteria was analyzed as a repeated measures split-plot block design, using log transformed data for normalization. Sampling date/wheat growth stage was the repeated factor; irrigation level was the main plot and wheat genotype the sub-plot (n=3). The analysis was conducted using the Proc Mixed procedure of SAS (v. 9.2, The SAS Institute, Cary, NC) with the first order autoregressive covariance structure, which assumes that repeated measures are more correlated when sampling dates are closer together than farther apart in time. Significantly different means ($P < 0.05$) were separated by the PDIFF option in SAS.

2.1.5. Sequence Processing and Analysis

All sequence read editing and processing was performed with Mothur Ver. 1.26 (Schloss et al., 2009). Briefly, sequence reads were trimmed (bdiff=0, pdiff=0, qaverage=30, minlength=150, maxambig=0, maxhomop=8) and aligned to the SILVA alignment available at the Mothur website (http://www.mothur.org). Filtered sequences were checked for chimeras using the uchime method and classified using the Silva database. Sequences were screened, filtered, and pre-clustered (diff =2) to remove potential pyrosequencing noise and then assigned to phylotypes. To mitigate the effect of sample size on measures of alpha and beta diversity, sub-samples of 1170 reads (based on the sample with the smallest number of high quality sequences) were randomly selected from each sample. Sample size independent values were calculated in Mothur for alpha diversity community descriptors such as observed species richness (S_{obs}), and Shannon's (H') and Simpson's diversity (1-D) indices. Beta diversity was assessed using phylotype-based clustering to compare community membership and structure between ACC+ bacterial communities.

2.2. Field study 2 – Fort Collins

A second field study was conducted to assess wheat genotype effects on ACC+ bacteria using culture-independent approaches. Potential ACC deaminase activity and PICRUSt analysis of 16S rDNA sequences were conducted on rhizosphere soils under 12 winter wheat genotypes grown under dryland or fully irrigated conditions. This study was conducted in 2013 on experimental research plots at the Agricultural Research, Development and Education Center (ARDEC), located near Fort Collins, CO (40°39'N, 105°00'W). Average annual precipitation is 400 mm (obtained from the Colorado Agricultural Meteorological Network). The soil at ARDEC is classified as a Nunn clay loam (fine, smectitic, mesic, Aridic Agriustoll). At this site two large

wheat trials were grown side-by-side in the same field. Both trials were planted in October 2012, at a seeding density of approximately 1,700,000 seed ha^{-1}. Row spacing was 23 cm between rows within a sub-plot, and 28 cm between sub-plots. One trial was fully irrigated, receiving 86 mm of precipitation from January through June and 210 mm of irrigation in May and June. The other trial (dryland) received the same amount of precipitation with no supplemental irrigation. Each trial included 299 genotypes (cultivars and advanced breeding lines), of which a subset was sampled for this study: a dryland and a fully irrigated plot for each of the 12 genotypes specified in Table 1. Wheat genotypes were planted in six-row plots 1.83 m long and 1.52 m wide.

2.2.1. Soil sampling

Rhizosphere soil was collected from each plot in June 2013, at mid-grain filling (approximate Zadoks growth stage 80-85; Zadoks et al. 1974). This growth stage corresponds to the growth stage of Field Study 1 when greatest relative abundances of ACC+ bacteria were measured (Table 4). Samples were collected by excavating roots and associated soil from at least three plants per plot with a trowel, to a depth of 20 cm. Samples from individual plants within each plot were composited into labeled Ziploc bags and stored on ice chests for transport back to the laboratory. Samples were homogenized by hand and stored at 4°C for ACC deaminase enzyme activity (see below). A subsample from each bag was frozen at -80° for molecular analysis.

2.2.2. Potential ACC deaminase enzyme activity

Potential ACC deaminase enzyme activity was determined according to Smaill et al. (2010), except that 1-g subsamples of soil were incubated in 5 mL of 0.1 M tris buffer (pH 8.5) and 20 mM ACC. Controls (for soil background absorbance) were prepared by adding soil to

buffer without ACC addition. Blanks were prepared that included all reagents but no soil (for baseline absorbance) Suspensions were incubated for 24 h at 30°C. After incubation, samples were vortexed and centrifuged at 480 × g. Supernatant (1 mL) was removed and mixed with 1.8 mL of 0.56 M HCl to end the reaction. Next, 0.3 mL of 0.1% (w/v) 2,4 dinitrophenylhydrazine in 2 M HCl was added to each sample. Samples were incubated for 15 min at 30°C, after which 2 mL of 2 M NaOH were added. The absorbance of each sample was read at 450 nm on a microplate reader (model 680, Bio-Rad Laboratories, Inc., Hercules, CA). The concentration of α-ketobutyrate developed (μg g^{-1} h^{-1}) was determined from a standard calibration curve of α-ketobutyrate (Sigma Aldrich, St. Louis, MO) prepared within a range of 0-220 μg mL^{-1} tris buffer.

2.2.3. 16S rDNA sequencing and PICRUS analysis

The abundance of ACC+ bacteria in the samples obtained from the Field Study 2 was assessed using a pyrosequencing/mapping approach. First, quantitative PCR (qPCR) was used to amplify the bacterial 16S rRNA genes (V1-V3 hypervariable region) using the 27F and 388R primer set (Lane et al., 1985; Marchesi et al., 1998). Each reaction contained 2 μl template DNA (diluted 1:20), 0.5 μM of each primer, and 1X Maxima SYBR™ Green master mix (cat # K0242, Thermo-Fisher Scientific); amplification was performed as follows: 1) 95 ºC for 8.5 min, 2) 95°C × 15 sec, 58°C × 30 sec, 72°C × 60 sec, repeated 35 times, 3) 72°C × 5 min. Genomic DNA isolated from *Pseudomonas putida* KT2440 was used as a positive control and molecular grade H_2O as a negative control. PCR amplicon products were purified using the Lonza FlashGel DNA Recovery System (Basel, Switzerland) and quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, CA). Amplified DNA samples were then pooled in equimolar ratios to create a single bacterial DNA library. The pooled library was re-quantified

using the KAPA Biosystems (Wilmington, MA) qPCR kits and subjected to pyrosequencing at four copies per bead with the Roche GS Junior Sequencing System (Branford, CT). All sequencing read editing and processing was performed with Mothur Ver. 1.35 (Schloss et al., 2009) using the default settings unless otherwise noted. Briefly, sequence reads were (i) trimmed (bdiff=0, pdiff=0, qaverage=25, minlength=100, maxambig=0, maxhomop=10); (ii) aligned to the bacterial-subset SILVA alignment available at the Mothur website (http://www.mothur.org); (iii) filtered to remove vertical gaps; (iv) screened for chimeras with UCHIME (Edgar et al. 2011); (v) classified using the RDP training set Vers. 9 75 and the naïve Baysian classifier (Wang et al., 2007) embedded in Mothur, after which all sequences identified as chloroplast or mitochondria were removed; (vi) sequences were screened (optimize=minlength-end, criteria=95) and filtered (vertical=T, trump=.) so that all sequences covered the same genetic space; and (vii) all sequences were pre-clustered (diff=2) to remove potential pyrosequencing noise and clustered (calc=onegap, coutends= F, method=average) into operational taxonomic units or OTUs (Huse et al., 2010). After processing, each 16S library was rarefied to 1000 sequence reads in order to standardize sampling effort for each sample.

The abundance of *acdS* (KEGG Orthology: KO1505 1-aminocyclopropane-1-carboxylate deaminase) genes in each 16S library was predicted using PICRUSt (Langille et al., 2013). Each edited and rarefied 16S library was (i) re-classified using the Green Genes database (Vers. 13_5_99) available from Mothur (classify.seqs, method=knn, numwanter=1), (ii) normalized by copy number (PiCRUSt: normalize_by_copy_number.py, and (iii) used to calculate metagenome contributions (PiCRUSt: metagenome_ contributions.py). Gene-specific relative abundances were calculated by dividing each gene count "CountContributedByOTU" by the total number of "normalized reads" for the sample of interest. Total gene-specific abundance (copies g^{-1} soil FW)

was calculated as the product of the relative abundance and the total 16S copies (copies g^{-1} soil FW) determine from the initial sample qPCR.

2.2.4. Data analysis

Potential ACC deaminase activity, averaged across the two plots per genotype, were regressed against predicted % *acdS* genes, based on PICRUSt analysis, using linear regression analysis in Excel (Microsoft Office 2010, Microsoft Corporation, Redmond, WA).

3. Results

3.1. Field study 1 – Greeley

In this study, we investigated the total and relative abundance of culturable ACC+ bacteria, under different wheat genotypes and irrigation practices, throughout a winter wheat growing season (2010-2011). Based on long-term weather data from a weather station installed at the LIRF site, the 2010-2011 growing season was dryer than average, with 184.7 mm of rainfall compared to the long-term average of 251.4 mm (from 1992 to 2011). While most months were drier relative to the long-term average, the rainfall in May 2011 was above normal (~2 fold the average precipitation, not shown). The average, minimum and maximum temperatures were within long-term average, ± 1 SD, in all months of the growing season, except for May 2011, when the monthly average maximum temperature was lower than the long-term values (Fig. 1B). Despite May 2011 being a wetter and cooler month than normal, the amount of cumulative rainfall + irrigation in dryland plots were less than half of the amount in fully irrigated plots (Fig. 1A)

We found that bacterial counts were affected by sampling date and irrigation interaction, whereas relative abundance was affected by a three-way interaction among sampling date,

irrigation regime, and wheat genotype. We selected the May sampling period, which corresponded to the anthesis (flowering) stage of winter wheat, to examine the diversity and composition of selectively enriched ACC+ bacteria under Ripper and RonL in greater detail. At this critical wheat growth stage, we found that the diversity and richness of ACC+ bacterial phylotypes were unaffected by wheat genotype or irrigation regime. However, composition and structure of ACC+ bacteria in the selected communities were strongly affected by wheat genotype. Results are presented in greater detail below.

3.1.1. Abundance of ACC positive bacteria

The abundance of culturable ACC+ bacteria in rhizosphere soil was relatively high, with numbers ranging from 1.69×10^7 to 3.28×10^9 CFU's g^{-1}. Abundance of ACC+ bacteria was affected by the interaction between sampling date and irrigation regime but not by wheat genotype. The number of ACC+ bacteria increased significantly from November to March under all irrigation treatments (Table 2). Under full irrigation, the number of ACC+ bacteria declined from March to mid-grain fill in June, in contrast to the increased or stable numbers over time under limited irrigation and dryland conditions. Irrigation in the fully irrigated plots did not begin until after the March sampling event, whereas irrigation in the anthesis-irrigated plots did not begin until just prior to the May sampling event. Thus, there was no irrigation effect within the first two sampling dates (November and March).

Abundance of ACC+ bacteria was divided by the total number of heterotrophic bacteria to obtain the relative abundance (%) of culturable ACC+ bacteria. This variable was affected by the three-way interaction between sampling date, irrigation regime, and wheat genotype. There were very few differences among wheat genotype × irrigation combinations in November 2010 and March 2011 (when no irrigation had occurred on any plots), so for simplicity, only the three-

way interaction data for May and June 2011 are presented. In May (anthesis), the % ACC+ bacteria were not significantly different among the varieties in the fully irrigated samples (Table 3). In limited irrigation soils, the % ACC+ bacteria were significantly lower under Baca than Hatcher but not Ripper and RonL. For dryland soils, the % ACC+ bacteria were significantly greater under RonL and Ripper than under Hatcher and Baca. Within RonL, the % ACC+ bacteria were greatest under dryland, followed by limited irrigation, and lowest under full irrigation. For Ripper, the % ACC+ bacteria were significantly greater under dryland than under full irrigation. In contrast, % ACC+ bacteria under Hatcher peaked when soils were under limited irrigated. Irrigation treatment had no effect on % ACC+ bacteria when Baca was grown.

By mid-grain filling stage in June, relatively high percentages of ACC+ bacteria were detected in limited irrigation and dryland soils (Table 4). As in May, the % ACC+ bacteria were not significantly different among the varieties in the fully irrigated samples. In limited irrigated and dryland soils, % ACC+ bacteria were significantly greater under RonL than under the other varieties (Baca, Hatcher, and Ripper). Within RonL, the % ACC+ bacteria were significantly greater under limited irrigation, followed by dryland, then full irrigation. For Ripper and Hatcher, the % ACC bacteria were significantly greater under dryland and limited irrigation than under fully irrigated soils. For Baca, % ACC bacteria were significantly greater under dryland soil, followed by limited irrigation, then fully irrigated soil.

3.1.2. Diversity and composition of ACC+ bacteria

Neither species richness nor Simpson's or Shannon's diversity indices of ACC+ bacteria differed significantly by wheat genotype or irrigation level (Table 5). Genotype affected ACC+ bacterial community composition, however, as evidenced by the separation of RonL and Ripper communities when analyzed by unweighted Jaccard's dissimilarity distances (J_{class}) that

compared phylotype membership (presence/absence) between communities. RonL ACC+ bacterial communities, regardless of irrigation treatment, showed a relatively higher degree of similarity to each other than was observed between the Ripper ACC+ bacterial communities (Fig. 2A). Using Analysis of Molecular Variance (AMOVA) this genotype effect was determined to be statistically significant (P=0.013). This genotype effect was not apparent when using Yue and Clayton's theta (ϴYC) distances to assess community structure (presence/absence and relative abundance of phylotypes) when all of the replicates were analyzed separately (data not shown); however, analysis of treatment differences in the combined replicates showed that samples clustered according to genotype rather than to irrigation regime (Fig. 2B). Weighted Unifrac distances (>5=community structure differs, <5= community structure is similar) also suggest that genotype (score= 0.583, $p<0.001$) was more important than irrigation regime (score= 0.438, $p<0.001$) as a driving factor of community structure.

Sequences were classified using the Silva database downloaded from the mothur website. Over 6,700 total sequences were classified to the genus level, and 99% of these sequences were distributed among 3 phyla (Bacteriodetes, Firmicutes, and Proteobacteria) and 23 known genera. The distribution of genera associated with Ripper and RonL, under full irrgation and dryland management, are shown in Fig. 3. Dominant genera included *Pseudomonas* (35-54% of OTUs), *Sphingobacterium* (3-13%), *Chryseobacterium* (5-10%), *Buttiauxella* (4-11%), *Stenotrophomonas* (~4%), and *Acinetobacter* (0-5%) (Fig. 2). A genotype effect was observed as trends for different distributions of *Pseudomonas, Buttiauxella* (phylum Proteobacteria)*, Bacillus* (phylum Firmicutes), and *Sphingobacterium* (phylum Bacteriodetes) between Ripper and RonL (Table 6). Compared to Ripper, RonL had greater proportions of species from *Sphingobacterium* (p=0.041), *Buttiauxella* (not significant), and *Bacillus* (not significant), and fewer species of

Pseudomonas (not significant) in its rhizosphere than did Ripper. The majority of sequences aligning to *Sphingobacterium* most closely aligned with *Sphingobacterium multivorum* using a BLAST search for classification.

3.2. Field study 2 – Fort Collins

A second field study was conducted to determine if the pattern of genotype effect, observed in the first study, would be consistent using culture-independent methods and at a different location/soil type. Potential ACC deaminase activity ranged from 190 to 729 nmol α-ketobutyrate g^{-1} day^{-1} (mean = 366, SD = 141, n=24). There was no significant effect of irrigation treatment on potential ACC activity when values were averaged across all genotypes. Mean values (±1 SD; n=12) were 388 ± 94 nmol g^{-1} day^{-1} for rhizospheres of dryland plots and αgenotype effect on potential ACC deaminase activity, averaged across the dryland and irrigated plots (n=2). The average potential ACC deaminase activity was lowest in Byrd rhizosphere soil (216 ± 37 nmol g^{-1} day^{-1}), intermediate in Ripper (324 ± 42 nmol g^{-1} day^{-1}) and greatest in rhizosphere soil of RonL (557 ± 242 g^{-1} day^{-1}).

PICRUSt analysis of 16S rDNA sequences from rhizosphere soils demonstrated that RonL rhizosphere was predicted to have the greatest abundance of bacteria containing the ACC deaminase gene, *acdS* (Fig. 4). The wheat genotype Settler had poor gene prediction results for not only *acdS* but for other functional genes in PICRUSt (data not shown), and so was excluded in the regression analysis with potential ACC deaminase activity. Regression analysis between predicted *acdS* and potential ACC deaminase activity was positive and significant ($p = 0.032$, $r = 0.65$, $r^2 = 0.42$, n=11), which was largely due to high potential ACC deaminase activity in RonL rhizosphere soil (Fig. 5).

4. Discussion

In most previous studies, ACC+ bacteria have been isolated from soil and studied as plant inocula for their abilities to promote plant growth under abiotic stress (e.g., Shaharoona et al., 2007; Naveed et al., 2008; Nadeem et al., 2010). This study is the first of its kind to describe the natural abundance and diversity of these beneficial bacteria in field soil, as influenced by winter wheat genotype, development stage, and irrigation regime/soil water availability.

In general, it was discovered that wheat genotypes influenced the relative amounts of ACC+ bacteria that accumulated in their rhizospheres as soil water availability declined in the limited irrigated and dryland plots at anthesis. Soil gravimetric water contents (0-20 cm depth) were similar across all LIRF plots at green up in March (32%) but fell to 10-11% in dryland and limited-irrigated plots in May (just prior to initiation of irrigation in limited-irrigated plots). The effects continued through mid-grain filling in June, when the greatest relative abundance of ACC+ bacteria was observed, especially in dryland plots (where gravimetric water content was only 3%). At the anthesis stage, irrigation had just started in the limited irrigation plots, so these plots experienced a period a water deficiency between green up and anthesis. The dryland plots, in contrast, were water limited throughout much of the growing season. It is known that ethylene plays a role in grain maturation in wheat, and that ethylene production increases from pre-anthesis stage to hard dough stage of grain (Beltrano et al., 1994). Therefore, an increased production of ACC as part of ethylene biosynthesis, combined with reduced soil water availability in the limited irrigation and dryland plots, could explain the increase in % ACC deaminase positive bacteria over time. Based on the results of this study, sampling for ACC+ bacterial abundance and diversity should be timed with the anthesis to mid-grain filling growth stages.

Phylogenetic analysis based on 16S rDNA sequences from LIRF showed that the diversity of ACC+ bacteria associated with RonL and Ripper at anthesis belonged to three major phylogenetic groups: Firmicutes, Bacteroidetes, and Proteobacteria. Bacteria within the Protobacteria, especially *Pseudomonas* species, were the most commonly detected ACC+ bacteria associated with Ripper and RonL. Compared to other genotypes, RonL rhizosphere was enriched in *Sphingobacterium*, particularly the species *Sphingobacterium multivorum*. This species is a Gram-negative rod that can be isolated from soil, water, and plants, including wheat rhizospheres (Yang et al., 2010). These results should be interpreted cautiously, however, as 16S rDNA sequence analysis was conducted on bacteria selectively enriched in the presence of ACC under laboratory conditions. Using this type of approach, others have also reported *Pseudomonas* to be a dominant genus of ACC+ bacteria in plant rhizospheres (e.g., Timmusk et al., 2011; Akhgar et al., 2014). Future studies should focus on developing and applying molecular-based methods to characterize the genetic diversity of non-culturable ACC+ bacteria in soil.

Under limited irrigated and dryland soils at LIRF in May and June, the four wheat genotypes showed differences in the relative percent and composition of ACC+ bacteria that accumulated in their rhizospheres. The percentage of ACC positive bacteria under RonL was greater compared to the other varieties (Baca, Ripper, and Hatcher), and RonL harbored as assemblage of ACC+ bacteria different from that of Ripper. Similar results were found at ARDEC, where RonL was predicted to have the greatest relative amounts of the ACC deaminase gene *acdS* and had the greatest potential ACC deaminase activity. RonL is a hard white winter wheat genotype that is medium in height, has medium to late maturity, and is reported to have "good" drought tolerance (Kansas Wheat Alliance, 2014). The ability of RonL to accumulate relatively high percentage of ACC+ bacteria could indicate that this genotype produces greater

concentrations of ACC and stress ethylene compared to the other genotypes tested when water stressed. Alternatively, RonL may be more effective at recruiting and selecting certain ACC+ bacteria than the other genotypes, presumably through production of root chemical signals. Soil microbial communities are known to vary under the influence of different genotypes of the same plant species, including wheat (Chanway et al., 1988; Mazzola et al., 2004; Micallef et al., 2009; Manter et al., 2010; Peiffer et al., 2013; Donn et al., 2015). Such biological interactions in the rhizosphere are primarily mediated by root exudates, which can serve as signals to promote chemotaxis (Gaworzewska and Carlile, 1982; de Weert et al., 2002), initiate symbiotic interactions (Oldroyd and Downie, 2008), and/or act as substrates promoting growth of specific microbes.

Drought is arguably the most devastating abiotic stress affecting crop production world-wide and the most difficult to address through crop genetic improvement. This is because of the inherent variability in drought severity, timing, and duration, and the many genes and mechanisms involved in the adaptive responses of plants to drought (Blum, 2011; Reynolds et al., 2009; Richards et al., 2010). RonL has some tolerance to drought, and compared to other genotypes studied, RonL showed the greatest potential to accumulate high relative abundances of ACC+ bacteria and potential ACC deaminase activity. Interestingly, Ripper, a tall semi-dwarf, early-maturing hard red winter wheat, is also considered drought tolerant (Haley et al., 2007). That both RonL and Ripper have shown drought tolerance yet different associations with ACC+ bacteria could be indicative of different strategies among winter wheat cultivars for coping with drought stress. Further studies are warranted to determine the role of root exudates and the relative contribution of ACC+ bacteria to drought tolerance among different winter wheat genotypes. With increased understanding, the exploitation of ACC+ bacteria's natural abilities

with appropriate host crops could help to improve food production and sustainable agriculture in water-scarce regions.

Table 2.1 Winter wheat genotypes used in Field Studies 1 and 2.

Genotype name	Study	Developer [†]	ID [‡]
Arlin	2	KSU	PI 564246
Baca	1	CSU	CItr 15891
Byrd	2	CSU	PI 664257
Hatcher	1, 2	CSU	PI 638512
HV9W03-1596R	2	WB	na
Onaga	2	WB	na
Ripper	1, 2	CSU	PI 644222
RonL	1, 2	KSU	PI 648020
Ruby Lee	2	OSU	PI 661991
Settler CL	2	UNL	PI 653833
TAM 107-R7	2	CSU, TAMU	GSTR 11601
TAM 202	2	TAMU	PI 561933
TX06A001281	2	TAMU	na

[†] CSU, Colorado State University; KSU, Kansas State University; OSU, Oklahoma State University; TAMU, Texas A&M University; UNL, University of Nebraska-Lincoln; WB, WestBred

[‡] Identifier in USDA-ARS National Plant Germplasm System database, http://www.ars-grin.gov/npgs/index.html (verified 17 Jan, 2016)

Table 2.2 The effect of wheat growth stage/sampling time and irrigation regime on log-transformed numbers of ACC+ bacteria g-1 dry soil (± 1 SE in parentheses) collected from rhizosphere soil and roots of winter wheat grown at LIRF, Greeley, CO. Data are averaged across four wheat genotypes (n=4).

	Irrigation level		
Growth stage	Full	Limited	Dryland
Emergence, November 2010	8.16 (0.10) b A	8.08 (0.09) c A	8.03 (0.10) b A
Green up,March 2011	8.53 (0.11) a A	8.58 (0.11) ab A	8.47 (0.11) a A
Anthesis,May 2011	7.76 (0.10) c B	8.23 (0.10) bc A	8.46 (0.10) a A
Mid-grain filling, June 2011	7.58 (0.63) c B	8.63 (0.06) a A	8.77 (0.06) a A

Within an irrigation treatment, means labeled with different lower case letters are significantly different ($P < 0.05$) among sampling dates. Within a growth stage, means labeled with different upper case letters are significantly different ($P < 0.05$) among irrigation treatment.

Table 2.3 The effect of winter wheat genotype and irrigation level on the percentage of culturable ACC+ bacteria (± 1 SE in parentheses), relative to the total culturable bacteria, at wheat anthesis stage in May 2011 at LIRF, Greeley, CO (n=3).

	Irrigation level		
Wheat genotype	Full	Limited	Dryland
Baca	1.22 (0.56) a A	4.05 (2.16) b A	6.87 (2.01) b A
Hatcher	1.29 (0.36) a B	13.5 (6.8) a A	6.85 (1.70) b B
Ripper	1.78 (0.41) a B	7.66 (4.13) ab AB	12.6 (2.3) ab A
RonL	1.70 (0.39) a C	7.98 (1.96) ab B	17.6 (9.3) a A

Within an irrigation treatment, means labeled with different lower case letters are significantly different ($P < 0.05$) among wheat genotypes. Within a wheat genotype, means labeled with different upper case letters are significantly different ($P < 0.05$) among irrigation treatments.

Table 2.4 The effect of winter wheat genotype and irrigation level on the percentage of culturable ACC+ bacteria (± 1 SE in parentheses), relative to the total culturable bacteria, at wheat mid-grain filling stage in June 2011 at LIRF, Greeley, CO (n=3).

	Irrigation level		
Wheat Variety	Full	Limited	Dryland
Baca	2.42 (1.18) a C	16.3 (4.7) c B	25.8 (3.1) bc A
Hatcher	2.98 (0.52) a B	24.3 (5.1) b A	19.9 (5.1) c A
Ripper	1.47 (0.43) a B	27.9 (4.0) b A	26.5 (7.8) b A
RonL	1.66 (0.39) a C	54.3 (13.6) a A	36.5 (7.9) a B

Within an irrigation treatment, means labeled with different lower case letters are significantly different ($P < 0.05$) among wheat genotypes. Within a wheat genotype, means labeled with different upper case letters are significantly different ($P < 0.05$) among irrigation treatments.

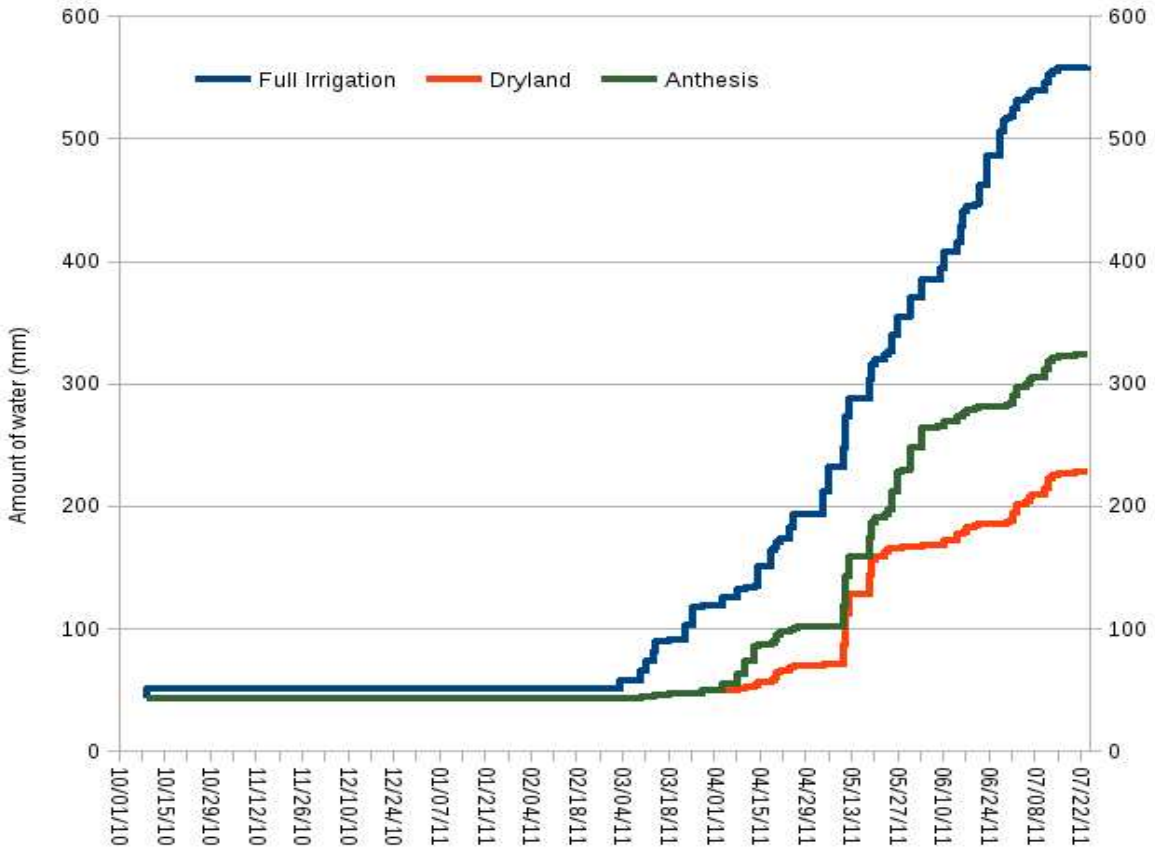
Table 2.5 Diversity measures (± 1 SE in parentheses) of ACC+ bacterial OTUs associated with Ripper and RonL winter wheat genotypes at the anthesis stage (May 2011), grown under full irrigation or dryland conditions at LIRF, Greeley, CO (n=3). The number of sequences analyzed per genotype in irrigation regime combination was 1,170. OTUs were classified at the 0.03 genetic distance. There were no significant differences based on two-way ANOVA with post hoc Bonferroni tests.

Wheat genotype and Irrigation	Coverage	Richness	Inverse Simpson's index	Shannon diversity index
Ripper, Full	0.961 (0.006)	28.99 (2.96)	3.43 (0.75)	1.77 (0.23)
Ripper, Dryland	0.976 (0.003)	20.29 (1.68)	2.41 (0.89)	1.33 (0.39)
RonL, Full	0.966 (0.004)	25.35 (0.88)	4.66 (0.46)	1.94 (0.09)
RonL, Dryland	0.963 (0.008)	25.68 (3.22)	4.07 (1.14)	1.73 (0.27)

Table 2.6 Proportion of dominant ACC+ bacterial OTUs (± 1 SE in parentheses) associated with Ripper and RonL winter wheat genotypes at the anthesis stage (May 2011), grown under full irrigation or dryland conditions at LIRF, Greeley, CO (n=3). Only the differences in Sphingobacterium between Ripper and Ron L are statistically significant (p=0.041, ANOVA with post hoc Bonferroni).

Genus	Ripper, Full	Ripper, Dryland	RonL, Full	RonL, Dryland
Bacillus	1.4% (0.47)	1.4% (0.41)	2.0% (0.71)	2.5% (2.46)
Buttiauxella	7.8% (2.92)	4.8% (3.85)	11.0% (4.27)	11.1% (0.78)
Pseudomonas	47.5% (4.48)	53.6% (6.02)	35.2% (6.34)	41.9% (10.03)
Sphingobacterium	3.6% (1.52)	7.9% (1.39)	14.9% (2.24)	13.1% (6.93)

A


Full Irrigation
Dryland
Anthesis
Amount of water (mm)
600
500
400
300
200
100
0
10/01/10
10/15/10
10/29/10
11/12/10
11/26/10
12/10/10
12/24/10
01/07/11
01/21/11
02/04/11
02/18/11
03/04/11
03/18/11
04/01/11
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06/24/11
07/08/11
07/22/11

B

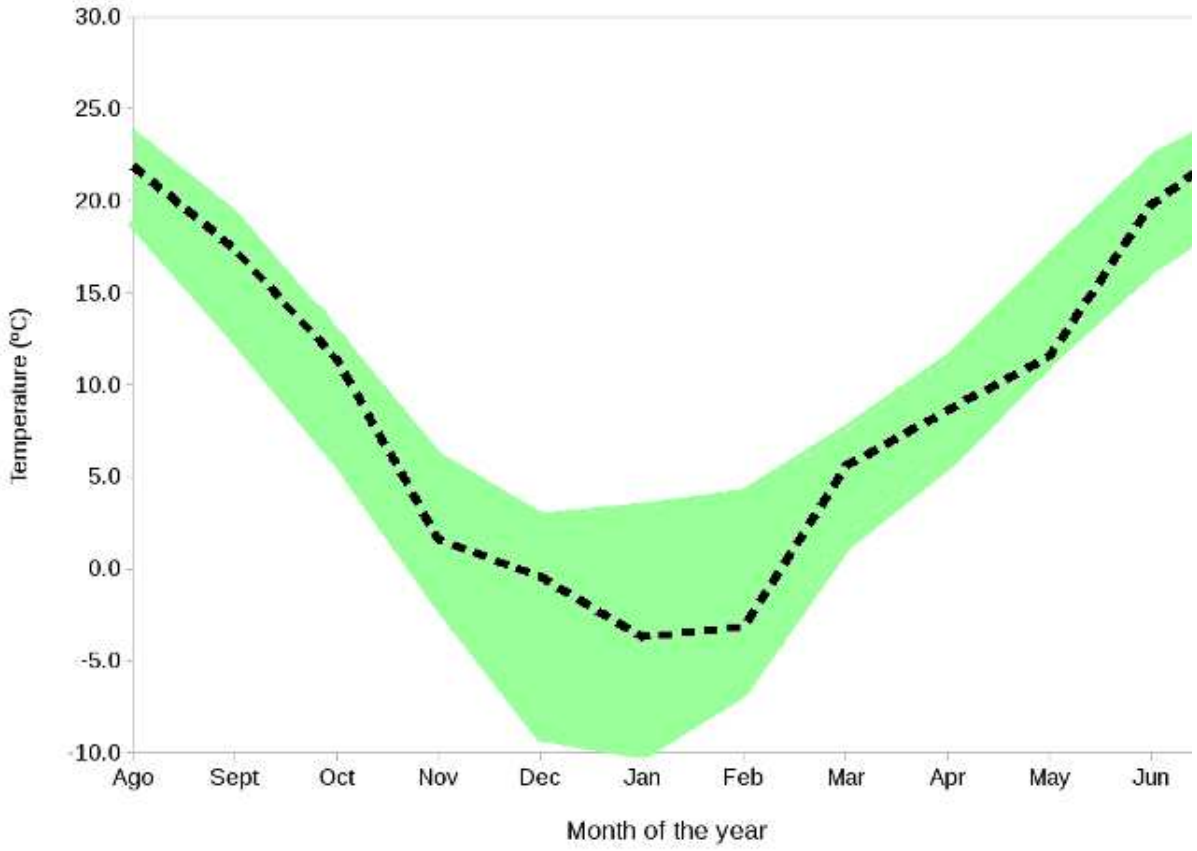
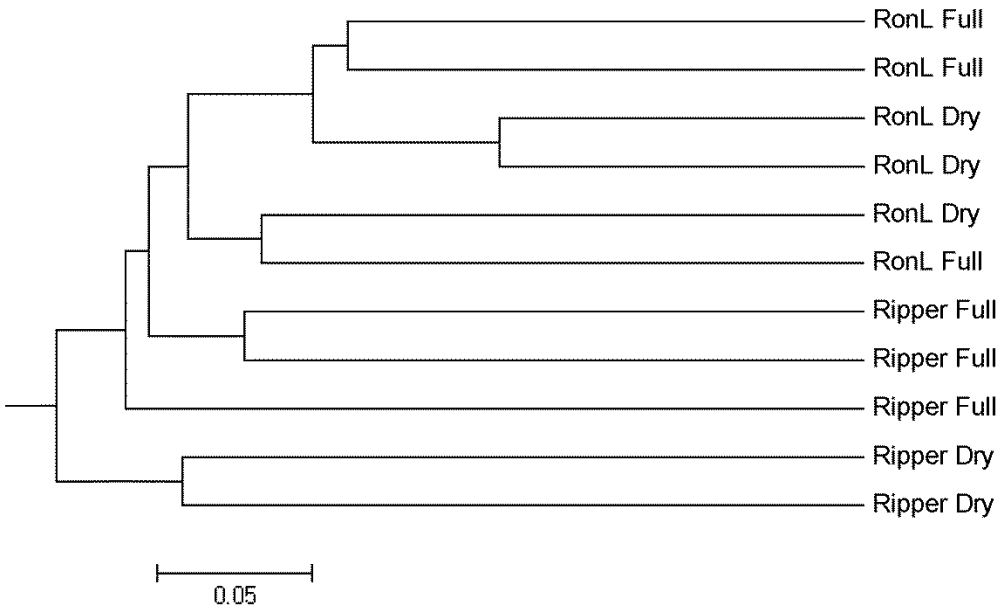


Figure 2.1 Weather conditions at the USDA LIRF near Greeley, CO during the 2010-2011 growing season. A) Cumulative rainfall + irrigation in three water treatments (full irrigation, limited irrigation until anthesis, and dryland), and B) 2010-2011 growing season monthly mean temperature (dashed line) and long term mean monthly temperatures ± 1 standard deviation (green area).

A



B

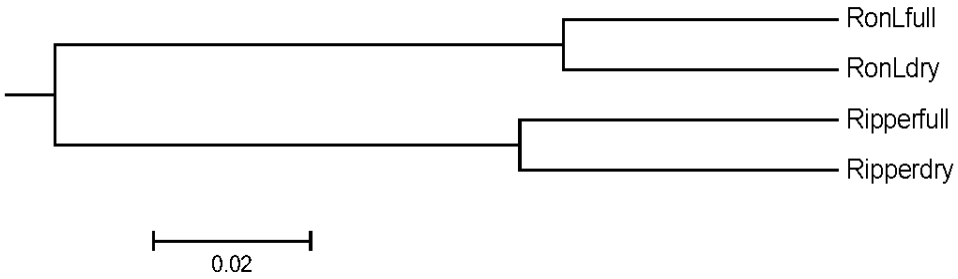


Figure 2.2 Cluster analysis of ACC+ bacterial OTUs associated with Ripper and RonL wheat genotypes at the anthesis stage (May 2011), grown under full irrigation or dryland conditions at LIRF, Greeley, CO. Tree distances were calculated at the 0.03 genetic distance using the (A) Jaccard Classification (individual replicates, membership presence/absence) and the (B) Yue and Clayton indices (ΘYC) (pooled replicates, membership presence/absence and relative abundance). In (A), one sample from the RonL, dryland treatment was removed from analysis due to insufficient numbers of high quality reads.

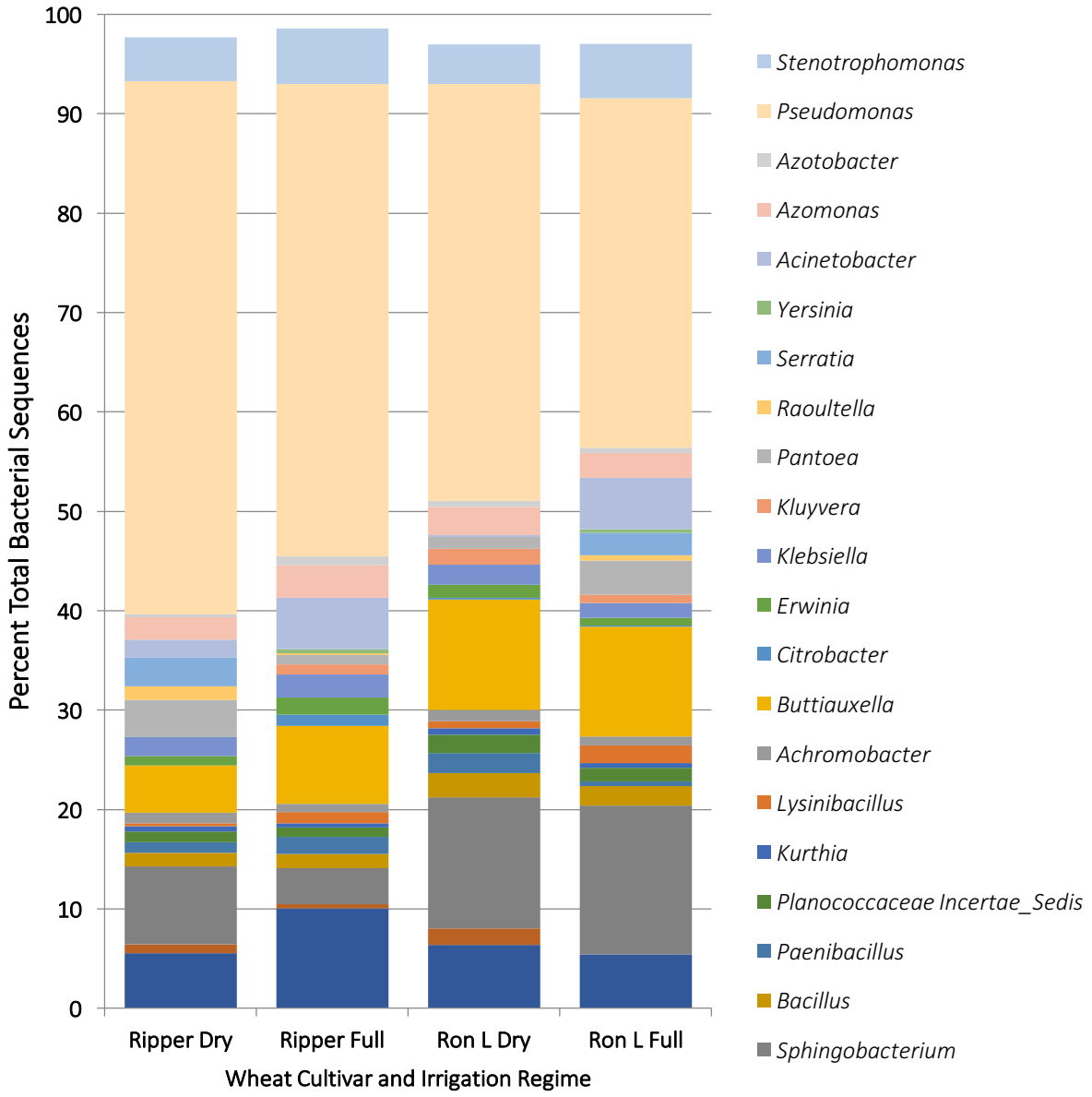


Figure 2.3 Composition of ACC+ bacterial genera associated with roots of Ripper and RonL wheat genotypes at the anthesis growth stage, under full irrigation or dryland conditions at LIRF, Greeley, CO (n=3).

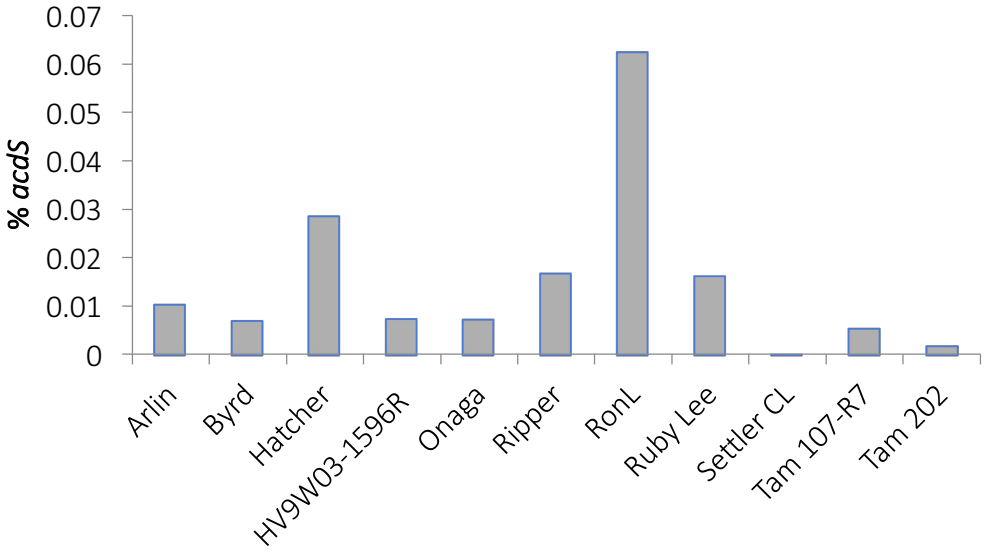


Figure 2.4 Predicted relative abundance of acdS genes in rhizosphere bacterial communities under different winter wheat genotypes, grown at ARDEC, Fort Collins, CO at mid-grain filling (June 2013), based on PICRUSt analysis of 16S rDNA sequences. Values are averaged across the full irrigation and dryland plots (n=2).

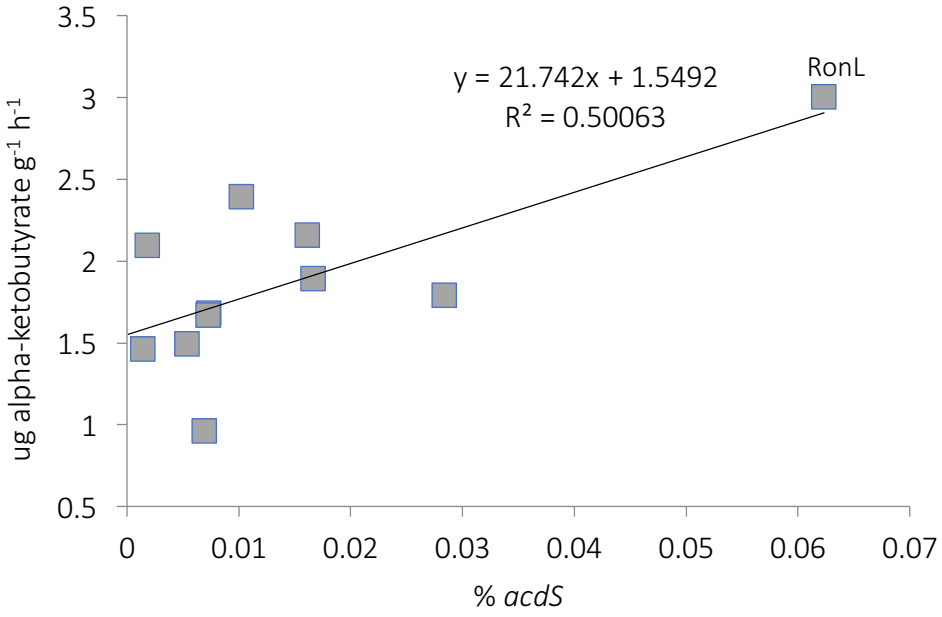


Figure 2.5 Regression analysis of predicted % acdS genes (from PICRUSt analysis) and potential ACC deaminase activity, averaged across irrigated and dryland plots, of rhizosphere bacterial communities under different winter wheat genotypes, grown at ARDEC, Fort Collins, CO at mid-grain filling (June 2013) (n=11). Activity level from RonL rhizosphere is shown.

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CHAPTER 3: CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) ASSOCIATED WITH WINTER WHEAT CULTIVARS (*TRITICUM AESTIVUM* L.)

1. Introduction

Plant growth promoting rhizobacteria (PGPR) have received much attention for their potential to improve plant growth and productivity in an environmentally friendly way. The term PGPR was introduced to soil science by Kloepper and Schroth (1978), and it describes root-associated bacteria (rhizobacteria) that enhance plant growth and production. A large number of studies have reported that rhizhobacterial species, including *Pseudomonas sp., Azospirillum sp., Azotobacter sp., Klebsiella sp., Enterobacter sp., Alcaligenes sp., Arthrobacter sp., Burkholderia sp., Bacillus sp.* and *Serratia sp.,* stimulate plant growth and productivity (Kloepper et al., 1989; Okon and Labandera-Gonzalez, 1994; Glick, 1995).

Plant growth promoting rhizobacteria enhance plant growth either directly through the ability to produce the plant growth hormone regulators such as indole acetic acid (IAA), gibberellins and cytokinins, and indirectly by increasing nutrient availability through nitrogen fixation and phosphate solubilization (Glick., 2012; Ahmad et al., 2008, and Taurian et al., 2010). They can also support plant growth by protecting plants from pathogens through production of antibiotics, siderophores**,** chitinase**,** and cyanide (Gururani et al., 2013, Haas and Défago, 2005, and Ribeiro and Cardoso, 2012,). Moreover, some PGPRs can enhance plant growth under abiotic stress through a variety of mechanisms, including the production of ACC deaminase, the enzyme that metabolizes 1-aminocylcopropane carboxylic acid (ACC), a plant-

produced precursor to ethylene (Gamalero, and Glick, 2015; Glick, 2015). These PGPRs are referred as ACC-deaminase positive bacteria.

In addition to these traits, PGPR must have other abilities to successfully promote plant growth under natural conditions. PGPR strains must be able to compete with native soil species for resources, be able to survive in their physical and chemical environment, and colonize the rhizospher (Cattelan et al., 1999). With these factors in mind, it is not surprising that application of PGPR in the field does not always give the desirable results as expected from laboratory or even greenhouse studies. This is because PGPR activities and traits are not always stable under natural field conditions and over time, and this has been largely attributed to competition with native strains and specific nutrient limitations. (Chanway and Holl, 1993; Zhender et al., 1997). As a result, soil inoculation with PGPR, under field conditions, may or may not affect plant growth and productivity.

Bacterial inocula for PGPR studies are usually developed by isolating culturable bacteria from plant roots and rhizospheres collected from various soils and environments, and testing each isolate for PGPR traits in laboratory and sometimes greenhouse studies (Khalid et al., 2004; Park et al., 2005; Flores-Vargas and O'Hara, 2006; Fischer et al., 2007; Senthilkumar et al., 2007; Abbasi et al., 2011). Characterization and selection of PGPR that carry multiple PGPR traits and are well adapted to a particular soil environment are expected to significantly support plant growth and yield, especially under harsh conditions. For example, in drought-prone regions of the world, where crop production is limited by water, it would be desirable to identify PGPRs that enhance crop growth under a variety of environmental conditions, including drought stress. Such is the case in eastern Colorado, where the climate is semi-arid and winter wheat is typically grown under rain-fed (i.e., without irrigation) conditions. Under these conditions, it may be

desirable to identify PGPRs for field inoculation of winter wheat and select PGPRs that possess a range of beneficial traits (e.g., ACC-deaminase positive strains that produce plant growth hormones, increase nutrient availability, and are tolerant to osmotic stress). Therefore, the objectives of the present study were (i) to isolate and identify ACC-deaminase positive bacterial strains from Colorado soils grown to winter wheat, and (ii) characterize each isolate for PGPR traits (ACC-deaminase activity, IAA production, tolerance to osmotic stress, phosphate solubilization, and siderophore production), and (iii) identify the desirable PGPR species with which may be useful as a soil inoculum to increase winter wheat growth and yield in Colorado soils.

2. Methods

2.1. Soil Sample Collection

Soil samples were collected from experimental research plots located at the USDA-ARS Limited Irrigation Research Farm (LIRF), located near Greely, Colorado. The soil at LIRF is classified as a fine sandy loam (mixed mesic, Ustic Haplargid) that contains 70% sand, 19.2% silt, 10.8% clay; 1.1% organic matter and has a pH of 8. Field plots were arranged in a split-plot block design with three replicate blocks. Main plots were irrigation levels (full irrigation or dryland), which were split into sub-plots of three winter wheat cultivars (*Triticum aestivum* L. Hatcher, Ripper, and RonL). Soil samples were collected at anthesis (flowering) in late May 2011, and mid-grain filling in late June 2011. Soil samples were collected by excavating roots and associated soil from at least three plants per plot with a trowel, to a depth of 20 cm from the surface of soil to avoid surface contamination. Samples from individual plants within each plot were composited into labeled Ziploc bags and stored on ice chests for transport back to the laboratory and stored at 4°C for prior to enrichment culturing.

2.2. Isolation of ACC deaminase- positive bacteria from soil samples

Subsamples (10 g) of rhizosphere soil and attached roots were suspended in 90 mL of sterile phosphate buffered saline (pH 7.2) to achieve a 1:10 dilution. Suspensions were blended on high speed for one minute in a Waring blender and serial, ten-fold dilutions were made in sterile phosphate buffered saline. Culturable ACC-positive bacteria were isolated by plating dilutions (10^{-3} to 10^{-5}) of the soil suspensions onto Dworkin and Foster (DF) minimal salts medium containing ACC as the sole N source (Penrose and Glick, 2003). Plates were incubated at 28°C for 4 -5 days. Afterwards, 12 representative colonies were selected per cultivar × irrigation treatment (6; for a total of 72 colonies), streaked onto fresh DF +ACC agar plates, and incubated to generate pure cultures. For each pure culture, a loopful of cells was collected with a sterile inoculating loop and transferred into a cryovial, along with a 1:1 mixture of sterile DF and glycerol. Vials were stored at - 80 °C for further work.

2.3. Identification of ACC deaminase-positive bacteria

Identification of the ACC deaminase–positive bacteria by molecular approach involved extracting total DNA, amplifying 16S rDNA by PCR, and sequencing the amplicons. First, DNA was extracted from the cell pellet using Fast DNA Spin kit. The 1500 bp region of 16S rDNA gene was amplified using 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGAATCCAGCC-3') primers. PCR amplifications were performed in 25 µL volumes containing 12.75 µL DI H_2O, 2.5 µL buffer (10x), 1.5 µL $MgCl_2$ (25 mM), 1 µL of each dNTPs (10 mM), 1.25 µL of each primer (10 mM), 0.5 µL Taq DNA polymerase (5U μL^{-1}), and 1 µL of DNA template. PCR cycles were as follows: (i) an initial denaturation at 94 °C for 5 min, (ii) 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, and (iii) a final extension at 72 °C for 10 min. The amplicons were

sequenced on an ABI 3130 Applied Biosystems Genetic Analyzer at the Proteomics and Metabolomics Facility at Colorado State University. Isolates were identified by comparing sequencing with sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. ACC deaminase activity assay

Potential ACC deaminase activity was determined for each isolate by measuring the production of α-ketobutyrate produced from ACC by ACC deaminase as described by Penrose and Glick (2003). Prior to the enzyme assay, isolates were cultured in liquid DF medium in the presence of ACC to induce ACC deaminase enzyme production, as described by Penrose and Glick (2003). Cultures were centrifuged at 8,000 g for 10 min at 4º C to pellet cells, and cells were washed in 0.1 M Tris-HCl, pH 7.6. After centrifuging to pellet cells again, cells were suspended in 600 µL of 0.1M Tris-HCl, pH 8.5, and 30 µL of toluene were added to prohibit cell growth during the assay. A 100-µL aliquot of the 'toluenized cells' was stored at -20 °C for protein assay (see below), and the remaining toluenized cell suspension was immediately used for ACC- deaminase activity assay.

For the ACC deaminase activity assay, 200 µL of toluenized cells were transferred into a 1.5-mL microcentrifuge tube and 20 µL of 0.5 M ACC were added to the suspension with vortexed, and the tubes were incubated at 30°C for 15 min. After incubation, 1 mL of 0.56 M HCl was added to the mixture and centrifuged for 5 min at 16,000 g. Then, 1 mL of the supernatant was transferred to a glass test tube, and vortexed with 800 µL of 0.56 M HCl. This was followed by the addition of 300 µL of 2,4-dinitrophenylhydrazine reagent (0.2% 2,4-dinitrophenylhydrazine in 2M HCl), to derivatize α-ketobutyrate into phenylhydrazone. The tube contents were vortexed and incubated at 30°C for 30 min. This was followed by the addition of 2 mL of 2 N NaOH to develop the color of the phenylhyrdazone. After mixing, the absorbance of

the solution was measured at 540 nm on a spectrophotometer. A standard curve of α-ketobutyrate was prepared from a stock solution of 1.76 mg α-ketobutyrate (Sigma-Aldrich Co., St Louis, MO, USA) and 5mL Tris-HCl, pH 8.5. The stock solution was diluted with the same buffer to achieve a standard curve in the range of 0-1.0 µmol α-ketobutyrate.

The protein content of the remaining toluenized cells was quantified in order to express ACC- deaminase activity as nmol α -ketobutyrate mg^{-1} of protein h^{-1}**.** The protein concentration of toluenized cells was measured according to Bradford (1976) with modifications. Dye reagent was prepared by diluting (1:4) dye in DI water, and then filtering through a Whatman #1 filter to remove particulates. A stock solution of bovine serum albumin protein (2 mg mL^{-1}) was diluted to achieve a standard curve in the range of 0-1 mg mL^{-1}. 10µL of each standard and cells (diluted 1:2 by mixing 5µL of cells and 5µl DI water) were added to microliter plate wells, along with 10 µL DI water as a blank. Then 200 µL of diluted dye reagent were added to each well, followed by a 1hour incubation at room temperature. Each microliter plate contained two replicates of each protein sample. Finally, the absorbance was measured at 595 nm to determine the concentration of protein in each sample.

2.5. Quantification of IAA production

Indole acetic acid (IAA) production was measured for each isolate following the method of Gordon and Weber (1951), as described by Cheryl and Glick (2002). Prior to the assay, each isolate was grown overnight in 15 mL tubes containg 5 mL Luria Bertani (LB) broth medium. After the incubation, 20 µL aliquots were transferred into new tubes contain 4.5 mL of LB broth medium, and 500 µL of 200 µg mL^{-1} of L-tryptophan (prepared in DI water) were added to each tube. The tubes and their contents were incubated in a shaker incubator at 200 r.p.m. at 28°C for 48 hours. The density of each culture was measured by a spectrophotometer at 600 nm. Cultures

were then centrifuged at 5,500 g for 10 min. One mL of the supernatant was mixed with 4 mL of Salkowski's reagent (150 mL of concentrated H_2SO_4, 250 mL of DI H_2O, 7.5 mL of 0.5 M $FeCl_3 \cdot 6H_2O$; Gordon and Weber**,** 1951) and allowed to rest for 20 min at room temperature. Finally, the absorbance of each solution was measured at 535 nm. A standard curve of IAA (in the range of 0-100 µg mL^{-1}) was prepared from a stock solution of 1000 µg mL^{-1} IAA, prepared in acetone. IAA production was expressed as µg mL^{-1}.

2.6. Tolerance to osmotic stress

Each isolate was tested for tolerance to water stress according to Sandhya et al., (2009). Bacteria were cultured in Trypticase Soy Agar (TSA) medium supplemented with 25% PEG6000 to achieve a water potential of -0.73 MPa. In brief, cultures from frozen glycerol stocks were streaked onto fresh TSA plates and incubated at 28°C for three days. Then, one colony was selected per plate and culture and transferred into 15 mL tubes containing 2 mL Trypticase Soy Broth (TSB), followed by incubation in a shaker incubation at 200 r. p.m. at 28°C for 24 hrs. 200 µL of the grown culture were then transferred to 15 mL tubes contain 1.8 mL TSB + 25 %PEG6000 followed by incubating at 200 r.p.m. 28 °C for 24 hours. Finally, culture growth was estimated by measuring the optical density at 600 nm using a spectrophotometer.

2.7. Phosphate solubilization assay

Each isolate was cultured onto fresh TSA plates from frozen glycerol stocks, as described above. After incubation at 28 ^{0}C for 48 hours, one colony per plate was streaked on to Pikovskaya's Agar Medium (PVK) to test for phosphate solubilization. The PVK medium was prepared as follows: glucose (10 g L^{-1}), yeast extract (0.5 g L^{-1}), $(NH_4)_2SO_4$ (0.5 g L^{-1}), $MgSO_4.7H_2O$ (0.1 g L^{-1}), $Ca_3(PO_4)_2$ (5 g L^{-1}), NaCl (0.2 g L^{-1}), KCl (0.2 g L^{-1}) , $MnSO_4.H_2O$ (2 mg L^{-1}), $FeSO4.7H_2O$ (2 mg L^{-1}), and 15 g agar per L DI water (Kumar et al., 2014). Plates were

incubated at 28 ^{0}C for 14 days. A positive result for phosphate solubilization was indicated by growth of bacterial colonies surrounded by a halo, or clear zone. Results were expressed as positive or negative for phosphate solubilization.

2.8. Siderophore production

Each isolate was tested for its ability to produce siderophores using the universal Chrome Azural S (CAS) assay described by Schwyn and Neilands, (1987). Prior to the assay, isolates were grown overnight on fresh TSA plates from glycerol stocks, as described above. All glassware were washed with 6 M HCl to remove any trace elements, and then rinsed with DI water. The following solutions were made to make the blue dye for CAS agar plates: for solution 1, 60 mg of CAS was dissolved in 50 ml of DI H_2O; for solution 2, 2.7 mg of $FeCl_3$-6 H_2O was dissolved in 10 ml of 10 mM HCl; for solution 3, 73 mg of HDTMA was dissolved in 40 ml of DI H_2O. To make the blue dye, solution 1 was mixed with 9 ml of solution 2. After mixing, this solution was added to all of solution 3. The final, CAS dye solution was autoclaved at 121°C for 15 min.

Fresh TSA plates were prepared, and after solidifying, the medium was cut into two halves, and the TSA medium was removed from one-half of each plate. Blue agar medium was then prepared by mixing 750 mL water, 15 g agar, and 30.24 g piperazine-N, N'-bis(2-ethanesulfonic acid) (PIPES), followed by 12 mL of NaOH (50% w/w with DI water) to raise the pH of the solution to 6.8. This solution was autoclaved at 121°C for 15 min. After cooling, the CAS dye solution (from above) was carefully added along the glass wall of the agar flask, and gently agitated to mix the solutions. Then, 15ml of the CAS blue agar medium was poured into the empty half of each TSA plate. After the CAS solidified, the halves containing TSA medium were inoculated with a bacterial isolate, carefully far away from the borderline between

the two media. Finally, the plates were incubated at 28°C for three weeks. A change in the CAS blue agar color from blue to orange, purple, or dark purplish-red (magenta) was interpreted as a positive result for siderophore production (Fe solubilizaiton).

2.9. Root elongation assay

The ability of selected isolates (seven total) to elongate roots of four winter wheat cultivars (RonL, Ripper, OK 06318, and Byrd) was determined, following the protocols of Penrose and Glick (2003). The isolates were selected based on their PGPR traits from previous assays. The isolates selected were three *Pseudomonas sp.* isolated from RonL rhizospheres in fully irrigated plots; one *Variovorax sp.* isolated from Ripper rhizosphere in a dryland plot, one *Pseudomonas sp*. from Ripper rhizosphere of a fully irrigated plot, and two *Pseudomonas sp.* isolated from Hatcher rhizospheres from a fully irrigated plot and a dryland plot (Table 2).

Bacterial isolates were cultured overnight in 50 mL tubes containing 5 mL of TSB, while shaking at 200 rpm at 28°C. The cultures were centrifuged at 8,000 x g for 10 min to pellet cells. Bacterial cell pellets were then suspended in 48 mL sterile 0.03 M MgSO4 buffer and placed on ice. The density of each cell suspension was adjusted to the same optical density after reading the absorbance of the sample at 600 nm by spectrophotometer.

Seed-pack growth pouches (Northrup King Co., Minneapolis, MN, USA) were used in this assay. The pouches were filled with 12 mL distilled water, were wrapped loosely in aluminium foil in groups of 10 to prevent water loss, and autoclaved at 121°C for 15 min.

Winter wheat seeds of the four cultivars were surface sterilized by soaking seeds in 70% ethanol for 1 min in Petri dishes followed by 1% sodium hypochlorite (bleach) for 10 min. Seeds were then rinsed five times with sterile DI water. The surface-sterilized seeds were then soaked in bacterial suspensions for 1 hr. at room temperature as follows: 12 seeds of each wheat cultivar

were inoculated with each bacterial culture. Surface-sterilized seeds were soaked in sterile 0.03 M $MgSO_4$ as a negative control. After the incubation, six seeds were placed in each of two growth pouches, terilized forceps. Sterile DI water (15 mL) was added to each pouch, and pouches were placed upright in pouch racks. The pouches were incubated in a growth chamber set at 28°C, with a light cycle of 12 h of dark followed by 12 h of light. Root growth was checked after 2 days of incubation, and length of the primary root was measured after six days of incubation. Seeds that had no root growth at two days were excluded from the analysis, as well as seeds that had root lengths of 1 cm or less.

2.10. Data analysis

The effect of isolate, cultivar, and isolate × cultivar interaction on root length was assessed by analysis of variance tests, using the PROC GLM procedure of SAS (SAS Institute, Cary, NC). When treatment effects were significant ($\alpha = 0.05$), mean comparison tests were conducted with the Dunnet's test.

3. Results

3.1. Screening of isolates for PGPR traits

Of the original 72 isolates, data on a suite of PGPR traits were collected from a total of 55 isolates. Of the remaining isolates, they either could not be revived from glycerol stocks or did not exhibit multiple PGPR trains. Most isolates were identified as species of *Pseudomonas*, but species of *Arthrobacter, Variovorax, Agrobacterium/Rhizobium, Ochrobacterium, Micrococcus, Rahnella*, and *Bacillus* species were represented as well (Table 3.1). Isolates were screened for potential ACC-deaminase activity, IAA production, osmotic stress tolerance, P-solubilization**,** and **s**iderophore production. The data regarding PGPR activity of each isolate are summarized in Table 3.1.

Overall, 47 (85.5 %) bacterial isolates tolerated osmotic stress, 42 (76.4 %) of isolates demonstrated ACC-deaminase activity, 16 (29.0 %) isolates were capable of solubilizing phosphate, 1 (1.8 %) isolate produced IAA in presence of 200 μg mL^{-1} tryptophan, and 0 isolates produced siderophores in vitro (Table 3.1).

Almost all of the isolates were tolerant of osmotic stress tolerance, when tested at an osmotic stress of -0.73 MPa water potential. Only eight isolates were unable to grow at -0.73 MPa water potential, which were two *Pseudomonas sp.* from Ripper rhizospheres (RD8 and RF9), *Variovorax sp.* RD12, *Agrobacterium sp*. RF2, and four *Pseudomonas sp*. isolated from RonL rhizospheres (RLD9, RLD10, RLF3, and RLF10) (Table 3.1).

Potential ACC-deaminase activity ranged from 0.012 to 4.36 nmoles mg^{-1} protein h^{-1}. Two *Pseudomonas sp.* (RLF9) and *Ochrobacterum sp.* (RipF7) isolated from RonL and Ripper rhizospheres of fully irrigated soil, had the highest ACC deaminase activity, compared to the other isolates (Table 3.1). Other isolates including *Arthrobacter sp.* RD3, *Variovorax sp.* RD5*,* and *Pseudomonas sp.* RF5, RF8, RF12, RLD1, RLD2, RLD10, RLD12, RLF6, RLF8, and HF1 had relatively high ACC deaminase activities compared with the other PGPRs under study (Table 3.1).

Out of the 55 isolates tested, 16 isolates demonstrated phosphate solubilizing abilities in vitro (Table 3.1). These isolates were isolated from dryland Ripper rhizosphere (RD5 and RD6), RonL rhizospheres (RLD12, RLF6, RLF8, RLF9, RLF12), and Hatcher rhizospheres (HD4, HD5, HD7, HD8, HD10, HD12, HF1, HF3, and HF9). In each case, the isolates produced a clearing of the calcium phosphate-based PVK medium, which resulted in a clear zone (halo zone) on the PVK plate, as shown in Fig 3.1.

Only the *Variovorax sp.* RD12 was positive for IAA production, although the concentration of IAA produced was low (Table 3.1). No isolates were positive for siderophore production under the assay conditions utilized.

3.2. Root elongation

Seven bacterial isolates were selected for the root elongation assay (Table 3.2). The isolates selected were three *Pseudomonas sp.* isolated from RonL rhizospheres in fully irrigated plots (RLF6, RLF9, and RLF12); one *Variovorax sp.* isolated from Ripper rhizosphere in a dryland plot (RD12), one *Pseudomonas sp*. from Ripper rhizosphere of a fully irrigated plot (RF12), and two *Pseudomonas sp.* isolated from Hatcher rhizospheres (HD8 and HF1). These isolates were selected because they had exhibited multiple PGPR traits, sometimes at relatively high activities (Table 3).

Results of the effect of inoculation with bacterial isolates on root length of each wheat cultivar are summarized in Table 3. 3. Overall, Ripper seedlings had the longest roots at 6 days of growth, whereas RonL had the shortest roots (Table 3.3, Fig 3.2, 3.3, and 3.7). Averaged across all cultivars, isolates RLF6 and RF12, both *Pseudomonas* sp., were found to be the most effective in increasing root length as compared with control (Fig 3.6). The effect of inoculation on root length, however, was dependent on cultivar type. RonL seedlings inoculated with *Pseudomonas sp*. HD8 or RLF9 developed significantly longer roots than control seedlings in growth pouch (Table 3.3). For Ripper and OK seedlings, the *Pseudomonas sp*. RLF6 treatment resulted in significantly longer roots compared to sterile control. For Byrd seeds, inoculation with either *Variovorax sp.* RD12 or *Pseudomonas sp.* RLF9 resulted in longer roots compared to sterile control.

4. Discussion

Plant Growth Promoting Rhizobacteria (PGPR) have beneficial activities that support plant growth and production (Glick, 1995). In the present investigation, a total of 55 bacterial isolates, originating from Ripper, RonL, and Hatcher wheat rhizospheres, were studied for their PGP traits including ACC-deaminase activity. In theory, PGPRs with ACC deaminase activity may be helpful in regulating concentrations of ethylene under drought stress, through the expression of ACC-deaminase enzyme that cleaves ACC, the precursor of ethylene, into α-ketobutyrate and ammonium, thereby leading to lower levels of ethylene in the plant. Moreover, the isolates studied here exhibited multiple PGPR traits, other than ACC deaminase activity, including tolerance to osmotic stress, phosphate solubilization, ability to elongate wheat seedling roots, and in the case of RD12, IAA production. Similarly, others have found that ACC-deaminase positive bacteria usually express more than one PGP trait such as phosphate solubiliziation and siderophore production (Cattelan et al., 1999; Taurian et al., 2010; Ahmad et al., 2008; Kuklinsky-Sobral, et al., 2004; Neilands and Leono, 1986, and Nautiyal, 1999). Yang et al. (2009) reported that PGPR with more than one PGP property can help plants tolerate abiotic stress, such as drought. Therefore, due to multiple PGP traits, these isolates may be considered as attractive choices for future plant-bacterial interaction studies investigating crop production under drought stress conditions.

The results showed that bacteria *Variovorax sp.* RD12 produced IAA in the presence of 200 μg mL^{-1} tryptophan. The level of the bacterial IAA is low, but it may support primary root elongation in wheat. It is known that low levels of bacterial IAA stimulate primary root elongation while high levels of IAA (either from strains that naturally secrete high levels or from high density inocula) stimulate the lateral and adventitious roots (Barbieri and Galli, 1993;

Beyerler et al., 1997; Loper and Schroth, 1986; Mayak et al., 2004). Our result agreed with Cheryl and Glick (2002), who reported that *Pseudomonas putida* produce IAA at concentrations in range at 50 - 500 μg mL^{-1}, in the presence of tryptophan. However; the rest of the isolates studied here were negative for IAA production. These isolates may need either higher concentrations of tryptophan for synthesis of IAA (500 μg mL^{-1} of tryptophan for example) or longer time for incubation (120 h for example) or both for IAA production.

Sixteen isolates produced a clear zone (halo zone) of phosphate solubiliziation surrounding the colonies, as an indicator of positive results for phosphate solubiliziation. In this study, the zone of clearing started to appear after 3 days of incubation on PVK agar plates, in agreement with Kumar and Ram (2014). On the other hand, other isolates were slower to develop the clear zone of phosphate solubilization; up to 13 days was the case for *Pseudomonas sp.* RF6. In 2009, Mahalakshmi and Reetha (2009) reported that 83.3% of *Pseudomonas* isolates tested were phosphate solubilizers.

In this study, no isolates had the ability to produce siderophores for Fe acquisition. The reason for this in not clear. Precautions were taken to eliminate contamination, including washing all glassware with 6 M HCl to remove any trace elements. A positive control was not included in the test, so it could be that the assay did not work. Many of the isolates were *Pseudomonas sp.,* which are known to produce siderophores.

Most (47 of 55) of the isolates in this study were considered tolerate to osmotic stress and could grow at -0.73 MPa water potential. These results contrast with those of Sandhya et al. (2009), who reported that of 212 pseudomonads studied, only 26 could tolerate maximum level of osmotic stress at -0.73 Mpa water potential. It is possible that a greater proportion of PGPRs

in the LIRF soil are tolerant to osmotic stress, given that the environment is semi-arid and prone to drought.

Overall, results from this study showed that most of the tested isolates exhibited more than three PGP traits, which may promote plant growth directly or indirectly or synergistically. In this study, plant growth promotion was studied by a root elongation assay. Results from the root elongation assay demonstrated the potential for selected isolates to elongate winter wheat roots. The assay furthermore demonstrated that root elongation by a particular isolate was cultivar dependent. The mechanism by which the inoculants stimulated root growth is not clear, but it is possible that the inoculants stimulated faster seed germination and or stimulated increased root growth due to PGP traits that were not measured. Two Isolates that significantly elongated roots (RLF9 and RLF6) had relatively high ACC-deaminase activity. ACC deaminase activity has been associated with root elongation in other studies. Based on the simple model (Glick et al., 1998), the association can be explained as follows: after bacteria bind to seed coats and during seed imbibition, ACC-deaminase positive bacteria hydrolyze ACC into α-ketobutyrate and ammonium, leading to lower levels of ethylene in the seedling. While ethylene is needed in the seed for stimulation of germination and breaking of seed dormancy (Esashi, 1991; Esashi and Leopold, 1969), high levels of ethylene after germination may inhibit root growth (Jackson, 1991). Therefore, ACC deaminase activity after germination may elongate roots by inhibiting the production of high concentrations of ethylene. In addition, while some PGPR synthesize and secrete indole acetic acid (IAA), ACC deaminase activity may prevent indole acetic acid (IAA) from activating ethylene synthesis by stimulating ACC synthase. In this study, IAA production did not occur in any isolate except one, *Variovorax* RD12. In this study, *Variovorax sp.* RD12 produced low levels of IAA and had moderate ACC deaminase activity;

however, RD12 did not support root elongation for all wheat cultivars, despite the potential to produce low concentrations of IAA.

5. Conclusions

The present study generated a set of bacterial isolates possessing multiple PGP traits and ability to stimulate plant growth, as evidenced by primary root elongation. Isolates with high ACC-deaminase activity and additional PGP traits may have potential as inoculants to stimulate crop production and possibly alleviate drought stress. The findings of this study are very attractive for further field studies, which would be helpful in extending isolate use as PGP inocula for wheat production, especially in Colorado and perhaps other semi-arid regions. Specifically, inoculation of winter wheat cultivars with PGPR-containing ACC-deaminase and other PGP traits could be an effective approach for successful crop production. Good selection of the combination between PGPR inoculant and wheat cultivars for obtaining optimum response should be considered in future studies. The ability of potential inoculants to survive in the rhizosphere, compete with indigenous microbial communities, and colonize roots should also be studied. Based on the study results, isolates RFL9 or RFL6 are proposed as potential inoculants for further study. Based on laboratory studies of potential PGPR activities, RLF9 could be a good inoculum selection to improve wheat growth and productivity under drought stress. Further research is needed to demonstrate the effectiveness of RLF9 as an inoculant in the field.

Table 3.1 Identification code, species match and plant growth-promoting traits of bacterial isolates from rhizospheres of three winter wheat cultivars (R=Ripper, RL=RonL, and H=Hatcher) grown under dryland (D) or fully irrigated (F) conditions at the Limited Irrigation Research Farm (LIRF) near Greeley, Colorado.

Isolate ID[1]	Species	% Match	ACC deaminase activity (nmoles mg^{-1} protein h^{-1})	IAA production (mg mL^{-1})	Osmostic stress (OD 600nm)	Phosphate solubilization (mm diam. of halo zone)	Siderophore production
RD1	*Pseudomonas sp.*	99%	1.684	-	0.045	-	-
RD2	*Arthrobacter sp.*	98%	0.124	-	0.0267	-	-
RD3	*Arthrobacter sp.*	99%	2.157	-	0.01	-	-
RD5	*Variovorax sp.*	99%	4.36	-	0.011	4	-
RD6	*Agrobacterium tumefaciens/ Rhizobium sp.*	99%	0.184	-	0.016	4	-
RD7	*Arthrobacter sp.*	82%	0.659	-	0.867	-	-
RD8	*Pseudomonas sp.*	99%	-	-	0	-	-
RD9	*Agrobacterium*	99%	1.462	-	0.008	-	-

	tumefaciens/ Rhizobium sp.						
RD10	*Pseudomonas sp.*	99%	-	-	0.025	-	-
RD11	*Pseudomonas sp.*	99%	2.576	-	0.049	-	-
RD12	*Variovorax sp.*	99%	1.42	0.071	-0.002	-	
RF1	*Agrobacterium tumefaciens/ Rhizobium sp.*	100%	0.013	-	0.023	-	-
RF2	*Agrobacterium sp./Rhizobium sp.*	99%	0.025	-	-	-	-
RF3	*Agrobacterium sp/ Rhizobium sp.*	100%	0.163	-	0.132	-	-
RF4	*Arthrobacter sp.*	99%	0.974	-	0.273	-	-
RF5	*Pseudomonas sp.*	99%	2.03	-	0.086	-	-
RF6	*Arthrobacter sp.*	98%	0.135	-	0.185	-	-
RF7	*Ochrobacterum sp.*	100%	3.329	-	0.128	-	-
RF8	*Pseudomonas sp.*	99%	1.938	-	0.019	-	-

RF9	*Pseudomonas sp.*	99%	0.053	-	0	-	-
RF10	*Agrobacterium tumefaciens/ Rhizobium sp.*	99%	1.48	-	0.019	-	-
RF11	*Pseudomonas sp.*	99%	0.122	-	0.024	-	-
RF12	*Pseudomonas sp.*	99%	1.574	-	2.527	-	-
RLD1	*Pseudomonas sp.*	99%	3.385	-	0.025	-	-
RLD2	*Pseudomonas sp.*	99%	3.031	-	.007	-	-
RLD3	*Agrobacterium tumefaciens/ Rhizobium sp.*	97%	1.764	-	0.02	-	-
RLD5	*Pseudomonas sp.*	97%	0.865	-	0.005	-	-
RLD6	*Pseudomonas sp.*	97%	0.104	-	0.009	-	-
RLD7	*Micrococcus sp.*	97%	0.012	-	0.024	-	-
RLD9	*Pseudomonas sp.*	97%	-		-	-	-
RLD10	*Pseudomonas sp.*	97%	2.828	-	-	-	-
RLD11	*Pseudomonas sp.*	97%	-	-	0.09	-	-
RLD12	*Pseudomonas sp.*	97%	2.033	-	0.035	3	-

RLF1	*Pseudomonas sp.*	97%	-	-	0.019	-	-
RLF3	*Pseudomonas sp.*	97%	-	-	-	-	-
RLF4	*Pseudomonas sp.*	97%	-	-	0.049	-	-
RLF6	*Pseudomonas sp.*	97%	2.828	-	2.845	3	-
RLF8	*Pseudomonas sp.*	97%	3.432	-	0.176	3	-
RLF9	*Pseudomonas sp.*	98%	3.19	-	1.367	3	-
RLF10	*Pseudomonas sp.*	97%	-	-	-	-	-
RLF12	*Pseudomonas sp.*	97%	0.974	-	2.453	3	-
HD1	*Rahnella aquatilis*	99%	0.145	-	2.236	-	-
HD3	*Rahnella aquatilis*	99%	-	-	1.534	-	-
HD4	*Pseudomonas fluorescens*	99%	0.654	-	0.867	3	-
HD5	*Rahnella aquatilis*	99%	-	-	2.273	3	-
HD6	*Pseudomonas brassicacearum/P. lini/ P. corrugate/ P. fluorescens*	78%	1.111	-	1.727	-	-
HD7	*Bacillus sp.*	99%	-	-	1.787	1	-

HD8	*Pseudomonas brassicacearum/P. corrugate/P. fluorescens*	99%	0.435	-	1.726	1	-
HD10	*Pseudomonas brassicacearrum/P. sp./P. fluorescens*	99%	2.352	-	3.755	1	-
HD11	*Rahnella aquatilis*	99%	-	-	1.768	-	-
HD12	*Pseudomonas brassicacearum/ P. sp./P. fluorescens*	100%	-	-	1.831	1	-
HF1	*Pseudomonas brassicacearum/ P. sp./P. fluorescens*	100%	1.733	-	2.066	1	-
HF3	*Pseudomonas brassicacearum/ P. sp./P. fluorescens*	100%	0.832	-	1.801	1	-

HF9	*Pseudomonas putida/ P. sp./P. fluorescens*	99%	0.133	-	2.345	2	-
HF11	*Pseudomonas putida/ P. sp./P. fluorescens*	100%	0.118	-	1.839	-	-

RD = Ripper dryland, RF = Ripper fully irrigated, RLD = RonL dryland, RLF = RonL fully irrigated, HD = Hatcher dryland, HF = Hatcher fully irrigated.

- = negative results.

Table 3.2 Plant growth-promoting traits of seven bacterial isolates selected for the root elongation assay.

Isolate Species and ID	ACC-deaminase activity (nmoles mg^{-1} protein h^{-1})	IAA (mg mL^{-1})	Osmotic stress tolerance (cell density absorbance at 600 nm)	Phosphate solubiliziation (mm diameter of halo zone)
Pseudomonas sp. RLF9	2.50	-	1.37	3
Pseudomonas sp. RLF6	1.80	-	2.85	3
Variovorax sp. RD12	1.42	0.071	-	-
Pseudomonas sp. RF12	1.58	-	2.53	-
Pseudomonas sp. RLF12	0.40	-	2.45	3
Pseudomonas sp. HD8	0.33	-	1.74	1
Pseudomonas sp. HF1	2.01	-	2.07	1

The symbol (-) representing the negative reaction (activity/ production) of PGP traits.
Pseudomonas sp. RLF9, *Pseudomonas sp.* RLF6, *Pseudomonas sp.* RLF12: Bacteria isolated from rhizosphere soil of RonL wheat cultivar under fully irrigation treatment.
Variovorax sp. RD12: Bacteria isolated from rhizosphere soil of Ripper wheat cultivar under dry-land treatment.
Pseudomonas sp. RF12: Bacteria isolated from rhizosphere soil of Ripper wheat cultivar under fully irrigated treatment.
Pseudomonas sp. HD8: Bacteria isolated from rhizosphere soil of Hatcher wheat cultivar under dry-land treatment.
Pseudomonas sp. HF1: Bacteria isolated from rhizosphere soil of Hatcher wheat cultivar under fully irrigated treatment.

Table 3.3 Lengths (mean ± 1 SD) of primary roots in 6-day-old winter wheat seedlings inoculated with bacterial isolates or sterile control.

Wheat Cultivar	Inoculum	Root length (cm)
RonL	*Pseudomonas sp.* HD8	8.0 ± 0.5 a
	Pseudomonas sp. HF1	4.5 ± 1.0 b
	Pseudomonas sp. RLF12	6.0 ± 0.9 b
	Pseudomonas sp. RLF6	5.0 ± 0.7 b
	Pseudomonas sp. RLF9	7.1 ± 1.2 a
	Pseudomonas sp. RF12	4.3 ± 0.5 b
	Variovorax sp. RD12	5.9 ± 0.5 b
	Control	5.4 ± 0.9 b
Ripper	*Pseudomonas sp.* HD8	8.7 ± 1.1 b
	Pseudomonas sp. HF1	7.6 ± 2.7 b
	Pseudomonas sp. RLF12	9.1 ± 1.3 b
	Pseudomonas sp. RLF6	11.2 ± 1.2 a
	Pseudomonas sp. RLF9	7.7 ± 2.4 b
	Pseudomonas sp. RF12	7.6 ± 0.7 b
	Variovorax sp. RD12	10.6 ± 1.6 b
	Control	7.0 ± 1.0 b
OK	*Pseudomonas sp.* HD8	8.1 ± 0.6b
	Pseudomonas sp. HF1	7.5 ± 1.7b
	Pseudomonas sp. RLF12	6.6 ± 0.8b
	Pseudomonas sp. RLF6	10.9 ± 0.3 a
	Pseudomonas sp. RLF9	8.8 ± 0.8b
	Pseudomonas sp. RF12	7.2 ± 0.6b
	Variovorax sp. RD12	9.7 ± 0.9b
	Control	7.3 ± 0.6 b
Byrd	*Pseudomonas sp.* HD8	5.9 ± 0.8 b
	Pseudomonas sp. HF1	7.2 ± 1.0 b
	Pseudomonas sp. RLF12	7.6 ± 1.3 b
	Pseudomonas sp. RLF6	7.6 ± 1.5 b
	Pseudomonas sp. RLF9	8.8 ± 2.3 a
	Pseudomonas sp. RF12	6.2 ± 0.7 b
	Variovorax sp. RD12	9.4 ± 1.0 a
	Control	6.6 ± 0.4 b

Means followed by the same letter in a column do not differ significantly at $P \leq 0.05$, as determined by analysis of variance tests and Dunnet's tests for mean comparisons (n=2).

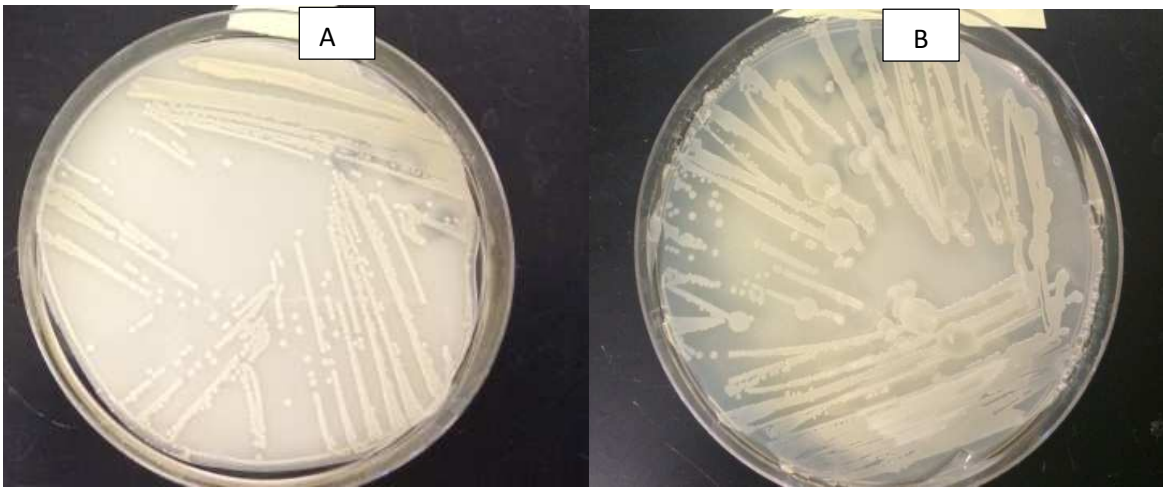

Figure 3.1 Bacterial growth on PVK agar plates showing A) a negative result for phosphate solubilization and B) a positive result for phosphate solubilization, as indicated by the zone of clearing.

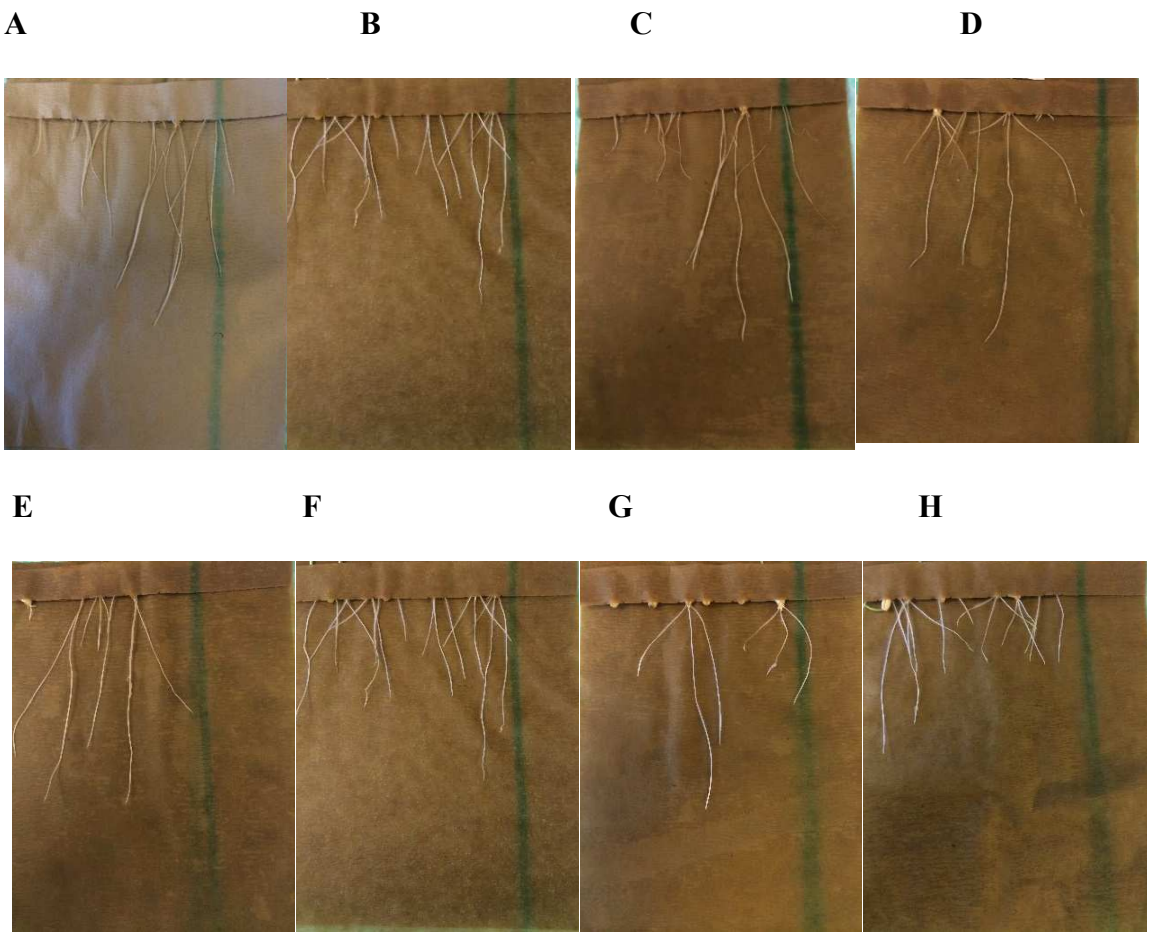

Figure 3.2 Primary root growth of RonL seedlings grown for 6 days in root pouches and with the following inoculation treatments: A) sterile control, B) RLF6, C) RF12, D) RLF9, E) HD8, F) RF12, G) HF1, and H) RD12.

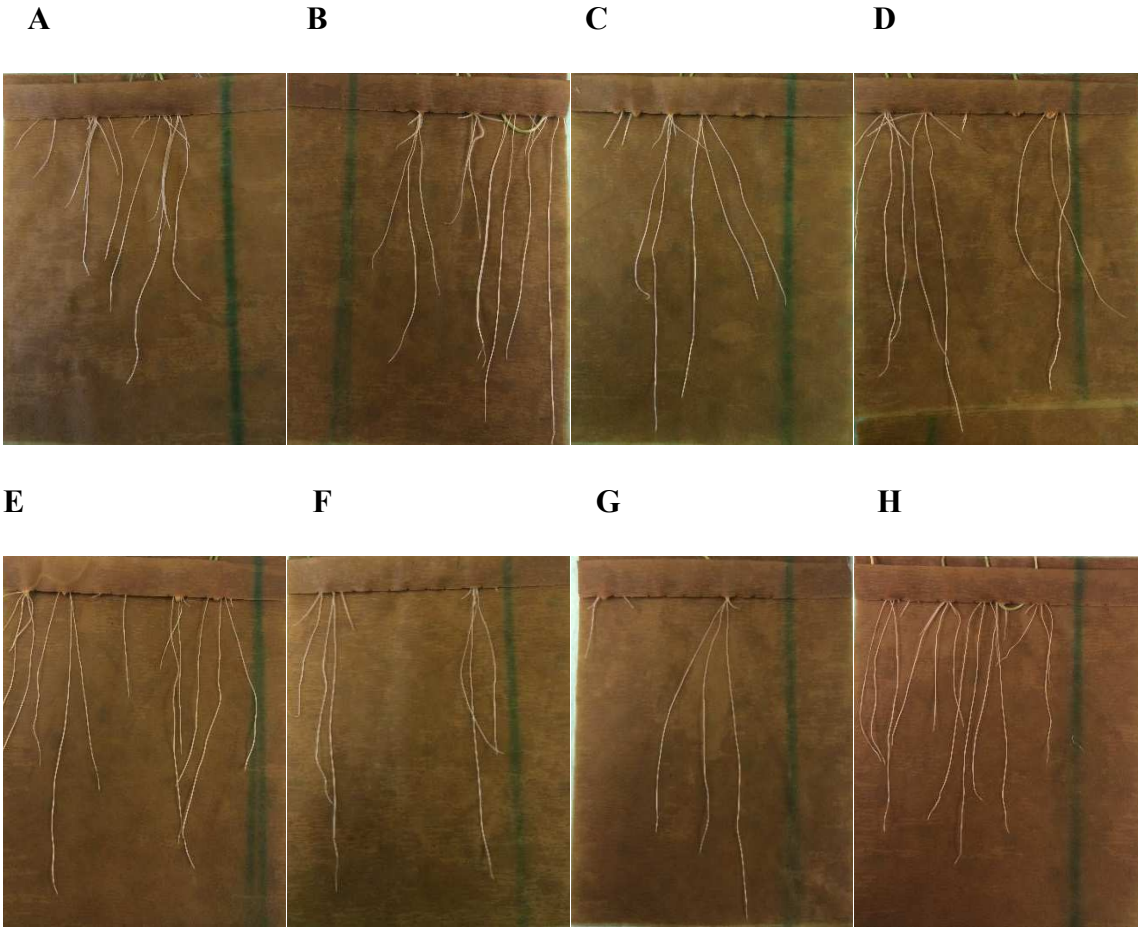

Figure 3.3 Primary root growth of Ripper seedlings grown for 6 days in root pouches and with the following inoculation treatments: A) sterile control, B) RLF6, C) RF12, D) RLF9, E) HD8, F) RF12, G) HF1, and H) RD12.

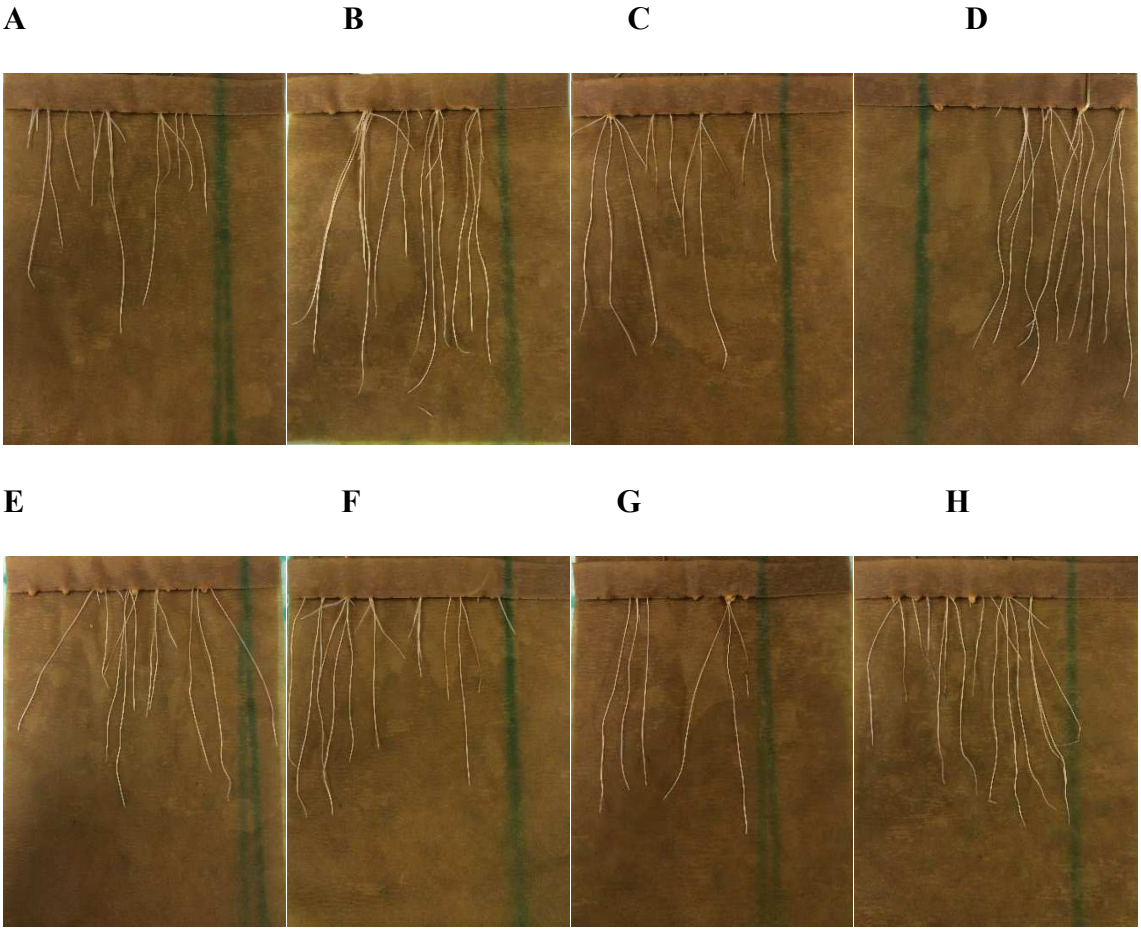

Figure 3.4 Primary root growth of OK seedlings grown for 6 days in root pouches and with the following inoculation treatments: A) sterile control, B) RLF6, C) RF12, D) RLF9, E) HD8, F) RF12, G) HF1, and H) RD12.

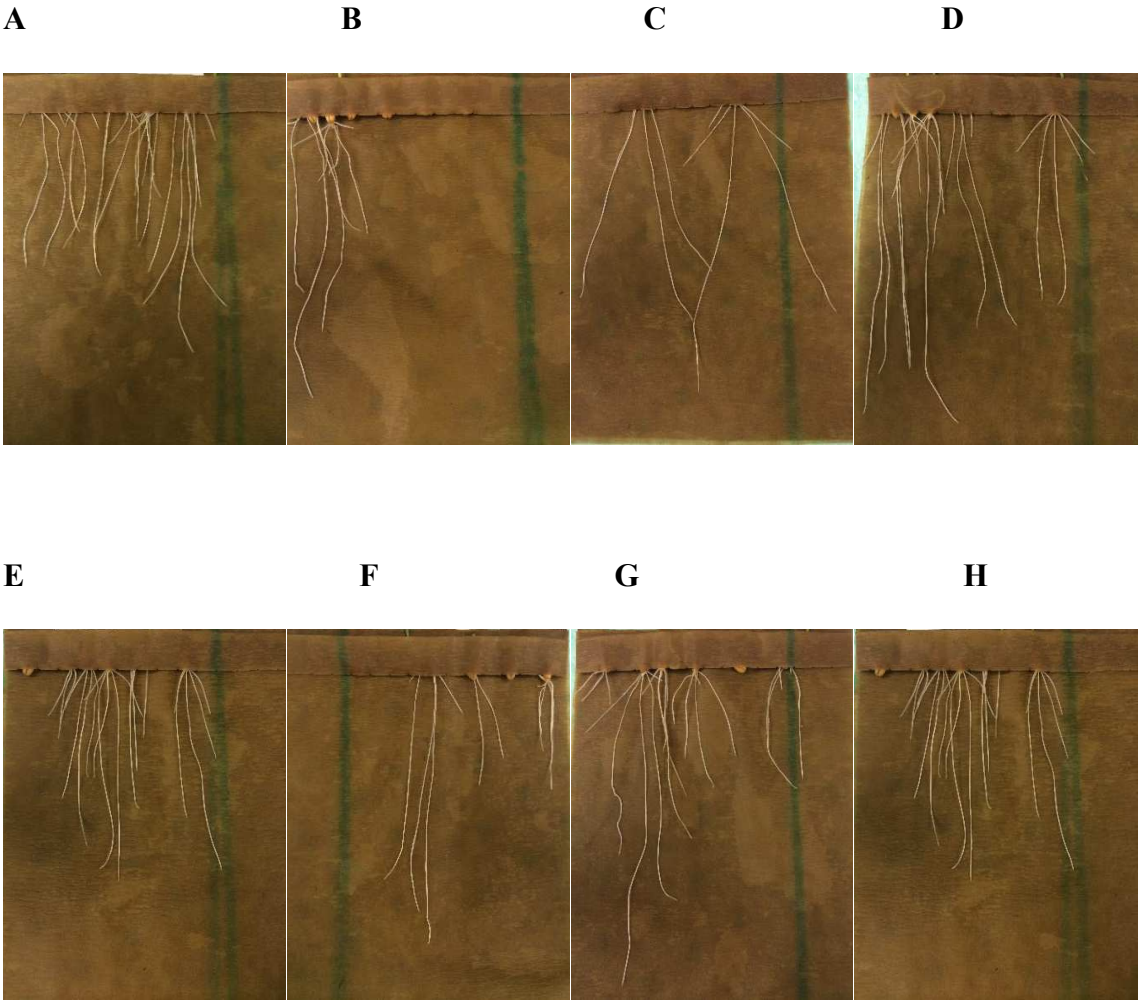

Figure 3.5 Primary root growth of Byrd seedlings grown for 6 days in root pouches and with the following inoculation treatments: A) sterile control, B) RLF6, C) RF12, D) RLF9, E) HD8, F) RF12, G) HF1, and H) RD12.

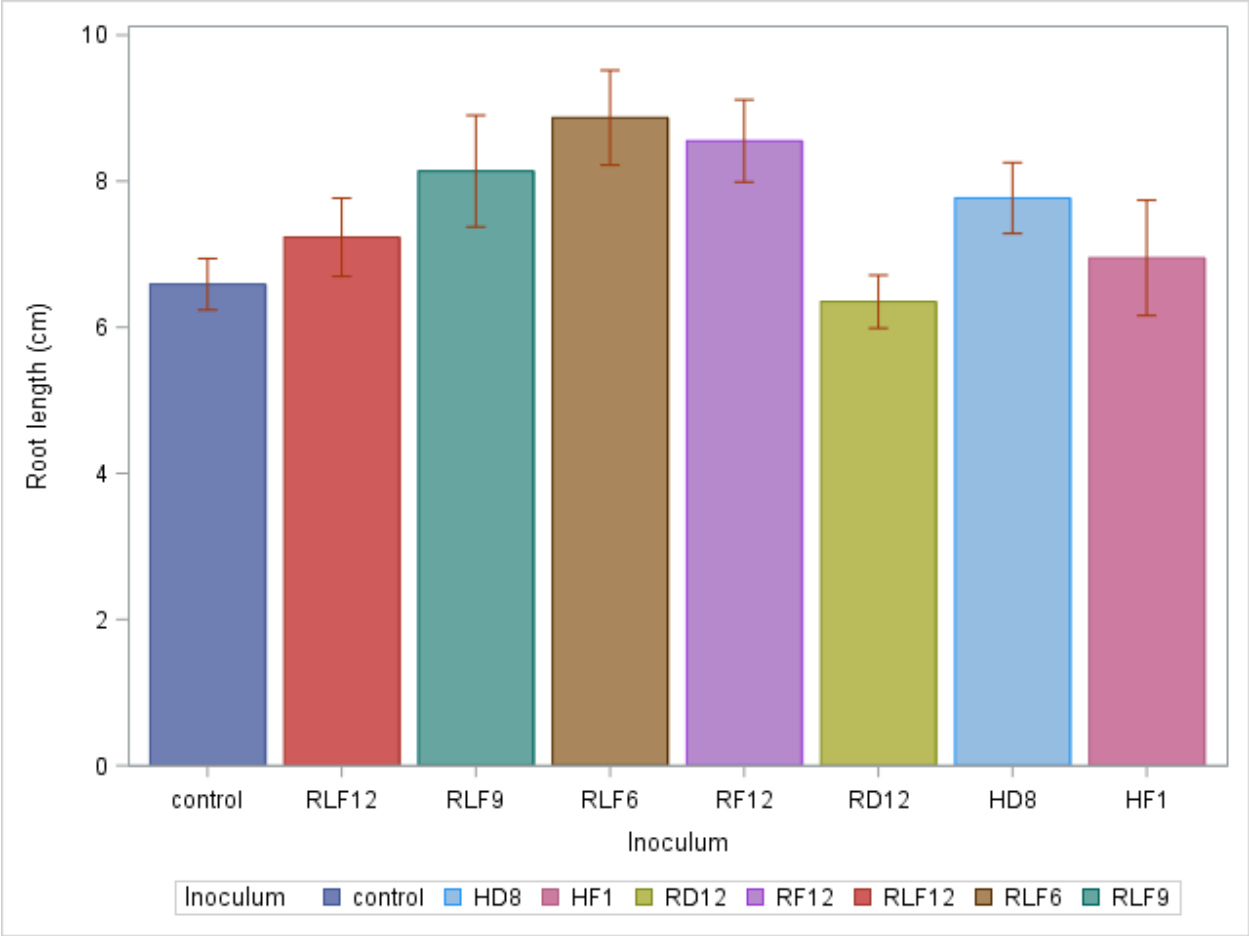

Figure 3.6 Root lengths (cm) of winter wheat seedlings, averaged across four cultivars, inoculated with seven bacterial isolates or sterile control. Histogram bars labeled with the same letter do not differ significantly at $P \leq 0.05$, as determined by analysis of variance tests and Dunnet's tests for mean comparisons (n=2).

RLF12, RLF9, RLF6: *Pseudomonas sp.* isolated from rhizosphere soil of RonL wheat cultivar under fully irrigation treatment.
RF12: *Pseudomonas sp.* isolated from rhizosphere soil of Ripper wheat cultivar under fully irrigated treatment.
RD12: *Variovorax sp.* isolated from rhizosphere soil of Ripper wheat cultivar under dry-land treatment.
HD8: *Pseudomonas sp.* isolated from rhizosphere soil of Hatcher wheat cultivar under dry-land treatment.
HF1: *Pseudomonas sp.* isolated from rhizosphere soil of Hatcher wheat cultivar under fully irrigated treatment.

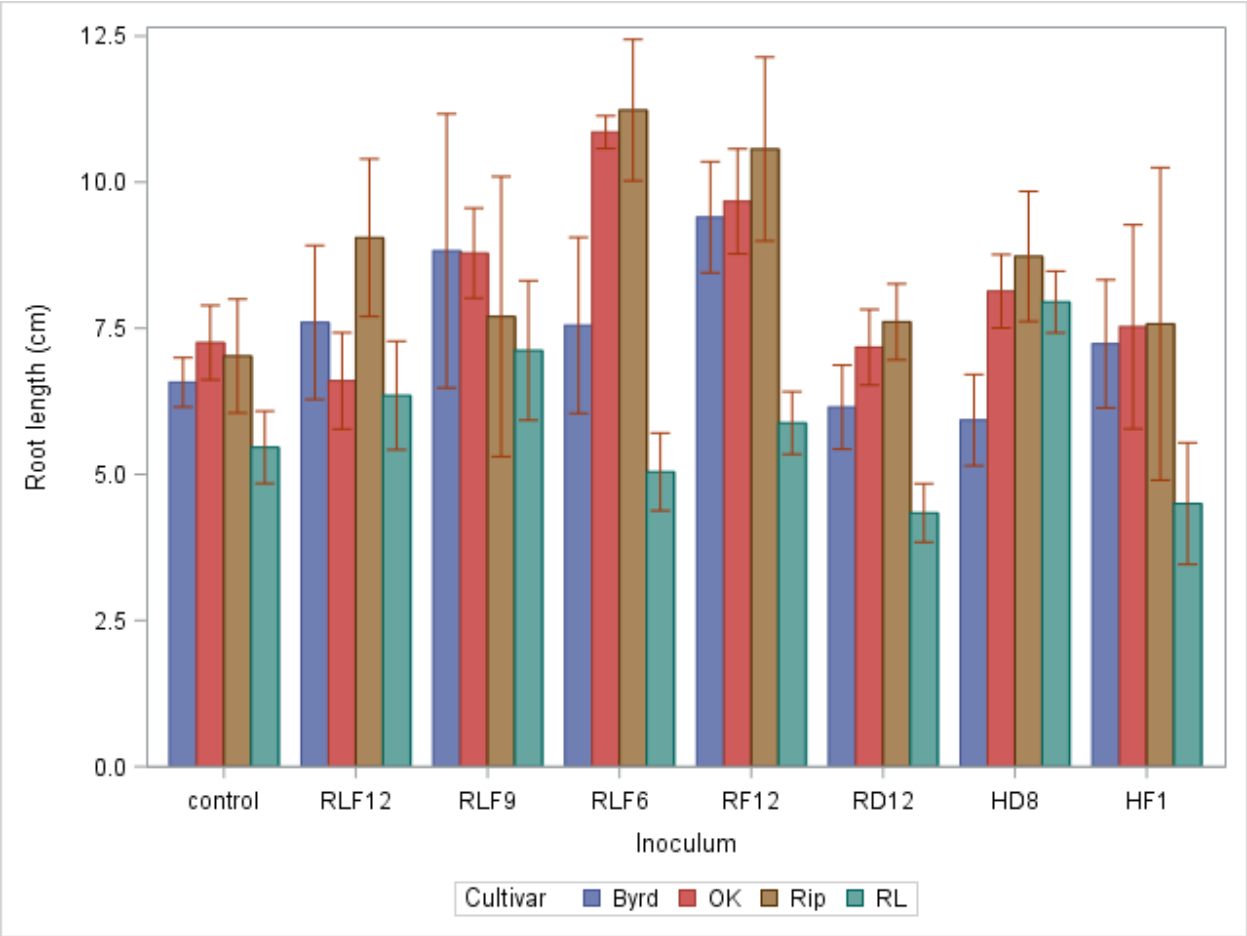

Figure 3.7 Root lengths (cm) of winter wheat seedlings (four cultivars) inoculated with seven bacterial isolates or sterile control.

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CHAPTER 4: SURVIVAL OF ACC-DEAMINASE POSITIVE BACTERIAL INOCULANT IN FIELD SOIL AND EFFECTS ON GROWTH OF WINTER WHEAT CULTIVARS

1. Introduction

Inoculation of plants with Plant Growth Promoting Rhizobacteria (PGPR) for crop improvement is currently an important area of research attracting attention of agronomists and soil microbiologists. There is a strong need for more research on the effect of PGPR inoculation on plant growth under field conditions, to achieve a better understanding of how to utilize PGPR inoculants to promote plant growth and increase yields.

Some studies have reported positive and desirable results on plant growth in response to PGPR inoculation when applied to soil, roots, or seeds (Mishustin and Naumova, 1962; Giddens et al., 1982; Dunigan et al., 1983; Diem et al., 1988; Sougoufara et al., 1989, Smolander et al., 1988; Belim, et al., 2002; Dey et al., 2004; Gamalero et al., 2008). However, variable results have also been obtained (Albrecht et al., 1981, Kapulnik et al., 1981; Han and Lee, 2006; Campo et al., 2010; Egamberdieva et al., 2010; Hungria et al., 2013). Lack of positive results has often been attributed to poor survival of inocula in the soil environment. Inoculum introduced into soil may have difficulties surviving over time due to the fact that the soil in nature is a heterogeneous and unpredictable environment for introduced organisms (Shaharoona et al., 2007). In fact, obtaining desirable results following the inoculation of beneficial rhizobactetia into the soil mainly depends on the capability of inoculated bacteria to survive in heterogeneous soil environment (Trabelsi and Mhamdi, 2013); therefore, an inoculation method that enhances inoculum survival in soil may be more effective in promotion positive plant response to inocula.

Alginate beads are one type of inoculant carrier that has been used in relatively few inoculation studies (Bashan and Gonzalez, 1999; Gonzalez and Bashan, 2000; Lebsky et al., 2001; Bashan and Hernandez, 2002). This method employs an alginate-based "gel", in the form of beads, to encapsulate living microbial cells. Alginate beads protect cells from direct exposure to the harsh environment, and release cells to the soil gradually as the alginate is degraded over time. Beads are usually amended to soil at the time of seed germination and seedling emergence (Bashan, 1986). The alginate beads method has many desirable advantages that make it attractive for use by growers; it is dry, easy to handle, uniform in size and shape, biodegradable, nontoxic and can contain a large uniform bacterial population. Alginate beads provide for the slow release of bacteria over long periods and is a source of carbon and nutrient to the inoculum (Bashan, 1986). Alginate is also less expensive compared to other inoculant carriers (Bashan and Gonzalez, 1999). The main goal of encapsulation of PGPR is to protect them from harsh soil environment, reduce microbial competition, and release them gradually to facilitate colonization of plant roots (Bashan et al., 2002; Vassilev et al., 2001). Use of the alginate bead method to introduce PGPR into the soil may be an effective strategy to promote crop growth and productivity and to achieve a better understanding of how to utilize PGPR inoculants to promote plant growth and increase yields under field conditions.

The main aim of the present study was to test the ability of an ACC-deaminase positive bacterial inoculant to survive in the soil and promote the growth and yield of winter wheat cultivars under field conditions. The inoculant was prepared in the form of calcium-alginate beads, and my hypotheses were that 1) alginate-forumulated inoculum is capable of surviving in soil, and 2) soil inoculation with *Pseudomonas sp.* RLF9 beads would promote the growth of winter wheat cultivars Byrd, Ripper, and RonL compared to non-inoculated wheat.

2. Methods

2.1 Isolation of ACC-deaminase positive rhizobacteria

An ACC deaminase-positive bacterium, *Pseudomonas sp.* RLF9, was isolated from a winter wheat rhizosphere and assayed for PGPR traits as described in Chapter 3. The bacterium was identified as a *Pseudomonas sp.,* and in addition to possessing ACC deaminase activity, this strain was also able to tolerate osmotic stress, solubilize rock phosphate, and enhance root elongation of Byrd and RonL seedlings.

2.2. Preparation of calcium–alginate beads

A 3 % (w/w) sodium alginate solution was prepared by adding 18 g of alginic acid sodium into 400 mL Milliq water in a 2-L beaker. The solution was stirred for approximately 2 h to ensure complete hydration, followed by autoclaving at 121 °C for 30 min. After autoclaving, 200 mL of sterile Milliq water was added to the alginate solution.

2.2.1. Inoculum preparation

Pseudomonas sp. RLF9 was grown in a 250-mL flask containing 50 mL sterile LB broth medium containing (per liter) 10 g tryptone, 5 g yeast extract, and 5 g sodium chloride (NaCl). The flask was placed in a shaker incubator at 100 rpm at 30 °C for 24 h. A 1-mL aliquot was removed from the LB broth culture and transferred to a 250-mL flask containing 50 mL sterile LB broth medium, and 50 µL of filter-sterilized rifampicin (50 mg mL^{-1}) was added. Rifampicin was added to select for antibiotic-resistant strains, for the purpose of tracking inoculum survival in soil over time. The culture was grown for 24 h in a shaker incubator 100 rpm and 30 °C. Afterwards, an aliquot of the rifampicin-resistant culture was transferred to 1 L of sterile LB broth media medium in a 2-L flask, to which 1 mL of filter-sterilized rifampicin (50 mg mL^{-1}) was added. The culture was grown for 24 h as described above.

2.2.2. Preparation of alginate inoculum

After the final incubation, 200 mL of inoculum was transferred into each of six sterile centrifuge tubes. To wash cells, the tubes were centrifuged at 5,000 rpm for 10 min. The supernatant of each tube was removed, and 25 mL of sterile LB with 2% glycerol medium were added to each centrifuge tube and vortexed to re-suspend the cell pellet. The washing process was repeated two more times. After the third centrifugation, and removal of the supernatants, cells were suspended in a final solution of 50 mL sterile LB with 2% glycerol medium. The volumes of each tube were combined (300 mL total) and added to the alginate beaker. Finally, the solution in the alginate beaker was stirred for 10 min.

2.2.3. Preparation of inoculant beads

The alginate beads were made at room temperature. The bacterial culture was incorporated into alginate beads that is considered as source of carbon and other nutrients to promote the survival of inoculant. The inoculated alginate was introduced dropwise from 23-gauge syringe needles through sterile pump tubing, at a rate of 10 rpm per 24 h, into a beaker containing 1 L of 1.5 % w/v calcium chloride solution being stirred at 500 rpm. Upon contact with the calcium chloride solution, the alginate drops gelled and formed into beads within the calcium chloride beaker. Once all the inoculated alginate was added, dropwise, the calcium chloride solution was pipetted out of the beaker, and the beads were washed with 200 mL of sterile Milliq water. After washing, the Milliq water was pipetted out of the beaker, and sterile LB with 2% glycerol medium was added to beaker to completely cover the beads. The beads were incubated overnight in a shaking incubator at 30 °C and 200 rpm. After the incubation, the LB medium was removed and the beads were spread onto a sterile, foil-lined cookie sheet and placed in biological cabinet to dry for overnight. The dry beads were then stored in the

laboratory in 50 mL sterile, conical bottom centrifuge tubes. Sterile, control beads were made following the same process, except that a 3 % w/v alginate sodium solution without inoculum was introduced into 1.5 % w/v calcium chloride solution.

To determine the inoculum density of the beads, 10 beads were diluted in 9 mL of sterile sodium citrate, pH 6.7, for 1 h and vortexed every 15 min. The solution was serially diluted to achieve a dilution range between 10^{-1} and 10^{-3}. 100 µl of each dilution was pipetted onto sterile LB agar plates containing 50 µl mL^{-1} rifampicin, and the plates were incubated at 30 °C for 48 h. Colonies were counted and the CFUs mL^{-1} was estimated, for the purposes of calculating inoculation rates into soil (described below).

2.3. Field study

The field experiment was conducted during 2015-2016 at the Agricultural Research Development and Education Center (ARDEC), located four miles north of Fort Collins, Colorado. The experimental design was a split-plot with three replications. Main plots were six genotypes, consisting of three winter wheat cultivars grown either as monocultures or bicultures (Byrd, Ripper, RonL, Byrd/Ripper, Byrd/RonL, and Ripper/RonL; Grogan et al., 2017). The bicultures were included because of previous greenhouse results showing a yield advantage when certain cultivars were grown together. The genotype plots were 1.83 m long and 12 rows wide, with rows spaced 23 cm apart. Seeding density was approximately 700,000 seeds ha^{-1}. Subplots were inoculation treatments (RLF9 inoculum or sterile control), which were applied in between two adjacent rows, with border rows of wheat separating the inoculation treatments. To inoculate soils, a shallow trench (~5 cm deep) was made using a hand rake, between the two rows of wheat, and alginate beads with or without the inoculum were added to achieve a density of 1×10^{10} CFUs m^{-2}. Plots were inoculated on April 21, 2016, when wheat growth was

approximately at Zadoks stage 32 (stem elongation). Two occurrences of this same design were planted in the same field separated by a border of wheat plants 6.1 m wide. One of these experiments was irrigated at weekly intervals from June 3 to 22 (corresponding to early to mid-grain fill), and the other was grown as a dryland crop without supplemental irrigation.

Soil samples were collected on June 2, 2016, 6 weeks after bead inoculation, and before the first irrigation treatment. Soil cores (9-mm wide and 5 cm deep), were collected using cork borers. Soil samples were placed in Ziploc bags on ice for transport back to the laboratory. Soil samples were further divided into three subsamples for measurements of inoculum Colony Forming Units (CFU**),** soil moisture content, and ACC-deaminase activity in soil**.** The samples were stored at 4 ^{o}C until analysis.

2.3.1. Inoculant survival in soil by Colony Forming Unit (CFU) determination

The number of viable RLF9 cells in each soil sample was estimated by counting rifampicin-resistant CFUs. Soil subsamples (1 g) were serially diluted in 9 ml of sterile sodium citrate, pH 6.7. Typically four dilutions in the range of 10^{-1} and 10^{-4} were used. 100 µl of each dilution was added onto LB agar plates containing 50 µL mL^{-1} rifampicin. Each dilution was plated with five replicates, and the plates were incubated at 30 °C for 3-6 days. The CFUs were counted, and data were expressed as the number of CFU's g^{-1} dry soil.

2.3.2. ACC-deaminase activity in soil

The ACC deaminase activity in soil was determined by quantifying the production of α-ketobutyrate produced from ACC by ACC-deaminase enzyme, according to the method of Smaill et al (2010) with modifications. Subsamples of soil (1 g) were added to 50-mL centrifuge tubes, along with 5 mL of 0.1M tris buffer, (pH 8.5), containing 20 mM ACC. Controls (soil blanks) were prepared in the same way, except that the tris buffer did not contain ACC. The

centrifuge tubes were incubated for 24 h at 28 °C. Afterwards, the tubes were centrifuged at 2,500 g at 25°C for 10 min. One mL of supernatant from each tube was transferred into a clean test tube, to which 1.8 mL of 0.56 M HCI and 300 µL of the 2,4-dinitrophenylhydrazine reagent (0.1% (w/v) in 2M HCl) were added. Reagent blanks were prepared as follows: 1 mL of 0.1M tris buffer, (pH 8.5), containing 20 mM ACC, 1.8 mL 0.56 M HCI, and 300 µL 0.1% (w/v) 2,4 dinitrophenylhydrazine in 2 M HCl. The tubes of soil solutions and blanks were incubated for 15 min at 28 °C. After incubation, 2 mL of 2 M NaOH were added to each tube. The absorbance of the mixture was measured at 540 nm on a spectrophotometer (Model 680, Bio-Rad Laboratories, Hercules, CA).

A standard curve of α-ketobutyrate,was prepared from a stock solution of 1.76 mg α-ketobutyrate (Sigma-Aldrich Co., St Louis, MO, USA) in 5mL tris-HCl, pH 8.5. The stock solution was diluted in the same buffer, to achieve a standard curve in the range 0-880 µg mL^{-1}. Aliquots of each standard were prepared by adding 300 µL of the 2,4-dinitrophenylhydrazine reagent. Solutions were incubated at 30 °C for 30 min, during which the a-ketobutyrate was derivatized as a phenylhydrazone. The color of the phenylhydazone was developed by the addition 2 mL NaOH, and the mixture was vortexed. The absorbance of the mixture was measured at 540 nm. ACC deaminase activity was expressed as the nmole of α-ketobutyrate h^{-1} g^{-1} dry soil.

2.3.3. Wheat growth and yield

Wheat was harvested in July 2016. Plants were carefully cut at the soil surface using a rice knife ensuring no roots were taken, and above-ground biomass from each plot was placed in labelled paper bags for transport to the laboratory for measurements of biomass, biomass grain, harvest index, and total grain at harvesting. Biomass was determined as above-ground biomass

of a 1-m strip of a single inoculated row, biomass grain was grain biomass obtained from the 1-m strip, harvest index was biomass grain divided by above-ground biomass, and total biomass grain was the sum of grain from the 1-strip plus remaining grain in the second inoculated row.

2.3.4. Statistical analyses

The effect of inoculation and cultivar on ACC-deaminase positive bacterial CFUs and soil ACC-deaminase activity were analyzed by two-way analysis of variance tests in SAS, version 6.1 (SAS Institute, Cary, NC). When inoculation and/or cultivar effects were significant, means were separated by the Tukey-Kramer post-test in SAS. For the harvest yield indices, wet and dry treatments were analyzed separately. Each irrigation treatment was analyzed separately as a split-plot block design with cultivar as the main plot factor and inoculum treatment as the sub-plot factor (α= 0.05)

3. Results

There was a significant inoculation × cultivar interaction effect on inoculant survival in soil, six weeks after inoculation (Table 4.1). For all cultivar treatments, numbers of rifampicin-resistant *Pseudomonas sp.* were greater in soil inoculated with RLF9 than sterile inoculant, except for the RonL/Ripper biculture treatment. Overall, soil under RonL accumulated the greatest ACC-deaminase positive bacterial populations (1.8×10^5 CFUs g^{-1} soil) compared to cultivar treatments (Table 4.1).

Soil ACC deaminase activity was highly variable but tended to be greatest in soil planted to RonL, RonL/Ripper and Ripper (93, 68, and 47 nmole α-ketobutyrate h^{-1} g^{-1} soil, respectively) (Table 4.2). Differences among inoculant × cultivar treatment combinations were not statistically significant, however. There was no significant correlation between inoculant abundance in soil and soil ACC-deaminase activity (p=0.811; data not shown).

Within an irrigation treatment, there were no significant differences in wheat yield by inoculation or by cultivar treatment (Tables 4.3 and 4.4). There was a trend for the RLF9 inoculant to enhance biomass (465.8 g) and biomass grain (113.2 g) of Ripper under dryland conditions, but the effect was not significant (Table 4.3). In the irrigated soil, there was a trend for greater yield and harvest indices among cultivars when soils were inoculated with RLF9; soil inoculated with *Pseudomonas sp.* RLF9 increased the biomass of the winter wheat over that of the uninoculated plants by 52%. However, this effect was not statistically significant (Table 4.4).

4. Discussion

Encapsulation is a useful and effective strategy for applying microbial inoculants in soil as it provides protection and nutrient sources. This field study demonstrated that the *Pseudomonas sp*. RLF9 survived in soil for at least up to six weeks after inoculation. The initial application rate was 1×10^{10} CFUs m^{-2}, and after six weeks, numbers of rifampicin-resistant bacteria ranged from 10^2 to 10^5 CFUs g^{-1} dry soil, except for treatment RonL/Byrd control, where rifampicin-resistant bacterial populations were below detection limits. Alginate beads help to reduce some of the main problems of inoculation of beneficial rhizosphere bacteria in agricultural soils. Since the optimal inoculation time is not always known, the slow release of the bacteria will ensure a constant supply of the bacteria over a relatively long period. The bead formulation also provides a source of C and N to sustain microbial inoculants until growing plants roots reach the zone of inoculant application. Therefore, this field study highlights the potential for using a relatively new agricultural technology, PGPB treatment applied in the form of alginate beads, to increase the biomass production of the winter wheat cultivars under drought stress.

There was no correlation between the inoculant CFU g^{-1} soil and ACC-deaminase activity It may that the bacteria have low enzyme activity or the activity of the enzyme changed. Large populations of ACC+ bacteria have been measured in Colorado soil (Chapter 2), so perhaps activity of indigenous bacteria made it impossible to detect differences in enzyme activity due to inoculation.

Despite the successful inoculant survival (at least up until six weeks after inoculation), inoculation did not significantly affect wheat growth under irrigated or dryland conditions. In late April, the inoculation may not have been placed deep enough in the soil to reach acrtively growing roots, so it could be the reason that inoculation failed to improve wheat yield over the sterile inoculant treatment. While the results were disappointing, there are several environmental factors which may have also affected the results. The field season was marked with abundant rainfall and cool temperatures during the spring, followed by outbreaks of pathogens, including stripe rust, that affected all field treatments and confounded late season drought and heat stress (Colorado Climate Center, 2016). The temperature in June ranged from 55.9 $^{\circ}$F - 86.7 $^{\circ}$F) and in July was (59.5 $^{\circ}$F - 89.2 $^{\circ}$F) (Colorado Climate Center, 2016). According to the Mountain Region Field Office of the National Agricultural Statistics Service (USDA), in the first week of June, heat units increased in frequency, resulting in steady to improved conditions while development of some crops was stimulated. Hot and relatively dry conditions persisted in the second week of June, sustaining significant gains in crop development and permitting widespread field activity. Hot conditions sustained the pace of crop development while limited, localized precipitation was generally received in Fort Collins, Colorado in June while no precipitation was recorded for July**.** As a result, there were no irrigation treatment or inoculation effects on wheat yield indices.

Other factors may have affected the results such as small plot size and late inoculation time. While bead encapsulation allows for slow release of microbes over time, future work should study optimal inoculation times. In this experiment, the inoculation occurred in late April during stem elongation, and may not have been carried out at the optimal time. Thus, part of the applied bacterial population may have died before root interception, causing less root colonization. Therefore, inoculating in wrong time and with inconstant bacterial level may lead to undesirable results in the field. Similarly, Bashan (1986) showed that late inoculation of soil with eight strains of rhizobacteria was ineffective of spring wheat growth.

Under irrigated treatment, inoculated soil with alginate beads increased the biomass of Ripper cultivar. This may due to the high ACC-deaminase activity (47 α-ketobutyrate nmol h g^{-1} soil), so the ACC enzyme cleaves ACC in to α-keytobutrate ammonia and, so the level of ethylene would be decreased under dry condition. This leads to enhanced the growth. For many plants, a low level of ethylene state is required to break seed dormancy (Esashi,1991) but, following germination, high ethylene state would inhibit root elongation (Jackson, 1991).

In conclusion, this study demonstrated the survival capability of *Pseudomonas sp.* RLF9 inoculant under field conditions. However, the inoculant did not affect wheat yield traits, which was unexpected. Many factors may have affected the results such as late inoculation, rainfall, level of bacterial population, competition, and size of plots. In addition, small size samples may affect the results. The experiment should be repeated over multiple years, when environmental conditions may be more favorable to the experiment. Certainly, more bacterial strains should be tested in the future to avoid the sensitivity of the suggested method. In addition, inoculation of plants with different bacterial strains at different stages of plant development may provide desirable results.

Table 4.1 Population density of ACC-deaminase positive bacteria in soil planted to six different winter wheat cultivar treatments, either uninoculated or inoculated with Pseudomonas sp. in the form of alginate beads. Values are means (n=6; ± 1 SE) of Colony Forming Units (CFU) g-1 dry soil.

Cultivar	Inoculum	CFU g^{-1} dry soil
Byrd	Uninoculated	16,400 ± 10,300 b
	Inoculated	69,400 ± 31,900 a
Ripper	Uninoculated	6,390 ± 4,190 b
	Inoculated	56,200 ± 30,400 a
Byrd/Ripper	Uninoculated	708 ± 708 b
	Inoculated	31,100 ± 24,200 a
RonL	Uninoculated	397 ± 397 b
	Inoculated	179,000 ± 113,000 a
RonL/Byrd	Uninoculated	BD[1]
	Inoculated	67,700 ± 35,700aab
RonL/Ripper	Uninoculated	26,100 ± 18,500 a
	Inoculated	25,100 ± 15,000aab

[1]BD = Below Detection limits.

Table 4.2. ACC-deaminase activity in soil planted to six different winter wheat cultivar treatments, either uninoculated or inoculated with Pseudomonas sp. in the form of alginate beads. Values are means (n=6; ± 1 SE) of enzyme activity, expressed as nmole α-keytobutrate h-1 g-1 dry soil. There were no significant treatment effects at $P \leq 0.05$, as determined by analysis of variance tests and Tukey tests for mean comparisons.

Cultivar	Inoculum	nmol α-keytobutrate hr^{-1} g soil
Byrd	Uninoculated	13 ± 3
	Inoculated	34 ± 10
Ripper	Uninoculated	25 ± 10
	Inoculated	47 ± 20
Byrd/Ripper	Uninoculated	13 ± 10
	Inoculated	14 ± 10
RonL	Uninoculated	13 ± 10
	Inoculated	93 ± 100
RonL/Byrd	Uninoculated	23 ± 20
	Inoculated	16 ± 4
RonL/Ripper	Uninoculated	12 ± 5
	Inoculated	68 ± 40

Table 4.3 Winter wheat above-ground biomass, biomass grain, total grain, and harvest index in field plots planted to six different winter wheat cultivar treatments, either uninoculated or inoculated with Pseudomonas sp. in the form of alginate beads and grown under dryland conditions. Values are means (n=3; ± 1 SE). There were no significance treatment effects at $P \leq 0.05$, as determined by analysis of variance tests and Tukey tests for mean comparisons.

Wheat cultivars	Inoculum	Total grain (g)	Biomass (g)	Biomass grain (g)	Harvest index
RonL	Uninoculated	330.6	438.0	102.5	0.233
	Inoculated	298.8	380.2	86.7	0.228
Ripper	Uninoculated	329.7	499.5	111.6	0.223
	Inoculated	347.0	465.8	113.2	0.243
Byrd	Uninoculated	389.4	483.8	130.9	0.271
	Inoculated	334.2	401.6	98.6	0.247
RonL/Ripper	Uninoculated	313.5	427.4	100.5	0.234
	Inoculated	323.3	382.9	82.2	0.213
RonL/Byrd	Uninoculated	387.0	467.6	119.1	0.253
	Inoculated	383.3	421.7	107.3	0.256
Ripper/Byrd	Uninoculated	358.4	453.6	120.6	0.267
	Inoculated	357.2	443.0	107.5	0.240

Table 4.4 Winter wheat above-ground biomass, biomass grain, total grain, and harvest index in field plots planted to six different winter wheat cultivar treatments, either uninoculated or inoculated with Pseudomonas sp. in the form of alginate beads and grown under irrigated conditions. Values are means (n=3; ± 1 SE). There were no significance treatment effects at $P \leq 0.05$, as determined by analysis of variance tests and Tukey tests for mean comparisons.

Wheat cultivars	Inoculum	Total grain (g)	Biomas (g)	Biomass grain (g)	Harvest index
RonL	Uninoculated	369.0	438.0	114.4	0.260
	Inoculated	361.6	477.7	122.0	0.256
Ripper	Uninoculated	401.5	473.6	133.1	0.280
	Inoculated	486.7	542.0	166.3	0.309
Byrd	Uninoculated	365.3	379.6	90.9	0.232
	Inoculated	412.8	385.7	105.2	0.265
RonL/Ripper	Uninoculated	353.8	528.4	125.6	0.239
	Inoculated	403.7	490.0	129.7	0.265
RonL/Byrd	Uninoculated	348.2	398.2	113.4	0.287
	Inoculated	366.0	434.8	123.8	0.284
Ripper/Byrd	Uninoculated	422.0	485.2	143.4	0.299
	Inoculated	445.0	505.8	139.8	0.279

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CHAPTER 5: CONCLUSIONS

My hypotheses were as follows: the richness of ACC-deaminase positive bacteria in Colorado soil will depend on winter wheat genotypes and irrigation treatment, the greatest diversity and activity of ACC deaminase positive bacteria will be associated with drought-tolerant winter wheat genotypes (e.g., Ripper), diversity will be greater under full irrigation compared to dryland management practice, and finally, drought tolerance in winter wheat can be improved through inoculation with ACC deaminase bacteria that possess multiple PGP traits and prepared as an alginate bead inoculum.

This study demonstrated that a Colorado soil accumulates relatively high numbers of ACC-deaminase positive bacteria, and populations increased under drought condition. The results reported that different winter wheat cultivars accumulated different relative amounts of ACC-deaminase positive bacteria, when under water stress. The study also found that wheat cultivar type influenced the community composition of ACC-deaminase positive bacteria.

This research supported all the hypotheses except the hypothesis, drought tolerance in winter wheat can be improved through inoculation with ACC deaminase bacteria that possess multiple PGP traits and prepared as an alginate bead inoculum, was not supported. However, alginate beads were an effective inoculant delivery method to ensure inoculant survival in soil, which is an important finding.

Drought is arguably the most devastating abiotic stress affecting crop production world-wide and the most difficult to address through crop genetic improvement. This is because of the inherent variability in drought severity, timing, and duration, and the many genes and mechanisms involved in the adaptive responses of plants to drought (Blum, 2011; Reynolds et

al., 2009; Richards et al., 2010). In our study, RonL has some tolerance to drought, and compared to other genotypes studied, RonL showed the greatest potential to accumulate high relative abundance of ACC+ bacteria and potential ACC deaminase activity. Interestingly, Ripper, a semi-dwarf, early-maturing hard red winter wheat, is also considered drought tolerant (Haley et al., 2007). That both RonL and Ripper have shown drought tolerance yet different associations with ACC+ bacteria could be indicative of different strategies among winter wheat cultivars for coping with drought stress. Further studies are warranted to determine the role of root exudates and the relative contribution of ACC+ bacteria to drought tolerance among different winter wheat genotypes. With increased understanding, the exploitation of ACC+ bacteria's natural abilities with appropriate host crops could help to improve food production and sustainable agriculture in water-scarce regions.

The present study generated a set of bacterial isolates possessing multiple PGP traits and ability to stimulate plant growth, as evidenced by primary root elongation. Isolates with high ACC-deaminase activity and additional PGP traits may have potential as inoculants to stimulate plant growth and crop productivity and possibly alleviate drought stress. The findings of this study are very attractive for further field studies, which would be helpful in extending isolate use as PGP inocula for wheat production, especially in Colorado and perhaps other semi-arid regions. Specifically, inoculation of winter wheat cultivars with PGPR-containing ACC-deaminase and other PGP traits could be an effective approach for successful crop production. Selection of PGPR inoculant and wheat cultivar combinations for obtaining optimum responses should be considered in future studies. The ability of potential inoculants to survive in the rhizosphere, compete with indigenous microbial communities, and colonize roots should also be studied. Based on the study results, isolates RFL9, and possibly RFL6, are proposed as potential

inoculants for further study. This is based on their relatively high ACC-deaminase activity, tolerance to osmotic stress, phosphate solubiliziation activity, and ability to elongate roots of more than one wheat cultivar.