

THESIS

THE ABUNDANCE OF ACC DEAMINASE-POSITIVE BACTERIA AND THEIR INTERACTION
WITH WINTER WHEAT IN A COLORADO SOIL

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2012

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ABSTRACT

THE ABUNDANCE OF ACC DEAMINASE-POSITIVE BACTERIA AND THEIR INTERACTION WITH WINTER WHEAT IN A COLORADO SOIL

Plant growth-promoting rhizobacteria (PGPRs) are known as beneficial bacteria for plant growth and yield. One PGPR group are the ACC deaminase-positive (ACC+) bacteria which degrade 1-aminocyclopropane-1-carboxylic acid (ACC), the plant-produced precursor to ethylene. Plants produce ethylene in elevated quantities under environmental stress (“stress ethylene”), and studies have shown that ACC+ bacteria, in association with plant roots, can improve plant growth under abiotic stress (e.g., drought, salinity, heavy metals) by reducing concentrations of stress ethylene. There are few studies that have examined the natural abundance and distribution of these bacteria as affected by plant genotype, plant growth stage, and agricultural management practice; and no studies have been conducted in the western United States. The objectives of my research were to determine the influence of winter wheat genotype and irrigation practice on the abundance of culturable ACC+ bacteria in a Colorado soil, and to determine the plant-growth-promoting effect of selected ACC+ on winter wheat varieties ranging in drought sensitivities under greenhouse conditions.

A field study was conducted at the Limited Irrigation Research Farm (LIRF) in Greeley, Colorado. Roots and root-associated soil (0-20 cm depth) were collected with a shovel under four winter wheat varieties (*Triticum aestivum* L. “Baca”, “Hatcher”, “Ripper” and “RonL”) managed by three different irrigation regimes: full irrigation, limited irrigation (irrigation commencing at the anthesis stage), and dryland. Samples were collected at four physiological growth stages during the 2010-2011 growing season: emergence (November 2010), green-up (March 2011), anthesis (May 2011) and mid-grain filling (June 2011). Total culturable bacteria were enumerated on TSB agar, and culturable ACC+ bacteria were enumerated on DF minimal salts agar media containing ACC as the sole N source. The abundance of ACC+ bacteria was relatively high in the Colorado soil (1.69×10^7 - 3.28×10^9 CFU's g^{-1}) and varied according by an interaction between sampling date and irrigation practice ($P < 0.0001$). Abundance of ACC+ bacteria in the fully irrigated plots peaked in March and declined throughout the rest of the growing season, whereas ACC+ bacterial numbers increased significantly in the late growth stages for the dryland and limited irrigation treatments. The percentage of ACC+ bacteria, relative to total culturable bacteria, was significantly affected by the three-way interaction between wheat variety \times irrigation treatment \times sampling date ($P= 0.0095$). Whereas few differences in % ACC+ bacteria were observed among treatments in November and March, % ACC+ bacteria in May and June were generally greater under dryland or limited irrigation compared to full irrigation management. Wheat variety had no effect on % ACC bacteria under full irrigation, whereas % ACC

bacteria generally was lowest under Baca and greatest under RonL under limited irrigation or dryland,

A greenhouse study was conducted to determine how interactions among specific ACC+ bacterial strains, winter wheat genotype, and water stress affect winter wheat productivity. Two strains of *Pseudomonas brassicacearum* (HD6 and HF1) were selected for inocula because of their high ACC-deaminase activity and numerical dominance in a Colorado soil. Two winter wheat varieties (Ripper and RonL) were grown under non-stressed (soil maintained at 80-100% water holding capacity) or water-stressed (soil maintained at 40-60% water holding capacity) conditions, with different ACC+ bacterial inoculum treatments (HD6, HF1, or a combination of HD6 + HF1). Inoculation with ACC+ bacteria increased stem height of both wheat varieties, and increased the biomass, number of seeds, and number of fertile heads of RonL. There was a negative effect of the strain HD6 on Ripper productivity under non-stressed conditions in the greenhouse study.

In conclusion, these studies demonstrate that the abundance of ACC+ bacteria and the potential effects of these bacteria on winter wheat productivity are dependent on winter wheat genotype and irrigation practice. Certain wheat varieties are able to accumulate more numbers of ACC+ bacteria in their rhizospheres under water stress, and certain strains of ACC+ bacteria can positively (RonL) or negatively (Ripper) impact winter wheat depending on the level of water stress.

ACKNOWLEDGMENTS

First, I'm so thankful to my supervisor, Dr. Mary Stromberger, who has always been supportive. I appreciate all her help, ideas, patience and encouragement that helped me through difficult times of analyzing the results and writing the thesis. I would like to thank my committee members, Dr. Marc Moragues, and Dr. Tiffany Weir who helped me to get more knowledge about many of the subjects related to my thesis. I would also thank my family in Libya. They support me to be patient to complete my thesis. Also, I would thank them for their help and encouragement, especially my beloved parents. Lastly, I would thank all of those who supported me in any respect during the completion of my project.

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Chapter 1

Introduction

Drought stress is the most frequent type of abiotic stress that reduces crop growth and yields (Zhu et al. 2002). The effect of drought stress on plants depends on the time of stress, the stress duration, and the severity and rapidity of the stress. Drought stress significantly decreases leaf water potential and photosynthetic rate in plants, and forces the stomata to be closed which leads to more respiration and less transpiration and then higher leaf and canopy temperature (Siddique et al. 2001). In addition, activities of the enzymes that control carbon assimilation are reduced and sometimes inhibited. Drought stress increases the production of reactive oxygen species in cell organelles, which also leads to less plant growth and productivity (Farooq et al. 2009).

Plants under biotic and abiotic stresses release high amounts of ethylene called “stress ethylene”. Drought stress increases the production of ethylene, especially when the drought has developed rapidly (Morgan and Drew 1997). Along with drought stress, high temperatures can lead to high ethylene production by the effect of temperature on the ethylene synthesis enzymes (Morgan and Drew 1997). High levels of ethylene inhibit stomatal closure and increase the rate of transpiration by inhibiting the Abscisic Acid (ABA) signaling pathway (Tanaka et al. 2005). Tamimi and Timko (2003)

reported that stress ethylene may also inhibit nitrogen fixation in legumes by inhibiting nodule formation and reducing rhizobia cell numbers during early stages of plant growth.

Plant growth promoting rhizobacteria (PGPRs) are known to support plant growth through several mechanisms when they associate with plant roots. Mechanisms include: increasing nutrient uptake efficiency by plants, producing plant growth hormones, and protecting host plants from the pathogens (Wu et al. 2005; Diaz-Zorita and Fernandez-Canigia 2008, and Gholami et al. 2009). Inoculating cereals with PGPRs can increase plant biomass, nutrient uptake, tissue N content, plant height, leaf size and root length (Bashan et al. 2004, Salantur et al. 2006, and Ahmed et al. 2006). ACC deaminase positive (ACC+) bacteria are one group of PGPRs that degrade 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor to ethylene. ACC+ bacteria have the ability to reduce ACC and ethylene levels between two-to-four fold, and studies have shown that ACC+ bacteria, in association with plant roots, can improve plant growth under abiotic stress (e.g., drought, salinity, heavy metals). The chemical structure of ACC is shown in Fig. 1.1 (http://en.wikipedia.org/wiki/1-Aminocyclopropane-1-carboxylic_acid).

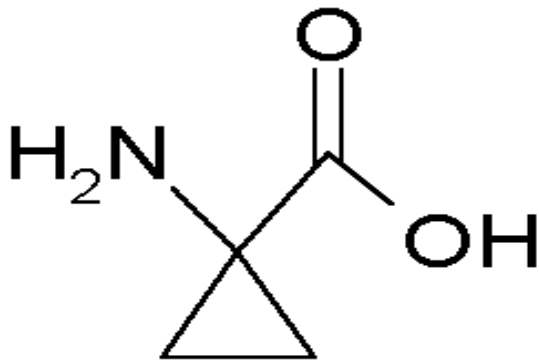


Figure 1.1 The chemical structure of ACC.

ACC deaminase is a multimeric enzyme with a monomeric subunit molecular mass of approximately 35–42 kDa (Glick et al. 2005). It is a sulfhydryl enzyme that utilizes pyridoxal 5-phosphate as an essential co-factor. Pyridoxal phosphate is tightly bound to the enzyme. It has been reported that D-serine and D-cysteine (D-amino acids) can be substrate for the ACC deaminase enzyme, while L-serine, and L- alanine can be competitive to ACC deaminase. ACC deaminase can catalyze the part of ACC that includes cyclopropane ring, and form a-ketobutyrate and ammonia. The ACC deaminase enzyme is found inside the cytoplasm of bacterial cells, and the ACC produced by the plants is taken up inside the bacterial cells and degraded by the ACC deaminase enzyme. The ACC deaminase enzyme has been found in different types of bacteria, such as Gram-negative bacteria, Gram-positive bacteria, rhizobia, and endophytic bacteria (Glick et al. 2005). Indole acetic acid (IAA) is produced by ACC⁺ bacteria, which stimulates plant

production of ACC. On the left figure, plants produce ACC which is then converted to ethylene. On the right figure, the ACC diffuses to ACC bacteria located on the seed or root surface. This prevents ACC from being made into ethylene. The mechanism of reducing the high levels of ethylene by the ACC+ bacteria is shown in (Fig. 1.2).

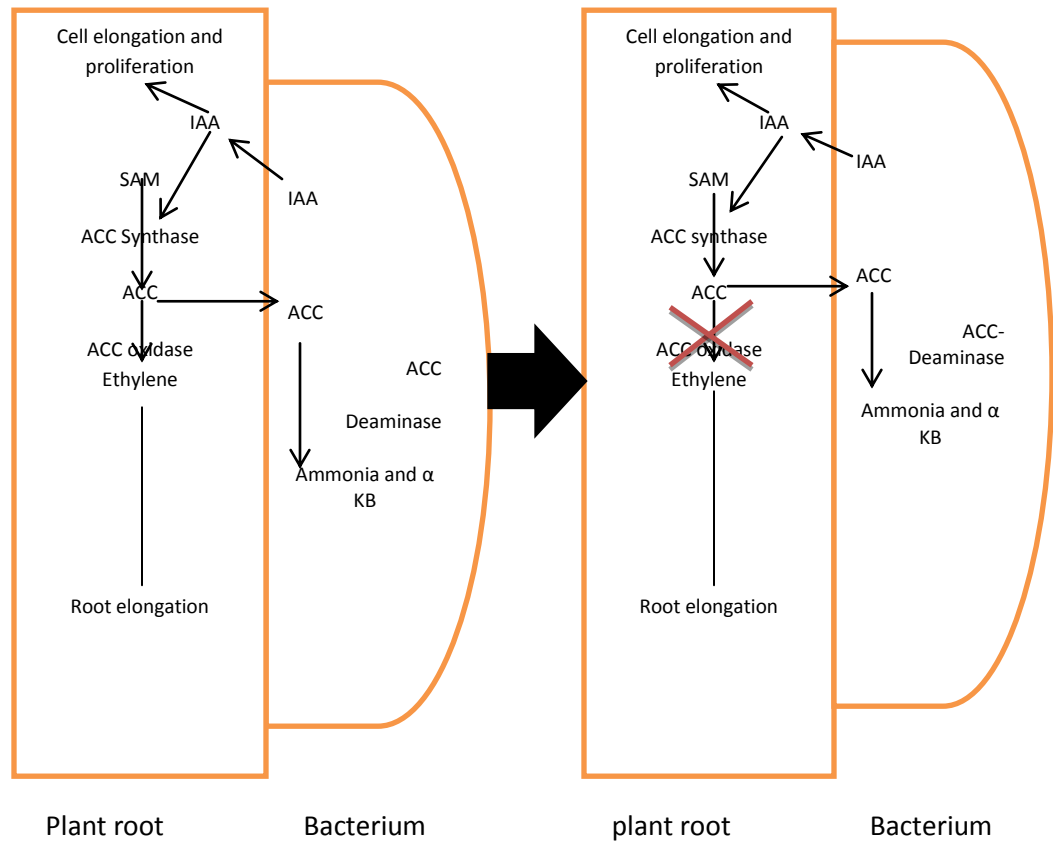


Figure 1.2 The mechanism of reducing the high levels of ethylene by the ACC+ bacteria.

One study showed that the presence of PGPRs containing ACC-deaminase improved the number of nodules, and the nodule weight of chickpea plants (Sahzad et al. 2008). Also, the root system of the plants was improved by the application of ACC+ bacteria, and these bacteria were able to adjust the ethylene and improve nutrient availability (Sahzad et al. 2008). Shaharoon et al. (2006) reported that ACC+ bacteria can decrease the effect of ethylene under both normal and stress conditions on plants because of their ACC-deaminase activity. The ACC+ bacteria increased the root elongation and the seedling length. It was also found that the production of ethylene by soybean roots was decreased by the inoculation of ACC+ *Bradyrhizobium japonicum*, which reduced the negative effect of ethylene on nodule formation (Suganuma 1995).

ACC+ bacteria can improve plants tolerance to heavy metals, fungal phytopathogens, and flooding (Nie et al. 2002). Under drought stress, plants treated with bacterial strains showed greater fresh and dry weights compared to non-inoculated plants. ACC deaminase activity improved peanut plant root growth in their early stages of growth (Mayak et al. 2004). In the late stages of growth, PGPR activity of ACC+ bacteria helped to increase plant biomass and yield through siderophore production, phosphorus solubilization, and nitrogen fixation. As a result of these PGPR activities, there was greater nutrient availability and greater production of nodules. ACC+ bacteria were also reported to increase plant height and shoot N and P content (Dey et al. 2004), as well as increase the resistance of the plants to salinity through the reduction of salinity-induced ethylene biosynthesis (Nadeem et al. 2009).

There are many strains of ACC+ bacteria that have been identified. One of these is *Pseudomonas fluorescens*, which was able to increase plant root and shoot elongation (Glick et al.1997). *Pseudomonas putida* was able to support the growth and germination of canola seeds under salinity stress by producing the enzyme ACC-deaminase (Jalili et al. 2009). Also, *Azospirillum brasilense* was found to be an ACC+ species that improve shoot and root growth (Holguin and Glick 2001). To my knowledge, no studies have been conducted on the ACC+ bacteria in western United States. In the western United States, agricultural crops are prone to drought stress, especially in the semi-arid Great Plains region.

There were two main objectives of my thesis research, which were addressed in one field and one greenhouse study. Chapter 2 describes a field study, in which my objective was to 1) quantify the effects of winter wheat variety, irrigation management, and sampling date on the abundance of culturable ACC+ bacteria in a Colorado soil. Chapter 3 describes a greenhouse experiment to address my second objective, which was to determine the plant-growth-promoting effect of selected ACC+ bacterial inocula on winter wheat varieties ranging in drought sensitivities grown with or without water stress. My hypotheses were that because of unique interactions between plant genotype and bacterial species, 1) the abundance of ACC+ bacteria would vary across different winter wheat varieties, and 2) the response of winter wheat to ACC+ bacterial inoculation during water stress conditions would be dependent on the wheat variety and specific inoculum.

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Chapter 2

Temporal and Water Management Effects on the Abundance of ACC Deaminase Positive Bacteria Associated with Winter Wheat

Summary of chapter 2

ACC deaminase-positive (ACC+) bacteria are plant growth promoting rhizobacteria (PGPRs) that degrade 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor to ethylene which is produced in high levels under abiotic stress (“stress” ethylene). The objective of this study was to determine the effects of winter wheat (*Triticum aestivum* L.) variety, irrigation regime, and sampling date on the abundance of culturable ACC+ bacteria in a Colorado soil. Wheat roots and adhering soil were collected during the 2010-2011 growing season from experimental field plots whose treatments included four winter wheat varieties (Baca, Hatcher, Ripper, and RonL), and three irrigation treatments (full, limited and dryland). Abundance of culturable ACC+ bacteria was relatively high, with numbers ranging between 1.69×10^7 - 3.28×10^9 CFU's g^{-1} . Abundance was significantly affected by the interaction between sampling date \times irrigation treatment; whereas the percent ACC+ bacteria, relative to total culturable bacteria, was significantly affected by a three-way interaction of wheat variety,

irrigation treatment, and sampling date. The abundance and relative % ACC+ bacteria increased from green-up in March to mid-grain filling in June under limited irrigation and dryland regimes, but not full irrigation. By the end of the growing season, RonL accumulated the greatest % ACC+ bacteria (up to 54%) under limited irrigation and dryland regimes, whereas there was no variety effect under full irrigation. In conclusion, different varieties of winter wheat accumulate different proportions of ACC+ bacteria, relative to the culturable microbial community, but the amount is dependent on water availability and wheat physiological growth stage. In addition, limited irrigation practice is one way that farmers can increase the abundance and relative % ACC+ bacteria while still providing water to wheat at critical growth stages.

Introduction

ACC deaminase-positive (ACC+) bacteria are plant growth promoting rhizobacteria (PGPRs) that degrade 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor to ethylene. Under biotic and abiotic stress, plants produce high levels of ethylene (“stress ethylene”) as a protective response. In mature wheat plants, increased ethylene production shortens grain filling period, decreases grain weight, hastens maturity, and triggers senescence and premature death (Bais et al. 2002). ACC+ bacteria have the ability to reduce ACC and ethylene levels between two-to-four fold, and

studies have shown that ACC+ bacteria in association with plant roots can improve plant growth under abiotic stress (e.g., drought, salinity, heavy metals) (Glick et al. 2005).

ACC deaminase is a multimeric enzyme with a monomeric subunit molecular mass of approximately 35–42 kDa (Glick et al. 2005). It is a sulfhydryl enzyme that utilizes pyridoxal 5-phosphate as an essential co-factor. Pyridoxal phosphate is tightly bound to the enzyme. It has been reported that D-serine and D-cysteine (D-amino acids) can be substrates for the ACC deaminase enzyme, while L-serine, and L-alanine can be competitive to ACC deaminase (Glick 2005). ACC deaminase can catalyze the part of ACC that includes the cyclopropane ring, and form α -ketobutyrate and ammonia. The ACC deaminase enzyme is found inside the cytoplasm of bacterial cells, and the ACC produced by the plants is taken up inside the bacterial cells and degraded by the ACC deaminase enzyme. The ACC deaminase enzyme has been found in different types of bacteria, such as rhizobia, and endophytic bacteria (Glick 2005).

Quantification of ACC deaminase+ has rarely been done in field soils, and studies instead have focused on the selective enrichment and isolation of ACC+ bacteria as inocula resources (e.g., Shaharoon et al. 2006, Gholami et al. 2009). The beneficial effect of plant growth-promoting bacteria expressing ACC deaminase on the host plant depends on the soil conditions, especially the free-living bacteria that live near the roots. Not all ACC+ bacteria have the same effect on plants; for example, Glick et al. (2005) reported that not all ACC+ *Pseudomonas putida* strains actively promote plant growth.

While inoculations have been conducted in the field, not much is known about the abundance of endogenous populations under natural conditions, and little is known of how their abundance is affected by season, environment (including water availability) or plant genotype. This information would be important, because abundance may be an important predictor of activity by native species in the field. Therefore, a field study was conducted to quantify the abundance of ACC+ bacteria associated with different winter wheat roots and under and across wheat physiological growth stages (emergence, green-up, anthesis, and mid-grain filling), and determine the effects of irrigation regimes (full irrigation, limited irrigation and dryland) on the abundance of the ACC+ bacteria.

Materials and Methods

Field study and soil sampling

Roots and adhering soil were collected from experimental research plots at the Limited Irrigation Research Farm (LIRF), located near Greeley, Colorado. The samples were collected from four winter wheat varieties (*Triticum aestivum* L. “Baca”, “Hatcher”, “Ripper”, and “RonL”) at the following physiological growth stages: emergence in November 2010, green-up in March 2011, anthesis in late May 2011, and mid-grain filling in late June 2011. Each variety was grown under three irrigation treatments (full irrigation, limited irrigation where irrigation commenced at anthesis, and dryland) in a split-plot block design, with triplicate blocks where irrigation treatment was the main

plot factor and wheat variety was the sub-plot factor. At least three samples of roots with the adhering soil (rhizosphere soil) were excavated with a shovel (0-20 cm depth) from each plot. Roots samples were transported to the laboratory on ice and stored at 4°C for bacterial enumeration.

Bacterial enumerations

Subsamples (10 g) of rhizosphere soil and attached roots were suspended in 90 ml of sterile phosphate buffered saline (PBS) to achieve 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 PBS. Total culturable bacteria were enumerated by plating diluted suspensions (10^{-4} to 10^{-6}) onto triplicate 10% TSBA plates (3g TSB, 18 g agar L⁻¹). Culturable ACC+ bacteria were enumerated by plating dilutions (10^{-3} to 10^{-5}) onto DF minimal salts medium containing ACC as the sole N source (Penrose and Glick 2003). Plates were incubated at 28^o for 4-5 days, and colonies were counted on plates. The abundance of culturable 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 get the proportion of ACC+ bacteria relative to total culturable bacteria.

Statistical analysis

Enumeration data were log transformed and analyzed by repeated measures analysis of variance using the Proc Mixed procedure of SAS (v. 9.2, The SAS Institute, Cary , NC). Time was the repeated factor; irrigation treatment was the main plot and wheat variety the sub-plot. I used the first order autoregressive covariance structure, which assumes that repeated measures are more correlated with sampling dates that are closer together than farther apart in time. Significantly different means ($P < 0.05$) were separated by the PDIFF option in SAS.

Results

Overall results summary

The number of the total culturable bacteria (expressed in Log^{10} values) was significantly affected by sampling date ($P = <0.0001$), but there was no effect of irrigation treatment or winter wheat variety. The log-transformed number of ACC+ bacteria was affected by the sampling date ($P = 0.0001$), irrigation treatment (<0.0001), and the interaction between sampling date \times irrigation treatment ($P = <0.0001$). The percentage of ACC+ bacteria, relative to total culturable bacteria, was significantly affected by sampling date, irrigation treatment, the interaction between sampling date \times irrigation treatment, the interaction between sampling date \times wheat variety, and the interaction between wheat variety \times irrigation treatment \times sampling date ($P = 0.0095$) (Table 2.1).

Table 2.1 Results of repeated measures analysis of variance tests on total culturable bacteria (Log Het), ACC+ bacteria (Log ACC) and percent ACC+ bacteria enumerated over the course of one growing season from rhizospheres of different winter wheat varieties grown under full, limited or dryland irrigation.

Model	Log Het		Log ACC		% ACC	
	F-value	P-value	F-value	P-value	F-value	P-value
Variety	0.08	0.9680	0.67	0.5793	1.31	0.2890
Irrigation	1.80	0.1823	22.76	<0.0001	13.01	<0.0001
Date	153.33	<0.0001	9.82	0.0001	22.56	<0.0001
Variety*Irrigation	0.54	0.7750	0.39	0.8824	0.56	0.7614
Variety*Date	1.40	0.2180	0.73	0.6758	2.50	0.0155
Irrigation*Date	1.02	0.4273	22.40	<0.0001	8.47	<0.0001
Variety*Irrigation*Date	0.97	0.5044	1.04	0.4425	2.22	0.0095

Abundance of total culturable bacteria

The number of total culturable bacteria changed significantly over the course of the growing season (Fig. 2.1). The number of culturable bacteria was lowest in November 2010 and peaked in March 2011, during the wheat green-up phase. The

number of culturable bacteria decreased from March to May and June 2011, when wheat reached its anthesis and physiological maturity stages respectively.

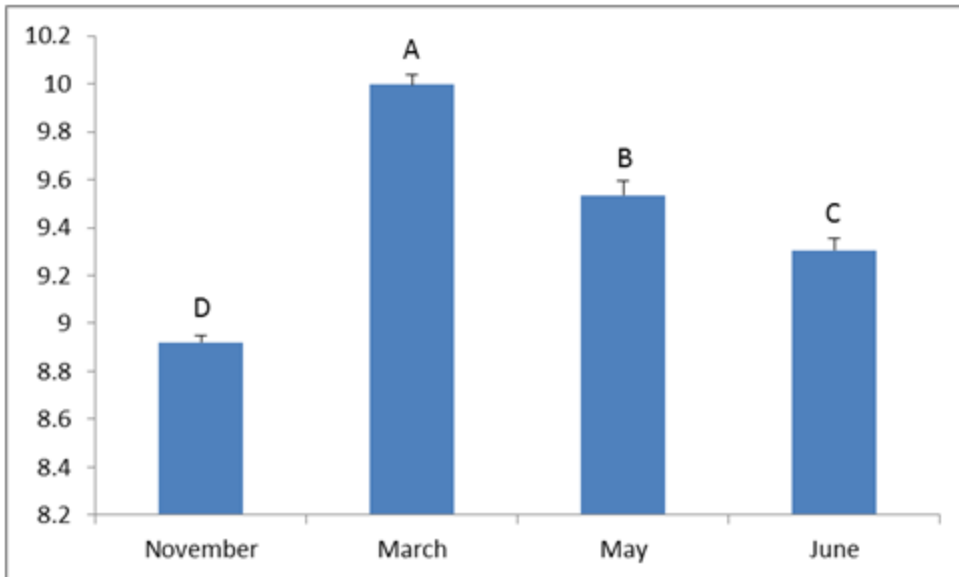


Figure 2.1 Log-transformed numbers of total culturable bacteria as affected by sampling date (LSD= 0.45). Error bars represent + 1 SE.

Abundance of ACC+ bacteria

The abundance of ACC+ bacteria was affected by the interaction between sampling date × irrigation treatment. The number of ACC+ bacteria increased significantly from November to March within all the irrigation treatments (Table 2). Abundance then decreased significantly over the last two sampling dates for the full irrigation samples, whereas abundances remained the same from March to June under limited irrigation or dryland management. Within the November or March sampling dates, numbers of ACC+ bacteria did not vary among the irrigation treatments, but in

May and June, abundances were significantly greater in the limited irrigation and dryland treatments than under full irrigation.

Table 2.2 Log-transformed numbers of ACC+ bacteria (± 1 SE) as affected by sampling date \times irrigation treatment interaction (LSD= 0.37).

Sampling Date	Irrigation Treatment		
	Full	Limited	Dryland
November 2010	8.16 \pm 0.10 b A	8.08 \pm 0.09 c A	8.03 \pm 0.10 b A
March 2011	8.53 \pm 0.11 a A	8.58 \pm 0.11 ab A	8.47 \pm 0.11 a A
May 2011	7.76 \pm 0.10 c B	8.23 \pm 0.10 bc A	8.46 \pm 0.10 a A
June 2011	7.58 \pm 0.63 c B	8.63 \pm 0.06 a A	8.77 \pm 0.06 a A

Within a column, means labeled with different lower case letters are significantly different ($P < 0.05$) due to irrigation treatment. Within a row, means labeled with different upper case letters are significantly different ($P < 0.05$) by sampling date.

Percent ACC+ bacteria

Overall, percent values of ACC+ bacteria, relative to total culturable bacteria, were lowest in March and May, intermediate in November, and highest at physiological maturity in June (Fig. 2). Values were significantly affected by a three-way wheat variety \times irrigation treatment \times sampling date interaction. There were very few differences among wheat variety \times irrigation treatment combinations in November 2010 and March

2011, so for simplicity, only the three-way interaction data for May and June are presented.

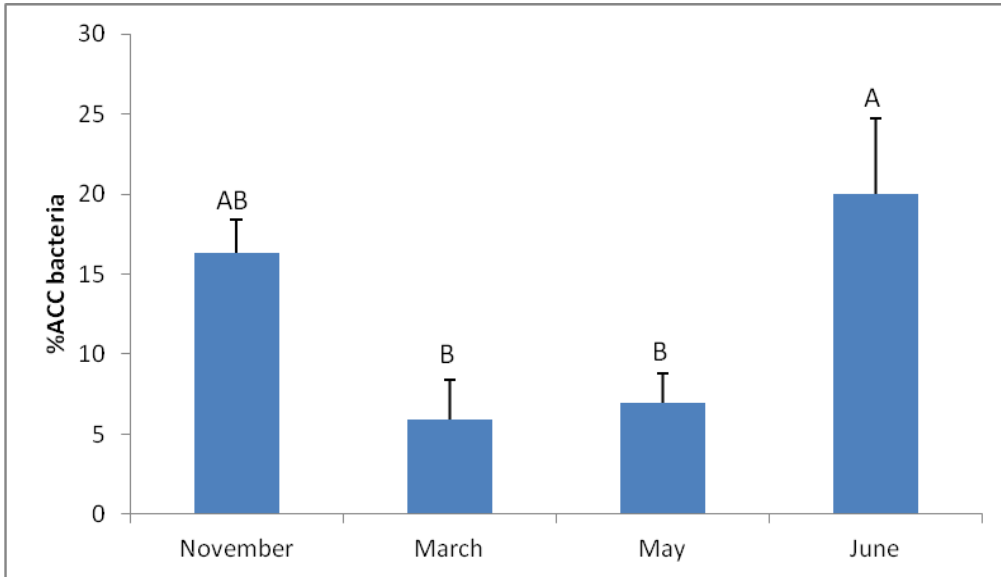


Figure 2.2 The effect of sampling date on the percentage of culturable ACC+ bacteria, relative to the total culturable bacteria (LSD= 10.7). Error bars represent + 1 SE.

In May 2011, the % ACC+ bacteria were not significantly different among the varieties in the fully irrigated samples. 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.

Table 2.3 The effect of winter wheat variety and irrigation treatment on the mean (\pm 1 SE) percentage of culturable ACC+ bacteria to the total culturable bacteria in May 2011 (LSD= 5.91).

Wheat Variety	Irrigation Treatment		
	Full	Limited	Dryland
Baca	1.22 \pm 0.56 a A	4.05 \pm 2.16 b A	6.87 \pm 2.01 b A
Hatcher	1.29 \pm 0.36 a B	13.5 \pm 6.82 a A	6.85 \pm 1.70 b B
Ripper	1.78 \pm 0.41 a B	7.66 \pm 4.13 ab AB	12.6 \pm 2.34 ab A
RonL	1.70 \pm 0.39 a C	7.98 \pm 1.96 ab B	17.6 \pm 9.33 a A

Within a column, means labeled with different lower case letters are significantly different ($P < 0.05$). Within a row, means labeled with different upper case letters are significantly different ($P < 0.05$).

By mid-grain filling stage in June, relatively high percentages of ACC+ bacteria were detected in limited irrigation and dryland soils. 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.

Table 2.4 The effect of winter wheat variety and irrigation treatment on the mean (\pm 1 SE) percentage of culturable ACC+ bacteria to the total culturable bacteria in June 2011 (LSD= 5.91).

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

Within a column, means labeled with different lower case letters are significantly different ($P < 0.05$). Within a row, means labeled with different upper case letters are significantly different ($P < 0.05$).

Discussion

To my knowledge, this is the first field study to describe the abundance of indigenous, culturable populations of ACC+ bacteria in winter wheat rhizospheres. In the present study, the abundance of the total culturable bacteria was between 2.68×10^8 - 3.89×10^{10} CFUs g^{-1} . Relative to total bacteria, the abundance of ACC+ bacteria was high, with numbers ranging between 1.69×10^7 - 3.28×10^9 CFU's g^{-1} . The % ACC+ bacteria was sensitive to sampling date, management practice (i.e., irrigation treatment), and winter wheat variety.

Temporal trends

The number of total culturable bacteria was the highest in March, and the lowest number was in November. Others have described similar seasonal patterns, where microbial biomass peaks at the beginning of the spring, following the harvest of crops and the incorporation of residue (Buchanan 1992). Lipson (2002) stated that the microbial biomass is greatest after the snowmelt period when the weather is warmer, and the plants begin to grow. Another possible cause for this seasonal trend is that some bacterial species isolated in a cold weather (November) are not able to grow well in the laboratory at 28°C (Smit 2001). In contrast, species isolated in warmer seasons were able to grow better at these incubating conditions. The ACC+ bacteria followed the same patterns of the total culturable bacteria only under the full irrigation treatment.

Otherwise, the highest number and greatest relative % ACC+ bacteria occurred as wheat reached mid-grain filling in June, especially for the dryland samples. Thus, abundances of ACC+ bacteria generally follow the abundance of total bacteria when water stress is absent, and peak during the spring. Otherwise, abundances of ACC+ bacteria under winter wheat peak during mid-grain filling, when ACC production is likely high.

Wheat variety and irrigation treatment interactions

The number of ACC+ bacteria changed over time following the irrigation treatment, with fully irrigated samples having higher numbers of ACC+ bacteria at the first sampling date than the other three dates. On the other hand, the limited irrigation and dryland samples showed an increase in the number and relative percent of ACC+ bacteria at the last two sampling dates. At the anthesis stage, irrigation had just commenced in the limited irrigation plots, so these plots had experienced a period a water deficiency. The dryland plots, in contrast, were water limited throughout much of the growing season. The increase in the relative percent of ACC+ bacteria in the limited irrigated and dryland plots at anthesis indicates that wheat plants were experiencing water stress, which continued on until mid-grain filling. 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, the increased production of ACC as part of ethylene biosynthesis, combined with reduced

soil water availability in the limited irrigated and dryland plots, explains the increase in abundance and % ACC+ bacteria over time.

In plots experiencing water stress (limited irrigated and dryland soils in May and June, the four wheat varieties showed differences in the relative percent ACC+ bacteria that accumulate in their rhizospheres. The percentage under RonL was greater compared to the other varieties (Baca, Ripper, and Hatcher). RonL is a hard white winter wheat variety that is medium in height and has a medium to late maturity. It is considered to be sensitive to drought, and in 2011, yields at the study site were 125 bu ac⁻¹ under full irrigation, 66 bu ac⁻¹ under limited irrigation, and only 45 bu ac⁻¹ under dryland. The ability of RonL to accumulate relatively high percentage of ACC+ bacteria could indicate that this variety produces greater concentrations of ACC and stress ethylene compared to the other varieties tested when water stressed. Ripper, in contrast, is a hard red winter wheat that matures early (140.5 day to heading) and has a short stem height (24 in). Ripper is also more tolerant to drought than RonL, with a yield of 127 bu ac⁻¹ under full irrigation, 84 bu ac⁻¹ under limited irrigation, and 54 bu ac⁻¹ under dryland at the study site in 2011. Although its physiological adaptations to drought have yet to be described, lower relative percent of ACC+ under Ripper than RonL indicates that Ripper produces lower concentrations of ACC and stress ethylene than RonL when water stressed.

Conclusion

This study demonstrated that Colorado soils have the potential to harbor relatively high numbers of ACC+ bacteria, and that abundance and relative percent of ACC+ bacteria are elevated in soil with reduced water availability. This study also showed that different varieties of winter wheat accumulate different relative amounts of ACC+ bacteria, depending on soil water availability and sampling date. Specifically, the percentage of ACC+ bacteria to total culturable bacteria was significantly increased under limited irrigation and dryland by anthesis and into mid-grain filling in the late growth stages of winter wheat than the first stages, but not under full irrigation. Limited irrigation is one way that farmers can increase the abundance and relative % ACC+ bacteria while still providing water to wheat at critical growth stages. At anthesis and mid-grain filling growth stages, drought-sensitive RonL accumulated greater % ACC+ bacteria than the other winter wheat varieties under limited irrigation and dryland management. This indicates that RonL produces greater amounts of ACC and stress ethylene under drought stress than the other varieties tested.

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Chapter 3

Interactions of ACC Deaminase Positive Bacteria and Winter Wheat in the Presence or Absence of Water Stress

Summary of chapter 3

ACC deaminase positive (ACC+) bacteria are plant growth promoting rhizobacteria (PGPRs) that degrade 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor to ethylene which plants produce in elevated quantities under environmental stress (“stress ethylene”). Studies have shown that ACC+ bacteria can reduce stress ethylene production in plants and promote growth under abiotic stress. The objective of this greenhouse study was to determine the effect of selected ACC+ bacteria on the growth and yield of different winter wheat in the presence or absence of water stress. The greenhouse experiment was conducted with two varieties of winter wheat (*Triticum aestivum* L. “Ripper” and “RonL”), two levels of soil water content (no water stress = soil moisture at 80-100% water holding capacity and water stressed = soil moisture at 40-60% water holding capacity) and four ACC+ bacterial inocula treatments: individual inoculum of one of two strains of *Pseudomonas brassicacearum* (HF1, and HD6), a 1:1

(vol) combination of both strains, and a sterile inoculum control. The strains selected represented the dominant species of ACC+ bacteria cultured from a Colorado soil. Inoculation with ACC+ bacteria increased the stem length for both winter wheat varieties. Inoculation with either HF1 or HD6 increased the number in RonL fertile heads regardless of soil water content, and in the water-stressed treatments, increased the biomass and seed number of RonL but not Ripper. In contrast, Ripper productivity was negatively impacted by strain HD6 under non-water stressed conditions. We hypothesize that certain strains of ACC+ bacteria can negatively interfere with ethylene production under non-stress conditions. In conclusion, positive or negative effects of ACC+ bacteria on plant productivity was dependent upon specific ACC+ bacterial strain, plant genotype, and presence/absence of abiotic stress.

Introduction

ACC deaminase positive (ACC+) bacteria are plant growth promoting rhizobacteria (PGPRs) that degrade 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor to ethylene which plants produce in elevated quantities under environmental stress ("stress ethylene"). In plants such as wheat, increased ethylene production shortens grain filling period, decreases grain weight, hastens maturity, and triggers senescence and premature death (Bais et al. 2002). The negative impacts of ethylene on plant growth can be decreased through the activity of ACC+ bacteria, and studies have

shown that these bacteria can increase plant root and shoot length and biomass under abiotic stress conditions, including drought, salinity and heavy metal toxicity (Gosh et al. 2003, Glick 2005, Reed 2005, Shaharoon et al. 2006).

Agronomic crops are exposed to many kinds of stresses by both biotic and abiotic factors. Most of these factors lead to ethylene production that inhibits plant growth through several mechanisms. Drought is one of the major environmental stresses that limit plant growth and productivity, and more than one-half of the arable land is susceptible to drought every year. Mayak et al. (2004) have reported that ACC+ bacteria significantly increased fresh and dry weights of tomato and pepper exposed to drought stress. In short term experiments, there were positive effects of ACC+ bacteria on root and shoot biomass, leaf area, and plant transpiration. In long term experiments, plants inoculated with ACC+ bacteria had greater seed yield, seed number, and seed nitrogen accumulation than in uninoculated plants. Temperature stress also leads to stress ethylene production (Saleem and Arshad 2007), and Bensalim et al. (1998) reported that ACC+ bacteria helped potato plants in maintaining normal growth under heat stress.

The inoculation of ACC+ bacteria can regulate levels of plant ethylene. Several studies have isolated ACC+ bacteria from plant roots, studied their ACC deaminase enzyme activity, and have developed inocula for greenhouse and field studies (e.g. Shaharoon et al. 2006, Shahzad et al. 2008, Gholami et al. 2009). These studies have mainly been conducted in Pakistan, India and Canada, but to my knowledge, no studies

have been conducted in the western United States where there is tremendous interest in improving winter wheat productivity under drought stress. Also, the above studies compared effects of several ACC+ bacterial isolates on a single plant genotype, and it is unknown if a specific ACC+ bacterial strain has similar or different effects on different plant genotypes of the same plant species, or if bacterial effects are independent of plant genotype.

Beneficial interactions between crops and PGPRs are agronomically important, and understanding the relationship between plant genotype and specific strains of ACC+ bacteria could lead to more efficient and sustainable management of agronomic cropping systems. Studies have shown that soil microbial communities vary under the influence of different plant species, or even varieties of the same species (Wieland et al. 2001, Buyer et al. 2002, Kowalchuck et al. 2002, Micallef et al. 2009). Therefore, I conducted a greenhouse study to determine the effect of ACC+ bacteria isolated from a Colorado soil on the growth of different winter wheat varieties when grown with or without water stress. Two varieties of winter wheat, with contrasting susceptibilities to drought, were inoculated with single or combined strains of two *Pseudomonas brassicacearum* (HF1 and HD6) that had contrasting ACC deaminase activities. I hypothesized that the response of winter wheat to ACC+ bacterial inoculation during water stress conditions would be dependent on the wheat variety and specific inoculum because of unique plant-microbial interactions.

Materials and Methods

Isolation and characterization of ACC deaminase positive bacteria

Roots and root-associated soil (0-20 cm depth) were collected with a shovel under winter wheat (*Triticum aestivum* L. "Hatcher") in June 2010, from three fully-irrigated and three dryland plots at the Limited Irrigation Research Farm (LIRF) in Greeley, Colorado. The soil of the plots at LIRF was sandy clay loam in texture (72% sand, 6% silt, and 22% clay) with a saturated paste pH of 7.5 and organic matter content of 1.1%. The samples were transported back to the laboratory on ice, where loose soil was shaken off the roots, and roots plus tightly adhering soil (rhizosphere soil) were stored at 4 °C for isolation of ACC+ bacteria. Subsamples of roots plus associated soil (10 g) were added to 90 ml of sterile phosphate buffered saline (PBS) and blended on high speed in a Waring blender for 1 min. The soil suspension was serially diluted ten-fold in sterile PBS. Culturable ACC+ bacteria were enumerated on DF minimal salts agar medium containing ACC as the sole N source (Penrose and Glick 2003). Plates were incubated for 72 h at 28 °C, after which the colony forming units (CFUs) were counted. Individual isolates were restreaked onto fresh DF plates to obtain pure cultures. After

incubating for 72 h, each pure culture was transferred to sterile 15 ml culture tube containing 2 ml of sterile tryptic soy broth (TSB) medium. Cultures were grown overnight on a shaker at 28 °C. The next day, 2 mL of culture were transferred to a sterile 2 mL microcentrifuge tube and centrifuged at maximum speed for 1 minute. The supernatant was decanted, and the pelleted cells were resuspended in 2 mL of fresh sterile TSB. A 0.5 ml subsample of resuspended cells were mixed with 0.5 ml of sterile TSB:glycerol (1:1) cryopreserved at -80 °C.

To prepare samples for molecular analysis, cryopreserved cells were revived on TSB agar plates. A single isolate was transferred to sterile TSB and grown overnight on a shaker as described above. DNA was extracted from pelleted cells using the MoBio PowerLyzer UltraClean Microbial DNA Isolation kits, and the nearly-full length 16S rDNA gene was amplified with universal primers 27f and 1541r (Weisburg et al. 1991). Amplicons were sequenced on an ABI 3130xL Genetic Analyzer (Applied Biosystems/Life Technologies Corporation, Carlsbad, California). Each sequence was subjected to a BLAST search in GenBank's nucleotide database to search for its closest matching sequence.

Representative isolates were then selected for functional characterization, whereby their ACC deaminase activities were determined by measuring the amount of α -ketobutyrate produced when the enzyme ACC deaminase reacted with ACC, following the methods in Penrose and Glick (2003). In brief, pelleted cells were incubated in the presence of ACC and toluene, and the concentration of α -ketobutyrate produced was

determined from a standard curve. Activity was expressed as nmole α -ketobutyrate mg⁻¹ cell pellet biomass h⁻¹.

Greenhouse experiment

The greenhouse experiment was conducted with two varieties of winter wheat (Ripper, and RonL), two levels of soil water content (no water stress and water stressed) and four inoculum treatments: individual inoculum of one of two strains of *Pseudomonas brassicacearum*, a 1:1 (vol) combination of both strains, and a sterile inoculum control. The strains selected (designated HF1 and HD6) represented the dominant species of ACC⁺ bacteria cultured from the soil with contrasting ACC deaminase enzyme activity (described below in the results section). The inocula were prepared by growing each strain to late log phase in 50 mL of sterile TSB, then mixing 50 ml of culture or sterile TSB (for control) with 50 g of ground, autoclaved peat. For the combined inoculum treatment, the peat was inoculated with 25 ml of each strain. The peat was incubated for 48 h at 28 °C, after which peat was stored at 4 °C for no more than two weeks. Seeds were treated with fungicide and vernalized at 2 °C for 8 weeks. Germinated seedling roots were coated with peat at a 1:1 (w:w) ratio using a 10% sterile glucose solution to assist with inoculum attachment. Non-inoculated control seedlings were coated with the peat treated with sterile TSB and 10% sugar solution. Replicate 15-cm pots (n=4) were filled with potting soil (Fafard 4P, Conrad Fafard Inc., Agawam, Massachusetts) and weighed. Each pot was moistened to 100% water holding capacity

and planted with one seedling per pot. The pots were arranged in complete block design across one greenhouse bench, and wheat was grown under ambient temperature and light. The pots were fertilized once with Osmocote[®] (Scotts-Sierra Horticultural Products Co., Marysville, Ohio) one week after planting (one teaspoon of fertilizer per pot). All pots were watered to maintain the soil water content at 80-100% water holding capacity. At jointing (stem elongation), pots of the water-stressed treatment were watered to maintain water soil content of 40-60% water holding capacity until physiological maturity. At maturity (~ 12 weeks after planting), the following productivity and yield components were measured: height of main stem, aboveground biomass (the aboveground biomass were cut and dried in the oven for 2 days and 80 °C), total number of heads, number of fertile heads, grain yield, grain number, and 1000-grain weight.

Statistical analysis

The experimental design was a randomized complete block design with three treatment factors (inoculum, wheat variety, and irrigation regime) and four replicates. Data were analyzed by a three-way analysis of variance (ANOVA) test in SAS (v. 9.2, SAS Institute Inc., Cary, North Carolina). When treatment effects were significant ($P < 0.05$), means were separated by the least significant difference (LSD) method.

Results

Selection of ACC+ bacteria for greenhouse inocula

The number of CFUs from the DF agar plates averaged $1.46 \times 10^7 \text{ g}^{-1}$ soil ($n = 6$). Twenty-four isolates were selected for molecular characterization; these isolates represented the diversity of colony morphologies among the pure cultures, with replicate isolates per morphology type. Of these, 53% were identified as *Pseudomonas brassicacearum* (99% match) and 27% were identified as *Rahnella aquatilis* (99% match). The remainder of the collection consisted of *Pseudomonas* species other than *P. brassicacearum*, and one species of *Bacillus*. Five distinct species and two different isolates of *P. brassicacearum* were then assayed in vitro for potential ACC deaminase enzyme activity. *Rahnella aquatilis*, *Bacillus* sp. and *P. umsongensis* displayed very low levels of enzyme activity, relative to *P. brassicacearum* and *P. tremae* (Table 1). Furthermore, two isolates of *P. brassicacearum* were highly contrasting, with activity of HD6 being twice that of HF1 (Table 1). Because of its numerical dominance in the soil studied, and because its ACC deaminase activity varied so widely, *P. brassicacearum* HD6 and HF11 were selected for inocula for the greenhouse experiment.

Table 3.1 Species identification and potential ACC deaminase activity (n=2) of representative isolates of ACC deaminase positive bacteria isolated from a Colorado soil.

Isolate #	Closest matching species	% match	ACC deaminase activity (nmol mg ⁻¹ h ⁻¹ ± SD)
HD1	<i>Rahnella aquatilis</i>	99%	149 ± 38
HD6	<i>Pseudomonas brassicacearum</i>	99%	6540 ± 250
HD7	<i>Bacillus</i> sp.	99%	129 ± 30
HF1	<i>P. brassicacearum</i>	99%	3280 ± 51
HF11	<i>P. umsongensis</i>	98%	72 ± 3
HF12	<i>P. tremae</i>	99%	5500 ± 330

Greenhouse study – summary of treatment effects

Stem length was the only variable that each treatment factor (inoculum, wheat variety, and soil water content) affected independently, with no interaction effects. The

number of fertile heads was significantly affected by the interaction between inoculum × wheat variety. Aboveground biomass, total number of heads and seed number were significantly affected by the three-way interaction of inoculum × wheat variety × irrigation regime. The remaining variables (grain yield and 1000-grain weight) were not influenced by inoculum treatment. The main effects of wheat variety and irrigation regime were significant for grain yield, whereas their interaction was significant for 1000-grain weight.

Inoculum effects

Stem length of winter wheat was differentially affected by the bacterial inocula. Ripper was significantly longer when inoculated with individual *P. brassicacearum* strains HF1 (66.38 cm) or HD6 (64.5 cm), then when inoculated with both strains (63.21cm) or with the sterile control (64.13 cm). Averaged stem length for winter wheat RonL was longer when inoculated with HF1 (63.5 cm) or HD6 (63.13 cm), than when inoculated with both strains (58.13 cm) or with the sterile control (53.4 cm).

The number of fertile heads varied between Ripper and RonL, depending on the inoculum treatment. Ripper yielded more fertile heads than RonL within the sterile control or the combined bacterial inoculum treatment (Table 2). When inoculated with either HF1 or HD6, both Ripper and RonL yielded similar numbers of fertile heads per plant. For either Ripper or RonL, the number of fertile heads was not significantly affected by inoculum treatment.

Table 3.2 Number of fertile heads (plant⁻¹) of Ripper and RonL winter wheat as affected by the ACC+ bacteria inoculation (n = 8, LSD = 2.89).

Variety	Inoculum Treatment			
	None	HD6+HF1	HF1	HD6
Ripper	9.13a	7.96a	7.96a	6.38a
RonL	5.38b	4.75b	6.88a	6.25a

Within a column, means followed by different letters are significantly different (P < 0.05).

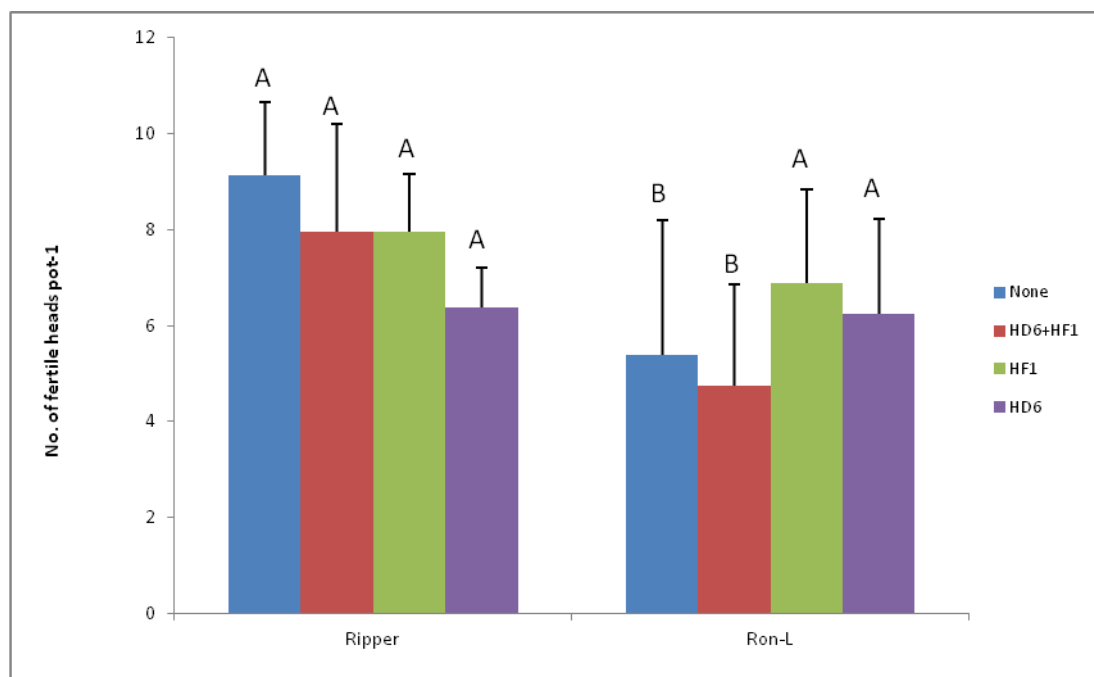


Figure 3.1 Number of fertile heads plant⁻¹) of Ripper and RonL winter wheat as affected by the ACC+ bacterial inocula (n = 8). Error bars are + 1 standard error Within each inoculation treatment, bars labeled with different letters are significantly different (P < 0.05, LSD = 2. 89).

The interaction among inoculum, wheat variety and soil water significantly affected aboveground biomass, total number of heads, and number of seeds per plant. Aboveground biomass production was generally greater under the wetter soil conditions than under water stress. For Ripper, biomass was significantly greater under wetter than drier conditions except when inoculated with HD6, where there was no difference in Ripper biomass between the two irrigation regimes (Table 3). This was because inoculation with HD6 significantly reduced Ripper biomass compared to the

other inocula under the wetter (but not drier) irrigation regime. For RonL, biomass was significantly different between the two irrigation regimes when inoculated with either HF1 or HD6, due to the relatively high biomass productivity achieved with these inocula under conditions of no water stress. Inoculation with either HF1 or HD6 alone increased RonL biomass by ~55% under wet conditions and by 50-65% relative to control under drier conditions, although the biomass increases were not statistically significant. Comparing the two varieties, RonL biomass was generally lower than the biomass of Ripper within an irrigation regime and bacterial inoculum treatment. Again, the exception occurred with the HD6 inoculum. Under the wetter conditions, inoculation with *P. brassicacearum* strain HD6 did not reduce RonL biomass as it did with Ripper, and as a result, RonL biomass was greater (but not significantly) than that of Ripper within this irrigation regime × inoculum treatment. This trend did not occur under conditions of water stress, however, where Ripper aboveground biomass was relatively high when inoculated with HD6, and significantly greater than the biomass of HD6-inoculated RonL.

Table 3.3 Aboveground biomass (g) of Ripper and RonLwinter wheat as affected by ACC + bacteria inocula and soil water content (n = 4, LSD = 5.08).

Variety × Irrigation	Inoculum treatment			
	None	HD6+HF1	HF1	HD6
Ripper				
Wet	18.9a	15.9a	20.4a	7.33a
Dry	11.3b	7.13b	7.97b	11.0a
RonL				
Wet	7.66a	7.70a	12.0a	11.4a
Dry	3.82a	3.28a	6.33b	5.71b

Wet = soil irrigated to 80-100% water holding capacity, Dry = soil irrigated to 40-60% water holding capacity.

Within each wheat variety, column means followed by different letters are significantly different (P < 0.05).

Trends for the number of total heads per plant were similar to those for aboveground biomass. The number of heads was generally greater with Ripper than RonL within an inoculum and moisture regime treatment, except with the HD6 inoculum (Table 4). Under the wet conditions, the number of heads for the Ripper was reduced

when inoculated with HD6 compared to the plants treated with HF1 and the control. There were no other significant effects of inoculum treatment on the number of wheat heads within a variety × irrigation treatment combination.

Table 3.4 Number of heads (plant⁻¹) of Ripper and RonLwheat as affected by ACC + bacteria inocula and soil water content (n = 4, LSD = 2.76).

Variety × Irrigation	Inoculum treatment			
	None	HD6+HF1	HF1	HD6
Ripper				
Wet	12.5a	11.3a	11.7a	6.25b
Dry	7.75b	5.67b	5.50b	8.00a
RonL				
Wet	8.50a	6.50a	9.00a	8.25a
Dry	5.00b	3.50b	5.25b	5.00b

Wet = soil irrigated to 80-100% water holding capacity, Dry = soil irrigated to 40-60% water holding capacity. Within each wheat variety, column means followed by different letters are significantly different (P < 0.05).

The number of seeds was greater with Ripper than with RonL within an inoculum and moisture regime, except with the HD6 inoculum under the wet conditions (Table 5).

Under the wet conditions, the number of seeds for the Ripper was significantly reduced when inoculated with HD6 compared to the plants treated with HF1 and the control. Within RonL, seed number was reduced under water-stressed versus non-stressed conditions in the sterile control but not when inoculated with HD6 (either alone or in combination with HF1). Overall, RonL seed number under water-stressed conditions was increased 95-130% by either HD6 or HF1 relative to the control, although these increases were not statistically significant.

Table 3.5 Number of seeds (plant⁻¹) of Ripper and RonL winter wheat as affected by ACC+ bacteria inocula and soil water content (n = 4, LSD = 98).

Variety × Irrigation	Inoculum treatment			
	None	HD6+HF1	HF1	HD6
Ripper				
Wet	393a	345a	408a	190a
Dry	224a	146b	183b	233a
RonL				
Wet	175a	162a	249a	212a
Dry	63b	93a	145b	123a

Wet = soil irrigated to 80-100% water holding capacity, Dry = soil irrigated to 40-60% water holding capacity.

Within each wheat variety, column means followed by different letters are significantly different (P < 0.05).

Wheat variety and irrigation regime effects

Stem height and grain yield were independently affected by wheat variety and irrigation regime. Ripper stems (64.6 cm) were longer than RonL stems (59.5 cm), and all stems were shorter (57.0 cm) under water stress compared to without water stress

(67.1 cm). Similarly, grain yield per pot was greater for Ripper (7.80 g) than RonL (4.94 g) under wetter conditions (7.94 g) than dry (4.83 g).

The weight of 1000 seeds was affected by the interaction between wheat variety and irrigation treatment. The weight of 1000 Ripper seeds was increased by 13% under the water stressed conditions compared to no water stress (Table 6). In contrast, weight of 1000 RonL seeds was unaffected by irrigation regime.

Table 3.6 Weight of 1000 seeds of Ripper and RonL winter wheat as affected by irrigation regime (n = 16, LSD = 2.5).

Variety	Irrigation regime	
	Dry	Wet
Ripper	31.1a	27.6b
RonL	30.7a	32.3a

Dry = soil irrigated to 40-60% water holding capacity, Wet = soil irrigated to 80-100% water holding capacity.

Within a column or row, means labeled with different letters are significantly different (P < 0.05).

Discussion

ACC+ bacteria

Pseudomonas brassicacearum is member of the fluorescent pseudomonad group and was first isolated from the rhizosphere of a brassica in France (Achouak et al. 2000). *Pseudomonas brassicacearum* was found to be a powerful species for colonizing and improving the root system of *Arabidopsis thaliana* and *Brassica napus* plants (Achouak et al. 2004). Others have isolated *P. brassicacearum* from Australian soils planted to winter wheat, and showed that this bacterium has biological control activity against the soilborne fungal pathogen responsible for take-all disease in wheat and barley (Ross et al. 2000).

Rahnella aquatilis is a genus of gram-negative, anaerobic, small, rod-shaped bacteria occurring in fresh water and soil. To my knowledge, this is the first report of *R. aquatilis* as a ACC+ bacterium. Also, *P. umsongensis* is a genus that was found in Korea, and it is gram negative, small rod, and is adapted to cold temperatures (4 °C) (Kwon et al. 2003). There is no previous evidence that *P. umsongensis* is an ACC+ bacterium. In addition, *P. tremae* was found in this study to have high ACC-deaminase activity, but up to this point it was not known to be an ACC+ bacterium.

The ACC- deaminase activities measured for most of the isolates in this study were in the range of ACC-deaminase levels reported in the literature. Holguin and Glick (2001) reported the level of ACC-deaminase activity in *Azospirillum brasilense* in their

study to be between 1,740 to 2,340 nmol α -ketobutyrate mg^{-1} cell protein h^{-1} . Onofre-Lemus et al. (2009) reported in their study that the ACC-deaminase activity for 18 bacterial species was in the range of 2,000 to 15,000 nmol α -ketobutyrate mg^{-1} protein h^{-1} . Three of the most abundant species that were found in this study had ACC deaminase activities that ranged from 3,280 (HF1) to 5,500 (HF12) to 6,540 (HD6) nmol α -ketobutyrate mg^{-1} protein h^{-1} (Table 1).

Greenhouse study

The two *P. brassicacearum* strains that were used in the greenhouse experiment (HF1 and HD6) showed significant effects on winter wheat stem height. They also affected aboveground biomass, number of fertile heads, total number of heads, and seed number, although their effects were dependent on wheat variety (fertile heads) as well as the presence or absence of water stress (biomass, total heads, and seed number). In a previous study, *Pseudomonas brassicacearum* was proven to enhance plant growth and yield under environmental stress (Achouak et al. 2004). The present study was conducted to show the different effect of the bacterial strains on two different winter wheat varieties (Ripper and RonL). Previous greenhouse studies (e.g. Shaharoon et al. 2006) were conducted to study the effects of different ACC+ bacteria on a single plant variety.

In contrast to what I expected, there were negative effects of *P. brassicacearum* HD6 on Ripper when grown without water stress. Perhaps HD6 interfered with growth

and development of Ripper because of its high ACC deaminase activity. In other words, the high ACC deaminase activity of HD6 may have prevented the synthesis of ethylene at levels necessary for optimum growth. Belimov et al. (2007) found that high densities of a *P. brassicacearum* strain (Am3) had negative effects on tomato root elongation and tomato biomass. They stated that the negative effect of ACC+ depends on plant species, growth conditions, and the concentration of bacteria. Similarly, we found the effect of HD6 on winter wheat productivity was dependent on winter wheat variety and presence or absence of water stress.

Although effects were not statistically significant, RonL benefited biologically from inoculation with either HF1 or HD6 alone, where the number of fertile heads was increased by 16-28% compared to the control, biomass by ~50%, and seed number by 95-130%. Interestingly, the combination of both inoculums (HD6+HF1) did not enhance RonL productivity, indicating no synergistic effect of the two strains on wheat productivity.

Conclusion

This study was conducted to determine the effects of ACC+ bacteria on different winter wheat varieties when grown with or without water stress. We found six bacterial strains that were abundant in the rhizosphere and roots of winter wheat that were positive for ACC deaminase (*Pseudomonas brassicacearum* HD6 and HF1, *P.*

umsongensis, *P. tremae*, *Rahnella aquatilis*, and *Bacillus sp.*), three of which were not previously known to be ACC deaminase producers (*Rahnella aquatilis*, *P. umsongensis*, and *P. tremae*). Two strains with high ACC deaminase activity were selected to test the effects on two winter wheat varieties (Ripper and RonL) grown with or without water stress. Inoculation with ACC+ bacteria increased stem height of both varieties. Inoculation also affected aboveground biomass, number of fertile heads, total number of heads, and seed number, although their effects were dependent on wheat variety (fertile heads) as well as the presence or absence of water stress (biomass, total heads, and seed number). While RonL showed improved productivity when inoculated with HF1 or HD6, Ripper was negatively affected by strain HD6 under non-stressed conditions. In conclusion, positive or negative effects of ACC+ bacteria on plant productivity was dependent on upon specific ACC+ bacterial strain, plant genotype, and presence/absence of abiotic stress.

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Chapter 4

Conclusions

There were two main objectives of my thesis research, which were addressed in one field and one greenhouse study. Chapter 2 describes the field study, in which my objective was to 1) quantify the effects of winter wheat variety, irrigation management, and sampling date on the abundance of culturable ACC+ bacteria in a Colorado soil. Chapter 3 describes a greenhouse experiment to address my second objective, which was to determine the plant-growth-promoting effect of selected ACC+ bacterial inocula on winter wheat varieties ranging in drought sensitivities grown with or without water stress. My hypotheses were that because of unique interactions between plant genotype and bacterial species, 1) the abundance of ACC+ bacteria would vary across different winter wheat varieties, and 2) the response of winter wheat to ACC+ bacterial inoculation during water stress conditions would be dependent on the wheat variety and specific inoculum.

This study demonstrated that Colorado soils have the potential to harbor relatively high numbers of ACC+ bacteria, and that abundance and relative percent of ACC+ bacteria are elevated in soil with reduced water availability. This study also showed that different varieties of winter wheat accumulate different relative amounts of ACC+ bacteria, depending on soil water availability and sampling date. Specifically,

the percentage of ACC+ bacteria to total culturable bacteria was significantly increased under limited irrigation and dryland by anthesis and into mid-grain filling in the late growth stages of winter wheat than the first stages, but not under full irrigation. Limited irrigation is one way that farmers can increase the abundance and relative % ACC+ bacteria while still providing water to wheat at critical growth stages. At anthesis and mid-grain filling growth stages, drought-sensitive RonL accumulated greater % ACC+ bacteria than the other winter wheat varieties under limited irrigation and dryland management. This indicates that RonL produces greater amounts of ACC and stress ethylene under drought stress than the other varieties tested.

The greenhouse study was conducted to determine the effects of ACC+ bacteria on different winter wheat varieties when grown with or without water stress. We found six bacterial strains that were abundant in the rhizosphere and roots of winter wheat that were positive for ACC deaminase (*Pseudomonas brassicacearum* HD6 and HF1, *P. umsongensis*, *P. tremae*, *Rahnella aquatilis*, and *Bacillus* sp.), three of which were not previously known to be ACC deaminase producers (*Rahnella aquatilis*, *P. umsongensis*, and *P. tremae*). Two strains with high ACC deaminase activity were selected to test the effects of these bacteria on two winter wheat varieties (Ripper and RonL) grown with or without water stress. Inoculation with ACC+ bacteria increased stem height of both varieties. Inoculation also affected aboveground biomass, number of fertile heads, total number of heads, and seed number, although their effects were dependent on wheat variety (fertile heads) as well as the presence or absence of water stress (biomass, total

heads, and seed number). While RonL showed improved productivity when inoculated with HF1 or HD6, Ripper in contrast was negatively affected by strain HD6 under non-stressed conditions. In conclusion, positive or negative effects of ACC+ bacteria on plant productivity was dependent on upon specific ACC+ bacterial strain, plant genotype, and presence/absence of abiotic stress.