DISSERTATION

ROOT MICROBIAL INTERACTIONS TO ENHANCE WHEAT PRODUCTIVITY UNDER WATER STRESS

Submitted by

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ABSTRACT

ROOT MICROBIAL INTERACTION TO ENHANCE WHEAT PRODUCTIVITY UNDER WATER STRESS

Water stress is one of the obstacles that most profoundly affects plant growth and crop yields worldwide. Water stress causes serious plant growth problems such as suppression of cell growth and photosynthesis, disturbance of plant water relations, and increased production of the plant hormone ethylene, which reduces plant root and shoot length, consequently hampering the growth and productivity of crop plants. Wheat (*Triticum aestivum* L.) is one of the most important staple food crops, and is one of the most widely cultivated crops worldwide. Water deficit is the major limiting factor for wheat productivity, affecting yield and crop productivity. As a consequence, studying the water stress tolerance and productivity of wheat is extremely important to cope with the issue of food security in the face of a changing climate. Microbial inoculants that improve plant performance offer an environmentally friendly and sustainable strategy to cope with water deficit. Plant growth-promoting rhizobacteria (PGPR) are defined as a group of beneficial bacteria capable of colonizing the rhizosphere and contribute to increased plant growth and crop productivity via different direct and indirect mechanisms, such as production of plant growth regulators such as cytokinins, auxins, and gibberellins, inhibition of stress ethylene by ACC deaminase enzyme, or suppression of soil-borne pathogens by induction of plant defense mechanisms such as production of antibiotics or induced systemic resistance to cope with microbial pathogen attack. The purpose of this research was to assess the performance of 1-aminocyclopropane-1-carboxylate (ACC) deaminase- positive bacteria (ACC+ bacteria)

inoculants isolated from Colorado soils on growth parameters and performance of different winter wheat genotypes grown in a greenhouse under water-stressed and well-watered conditions (Chapter 2). The results of this study showed that under water stress, leaf relative water content was improved among genotypes in response to inoculation, whereas the growth responses of winter wheat to inoculation with ACC+ bacteria depended on the wheat genotype tested. Understanding of the biological underpinnings of the relationships between different genotypes and PGPRs could be clarified by exploring the chemical signals that mediate these interactions. Therefore, in chapter 3, a global metabolomics analysis of rhizosphere-associated metabolites was conducted to identify chemical signals that may be important in these interactions. The prime objective of this chapter was to identify whether specific root metabolites were associated with improved resistance to water stress. Root exudates from three winter wheat genotypes, under well-watered or water-stressed conditions and with or without inoculation by ACC+ bacteria, were collected and analyzed for global metabolite profile differences. By using untargeted UPLC-MS/GC-MS models for studying root exudate profiles of winter wheat genotypes that differ in their ability to withstand water stress combined with multivariate statistical techniques (PCA and OPLS-DA), we were able to identify statistically and potentially significant biochemical compounds that may contribute to improvement the stress tolerance in specific winter wheat genotypes. The results revealed that metabolite profiles were most influenced by irrigation status, with global differences between water stressed and well-watered plants evident from both unsupervised (PCA) and supervised (OPLS-DA) ordination plots of the data. In particular, water stress plants had higher levels of the citric acid cycle intermediate, succinate, as well as organic acids such as lactic acid. These compounds have been shown to be produced by bacteria and to enhance plant growth under varying environmental stress conditions.

The data also illustrate that metabolomic profiling is a powerful tool for generating specific hypotheses related to novel mechanisms of plant-microbe interactions for attenuation of water stress in winter wheat.

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Finally, I dedicate this dissertation to the spirit of my mother, whom I love and whose memory will never fade from the mind or soul.

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW

Introduction: Water Stress

Water stress is considered to be one the most crucial environmental stresses in agricultural systems that restricts plant growth and crop productivity in many parts of the world (Cattivelli et al., 2008; Seghatoleslami, 2008; Efeoğlu et al., 2009; Salekdeh et al. 2009; Alqudah et al., 2011; Farooq, 2012; Shukla et al., 2011; Kasim et al., 2013; Hu and Xiong, 2014). Water stress can be defined as a period without significant rainfall, which in turn leads to depletion of soil water and therefore restrain plant growth (Hanson and Weltzin, 2000). In addition, water stress can also be defined as the alteration in water potential gradients between soil and plant, which impairs the ability of roots of the plants to take up water, thus leading to loss of cell turgor (Marco et al., 2015), decline in cell volume (Martínez et al., 2007; Sobrado, 2015), protein denaturation, and changes in various physiological and molecular constituents as well as membrane integrity (Marco et al., 2015).

Drought-limited crop production in the world is associated with global warming and concomitant increase in drought-prone areas (Farshadfar et al., 2012; Fang and Xiong, 2015). The influence of water stress conditions on grain development and crop yield depend upon the duration of stress, rapidity of the stress, stress severity and the stage of plant growth during which drought stress occurs (Bayoumi, 2008; Farooq et al., 2009; Jaleel et al., 2009; Lata et al., 2015). Keshavarzi et al. (2013) have demonstrated that short-term drought can affect considerable losses in crop productivity as well.

The diverse crop developmental stages exhibit different sensitivity to water stress. In wheat, the majority of the floret primordia that access the fertile floret phase become grains after

anthesis (Cattivelli et al., 2008). Water stress can happen at various periods through the growing season, and plants respond differently to drought stress depending upon developmental stage. For instance, drought has the considerable adverse impact on maize yields when experienced during flowering (Deikman et al., 2012) and on wheat crops during booting and flowering (Alghabari et al., 2014) (Table 1.1 Farooq et al., 2012). In field, drought can assist in initiating or increasing pest and pathogen and simultaneously pathogens can severely impact plant water relations and thereby diminishing water potential in plant cells (Atkinson et al., 2015).

Hasanuzzaman et al. (2012) described how plants suffer from water deficit because of unavailability of water to the root zone or increasing transpiration rate. However, the adverse impact of the desiccation on plant growth and crop development are varied in nature, and therefore, unraveling the impacts of drought on plants is critical for enhanced crop management practices and breeding efforts in agricultural systems. Deficit of water not only leads to a reduction in crop productivity, but also contribute to ecological damage, land desertification, and soil erosion. Consequently, water stress has been considered as a critical ecological and global problem (Fang and Xiong, 2015).

Plant Responses to Water Stress

Water stress impairs a variety of morphological, physiological, and molecular mechanisms in plants including loss of turgor, suppression of cell growth, inhibition of photosynthetic activity, and activation of respiration (Shinozaki and Yamaguchi-Shinozaki, 2007; Sidana et al., 2015). Effects occur at both the cellular and whole plant levels, which in turn lead to specific and nonspecific phenotype and physiological responses. Kim et al. (2012) reported that decreases of photosynthetic activity is considered to be one of the biochemical and physiological responses to water stress due to various factors such as stomatal closure and

lowering of photosynthetic enzyme efficacy. Closure of stomata allows plants to restrict transpiration, but it also restricts CO_2 absorption, which in turn leads to a diminished photosynthetic activity (Efeoğlu et al., 2009).

Deficiency of water can diminish the availability of CO_2 for C fixation that causes the accumulation of reactive oxygen species (ROS) such as superoxide radical hydrogen peroxide and hydroxyl radicals (Selvakumar et al., 2012). Under water stress, ROS can attack the most sensitive biological macromolecules in plant cells and impose their deleterious impact through lipid peroxidation, protein denaturation, and DNA damage (Choudhary et al., 2015; Dutta and Khurana, 2015; Fang and Xiong, 2015). Water deficit can disturb the balance between generation of ROS and the antioxidant defense causing accumulation of ROS, which lead to induce oxidative stress (Farooq et al., 2012). Furthermore, under water-limiting conditions, nitrate reductase (NRase) activity diminishes in plants because of the lower uptake of nitrate from the soil by the roots (Selvakumar et al., 2012).

Cattivelli et al. (2008) reported that physiological traits that associate with the responses to water stress and/or are modulated by water deficits include a wide range of pivotal processes (Table 1.2). When a decline in water potential develops, responses of a wide range of physiological processes are stimulated. Some of these responses are directly caused via altered water status of the tissues whereas others are brought by phytohormones that signal changes in water conditions. Consequently, it can be anticipated that there is no unique response pattern that is robustly correlated with yield under all drought conditions.

One of the most impacting effects of ethylene on plant growth occurs when a plant is exposed to stressful conditions (Nascimento et al., 2016). Under unfavorable environmental conditions such as drought, a higher plant produces high concentrations of ethylene, a gaseous

plant-growth regulator produced endogenously in most plants (Saleem et al., 2007). Despite its simple two-carbon atom molecule, the ethylene is a robust modulation of plant growth and development with deep influences on plants (Dandekar et al., 2004; Van de Poel and Van Der Straeten, 2014). The plant hormone ethylene is associated with multiple aspects of the plant life cycle, encompassing seed germination, root hair development, root nodulation (Wang et al., 2002), flower opining and senescence, leaf and fruit abscission (Saraf et al., 2010), and fruit ripening (Wang et al., 2002: Saraf et al., 2010), as well as all growth stages of plant development are influenced via ethylene (Glick, 2014). Furthermore, ethylene has both adverse and advantageous impact on plant growth, depending on its concentrations relative to growth stages of plant (Shahzad et al., 2013). Commonly, ethylene is considered to be as an inhibitor of plant growth; however, lower levels of ethylene can enhance plant growth (Glick, 2005; Van Loon, 2007).

Under abiotic stress, increased ethylene production ("stress ethylene") can stimulate defense response, for instance reduced plant root and shoot length, which eventually leads to reduced plant growth and diminished crop productivity (Van Loon, 2007; Shahzad et al., 2013). A few review papers have covered all portions of ethylene biology in plants, including its biosynthesis, signaling and physiology (Van de Poel and Van Der Straeten, 2014). Its biochemical precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) is also a quite simple structure, however, probably its role in plant biology is earnestly underestimated (Van de Poel and Van Der Straeten, 2014). The ethylene biosynthesis initiates with enzyme ACC synthase that converts S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC). The next step is the transformation of ACC to ethylene by ACC oxidase (Figure 1.1) (Dandekar et al., 2004; Singh et al., 2015).

Understanding of biosynthetic pathways of ethylene production in plants has made it possible for plant physiologists to demonstrate the mechanisms via which plants modulate the endogenous ethylene concentration for their normal growth and development (Saleem et al., 2007). Fundamentally, the enzymes that degrade S-adenosylmethionine (derived from L-methionine) or ACC have been described to effectively diminish ethylene concentrations without immensely changing the physiology of the plant. In this context, a number of enzymes have been examined which assist in reducing ethylene levels in the plants. Among these, the enzymes S-adenosylmethionine (SAM) hydrolase and SAM decarboxylase have been studied to a lower extent in relation to regulation of ethylene in plants whereas ACC synthase and oxidase have been extensively addressed in various plant species (Saleem et al., 2007).

Adaptation Strategies to Water Stress

An understanding of plant responses to drought is of significant importance for developing stress tolerance in agronomic crops (Jaleel et al., 2009). As a consequence of drought, plants have evolved specific or adaptive strategies to cope or alleviate water deficit conditions for improved plant growth and development, which are strategies for (1) stress escape (2) avoidance (3) tolerance (Farooq et al., 2012; Fang and Xiong, 2015; Marco et al., 2015) and (4) drought recovery (Fang and Xiong, 2015). *Drought escape* refers to the capacity of a plant to complete its life cycle before the beginning of drought and enter dormancy before the commencement of dry season. Generally, drought escape occurs in some desert plants (Farooq et al., 2012; Marco et al., 2015). *Drought avoidance* implies the capacity of a plant to maintain high plant water status or cellular hydration under water deficient. Plants achieve this mechanism either via taking more water from the soil or by diminishing water loss through transpiration (Farooq et al., 2012). *Drought tolerance* refers to the capability of plants to retain a certain level

of physiological activities under critical drought stress conditions, through the organization of several genes and series of metabolic pathways to decrease or restore the resulting stress damage (Fang and Xiong, 2015). Fang and Xiong (2015) have described an additional strategy to adapt to drought stress, which is *drought recovery*. This refers to the ability of the plants to continue growth and gain yield after exposure to harsh drought stress that causes an entire loss of turgor pressure and leaf desiccation.

One way that plants avoid, tolerate or recover from drought is through osmotic adjustment (Hu and Xiong 2014). Osmotic adjustment (OA) or osmoregulation refers to the accumulation of organic and inorganic solutes in tissue cells under drought and/or salinity, which in turn leads to lower water potential without diminishing actual water content (Farooq et al., 2012). Those compounds, often called osmoprotectants or compatible solutes, are commonly utilized to improve drought stress tolerance in plants under water deficit environments (Marco et al., 2015). Furthermore, these compounds do not have any deleterious impacts on membranes, enzymes, and other macromolecules, even at higher concentrations (Farooq et al., 2012; Marco et al., 2015). A wide variety of metabolites have been identified as osmoprotectants, including certain amino acids such as proline, quaternary and other amines such as glycine betaine and polyamines, and a wide range of sugars and sugar alcohols such as mannitol and trehalose (Marco et al., 2015). In general, analysis of osmotic adjustment (OA) in wheat under various drought stress conditions has demonstrated that osmoregulation can be an efficient selection criterion for drought tolerance, and become of its role in diminishing drought dependent yield loss, in particular when deficiency of water occurs through the reproductive growth stage (Cattivelli et al., 2008).

Wheat is regarded as one of the most important staple food crops (Ali et al., 2008; Semenov and Shewry, 2011; Verma et al., 2015), and is one of the most widely cultivated crops in throughout the world (Ran et al., 2015). Dhanda et al. (2004) and Ran et al. (2015) stated that almost one-third of the world's population mainly feeds on wheat. Because wheat is commonly grown in rainfed agricultural systems, without irrigation, wheat is susceptible to drought and water scarcity. Deficiency of water is a decisive environmental factor that leads to restrict wheat productivity in many portions of the world (Shukla et al., 2015). Moreover, the influence of deficit rainfall through the sowing period appears to be one of the core points which in turn lead to decrease in wheat productivity (Farshadfar et al., 2012).

Among crop plants, wheat is an attractive study system due to the variation in its genetic traits associated with drought tolerance (Khanna-Chopra and Selote, 2007). Gupta et al. (2011) have demonstrated that wheat genotypes that possess the capability to synthesize and store a high concentration of water soluble carbohydrates in the stems before to anthesis are more presumably to exhibit enhanced grain yield under water stress conditions.

This would lead to allow the tolerant cultivar to exhibit promoted grain yield even through stress conditions. Khanna-Chopra and Selote (2007) illustrated that variation in wheat genotypes in drought tolerance could be attributed to the ability of plants to acclimate and stimulate antioxidant defense under drought stress.

As a consequence, studying the drought tolerance and productivity of wheat is extremely important to overcome the issue of food security under climate change (Gong et al., 2003). Therefore, understanding mechanisms of drought stress tolerance in wheat is considered to be of great value for ensuring continued wheat production in drought-prone regions of the world. Ray et al. (2012) have reported that wheat yields are stagnate in the Khyber Pakhtunkhwa and

Balochistan states of Pakistan (starting after ~2000), and in Australia, wheat yields are stagnating in ~ 44% of its areas. However, 61% of global wheat areas are witnessing yield increases. Most of Canadian wheat areas, ~ 64% of United States wheat areas, Russian wheat, most African wheat areas, some areas of Asian wheat and ~ 40% of Australian wheat areas are still witnessing yield increases (Figure 1.2).

Microbial Interactions for Water-Stress Tolerance

The beneficial interactions between plants and soil microbes are of great agronomical importance through their capacity to potentially mitigate water deficit conditions. Plant growth promoting rhizobacteria (PGPR) are a group of beneficial bacteria that improve plant growth and development via their ability to alleviate biotic and abiotic stress in crop plants (Kasim et al., 2013; Choudhary et al., 2015; Dutta and Khurana, 2015; Glick, 2015). PGPR encompass diverse group of bacteria that colonize the rhizosphere around the root and improve or facilitate plant growth through several activities. Kasim et al. (2013) reported that many PGPR strains are known to stimulate abiotic stress tolerance in some plants such as drought stress in wheat. Singh et al. (2011) reported that *Bacillus* and *Pseudomonas* are the predominant genera among the varied bacteria identified as PGPR.

A variety of direct and indirect mechanisms of plant growth and development have been addressed by PGPR (Singh et al., 2011; Kim et al., 2012; Kasim et al., 2013; Shahzad et al., 2013; Glick, 2014; Dutta and Khurana, 2015). Among the indirect mechanisms, PGPR may facilitate plant growth by diminishing the detrimental effects of certain microbial pathogens by stimulating host resistance mechanisms (Singh et al., 2011;Shahzad et al., 2013). Directly, PGPR can beneficial by either supplying plants with the compounds synthesized via the bacterium or increasing nutrient uptake from the environment (Singh et al., 2011). Furthermore, plant growth

can be improved by some PGPR that facilitate root development and alter root architecture through production of phytohormones such as indole acetic acid (IAA) (Yang et al., 2009). The beneficial rhizobacteria in crop plants can modify and alter the plant cell wall through combination of organic compounds that are involved in plant defense traits (Shahzad et al., 2013). Similarly, Kasim et al. (2013) have shown that PGPR possess the capacity to modify plant metabolism under both stress and natural conditions. This effect is often attributed to various mechanisms such as ACC deaminase activity, indole-acetic acid production, production of antioxidant compounds, and nitrogen fixation.

Glick (2007) has indicated that the direct route via PGPR involves either providing plant hormones such as auxin or cytokinin, or minimizing plant ethylene levels through the action of the enzyme ACC deaminase which can regulates ethylene levels in plants by hydrolyzing ACC (the immediate precursor of ethylene in higher plants) into ammonia and α-ketobutryate. The ACC deaminase enzyme has been found in various bacterial and fungal species from soil or as endophytes, but this enzyme is most commonly found in PGPRs (Arshad et al., 2007). Moreover, under water stress PGPR can work through specific mechanisms such as production of exopolysaccharides (EPS) and improving the activity of antioxidant enzymes (Nadeem et al., 2015).

ACC Deaminase-Positive Bacteria

ACC-deaminase positive (ACC+) bacteria can stimulate ACC exudation from plant root and hence are provided with a unique source of carbon and nitrogen, and thereby the growth of ACC+ bacteria is accelerated in the near vicinities of plant roots as compared to other soil organisms. Therefore, the level of ACC is reduced within the plant, which in turn leads to reduced endogenous biosynthesis of ethylene (Arshad et al., 2007; Saleem et al., 2007). ACC+

bacteria hydrolyze ACC into ammonia and α-ketobutryate, which can be further degraded as a carbon source. By acting as a sink for ACC, ACC+ diminish levels of stress ethylene, and as a result, plant growth is improved (Glick, 2005; Arshad et al., 2007; Shaharoona et al., 2007; Glick, 2007; Van Loon, 2007; Singh et al., 2011; Kim et al., 2012; Selvakumar et al., 2012; Shahzad et al., 2013; Glick, 2014; Van de Poel and Van Der Straeten, 2014; Glick, 2015). In the presence of ACC+ bacteria, plant ACC is sequestered and hydrolyzed via bacterial cells to provide nitrogen and energy (Figure 1.3).

By eliminating ACC, the bacteria diminish the deleterious impact of ethylene, mitigating plant stress and improving plant growth (Choudhary et al., 2015). In addition, ACC+ bacteria can diminish the absorption and uptake of Na^+ (Nadeem et al., 2015). The reduction in Na^+ uptake is presumably due to the reduced passive flow of Na^+ into the vascular tissues. Moreover, ACC+ bacteria can increase nitrogen, phosphorus, and potassium concentration under both salt and drought stresses (Nadeem et al., 2015). Furthermore, ACC deaminase-producing rhizobia can enhance nodulation in legumes by inhibiting of ethylene biosynthesis, and thereby promoting symbiosis and nitrogen fixation in plants (Van de Poel and Van Der Straeten, 2014; Nadeem et al., 2015). Bhattacharyya et al. (2012) demonstrated that the genetic modification of ACC+ bacteria is beneficial to biological control of diverse plant diseases. Plants that were treated with ACC+ bacteria have also shown increased resistance to the harmful impacts of the high ethylene levels that is produced as a result of stressful environments such as flooding, heavy metals, phytopathogens, salinity, drought (Penrose and Glick, 2003; Saleem et al., 2007; Kang et al., 2010; Saraf et al., 2010), the presence of organic and inorganic toxicants (Saraf et al., 2010).

Choudhary et al. (2015) have been reported that the microbial strain, *Achromobacter piechaudii* ARV8, which expressed ACC deaminase, conferred induced systemic tolerance (IST)

against drought and salt in pepper and tomato. Saleem et al. (2007) demonstrated that genetic modification of PGPR having ACC deaminase genes assisted in modulation of nodulation in legumes and biological control of plant disease. Consequently, these advantages make the selection of PGPR having ACC deaminase more reliable than any other alternative (Saleem et al., 2007). Accordingly, utilization of ACC+ bacteria is currently attracting considerable attention among researchers as a means to improve plant growth and development under stress and non-stressed conditions (Singh et al., 2011). Van de Poel and Van Der Straeten (2014) have reported that ACC+ bacteria are a biotechnological tool to control endogenous ACC levels and ultimately decrease ethylene levels in plants. Based on these studies, inoculation with ACC+ bacteria may be an effective means to increase tolerance to stress in plants that are more susceptible to the impacts of ethylene, particularly under stressful environments such as flooding, drought and phytopathogens (Dutta and Khurana, 2015).

Plant Selection of ACC Deaminase-Positive Bacteria

Plant roots have the ability to release a wide range of compounds, called root exudates, into the rhizosphere (Walker et al., 2003). Based on the components of their root exudates, plant select and enrich the type of bacteria in the rhizosphere (Choudhary et al., 2015). Plant root exudates are generally composed of low molecular weight compounds such as sugars, organic acids, and amino acids and high molecular weight compounds such as proteins and mucilage (Bais et al., 2006; Badri and Vivanco, 2009; Shi et al., 2011; Huang et al., 2014). These compounds can be degraded as a bacterial food source, which is the main reason why the numbers of bacteria surrounding the plant roots are more abundant than the bulk soil (Gray and Smith, 2005; Glick, 2014; Nadeem et al., 2015). Due to the variety of these compounds, soil microbial population including PGPR, are highly abundant in the area surrounding the plant

roots and utilize these compounds as food sources to increase their growth and development (Nadeem et al., 2015).

Root exudates have a significant role in in determining the diversity and activity of soil microorganisms, which perhaps influence plant growth (Shi et al., 2011). Bertin et al. (2003) reported that production of root exudates could modify the species composition of the microorganisms in the rhizosphere. Consequently, nutrient condition through decomposition and mineralization of organic matters are altered via the formation of soil organic matter (Bertin et al., 2003). Root exudates might play considerable indirect roles in resource competition via changing soil chemistry, soil processes, and microbial communities (Bais et al., 2006; Shukla et al., 2011). Certain root exudates, including amino acids and carbohydrates, have been found to act as chemo-attractants because they attract and stimulate growth microbial growth (Huang et al., 2014; Kudoyarova et al., 2014).

Yuan et al. (2015) have expressed that root exudates are not only utilized by PGPR as nutrient sources, but also act as signaling compounds that can attract and/or repel soil microbial communities. Likewise, the same study showed that low molecular weight organic acids that were released via roots such as malic, citric and fumaric acid could play precise roles in recruiting of PGPR to the roots serving, for instance as carbon substrates and signaling molecules. Studies reported that increases in root exudates resulted in more ACC+ bacteria colonizing the rhizosphere surrounding roots (Siddikee et al., 2011; Van de Poel and Van Der Straeten, 2014). The more ACC that is utilized via bacteria, the lower the amount of ACC that is converted by ACC oxidase into ethylene, leading to reduced adverse impacts of stress ethylene and thereby enhancing plant growth. Therefore, plant growth promotion under stressful growth

conditions is an immediate consequence to the presence of ACC deaminase activity in PGPR (Siddikee et al., 2011).

Microbial Inoculation Strategies

Novel strategies need to be established to diminish the risk of drought stress in plants. These include of the creation of innovative biological strategies in agricultural systems, and also assessment of microbial inoculants. Specifically, inoculation of agricultural crops with PGPR are a promising strategy to cope with or mitigate environmental stresses because of the ability of PGPR to improve plant growth, enhance nutrient availability and uptake, and improve the health of plants (Gallarato et al., 2015; Gunes et al., 2015; Nadeem et al., 2015). Examples of studies that involved plants inoculated with ACC deaminase containing bacteria and their beneficial effects on host plants are summarized in (Table 1.3 and 1.4). Inoculation benefits might due to a wide array of morphological, physiological, and metabolic impacts on the host plant brought via the beneficial effects of these bacteria (Kasim et al., 2013). Choudhary et al. (2015) demonstrated that these soil microorganisms could supply significant models for understanding stress tolerance mechanisms that could be subsequently applied into crop plants.

Glick (2015) has shown that the diverse impacts of several stresses can be averted via PGPR inoculants that have ability to provide the plant with varying approaches to alter plant metabolism and therefore diminish the severity of the stress. Zahir et al. (2008) have reported that pea plants inoculated with ACC+ bacteria were dramatically more resistant to the inhibitory impacts of stress ethylene on plant growth, which is synthesized as a consequence of stressful environmental conditions such as salinity and drought. Saleem et al. (2007) demonstrated that ACC + bacteria *Achromobacter piechaudii* ARV8 had greater the fresh and dry weights of both tomato and pepper seedlings under water stress.

Consequently, further research is needed for a deeper knowledge of ACC+ bacteria establishment and survival requirements. These interactions can, in turn, lead not only to increased knowledge on crop-microbe interactions but also to greater effectiveness and reliability of utilizing microbial inoculants to enhance the sustainable production of cultivable crops under water deficit conditions. Furthermore, the identification of effective ACC+ bacteria is critical for accomplishing an optimal/maximum benefits in terms of improved plant growth and tolerance.

Summary and Conclusion

Drought can be defined as a condition of deficiency of water that has drastic impacts on plant growth and productivity and thereby decreases crop yields substantially. Drought can induce various morphological, physiological, and molecular changes in plants. Some of these alterations include reduction of water potential in tissues, cell growth, stomatal closure, transpiration and inhibition of photosynthesis. Water deficit leads to the accumulation of reactive oxygen species that causes damage to lipid, proteins, carbohydrates, and DNA. Water deficit imposes considerable constraints on wheat. Therefore, enhancing drought tolerance of wheat under water stress is vital for crop improvement. Consequently, plants display a variety of mechanisms to overcome or withstand drought stress including drought escape, avoidance, tolerance, and recovery.

Furthermore, soil microorganisms including plant growth-promoting rhizobacteria (PGPR) could also play a significant role in stress tolerance. Due to the presence of a wide range of compounds such as carbohydrates, organic acids, and amino acids in the root exudates, PGPR are present in high concentration around the plant roots.

PGPR play a precise role in improving plant growth and development by a range of direct and indirect mechanisms. The mechanisms that enhance plant growth include: inhibition of the

deleterious effects to plants via pathogenic organisms, facilitating the acquisition of nutritional resources, for instance, nitrogen and phosphorus, stimulating plant growth via either providing plant hormones, such as auxin, cytokinin, and gibberellin, or decreasing plant ethylene levels through the action of the enzyme ACC deaminase. PGPR expressing ACC deaminase are vital to regulate ethylene production by hydrolyzing ACC (the immediate precursor of ethylene) into ammonia and α –ketobutyrate. Plants treated with PGPR having ACC-deaminase might have relatively extensive root growth due to reduced ethylene and can better cope various stressful environment conditions such as extremes of temperature, waterlogging, drought, and salinity. Due to root colonization ability and their interaction with plant, PGPR have significant potential to enhance plant growth and increase crop yields substantially.

In recent years, there has been an increasing interest in the interaction between PGPR and plants to ensure survival of agricultural crops and sustainable food production. Nevertheless, which mechanisms are important under abiotic stresses such as drought for enabling these microbes to interact with plants remain poorly understood. Furthermore, which strains of PGPR are most effective to obtain maximum benefits to enhance plant growth are yet to be determined. Therefore further investigations are needed to employ certain mechanisms for understanding the interactions that occur between microorganisms and plants. In addition, the selection of a particular strain or strains of PGPR are vital for accomplishing maximum benefits in terms of improved plant growth and tolerance against several stressful environmental conditions and thereby enabling plants to withstand and survive in such extreme conditions.

Figures

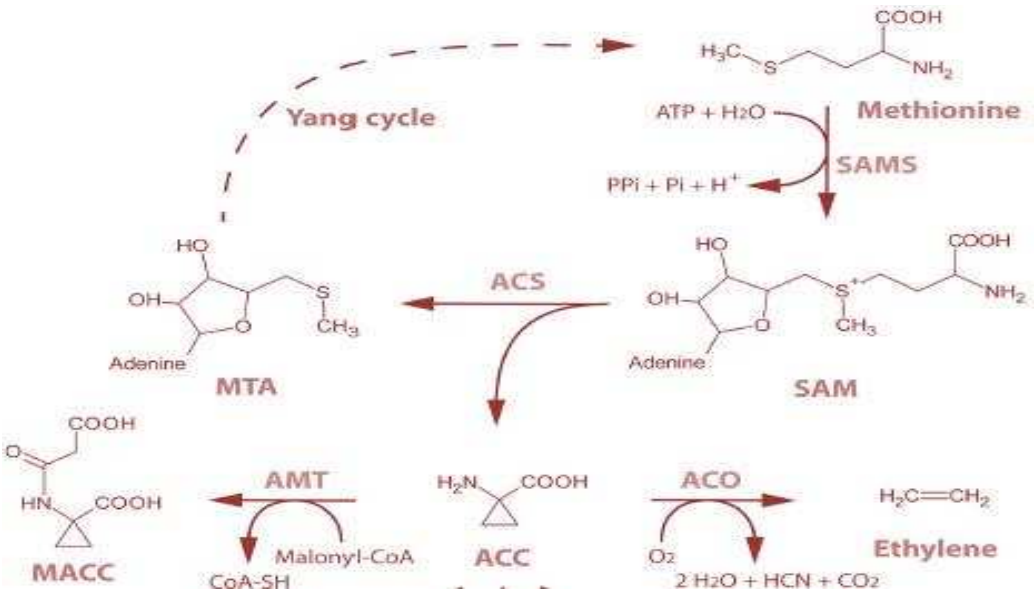


Figure 1.1. The Ethylene Biosynthesis Pathway.

The amino acid methionine is catalyzed by SAM synthetase (SAMS) to S-adenosyl-L-methionine (SAM) with the requirement of ATP. The general precursor SAM is catalyzed by ACC synthase (ACS) to 5′-methylthioadenosine (MTA). MTA can be recycled back to methionine by the Yang cycle (dashed arrows show various enzymatic steps). SAM can also be converted to ACC (the immediate precursor of ethylene) via ACS. ACC can be metabolized to ethylene by ACC oxidase (ACO) in the presence of oxygen. ACC can also be converted to 1-malonyl-ACC (MACC) by the yet uncharacterized ACC-N-malonyl transferase (AMT) with the requirement of malonyl-Coenzyme-A. Modified figure adapted from the reference (Van de Poel and Van Der Straeten, 2014).

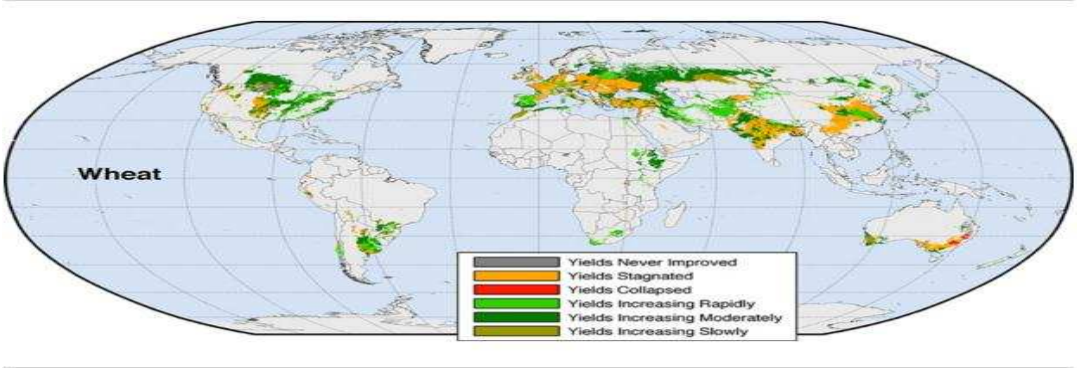


Figure 1.2. Global map of wheat yield trends. The trends divided into the six categories and colour coded (*Source;* Ray et al., 2012).

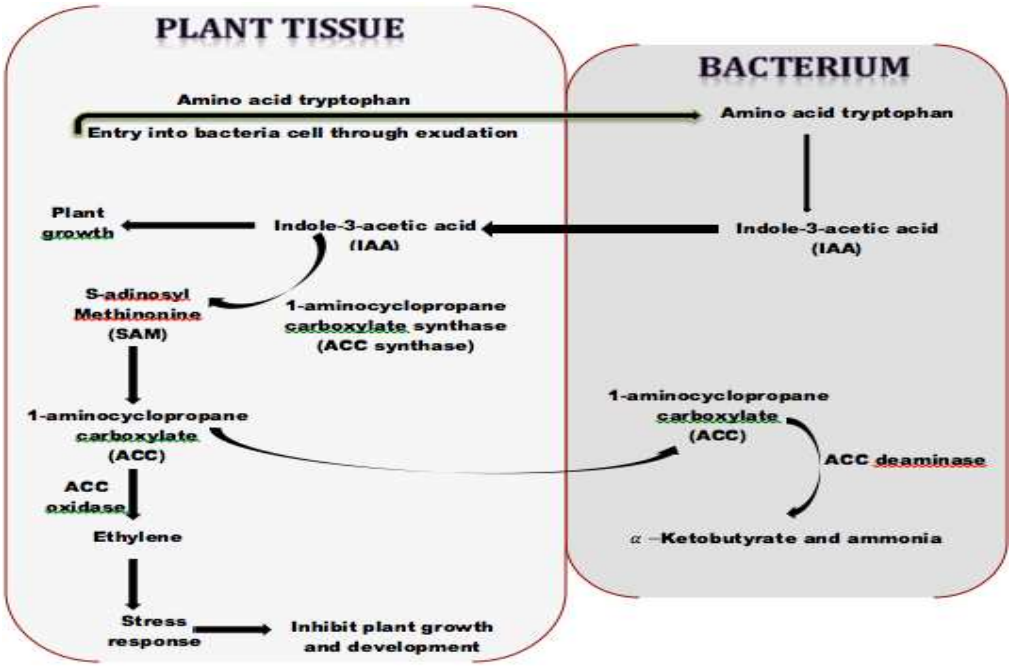


Figure 1.3. Schematic model of how a plant growth promoting rhizobacterium (PGPR) that both produce ACC deaminase and synthesize IAA lowers the ethylene concentration and thereby promoting plant growth. (Modified figure adapted from the source Choudhary et al., 2015). In response to amino acid such as tryptophan and other small molecules in the plant root exudates, the bacteria synthesize and secrete the phytohormone indole-3-acetic acid (IAA), some of which is taken up by the plant. This IAA, together with endogenous plant-synthesized IAA can either stimulate plant growth or induce the enzyme of ACC synthase to convert SAM to ACC (the immediate precursor of ethylene). Plants secrete some ACC that is taken by rhizosphere bacteria (PGPR) and then degraded by the enzyme ACC deaminase into ammonia and α-ketobutryate.

Tables

Table 1.1. Diminish in grain yield in various crops by water deficit (Farooq et al., 2012).

Crop	Growth stage	Type of stress	Reduction in grain yield (%)
Rice	Reproductive	Mild stress	54
Rice	Reproductive	Severe stress	94
Rice	Reproductive	Severe stress	24-84
Rice	Flowering	Short severe stress	54
Rice	Flowering and grain filling	Prolonged severe stress	84
Rice	Flowering and grain filling	Prolonged mild stress	52
Wheat	Reproductive	Prolonged mild stress	50-66
Wheat	Pre-anthesis	Prolonged mild stress	18-53
Wheat	Post-anthesis	Prolonged mild stress	13-38
Wheat	Terminal	Prolonged mild stress	32-63
Wheat	Flowering and grain filling	Prolonged mild stress	58-92
Wheat	Stem elongation	Mild stress	18
Wheat	Anthesis	Mild stress	8
Wheat	Stem elongation + anthesis	Mild stress	22
Mungbean	Vegetative growth stage	-	40
Mungbean	Reproductive growth stage	-	4
Sunflower	Immediately prior anthesis	Mild stress	5-56

Table 1.2. Physiological traits relating in response to drought conditions (Cattivelli et al., 2008).

Plant traits	Impacts relevant for crop	Modification under stress
Stomatal conductance/leaf temperature	More/less rapid water consumption. Leaf temperature reflects the evaporation and thus is a function of stomatal conductance	Stomatal resistance increases under stress.
Photosynthetic capacity	Modification of concentration of Calvin cycle enzymes and elements of the light reactions	Decrease under stress.
Timing of phenological phases	Early/late flowering. Maturity and growth duration, synchrony of silk emergence and anthesis, reduced grain number	Wheat and barley advanced flowering, rice delayed, maize asynchrony.
Anthesis-silking interval (ASI) in maize	ASI is negatively associated with yield in drought conditions	Drought stress at flowering causes a delay in silk emergence relative to anthesis.
Starch availability during ovary/embryo development	A reduced starch availability leads to abortion, reduced grain number	Inhibition of photosynthetic activity reduces starch availability.
Single plant leaf area	Plant size and related productivity	Diminished under stress (wilting, senescence, abscission).
Rooting depth	Higher/lower tapping of soil water resources	Reduced total mass but increased root/shoot ratio, growth into wet soil layers, regrowth on stress release
Osmotic adjustment	Accumulation of solutes: ions, sugars, poly-sugars, amino acids, glycinebetaine	Slow response to water potential.
Membrane composition	Increased membrane stability and changes in aquaporine function	Regulation in response to water potential changes.
Antioxidative defense	Protection against active oxygen species	Acclimation of defense systems.

Table 1.3. Drought stress tolerance in some plants induced through rhizosphere bacteria (Nadeem et al., 2015)

Plants	Rhizobacterial strain	Mechanism utilized/processes regularized
Wheat	*Burkholderia phytofirmans* PsJN	Modification of metabolism, enhancing ionic balance
	Bacillus amyloliquefaciens 5113 and A. brasilense NO40	Amelioration in homeostatic mechanisms and might be due to a combination of morphological, physiological, and metabolic impacts.
	Azospirillum	Morphological modulations of the coleoptile xylem architecture, upregulation of its own indole-3- pyruvate decarboxylase gene, and improved bacterial IAA synthesis.
Rice	*A. brasilense*	Arbuscular mycorrhizae colonization through promotion of fungal propagule germination, stimulation of mycelial growth, or changes in the root architecture through the production of growth factors.
Maize	*B. phytofirmans* PsJN, *Enterobacter sp.* FD17	Amendments of metabolism during endophytic colonization.
	Pseudomonas putida	Inhibition of ethylene production due to ACC deaminase activity.
	Pseudomonas entomophila BV-P13, *Pseudomonas stutzeri* GRFHAP-P14, *P. putida* GAP-P45, *P. syringae* GRFHYTP52, *and Pseudomonas monteilii* WAPP53	Regulation of activities of antioxidant enzymes, ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX).

Table 1.4. Inoculation with PGPR having ACC deaminase and subsequent physiological changes in plants. Modified table adapted from the reference (Saleem et al., 2007)

Plant	PGPR	Physiological changes in plants
Canola	*Methylobacterium fujisawaense*	Bacterium promoted root elongation in canola.
	Bacillus circulans DUC1, *Bacillus Wrmus* DUC2, *Bacillus globisporus* DUC3	Bacterial inoculation enhanced root and shoot elongation.
	Alcaligenes sp. *Bacillus pumilus* *Pseudomonas sp.* *Variovorax paradoxus*	Inoculated plant demonstrated more vigorous growth than the control (uninoculated).
	Enterobacter cloacae	A significant increase in the root and shoot lengths was observed.
Carnations	*Azospirillum brasilense* Cd1843	Inoculated cuttings produced longest roots
Soybean	*Pseudomonas cepacia*	Rhizobacterium caused an early soybean growth.
Pea	*Rhizobium leguminosarum bv. viciae* 128C53K	Bacterium enhanced nodulation in plants.
Mung bean	*Pseudomonas sp.* *Bradyrhizobium sp.*	Bacterium promoted nodulation in mung bean.
Mung bean	*Pseudomonas putida*	The ethylene production was inhibited in inoculated cuttings.
Maize	*Enterobacter sakazakii* 8MR5 *Pseudomonas sp.* 4MKS8 *Klebsiella oxytoca* 10MKR7	Inoculation increased agronomic parameters of maize.
Maize	*Pseudomonas sp.*	Bacterium caused root elongation in maize.

CHAPTER 2: WATER STRESS TOLERANCE OF WINTER WHEAT (*TRITICUM AESTIVUM* L.) GENOTYPES IS IMPROVED BY 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC)-DEAMINASE POSITIVE BACTERIA

Summary

Water stress is a major factor limiting wheat production in rain-fed areas around the world. Wheat tolerance to water stress may be enhanced through genotypic selection, but recently, there has been interest in manipulating wheat-microbial interactions to promote water stress tolerance. The prime objective of the study was to examine the effects of inoculation with 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase containing (ACC+) bacteria on different winter wheat genotypes under water-stressed and well-watered conditions as determined by root length, above- and below-ground biomass, and leaf relative water content. The results revealed that inoculation significantly increased leaf relative water content across all genotypes under the water-stressed condition. Irrespective of soil water status, inoculation significantly increased aboveground biomass and root biomass in the deepest tube section (67-99 cm depth increment) of the wheat genotype RonL, by 25% and 145% respectively, as compared to non-inoculated controls. Inoculation with ACC+ bacteria affected root lengths within certain root diameter classes, but did not affect total root length. Specifically, inoculation increased root length by 44% within the 0.50-0.75 mm diameter class for RonL variety, in comparison with non-inoculated control, regardless of irrigation treatment. Furthermore, under water-stress conditions, inoculation increased root length by 129% within the 0.75-1 mm diameter class for RonL, in comparison with non-inoculated control. The results of the present study showed that under water stress, leaf relative water content was improved among genotypes, in response to inoculation, whereas the growth response of winter wheat to inoculation with ACC+ bacteria was

genotype dependent. The genotype RonL appeared therefore to be a good plant model to study wheat interactions with ACC+ bacteria. Finally, the variation among the genotypes might be of significant benefit for choosing traits for improved water stress tolerance of wheat genotypes.

Introduction

Water stress is one of the most devastating abiotic stresses, restricting plant growth and crop productivity throughout the world (Cattivelli et al., 2008; Seghatoleslami, 2008; Efeoğlu et al., 2009; Salekdeh et al., 2009; Alqudah et al., 2011; Anjum et al., 2011; Farooq, 2012; Shukla et al., 2011; Kasim et al., 2013; Hu and Xiong, 2014; Tiwari et al., 2016). Plant response to water stress is complex because it is characterized by various morphological and physiological traits that interact and vary in their individual response based on the severity and duration of water stress (Witcombe et al., 2008; Nezhadahmadi et al., 2013).

Impacts of water stress include diminished photosynthetic activity due to decreased stomatal conductance, as well as reductions in leaf size, stem extension, root proliferation, turgor pressure, and water-use efficiency (Shinozaki and Yamaguchi-Shinozaki, 2007; Anjum et al., 2011; Sidana et al., 2015). Exposure of plants to water stress has been shown to significantly reduce the leaf water potential, relative water content and transpiration rate, with a simultaneous increase in leaf temperature (Siddique et al., 2000). Additionally, under unfavorable environmental conditions such as water stress, plants can produce relatively high concentrations of ethylene, an endogenous gaseous plant-growth regulator (Saleem et al., 2007). Ethylene is associated with multiple aspects of the plant life cycle, including seed germination, root hair development, root nodulation, flower opening and senescence, and leaf and fruit ripening (Wang et al., 2002; Saraf et al., 2010). Under abiotic stress, including water stress, increased ethylene

production ("stress ethylene") can lead to reduced plant root and shoot length, which eventually reduces plant growth and diminishes crop productivity (Van Loon, 2007; Shahzad et al., 2013).

Wheat (*Triticum aestivum* L.) is regarded as one of the world's most important staple food crops (Ali et al., 2008; Semenov and Shewry, 2011; Verma et al., 2015), providing primary sustenance for almost one-third of the world's population (Dhanda et al., 2004; Ran et al., 2015). Among crop plants, wheat is relevant for studies of water stress tolerance because it is typically grown under rain-fed conditions, often experiences water deficits, and is known to vary in genetic traits associated with water stress tolerance (Khanna-Chopra and Selote, 2007).

Recent evidence has indicated a role for soil microbial communities in mediating water stress (Glick, 2005; Yang et al., 2009). Plant growth promoting bacteria (PGPR) are beneficial bacteria in the soil microbial community that improve plant growth and development via their ability to alleviate biotic and abiotic stress in crop plants (Kasim et al., 2013; Choudhary et al., 2016; Dutta and Khurana, 2015; Glick, 2015). Many PGPR strains are known to stimulate abiotic stress tolerance in plants, including water stress in wheat (Kasim et al., 2013). Plant growth is improved by PGPR through various mechanisms which include production of phytohormones such as indole acetic acid (IAA) and cytokinin, facilitation of nutrient uptake, and enhancement of nitrogen fixation.

The facilitation of plant growth in the presence of water stress also occurs when seeds or roots are inoculated with PGPR having the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme (ACC+ bacteria). ACC deaminase hydrolyzes the ethylene precursor ACC to α-ketobutyrate and ammonia, thereby diminishing ethylene levels in stressed plants (Glick 2005; Saleem et al., 2007; Zahir et al., 2008; Choudhary et al., 2016; Dutta and Khurana, 2015; Gallarato et al., 2015; Gunes et al., 2015; Nadeem et al., 2015; Glick, 2015). In addition,

Saleem et al. (2007) suggest that bacterial strains having ACC deaminase activity are beneficial because they are more likely to survive and colonize plant roots. ACC deaminase potential has subsequently been detected in a wide array of soil microorganisms, particularly in different species of *Pseudomonas* (Magnucka and Pietr, 2015).

While it is known that water stress can disrupt root–microbial associations (Selvakumar et al., 2012), it is interesting that under water stress, ACC+ bacteria might benefit plants by reducing sensitivity to water stress. However, neither the potential for this association to benefit winter wheat nor whether such associations can be promoted across multiple genotypes is known. Current studies have focused on genotype-specific associations between wheat and PGPRs, such as Bacillus sp. (Chanway et al., 1988), fluorescent pseudomonads (Mazzola et al., 2004), and recently, ACC+ bacteria (Stromberger et al., 2017). Therefore, we conducted a greenhouse study with the following objectives: (1) to evaluate the potential beneficial effect of ACC+ bacterial inoculation on physiologic traits of winter wheat (*Triticum aestivum* L.) under contrasting water regimes; and (2) to characterize wheat genotypes that vary in their sensitivity to water stress treatments, with and without ACC+ bacterial inoculation.

Because of the wide genetic variation in winter wheat for water stress tolerance, we hypothesized that sensitivity to water stress would vary by genotype, as measured in above- and below-ground biomass, root length, and leaf RWC. We also hypothesized that inoculation of winter wheat with ACC+ bacteria would increase wheat tolerance to water stress, by increasing root biomass and length, and leaf RWC, under water stress. Finally, we predicted that the response of winter wheat to inoculation would be genotype specific.

Material and Methods

Preparation of ACC+ Bacterial Inoculum

A mixed-culture inoculum of ACC+ bacteria was developed from a water stress-prone soil in Walsh, Colorado (37.23ºN, 102.17ºW). The soil at this location is a loamy sand (fine-loamy, mixed, mesic Ardic Ustochrept) with an organic carbon content of 2.7 g kg^{-1} soil. The annual precipitation is 38 cm, the growing season open-pan evaporation is 190 cm, and on average, the air temperature exceeds 32ºC (90ºF) 64 days a year (Sherrod et al., 2005). Previous studies have described the microbial community structure and activity of the Walsh soil as being particularly active for the size of its biomass, and showing no increased signs of physiological stress, compared to microbial communities from sites in Colorado with lower evapotranspiration potentials (Stromberger et al., 2007, 2011). For this study, we capitalized on an opportunity to select a water stress-adapted culture of ACC+ bacteria by enriching bacteria from a subsample of Walsh soil (0-10 cm depth) that had been collected and air-dried in 2009, and then stored at room temperature for nearly five years prior to this study. ACC+ bacteria were selectively cultured from this subsample according to the method of Penrose and Glick (2003). In brief, 1 g of stored, air-dried soil was incubated in sterile *Pseudomonas* Agar-F (PAF) medium to selectively enrich pseudomonads and similar bacteria. A 1-mL aliquot of culture was then transferred to sterile Dworkin and Foster (DF) minimal salts medium containing $(NH_4)_2SO_4$ as the N source and incubated. After 24 h, a 1-mL aliquot was transferred into fresh sterile DF minimal salts medium containing 3.0 mM of ACC as the sole N source and incubated for 24 h. This culture was maintained in liquid DF minimal salts medium with ACC as the sole N source.

To determine the species composition of the culture, an aliquot of medium was centrifuged to pellet cells. DNA was extracted and the 16S rRNA gene was amplified and

pyrosequenced following the methods described by Zhu et al. (2016) and Stromberger et al. (2017). Dominant species included *Pseudomonas stutzeri* (95%), *Erwinia* sp. (2%), *Azomonas* sp. (1.5%), and other *Pseudomonas* sp. (1.5%).

Plant materials and seedling inoculation

Seven winter wheat genotypes (cultivars or advanced breeding lines developed in the U.S. Great Plains) were selected for this study: 'Byrd', 'Hatcher', OK06318, 'Ripper', 'RonL', 'TAM112', and 'WB Cedar' (Table 2.1). These genotypes were selected to achieve a range in drought sensitivities, as determined from previous field and greenhouse observations (Becker et al., 2016; P. Byrne, unpublished data; S. Haley, Colorado State University, personal communication). Seeds (~60) of each genotype were added to 250 mL Erlenmeyer flasks (1 flask per genotype) and immersed in 70% ethanol for 30 seconds and rinsed with sterile physiological saline (0.85% NaCl). The seeds were then immersed in 10% bleach (0.5% sodium hypochlorite final concentration) with a drop of Tween for 3 min and rinsed six times in sterile physiological saline. Seeds of each genotype were divided into sterile petri dishes, with ~30 in one dish for bacterial inoculation and ~30 in a second dish for a control.

The inoculum was grown overnight in fresh tryptic soy broth (TSB), after which the culture was centrifuged at 4,000 rpm for 10 min. The supernatant was removed and pelleted cells were resuspended in 45 mL of sterile physiological saline so that the final density of cells was ~ 5 x 10^8 cells mL^{-1}. Approximately 5 mL of inoculum was added into petri dishes of surface sterilized seeds. Sterile physiological saline was added in control petri dishes. The seeds were imbibed with the inoculum or sterile physiological saline for 1 h just prior to planting in root tubes (2 seeds per tube). Confirmation of inoculum colonization on roots of inoculated winter

wheat genotypes was assessed by quantitative PCR (qPCR) of the *acdS* gene using the method as described previously by Stromberger et al. (2017).

Experimental design and treatments

The experiment was performed in a greenhouse at Colorado State University (Fort Collins, CO) from December 2013 to February 2014. Plants were grown in polyvinyl chloride (PVC) root tubes (1 m tall × 10 cm diameter), as explained in Becker et al. (2016). For easy removal of root masses, tubes were lined with a polytube liner and filled with Greens Grade fritted clay (Profile Products LLC, Buffalo Grove, IL), a growing medium that allows entire root masses to be isolated and characterized.

The experiment consisted of two adjacent blocks of 60 tubes for water-stressed (WS) treatment and 59 tubes for well-watered (WW) treatment. The unbalanced design was due to plant death in one of the well-watered tubes. Within each irrigation treatment, genotype-inoculation combinations were distributed in a completely randomized design. Each tube was planted with two seeds, inoculated either with bacteria or sterile physiological saline as the control. Plants were grown under greenhouse conditions with a photoperiod of 16 h of light and 8 h of darkness, at a temperature range from 18.3 to 25.5 ^{o}C. Plants were irrigated daily until week 4, after which daily irrigation was stopped for tubes in the WS treatment. Three weeks later, plants were evaluated for RWC, above- and below-ground biomass, and root traits. Except where stated otherwise, all measurements refer to the sum of both plants in a tube.

Physiological and Morphological Measurements

Leaf relative water content was measured according to the method of Barrs and Weatherly (1962). Approximately 2-cm segments were excised from the fifth leaf of one plant per tube, and weighed to obtain the fresh weight (FW). The leaf segments were placed in

distilled water for 24 h at 4°C in darkness, after which the turgid weight (TW) was recorded. The dry weight (DW) was obtained after drying for 24 h at 70 °C. The RWC was then calculated as (FW– DW) / (TW– DW) × 100.

Above-ground biomass samples were collected at the termination of the experiment (Zadoks growth stage 31, jointing stage; Zadoks et al. 1974) and dried at 70 ^{o}C for at least 24 h prior to weighing. Following collection of the above-ground biomass, the root systems were removed from the polytube liners, washed free of growth medium, and measured for the longest seminal root length. The roots were divided into the top (0–33 cm depth), middle (34–66 cm depth), and bottom (67–99 cm depth) sections of the tube. Individual root sections were floated on approximately 1 cm of water in a 30 x 40.5 cm plexiglass tray and scanned with a MicroTek Scanmaker 9800XL (Microtek, Santa Fe Springs, CA). Digital images were analyzed for length and diameter with WinRhizo Regular software (Regent Instruments Inc., Quebec, Canada). Root morphology measurements recorded by WinRhizo included total root length, average root diameter, and root length of the following diameter classes: 0.00–0.25, 0.25–0.50, 0.50–0.75, 0.75 1.00, and >1.00 mm. Root sections were collected after scanning and dried at 68° C for at least 48 h prior to weighing to obtain root biomass weight.

Enumeration of ACC Deaminase-Positive Bacteria

The rhizosphere soil adhering to the root was collected from syringe tubes that were buried within root tubes in the greenhouse (see description in Chapter 3). Soil samples were mixed with water at 9ml/gram of soil, serially diluted and drop-plated on minimal media containing ACC as sole carbon source to enumerate the colony forming units (CFU) of ACC+ bacteria as described by Penrose and Glick (2003). Briefly, DF minimal medium was prepared as follows: trace elements (10mg H_3BO_3, 11.19 mg $MnSO_4 \cdot H_2O$, 124.6 mg $ZnSO_4 \cdot 7H_2O$, 78.22

mg $CuSO_4 \cdot 5H_2O$, and 10mg MoO_3) were dissolved in 100 mL sterile distilled water and then stored in the refrigerator prior to use. In addition, a solution containing $FeSO_4 \cdot 7H_2O$ (100 mg) was dissolved in 10 mL sterile distilled water and was stored in the refrigerator prior to use. The following ingredients (4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g glucose, 2.0 g gluconic acid, 2.0 g citric acid, and 18 g of Bacto-Agar, Difco), and 0.1 ml of each of the solutions of trace elements and $FeSO_4 \cdot 7H_2O$ were then dissolved in 1 L distilled water and autoclaved for 20 minutes. Finally, a 0.3 M solution of ACC in distilled water was filter sterilized and 100 µL of this solution was pipetted onto prepared plates and spread on the entire surface using a sterilized glass rod. The plates were inoculated with diluted soil suspensions using a drop-plating method, where 20 ul of the suspension is plated in a droplet and allowed to air dry, for enumeration of ACC deaminase-positive bacteria. Once inoculated, plates were stored in an incubator at 28°C for 3-10 days. After incubation period, the numbers of colony forming units (CFU) of the ACC+ bacteria were counted under water-stressed and well-watered conditions and multiplied by 5 to replicate colony forming units obtained from traditional spread-plate methods.

Statistical analysis

The greenhouse experiment was conducted as a completely randomized design. The statistical analysis was performed by three-way-ANOVA using SAS software version 9.2 PROC MIXED procedure (SAS Institute, Cary, NC). All treatment effects were considered as fixed. The differences among various treatment means were compared using Tukey HSD test. Significant differences were considered at probability of $P \leq 0.05$. The unequal variance model was employed for leaf relative water content, below- and above-ground biomass, total root length, and root length of diameter classes before the statistical analysis was applied to ensure

that the assumptions of the analyses of variance (ANOVA) were not violated. Pearson phenotypic correlation analysis was employed using SAS PROC CORR (SAS Institute, Cary, NC) to assess the significance of all pairwise trait correlations.

Results

Effect of ACC+ bacterial inoculation on leaf relative water content (RWC)

Analysis of variance revealed that leaf RWC was affected by irrigation condition, inoculation, and irrigation x inoculation interaction (Table 2.2). Water stress caused ~ 60% reduction in leaf RWC of winter wheat genotypes (Supplemental Figure S2.1). However, wheat inoculated with ACC+ bacteria had greater average leaf RWC compared to non-inoculated controls under water-stressed condition (Figure 2.1). By contrast, the genotypes showed no significant differences in their relative water content when inoculated with ACC+ bacteria under well-watered condition. Although there was no significant genotype x inoculation interactions (Table 2.2), the individual responses of wheat genotype to inoculation appeared to vary; for example, the difference in leaf RWC between inoculated and non-inoculated plants tended to be greater in some genotypes, such as 'RonL' and 'OK06318', than others like 'WB Cedar' and 'Byrd' (Figure 2.1).

Effect of ACC+ bacteria on above- and below-ground biomass

Above-ground biomass was significantly affected by irrigation conditions, genotype, and the genotype x inoculation interaction (Table 2.2). As expected, significant variation among genotypes was detected for aboveground biomass under water-stressed treatment compared to well-watered treatment (Supplemental Figure S2.2).

In addition, inoculation with ACC+ bacteria significantly increased aboveground biomass of certain wheat genotypes, named RonL and TAM112 by 25 and 23% respectively, as compared to uninoculated controls, irrespective of irrigation treatments (Figure 2.2).

The results of the study further showed that there were significant ($P \leq 0.05$) genotype and inoculation x genotype interaction on root biomass in the bottom section (67-99 cm depth) of the root tubes (Table 2.2). Irrespective of soil water status, inoculation with ACC+ bacteria considerably increased RonL root biomass by 145% in the bottom tube section, compared to the non-inoculated control (Figure 2.3).

Effect of ACC+ bacterial inoculation on the total root length and root length of diameter classes

Analysis of variance revealed that total root length varied among genotypes and irrigation conditions (Table 2.2). According to the results of the present study, genotypes varied significantly throughout the entire root tube sections under water-stressed condition as compared to well-watered condition (Supplemental Figure S2.3). The results further indicated that there was no significant effect of inoculation or inoculation x genotype interaction on total root length in any of the root tube sections (Table 2.2). Analysis of variance revealed significant differences of genotypes on root lengths within different root diameter size classes in the bottom tube section under water-stressed condition, except for 0.00-0.25 mm in diameter (Supplemental Table S2.1). In addition, there were significant ($P \leq 0.05$) genotype and inoculation x genotype interaction effects on root length within the 0.50-0.75 mm diameter class in the bottom section (67-99 cm depth) of the root tubes (Table 2.3). Regardless of irrigation condition, inoculation with ACC+ bacteria significantly increased root length within the 0.50-0.75 mm diameter class for RonL, in the bottom tube section, by 44% when compared to non-inoculated control (Figure 2.4).

Although there were no significant effect of genotype x inoculation interaction on root length in the 0.75-1 mm root diameter class in the bottom section (67-99 cm depth) of the root tubes (Table 2.3), inoculation with ACC+ bacteria increased RonL root length by 129% in this root diameter class under water-stressed condition, as compared to the non-inoculated control (Figure 2.5). Pearson's correlation analysis revealed that nearly all root traits were significantly ($P \leq 0.05$) and positively correlated with above-ground biomass when inoculated with ACC+ bacteria under water-stressed conditions (Table 2.4). Root traits were generally not correlated with leaf RWC, however.

Counts of ACC Deaminase-Positive Bacteria

At the end of the experiment the numbers of ACC+ bacteria on soil samples were enumerated from soil samples from both water-stressed and well-watered treatments. Results detected a significantly higher number of ACC+ bacteria in soils that had been intentionally inoculated relative to those that were non-inoculated (Table 2.5). In addition, the number of ACC+ bacteria was more than doubled under well-watered conditions relative to water stressed soils.

Discussion

The current study hypothesized that ACC+ bacteria would reduce the sensitivity of root and leaf growth to water stress among certain wheat genotypes, consequently allowing wheat to utilize soil moisture from deeper profiles, improve root proliferation, and ultimately contribute to improved leaf water status in the plant. The current study partially supported this hypothesis. The water stress treatment had drastic effects on growth of winter wheat genotypes. However, wheat inoculated with ACC+ bacteria resulted in significant protection against water-stress, as evidenced by greater leaf RWC across all genotypes. Moreover, in the case of the genotype

RonL, greater root length of certain root diameter size classes (0.50-0.77 mm and 0.75-1 mm) was observed in the bottom section of the root tubes when inoculated with ACC+ bacteria. Inoculation also significantly increased root biomass in the bottom tube section, for RonL, compared to non-inoculated control, regardless of soil water status. Furthermore, our results showed that inoculation with ACC+ bacteria significantly increased above-ground biomass for RonL and TAM112, regardless of irrigation conditions.

Ashraf et al. (1994) reported that wheat genotypes with greater leaf RWC were more water-stress tolerant in comparison to those with lower RWC. In the present study, variation among the genotypes in leaf RWC was observed when inoculated with ACC+ bacteria and subjected to water stress (Figure 1). Specifically, RonL and OK06318 had greatest leaf RWC when inoculated with ACC+ bacteria. Overall, RonL was most responsive among the genotypes to ACC+ bacterial inoculation, in terms of above-ground and below-ground biomass and root length production. For RonL, greater water-stress tolerance may be due to the enhanced growth response of deep roots, in response to inoculation with ACC+ bacteria, and presumably greater ability of RonL to acquire water at depth. However, other mechanisms may be at play, as inoculation increased leaf RWC in several genotypes, including OK06318, without affecting root biomass and length. It is known that PGPR containing the enzyme ACC deaminase could confer resistance to water stress in inoculated plants by a variety of mechanisms, such as by improving the activity of antioxidant enzymes (Habib et l., 2016), which suppress diverse plant diseases compared to strains without this enzyme. For instance, transformation of ACC deaminase genes into *Pseudomonas fluorescens* strain CHA0 exhibited a significant reduction in disease symptoms by protecting cucumber against Pythium damping-off and potato tuber against *Erwinia* soft rot (Wang et al., 2000).

Other studies have shown that inoculation of plants with ACC+ bacteria can stimulate above-ground biomass and root growth, irrespective of soil water regime. For example, inoculation with ACC+ bacteria resulted in increased above-ground biomass of wheat seedlings (Belimov et al., 2009a) and root biomass of pea and sunflower seedlings (Dodd et al., 2004; Belimov et al., 2009a; Sandya et al., 2009). In contrast, others reported that root elongation response to ACC+ bacterial inoculation was dependent on soil moisture regime, or that inoculation did not affect root biomass or elongation. Studies conducted on crops including tomatoes (*Solanum lycopersicum* L.), peppers (*Capsicum annuum* L.), and peas (*Pisum sativum* L.) found that inoculation with ACC+ bacteria resulted in significant increases in root elongation under water stress (Mayak et al., 2004; Arshad et al., 2008; Zahir et al., 2008, 2009; Belimov et al., 2009a), whereas Jiang et al. (2012) found no effect of inoculation with ACC+ bacteria on root length of pea. These contrasting results might be due to differences in PGPR strains used in these experiments, as PGPR bacteria display varied capability to enhance plant growth due to the variation in ACC deaminase activity (Penrose and Glick, 2003; Shaharoona et al., 2006) or by the variations in plant genotypes studied, and variations in root exudate compounds secreted by these varieties and their ability to recruit and assist in colonization of particular species of ACC+ bacteria (Belimov et al., 2009b).

Following germination, the capability of deep root growth diameters in maintaining plant productivity under water stress conditions, particularly at depths in the soil profile might promote water acquisition when water at depth is available (Arshed et al., 2008; Comas et al., 2013). With respect to root length of diameter classes, the results indicated that root length within specific diameter classes (0.50-0.75 and 0.75-1 mm) for RonL genotype in the bottom tube section was significantly increased in response to inoculation with ACC+ bacteria, regardless of soil water

status. By contrast, Jiang et al. (2012) reported that inoculation with ACC-deaminase containing rhizobacterium *Variovorax paradoxus* 5C-2 had no significantly effects on root length distribution according to diameter class.

The intention of the inoculum development procedure was to selectively enrich a bacterial population capable of long-term survival in dry soil and degrading ACC. This was achieved by using a soil sampled from an area of Colorado (Walsh) that experiences a large water deficit in most growing seasons, relative to other areas of Colorado. Furthermore, the soil was stressed by being stored under air-dried conditions for nearly five years prior to culturing, further creating a selective pressure on the bacterial community. The criteria for ACC deaminase activity was selected by culturing bacteria in the presence of ACC as the soil source of organic N. While this method may also select for N_2 fixing bacteria in theory, the culturing conditions likely limited the selection of bacteria utilizing N_2 as their preferential source of N (rapid growth in the presence of abundant organic N and O_2). There was no intention to develop a pure culture of a single ACC+ bacterial species. Nevertheless, the enrichment procedure (long-term storage at air-dried conditions, followed by medium enrichment) resulted in a mixed culture that was dominated largely by one species, *Pseudomonas stutzeri*. This particular species is a soil bacterium with known ACC deaminase activity (Govindasamy et al., 2008), genes involved in osmotic protection, and the capacity to colonize the surface and superficial layers of rice and wheat roots (Yan et al., 2008). It also has several plant-growth promoting properties, including siderophore production, P solubilization, indole acetic acid production (Trivedi et al., 2011), N_2 fixation under low O_2 concentration (Yan et al., 2008), and oxidative stress resistance (Rediers et al., 2003).

As hypothesized, genotypes varied significantly in their above- and below-ground responses to water stress. Within different genotypes, the genotype Hatcher was able to achieve high mean values for nearly all investigated traits under water stress treatment, indicating that Hatcher displayed relative tolerance to water stress, compared to the other genotypes studied. On the other hand, the genotype 'RonL' displayed the highest sensitivity to water stress conditions in most investigated traits, including root biomass and root length. However, the results also showed that among all winter wheat genotypes studied, RonL showed significant increases in root biomass and root length of diameter classes 0.50-0.75 and 0.75-1 mm in the bottom tube section when inoculated with ACC+ bacteria, increasing by 145%, 44%, and 129%, respectively, regardless of soil water regime. Perhaps not coincidentally, RonL was shown to accumulate greater relative percentages of ACC+ bacteria, and had greater ACC deaminase activity in its rhizosphere, compared to other winter wheat genotypes grown under field conditions (Stromberger et al., 2017). Similarly, Safronova et al. (2006) and Chen et al. (2013) demonstrated that inoculation effects with PGPR containing ACC deaminase were dependent on plant genotype. Given the potential for genotype-specific responses to inoculation, Moutia et al. (2010) reported that the plant genotype should be considered when recommending bacterial inoculation for improved plant growth. Accordingly, utilization of ACC+ bacteria is currently attracting considerable attention among researchers as a means to improve plant growth and development under stress and non-stressed conditions (Saleem et al., 2007; Singh et al., 2011), but the effectiveness of ACC+ bacterial inoculation technology may likely be dependent on the genotype grown.

Conclusion

The study presented here demonstrated the effectiveness of ACC+ bacteria for inducing water stress tolerance and consequently improving the growth of winter wheat genotypes under water-stressed condition. However, the ability of ACC+ bacteria to promote water stress tolerance was genotype-specific. This variation in growth promotion effects among genotypes might be either due to a unique root exudate profile for certain wheat genotypes or by the genotypic differences among the genotypes and their abilities to preserve relatively large proportions of ACC+ bacteria in the rhizosphere, and should be further examined. According to the results obtained, inoculation of certain wheat genotypes with ACC+ bacteria has the potential to improve root growth in the bottom tube section (67-99 cm depth) and presumably improve water uptake from deep soil layers, thereby reducing the deleterious effects of water stress on the growth of wheat genotypes. However, a better understanding of this variation and how it improves root acquisition of water at depth to increase crop productivity under water stress is needed.

Figures

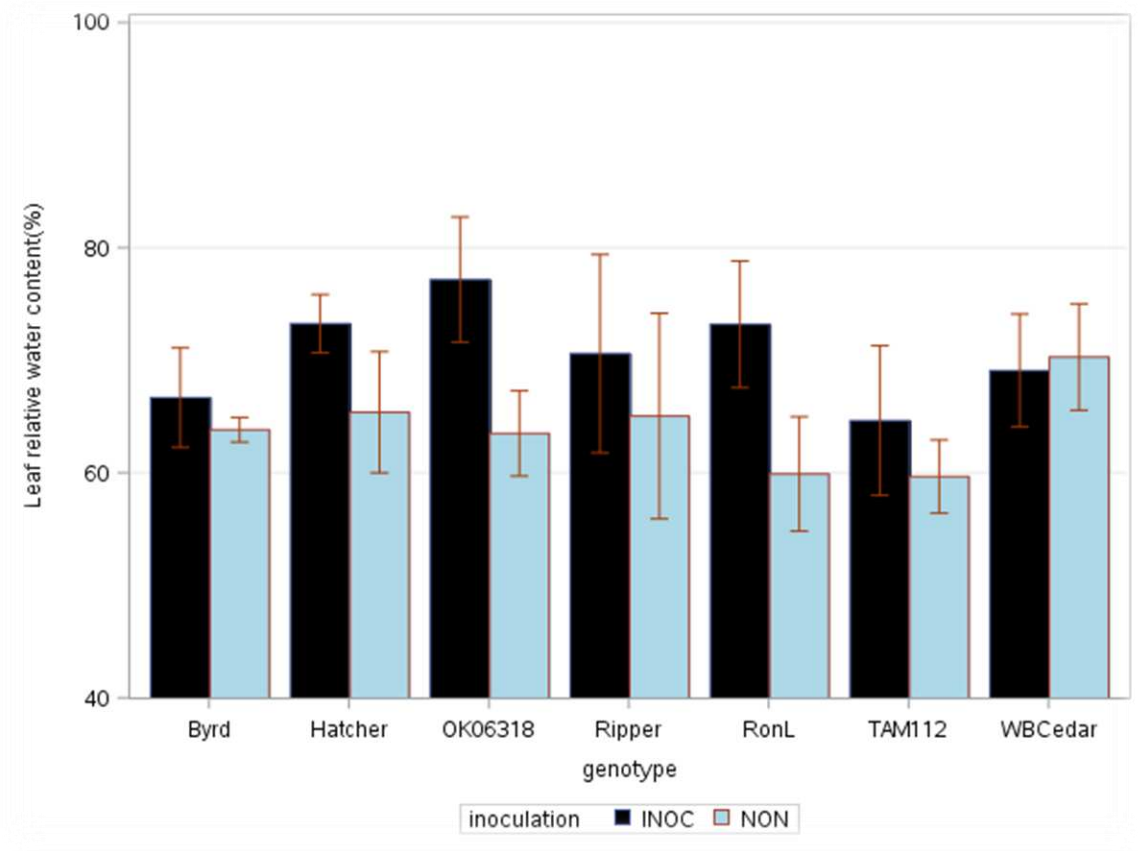


Figure 2.1. Relative leaf water content of seven winter wheat genotypes, grown under water-stressed (WS) conditions in the greenhouse, with or without inoculation by ACC+ bacteria. Bars represent the mean $\pm$ 1 SE (n=3-5).

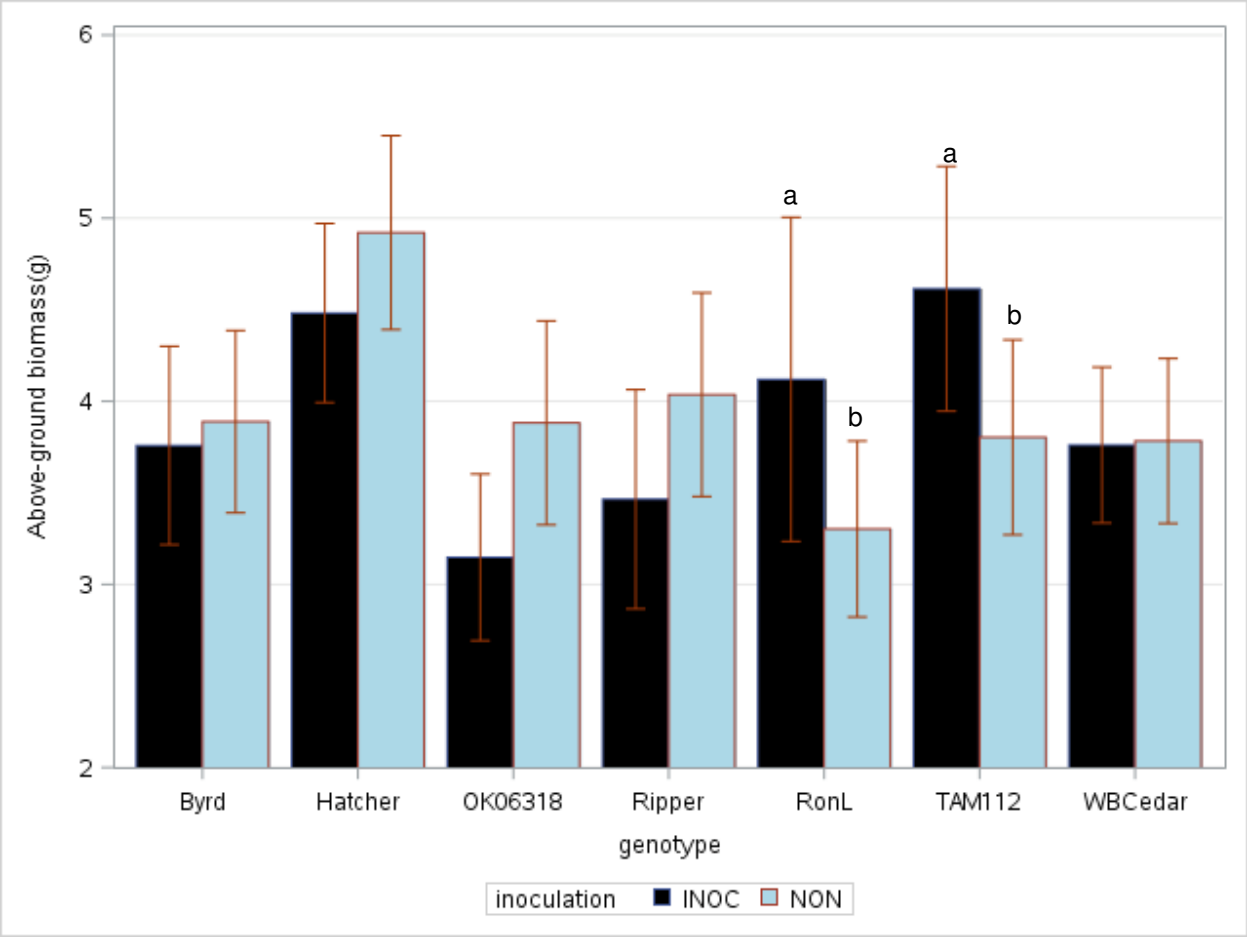


Figure 2.2. Aboveground biomass of seven winter wheat genotypes, grown in the greenhouse, with or without inoculation by ACC+ bacteria, averaged across irrigation treatments. Bars represent the mean $\pm$ 1 SE (n=6-10). Within each genotype, bars with different letters indicate significant differences between inoculated (INOC) and non-inoculated (NON) treatments according to Tukey's test at $P \leq 0.05$.

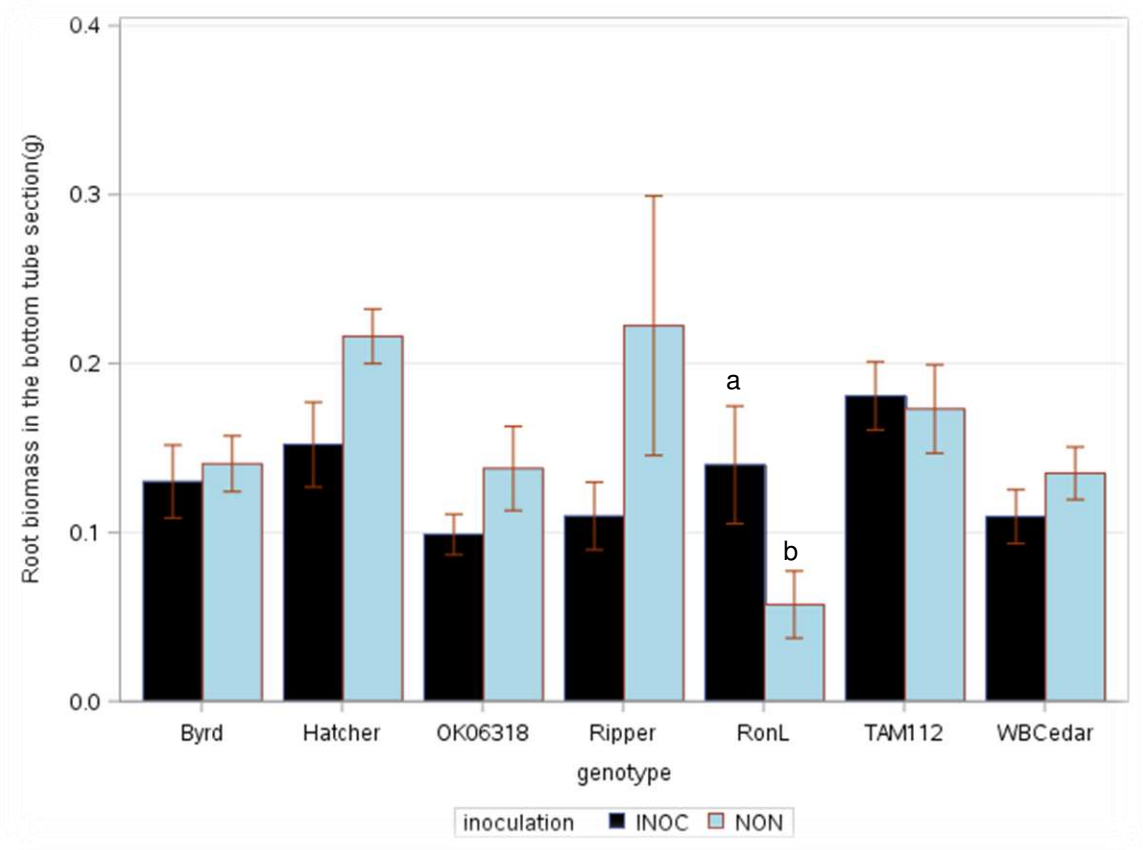


Figure 2.3. Root biomass of the bottom tube section (67-99 cm depth) of seven winter wheat genotypes, grown in the greenhouse, with or without inoculation by ACC+ bacteria, averaged across irrigation treatments. Bars represent the mean $\pm$ 1 SE (n=6-10). Bars with different letters indicate means that are significantly different at $P \leq 0.05$ according to Tukey's test.

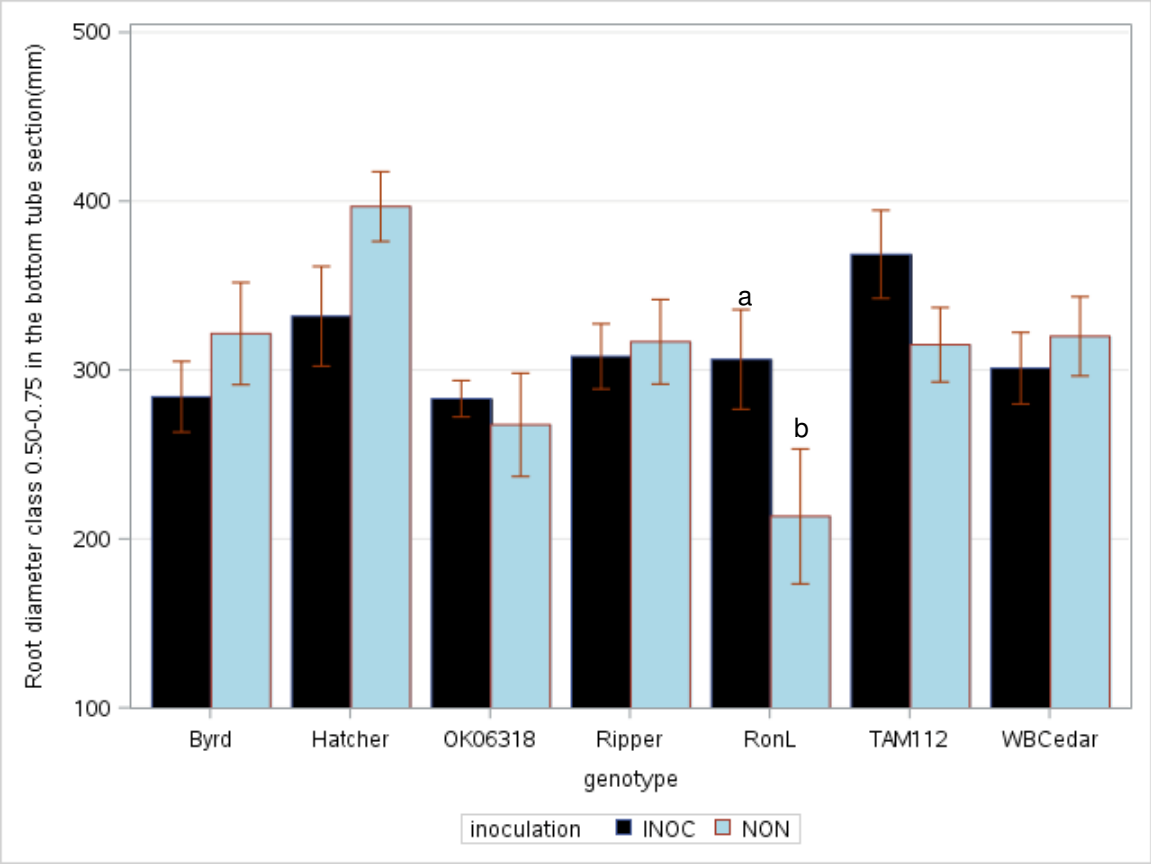


Figure 2.4. Root length of the diameter class 0.50-0.75 mm in the bottom tube section (67-99 cm depth) of seven winter wheat genotypes, grown in the greenhouse, with or without inoculation by ACC+ bacteria, averaged across irrigation treatments. Bars represent the mean $\pm$ 1 SE (n=6-10). Bars with different letters indicate means that are significantly different at $P \leq 0.05$ according to Tukey's test.

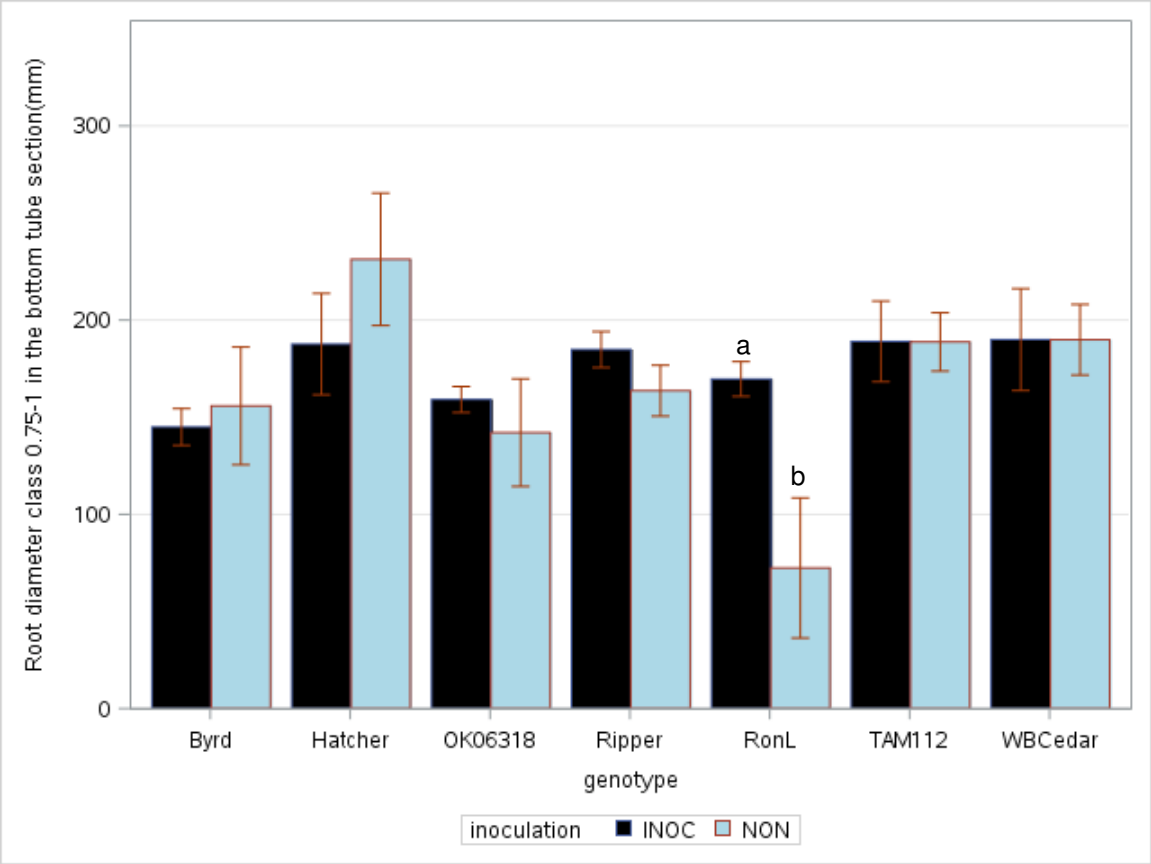


Figure 2.5. Root length of the diameter class 0.75-1 mm in the bottom tube section (67-99 cm depth) of seven winter wheat genotypes, grown under water-stressed (WS) conditions in the greenhouse, with or without inoculation by ACC+ bacteria. Bars represent the mean $\pm$ 1 SE (n=3-5). Bars with different letters indicate means that are significantly different at $P \leq 0.05$ according to Tukey's test.

Tables

Table 2.1. Wheat genotypes used in this study.

Genotype Name	GRIN Identifier	Year Derived	Year Released	Developer	Reference
Byrd	PI 664257	2006	2011	CSU	Haley et al., 2012
Hatcher	PI 638512	1998	2004	CSU	Haley et al., 2005
OK06318	NA	2006	NA	OSU	NA
Ripper	PI 644222	2000	2006	CSU	Haley et al., 2007
RonL	PI 648020	2003	2007	KSU	Martin et al., 2007
TAM112	PI 643143	1998	2007	TAMU	Rudd et al., 2014
WB Cedar	NA	NA	NA	WB	NA

PI, Plant Introduction, USDA, USA; GRIN, Germplasm Resources Information Network; CSU, Colorado State University; OSU, Oklahoma State University; KSU, Kansas State University; TAMU, Texas A&M University; WB, WestBred; NA, Not available

Table 2.2. *F*-and *P*-values of three-way ANOVA for leaf relative water content (RWC), above- and belowground biomass, and root length of root tube sections of seven winter wheat genotypes, grown under greenhouse conditions, with full or limited irrigation, and with or without ACC+ bacterial inoculation.

Effect	RWC		Above-ground biomass		Root biomass in three tube sections						Root length in three tube sections					
					Bottom (67-99 cm)		Middle (34-66 cm)		Top (0-33 cm)		Bottom (67-99 cm)		Middle (34-66 cm)		Top (0-33 cm)	
	F value	P value	F value	P value	F value	P value	F value	P value	F value	P value	F value	P value	F value	P value	F value	P value
Main effects																
Irrigation	303.24	***	345.85	***	0.04	ns	0.07	ns	344.32	***	0.11	ns	3.01	ns	511.12	***
Inoculation	5.05	*	0.00	ns	2.03	ns	1.25	ns	0.32	ns	0.17	ns	0.60	ns	0.64	ns
Genotype	0.70	ns	4.48	***	3.97	**	2.13	ns	1.55	ns	2.49	*	3.80	**	2.17	ns
Two-way interactions																
Irrigation*inoculation	4.91	*	0.89	ns	1.43	ns	1.81	ns	1.40	ns	0.00	ns	0.10	ns	0.67	ns
Irrigation*Genotype	0.52	ns	0.62	ns	1.87	ns	0.54	ns	0.44	ns	1.54	ns	1.10	ns	0.67	ns
Inoculation* Genotype	0.48	ns	2.47	*	2.45	*	1.10	ns	1.92	ns	1.37	ns	0.43	ns	0.35	ns
Three-way interactions																
Irrigation*Inoculation*Genotype	0.57	ns	1.54	ns	1.55	ns	0.97	ns	1.73	ns	0.36	ns	0.99	ns	0.63	ns

Significant effects are indicated by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and ns, not significant.

Table 2.3. *F*-and *P*-values of three-way ANOVA for root diameter classes (mm) in the bottom tube section of seven winter wheat genotypes, grown under greenhouse conditions, with full or limited irrigation, and with or without ACC+ bacterial inoculation.

Effect	Root diameter classes (mm) in the bottom tube section									
	0.00-0.25		**0.25-0.50**		**0.50-0.75**		**0.75-1**		**>1**	
	F-value	*P*-value	F-value	*P*-value	F-value	*P*-value	F-value	*P*-value	F-value	*P*-value
Main effect										
Irrigation	0.12	ns	1.95	ns	0.39	ns	0.10	ns	0.74	ns
Inoculation	0.18	ns	0.03	ns	0.17	ns	1.00	ns	0.05	ns
Genotype	1.03	ns	2.90	**	3.86	***	3.38	**	3.23	**
Two-way interactions										
Irrigation*inoculation	0.14	ns	0.07	ns	0.03	ns	0.11	ns	2.62	ns
Irrigation*Genotype	0.68	ns	2.02	ns	1.69	ns	1.82	ns	1.12	ns
Inoculation* Genotype	1.01	ns	1.77	ns	2.17	*	1.60	ns	1.37	ns
Three-way interactions										
Irrigation*Inoculation* Genotype	0.49	ns	0.44	ns	0.47	ns	0.62	ns	0.82	ns

Significant effects are indicated by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and ns, not significant

Table 2.4. Correlations coefficients (*r*) between root traits in the bottom tube section (67-99 cm depth) of winter wheat genotypes inoculated with ACC+ bacteria and grown under water-stressed conditions in the greenhouse (n=30).

Root trait	BRD1	BRD2	BRD3	BRD4	BRD5	RLB	RBB	AGB	RWC
BRLD1	-	0.83***	0.64***	0.50**	0.48**	0.91***	0.64***	0.52**	-0.28
BRLD2		-	0.88***	0.74***	0.75***	0.97***	0.88***	0.61***	-0.23
BRLD3			-	0.94***	0.81***	0.87***	0.82***	0.53**	-0.21
BRLD4				-	0.84***	0.75***	0.70***	0.44**	-0.15
BRLD5					-	0.74***	0.74***	0.35	0.09
RLB						-	0.83***	0.59***	-0.24
RBB							-	0.67***	-0.34
AGB								-	0.45**
RWC									-

BRLD1-BRLD5: root lengths of 5 diameter classes in the bottom tube section. BRLD1, 0-0.25 mm diameter; BRLD2, 0.25-0.50 mm diameter; BRLD3, 0.50-0.75 mm diameter; BRLD4, 0.75-1 mm diameter; BRLD5, >1 mm diameter; RLB: total root length in the bottom tube section, RBB: root biomass in the bottom tube section, AGB: above-ground biomass, RWC: leaf relative water content. Significant correlations are indicated as follows: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Table 2.5. Colonization of winter wheat (*Triticum aestivum*) roots by ACC+ bacteria under well-watered and water-stressed conditions.

ACC+ bacteria/treatment	Number of ACC+ bacteria (CFU/g rhizosphere soil)	
	Well-watered (WW)	Water-stressed (WS)
Inoculation	1182.6±462.2	518.5±136.6
Non-inoculation	86.7±14.5	ND

Values represent means ± SE. ND; Not detected

CHAPTER 3: DETECTION OF GLOBAL RHIZOPHERE METABOLITE PROFILES DISCRIMINATE WATER STRESS AND WELL-WATERED REGIMES IN WINTER WHEAT GENOTYPES

Summary

Metabolomics, the comprehensive profiling of small molecules in a biologic sample, can provide insight into how multiple components of an ecosystem interact under varying conditions. In the present study, we examined rhizosphere metabolite profiles to determine how root exudates, soil nutrients, and microbial metabolite profiles differ by plant genotype and under varying physiological conditions (ie. water stress and inoculation with ACC+ bacteria). In particular, we aimed to identify whether specific root exudate chemicals were associated with improved resistance to water stress. Root exudates from three winter wheat genotypes (*Triticum aestivum* L.), under well-watered or water stress conditions and with or without inoculation by ACC-deaminase positive bacteria, were collected and analyzed for global metabolite profile differences. Due to a wide array of chemical properties of metabolites, profiling was conducted using both GC-MS and LC-MS (negative and positive ionization modes) platforms to enhance coverage of chemical compounds detected. Multivariate statistical analyses were used to identify metabolites associated with each of the test conditions. Metabolite profiles were most influenced by irrigation regime, with global differences between water stressed and well-watered plants as evidenced from both unsupervised (PCA) and supervised (OPLS-DA) ordination plots of the data. Cross validation score plots, p CV-ANOVA values, and permutation test were performed for their further validities in this study. The results of such validation analyses confirmed the class separation for irrigation treatments (WS and WW) and indicated the stability and reliability of OPLS-DA model. By analyzing S-plots and VIP >1 of OPLS-DA model generated with the GC-MS platform, we identified metabolites important in discriminating between irrigation

treatments. Exudates from well-watered plants had higher levels of phosphate in the soil compared to water stressed plants. Under water-stressed, the discrimination was driven by metabolites such as succinic acid, lactic acid, and an indazol-amine-like compound. Organic acids that are more abundant in the water stressed soils may be assisting in mobilization of phosphates, explaining the distribution of these metabolites under our experimental conditions. In addition, succinic acid, a microbial fermentation product, was higher in extracts from the historically drought tolerant cultivar RonL, when inoculated with ACC+ bacteria and under water-stressed conditions. We suggest that ACC+ bacteria may secrete this compound, which could play a role in plant nutrient acquisition and growth regulation. These data demonstrate that metabolomic profiling is a valuable tool for generating specific hypotheses related to novel mechanisms of plant-microbe interactions for mitigation of water stress in winter wheat.

Introduction

Plant roots secrete a wide variety of chemical compounds into the rhizosphere. These compounds are broadly referred to as root exudates. Plant roots exude approximately 5%-21% of total photosynthetically fixed carbon into the rhizosphere via exudation (Badri and Vivanco, 2009; Chaparro et al., 2014). These plant metabolites can typically be classified into two categories of compounds: 1) low molecular weight compounds such as amino acids, organic acids, and sugars and 2) high molecular weight compounds such as proteins and mucilage (Bais et al., 2006; Badri and Vivanco, 2009). Plant roots can also release protons, oxygen, and water (Bertin et al., 2003; Bais et al., 2006; Hartmann et al., 2009). It is generally acknowledged that the composition and quantity of root exudates is influenced by plant species, plant growth stage, and environmental factors like biotic and abiotic stresses (Badri and Vivanco, 2009; Lou et al., 2017). Henry et al. (2007) demonstrated that nutrient and water stress caused significant changes

on the quantity and composition of root exudates of crested wheatgrass (*Agropyron cristatum*). There is evidence to suggest that root exudates also vary considerably between plant cultivars within a species (Wu et al., 2001; Aira et al., 2014; Huang et al. 2014). For instance, Wu et al (2001) demonstrated variability in seven different root-excreted phenolic acids across 58 different wheat accessions.

Root exudation, also known as rhizodeposition, influences plant growth and soil ecology, and contributes to rhizospheric processes such as nutrient acquisition and pathogen protection (Bertin et al., 2003). Root exudates play an important indirect role in resource competition by mediating changes to soil chemistry and stimulating rhizosphere microbial communities (Bais et al., 2006; Shukla et al., 2011). Root exudates are a significant contributor to the diversity and activity of soil microorganisms, which subsequently can produce metabolites that influence plant growth and health (Shi et al., 2011).

Bertin et al. (2003) reported that secretion of root exudates modifies the species composition of rhizosphere microorganisms by secreting chemoattractant compounds that are important in establishment, colonization, and maintenance of rhizosphere microbial communities (Raja et al., 2006). For example, there is evidence that root exudates of maize release secondary metabolites such as 2,4- dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA) that recruit and attract plant beneficial rhizobacteria through the relatively young and vulnerable growth stages (Neal et al., 2012). In turn, plant growth promoting rhizobacteria produce metabolites that stave off pathogen infections and stimulate plant growth (Yoshikawa et al., 1993; Gouda et al., 2017)

Soil microorganisms can also influence the composition and quantity of root exudates (Huang et al., 2014). For instance, Matilla et al. (2010) reported that *Pseudomonas putida*

KT2440 induced changes in root exudate profiles of *Arabidopsis thaliana* when compared to exudates from plants that were not exposed to *Pseudomonas putida* KT2440. In addition, wheat root exudates induced changes in the lipopolysaccharide profile of *Azospirillum brasilense* Cd both under normal and saline stress conditions (Fischer et al., 2003). Finally, soil microbes can impact root exudates found in the soil by degrading and metabolizing exudate components (Faure et al., 2009). Plant roots also secrete metabolites utilized as carbon source that attract beneficial soil microorganisms which function as suppression of plant disease directly by the production of antimicrobial compounds or indirectly by the induction of plant stress resistance (Haichar et al., 2014).

Based on the bidirectional interactions between plants and soil microbes, signaling mediated by root exudates and microbial metabolites may play a role in determining winter wheat cultivar-specific responses to water stress. In this exploratory study, we examined whether the type and quantity of root-associated metabolites would vary by cultivar and treatment variables. To this end, we established a unique collection system to capture root-associated metabolites (includes root exudates and microbial products) from greenhouse grown winter wheat cultivars.

The objectives of the present study were (1) to determine how root-associated metabolite profiles differed by plant genotype and inoculation status, and in response to water stress, (2) to identify specific metabolites that can be further explored to determine their role in improved resistance to drought stress. Specifically, we hypothesized that water stress would be an important factor in determining root exudate profiles. We also hypothesized, based on previous studies suggesting that RonL displays increased colonization of ACC-deaminase positive bacteria, that chemicals associated with inoculated RonL may contribute to protection against

water stress. Finally, we describe a novel method of root exudate collection that more closely mimics actual plant growth conditions in the soil. One of the major challenges of studying root exudates is mimicking actual plant growth situations (ie. field or greenhouse) and accessing the rhizosphere without disturbing or damaging plant root systems (Phillips et al., 2008). Many in vitro studies examining root exudates rely on growth of plants in sucrose-rich liquid medium and maintained under sterile and aerated conditions. Field and greenhouse study exudate profiles have utilized soil extraction methods, which may bias against exudates that adhere to soil particles or are rapidly modified/degraded.

Here, we describe a unique collection approach that allowed us to grow plants in a standard greenhouse substrate (fritted clay) while allowing for collection of exudates in inert silica tubes to obtain a greater understanding of root exudate profiles of winter wheat genotypes under greenhouse conditions. We utilized a non-targeted metabolomics approach, which is becoming one of the most important tools for analysis and identification of a wide array of different plant metabolites and root exudates. To maximize coverage of different types of chemical compounds, we used gas and liquid chromatography-coupled with mass spectrometry (GC/MS; LC/MS).

Material and Methods

Plant cultivars and experimental conditions

For this study, three winter wheat genotypes were selected: 'Byrd', 'OK06318' and 'RonL'. These genotypes were selected based upon the variation in response to inoculation with ACC+ bacteria under water stress through the maintenance of leaf relative water content and other traits (Chapter 2; Stromberger et al., 2017). Growth conditions and inoculation methods are reported in Chapter 2.

Rhizosphere metabolite collection

Root exudates and other rhizosphere metabolites were collected in 60 mL syringes filled with inert sand and capped with glass wool. Syringes were buried at 15-30 cm depth in root tubes filled with a fritted clay a growing medium (Figure 3.1). Briefly, sterilized or inoculated seedlings of different winter wheat cultivars were planted in the root tubes and allowed to grow for 7 weeks as described in Chapter 2. As the plants matured, sections of the roots grew into the buried syringes containing silica (sand) and plugged with glass wool. This allowed the plants to grow normally during the experimental period but provided a means for collecting root metabolites from a medium more amenable to extraction. At termination of the experiment, the syringes were collected and those containing roots were extracted by addition of 20 mL sterile distilled water per syringe, which was then vacuum filtered into 50 mL glass tubes (Figure 3.2). The collected material was lyophilized, extracted with 80% MeOH, concentrated under N_2 and resuspended in 100% MeOH for ultra high pressure liquid chromatography-coupled with mass spectrometry (UPLC-MS) or derivitized prior to analysis by gas chromatography coupled with mass spectrometry (GC-MS) at the Proteomics and Metabolomics Facility at Colorado State University.

GC-MS Analysis

Extracts were dried using a speedvac, resuspended in 50 µL of pyridine containing 50 mg/mL of methoxyamine hydrochloride, incubated at 60ºC for 45 min, sonicated for 10 min, and incubated for an additional 45 min at 60ºC. Next, 25 µL of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Thermo Scientific) was added and samples were incubated at 60 ºC for 30 min, centrifuged at 3000xg for 5 min, cooled to room temperature, and 80 µL of the supernatant was transferred to a 150 µL

glass insert in a GC-MS autosampler vial. Metabolites were detected using a Trace GC Ultra coupled to a Thermo ISQ mass spectrometer (Thermo Scientific). Samples were injected in a 1:10 split ratio twice in discrete randomized blocks. Separation occurred using a 30 m TG-5MS column (Thermo Scientific, 0.25 mm i.d., 0.25 μm film thickness) with a 1.2 mL/min helium gas flow rate, and the program consisted of 80 ºC for 30 sec, a ramp of 15 ºC per min to 330 ºC, and an 8 min hold. Masses between 50-650 m/z were scanned at 5 scans/sec after electron impact ionization (Fleischmann et al., 2017). The ionization source was cleaned and retuned and the injection liner replaced between injection replicates.

UPLC-MS Analysis

UPLC-MS analysis was performed as previously described (Heuberger et al., 2014) with slight modifications. Briefly, a volume of 100 uL of extract was transferred to an autosampler vial, and 3 μL of extract was injected twice (n=2 technical replicates) onto a Waters Acquity UPLC system in discrete, randomized blocks, and separated using a Waters Acquity UPLC CSH Phenyl column (1.7 μM, 1.0 x 100 mm). Separation was achieved by using a gradient from solvent A (water, 0.1% formic acid) to solvent B (Methanol, 0.1% formic acid). Injections were made in 98% A, held at 100% A for 0.2 min, ramped to 40% B over 0.9 minutes, 70% over two minutes, and 98% B over 8 minutes 98% B was held for 6 minutes, and then returned to starting conditions over 0.05 minutes and allowed to re-equilibrate for 5 minutes, with a 140 μL/min constant flow rate. The column and samples were held at 50 °C and 5 °C, respectively. The column eluent was infused into a Waters Xevo G2 Q-TOF-MS with an electrospray source in positive mode, scanning 50-1200 m/z at 0.2 seconds per scan, alternating between MS (6 V collision energy) and MSE mode (15-30 V ramp). Calibration was performed using sodium

formate with 1 ppm mass accuracy. The capillary voltage was held at 2200 V, source temp at 150 °C, and nitrogen desolvation temp at 350 °C with a flow rate of 800 L/hr.

Data Analysis and Statistics

Non-targeted Data acquisition (GC-MS and UPLC-MS):

For each sample, raw data files were converted to .cdf format, and a matrix of molecular features, as defined by retention time and mass (m/z), was generated using XCMS software in R (Smith et al., 2006) for feature detection and alignment. Raw peak areas were normalized to total ion signal in R, outlier injections were detected based on total signal and PC1 of principle component analysis, and the mean area of the chromatographic peak was calculated among replicate injections (n=2). Features were grouped based on a novel clustering tool, RAMClustR (Broeckling et a., 2014), which groups features into spectra based on co-elution and covariance across the full dataset, whereby spectra are used to determine the identity of observed compounds in the experiment. Compounds were annotated based on spectral matching to in-house, NISTv12, Golm, Metlin, and Massbank metabolite databases (Heuberger et al., 2014). The peak areas for each feature in a spectrum were condensed via the weighted mean of all features in a spectrum into a single value for each compound. The confidence levels of an annotation were reported based on the guidelines provided by the Metabolites Standards Initiative (Sumner et al., 2007).

For multivariate statistical analyses, root exudate metabolite profiles were visualized by principal components analysis (PCA) and orthogonal projection to latent structures - discriminate analysis (OPLS-DA). PCA unsupervised method, it is a statistical procedure based on no *a priori* knowledge of class membership. While OPLS-DA is a supervised, class-based method where class membership is assigned and aimed to obtain maximum data separation (Matthews et al.,

2012; Robotti et al., 2014). The data set was input into SIMCA version 14.1 software package (Umetrics AB, Umea, Sweden). All variables were pareto scaled (Par), which uses the square root of the standard deviation as a scaling factor. A loadings S-plot and variable importance in the projection (VIP) derived from the OPLS-DA model were employed to identify metabolites that contributed to discrimination between the treatments. The robustness and validation of the OPLS-DA model against overfitting were evaluated by the cumulative (cum) R^2Y [R^2Y (cum)] which represents the total variation of Y (class discrimination) explained by the model, Q^2 (cum) describes the predicative capability of the model by cross-validation. In addition, cross validation (CV), analysis of variance testing of cross-validated predictive residuals *p*[CV-ANOVA], and a permutation test with 200 iterations were conducted (Zhong et al., 2011; Yan et al., 2012; Del Coco et al., 2014; Musharraf et al., 2016; Subramani et al., 2016; Tugizimana et al., 2016; Worley and Powers, 2016). Furthermore, dot plots representing individual observations were further constructed to describe distributions in a group of data and avoid the risk of overfitting (Wilkinson, 1999; Tugizimana et al., 2016).

Additionally, a Bonferroni-corrected 3-way analysis of variance (ANOVA) was implemented to investigate whether the peak values of the differential metabolites derived from PCA and OPLS-DA techniques had significantly different abundance between treatments (irrigation status, inoculation, and cultivar). An adjusted $P < 0.05$ was considered to be statistically significant.

Results

Metabolomic profiling by GC-MS using principal component analysis (PCA)

A total of 497 compounds were detected in roots exudates of different wheat genotypes using the GC-MS platform. Sometimes multiple compound profiles were annotated to the same

match in the database, despite having different retention times. Among these, 48 compounds were annotated based on the compound mass and peak matches to chromatograms of pure compounds found in public databases. Included among the annotated compounds were sugars, amino acids, organic acids, and fatty acids (Table 3.1). Global metabolite profiles generated by GC-MS were first visualized using an unsupervised Principal Components Analysis (PCA) method to identify major sources of variation under the experimental conditions: water-stressed (WS) and well-watered (WW), inoculation (inoculation and non-inoculation), and among different genotypes (Byrd, OK06318 and RonL).

The PCA scores plot of the first and second components (Figure 3.3A) showed partial discrimination between irrigation conditions (WS and WW). The first two principal components (PC1 and PC2) cumulatively explain 59.9% of the total variation in the dataset. Samples clustered by irrigation treatments along the first principle component (PC1=45.6%; Figure 3.3A). An analysis of variance (ANOVA) showed highly significant differences (P=0.0017) between irrigation treatments in the first principal component (PC1).

A loading scores plot of the PCA data (Figure. 3.3B) for each metabolite demonstrated which metabolites were most strongly associated with each quadrant. Based on this plot, phosphate appeared to be a key metabolite associated with irrigated treatments (right of the PCA; blue dot), and several organic acids, including succinic and lactic acids, were more highly associated with the water stress treatment (left of the PCA; red dot). In contrast, there was no clear clustering of samples by inoculation treatment or genotype along any of the calculated principal components (data not shown), suggesting that irrigation condition was the primary factor responsible for differences in metabolite profiles.

Metabolite profiling by LC-MS using principal component analysis (PCA)

Using negative and positive ionization mode mass spectrometry, coupled with separation by UPLC, a total of 70 compounds were detected for the negative ionization mode and 349 compounds in the positive ionization mode. The results revealed that scores plots derived from PCA of LC-MS data exhibited separation between irrigation treatments (WS and WW) in the negative ionization mode (Figure 3.4A). Moreover, the results showed that the first two principal components (PC1 and PC2) represented 44.9% of the total variability contained in the dataset. In addition to this, an analysis of variance (ANOVA) showed highly significant differences (P<0.0001) between irrigation treatments in the first principal component (PC1). On the other hand, the positive ionization mode showed no significant separation of global root exudate profiles along these components (PC1 and PC2) by water status (Figures 3.4B). The results also detected that neither genotype nor inoculation conditions were significant sources of variation among the metabolite profiles.

Orthogonal projection to latent structures - discriminate analysis (OPLS-DA)

To maximize class discrimination and identify potential metabolites associated with each treatment condition, the data were analyzed using the OPLS-DA technique with pareto scaling and mean-centering.

The score plot of OPLS-DA derived from GC-MS data showed an obvious discrimination between the irrigation treatments (WS and WW). The values of R^2Y (cum) and Q^2 (cum) were 0.829 and 0.712, respectively using one predictive and one orthogonal component (Table 3.2). The results revealed that the model explains 82.9% of the variation of Y, with a predictive quality (Q^2) of 71.2%, indicating that the model was stable and good for fitness and prediction (Figure 3.5A). Moreover, the results indicated no separation was achieved based on inoculation

status or plant genotype associated with the samples. The cumulative variation (R^2Y) and predictive quality (Q^2) of the OPLS-DA model were 0.438 and -0.434, respectively, between plant genotypes, and 0.322 (R^2Y) and –0.421 (Q^2) between inoculation conditions (Figure 3.5 B and C). These values suggest overfitting and a high level of stress in these models.

The results further revealed that scores plots of OPLS-DA of the UPLC-MS data obtained with negative and positive ionization modes also showed a significant separation based on irrigation conditions (Figures 3.6 A and B). The cumulative variation (R^2Y) and predictive quality (Q^2) of the model were 0.843 and 0.616 using one predictive and one orthogonal component, respectively, in the negative ion mode (Table 3.2). In the positive ion mode, these values were 0.804 (R^2Y) and 0.42 (Q^2) using one predictive and one orthogonal component, respectively (Table 3.2). On the other hand**,** the results obtained from inoculation conditions or plant genotypes showed no group separation is visible in OPLS-DA scores plots based on the lower values of R2 and Q2 in both negative and positive ionization modes. The R^2Y (cumulative) and Q^2 (cumulative) of the OPLS-DA model were 0.438 and -0.158, respectively, between plant genotypes, and 0.387 (R^2Y) and –0.314 (Q^2) between inoculation conditions in the negative ion mode (Figure 3.7 A and B), while the R^2Y (cum) and Q^2 (cum) of the OPLS-DA model were 0.679 and -0.0966, respectively, between plant genotypes, and 0.577 (R^2Y) and –0.666 (Q^2) between inoculation conditions in the positive ion mode (Figure 3.8 A and B). The results indicated overfitting and a high level of stress in these models. These data support our hypothesis that irrigation status is an important factor driving soil metabolite profiles.

To assess the discrimination obtained from OPLS-DA model between irrigation treatments and to validate the model against over-fitting, cross validation (CV-ANOVA p-values), and permutation test were performed to evaluate the results obtained. For both GC-MS

and LC-MS data, the results from such validations confirmed the class separation for irrigation treatments (WS and WW) and indicated the stability and reliability of OPLS-DA model. For the GC-MS dataset, the cross validation and the cross-validated residuals (CV)-ANOVA for testing the OPLS-DA model were highly significant (CV p-value= $5.73x10^{-5}$) and OPLS-DA model displayed separation between two groups (WS and WW) (Figure 3.9 A and Table 3.2). Musharraf et al. (2016) reported that lower p-value indicate the group discrimination is significant. The permutation test was also performed to help assess the validity of the model against overfitting via randomly permuting class labels and refitting a new model and then compared to the values of the original model (Yan et al., 2012; Tugizimana et al., 2016). Chang et al. (2007) reported that well-fit models will have higher R2 (green) and Q2 (blue) values to the right than that of the permuted model to the left, while the intercepts of R^2 below 0.4 and that of Q^2 below zero or not exceed 0.05, suggesting the robustness of the original model (Eriksson et al., 2003; Yan et al., 2012; Zhu et al., 2015). In the present study, the values of R^2 and Q^2 intercepts in the permutation test were 0.31 and –0.42, respectively, after 200 permutations (Figure 3.9 B), indicating that the original OPLS-DA model was valid. The significant discrimination between irrigation conditions was further conducted using a dot plot. An obvious separation was observed, demonstrating a robust metabolic difference between water stress and well-watered regimes (Figure 3.9 C).

Similar results from LC-MS data were observed in both negative and positive ionization modes from these additional validation analyses. In the negative ion mode, the cross validation and the cross-validated residuals (CV)-ANOVA for testing of OPLS-DA model were highly significant (CV p-value=0.0001) and OPLS-DA model exhibited discrimination between the two irrigation treatments (WS and WW) (Figure 3.10 A and Table 3.2). Further model validations of

OPLS-DA with the number of permutations equaling 200 generated intercepts of R^2 = 0.35 and Q^2 = -0.48 (Figure 3.10 B), suggesting that the original model was not overfit and highly predictive. The significant separation between irrigation conditions was further evaluated using a dot plot. An obvious separation was observed between the two groups of irrigations (Figure 3.10 C). Additionally, in the positive ion mode, the cross validation and the cross-validated residuals (CV)-ANOVA for testing of OPLS-DA model were highly significant (CV p-value =0.011) and OPLS-DA model exhibited discrimination between the two irrigation treatments (WS and WW) (Figure 3.11 A and Table 3.2). Furthermore, the permutation tests with (n=200) generated intercepts of R^2 and Q^2 values were 0.64 and –0.41, respectively (Figure 3.11 B). In the positive ion mode, the values of R^2 and Q^2 intercepts in the permutation test (n=200) showed a much more reliable Q^2 than R^2. The significant separation between irrigation conditions was further evaluated using a dot plot. An obvious discrimination was observed, indicating no overlap between the two groups of irrigations (Figure 3.11 C). These results demonstrated the stability and reliability of the OPLS-DA models obtained on all of the separation and analysis platforms (GC-MS, and negative and positive ionization UPLC-MS) and confirmed that the irrigation regimes were a key driving factor of the global rhizosphere metabolite profiles.

Identifying specific metabolites associated with the various experimental conditions is useful for providing clues regarding the interactions between plants and their environment. It also facilitates the generation of testable hypotheses to further explore plant-environment interactions. Therefore, in the present study, S-plots of the OPLS-DA were generated to identify potentially significant biochemical variables responsible for discriminating between the two irrigation regimes. S-plots of the OPLS-DA derived from GC-MS were most important in discriminating between the various experimental treatments. Under water-stressed condition, the

metabolites that mainly contributed to the discrimination were succinic acid, lactic acid, urea, and an indazol-amine-like compound, while benzohydroxamic acid and phosphate were the primary metabolites associated with irrigation (Figure 3.12). The predictive potential of these compounds as biomarker metabolites of the differing irrigation treatments was further validated based on calculation of the Variable Importance in Projection (VIP) values (VIP > 1) combined with one-way analysis of variance (ANOVA). Consistent with the results from S-plots of OPLS-DA model, seven metabolites increased in the wheat root exudates when subjected to water stress while three decreased by water stress treatment (Table 3.3).

Considering irrigation treatments, inoculation status and genotype as three independent factors the possible interaction between them was explored using a 3-way ANOVA. The ANOVA analysis revealed that the accumulation of organic acids, notably succinic acid was significantly higher in inoculated exudates of RonL and OK03618 cultivars relative to exudates from Byrd under water-stressed condition (Figure 3.13).

Discussion

Development of novel strategies to mitigate water stress in agricultural crops for increased yields is critical if we hope to maintain an abundant food supply to feed a growing population. Understanding rhizosphere interactions and the chemical signaling components involved in water stress responses will be a key factor in developing strategies for water stress abatement. Root exudates and other root-associated metabolites differ with plant genotype, and in response to water stress and bacterial inoculation (Wu et al., 2001; Badri and Vivanco, 2009; Dennis et al., 2010; Chaparro et al., 2013; Aira et al., 2014; Huang et al. 2014; Lou et al., 2017). These variations attract soil microbial populations into the rhizosphere, which have a certain degree of specificity for each plant species (Huang et al., 2014). The analysis of metabolites

could contribute to the understanding to water stress tolerance in plants through the identification of a wide array of chemical compounds in metabolic profiles (Silvente et al., 2012).

In the present study, different metabolite-profiling approaches combined with multivariate statistical analyses, such as PCA and OPLS-DA, were performed to identify the differences between experimental conditions with the aim of identifying metabolites useful for determining water stress response. This approach is one of the strengths of the study. While use of a single metabolite profiling restricts the coverage of metabolites detected (Chen et al., 2011), our multiple analysis platform approach allowed for better coverage of various chemical classes. In addition, the use of supervised and unsupervised multivariate statistical methods allowed us to detect meaningful drivers of metabolite diversity while still being able to detect specific metabolites important under the various test conditions. Based on the score scatter plots of PCA, clear differentiation in the composition and quantities of the root-associated metabolites were detected between irrigation conditions. Similarly, Silvente et al. (2012), who studied the metabolic changes in response to drought treatments in soybean (*Glycine max* L.) genotypes, reported that in the case of leaves, the PCA model illustrated that the main discrimination in metabolites levels was associated with PC1 due to the irrigation conditions (well-watered versus water stress). Furthermore, Dai et al. (2010) also reported that drying conditions caused significantly changes on the metabolite compositions of *Salvia miltiorrhiza* Bunge (SMB) roots. Another study demonstrated that the quantity and composition of crested wheatgrass (*Agropyron cristatum*) root exudates have significantly altered in response to water stress (Henry et al., 2007). Furthermore, our results illustrated that the scores plots of PCA derived from GC-MS/LC-MS data showed that variation between the water stressed and well-watered conditions is more pronounced on the PC1 that counts for the largest variation in the models.

In order to maximize the differentiation among samples and identify important discriminatory metabolites, an OPLS-DA approach was constructed. The S-plot of OPLS-DA showed the metabolites responsible for maximum separation under conditions of irrigation. Based on the score scatter plots of PCA and OPLS-DA analyses and also CV-ANOVA and permutation methods using GC-MS/LC-MS data, obvious discrimination in the compositions and quantities of the root exudates released from wheat genotypes were detected under the supervised OPLS-DA model, in particular between irrigation conditions. The use of CV predictive residuals makes the CV-ANOVA more reliable than ordinary ANOVA (Musharraf et al., 2016). In this study, the CV-ANOVA p-value was significantly higher, suggesting the stability and reliability of the model. The obtained results using the GC-MS associated with PCA and OPLS-DA indicated that organic acids, fatty acids, and sugar were the main components of root exudates. In the leaves of two soybean (*Glycine max* L.) varieties, water stress triggered the accumulation of succinic acid, which its concentration was doubled after plants were subjected to water stress (Silvente et al., 2012). In accordance, the results showed the enhanced accumulation of succinic acid in the rhizosphere of RonL and OK06318 under water-stressed conditions and when inoculated with ACC+ bacteria. This suggests that ACC+ bacteria exude this compound and might play a crucial role in tolerating to water stress, although metabolomics analysis cannot provide information regarding compound origination (ie. plant or microbial origin). Collectively, the results showed that the RonL and OK06318 genotypes had higher amounts of organic acid, notably succinic acid as compared to the genotype Byrd.

Yoshikawa et al. (1993) reported that exudation of organic acids such as succinic and lactic via some PGPR strains may increase plant growth when their accumulations in the rhizosphere are appropriate. In the present study, inoculation of certain winter wheat genotypes,

particularly RonL with PGPR containing ACC deaminase increased most notably succinic acid under water water-stressed condition. Stromberger et al. (2017) reported that the RonL genotype might be able to accumulate a relatively high percentage of ACC+ bacteria in the rhizosphere presumable through producing greater concentrations of ACC and stress ethylene than the other genotypes when subjected to water stress. Furthermore, RonL genotype might be more effective in recruiting and selecting certain ACC+ bacteria than the other genotypes, presumably through releasing of root chemical signals. Organic acids are a crucial class of exudate compounds (Henry et al., 2007). In addition, exudation of organic acids into the rhizosphere could contribute to improve plant growth in several approaches, including solubilization of inorganic phosphates (Gilbert et al., 1999; Vyas and Gulati, 2009). Vazquez et al. (2000) demonstrated that the production of organic acids by several mangrove rhizosphere microorganisms might have involved in the solubilization of insoluble calcium phosphate. Jin et al. (2014) reported that a sufficient phosphorus (P) supply resulted in significantly improved drought tolerance in field pea (*Pisum sativum*) as a consequence of increased concentrations of soluble sugars, inorganic P content, and root length distribution in deeper soil layers. Moreover, the functional role of succinic acid could be related to improve physiological and metabolic activities related to photosynthesis, osmotic regulation, antimicrobial activities, antioxidant defense, and phosphate solubilization (Mokbel and Hashinaga, 2005; Vyas and Gulati, 2009; Song et al., 2012).

In the present study, both GC-MS and LC-MS platforms were implemented to conduct non-targeted profiling of rhizosphere metabolites. The GC-MS platform provided the strongest separation in metabolite profiles of water stress and well watered plants and many of the metabolites responsible for this separation were able to be annotated. Metz et al. (2007) reported that the GC–MS analytical method has larger commercial and public libraries of mass spectral

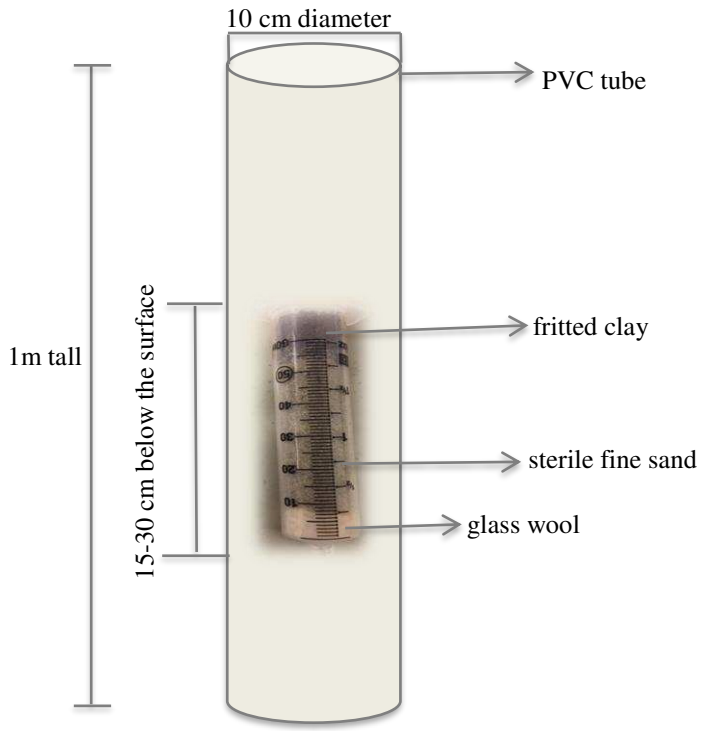
data. GC–MS also provides more reproducible retention times relative to the generated by LC-MS.

While the collection method and analytical approach were strengths of this study, it is important to acknowledge several weaknesses as well. First, the lack of a well-characterized chemical library for metabolomic data annotation limited our ability to assign chemical identifications to many of our compounds. In fact, less than 10% of the total compounds we identified could be annotated, and therefore, there are likely key metabolites driving rhizosphere processes that we fail to acknowledge. In addition, the silica tubes were flushed with sterile water to remove metabolites prior to an 80:20 methanol: water extraction; which likely biased our captured compounds towards water soluble polar compounds. This may be why organic acids are disproportionately represented in our annotated dataset. Finally, while this study had sufficient replication to broadly classify profiles between water stressed and well-watered conditions, there was insufficient replication, particularly with 2- and 3-way interactions (ie. cultivar x inoculation) to draw meaningful conclusions regarding their effects on metabolite profiles. Despite these limitations, the present study did gain valuable hypothesis-generating insights into rhizosphere interactions that can be further tested in future studies.

Conclusion

Global metabolic profiling with GC-MS and UPLC-MS, and multivariate statistical data analysis such as PCA and OPLS-DA models were implemented to identify differentiating metabolites and provide complementary information. The present study utilizing the combined MS-based metabolomic profiling techniques revealed that the irrigation status significantly changes the composition and quantity of root associated metabolites. The data obtained by GC-MS/LC-MS models derived from OPLS-DA analysis showed an obvious

discrimination between irrigation conditions (WW and WS) compared to inoculation status and plant genotype associated with the samples. Additionally, CV-ANOVA and permutation methods confirmed the results of group separation between the two levels of irrigation regimes. The consistent results derived from the combined of two platforms (GC-MS and UPLC-MS) illustrate the robustness of the metabolomic procedure utilized in the present study. Therefore, implementation of metabolomics combined with multivariate statistical techniques offer a promising tool for understanding of the underlying mechanisms of soil-plant-microbe interactions under varying environmental conditions. From the results presented in this study it can be demonstrated that response to water stress drives soil metabolite profiles. While higher replication is needed to identify whether these responses vary by inoculation status or plant genotype, this study provides a proof of concept for use of these technologies in studying wheat responses to water deficit.



Figures 3.1. A schematic diagram showing the syringe placement in the tube. PVC, polyvinyl chloride tube. *Note*: Drawing not to scale.

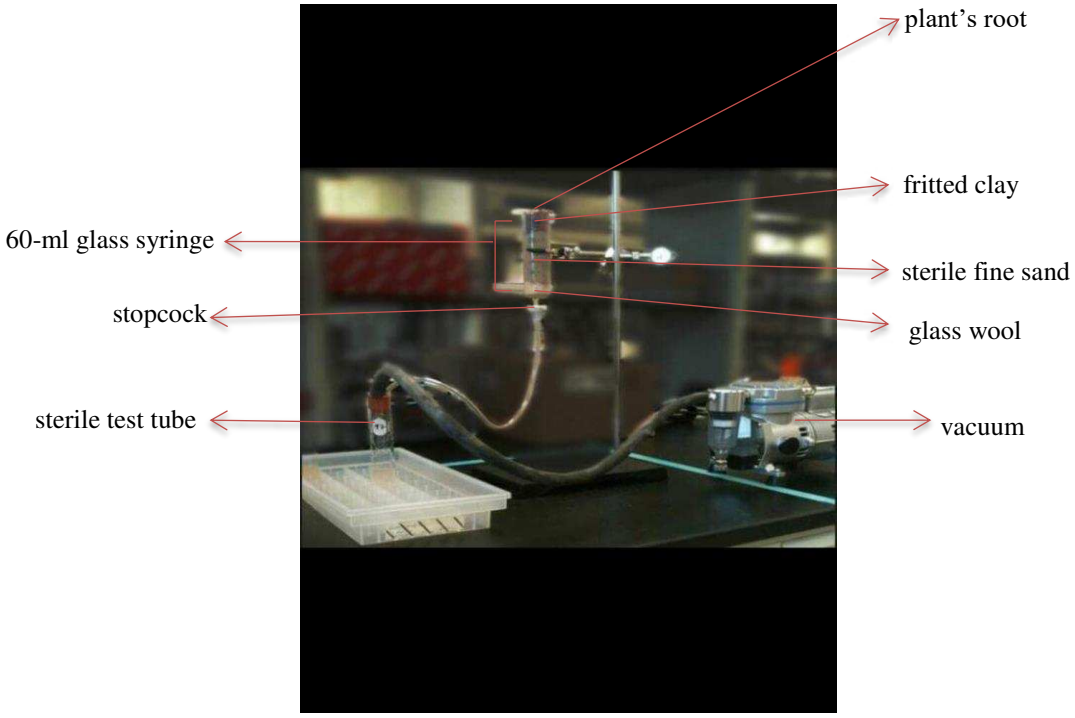


Figure 3.2. Technique for collection of root exudates from plants grown in 60 mL buried syringes.

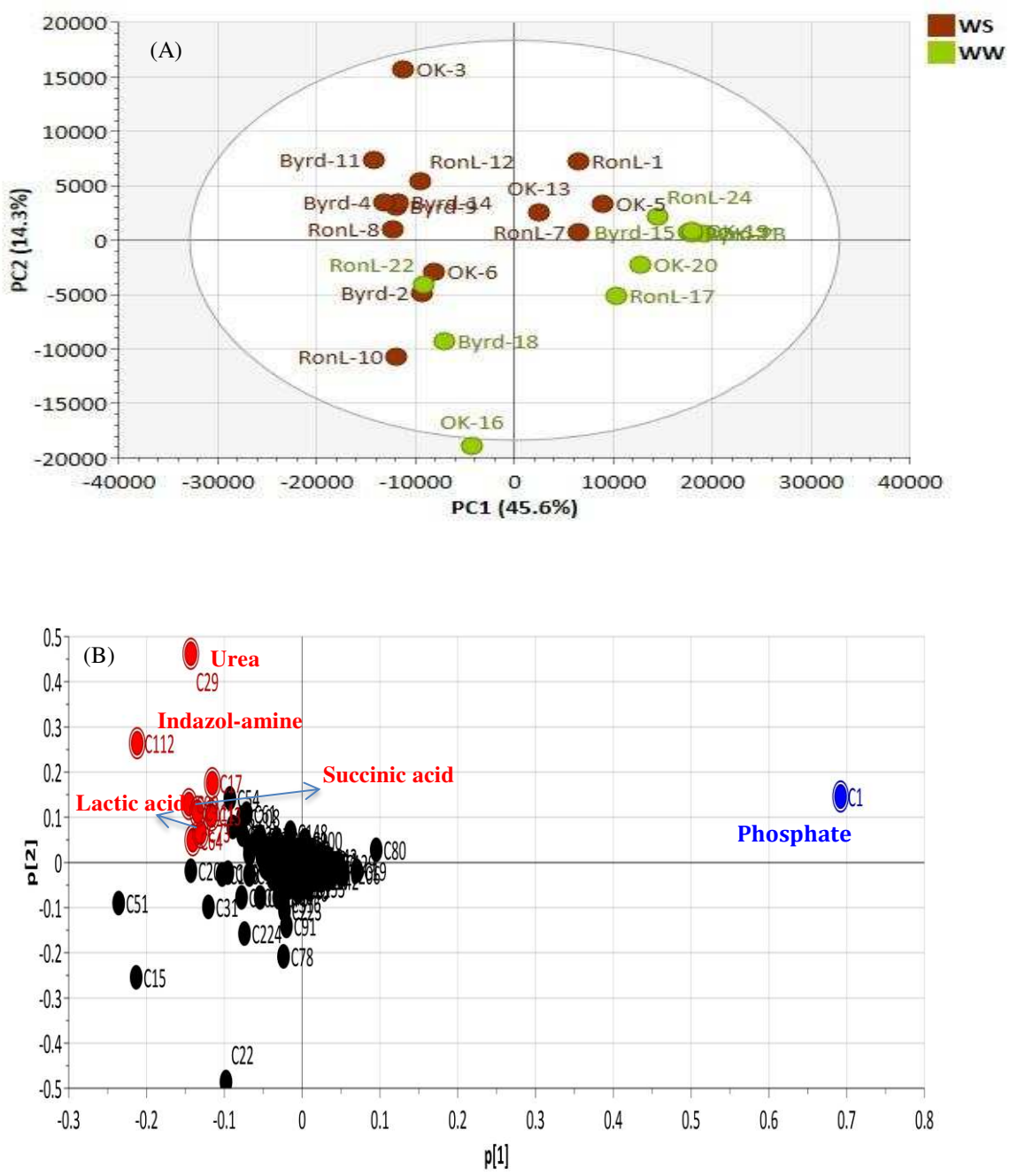


Figure 3.3. Principal component analysis (PCA) of root exudate profiles of three wheat genotypes grown under water-stressed (WS) and well-watered (WW) conditions obtained by GC-MS model. (A) PCA score plots (B) Loading plot of PCA. PC1, the first principal component; and PC2, the second principal component. Red dots represent the samples under WS treatment. Blue dot represent the sample of WW treatment.

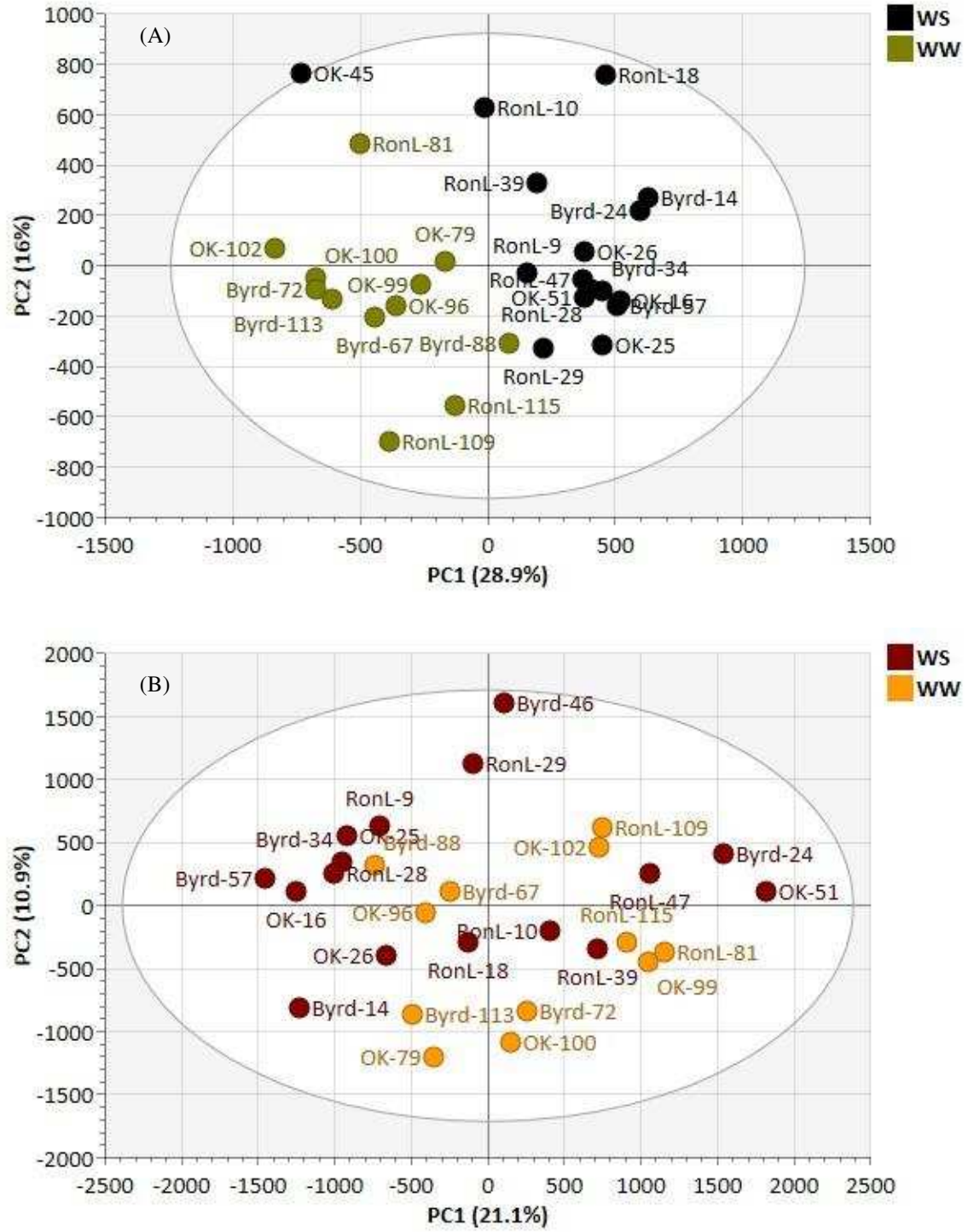


Figure 3.4. Principal component analysis (PCA) of root exudate profiles of three wheat genotypes grown under water-stressed (WS) and well-watered (WW) conditions derived from LC-MS model. (A) PCA score plots of the negative ion mode (B) PCA score plots of the positive ion mode. PC1, the first principal component; and PC2, the second principal component.

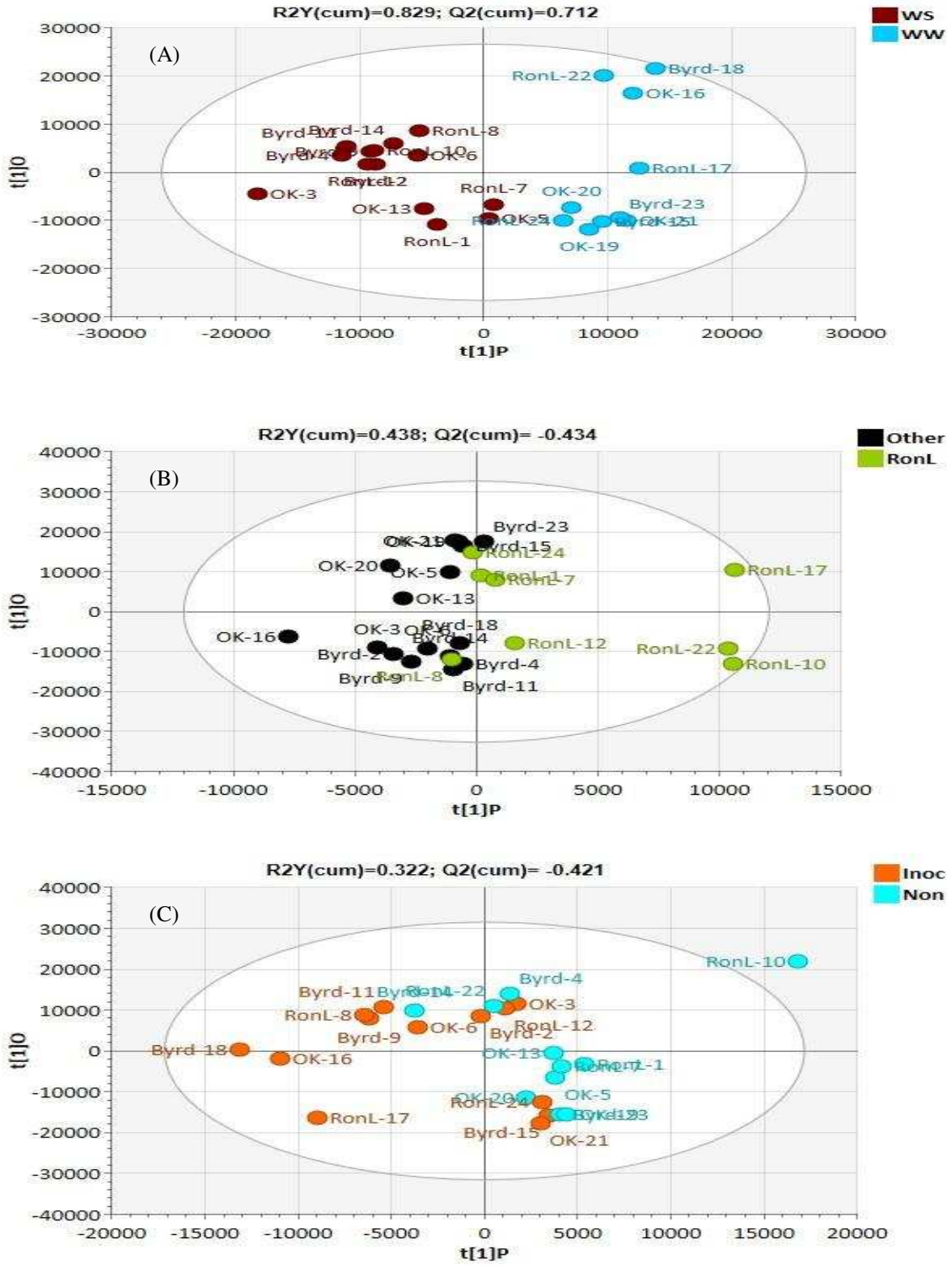
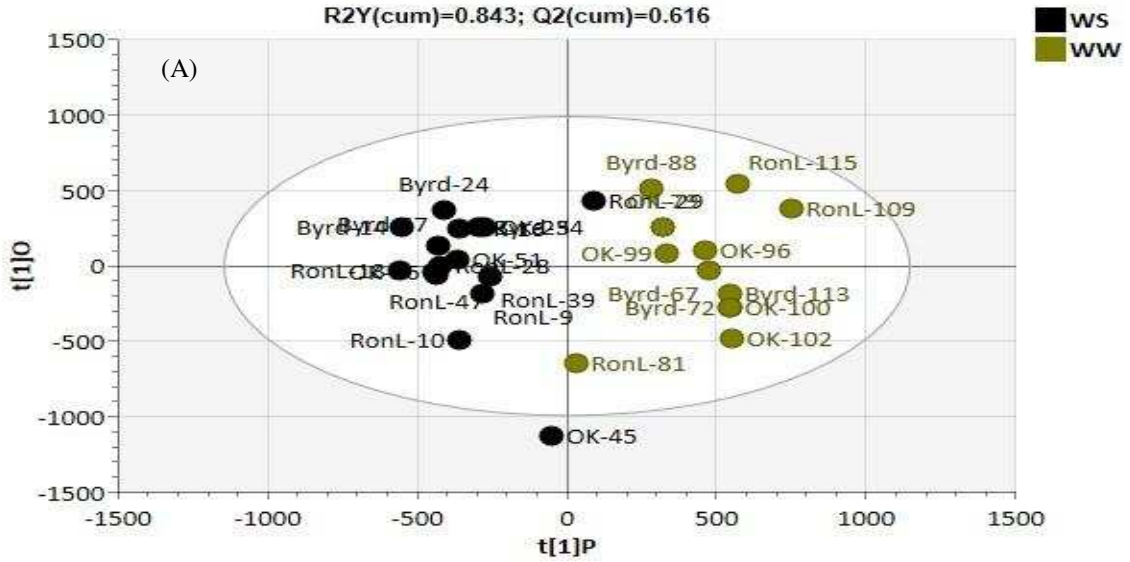


Figure 3.5. OPLS-DA analysis of root exudate profiles of three wheat genotypes derived from GC-MS model. (A) OPLS-DA score plots of GC-MS model between irrigation treatments (B) OPLS-DA score plots of GC-MS among wheat genotype (C) OPLS-DA score plots of GC-MS between inoculation status.



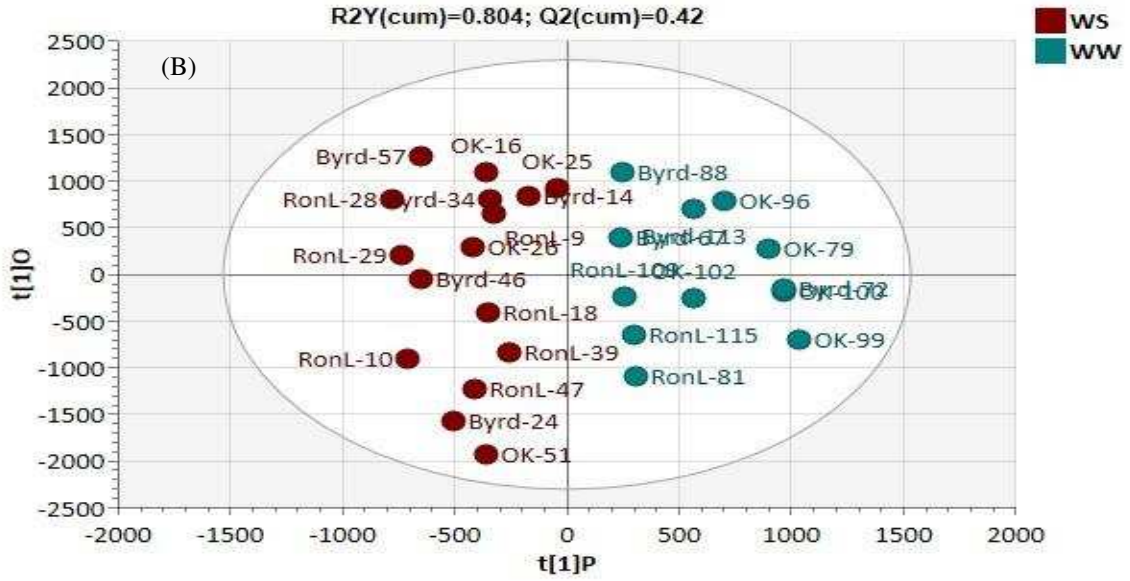


Figure 3.6. OPLS-DA analysis of root exudate profiles of three wheat genotypes grown under water-stressed (WS) and well-watered (WW) conditions derived from LC-MS model. (A) OPLS-DA score plots of LC-MS data in the negative ion mode (B) OPLS-DA score plots of LC-MS data in the positive ion mode.

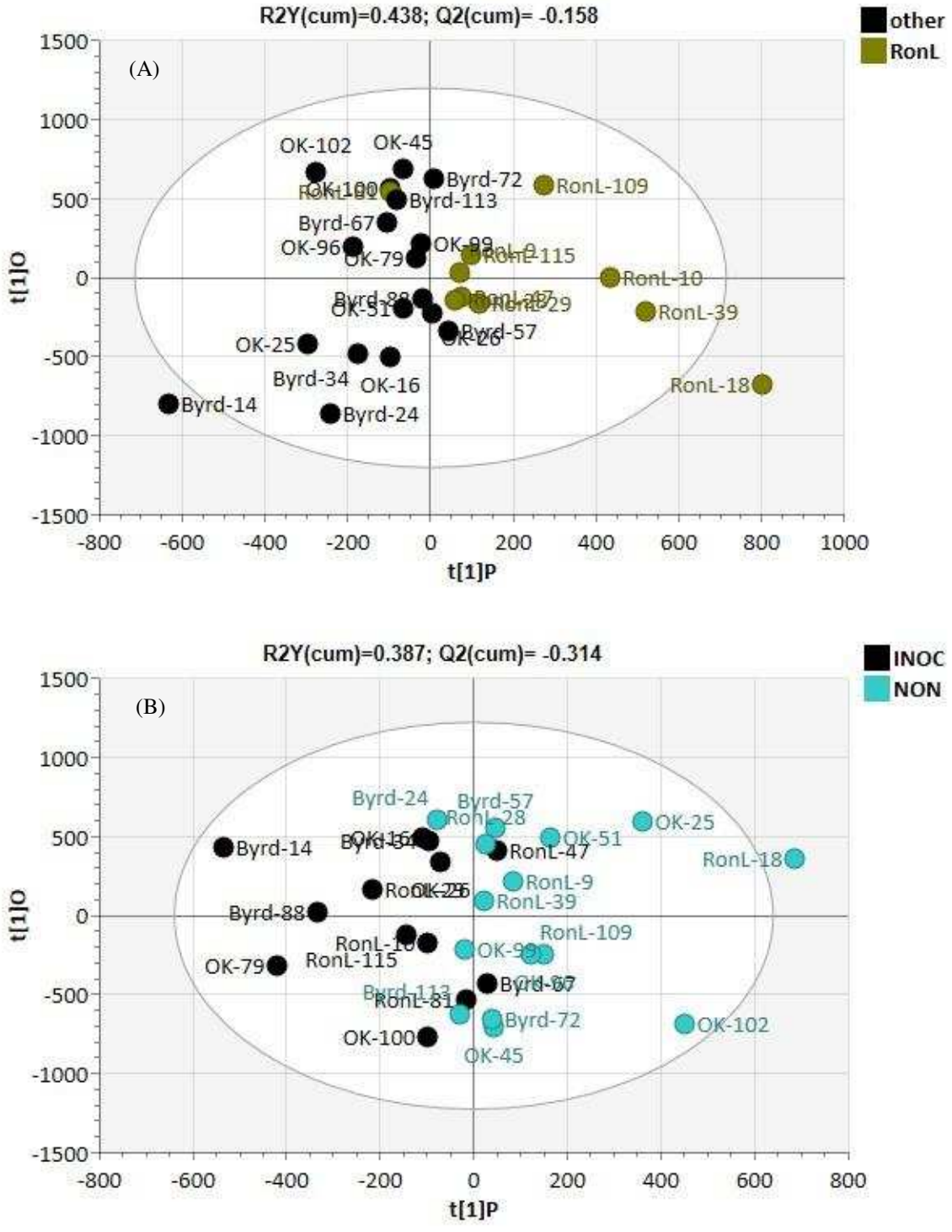


Figure 3.7. OPLS-DA analysis of root exudate profiles of three wheat genotypes derived from LC-MS model in the negative ion mode. (A) OPLS-DA score plots of LC-MS data between wheat genotypes (B) OPLS-DA score plots of LC-MS data between inoculation conditions.

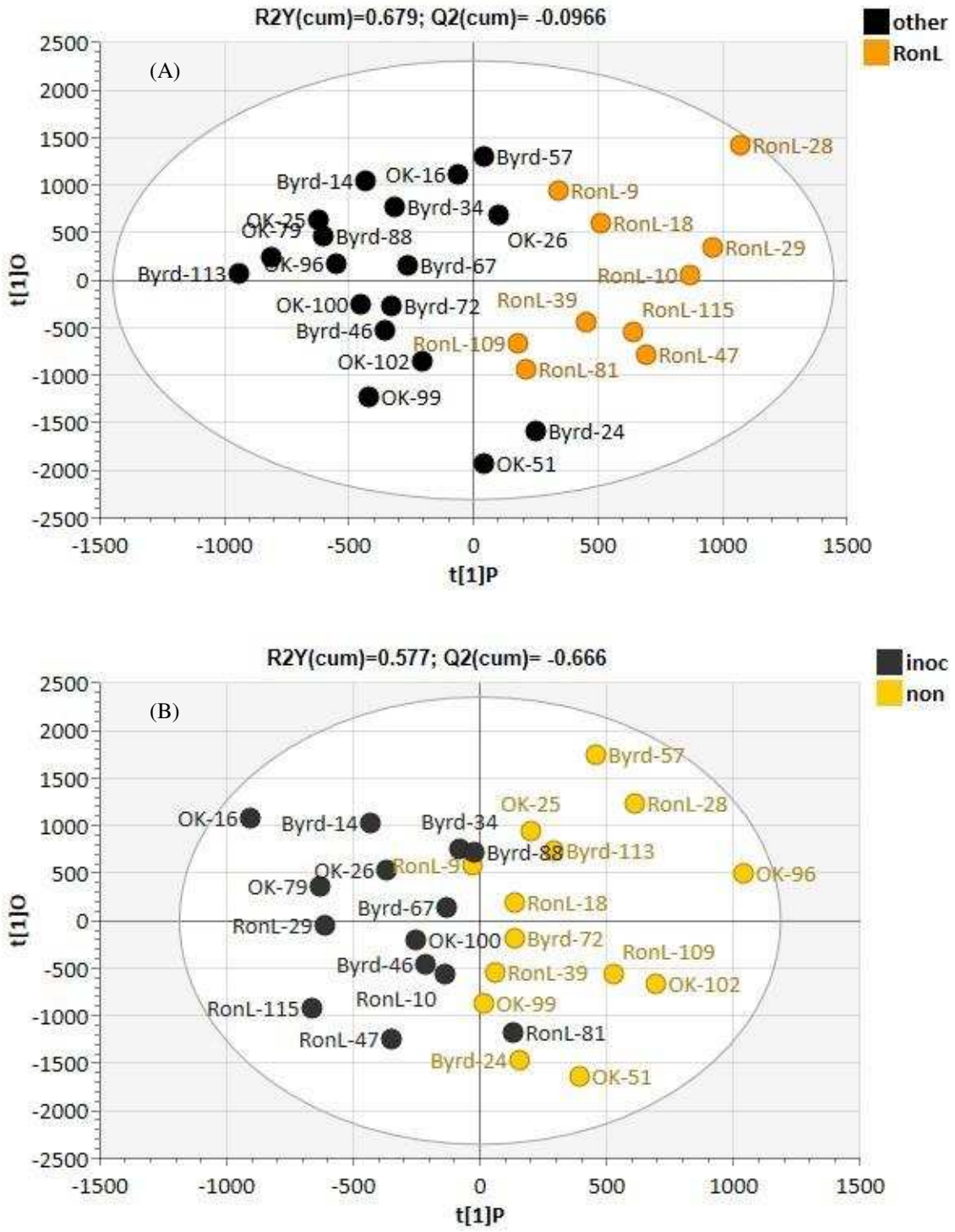


Figure 3.8. OPLS-DA analysis of root exudate profiles of three wheat genotypes derived from LC-MS model in the positive ion mode. (A) OPLS-DA score plots of LC-MS data between wheat genotypes (B) OPLS-DA score plots of LC-MS data between inoculation conditions.

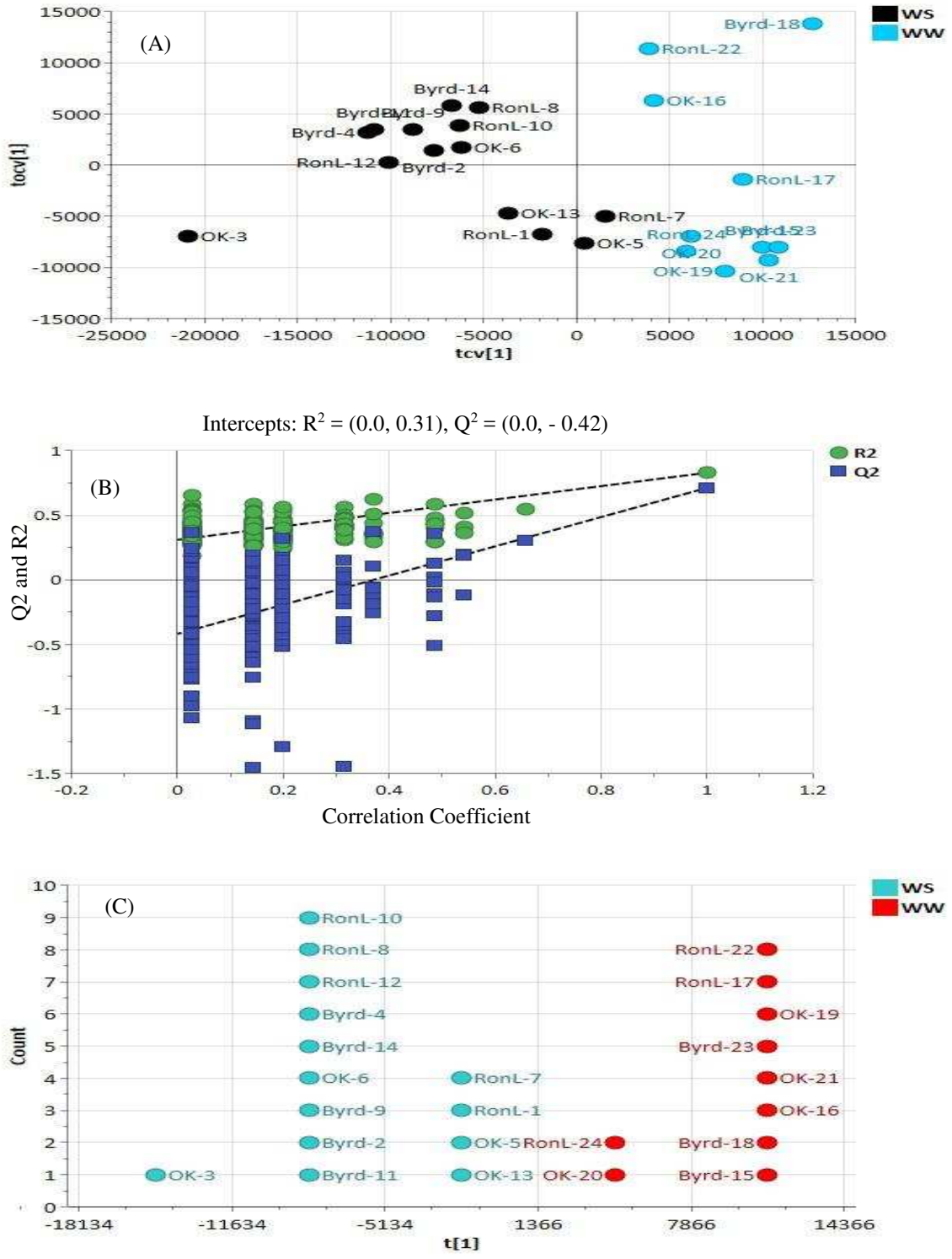
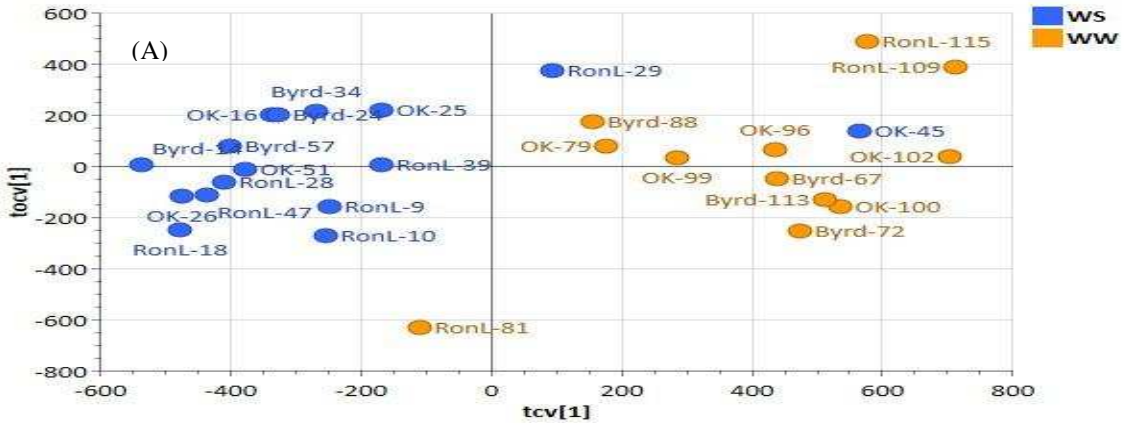
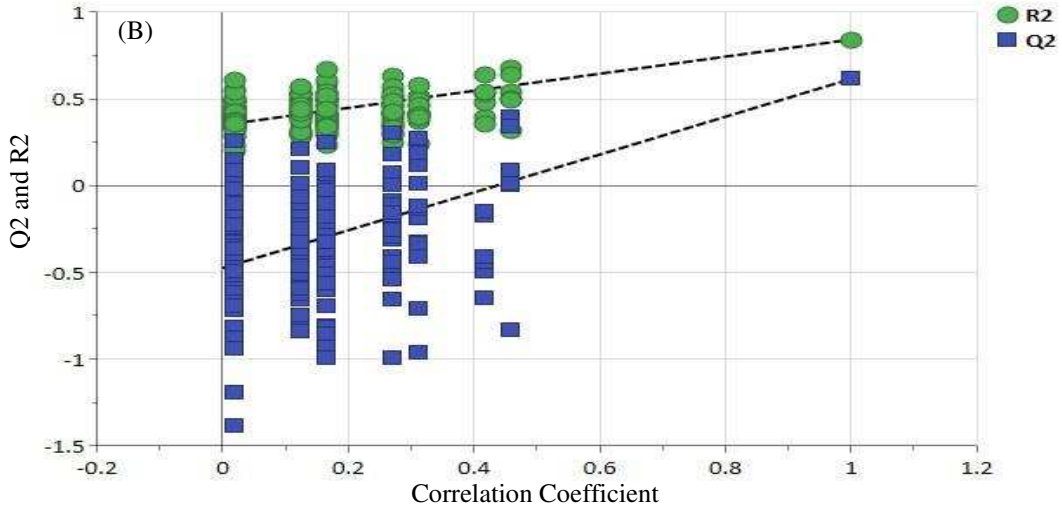


Figure 3.9. Cross Validation scores plots (A) of OPLS-DA model derived from GC-MS data showing group separation between (WS and WW) treatments; (B) the permutation plot for the OPLS-DA model. Green circle: R^2; blue square Q^2; (C) the dot plot of the irrigation regimes (WS and WW) showing that irrigation conditions clearly separated the two groups of irrigations and it has no overlap between two classes.



Intercepts: R^2 = (0.0, 0.35), Q^2 = (0.0, - 0.48)



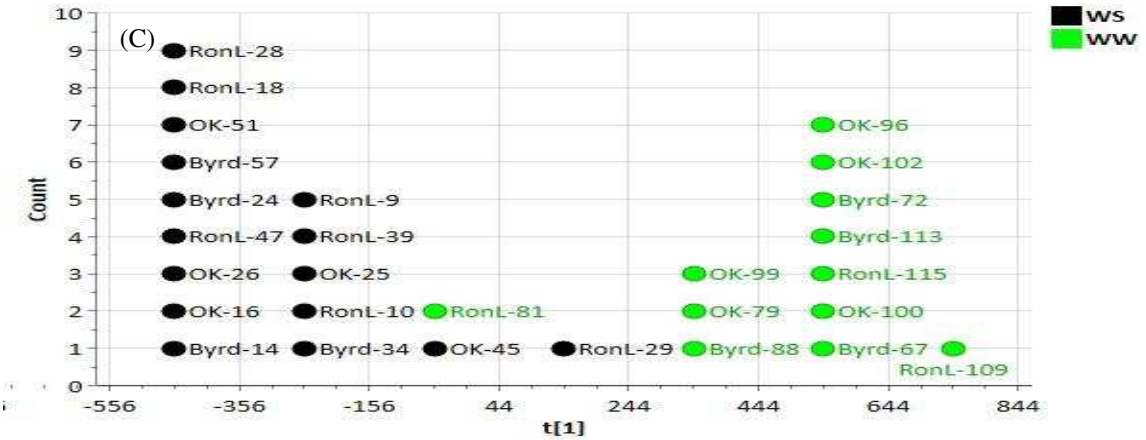


Figure 3.10. Cross Validation scores plots (A) of OPLS-DA model derived from LC-MS data in the negative ion mode showing group separation between (WS and WW) treatments; (B) the permutation plot for the OPLS-DA model. Green circle: R^2; blue square Q^2; (C) the dot plot of the irrigation regimes (WS and WW) showing that irrigation conditions clearly separated the two groups of irrigations and it has no overlap between two classes.

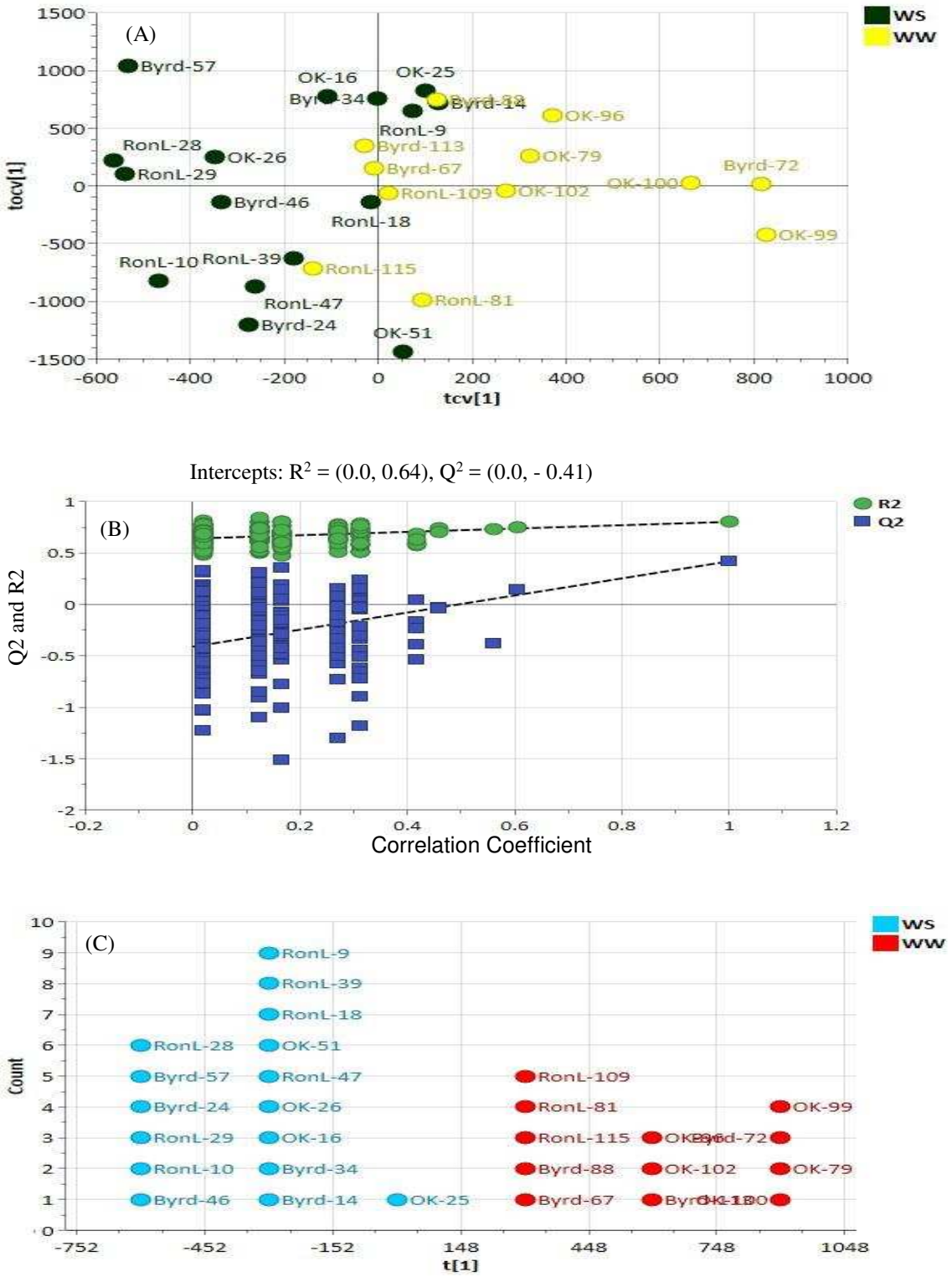


Figure 3.11. Cross Validation scores plots (A) of OPLS-DA model derived from LC-MS data in the positive ion mode showing group separation between (WS and WW) treatments; (B) the permutation plot for the OPLS-DA model. Green circle: R^2; blue square Q^2; (C) the dot plot of the irrigation regimes (WS and WW) showing that irrigation conditions clearly separated the two groups of irrigations and it has no overlap between two classes.

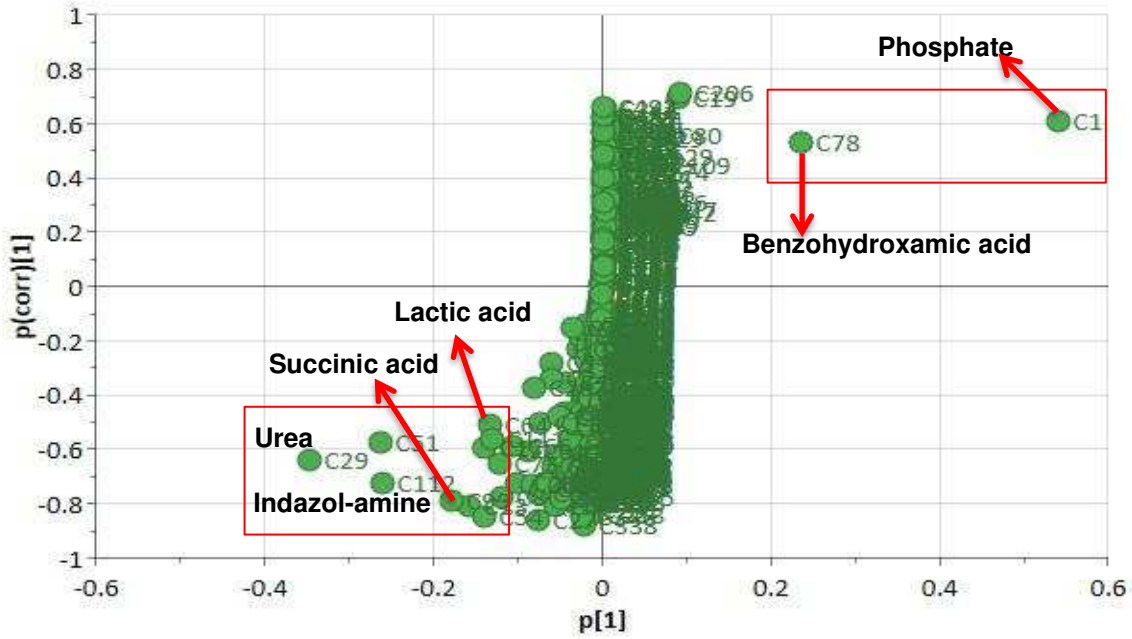


Figure 3.12. OPLS-DA on GC-MS spectra of root exudate profiles of three wheat genotypes grown under water-stressed (WS) and well-watered (WW) conditions, S- plots, which indicated the potential biomarkers between irrigation treatments (red boxes; (right up) represent metabolites under well-watered treatment and (left below) for the metabolites found under water-stressed treatment.

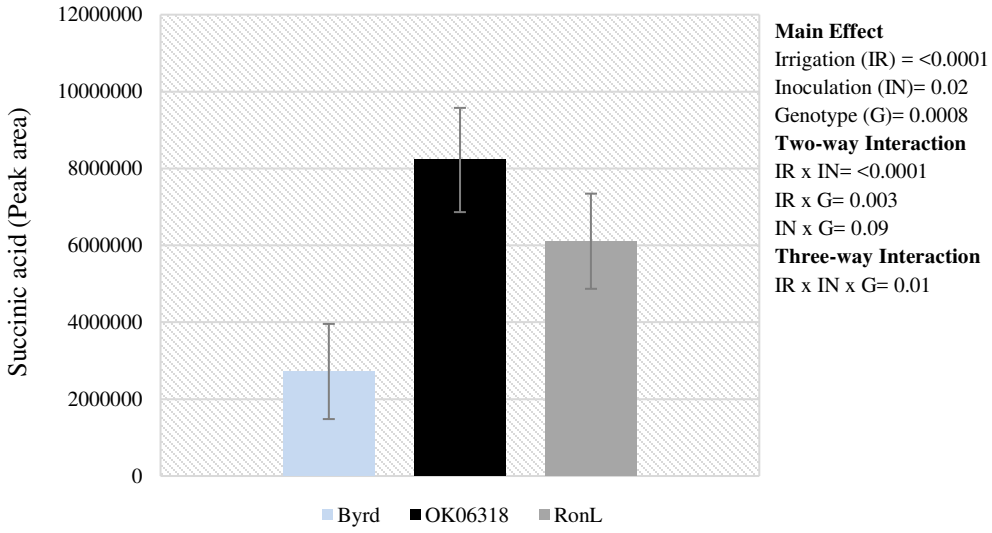


Figure 3.13. Accumulation of succinic acid in the root of three winter wheat genotypes. Within each genotype, bar represents the differences in the mean of inoculated from those of uninoculated under water-stressed treatment and the standard error (SE) represents standard error of the mean difference. *P*-values determined by three-way ANOVA for irrigation, inoculation, and genotype and their interaction are shown according to Bonferroni correction test (P <0.05).

Tables

Table 3.1. List of annotated metabolites from GC-MS profile of wheat root exudate, representative of different metabolite classes.

Name of the compound	Retention time	ID Confidence	Name of the compound	Retention time	ID Confidence
Phosphate	329	2	Malic acid	440	2
Phosphenodiimidic amide	425	2	Glycerate	359	2
Carbonate, putative	256	2	Hexanoic acid, 2-ethyl	378	3
Hydroxylamine	245	2	Galactose	646	2
Trehalose	927	2	Citric acid	594	2
Saccharide	895	3	Furanose	607	3
Palmitic acid	678	2	Adipic acid, hexyl-methylphenyl ester	576	2
Urea	306	2	Tartronic acid	385	3
Xylobiose	708	2	Vanillic acid	570	2
myo-inositol phosphate	824	2	Sterol	525	3
Fatty acid	815	2	Dodecanol	476	2
1,5-anhydroglucitol	557	2	N-carboxyglycine	431	2
Sorbitol	449	3	Indazol-amine	182	3
Stearic acid	751	2	Succinic acid	349	2
Galactopyranoside	1031	3	ketobutyric acid	297	2
Lactic acid	215	2	Pyranose	592	3
Nonanoic acid	371	2	Glycolic acid	222	2
Benzohydroxamic acid	198	2	Uracil	386	2
Bis(tert-butyl)phenol	467	3	Glucopyranose	662	2
Phthalate	405	2	Benzoic acid	315	2
p-Toluic acid	377	2	myo-inositol	708	2
Boric acid	180	2	Monosaccharide	622	3
Pyranoside	635	3	Glycerol-phosphate	570	2
Pyroglutamate	459	2	Glucose	632	2

Table 3.2. Statistics of computed OPLS-DA model for the irrigation treatments demonstrating the quality and validation of the model.

Data Set	**Model quality and Description**					
	OPLS-DA			CV-ANOVA *p*-value	Permutation Test (with *n*=200)	
	No.	R^2Y (cum)	Q^2 (cum)		R^2	Q^2
GC-MS	1P+1O	0.829	0.712	$5.73x10^{-5}$	(0.0, 0.31)	(0.0, -0.42)
LC-MS (–) ion mode	1P+1O	0.843	0.616	0.0001	(0.0, 0.35)	(0.0, -0.48)
LC-MS (+) ion mode	1P+1O	0.804	0.42	0.01	(0.0, 0.64)	(0.0, -0.41)

(–), negative ion mode; (+), positive ion mode; 1P + 1O, one predictive component and one orthogonal component for establishing the OPLS-DA model. No, the number of components

Table 3.3. A list of potential important metabolites based on the variable importance (VIP) from OPLS-DA modeling of GC-MS data under water-stressed and well-watered conditions.

Metabolites	VIP	Effect with irrigation (FC)
Phosphate	12.0	-1.76**
Urea	7.7	3.28*
Pyridinol	5.8	1.31^{ns}
Indazol-amine	5.8	1.13**
Benzohydroxamic acid	5.2	-1.42***
Succinic acid	4.0	0.92***
Hydroxylamine	3.1	1.15*
Lactic acid	3.0	0.54*
Boric acid	1.5	1.65***
Glucose	1.1	-3.64^{ns}

VIP was obtained from OPLS-DA model with the criterion of VIP >1. *P*-values were calculated from Bonferroni Correlation test $P < 0.05$ (one-way ANOVA). Significant effects between two levels of irrigation conditions are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and ns, not significant. VIP, variable importance in the projection. FC, the fold changes in the concentrations of each metabolite between the two levels of irrigation conditions was calculated using the formula $\log_2$ (water-stressed/well-watered).

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Wheat is an important grain crop plant that is continually threatened by environmental stresses, in particular water stress. As a consequence, development of improved water stress tolerance in wheat mediated by the interaction with soil microorganisms surrounding the plant roots plays an essential role in increased plant growth and productivity. Therefore, this research was undertaken to assess the effects of inoculation with 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase containing (ACC+) bacteria on different winter wheat genotypes, grown in greenhouse under water-stressed and well-watered conditions as determined by root length, root length of diameter classes, above- and below-ground biomass, and leaf relative water content (Chapter 2).

Results from this greenhouse experiment (Chapter 2) revealed that inoculation with ACC+ bacteria significantly increased leaf relative water content across all genotypes under water stress. Furthermore, wheat cultivar RonL, when inoculated with ACC+ bacteria, displayed increased aboveground biomass and root biomass in the deepest section of the root tube, irrespective of irrigation status. In addition, inoculation with ACC+ bacteria affected root lengths of cultivar RonL within certain root diameter classes, regardless of soil water status, but did not affect total root length. The results of the present study revealed that there were significant genotypic differences in water stress tolerance and the genotype RonL exhibited improved water stress tolerance after inoculation with ACC+ bacteria. Consequently, the RonL genotype may be useful in studying and/or evaluating the beneficial effects of PGPR on tolerance to water stress and crop performance in wheat genotypes. Finally, understanding how the water-stress tolerance varies among genotypes might be of benefit when choosing traits for improved water stress

tolerance of wheat genotypes. Genotypes that vary in water stress resistance mechanisms have become an important subject to study the variation in water stress tolerance to increase yields in crop plants (Basu et al., 2016).

In Chapter 3, the research examined rhizosphere metabolite profiles for three wheat genotypes "Byrd, OK06318, and RonL" on the basis of their variation in response to inoculation with ACC+ bacteria under water stress through the maintenance of leaf relative water content and other root traits (Chapter 2; Stromberger et al., 2017) to identify how root exudates, soil nutrients, and microbial metabolite profiles differ by plant genotype and under varying physiologic conditions, for instance, water stress and inoculation with ACC+ bacteria. In particular, this study aimed to determine whether specific root exudate chemicals were associated with improved resistance to water stress. Root exudates from three winter wheat genotypes (*Triticum aestivum* L.), under well-watered or water stress conditions and with or without inoculation by ACC-deaminase positive bacteria were collected and analyzed for global metabolite profile differences. To better grasp mechanisms of water stress tolerance, it is critical to understand which components in root exudates may be involved in mitigating water stress. Studying root exudates can be challenging, particularly because it is difficult to access secreted compounds in situations that mimic actual plant growth situations (ie. field or greenhouse) without disturbing or damaging plant root systems, which can modify profiles of secreted compounds (Phillips et al., 2008). To overcome this challenge, it has employed a unique collection approach that allowed us to obtain a greater understanding of root exudate profiles of winter wheat genotypes from intact toot systems under greenhouse conditions (Chapter 3).

Zhu et al. (2015) reported that metabolite methods could detect the functional state of the organism in a particular environment. Furthermore, combination of two analytical platforms such

as GC–MS and LC–MS could take advantage of complementary outcomes and thereby providing an enhanced analytical means for demonstrating the biological discrimination in living systems (Ni et al., 2007). In the present study, the metabolomic profiles of three winter wheat genotypes were analyzed by GC-MS and LC-MS in combination with multivariate statistical analyses such as principal component analysis (PCA) and orthogonal projection to latent structure-discriminant analysis (OPLS-DA). The results revealed that metabolite profiles derived from two analytical platforms (GC–MS and LC–MS) were most influenced by irrigation regime, with global differences between water stressed and well-watered plants evident from both unsupervised (PCA) and supervised (OPLS-DA) ordination plots of the data. No clear influence of genotype or inoculation conditions on global root exudate composition were seen in PCA and OPLS-DA models derived from both platforms based on the scores plots of PCA approach with cross validation and CV-ANOVA p-value of the OPLS-DA model.

The discrimination among samples by irrigation treatments was driven by metabolites such succinic acid, lactic acid, urea, and an indazol-amine-like compound under water-stressed treatment, while phosphate was the primary metabolite associated with irrigation (well-watered) treatment. According to the analysis of variance (Three-way ANOVA), the level of succinic acid was significantly higher in the genotypes OK06318 (P <0.001) and RonL (P <0.01) when inoculated with ACC+ bacteria, but no significance was observed in the genotype Byrd (P >0.05) under water-stressed condition as compared to well-watered condition. For measuring water stress tolerance, several researchers considered leaf relative water content as meaningful indicator to evaluate the plant water status and reflect the metabolic activity in leaf tissues (Teulat et al., 2003; Rampino et al., 2006; Anjum et al., 2011; Bano et al., 2013). These studies support the results of the current study. The metabolomics study were in consistent with the

greenhouse experiment which showed that the individual responses of wheat genotype to inoculation appeared to vary; for instance, the difference in leaf RWC between inoculated and non-inoculated plants tended to be greater in some genotypes, such as 'RonL' and 'OK06318', than others like 'WB Cedar' and 'Byrd'.

There is evidence to suggest that ACC+ bacteria secrete succinic acid (Yoshikawa et al., 1993) and that it might be playing an integral role in tolerance to water stress. Succinate is a citric acid cycle (TCA) intermediate, potentially feeding into this cycle to stimulate plant growth through enhanced energy production. An et al. (2014) demonstrated that foliar application of succinic acid was effective in alleviating aluminum stress in aluminum. With regard to water stress, Witt et al (2012) noted a strong negative correlation between succinic acid and leaf temperature (indicative of water stress) and a positive correlation between succinic acid and stomatal conductance in greenhouse grown maize. Therefore, the accumulation of the succinic acid, notably in RonL under water stress treatment, is in accordance with our results from the greenhouse study showing increased relative water content and specific root length for RonL when subjected to water stress.

These observations, combined with our findings, suggest that further experiments to understand whether soil applied succinic acid or inoculation with succinate-producing growth promoting rhizobacteria would be a viable approach to alleviating water stress in winter wheat. Additionally, examination of whether succinate levels in rhizosphere soils could serve as a biomarker for water stress tolerance are also warranted. Therefore, a holistic research approach to understanding how to improve crop productivity under water stress conditions could involve the utilization of morpho-physiological responses with a combination of multivariate statistical analyses of metabolomics data. This information would increase our understanding of the

mechanistic underpinnings of plant-microbe interactions and how they influence responses to water stress.

Summary and Future research

The application of analytical platforms including gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC-MS) in the untargeted data collection mode, combined with multivariate statistical analysis such as principal components analysis (PCA) and the orthogonal projection to latent structures - discriminant analysis (OPLS-DA), provide a useful tool for examination of rhizosphere metabolite profiles that could provide clues to mechanisms of plant water stress tolerance. Among treatment variables and plant genotype, irrigation status was a powerful driver of global soil metabolite profiles, with multiple analysis approaches demonstrating distinct separation between water-stressed and well-watered treatments. These results highlight the power and potential of these metabolomics profiling tools to contribute to our understanding of the beneficial interactions between plants and rhizobacteria and potentially to elucidate how plant genotypes vary in their response to inoculation with PGPRs under varying physiologic conditions, including water stress.

One limitation of metabolomics is that it is impossible to determine the source of origin of the metabolites (ie. plant or microbe) which confounds interpretation of results. Additionally, Minai-Tehrani et al. (2016) and Zheng et al. (2017) reported that the metabolite identification via LC-MS is more difficult, due to the shortage of comprehensive spectral libraries, than GC-MS which has larger commercial and public libraries. Accordingly, combining the results obtained from two analytical approaches together, GC–MS was able to identify the metabolites accountable for such significant discrimination by using S-plot of the OPLS-DA model and the variable importance in projection (VIP) based on the criterion for VIP exceeding 1.0. Further

studies are required to address root colonization by PGPR in order to better understand how PGPR interact with plant roots to successfully colonize the root zone, thereby providing beneficial effects and promoting plant growth.

In addition, it is acknowledged that soil microorganisms in the rhizosphere can influence plant growth and productivity through root-microbe interactions; however, the chemical signaling compounds in initiating these interactions are not well documented. Yuan et al. (2015) reported that organic acids like malic and fumaric acids secreted from root exudates of banana play a pivotal role in attracting and initiating PGPR colonization on the host roots, which might be attributed to induction of chemotaxis and biofilm formation by these organic acids. Yuan et al. (2015) further showed that even though the concentration of oxalic acid was significantly higher in banana root exudates, oxalic acid did not stimulate chemotaxis or biofilm formation. Furthermore, the chemotactic response of the *Pseudomonas fluorescens* strain WCS365 was induced in the presence of the five organic acids including succinic acid, which was identified in the tomato root exudates (de Weert et al., 2002). Even though the colonization assays such as chemotaxis and biofilm formation of the ACC+ bacteria towards the surface of wheat roots were not investigated in this research, our results revealed a critical role of organic acids, especially succinic acid in improved water stress tolerance in winter wheat genotypes, in particular RonL. These compounds might play a role as the chemo-attractants for ACC+ bacteria in the wheat rhizosphere. Further research should focus on the critical role of these chemical signaling compounds in communication between soil microorganisms and their hosts.

Based on results of the included studies, it is hypothesized that ACC+ bacteria will sense and migrate toward organic acids (succinic acid) to establish beneficial relationships with host plants. To test this, in vitro assays of chemotaxis, such as a drop, Petri dish, and capillary assays

could be utilized. To further confirm chemotaxis in vivo, succinic acid could be added directly to soils with fluorescently tagged PGPR bacteria and imaged to determine root colonization. Negative controls would include inoculated plants with no added succinic acid and succinic acid with no inoculation. The latter control would demonstrate whether succinic acid has direct effects on plant growth. Combining the chemotactic response and biofilm formation of PGPR towards plant root exudates, will explicitly identify the importance of root exudates as a mechanism to recruit PGPR bacteria to enhance water stress tolerance, and therefore, understating the chemical signaling compounds will play an important role in plant-PGPR interaction.

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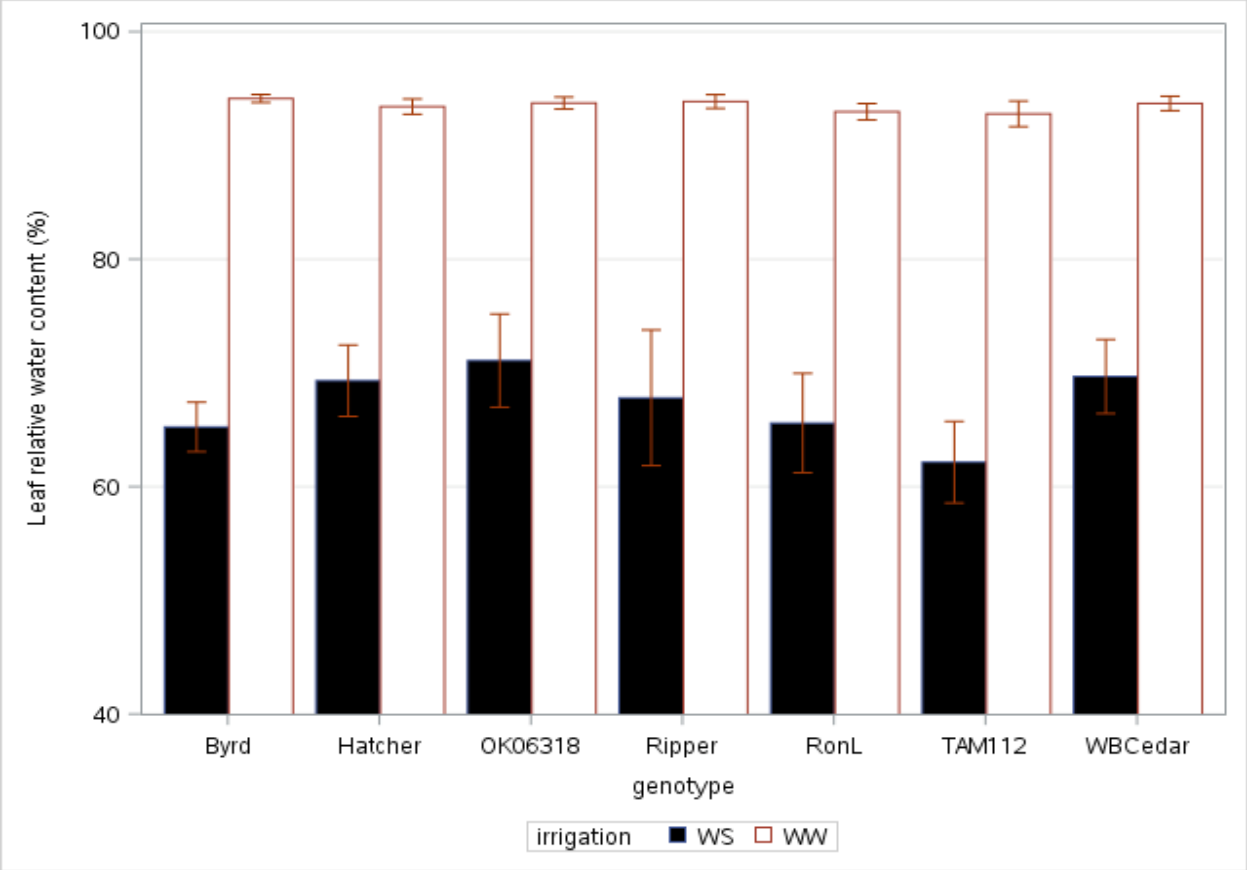
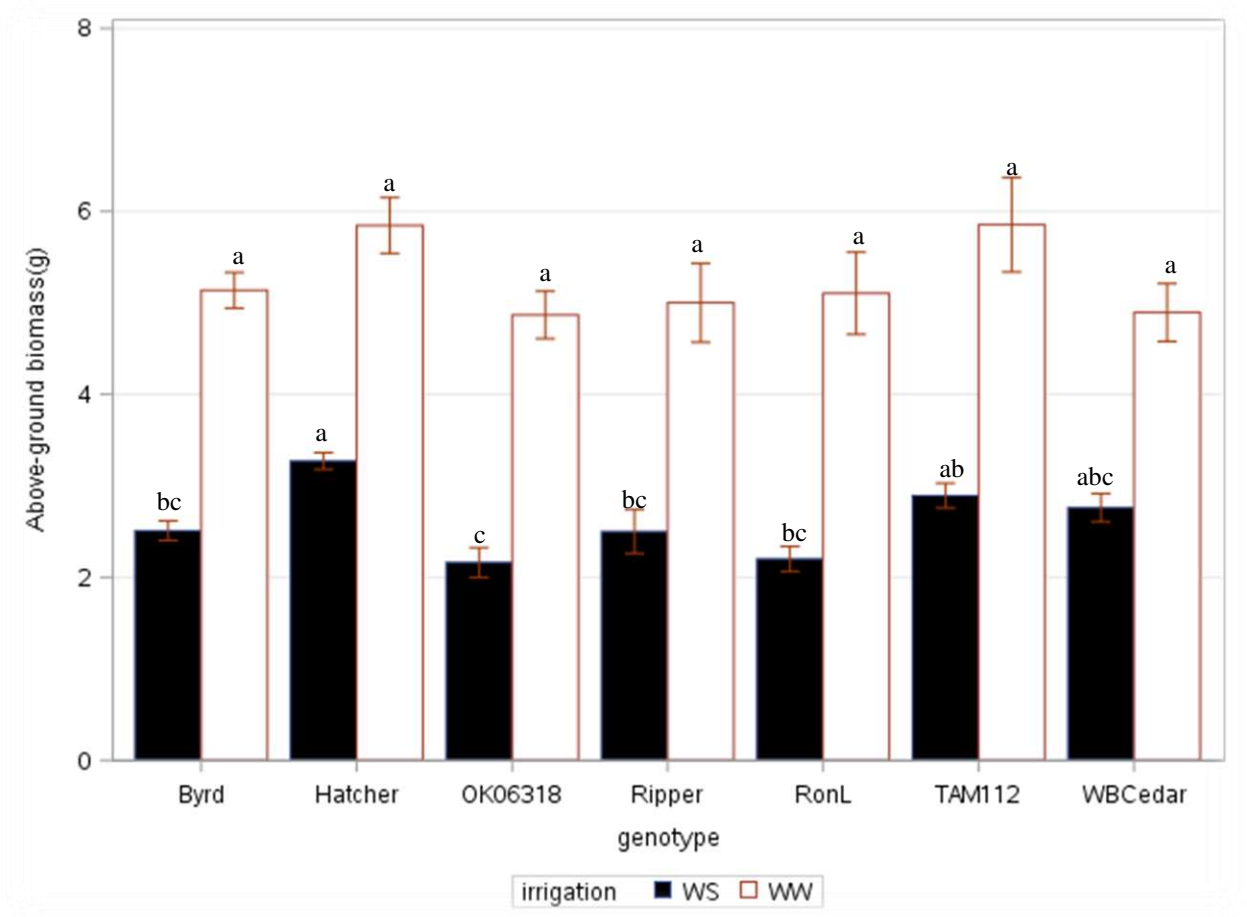
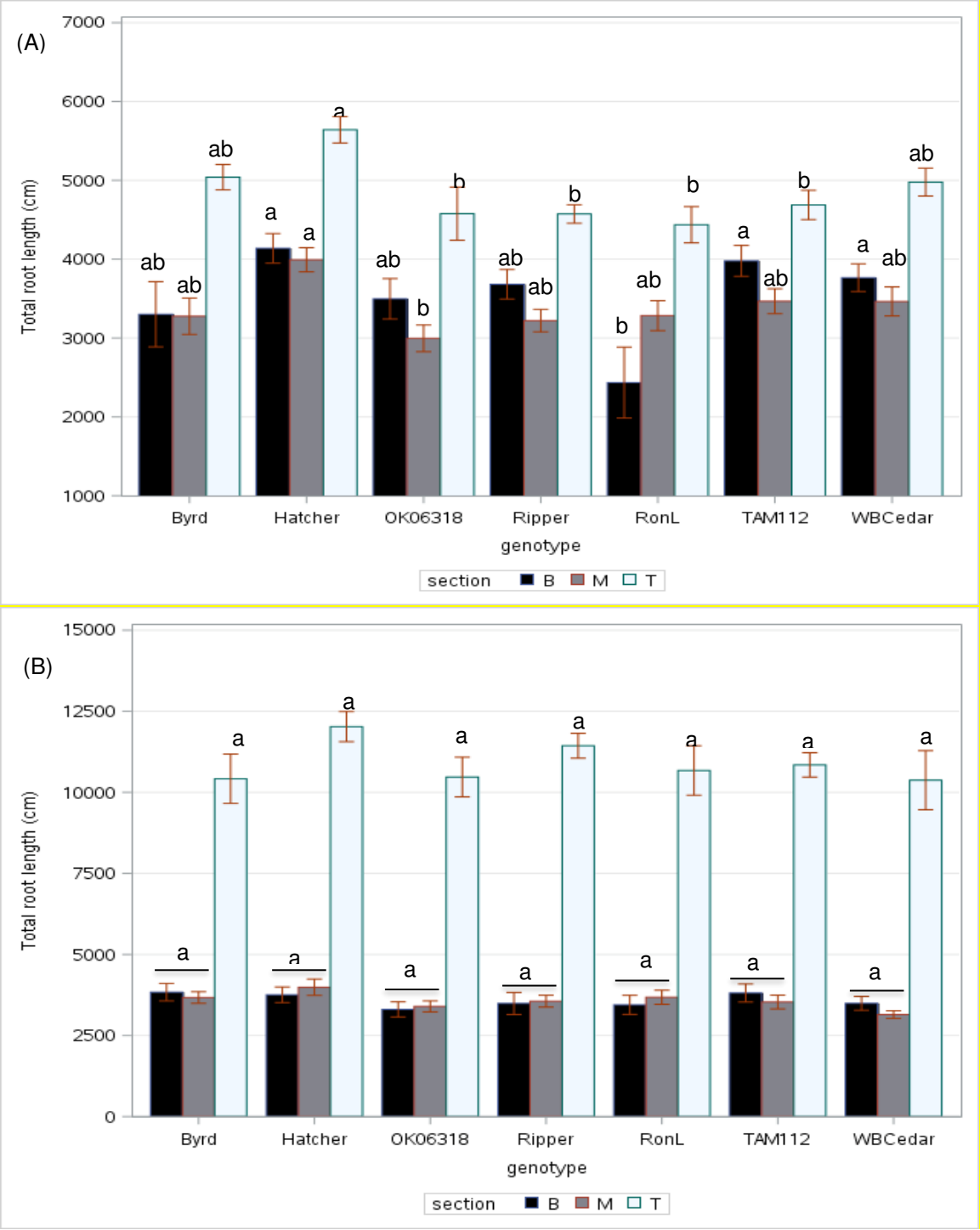
Supplemental Figures



Supplemental Figure S2.1. Leaf relative water content of seven winter wheat genotypes, grown under water-stressed (WS) and well-watered (WW) conditions. Bars represent the mean $\pm$ 1 SE, averaged across inoculation conditions (n=6-10).



Supplemental Figure S2.2. Above-ground biomass of seven wheat genotypes, grown under water-stressed (WS) and well-watered (WW) conditions. Bars represent the mean $\pm$ 1 SE, averaged across inoculation conditions (n=6-10). Within each irrigation treatment, bars followed by the different letter are statistically different (according to Tukey's test at $P \leq 0.05$).



Supplemental Figure S2.3. Total root length of the three-root tube sections top (T), middle (M), and bottom (B) of seven winter wheat genotypes, grown under (A) water-stressed and (B) well-watered (WW) treatments in the greenhouse. Bars represent the mean $\pm$ 1 SE, averaged across the inoculation conditions (n=8). Within each root tube section, means labeled with different letters are significantly different ($P \leq 0.05$; Tukey's test).

Supplemental Table

Supplemental Table S2.1. Means values for root length of all diameter classes of winter wheat genotypes computed in the bottom tube section under water-stressed treatment

Winter Wheat Genotype	Root diameter classes (mm) in the bottom tube section under water-stressed treatment				
	0.00-0.25	**0.25-0.50**	**0.50-0.75**	**0.75-1**	**>1**
Byrd	1814.0 a	965.6 ab	275.3 ab	150.4 ab	96.5 ab
Hatcher	2128.0 a	1270.3 a	371.4 a	209.5 a	155.5 a
OK06318	2034.2 a	955.0 ab	270.1 ab	150.6 ab	71.7 ab
Ripper	2014.4 a	1091.6 a	321.6 ab	174.2 ab	80.1 ab
RonL	1515.4 a	615.7 b	212.1 b	119.0 b	46.5 b
TAM112	2103.0 a	1213.1 a	351.0 a	189.0 a	122.8 ab
WBCedar	2001.1 a	1127.7 a	331.0 a	189.1 a	117.0 ab

Within each column, means labeled with different letters are significantly different ($P \leq 0.05$; Tukey's test)