

BACTERIALLY MEDIATED WATER STRESS TOLERANCE IN WHEAT
CONFERRED BY PHENAZINE-PRODUCING RHIZOBACTERIA

A Thesis

by

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ABSTRACT

The plant microbiome is the community of microorganisms living in association with plants and are considered to be a “second genome,” capable of directly modifying the plant’s biotic and abiotic environment. Predicted changes in the climate suggest that it is important to increase the plant’s ability to survive and recover from water stress. Plants recruit communities of plant growth promoting microorganisms (PGPMs) with functionalities that enhance their health. Previously, researchers showed that populations of phenazine-producing bacteria are higher in the rhizospheres of dryland wheat compared to irrigated wheat. My research investigates the selection by wheat of PGPMs with the functional capacity to produce phenazines. Phenazine-producing rhizobacteria are hypothesized to increase plant water stress recovery and root growth. I studied the interactions between drought tolerant winter wheat cultivars and the PGPM *Pseudomonas chlororaphis* 30-84.

In water-stress trials, the presence of the wild-type phenazine-producing bacteria almost doubled the survival rate of wheat seedlings and an enhanced phenazine-producer tripled the survival of wheat seedlings compared to seedlings treated with a phenazine-deficient mutant or the non-inoculated control plants. The presence of phenazine-producing bacteria improved root system architecture and seedling health following water stress. Seedlings colonized by phenazine-producing bacteria had 2 fold more root tips than the two controls. These results suggest that the presence of the phenazine-producing bacteria enabled plants to survive water stress and enhanced recovery, in part, via their influence on root system architecture.

I also investigated the composition of rhizosphere communities recruited by cultivars of winter wheat with different levels of drought tolerance. The role of soil legacy was investigated by collecting soils from adjacent fields with different long-term land use histories, e.g. dryland versus irrigated wheat production. The role of water stress on recruitment was examined by subjecting cultivars grown in soil with different land use histories to water stress. I showed that cultivars with higher drought tolerance

had increased recruitment of phenazine-producing bacteria and did so more effectively from dryland soils. Given the potential for phenazine-producers to enhance plant adaptation to water stress, breeding for wheat cultivars that recruit indigenous soil phenazine-producing bacteria could increase water stress tolerance without need for application of microbial inoculum.

DEDICATION

To my dear nieces and nephew,
Celina, Julia, and Nicholas Ries.

For helping make my life:

Joyful,

Fun,

&

Full of Love

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This work was supervised by a thesis committee consisting of Professor Elizabeth Pierson of the Department of Horticultural Sciences, Professors Leland Pierson of the Department of Plant Pathology and Microbiology, and Professors Shuyu Liu and Endang Septiningsih of the Department Soil and Crop Sciences.

All work for the thesis was completed by the student, in collaboration with Pierson lab members of the Department of Plant Pathology and Microbiology, Horticultural Sciences, and Molecular and Environmental Plant Sciences. The enhanced phenazine-producing strain (30-84 ENH) was constructed by Dr. Jun Yu.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

In order to provide sufficient background information for my thesis the following topics will be discussed:

- Phytobiome
- Climate Change and Drought
- Plant Water Stress Response
- PGPMs and Water Stress
- Rhizosphere Community Development
- Synthesis

Key concepts within each topic will be discussed. The phytobiome section will introduce key terms such as rhizosphere, and overview the importance of harnessing the phytobiome to improve plant health. The climate change and drought section will underline one of the grand challenges of the 21st century: improving food production under water-stressed conditions. Within the plant stress response section the following concepts will be discussed: root growth and functionality; plant responses to water stress; plant water stress response strategies; characterizing and quantifying plant responses; response variables; and reactive oxygen species (ROS) and water stress. The root growth and functionality section will include an explanation of root growth zones, water-uptake, and morphological changes in roots in response to water stress. In the plant water stress response strategies section the four main water stress response strategies will be described: drought escape, stress avoidance, dehydration tolerance, and drought recovery. Within the response variables section measurable root changes are discussed: root architecture and root morphology. In this section I describe the response variables that are most informative in quantifying plant water stress response strategies. *In this study, I measure the three root architecture measurements described: allometry, surface area, and root number.* The plant growth promoting microorganisms (PGPMs) and water stress section will describe the various ways that microorganism can improve

water stress response such as via their influence on plant growth through production or alteration of phytohormones, assistance to the plant in maintaining high relative water content, enhancing osmotic adjustment, and reducing the negative effects of ROS by inducing antioxidant systems. The rhizosphere community development section will be a narrative on what we know about plant investment in root exudation and rhizodeposition as well as a distinction between short, medium, and long term plant investment in the rhizosphere. This section will also cover: temporal and spatial root exudation and rhizodeposition patterns; genotypic variation in root exudation and root exudation plasticity; and microbial community assembly. The factors effecting the selection of microorganisms will also be discussed such as microbial functionality. The synthesis section will provide a brief overview of the gaps in our current understanding, questions that should be addressed, and the ideal biological system for studying these questions. The introduction concludes with the approaches for answering my specific research questions for my two chapters.

PHYTOBIOME

The “phytobiome” is the community of organisms that live in intimate association with plants. The phytobiome includes all organisms that interact with the plant, including insects, nematodes, arthropods, etc. In this thesis, I focus on the microorganisms that colonize plant surfaces, residing within plant organs either in the extra- or intra-cellular domains, or dwelling within the plant’s zone of influence. These plant-associated microorganisms are considered to be a “second genome” for plants (Reviewed in: Berendsen et al. 2012; Turner et al. 2013), providing the capability to directly modify the plant’s biotic and abiotic environment, as well as influence phenotypic changes in plants to enhance their ability to respond to their environment. The rhizosphere is the plant’s zone of influence in the soil and this interface between plant roots and the soil is a dynamic environment that is the site of a majority of phytobiome services. The term “rhizosphere” was defined more than a century ago by

Hiltner (1904) as “the soil compartment influenced by plant roots” and has since been a fascinating focal point of plant-microbe research (Hartmann et al. 2009; Bakker et al. 2013). Evidence supports the hypothesis that plants recruit a specific microbial community to their rhizospheres (Bergsma-Vlami et al. 2005; Haichar et al. 2014; Mendes et al. 2013; Yan et al. 2017). Moreover rhizosphere microbial populations are much larger than bulk soil populations (Foster et al. 1983; Bakker et al. 2013) due to the substantial carbon investment by the plant (Hartmann et al. 2009; Bais et al. 2006; Bakker et al. 2013; Walker et al. 2003). The benefits to the plant of having the capacity to fine tune microbial selection based on recruitment of specific microbial services and functions, would include enhancement in the establishment of plant growth-promoting microorganisms (PGPM) potentially at the expense of deleterious ones, resulting in improved plant health.

PGPM provide a plethora of ecological services that bolster plant health. These microorganisms are able to improve nutrient and water acquisition, modulate plant hormone levels, produce enzymes and metabolites, protect against pathogens, and suppress the negative impacts of biotic and abiotic stressors (Weller 1988; Kloepper and Bay-Peterson 1991; Naveed et al. 2014; Yang et al. 2009). Agricultural production is highly dependent on the services provided by these microbial symbionts. With regard to water acquisition and water stress tolerance, rhizosphere bacteria can elicit plant responses in roots that enhance water uptake, in shoots that alter growth characteristics or transpiration rates, and in plant tissues that alter plant relative water content, adjust osmotic capabilities to increase drought tolerance, or improve antioxidant metabolism (Hoekstra et al. 2001; Gururani et al. 2013; Grover et al. 2014; Berendsen et al. 2012; Timmusk et al. 2014a; Vardharajula et al. 2011; Ngumbi and Kloepper 2016).

CLIMATE CHANGE AND DROUGHT

Drought is an exceedingly important concern globally and is expected to be a chronically serious problem for more than 50% of the arable lands by 2050 (Vinocur and Altman 2005; Naveed et al. 2014). Moreover, elevated temperatures predicted from climate change will increase the rate of soil drying in agricultural land, resulting in the more rapid onset of water stress with higher intensity (Trenberth et al. 2013; Fischer and Knutti 2015). Furthermore, because warmer air temperatures increase moisture holding capacity, the intensity of rains may be greater (Trenberth et al. 2014). Predicted changes in climate suggest that it is not only important to increase the ability of plants to withstand water stress, but to enhance the potential of plant root systems for water-uptake. More intense, sporadic rainfall events will likely exacerbate the need for deeper soil exploration by roots in order to reach available water. Thus, breeding selection for deeper rooting and altered plant allometry, or investment in root vs. shoot, may be necessary in order to keep pace with the anticipated changes in soil water availability accompanying climate change (Schenk and Jackson 2002).

The predictions for the need to increase global food production to feed the continued growth in the world population combined with predicted climate changes means that increases in agricultural production must occur under predominantly water-stressed conditions. To meet global food production needs it is imperative to work across disciplines to explore all feasible solutions. Recognizing and realizing the potential of the phytobiome may be vital to increasing agricultural productivity under water-stressed conditions. As described in a recent review by Ngumbi and Kloepper (2016), keys to healthier utilization of bacterially mediated drought tolerance will be better knowledge of: the mechanisms plants use to survive and grow during and after episodes of water stress, the ways rhizosphere bacteria survive drought stress, and the plant physiological processes PGPM can influence that would result in enhanced water stress tolerance. Ultimately, this knowledge could inform breeding strategies to select for plants more capable of recruiting and utilizing PGPM and the services they provide.

In order to better understand plant response to water stress, I first describe root growth and functionality, plant strategies for dealing with water stress, and metrics for characterizing and quantifying plant responses.

PLANT WATER STRESS RESPONSE

Root growth and functionality

The functionality of specific root tissues changes throughout the stages of root growth and development. Root tissue originates at the rapidly maturing root tip, where diversity among adjacent tissues is most readily visible, corresponding to the well described root tip zones (Fig. 1.1): cell division, elongation, and maturation (Baluska et al. 1996; Ishikawa and Evans 1993, 1995). At the apex within the zone of cell division, are the root cap meristem, giving rise to the border cells of the root cap, and the apical meristem, giving rise to the new root tissue. Just above the zone of cell division is the zone of elongation. The elongation of recently divided cells in this zone pushes the developing root through the soil. The zone of maturation is just beyond the zone of elongation. In this zone, root hairs form, lateral roots initiate, and the vascular tissues become mature, including the maturation of the Casparian strip, which is essential for water uptake. Root hairs, the primary structures involved in water uptake, develop from epidermal cells and persist for days to weeks. Their longevity depends in part on the environmental conditions, but also on the quantifying technique used to study them (Fusseder 1987; Henry and Deacon 1981; McElgunn and Harrison 1969). Cells in the zone of maturation typically are responsible for greater than 95 percent of the water-uptake capacity of the root system. As the cells that were previously involved in water acquisition mature, they become re-programmed functionally and morphologically, including changes in membrane composition and permeability via suberization and lignification (Kramer and Boyer 1995; Segal et al. 2008). The re-programming shifts cells developmentally from having a water-acquisition functionality to being sturdy conduits for long distance transport of water and minerals. Thus the roots cells situated in a

specific locale are continually changing in functionality and structure, and the sites of water and nutrient uptake are continually shifting as the root grows (Sander 1960; Segal et al. 2008).

Plants possess multiple mechanisms to manage water shortage, such as by altering root architecture and remodeling root morphology. Given the importance of root tips and specifically root hairs in the zone of maturation for water-uptake, one of the greatest benefits to the plant's capacity for water acquisition is an increase in the number of root tips by branching. Remodeling of root morphology via cortical cell death and suberization are also common responses. It has been speculated that drought-induced cortical cell death is a mechanism to decrease the radial resistance to water conduction facilitating maintenance of transpiration as water becomes more limited (Jupp and Newman 1987). For instance in *Lolium perenne*, in response to water deficit, the formation of new lateral roots originating from the pericycle were observed and death of cortical cells and root hairs also was higher in plants grown under water deficit as compared to well-watered controls (Jupp and Newman 1987). In addition to cortical cell death, plants exposed to water stress also may exhibit increased endodermis and epidermis suberization, thus protecting the stele and the root as a whole from water loss (Clarkson et al. 1968; Jupp and Newman 1987). These changes facilitate maintenance of root tip growth and the initiation and elongation of new lateral roots under stressed conditions (Ktitorova et al. 2002). Thus, root systems have the capacity for significant phenotypic plasticity both in the development of new roots and the remodeling of existing roots.

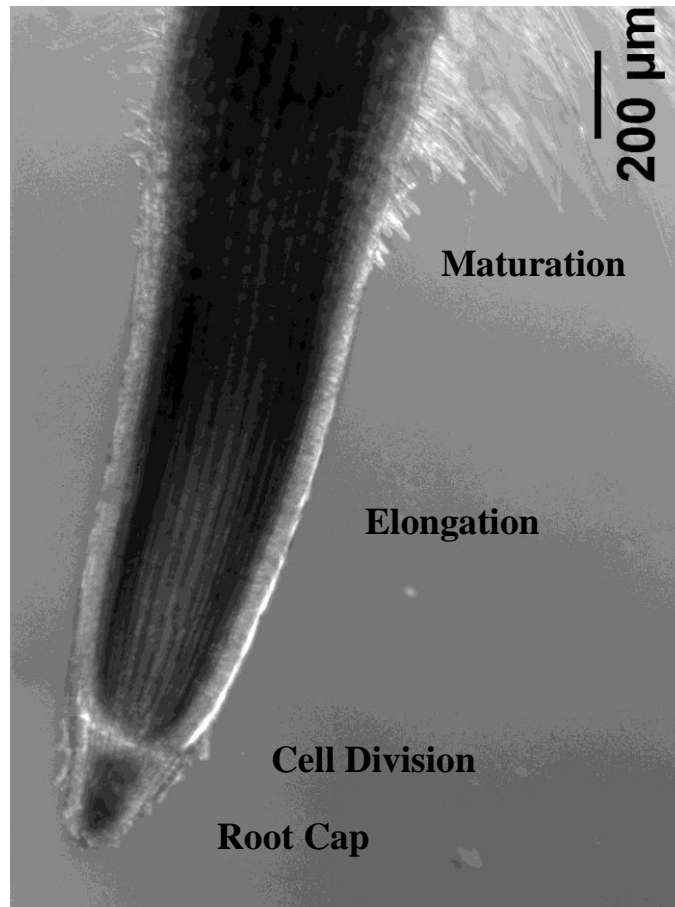


Figure 1.1: Zones of root tip development. Sterilized seeds were germinated on filter paper and were imaged 3 days after germination. Roots were rinsed with distilled water and mounted in water. Primary root of winter wheat seedling was imaged with Zeiss Axiophot using Differential Interference Contrast (DIC)-Nomarski. Objective 2.5X./0.075 dry. Scale bar = 200 μm . Image taken by Tessa Ries at the Texas A&M Microscopy and Imaging Center (MIC).

Plant responses to water stress

The onset of water stress initiates downstream signaling processes and transcription controls, which activate cascades of responses that lead to either stress tolerance or stress avoidance (Vinocur and Altman 2005). Water stress response cascades have been extensively reviewed (Akpinar et al. 2012; Kohli et al. 2013; Singh and Laxmi 2015; Verma et al. 2016; Wang et al. 2003). In brief, the primary plant stress caused by drought, salinity, heat, or the combination of these instigates cellular damage and secondary stresses, such as osmotic and oxidative stress. Osmotic stress occurs when the cellular solute concentration drastically changes. Oxidative stress occurs as ROS are overproduced and accumulate. These secondary stresses then initiate a suit of downstream signaling processes (including hormone signaling) and transcriptional and other regulatory controls, which activate stress-responsive mechanisms (Vinocur and Altman 2005; Wang et al. 2003; Jung and McCouch 2013).

The plant hormone abscisic acid (ABA) is the most reactive to water stress and plays a role in coordinating plant responses (Fang and Xiong 2015), although ethylene, and cytokinins also play rolls in root-shoot signaling (Steudle 2000; Sharp and LeNoble 2002; Verma et al. 2016). In response to water shortage, ABA is produced in the roots and transported to the above-ground parts of the plant in the xylem to alter physiology and growth (Sauter et al. 2001; Zhang et al. 1987). ABA triggers a cascade of physiological responses including stomatal closure, photosynthetic alterations, and altered root growth. Transcriptional reprograming and alterations in carbon investment are also important responses (Sharp and LeNoble 2002; De Smet et al. 2006; Osakabe et al. 2014). Increased ABA accumulation also is hypothesized to increase the accumulation of ROS, thus initiating the upregulation of antioxidant defense system (Jiang 2002). Plant water stress response cascades are complex and dynamic and allow the plant to adjust growth to adapt to and withstand periods of reduced water between precipitation events.

Plant water stress response strategies

The most efficient strategy for dealing with water stress depends on the characteristics of the stress, which can change throughout the growing season and the developmental stage and health of the plant. Since, plants do not have a specific weather prediction system, it is necessary for them to react to the environmental conditions in “real time.” Plants have both constitutive and adaptive phenotypes, especially root phenotypes, which often complicate the characterization of root QTLs (Collins et al. 2008). The nature of the water stress will significantly alter which root system phenotype is favorable, and thus the strategies that will increase productivity under water stress. *Plant species and even specific genotypes differ significantly in root water stress response strategies.* The water stress response strategies initiated can be divided into four major mechanisms: escape, stress avoidance, dehydration tolerance, and recovery (Fang and Xiong, 2015; Huang et al. 2014; Lawlor, 2013; Turner 1979; Yue et al. 2006)

Escape

Some plants are able to **escape** water stress by completing their life cycle before the onset of drought or by becoming dormant during the stress and resuming growth after the soil water is replenished (Turner 1986; Huang, et al. 2014). Breeding for crops with the right germination and maturity characteristics to target windows of water availability is one way to make use of this innate drought avoidance capacity.

Stress avoidance

Strategies that enable plants to **avoid** water stress typically involve:

- Leaf modifications such as: reducing water loss via stomatal closure (Campalans et al. 1999; Reddy et al. 2004; Wilkinson and Davies 2002), leaf rolling (Tardieu 2013), and increasing wax accumulation on the leaf surface (Zhang et al. 2005).
- Root modifications such as: enhancing water uptake capacity by increased rooting depth or production of fine roots and branching, or allometric shifts e.g. in root/shoot ratio (Price et al. 2002; Fulda et al. 2011; Hu and Xiong 2014).

Phenotypic flexibility resulting from changes in root architecture, allometric shifts in root:shoot ratios, or regulation of stomata enable plants to take up more water and/or minimize water loss via transpiration (Blum 2005; Tardieu and Allakhverdiev 2013). Improving the size, architecture, or hydraulic conductance of the root system is an important way plants avoidance stress (de Dorlodot et al. 2007). For example, increasing the volume of soil explored and the water acquisition capacity of the root system via the proliferation of root tips and fine roots (root surface area), or by changes in root growth patterns to favor exploration of greater soil depths are important adaptations for maintaining water uptake during periods of limited rainfall. Plants are able to avoid water stress by accessing available soil moisture either by having an extensive shallow root system in soils or by deep rooting depending on the location of available water. Moreover, increasing surface area by increasing the density of fine roots and root hairs improves soil exploration while reducing carbon investment (Comas 2008).

Dehydration tolerance

Dehydration tolerance refers to the capacity of plants to maintain function under low leaf water status (Luo 2010). Dehydration tolerance strategies such as osmotic adjustment, maintenance of root viability under dehydration, stabilization of structures and metabolic processes, and management of antioxidant metabolism may enable nearly normal plant growth and metabolic activities even under water stress (Huang et al. 2014; Ngumbi and Kloepper 2016). Sustaining a certain level of physiological activities entails the regulation of thousands of genes in multiple metabolic pathways to decrease or repair stress damage (Mitra 2001; Yue et al. 2006; Fang and Xiong 2015).

Recovery

Drought recovery refers to the plant capability to recover growth after severe drought (Luo 2010; Lawlor 2013; Fang and Xiong 2015). Certain metabolic adjustments or damage caused by water stress are reversible during a recovery phase, while other effects are not or only partially reversible (Salekdeh et al. 2002). The factors effecting the reversibility of the effects and consequences of water stress are reviewed by Feller (2016). There are a number of factors effecting the reversibility of the water stress including the extent of the damage to enzymes, organs, and whole plant (Feller 1998; Gilgen and Feller 2014). Certain tissues such as leaves may be irreversibly damaged but may be rapidly replaced during a recovery phase after the water stress (Blösch et al 2015). Even though certain enzymes may be inactivated (irreversibly), these can be produced de novo as long as basic cellular functions including gene expression and protein synthesis are maintained (Cartagena et al. 2015; Fu et al. 2008; Wang et al. 2015). During the water stress and recovery period, protein synthesis is vital for the adaptation of the metabolism (Feller 2016), particularly production of:

- Protective proteins (such as dehydrins) (Close 1997; Feller 2016; Vaseva et al. 2014; Volaire and Lelievre 2001).
- ROS detoxification enzymes (Ahmed et al. 2015; Jain et al. 2015; Feller 2016).
- Compatible solutes and secondary metabolites (Feller 2016; Jain et al. 2015; Simova-Stoilova et al. 2015).

The maintenance of the basic cellular functions during and after water stress is vital for water stress resilience and productivity after recovery. The capacity of the plant to recover, water stress resilience, depends on the pre-stress state and response during and after the stress; thus the whole life cycle is relevant (Feller 2016). The ability of the plant to recover from water stress relies on the plants capabilities for systemic phenotypic changes (avoidance) and dehydration tolerance. Thus a plant that increased root area during the early onset of water stress is more likely to recover after an extreme drought stress.

Characterizing and quantifying plant responses

Recent studies have pointed the direction to more effective metrics for documenting differences in root growth, production, and architecture under different circumstances. However, the overall “best” measurements and most predictive root phenotypes are likely to depend on the specific crop, age, and nature of water stress. Advances in our knowledge of root biology have refashioned the way we approach root data collection and experimental design. Moving away from root biomass as the only crude estimator of root fitness toward response variables that measure root architecture (e.g. root surface area, root length, and lateral root formation), root morphology or other physiological traits is critical for understanding what attributes of the root system are contributing to plant health and productivity (Comas et al. 2013). Here are some examples of useful response variables for studying water stress.

Response variables

- **Allometry**

Root allometry, the comparison of the investment in the roots vs. aboveground parts (e.g. root/shoot ratio), is a parameter helpful for selection of water stress tolerant plants (Karcher et al. 2008). Comas (2013) emphasizes the importance of this ratio with a horse and cart analogy wherein the above ground portion of the plant (horse) is driving uptake from the root system (cart). The “cart” or root system capacity or size limits the capacity for uptake whereas the “horse” or above ground portion of the plant can only pull/support a certain sized cart. Careful consideration of this ratio must be taken into account since the two limit each other. This ratio is usually depicted as a ratio of mass, but biomass does not accurately depict water uptake potential or photosynthetic capacity. Comparison of surface areas is likely to be more accurate, especially since investment in fine roots adds little to the biomass of the root system, despite greatly facilitating the capacity of the root system for water and nutrient acquisition.

- **Surface area**

Increased root surface area occurs via the growth and development of fine roots especially the differentiation of new roots via branching. Initiation of lateral roots (LRs) is one of the greatest overall way plants generate increased surface area (Manzano et al. 2014). Measuring traits such as surface area provides a correlation between soil exploration and water acquisition potential. Since exploration of soil in areas with available soil water is important under water-stressed conditions, it is beneficial to measure the surface area of roots in samples from different depths, as an indicator of a plant's having successful strategies to acquire water from different depths (Comas 2008). High root surface area in the upper soil layers may be of little benefit in a cropping system where available water is deep in the soil profile. Thus, sampling considerations of the deployment of surface area will depend on the specific water stress of interest. Moreover, although quantifying the root architecture of seedlings in a controlled environment is economical, repeatable, rapid, and accurate compared to adult root systems, such studies may not be good predictors of root system responses under field conditions. Previous work indicated the correlation between seedling and adult plant root systems may vary from study to study, ranging from predictive, positive correlations to no correlation (Watt et al. 2013; Caradus 1977; Comas et al. 2013), emphasizing the need to assess root system behavior under field conditions with specific attention to the life stage of interest.

- **Root number**

Since water uptake occurs primarily at the root tips, quantifying root tip number is another important parameter for predicting water uptake and hydraulic conductance. Increases in fine (small-diameter) lateral roots may improve the drought tolerance of the plant by enhancing hydraulic conductance through increased sites of water uptake (Comas et al., 2008).

A number of software programs facilitate the estimation of these root architecture parameters. In the current study, WinRhizo was used for root quantification (Arsenault et al. 1995).

- **Root morphology**

Plants are also equipped with other morphological approaches that increase water uptake efficacy and prevent cavitation under very low soil moisture. Root morphological changes such as decreased xylem diameter reduce the risk of cavitation (Comas et al. 2013; Tyree et al. 1994). The reduced risk for cavitation may also increase plant capacity to recover after extreme water stress. Remodeling of root morphology via cortical cell death and suberization are also common responses. Measuring these morphological changes is usually done via microscopic observation of roots in cross section or measuring hydraulic conductance. Despite the difficulty in obtaining these measurements, they are important for understanding root responses.

- **ROS and water stress**

The increase in hydrogen peroxide (H_2O_2) during and after water stress may be involved in the root remodeling and morphological changes that occur. Both lignification and suberization of plant cell walls is dependent on ROS signaling in a peroxidase-mediated free radical coupling process (Barceló 2009). ROS production in roots is highest in epidermal root cells and vascular tissues, where most of the ROS-dependent reactions for cell wall lignification and suberization take place (Rodríguez-Serrano et al. 2006; Barceló 2009). Lateral root initiation, emergence, and development are regulated by auxin and ROS signaling (Casimiro et al. 2001; Manzano et al. 2014). H_2O_2 accumulates in the lateral root primordium (LRP), and the peroxidase activity is proposed to transition cells from proliferation to differentiation (Manzano et al. 2014). The alterations in ROS are usually measured using staining techniques and microscopically visualized. (Juárez et al. 2015).

PGPMS AND WATER STRESS

To date, plant growth promoting microorganisms (PGPM) have been shown to help plants avoid or tolerate water stress by influencing a variety of water stress related issues affecting plant growth, survival, and yield. Ngumbi and Kloepper (2016) describe the primary ways that PGPM's influence plant water stress response in their review, and these mechanisms are summarized here.

Mechanisms

- **Root growth**

As discussed, root system architecture is one of the most important adaptations for drought (Yu et al. 2007; Huang, DaCosta, and Jiang 2014; Ngumbi and Kloepper 2016). PGPM may promote root growth (Kloepper and Bay-Peterson 1991; Kloepper et al. 2004; López-Bucio et al. 2007), and alteration in root architecture may lead to an increase in total root surface area thus enhanced water and nutrient uptake (Somers and Vanderleyden 2004; Timmusk et al. 2014; Ngumbi and Kloepper 2016).

- **Allometry**

Water stress results in an inhibition of above ground growth to reallocate carbon to root growth often leading to yield loss. PGPM may help plants maintain near-normal above ground growth to minimize yield loss (Vardharajula et al. 2011; Timmusk et al. 2014). PGPM are able to influence plant growth through production or alteration of phytohormones (Puga-Freitas and Blouin 2015; Boiero et al. 2007; Castillo et al. 2013; Belimov et al. 2009).

- **Relative water content**

Maintenance of relative water content (RWC) or plant water status is an important drought tolerance mechanism (Ashraf 2010). PGPM are capable of helping plants maintain high RWC during water stress thus maintaining cell turgor necessary for cell expansion and growth (Grover et al. 2014; Sandhya et al. 2010).

- **Osmotic adjustment**

Osmotic adjustment and the accumulation of proteins and other metabolites are important for maintaining structural and metabolic stability. Treatment with PGPM can alter plant accumulation of solutes (e.g. proline) enhancing osmotic adjustment and thereby drought tolerance (Hoekstra and Buitink 2001; Vardharajula et al. 2011; Wang et al. 2012).

- **ROS alteration**

Water stress typically results in the production of damaging reactive oxygen species (ROS). The production of scavenging enzymes has been correlated to drought tolerance (Contour-Ansel et al. 2006). PGPM are also able to induce antioxidant systems (Gururani et al. 2013; Saravanakumar et al. 2011), thereby priming the plant for water stress and/or reducing the negative effects of ROS.

PGPM are able to increase water stress avoidance, dehydration tolerance, and recovery through the mechanisms discussed above. The physiological, transcriptional, morphological changes associated with plant tolerance and avoidance of water stress can therefore not be separated from the services provided by the phytobiome. Increasing production under water-stressed conditions must then include enhancing the ability of the plant to take advantage of phytobiome services.

RHIZOSPHERE COMMUNITY DEVELOPMENT

Recently, there has been interest in breeding crops capable making use of phytobiome services (Bakker et al. 2012; Gopal and Gupta 2016; Sessitsch and Mitter 2015; Wei and Jousset 2017; Wissuwa et al. 2009). In thinking about the development of rhizosphere communities, it is important to consider the plant, microbial, and our viewpoints, i.e. to take a balcony view of the ecological reasons for the involvement of all bionts (plant and a plethora of microorganisms) that form symbiotic communities on which agricultural productivity hinges. From our point of view, the goal of these established communities is plant health that leads to enhanced productivity. The driver

for the symbionts is individual fitness. As discussed above, the phytobiome may have profound effects on plant fitness. However, soil residing microbes that are adapted to occupy the rhizosphere niche and are capable of colonizing roots also may have increased fitness because of the nutrients and protection provided by the plant. The microbial influence on root architecture and physiology, while potentially providing enhancements in plant health, increase the availability of suitable niches for the microbe, which may be the microbial driver for these enhancements. Microbes also benefit from increased plant health potentially via further plant investment in nutrients into the niche. Thus, cultural practices that seek to improve the productivity of plant communities by enhancing the establishment of beneficial phytobiomes are likely to result in forces that sustain both the productivity of the plant and the phytobiome.

If our goal is to optimize beneficial symbiosis to improve production, we must understand factors that influence this dynamic process. The application of specific microbes to enhance plant health has had limited success. Practices that result in shifts in the entire soil community toward a plant microbiome that yields enhanced plant productivity have much larger, long-term implications for improving food security. Given the importance of plant investment in root exudation for shaping the rhizosphere microbiome (Badri and Vivanco 2008; Berg et al. 2014; Reinhold-Hurek et al. 2015; Bais et al. 2006), it is important to understand exudation patterns: what factors influence them and how they vary spatially and temporally.

Plant investment in root exudation and rhizodeposition

Plants invest a substantial amount of total net fixed carbon into below ground structures and processes, including rhizodeposition defined as the release of carbon into the rhizosphere (Jones et al. 2009). This investment varies among plant species and during the course of plant development and maturation. For example, in one study it was estimated that of investment in total net fixed carbon to root biomass, rhizosphere respiration, rhizodeposition, and soil residues were 19, 12, 11, 5%, respectively, with a minimal amount lost to leaching and runoff (Jones et al. 2009). Rhizodeposition includes release of border cells and mucilage, death and lysis of root

cells, production volatile organic carbon, and root exudation (extensively review by Jones et al. 2009). Investment of photosynthetically fixed carbon by plants as rhizodeposition is a significant carbon cost for the plant (Badri et al. 2009; Baetz and Martinoia 2014). As a result of the carbon investment, carbon is not as limited in the rhizosphere as it is in the bulk soil (Bakker et al. 2013; Overbeek and Elsas 1997; Koch et al. 2001). This investment mediates symbiotic associations with beneficial microbes, including their inhibition of deleterious and pathogenic microbes (Baetz and Martinoia 2014; Bais et al. 2006; Haichar et al. 2008; Philippot et al. 2013).

Carbon investment by the plant can be viewed as a necessary investment in the short, medium, and long-term (Haichar et al. 2008; Haichar et al. 2014). In the short term, root exudates attract soil microorganism, after which they may colonize this carbon rich environment (Lugtenberg and Kamilova 2009). The boost in carbon availability leads to increased microbial populations and may both encourage intimate symbiosis (such as colonization of root surface and endosphere) (Hardoim et al. 2008) and alter the soil environment by encouraging microbial soil organic matter decomposition. Together these outcomes may further increase nutrient availability and thus lead to medium and long term gains (Churchland and Grayston 2014; Scott-Denton et al. 2006; Subke et al. 2004). After establishment of symbiosis, the plant may continue to invest in these microbial partners and may be thought of as a long term investment in a partnership, although the return on investment may not be immediate. For example in the case of arbuscular mycorrhization, plant investment in carbon may lead to a long-term return on investment in the form of nutrient availability and acquisition (reviewed by Lanfranco et al 2016). However, the return on investment is not guaranteed: plants may not reap the benefits of symbiosis with a microbe capable of inhibiting a plant pathogen unless favorable environmental conditions occur for pathogen invasion occurs. The benefits of maintained symbiosis and investment may extend beyond the current season, promoting soil health in the next cropping system or even for years to come. This may include the long-term buildup of soil organic matter (Clemmensen et al. 2013),

soil nutrients (this is well documented for nitrogen producing rhizobia), and microbial populations.

Temporal and spatial root exudation and rhizodeposition patterns

Root exudates include amino acids, organic acids, sugars, phenolics and other secondary metabolites (such as antimicrobials and phytotoxins). Root exudate components have been extensively summarized and reviewed (Badri and Vivanco 2008; Dennis et al. 2010; Haichar et al. 2014; Jones et al. 2004). Root exudation patterns are not uniform along the entire root surface given the unique physiological process occurring within each root zone. Different sources and compounds may be released from distinct zones of the root system (Frenzel 1960; Badri and Vivanco 2008).

At the root apex, root carbon deposition is composed primarily of mucilage and root border cells. As the root tip grows through the soil it is protected by the cells of the root cap and the sheath of mucilage they produce. The living, detached root cap cells called border cells are metabolically active (Hawes 1991), and influence rhizosphere communities in a variety of ways. These include their effects on pathogenic (Gochnauer et al. 1990; Gunawardena and Hawes 2002; Hawes et al. 1998, 2000), and plant-beneficial microorganisms (Hawes et al. 1998). Immediately behind the root cap is the meristematic zone where the vast majority of root exudates are thought to be released (Dennis et al. 2010; McDougall and Rovira 1970; Norton et al. 1990; Darwent et al. 2003). The meristematic zone is thought to be a site of exudation of strigolactones (attract arbuscular mycorrhizal fungi (Akiyama et al. 2005) and flavonoids attract rhizobia (Spaink 1995; Hirsch et al. 2003). In the meristematic or cell-division zone, the plant actively allocates carbon to maintain cell division and the high carbon allocation to this area is thought to alter electrochemical gradients to produce passive exudation (Dennis et al. 2010; McDougall and Rovira 1970). The meristematic zone transitions into the zone of elongation, where cells expand 10–20 times of their original length, thus approximate growing at a rate of 0.2–1.0 $\mu\text{m s}^{-1}$ (Dennis, Miller, and Hirsch 2010). Using a creative microfluidic live-imaging technique called TRIS (tracking root interactions system), Massalha et al. (2017) showed that *Bacillus subtilis* is

chemotactically recruited to the root elongation zone. After initial colonization of the elongation zones, colonization was also visualized in the maturation zone (Poole 2017; Massalha et al. 2017). For example, root hairs are also known to secrete mucilage at their tips (Scott et al. 1958; Curl and Truelove 1986).

Although root exudation may be highest at the actively growing root tips (García et al. 2001), older roots also exude organic compounds and are sites of microbial colonization (Badri and Vivanco 2008; Bowen 1968; Mcdougall and Rovira 1970; Pearson and Parkinson 1960; Rovira 1969). For example, Frenzel (1960) showed using mutants of *Neurospora* with specific nutrient requirements that different organic compounds were available uniquely along the root surface. Threonine and asparagine were available at the root apex, while leucine, valine, phenylalanine, and glutamic acid were available in the root hair zone (maturation zone), and aspartic acid was available along the whole root (Rovira 1969; Frenzel 1960). Exudates are also high where lateral roots emerge (Van Egeraat 1975). Since the root becomes suberized in the maturation zone, it is expected that exudation is decreased however evidence suggests that the plant is still investing in these areas. At first glance these results suggest that the previous hypotheses regarding root exudation being derived mainly from the meristematic region are incorrect. However given the rapid nature of root growth and development at the root tip it is not surprising that root exudates and signals produced at one developmental stage are utilized by microbial population when the root is slightly older, owing to the time it takes for microbes to arrive and their relative ability to travel with the developing root tip. Moreover, the exact source of exudation is difficult to pin point as it may be produced by the plant de novo or it could have been deposited by cells at the root tip and is now available on older tissues or in another form.

Genotypic variation in root exudation and root exudation plasticity: effects on community assembly

Root exudation patterns are influenced by plant genotype, developmental stage, and environment. Importantly, the exudation of some compounds is an active process that requires ATP and/or is mediated by specific transporters (Jones et al. 2004; Loyola-

Vargas et al. 2006; Badri et al. 2008), suggesting purpose behind exudation patterns. Increasingly, evidence suggests that the phytobiome composition is strongly determined by plant species (Haichar et al. 2008; Lemanceau et al. 1995; Miethling et al. 2000; Costa et al. 2006; Garbeva et al. 2008), and plant genotype/cultivar (Rengel et al. 1998; Berg et al. 2002, 2006; Miethling et al. 2000; İnceoğlu et al. 2012; Mazzola et al. 2004; Kuklinsky and Sobra 2005) presumably via rhizodeposition patterns. For example, comparison of rhizosphere bacteria communities of different plants grown in the same soil showed that plant species was a strong selective determinant of bacterial community composition (Dohrmann and Tebbe 2005). The diversity of rhizosphere bacteria was also found to be different between ancient land races and modern wheat cultivars. Interestingly, pseudomonads were more abundant in the rhizospheres of the land races, but were the most dominant endophytes in the modern cultivars, which may have been due to differences in rhizodeposition patterns, root morphologies, or both (Germida and Siciliano 2001). The rhizosphere microbiome is also modulated by phytohormones, mainly salicylic acid (Balachandar et al. 2006; Lebeis et al. 2015), as well as phenolics (Badri et al. 2013) released from the roots, potentially in a genotypically distinct manner (Gopal and Gupta 2016).

Root exudation and rhizodeposition patterns also are affected by environment, especially plant stress (Baudoin et al. 2003) including: water supply (Henry et al. 2007; Song et al. 2012; Calvo et al. 2017) temperature (Rovira 1959), light (Hodge et al. 1997), atmospheric CO₂ concentration (Calvo et al. 2017; Cheng and Johnson 1998; Paterson et al. 1996), and nutrient availability (Carvalhais et al. 2011; Yang and Crowley 2000). For example, total organic carbon exuded by wheat grass exposed to drought stress increased by 71% compared to the well-watered control (Henry et al. 2007). Root exudation or rhizodeposition has been shown to significantly influence rhizosphere bacterial diversity (Latour et al. 1996; Rovira 1965). Environmental influences on root exudation patterns may allow plants to select for a rhizosphere microbiome capable of supporting them during these different stresses. For example, Santos-Medellín et al. (2017) found that rice rhizosphere communities were significantly altered by drought

stress. By sequencing (16S rRNA region- bacteria and ITS1- fungi) rhizosphere and endosphere (the root interior) samples, they found that drought significantly altered the overall bacterial and fungal compositions in the maturation zone (Santos-Medellín et al. 2017). They used older roots to determine how already existing microbial populations shift following drought. This work provided strong evidence that plants are able to reconstruct the rhizosphere and endosphere microbiome *de novo* (under drought pressure) even in older regions of the root, where the root invests less carbon. Rhizosphere community assembly also has been shown to be affected by plant physiology (Kniskern and Traw 2007; Long et al. 2010), plant growth stage (Lundberg et al. 2012), soil type (Latour et al. 1996; Berg and Smalla 2009) and soil history (Garbeva et al. 2008; Lupwayi et al. 1998), suggesting exudation plasticity may be affected by both internal and external influences.

Strategies for utilizing the plants influence on microbial community assembly

Genetic by environmental influences on microbial community assembly presumably via alterations in root system growth patterns, rhizodeposition patterns, or both, suggest that selecting lines for enhanced capacity to attract beneficial *rhizosphere microbiomes* may be a promising breeding target (Bakker et al. 2012). The exact mechanistic approach to screening for “intelligent” phytobiome selection is complicated by the complexity of the phytobiome, and it is unclear whether analyses should be based on characterizations of the taxonomic structure or functionalities of these microbial communities. Although many studies have focused on the former, aided by next generation sequencing techniques, Yan et al. (2017) found that microbial functionalities may be more likely to be conserved in community assemblies. In other words, it is likely that plants are actively recruiting microbes that have functional capacities that are favorable under certain conditions (Mendes et al. 2014; Yan et al. 2017). As such root exudation patterns may be an important target for altering microbial communities, providing a potential target for screening in a breeding program (Jones et al. 2009).

The recruitment of specific microbial symbionts and their effect on the host have been found to be highly specific. For example, Meyer et al. (2010) showed that the

growth promotion effects of an inoculant containing *Pseudomonas* were dependent on the wheat cultivar with which it was paired and other environmental factors. In this study, different Swiss cultivars of winter wheat (*Triticum aestivum*) including Arina, Zinal, and Cimetta were tested for their ability to recruit plant-beneficial pseudomonads from the rhizosphere. Rhizosphere populations were screened for the potential to produce 2,4-diacetylphloroglucinol (DAPG), a well-characterized secondary metabolite important for suppression of disease caused by *Pythium* species. Significant differences were found among cultivars in the recruitment of DAPG-producing microbes in the presence and absence of the *Pythium* (Meyer et al. 2010).

The genetic bases of these interactions is of interest, and statistical methods such as quantitative trait locus (QTL) can be used to correlate a plant trait (such as disease resistance) and plant genotypic data (usually molecular markers). These statistical methods that link the trait of interest, such as microbial colonization, with a few or single genetic loci provide a useful approach for rapidly screening for the trait or symbiosis of interest. For example, Smith and Goodman (1999) used a QTL analyses to characterize plant traits important for interactions between tomato and a disease-suppressive bacterial species, *Bacillus cereus*. Three QTL were found to explain 38% of the phenotypic variation associated with disease suppression by *B. cereus*, in the recombinant inbred lines (Smith and Goodman 1999). These results demonstrate the role of host genotype in influencing the colonization of plant-beneficial bacteria and ultimately the success of the plant-microbe interaction in improving plant health.

Effort has also been made to alter the genetics of plants to enhance microbial recruitment and symbiosis with a specific microbial partner. One example is engineering the plant to produce novel carbon sources which favor the growth of an inoculant strain (Bakker et al. 2013). The presence of a particular substrate may favor the growth of one microbe and inhibit others. For example, benzoxazinoids (BX), antibiotics found in maize root exudates, have been shown to attract the BX-insensitive PGPM, *Pseudomonas putida* KT2440 (Neal et al. 2012). It is important to understand that altering plant investment strategies may have off target effects given the high diversity

of microbes in the soil. Moreover, there is a tendency to focus on plant interactions with single isolates or a consortium containing a few microbes, which may not provide a complete picture of the structure and function of plant-beneficial rhizosphere communities. For example, disease control is not always dependable when single isolates are re-introduced into field conditions since other soil microbes can inhibit the disease-suppressing capacity of the biological control agent (Morello et al. 2004). The application of specific inoculants undeniably has the potential to increase plant production and facilitate a solid understanding of the services and mechanisms of action provided by the inoculant. However, a more sustainable approach is to develop plants that recruit **indigenous** microbes having the beneficial qualities of the inoculant, rather than rely on exogenous application. Taking advantage of naturally-occurring rhizosphere microbes may hold exceptional potential to increase agricultural production in the future.

This relationship between plant genotype-specific root exudation patterns and rhizosphere phytobiome composition suggests that it may be possible to breed for certain root exudation patterns, which in turn create beneficial rhizosphere microbiomes. Although the exact microbial services and mechanisms of action need for a specific benefit may not be known, it is important to study the microbial functionalities that are selected under the growing conditions of interest. Selecting lines capable of taking advantage of soil microorganisms with the capacity to improve plant health may be a viable solution to addressing agricultural problems such as water stress. *Evidence presented in my study suggests that plants are selecting for microbial functionalities that are a benefit to them under water-stressed conditions.*

SYNTHESIS

Given the current understanding of predicted climatic changes, the potential of PGPM for improving plant productivity under water deficit, and the role of the plant in rhizosphere microbiome selection, it is imperative that we begin to incorporate strategies for harnessing the plant microbiome into our plant breeding efforts. Over all, there is a

need to select for genotypes capable of selecting for microbial partners with functionalities that enhance plant fitness under water stress.

What is the best way to get there? One strategy is to study the microbial functionalities that are selected under the growing conditions of interest—in this case water stress tolerance. Some important questions to consider are:

- What bacterial functionalities are selected for under water stress?
- Do these functionalities increase water stress tolerance? What plant strategies (escape, avoidance, dehydration tolerance, recovery) or phenotypes are being improved by the presence of the microbe or functionality under water stress conditions?
- Is there plant genetic variation in the selection of microbes with these functionalities?
- What mechanisms are underpinning how plant-microbiome assembly occurs and to what extent are they influenced or independent of environment, including land use history?
- How can we work across disciplines to incorporate strategies for the enhancement of phytobiomes into production systems to improve production in dryland agriculture?

In order to address these questions it is necessary to have a good **biological system**.

Requirements of a good biological system include:

1. Genetic resources including plant genotypes with known drought response as well as microbial agents for which mutants deficient in specific microbial functionalities already exist.
2. The symbiosis between the partners must be prevalent in nature, especially in dryland agriculture.
3. Both symbionts should be well adapted to water deficit conditions, e.g. have phenotypes that could be modified or enhanced by each partner.

4. Microbial colonization must have plasticity in response to environmental conditions, e.g. there should be a correlation between presence of the microbe and ability to grow in the desired condition.
5. The symbiosis must result in an increase in plant fitness under water deficit i.e., the presence of the microbe should provide a functionality that improves plant water stress tolerance.

In this study, I focused on winter wheat (*Triticum aestivum*) as the plant host because it is a vital food crop that is grown in regions subject to extreme drought. Specifically, I focused on Texas A&M (TAM) winter wheat lines and cultivars that are widely grown in the USA great plains and have either been selected for their water stress tolerance or their ability to grow in the same climatic regions under irrigation (**Requirement 3**). Their well-characterized variance in drought tolerance and the genetic and physiological resources for breeding using this material makes TAM winter wheat an ideal crop for studying/enhancing Plant-PGPM interactions (**Requirement 1**).

Recently, researchers reported that rhizosphere bacterial communities differed for wheat plants grown in dryland production compared to irrigated fields (Mavrodi et al., 2012a, b). These studies focused on the abundance of microorganisms known to be antagonistic to soilborne fungal pathogens, such as *Gaeumannomyces graminis* var. *tritici* (Ggt). The studies focused primarily on the relative abundance of *Pseudomonas* strains capable of producing redox-active phenazines or the polyketide 2,4-diacetylphloroglucinol (2,4-DAPG), both broad spectrum antibiotics effective against Ggt. Mavrodi et al. (O. V. Mavrodi et al. 2012-2012b) reported that indigenous phenazine-producing bacteria were detected at high frequencies (67 to 100% of plants sampled) on dryland winter wheat roots as compared to (8 to 50% of plants sampled) in irrigated fields. Populations of phenazine-producing strains were substantial on wheat roots from dryland production ($>10^5$ CFU g⁻¹ fresh weight of root). The abundance of phenazine-producing bacteria in natural wheat soils suggest that this symbiosis is wide spread in dryland wheat production (**Requirement 2**). The frequency and abundance of phenazine-producers were determined from the presence of genes responsible for the

production of phenazine-1-carboxylic acid (PCA). These indigenous populations included at least 31 *Pseudomonas* genotypes (Parejko et al. 2012). Moreover, in a companion study (D. V. Mavrodi et al. 2012- 2012a) they found that the frequency of wheat root systems colonized by phenazine-producing (Phz⁺) pseudomonads was inversely related to annual precipitation, concluding that Phz⁺ pseudomonads flourish in the rhizospheres of wheat experiencing low soil moisture. However the mechanisms underlying this relationship were unknown. The variation in the frequency and abundance of phenazine-producing bacteria along a soil moisture gradient suggests that there is plant phenotypic plasticity in the selection of the microbiome correlated to environment (**Requirement 4**). Previous work by the Pierson laboratory group and others demonstrated that phenazine production is a strong determinant of the inhibition of soilborne pathogens, biofilm production and architecture, and the competitive survival of the phenazine-producing strains in the rhizosphere (Pierson III and Thomashow 1992;Weller 2007; Pierson and Pierson 2010).The production of phenazine is thus a microbial trait that aids microbial survival in water deficit conditions (**Requirement 5**).

The functional benefit provided to the plant by phenazine production was studied using a well-characterized phenazine producer, *Pseudomonas chlororaphis* 30-84. *P. chlororaphis* 30-84 was isolated from the wheat rhizosphere and selected for its ability to suppress take-all disease of wheat (Weller 1988; Pierson III and Thomashow 1992; Thomashow et al. 1990). The rhizosphere competence of *P. chlororaphis* 30-84 makes it an ideal PGPM for studying the recruitment of phenazine-producers by drought-adaptive wheat cultivars. The availability of *P. chlororaphis* 30-84 mutants deficient in or enhanced in phenazine production also enabled me to look specifically at the effect of phenazine production on water stress tolerance (**Requirement 1**).

System specific questions

Using this ideal biological system, the aim of my thesis was to address several questions:

- Is phenazine-production a functional trait that is selected for by TAM Winter wheat? (**Chapter II**)

- How does the presence of the phenazine-producer *P. chlororaphis* 30-84 affect plant water stress response? What plant water stress management strategies are enhanced? For example does colonization of wheat roots by phenazine-producers enable wheat to escape or tolerate dehydration stress and/or recover from water stress events? What phenotypes contribute to increased health under water-stressed conditions? Is the effect growth stage dependent? (**Chapter II**)
- Do TAM cultivars vary in the selection of phenazine producers? What mechanism are underpinning plant-phytobiome assembly? Is this a phenotype that can be selected for? (**Chapter III**)
- Is selection of phenazine producers effected by environmental factors such as land use and water stress? (**Chapter III**)
- How can we work across disciplines to incorporate enhancement of phytobiome into production systems to improve production in dryland agriculture? (**Future work**)

Chapter II approach

To determine the ability TAM 112 and TAM 111 to select for *P. chlororaphis* 30-84, wheat seeds were planted in soil pre-inoculated with the bacteria and colonization was quantified. The functional benefit of phenazine production was analyzed by conducting water stress trials. The availability of mutants deficient in or enhanced in phenazine production enabled me to look specifically at the effect of phenazine production on water stress tolerance, especially root architecture. I hypothesized that bacterial production of phenazines may facilitate resilience following extreme water stress by influencing phenotypic changes that may contribute to water stress avoidance.

Chapter III approach

The effect of cultivar, land use, and water stress were analyzed by growing winter wheat in natural field soil with different production histories and under different soil moisture regimes. Using two Texas winter wheat cultivars bred for drought tolerance, TAM 111, TAM 112, and a drought-sensitive cultivar TAM 304 bred for use with irrigation allowed me to determine whether selection was cultivar dependent. I

hypothesized that cultivars with higher drought tolerance would have increased recruitment of phenazine-producing bacteria and because of this, land use history where cultivar selection may come into play, may also influence community composition. Moreover, I hypothesized that water stress may be important in shaping rhizosphere communities.

Future work

Breeding for lines capable of selecting indigenous phenazine-producing bacteria may increase drought tolerance by taking advantage of the functional capacity of soil organisms. Can specific wheat QTLs be correlated with the enhancement of colonization by phenazine-producers in drought tolerant cultivars? Does the selection of lines capable of recruiting this microbial function (phenazine-production) lead to enhanced plant water stress tolerance via improvements in water use efficiency, favorable root phenotypes, or plant growth patterns under field conditions?

CHAPTER II
BACTERIALLY MEDIATED WATER STRESS TOLERANCE IN WHEAT
CONFERRED BY PHENAZINE-PRODUCING RHIZOBACTERIA

INTRODUCTION

Drought is an exceedingly important global concern and is expected to be a chronically serious problem for more than 50% of arable lands by 2050 (Vinocur and Altman 2005; Naveed et al. 2014). Moreover, elevated temperatures predicted from climate change will increase the rate of soil drying in agricultural land, resulting in the more rapid onset of water stress with higher intensity (Trenberth et al. 2013; Fischer and Knutti 2015). Predicted changes in climate suggest that it is not only important to increase the ability of plants to withstand water stress (dehydration tolerance), but to enhance the potential of plant root systems for water-uptake (water stress avoidance) and recovery after extreme water stress. Plant water stress tolerance is a complicated phenotype controlled by many genes and traits that in turn are influenced by numerous environmental factors. Given the complexity of breeding for crop improvements in water stress tolerance and the length of time required for the release of new varieties, there is urgency for identifying additional solutions such as the utilization of the plant's microbiome for enhancing the plant's capacity for stress tolerance. The plant microbiome includes the microorganisms colonizing plant surfaces, residing within plant organs either in the extra- or intra-cellular domains, or dwelling within the plant's zone of influence. These plant-associated microorganisms are considered to be a "second genome" for plants (reviewed in: Berendsen et al. 2012; Turner et al. 2013), providing the capability to directly modify the plant's biotic and abiotic environment, as well as influence phenotypic changes in plants to enhance their ability to respond to their environment. The rhizosphere is the plant's zone of influence in the soil and this interface between plant roots and the soil is a dynamic environment that is the site of a

majority of ecosystem services provided by beneficial, plant growth promoting microorganisms (PGPM) (Wei and Jousset 2017).

PGPM have been shown to increase the plant's capacity to avoid or tolerate water stress through a variety of mechanisms (review by Ngumbi and Kloepper 2016) including: increases in root growth (Kloepper and Bay-Peterson 1991; Kloepper et al. 2004; López-Bucio et al. 2007), influences on plant growth through production or alteration of phytohormones (Puga-Freitas and Blouin 2015; Boiero et al. 2007; Castillo et al. 2013; Belimov et al. 2009), maintenance of high relative water content (Grover et al. 2014; Sandhya et al. 2010), enhancing osmotic adjustment (Hoekstra, Golovina, and Buitink 2001; Vardharajula et al. 2011; Wang et al. 2012), and reducing the negative effects of ROS by inducing antioxidant systems (Gururani et al. 2013; Saravanakumar et al. 2011). Agricultural production is highly dependent on the services provided by *indigenous* PGPM and making use of opportunities afforded by these microbial partners is an important dimension of crop improvement.

Evidence suggests that plants recruit a specific microbial community to their rhizospheres (Bergsma-Vlami et al. 2005; el Z. Haichar et al. 2008; Mendes et al. 2014; Yan et al. 2017), mainly through alterations of rhizodeposition (Latour et al. 1996; Rovira 1965). Rhizodeposition is a variable trait influenced by plant genotype (Rengel et al. 1998; Berg et al. 2002, 2006; Miethling et al. 2000; İnceoğlu et al. 2012; Mazzola et al. 2004; Kuklinsky and Sobra 2005) and environmental conditions (Baudoin et al. 2003; Calvo et al. 2017; Henry et al. 2007; Song et al. 2012). In other words, plants recruit higher populations of rhizosphere microbes under certain conditions, and evidence suggests that this selection is highly dependent on the microbial functional capacities rather than microbial taxonomy (Mendes et al. 2014; Yan et al. 2017). But, does recruitment of microbial communities with distinct microbial functionalities occur at landscape scales where plants are subject to chronic water-stressed conditions? How do these microbial functionalities enhance plant water stress response? What bacterial mechanisms underpin the enhancement of plant water stress tolerance?

Recently, researchers reported that rhizosphere bacterial communities differed for wheat plants grown in dryland production compared to irrigated fields (Mavrodi et al., 2012a, b). These studies focused on the abundance of microorganisms known to be antagonistic to soilborne fungal pathogens, such as *Gaeumannomyces graminis* var. *tritici* (Ggt). The studies focused primarily on the relative abundance of *Pseudomonas* strains capable of producing redox-active phenazines or the polyketide 2,4-diacetylphloroglucinol (2,4-DAPG), both broad spectrum antibiotics effective against Ggt. Mavrodi et al. (2012b) reported that indigenous phenazine-producing bacteria were detected at high frequencies (67 to 100% of plants sampled) on dryland winter wheat roots as compared to (8 to 50% of plants sampled) in irrigated fields. Moreover, in a companion study (2012a) they found that the frequency of wheat root systems colonized by phenazine-producing (Phz⁺) pseudomonads was inversely related to annual precipitation, concluding that Phz⁺ pseudomonads flourish in the rhizospheres of wheat experiencing low soil moisture. In addition to inhibiting other organisms via biocontrol and competition, phenazines have also been shown to act as: electron shuttles with the potential to both generate reactive oxygen species (ROS) or mediate redox stress caused by ROS, contribute to biofilm formation and architecture, enhance rhizosphere competence, and influence metabolic activities of other organisms, both prokaryotic and eukaryotic (Mavrodi and Blankenfeldt 2006; Pierson and Pierson 2010; Pierson III and Thomashow 1992; Weller 2007; Xu et al. 2015). The abundance of phenazine producers in dryland agriculture and the roles of phenazines in increasing bacterial water stress tolerance, lead me to hypothesize that the production of phenazines by rhizosphere bacteria is an important functional trait selected by wheat under water stress, leading to increased plant water stress tolerance. I hypothesize that phenazine-producing pseudomonads enhance water stress tolerance through alterations of plant water stress tolerance mechanisms such as: changes in root growth (avoidance); dehydration tolerance (through the amelioration of ROS stress); or increasing water stress resilience or recovery after water stress.

The aim of the present study was to address several questions. Is phenazine-production a functional trait that is selected for by drought tolerant lines of TAM winter wheat? Does colonization of wheat roots by phenazine-producers enable wheat to tolerate and recover from extreme water stress events? Is the effect growth stage dependent? If phenazine production does enhance water stress tolerance, are there easily observable root phenotypes associated with microbial phenazine production that could be used as predictors of responsive plant-microbe interactions? In the present study, the effect of the well-characterized phenazine-producing biological control strain, *Pseudomonas chlororaphis* 30-84, on water stress tolerance and recovery of wheat was evaluated. The rhizosphere competence of *P. chlororaphis* 30-84 makes it an ideal PGPM for studying the recruitment of phenazine-producers by drought-adapted wheat cultivars. The availability of mutants deficient in or enhanced in phenazine production enabled me to look specifically at the effect of phenazine production on water stress tolerance, especially root morphology and plant allometry. I hypothesized that bacterial production of phenazines may facilitate survival and recovery of plants following extreme water stress by influencing plant phenotypic changes that may contribute to water stress avoidance.

RESULTS

***P. chlororaphis* 30-84 recruitment from soil by TAM 111 and TAM 112**

P. chlororaphis 30-84 recruitment from soil by drought-adapted winter wheat cultivars TAM 111 and TAM 112 was examined. Wheat seeds were sown in soil inoculated with bacteria, allowed to germinate, and then stored at 5 C for 8 weeks. Roots were harvested after 8 weeks of vernalization and bacterial colonization was measured by dilution plating. Colonization of both cultivars exceeded 10^5 and was relatively uniform over the entire length of the root (Fig. 2.1). Bacterial colonization of TAM 112 was slightly higher on all three root segments [proximal roots near the crown, the maturation zone, and the meristematic zone (root tip)], compared to TAM 111, and

these differences were statistically significant for the maturation and meristematic segments. The average population per centimeter on maturation root segments was 5.2×10^4 for TAM 111, and 1.9×10^5 for TAM 112. Meristematic root segment populations were 7.6×10^4 and 2.1×10^5 for TAM 111 and TAM 112, respectively (Fig. 2.1).

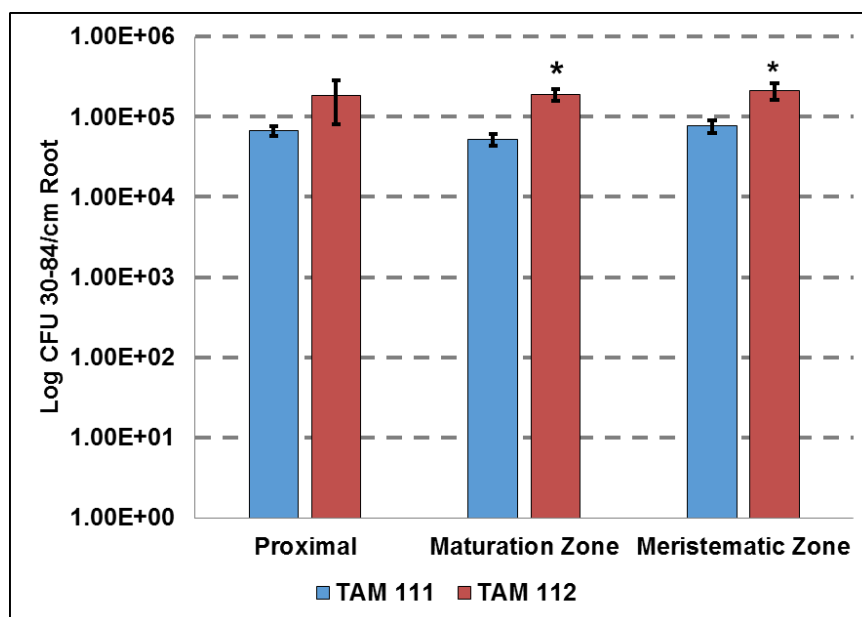


Figure 2.1: Recruitment of *Pseudomonas chlororaphis* 30-84 by TAM 111 and TAM 112 after eight weeks of vernalization. Log colony forming units/cm root isolated from the proximal roots, maturation zone, and cell division zone when grown in autoclaved soil with 10^7 CFU *Pseudomonas chlororaphis* 30-84/gram soil at planting. Values with * differ significantly using T-test ($P > 0.05$). $n=4$.

Plant water stress recovery is improved by the presence of phenazine-producing bacteria

I hypothesized that bacterial production of phenazines may facilitate recovery of plants following extreme water stress. To test this hypothesis, winter wheat (cultivar

TAM 112) was grown in soil inoculated with *P. chlororaphis* 30-84 wild-type (30-84WT), the *P. chlororaphis* 30-84 enhanced phenazine-producer (30-84ENH), the *P. chlororaphis* 30-84 phenazine-deficient mutant (30-84ZN), or soil without bacteria (control) for 3 weeks with adequate water. Plants were subsequently water stressed by withholding water for 11 days (the maximum period after which plants were able to recover from water stress as determined in a preliminary experiment). After 7 days of recovery (e.g. following rewatering), plants grown in soil inoculated with 30-84ENH had significantly higher survival rates compared to 30-84 wild-type inoculated soil, and both of these phenazine-producers survived better than plants grown in soil inoculated with 30-84ZN or the non-inoculated control plants (Fig. 2.2A). Recovery from water stress also was evaluated using a Recovery Index (RI) based on the amount of above ground tissue that recuperated after extreme wilting, where RI-0 = no recovery, RI-1 = slight new growth, RI-2 = recovery of partial leaf, RI-3 = recovery of one or more entire leaves (Fig. 2.2B). Similar to the survival rates, the RI of 30-84ENH-inoculated plants was significantly higher than plants inoculated with 30-84WT, and both phenazine-producers recovered better than 30-84ZN or the control plants (Fig. 2.2C). Of note in all experiments, the survival rates and RIs of 30-84ZN-inoculated plants and control plants were not significantly different, but were significantly less than both phenazine-producers (Fig. 2.2A,C), suggesting that bacterial phenazine production functions in water stress resilience. Higher water stress resilience with enhanced phenazine production compared to wild type also supports this hypothesis.

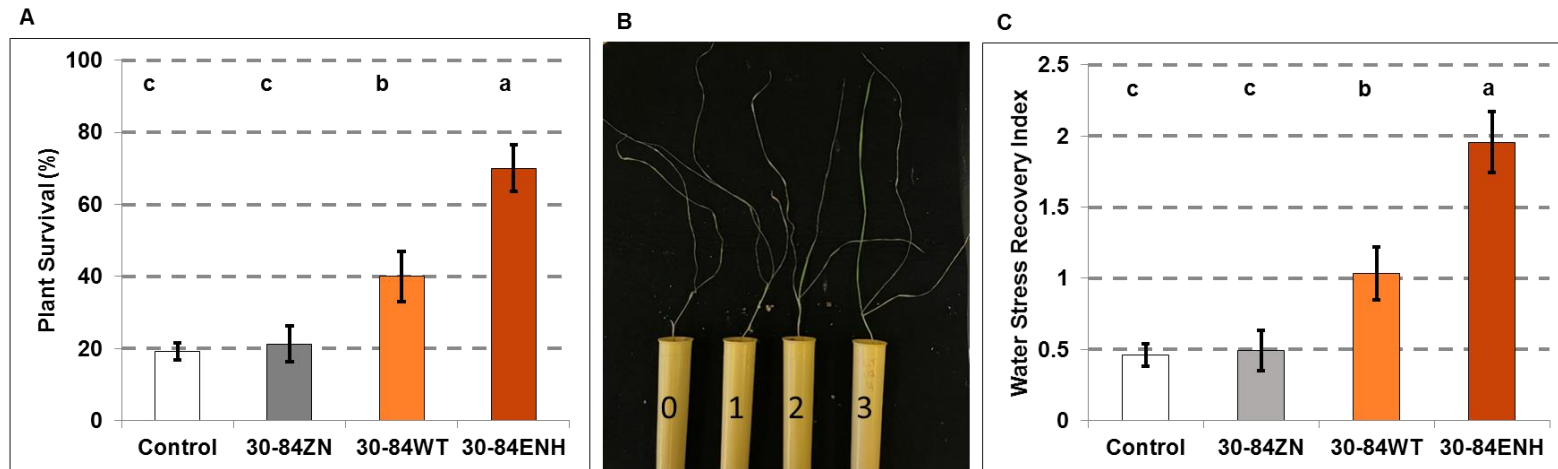


Figure 2.2: Effect of enhanced phenazine production on water stress tolerance. A. Plant survival. B and C. Recovery index (RI) and Recovery following water stress. RI evaluated from the amount of the above ground tissue recuperated after extreme wilting (RI-0 = no recovery, RI-1 = slight new growth, RI-2 = recovery of partial leaf, RI-3 = recovery of one or more entire leaves). Wheat seedlings were sown either in bacterial inoculated soil (30-84WT, 30-84ZN and 30-84ENH) or non-inoculated soil (control). After 3 weeks of growth, plants were water stressed for 10-11 days, and re-watered. After 7 days of re-watering, plants were evaluated and measurements taken. These experiments were repeated once. Values with the same letter do not differ significantly as determined by a Fishers protected Least Significantly Difference (LSD) test ($P > 0.05$).

Phenazine-producing bacteria influence root tip production and plant architecture

The effects of *P. chlororaphis* derivatives (30-84WT, 30-84ENH, and 30-84ZN) on wheat root morphology were examined in order to understand the mechanisms underpinning the increased water stress resilience of wheat seedlings in the presence phenazine-producers. It was important to capture the effect of the phenazine-producing bacteria on root morphology at two unique stages of plant development, e.g. seedlings and older, vernalized plants in the jointing stage. Because TAM 112 is a winter wheat variety, vernalization was required to promote plant development beyond the vegetative stage. The seedlings used in this experiment were the same plants from the previous experiment, and roots were analyzed after exposure to a second water stress. After the second water stress, roots were harvested, washed, scanned, and a representative picture of each treatment is included before and after washing Fig. 2.3 and Fig. 2.4, respectively. For the older plants, winter wheat seeds were sown in soil inoculated with bacteria (30-84WT, 30-84ZN, or 30-84ENH) or without (control), allowed to germinate, and then stored at 5 C for 8 weeks. After vernalization, plants were transferred to soil with the same soil inoculation treatment and watered well until jointing stage. Plants were then water stressed for 15 days and harvested. Because many of the vernalized plants did not recover from the water stress treatment, only one water stress/recovery cycle was performed.

Whinrhizo software was used to analyze root architecture: e.g., root surface area, root length, and number of root tips. As expected, seedling plants had significantly less root development compared to roots of older vernalized plants regardless of the presence or absence of bacteria. Average root surface area for seedlings ranged from 2.9 to 6.1 cm² compared to 15.5 to 23.9 cm² for vernalized plants (Fig. 2.5). Total root length also was much lower for seedlings ranging from 48.6 to 85.7 cm, compared to 201.2 to 293.1 cm for vernalized plants (Fig. 2.5). The average number of root tips for seedlings ranged from 182.4 to 376.5 and from 569.3 to 997.9 for vernalized plants (Fig. 2.5).

The main effect of bacterial phenazine-producers was an enhancement in the branching of the roots. Both seedlings and vernalized plants inoculated with 30-84ENH

and 30-84WT had a significantly greater numbers (e.g., ~2 fold more) of root tips, compared to the 30-84ZN and the non-inoculated control plants (Fig. 2.5). In addition, for seedlings root surface area and root length were significantly greater for the plants treated with 30-84ENH and 30-84WT compared to plants treated with 30-84ZN or the non-inoculated control plants (Fig. 2.4 and Fig. 2.5). However, for the older plants difference in surface area and root length were not significant. These data suggest that bacterial production of phenazines influences tip production and seedling root growth, but as plants age the effect on root growth becomes less pronounced, whereas the effect on tip production persists. However, more phenazine production by the enhanced phenazine-producer does not lead to more prominent changes in any of these parameters (Fig. 2.3-2.5).

Root biomass and root/shoot ratio are standard measurements of resource allocation. Turgor weight (water-soaked fresh weight) was used to assess root and shoot production in older vernalized plants because it provides a better estimate of living biomass, e.g. the more living tissue, the greater the turgor weight, as compared to dry biomass. There were no differences among treatments in shoot turgor weight, indicating a similar investment in above ground production regardless of treatment (Fig. 2.6A). The root turgor weight was significantly greater for plants treated with 30-84ENH compared to plants treated with 30-84ZN or the non-inoculated control plants (Fig. 2.6B); root turgor weight for plants treated with the wild type was intermediate. The difference in investment in roots translated into a significantly greater root/shoot ratio for the plants treated with the enhanced phenazine-producing strain and the ratio was intermediate for plants treated with the wild type to those treated with 30-84ZN or the non-inoculated control plants (Fig. 2.6C).



Figure 2.3: Effect of phenazine-producers on seedlings Winter wheat seedling were either grown in bacterial inoculated soil (30-84ZN, 30-84WT, and 30-84Enh) or non-inoculated soil (control) for 3 weeks (well-watered), and then exposed to two water stress cycles. After 7 days of recovery from the second water stress cycle roots were harvested and scanned with EPSON Perfection V700. Pictures are representative samples of each treatment.

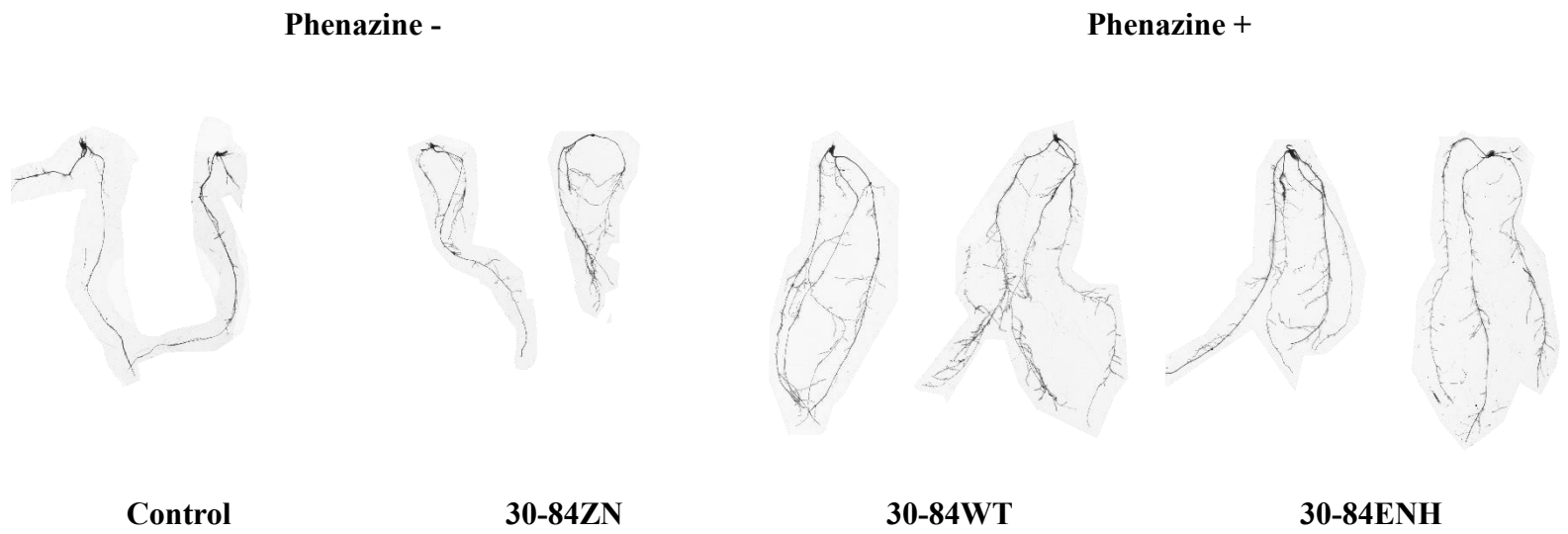
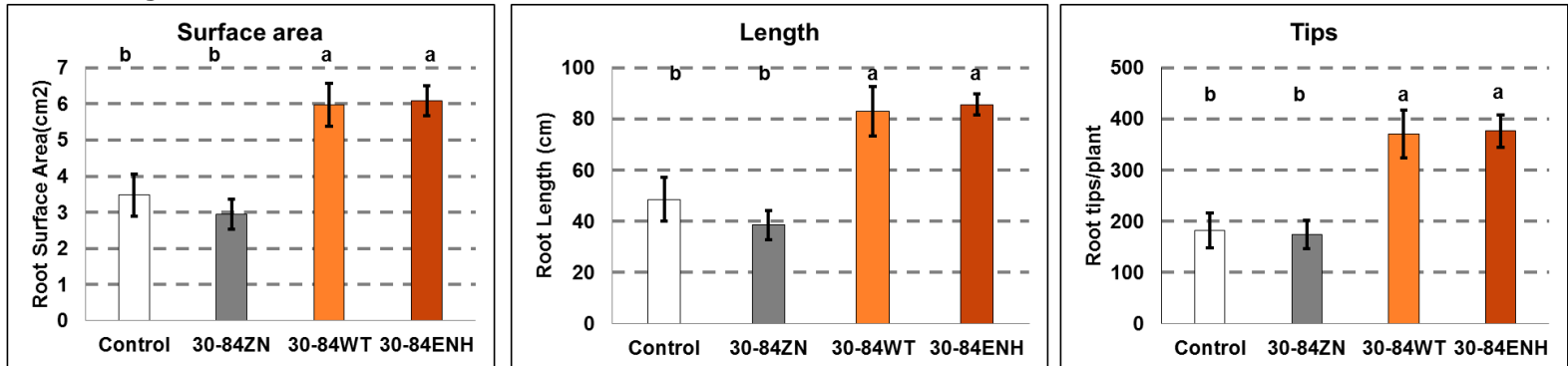


Figure 2.4: Effect of phenazine-producers on seedling root architecture. Winter wheat seedling were either grown in bacterial inoculated soil (30-84ZN, 30-84WT, and 30-84ENH) or non-inoculated soil (control) for 3 weeks (well-watered), and then exposed to two water stress cycles. After 7 days of recovery from the second water-stress cycle roots were harvested and scanned with EPSON Perfection V700. Pictures are representative samples of each treatment.

A: Seedling



B: Vernalized

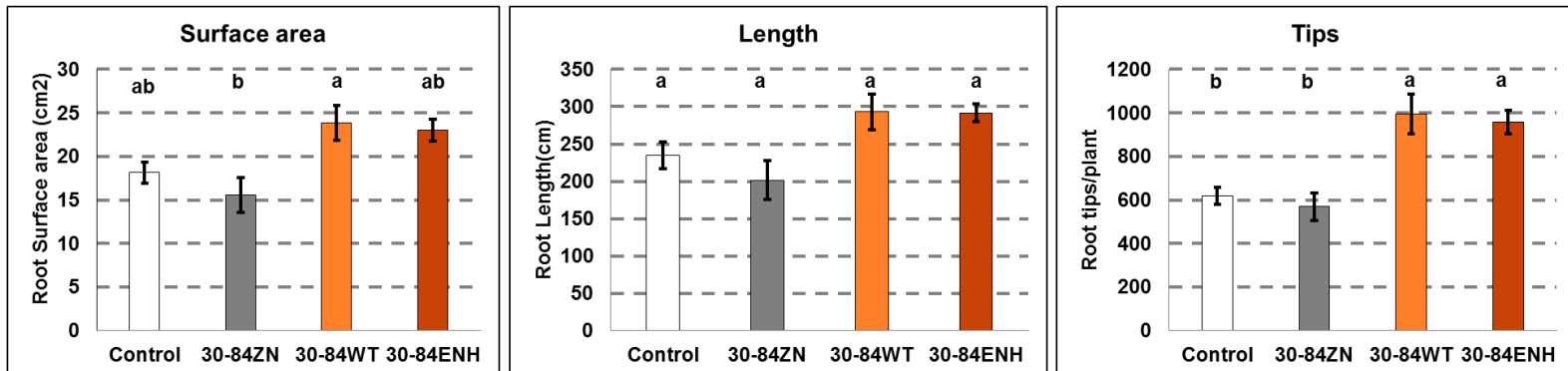


Figure 2.5: Effect of phenazine-producers on root development. Seedling and vernalized roots were either grown in bacterial inoculated soil (30-84WT, 30-84ZN and 30-84ENH) or non-inoculated soil (control), and were scanned and analyzed using WhinRhizo software package (Regent Instruments Inc., Quebec, Canada) after water stress. Seedling plants were exposed to two water-stressed cycles (10 and 7 days) and vernalized plants were water stressed for 15 days at the jointing stage. These experiments were repeated once. Values with the same letter do not differ significantly as determined by a Tukey test ($P > 0.05$), $n=5$ (three replicate plants per scan).

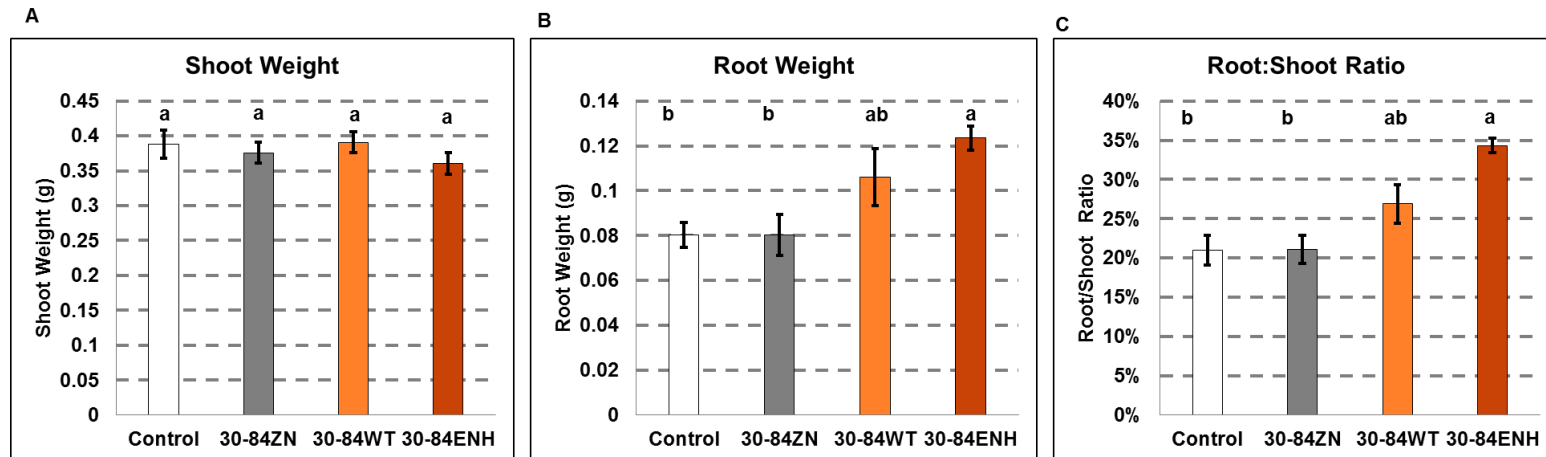


Figure 2.6: Plant investment is altered by enhanced phenazine-producing bacteria. A. Shoot turgor weights B. Root turgor weights C. Root/shoot turgor weight ratio. Vernalized winter wheat (cultivar TAM 112) were sown in either bacterial inoculated soil (30-84WT, 30-84ZN and 30-84ENH) or non-inoculated soil (control). At jointing stage, plants were water stressed for 15 days and provided time to recover (7 days). Roots were harvested, soaked in water for ~16 hrs, blotted dry, and weighed. Values with the same letter do not differ significantly as determined by a Tukey test ($P > 0.05$). $n=5$ (three plants per replicate)

DISCUSSION

This study investigated the functional capacity of phenazine-producing bacteria to promote water stress tolerance and resilience in water stress recovery trials with drought tolerant winter wheat cultivars. The presence of the wild type phenazine-producing bacteria almost doubled the survival rate of wheat seedlings after the extreme 11-day water-stress period and the enhanced phenazine-producer more than tripled the survival of wheat seedlings compared to seedlings treated with the phenazine deficient mutant (30-84ZN) or the non-inoculated control plants. Phenazine-producing bacteria also enhanced seedling health following the water stress, as determined from the recovery index. Plants inoculated with the phenazine deficient mutant (30-84ZN) or non-inoculated (control) had an average recovery index of 0.5, indicating that most plants did not recover at all or had slight regrowth, usually only near the crown of the stem. The plants inoculated with wild type (30-84WT) had an average recovery index of 1, indicating that most had modest regrowth. Plants inoculated with the enhanced phenazine-producer (30-84ENH) had an average recovery index of 2.5 indicating that on average, plants had good to complete leaf recovery. These results suggest that the presence of the phenazine-producing bacteria enabled the plants to not only survive water stress, but also enhanced the ability of the plants to recuperate after the stress. Moreover, the results demonstrate that the capacity of the root colonizing bacteria to produce phenazines is necessary for the increased water stress recovery and health after recovery, since the roots of seedlings treated with the phenazine-deficient mutant (30-84ZN) are indistinguishable from the roots of the untreated control plants.

In order to understand the plant traits contributing to the enhanced survival and recovery, I focused on important root system response variables associated with water stress avoidance such as root tip number (indicative of the abundance of water acquisition sites), root surface area (indicative of the level of soil exploration), and root allometry (root/shoot ratio indicative of the relative investment in roots versus shoots). Because water and nutrient uptake is limited mainly to root tips, increased root tip

formation should be more important for stress resilience than other parameters such as root length or surface area (Comas et al. 2013). One of the most significant findings of my study was that phenazine-producing strains strongly increased the number of root tips produced by both seedlings and older plants. Interestingly seedlings colonized by phenazine-producing bacteria had two fold more root tips than seedlings colonized by 30-84ZN or the untreated controls (e.g., almost 400 root tips compared to almost 200, respectively). For older plants, colonization by phenazine producing bacteria resulted in an average of almost 1000 root tips compared to an average of about 600 root tips on plants colonized by 30-84ZN or the untreated controls. I hypothesize that facilitating this increase in root tip production may be ecologically important for phenazine-producing bacteria since enhancement of root tip production potentially provides more sites of active plant investment in microbial populations.

Treatment of seedlings and older plants with phenazine-producing bacteria also resulted in more root surface area and length, although this was not significant in older plants. As plants age and root systems become larger, new growth becomes an increasingly smaller percentage of the established root system. This may explain why apparent differences in root system development among older plants having different inoculation treatments were not significantly different. Although none of the plants appeared pot bound, restrictions on root growth imposed by container size and shape may affect root system architecture and thus limit root development (Bengough and Mullins 1991; Falik et al. 2005). Because the roots established early in the season serve as the foundation for deeper root development later in the growing system, root system vigor early in the season increases the overall capacity of the plant to uptake water and nutrients thus favoring crop establishment and subsequent yield (Liao et al. 2006). By increasing investment in root development early in the season, the effect of phenazine-producing bacteria on root development may be an important mechanism for increasing the plant's capacity for water stress tolerance. Moreover this may insure the bacteria of a more reliable niche.

For the older plants, although they “invested” the same amount of resources into shoot production regardless of bacterial treatment, root investment was significantly greater for plants treated with the enhanced phenazine producing strain and intermediate for plants treated with the wild type, resulting in higher root/shoot ratios compared to the other treatments. It is now well recognized that water stress often results not just in an increase in root production, but an overall increase in investment in roots as compared to shoots, resulting in a change in the allometric relationship between root and shoot production (Xu et al. 2015b). Although plants are capable of responding to water stress by altering root systems, this functionality may be lost in extreme stressed conditions (Xu and Shimizu 2010). When Xu and Zhou measured allometry of *Leymus chinensis* under moderate and extreme water stress, they found higher belowground investment under moderate stress, whereas they observed the opposite, decreased root dry mass, in extreme stress (Xu and Zhou 2005). The influence of phenazine-producing bacteria on this ratio, especially under extreme water stress, could have profound impacts on stress tolerance since root versus shoot investment may improve hydraulic status (Comas et al. 2013). The ability of phenazine-producing bacteria to rapidly and reliably bring about this altered root investment in response to water stress may be an enhancement of the plants innate capacity for this response.

My results indicate that phenazine-producing bacteria also facilitate recovery following water stress. The influence of phenazine production on root branching and investment in root growth were the most profound effects I observed in this study, and these enhancements in the plant’s ability to avoid stress probably explain much of the improvement in water stress recovery. But other factors may have contributed. Here are a couple of ways I speculate that phenazine production may have contributed to the plant’s ability to recover following extreme water stress:

- Heightened biofilm formation by phenazine producers and more specifically the production of biofilm matrix, which acts like a humectant, may indirectly have contributed to water stress recovery. The biofilm matrix produced by phenazine-producers may increase the soil moisture holding capacity and thus water

availability in proximity to rhizosphere populations. This enhancement in soil moisture may delay the onset of extreme water stress, thus enabling a longer adjustment period for innate water stress responses and a more pronounced water stress avoidance phenotype.

- Phenazine-production also may have affected the plant's ROS signaling and antioxidant systems, both of which have been shown to play important roles in plant response to water deficit by stimulating/modulating plant global stress responses (Sewelam et al. 2016). Additionally, lateral root initiation and emergence is regulated by auxin and ROS signaling (Casimiro et al. 2001; Manzano et al. 2014). Hydrogen peroxide accumulates in the lateral root primordia, and peroxidase activity is proposed to transition cells from proliferation to differentiation (Manzano et al. 2014). I propose that phenazines have a dual role in altering ROS signaling by increasing/altering the availability of ROS and by inducing the plant's antioxidant systems. Thus the production of redox active phenazines could theoretically alter ROS production in the roots leading to water stress response priming and/or increased lateral root production.

As discussed in the introduction, Mavrodi et al. (2012a and b) reported that indigenous phenazine-producing bacteria were detected at high frequencies and population sizes on dryland winter wheat roots as compared to roots from irrigated fields. The authors attributed the potential benefits of phenazine producers primarily to the protection of seedlings from soilborne pathogens. These observations stimulated our interest in studying the role of phenazine-producers in water stress tolerance. Our work, albeit limited to container studies, suggests another ecological role for phenazine producers: the enhancement of wheat seedling water stress tolerance.

As Mavrodi et al. (2012b) reported the recruitment of phenazine producers by plants grown without irrigation may be due in part to enhanced rhizodeposition under water-stressed condition. Root exudation patterns are effected by environment, especially plant stress (Baudoin et al. 2003) including: water supply (Henry et al. 2007; Song et al. 2012; Calvo et al. 2017) temperature (Rovira 1959), light (Hodge et al.

1997), atmospheric CO₂ concentration (Calvo et al. 2017; Cheng and Johnson 1998; Paterson et al.1996), and nutrient availability (Carvalhais et al. 2011; Yang and Crowley 2000). For example, total organic carbon exuded by wheat grass exposed to drought stress increased by 71% compared to the well-watered control (Henry et al 2007). A deeper analysis of whether wheat plants select for phenazine-producers under dryland condition is the subject of Chapter III.

The effect of phenazine-producers on root growth is especially important given the need for heightened food production under water-limited conditions. Since rains are predicted to be more sporadic with climate change, the ability to produce more extensive root systems with a greater capacity for water uptake (i.e., root tips) will be favorable. Furthermore, the ability to recover from extreme water stress will be especially important for dryland agriculture where precipitation is less predictable and seasons are typically punctuated by episodic periods of extreme water stress (Mertz et al. 2009). The enhancement of the root/shoot ratio may also be an important parameter since root/shoot ratio has emerged as an important predictive metric. In wheat, root to shoot ratio has previously been shown to increase in response to water stress (Reynolds et al. 2007; Blum et al. 1983). Karcher et al. (2008) found that selection of tall fescue plants with high root/shoot ratios was an effective strategy for breeding lines with greater drought tolerance and resilience. Of significance, my results indicate that both root tip formation and root/shoot ratio are phenotypes that may be altered by plant-microbe interactions and thus breeding for microbial symbiosis may improve water stress resilience.

My study showed that drought tolerant winter wheat cultivars highly utilized in Texas dryland production recruited phenazine producing bacteria, expanding on the previous observations for dryland wheat production in Washington State reported by Mavrodi et al. (2012 a, b). Cultivar TAM 112 was found to have slightly, but significantly higher populations of the phenazine producing microorganism near the root tip in the meristematic and maturation zones than TAM 111, however bacterial populations in the older areas of the root near the crown (proximal) did not differ significantly. The root tip and the maturation zones (which have lateral roots), are areas

of active rhizosphere investment and “rhizodeposition”. Given the potential for phenazine-producers to enhance plant water stress tolerance, breeding for wheat cultivars that recruit and are responsive to the influence of phenazine-producing bacteria *naturally occurring in the soil* could increase water stress tolerance without need for application of microbial inoculum.

The relationship between low soil moisture and high rhizosphere populations of phenazine-producing pseudomonads may be a function of both enhanced microbial survival under these conditions and enhanced fitness of plants with phenazine-producers as symbionts, resulting in better plant recruitment of these PGPM. Phenazine production may facilitate microbial survival via water stress avoidance strategies such as biofilm production or other stress tolerance mechanism including managing microbial redox stress. Although the mechanism underlying the role of phenazines remain unclear, my results are the first to demonstrate that phenazine-producing bacteria significantly increase wheat water stress tolerance and resilience, at least in part by influencing increased root branching, resulting in a doubling of the number of available root tips for water and nutrient uptake. Phenazine producing bacteria in dryland soils may be providing an ecological benefit to wheat especially in water-stressed conditions by increasing water stress tolerance via their influence on root development and other morphological changes. These data suggest that phenazine-producing bacteria play important roles in addition to their well-established roles in protecting wheat from soilborne diseases. They also provide plant’s with the ability to tolerate abiotic stress related to water stress. Future work should focus on breeding plants capable of taking full advantage of this microbial functionality to improve water stress tolerance, and this study provides evidence for appropriate root phenotypes on which to base screening.

MATERIALS AND METHODS

Soil and plant material

Winter wheat seeds (cultivars TAM 112 and TAM 111) and soil was provided by Dr. Shuyu Liu. The soil used for these experiments is classified as a Pullman clay loam soil and was collected from the USDA-ARS, Bushland, TX dryland wheat plots at a depth of 1 to 15 cm. Prior to use in pots, it was necessary to sieve (2mm) and mix soil with sand (soil: sand, 2:1, v:v) to facilitate drainage. The soil-sand mix, hereafter referred to as soil, was autoclaved twice at 121 C, 15 PSI, 1 hour with a 24 hour break between cycles.

Root colonization assay

To determine the ability of *P. chlororaphis* 30-84 to colonize TAM 112 and TAM 111, wheat seeds were surface sterilized and planted in soil pre-inoculated with the bacteria (as described next section). After germination (4 days after planting), plants were stored in 5 C for 8 weeks. Following vernalization, seedling roots were carefully washed and dissected. One cm sections of the root were taken from the root tip (meristematic zone), the maturation zone (2.5-4 cm from tip where lateral roots and root hairs are found), and proximal roots (near the crown). Samples were immersed in 1 ml phosphate buffered saline (PBS). Bacteria were removed from root segments by vortexing and sonication, and populations were determined by serial dilution on LB agar amended with rifampicin.

Water stress tolerance assay

These assays were conducted by growing wheat seedlings in plastic tubes (2.5-cm diameter × 16.5-cm long) filled with soil that had either been inoculated with bacteria or non-inoculated (control). The *P. chlororaphis* 30-84 enhanced phenazine-producer (30-84ENH) and the *P. chlororaphis* 30-84 phenazine-deficient mutant (30-84ZN) were derived from the *P. chlororaphis* 30-84 wild-type (30-84WT) as described previously (Wood et al. 1997; Maddula et al. 2006; Unpublished Yu). Inoculum of the three strains were grown separately in LB broth for 24 hrs at 28 °C with rapid agitation.

Cultures were washed three times with sterilized deionized water, and bacterial populations were adjusted to an OD₆₂₀ of 0.8. The autoclaved soil was pre-inoculated with bacteria by mixing the inoculum thoroughly with the soil (1.5 ml inoculum in 20 ml water to 500 gm soil) and allowing bacterial populations to equilibrate to soil conditions for 4-6 days. For the negative control, the same volume of sterilized water was used to treat the soil. Fifty grams of bacterial inoculated (ca. 10⁷⁻⁸ CFU/g of soil) or non-inoculated soil was added to each container.

Wheat seeds (cultivar TAM 112) were surface sterilized using 0.6 % NaClO (10% of commercial bleach) for 10 min, followed by multiple rinses in sterile-distilled water. Seeds were pre-germinated on sterilized germination paper and two 2-day-old seedlings were sown into each container and covered with autoclaved vermiculite. A total of 60 plants of each treatment were arranged in a randomized complete block design (4 blocks) and watered every three days for three weeks with 5 ml sterile deionized water. After 2 days establishment, plants were thinned to 1 plant/container.

To induce water stress, water was withheld for 10-11 days depending on relative humidity (approximately 2% soil moisture). Plants were re-watered and allowed to recover for seven days and plant survival rate was determined. Water stress Recovery Index (RI) was evaluated using a scale of 0 to 3, where 0 = no recovery (dead), 1 = slight new growth in stem, 2 = recovery of partial leaf, 3 = recovery of one or more entire leaves.

Root morphology assessment

The effects of *P. chlororaphis* derivatives (30-84WT, 30-84ENH, and 30-84ZN) on the morphology of roots were assessed for seedlings and older, vernalized plants in the jointing stage. The seedlings used in this experiment were the same plants from the previous experiment, and were analyzed after exposure to a second water stress (7 days) and a seven day recovery period. For the older plants, winter wheat seeds were sown in soil inoculated with bacteria (30-84WT, 30-84ZN, or 30-84ENH) or without (control), allowed to germinate, and then stored at 5 C for 8 weeks. After vernalization, plants were transferred to larger pots (4-cm diameter × 21-cm long) containing soil with the

same soil inoculation treatment and watered well (10 ml sterile deionized water every 3 days) until jointing stage. Plants were then water stressed for 15 days and harvested. Because many of the vernalized plants did not recover from the water stress treatment, only one water stress/recovery cycle was performed. Intact plants of both seedling and vernalized plants were harvested by carefully washing roots to remove adhering soil. To calculate fresh shoot and root turgor weights intact plants were wrapped in a paper towel and allowed to soak for 16 hour in sterile deionized water. Intact plants were then separated into above and below-ground parts, blotted dry, and weighed separately. Roots were then added to a clear box filled with ~1cm water placed on a scanner (EPSON Perfection V700), and then carefully arranged to minimize overlap prior to scanning. Photoshop was used to remove shadows from the scanner and loose soil particles. Whinrhizo software was used to analyze root morphology and compute root surface area, root length, and number of root tips.

Statistical analysis

All data presented are mean \pm the standard error of the mean (SEM). Data were analyzed by ANOVA and Fisher's protected Least Significant Difference (LSD) or Tukey test ($P < 0.05$) with GraphPad Prism software (GraphPad Software, San Diego, CA).

CHAPTER III
SELECTION FOR PHENAZINES-PRODUCING BACTERIA: ROLE OF CULTIVAR,
LAND USE HISTORY, AND WATER STRESS

INTRODUCTION

The rhizosphere microbiome serves as the plant's second genome and has the potential to increase plant tolerance of biotic and abiotic stress (reviewed in Berendsen et al. 2012; Turner et al. 2013). The majority of the plant's phytobiome is recruited from the soil and lives on or within plant tissues or within the plant's zone of influence—the root rhizosphere. Thus, knowledge of the structure and function of rhizosphere communities, the spectrum of services microbes provide the plant, and the dynamic nature of the interactions determining both, may be crucial for improving crop productivity under stressful conditions. It is now well established that the composition of root exudates differs among plant species and even cultivars, and that exudate composition is a strong determinant of the rhizosphere community composition and functionality (Dalmastri et al. 1999; Kuklinsky-Sobral et al. 2004; Lemanceau et al. 1995; Mazzola et al. 2004). Genetic variation in root exudation suggests that it may be possible to breed varieties with particular root exudation patterns capable of altering the rhizosphere microbiome composition (Badri et al. 2008; Wissuwa et al. 2009; Philippot et al. 2013; Wei and Jousset 2017). Moreover, enhancing populations of plant growth promoting microorganisms via continuous culture of particular crops or cultivars may increase the ability of current and subsequent crops to withstand stressful conditions, a concept referred to as soil legacy. Thus previous land use conditions may have a profound influence on the indigenous microbial populations in soil that may be recruited to the rhizosphere of the current crop, thereby contributing to the growth and fitness of plants in the current growing season, and potentially subsequent seasons (Bever et al. 2012; Bakker et al. 2013; Monger et al. 2015). The composition of rhizosphere populations are also affected by plant stresses, because rhizodeposition patterns changes

in response to stress (Henry et al. 2007; Marasco et al. 2012; Bogino et al. 2013). Thus the development of rhizosphere microbiomes should be thought of as a dynamic process influenced by the plant genotype and environmental conditions ($G \times E$), wherein the environmental component must take into account land use history, as well as ongoing abiotic and biotic conditions.

Selecting plant lines capable of taking advantage of the rhizosphere microorganisms with the capacity to improve plant health under stress conditions may be a viable solution to meeting future agricultural challenges. In particular, this includes dealing with climate variability as it relates to the frequency and duration of water stress events. My study was motivated in part by previous research indicating that rhizosphere bacterial communities differed for wheat plants grown in irrigated fields, as compared to dryland production (Mavrodi et al., 2012a, b). These studies focused on the relative abundance of specific populations of rhizosphere microorganisms known to be antagonistic to soilborne fungal pathogens, such as *Gaeumannomyces graminis* var. *tritici* (Ggt), the causative agent of take-all disease, and *Fusarium* species, the causative agents of crown rot and wilt diseases. Specifically, the study focused on the relative abundance of *Pseudomonas* strains capable of producing redox-active phenazines or the polyketide 2,4-diacetylphloroglucinol (2,4-DAPG), both broad spectrum antibiotics effective against Ggt. Mavrodi et al. (2012b) showed that phenazine-producing bacteria were detected at high frequencies (67 to 100% of plants sampled) on dryland winter wheat roots as compared to (8 to 50% of plants sampled) in irrigated fields, where 2,4-DAPG-producing bacteria were abundant. Populations of phenazine-producing strains were substantial on wheat roots from dryland production and ranged from 4.8 to 6.3 log CFU g of root fresh weight. In this study, frequency and abundance were determined from the presence of genes responsible for the production of each compound. Moreover, in a companion study Mavrodi et al. (2012a) showed that there was a strong inverse relationship between annual precipitation and the proportion of plants colonized by phenazine-producing *Pseudomonas* and that the abundance of rhizosphere microbes with phenazine genes correlated with phenazine production in the rhizosphere. Together,

these observations are of interest because they are the first to show a strong inverse correlation between soil moisture and the abundance of phenazine-producing microbes in the wheat rhizosphere. They also illustrate how crop production practices influence indigenous populations of antibiotic-producing pseudomonads with the capacity to suppress soilborne wheat diseases (Mavrodi et al. 2012a, b). Regarding this relationship between soil moisture limitation and the rhizosphere abundance of phenazine-producing *Pseudomonas*, there are many questions that remain unanswered. For example, what role does phenazine production play for the producing bacteria and what, if any, role does it play in the fitness of the plant host? It is well established that phenazines are inhibitory to a broad spectrum of microbes potentially competing for the same rhizosphere niche (Mazzola et al. 1992). They also have been shown to enhance biofilm production, biofilm architecture, and competitive rhizosphere survival (Maddula et al. 2006; Maddula et al. 2008; Mazzola et al. 1992). Does this correlative relationship between low soil moisture and large phenazine-producing populations then merely reflect the capacity of phenazines to enhance the survival of phenazine-producing pseudomonads under dry conditions? It is intriguing to speculate that phenazines also provide services that directly or indirectly alter the innate capacity of the plant to tolerate water stress, and thus enhance the fitness of wheat under dryland production, not only via the inhibition of wilt pathogens. My previous results (Chapter II) were the first demonstration that phenazine-producing bacteria significantly increased wheat water stress tolerance and resilience, at least in part by influencing increased wheat root branching, resulting in a doubling of the number of available root tips for water and nutrient uptake. Thus, is it possible that plants are actively recruiting phenazine-producers under conditions where water availability is unreliable—i.e., does community composition reflect both plant recruitment of microbes and microbial survival? Given that certain cultivars of wheat are bred for particular environments, how might cultivar usage play a role in the selection of phenazine-producing strains in dryland agriculture—do cultivars bred for dryland production in Texas also recruit indigenous phenazine-

producers from Texas soils? What role does land use history play? Is water stress needed to produce differences in community composition?

In this study, I investigated the composition of rhizosphere communities recruited by cultivars of winter wheat that either were or were not bred for drought tolerance. The role of soil legacy was investigated by collecting soils from adjacent fields with different long-term land use histories, e.g. dryland versus irrigated wheat production. The role of water stress on community composition was examined by subjecting cultivars grown in soils with different land use histories to extreme water stress. I hypothesized that cultivars with higher drought tolerance would have increased recruitment of phenazine-producing bacteria and because of this, land use history where cultivar selection may come into play, may also influence community composition. Moreover, I hypothesized that water stress may be important in shaping rhizosphere communities.

RESULTS

Rhizosphere populations

Total culturable aerobic bacteria populations were comparable for all three cultivars regardless of whether the soil had been collected from dryland or irrigated fields or whether the plants had been water stressed or not (Fig. 3.1). Colonization of plant roots by indigenous *Pseudomonas* also was high for all treatments (10^5 - 10^7 CFU per gram fresh weight of root) (Fig. 3.2A). However, the percentage of the total population that was *Pseudomonas* was higher in the rhizosphere of the two drought tolerant cultivars TAM 111 and TAM 112 when they were subjected to water stress as compared to well-watered plants (Fig. 3.2B). This was particularly true for the plants grown in soil collected from the non-irrigated fields. There was no difference in the percentage of the population that was *Pseudomonas* for drought sensitive TAM 304 under any treatment condition. These results suggest that for TAM 111 and TAM 112,

water stress played a significant role in the composition of the rhizosphere community, resulting in *Pseudomonas* strains being a greater percentage of the rhizosphere colonizing bacteria. The frequencies and densities of Phz+ *Pseudomonas* strains were generally higher in the rhizospheres of the drought tolerant cultivars TAM 111 and TAM 112 compared to TAM 304 (Fig. 3.3A,B). Phenazine-producing pseudomonads were detected in all TAM 111 treatments, and in both of the dryland soil treatments for TAM 112, whereas phenazine-producing pseudomonads were only detected on the drought sensitive cultivar (TAM 304) in the irrigated soil, water-stressed treatment (Fig. 3.3A). For the roots having detectable levels of phenazine-producing pseudomonads, population densities varied from 10^3 - 10^6 CFU per gram fresh weight of root. The percentage of pseudomonads that was Phz+ for TAM 111 ranged from 25 to almost 100 percent for all treatments, whereas the percentage was less than 10% for the other two cultivars (data not shown). These results clearly indicate a cultivar preference for the recruitment of indigenous Phz+ *Pseudomonas* strains from soil for TAM 111.

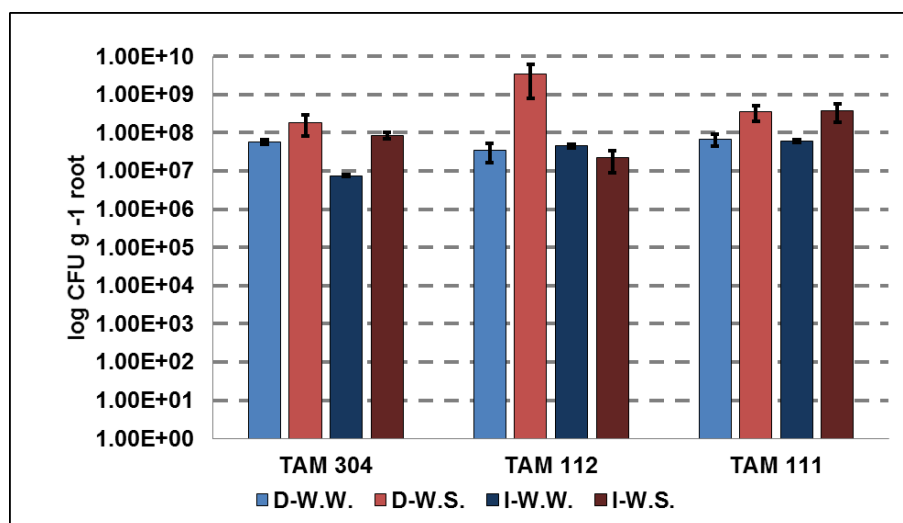


Figure 3.1: Population densities of total culturable aerobic bacteria from the rhizoplane and rhizosphere of wheat plants. Wheat seedlings were grown for 3 weeks in soil collected from a dryland soil (D) or an irrigated soil (I) in the growth chamber. Treatments were then well-watered (W.W.) or water-stressed (W.S.) for 8 days. Plants were re-watered, and plant roots and loosely adhering soil were collected. Bacterial population sizes were determined by diluting the root wash and observing which dilution(s) grew in 1/10 TSA broth after 3 days. Predicted CFU was standardized to root fresh weight. n=4

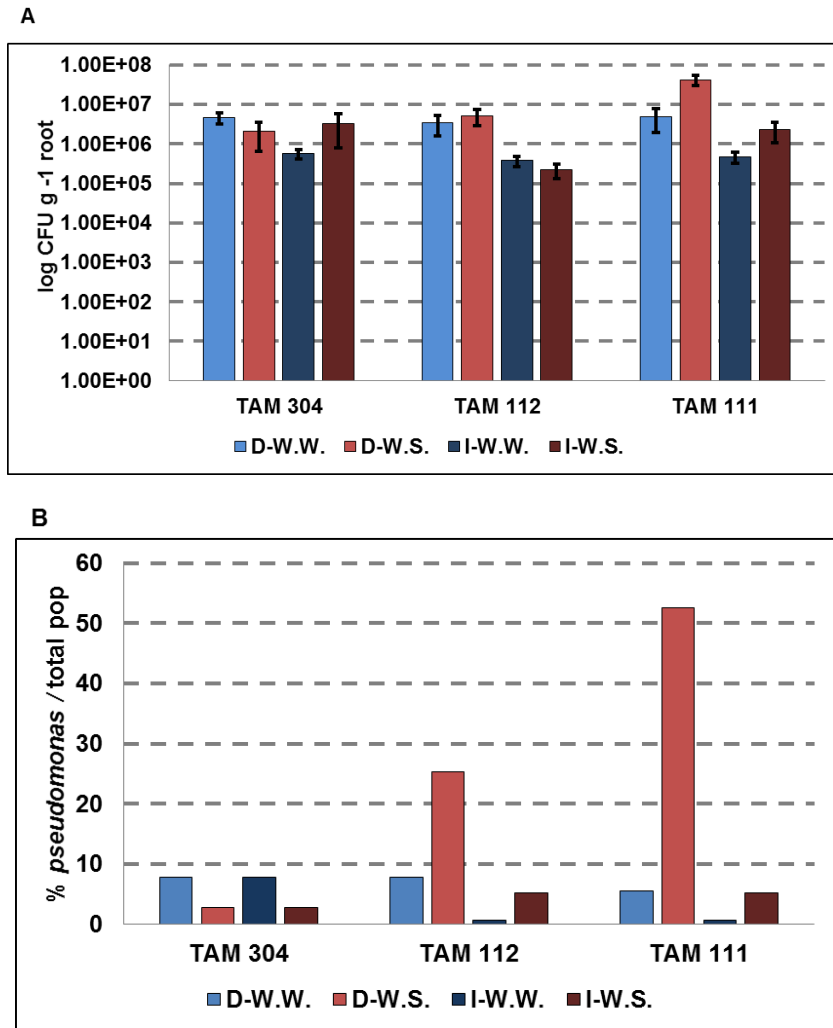


Figure 3.2: Population densities of *Pseudomonas* from the rhizoplane and rhizosphere of wheat plants. A. Log *Pseudomonas* populations per gram fresh root. B. Percent of population represented by *Pseudomonas* / total culturable aerobic bacteria. Wheat seedlings were grown for 3 weeks in soil collected from a dryland soil or an irrigated soil. Treatments were then well-watered or water-stressed for 8 days. Plants were re-watered and plant roots and loosely adhering soil were collected. The population size of the *Pseudomonas* component of the community was determined by diluting the root wash and observing which dilution(s) grew in a semi-selective growth medium for *Pseudomonas*, 1/3 KMB augmented with cycloheximide (100 $\mu\text{g/ml}$), chloramphenicol (13 $\mu\text{g/ml}$), and ampicillin (40 $\mu\text{g/ml}$), after 3 days. Predicted CFU was standardized to root fresh weight. n=4

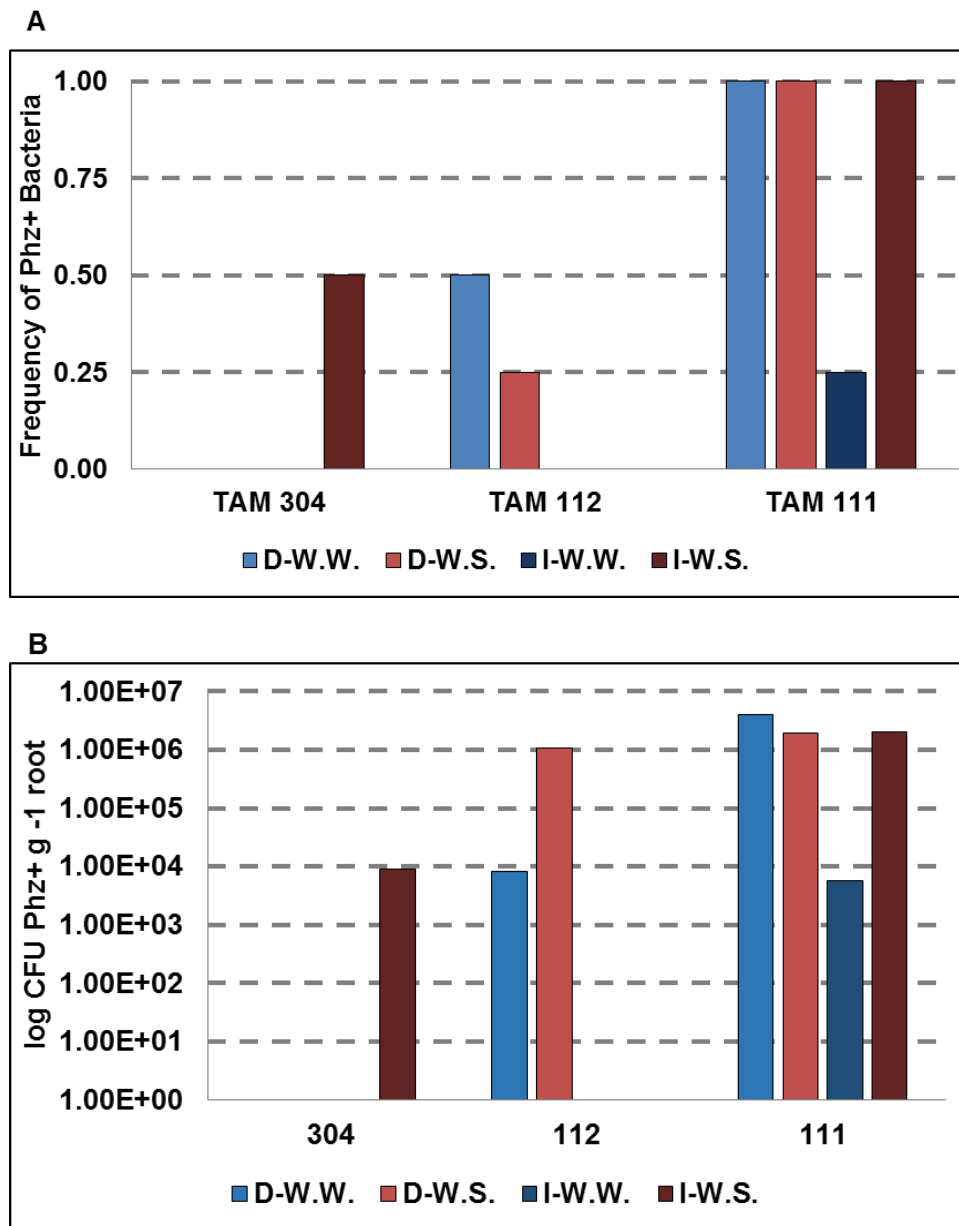


Figure 3.3: Colonization of wheat roots by phenazine-producing *Pseudomonas*.
 A. Frequency of root systems of individual plants colonized by *pseudomonas* that were Phz+. B. Population densities of *pseudomonads* that were Phz+ in samples that had Phz+ colonization. Wheat seedlings were grown for 3 weeks in soil collected from a dryland soil (D) or an irrigated soil (I). Treatments were then well watered (W.W.) or water-stressed (W.S.) for 8 days. Samples used to detect *Pseudomonas* (Fig. 3.2) that were positive for growth were screened for the presence of the *phzF* gene via PCR. Predicted CFU was standardized to root fresh weight. n=4

DISCUSSION

This research investigated the effect of plant genotype, land use history, and water stress on rhizosphere bacteria populations. My results indicate that the cultivars recruited rhizosphere populations that differed in their composition, and that rhizosphere composition also differed by the soil history and stress condition in which plants were grown. Colonization of wheat roots by *Pseudomonas* was significantly higher for the two drought tolerant cultivars TAM 111 and TAM 112 when grown in the soils from the dryland field, and especially when they were subjected to water stress. The frequencies and densities of phenazine-producing pseudomonads also were generally higher in the rhizospheres of TAM 111 and TAM 112 compared to drought sensitive TAM 304. Phenazine-producing bacteria were detected on the roots of all TAM 111 treatments, where they accounted for a large percentage of the pseudomonad population and often reached populations above the threshold of 10^5 CFU g⁻¹ of root, a density considered necessary for biological activity in the rhizosphere such as production of antibiotics at a level significant for biological control activity (Pierson et al. 1994; Khan et al. 2005; Maddula et al. 2006). Phenazine-producing bacteria also were detected on the roots of TAM 112 grown in the dryland soil, but with reduced frequency. Although present on a few TAM 112 roots, the limited number of samples showed that populations reached 10^3 to 10^6 CFU per gram fresh weight of root, but that phenazine-producers were not the major type of pseudomonad present. That both TAM 111 and TAM 112 recruit phenazine-producing *Pseudomonas* strains was not unexpected given the findings from Chapter II that both recruited *P. chlororaphis* 30-84 from autoclaved soil resulting in the establishment of rhizosphere populations of almost 10^5 CFU per cm of root length, although establishment was slightly, but significantly higher on TAM 112 root tips. The drought sensitive cultivar TAM 304 also had detectable levels of phenazine-producing bacteria, but only on the roots of some plants grown in the irrigated soil following water stress treatment.

One of the goals of the study was to consider whether the correlative relationship between low soil moisture and frequency/density of phenazine-producing pseudomonads in wheat rhizosphere communities was primarily a function of the capacity of phenazine-producers to survive water stress better than other pseudomonads or related in part to plant recruitment of phenazine-producers. If production practices such as dryland or irrigated farming dictate which cultivars are likely to be grown as well as the probability of water stress, to what extent would the influence of land use practice on the *soil microbiome* affect the establishment of *rhizosphere communities*? How important would a recent water stress event be relative to the potential for differential cultivar recruitment or soil legacy? I hypothesized that if microbial survival of water stress were the major determinant of rhizosphere composition, I would expect all cultivars to have large populations of phenazine-producing pseudomonads under water-stressed conditions and especially when grown in soils from dryland production, i.e., previously conditioned by G × E interactions related to water stress events. Given the differences among cultivars in the composition of the rhizospheres, especially in the proportion of pseudomonads and phenazine-producing pseudomonads present, it appears that cultivar recruitment plays a strong role in which microbes are recruited and ultimately establish in the wheat rhizosphere. However, populations of pseudomonads also were found to be influenced by land use history and water stress. Pseudomonads were present in the rhizospheres of plants grown in both dryland and irrigated soils, however the pseudomonad populations were greater on plant roots of TAM 111 and TAM 112 roots grown in dryland soils and exposed to water stress. These results suggest that plant selection for pseudomonads may increase in response to reduced soil moisture (i.e., water stress) and this selection may be more pronounced when plants are grown in a soil preconditioned by dryland agriculture. The effect of land use preconditioning may be to enrich the available pool of microbes for those having the functional capacity to colonize wheat under water-deficit conditions. Interestingly the altered selection for pseudomonads was only observed in the drought tolerant cultivars TAM 111 and TAM 112, and not TAM 304, suggesting

that drought tolerant cultivars may have an increased capacity to differentially select for these microbial partners under water-stressed conditions.

Mavrodi et al. suggested the mechanisms underlying the relationship between low soil moisture and high populations of Phz+ *Pseudomonas* species on wheat may be a function of both plant and environmental recruitment and differential survival of microbes (Mavrodi et al. 2012b). For example they suggested that soil moisture may alter the amount and/or composition of root exudates, thereby altering the recruitment and establishment of different microbes. However, in their study they did not consider which cultivars were being grown at each site of their dryland or irrigated fields and thus may have overlooked cultivar specific selection for rhizosphere constituents. Another factor they considered was the relative abundance of fungal pathogens and specifically *Gaeumannomyces graminis* var. *tritici* which causes root lesions that may influence the relative abundance of pseudomonads differing in their competitiveness for these pathogen-induced niches (Mavrodi et al. 2012b). The production of phenazines in the rhizosphere also may increase the fitness and survival of microbial producers because of the important role phenazines play in biofilm development. Biofilm formation is one of the physiological mechanisms of bacteria for protection against physical and chemical stresses and an adaptation to survival in low-moisture habitats (Chang and Halverson 2003). Phenazines have been directly linked to biofilm formation (Harris 1981; Maddula et al. 2006). In other words, the phenazine-producers may have an enhanced capacity to withstand water deficit due to heightened biofilm formation. Similar to the findings of the Washington group, my results suggest that populations of phenazine-producing bacteria may be recruited more reliably from soils with a history of dryland production. Moreover my results add to their findings showing that the relationship is robust enough to include other wheat producing areas such as Texas. However I found that the recruitment effect is most pronounced for wheat cultivars bred to be drought tolerant. These data suggest there are populations of indigenous phenazine-producing bacteria that are colonizing winter-wheat in a cultivar-dependent manner.

Rhizosphere phytobiome communities can greatly influence plant health. The ability of plants to influence this microbial community development is an important phenotype to consider especially in terms of breeding cultivars for improved agricultural productivity under water limited conditions. Plant selection of soil bacteria has been found to be driven more by selection for services the microbe may provide than microbial taxonomy. Such services include indirect effects on plant health such as enhancement of nutrient availability, control of root pathogens, or influence on water availability in the soil/rhizosphere interface (Mendes et al. 2014; Yan et al. 2017). Microbes also may directly affect plants via hormone production, signaling, or in the case of phenazines, potentially balancing reactive oxygen stress or electron shuttling. In Chapter II, I demonstrated that one of the outcomes of plant microbe interactions was enhancement of root growth correlated with the presence of phenazine-producing *Pseudomonas*, which served to both increase water stress tolerance and resilience.

What does this research mean for dryland agricultural production? Given my results demonstrating that certain drought adapted cultivars can recruit indigenous phenazine-producing pseudomonads and the importance of bacterial phenazine production for enhancing drought tolerance, future work should be directed toward selecting drought tolerant lines capable of taking advantage of the indigenous phenazine-producers to enhance innate water stress tolerance. Ultimately, focusing on the capability of plants to recruit/select phytobiomes with the functional capacity to increase water stress tolerance and resilience may be crucial for meeting long term goals of increasing global food productivity in areas experiencing water stress.

MATERIALS AND METHODS

The objective of this study was to determine whether there were differences in the composition of the rhizosphere communities of wheat seedlings recruited by different cultivars, when plants were grown in soils with different production histories and under different soil moisture regimes. I was particularly interested in the recruitment

of phenazine-producing *Pseudomonas* strains under these conditions. For the study I used two Texas winter wheat cultivars bred for drought tolerance, TAM 111, TAM 112, and a drought-sensitive cultivar TAM 304. Plants grown in a Pullman clay loam soil collected from adjacent fields previously used during multiple preceding years for dryland or irrigated wheat production (S. Liu personal communication). Natural field soil was mixed with autoclaved sand as described above (soil: sand, 2:1, v:v). Plants were sown in plastic tubes (2.5-cm diameter × 16.5-cm long), Initially 2 seeds/pot were planted and the density was thinned to 1 plant/pot after emergence and plants were allowed to establish for 3 weeks. For the water stress treatment, plants were water stressed by withholding water for 8 days whereas for the irrigated treatment (well-watered) plants were watered to field capacity every three days. At the end of the 8 week water stress or well-watered treatment period, all plants were re-watered and harvested (2 days after re-watering). Roots and loosely adhering soil were used to determine the sizes of cultural aerobic bacteria, *Pseudomonas*, and phenazine-positive (Phz+) *Pseudomonas* populations as described previously (Mavrodi et al 2012). Briefly, loosely adhering soil was removed and roots were transferred to falcon tubes with sterile ddH₂O. Samples were vortexed and sonicated, and used to make dilutions in 96 well plates. Dilutions then were used to inoculate 96-well microtiter plates with either 200 microliters of one-tenth-strength tryptic soy broth (1/10 TSB) supplemented with cycloheximide (100 µg/ml) to inhibit fungal growth to determine total culturable aerobic rhizosphere bacteria or one-third-strength King's medium B (1/3 KMB) liquid medium supplemented with cycloheximide (100 µg/ml), chloramphenicol (13 µg/ml), and ampicillin (40 µg/ml), a semiselective growth medium for fluorescent *Pseudomonas*. Microtiter plates were incubated in 28 C with shaking at 200 RPM for 72 hrs, and then optical density OD₆₂₀ was recorded for each well. Wells were considered positive for bacterial growth if OD was 0.1 or greater. All samples with positive growth in the KMB media were screened for the presence of Phz⁺ pseudomonads by PCR. Primers (Ps_up1 and Ps_low1) were used to target the *phzF* (biosynthesis genes in the phenazine operon) (Mavrodi et al. 2010). The core biosynthesis gene, *phzF*, was used because it is common

to all known phenazine-producers (Mavrodi et al. 2010). After the PCR reaction, samples were ran on a 0.8 agarose gel and quantified for the presence or absence of a band compared to a control (known phenazine producer). The final dilutions that were positive for growth and were positive for *phzF*, were used to calculate phenazine-producing *pseudomonas* populations.

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

SUMMARY OF CONCLUSIONS

The novel contributions of my study are the expansion of our understanding of the ecological role of phenazines-producing *Pseudomonas* in the wheat rhizosphere to enhance water stress tolerance. Phenazine production improves the producer's capacity to: colonize and persist in the plant rhizosphere and compete with other rhizosphere and soil dwelling organisms (e.g. compete for resources or inhibit the growth other microbes such as fungal pathogens). Moreover under water-stressed conditions phenazine-induced biofilm formation may provide microbial communities with some protection from desiccation (Pierson and Pierson 2010; Weller 2007). In addition to the ecological benefit to the producer, my results are the first demonstration that phenazine-producing bacteria significantly increase water stress tolerance and resilience in wheat. Significant findings from Chapter II are summarized in the bulleted points below:

- The well characterized phenazine-producer, *P. chlororaphis* 30-84, colonized Texas A&M winter wheat cultivars and led to an enhancement in the water stress-tolerance of wheat seedlings. The presence of the wild type, phenazine-producing bacteria nearly doubled the survival rate of wheat seedlings after the extreme water-stress period compared to seedlings treated with a phenazine deficient mutant (30-84 ZN) or the non-inoculated control plants. Interestingly, colonization by an enhanced phenazine-producer more than tripled the survival of wheat seedlings compared to the appropriate controls. Phenazine-producing bacteria also promoted seedling recovery following the water stress.

This increase in plant survival and recovery is due in part to the enhancement of water stress avoidance mechanisms:

- Phenazine-producing strains were shown to alter root architecture by increasing the number of root tips produced by both seedlings and older plants after water stress. Increased root tip production by phenazine-producing strains may be an important mechanism for promoting water acquisition and prolonging avoidance of extreme water stress, as well as fostering acquisition potential following re-watering.
- Enhanced phenazine-producers altered the root/shoot ratio in adult plants compared to the control plants, suggesting that phenazine production may influence resource allocation. There were no differences among treatments in shoot turgor weight, however root turgor weight was significantly greater for plants treated with 30-84ENH compared to plants treated with 30-84ZN or the non-inoculated control plants. These results indicate that a change in resource investment in below ground growth occurred in plants grown in soil inoculated with enhanced phenazine-producing bacteria.

Alterations of root architecture and investment strategies are important plant traits that have been correlated previously with drought tolerance (Comas et al. 2013; Ngumbi and Kloepper 2016). Although the exact mechanisms underpinning the enhanced water stress tolerance are unknown, however, the presence of phenazine-producing bacteria are directly or indirectly altering the innate capacity of the plant to tolerate water stress. Possible water stress tolerance mechanisms include:

- The biofilm matrix produced by phenazine-producers may increase water potential thus increasing local soil moisture. This alteration of soil moisture may change the water potential of the rhizosphere soil interface thus delaying the onset of extreme water stress and protecting the roots from the adverse effects associated with complete drying.
- Biofilm induced changes in soil moisture (increased water potential) of the rhizosphere soil interface can influence root growth. It is well known that roots grow towards areas of higher water potential, which is termed hydrotropism

(Henry 1915; Krieger et al. 2016). The change in water potential may be encouraging localized root growth.

- The redox activity of phenazines is also proposed to play an important role in altering root morphology. Lateral root initiation, emergence, and development are regulated by auxin and ROS signaling (Casimiro et al. 2001; Manzano et al. 2014). Hydrogen peroxide (H₂O₂) accumulates in the lateral root primordium (LRP), and the peroxidase activity is proposed to transition cells from proliferation to differentiation (Manzano et al. 2014). I propose that phenazines have multiple roles in altering ROS signaling e.g., by varying the abundance of ROS (via serving as an electron donors or acceptors) and/or enhancing plant antioxidant activities. Thus, the production of bacterial phenazines may lead to increased lateral root emergence via their influence on ROS signaling pathways.
- Induction of the plant's antioxidant systems to reduce the negative effects of ROS in the plant also has been correlated to drought tolerance (Contour-Ansel et al. 2006). Overall the redox-activity of phenazines is intriguing given the importance of ROS signaling stimulating global stress responses of the plants (Sewelam et al. 2016).

Taken together, my results suggest that bacterial phenazine production is an important microbial functionality that increases water stress tolerance and resilience in wheat.

How can we incorporate the functional benefit provided by phenazine-producing bacteria into strategies to enhance dryland wheat production? Although it may be intriguing to speculate that *the application* of phenazine-producing PGPM may reduce drought associated yield loss, the complexity of the rhizosphere interface frequently confounds the benefits of PGPM inoculations making it difficult for such applications to have consistent benefits in a production system. Moreover, the cost associated with applications, challenges to providing efficacious delivery systems, shelf-life of the product, and potential negative effects on the rhizosphere community structure, leads me to the conclusion that utilization of *indigenous* phenazine-producing bacteria may be most effective and efficient.

This idea led me to the second question addressed in this study:

Do indigenous phenazine-producing bacteria colonization Texas A&M winter wheat cultivars and, if so, is colonization effected by cultivar, land use history, or water stress? In other words, are wheat cultivars bred for dryland agriculture able to recruit these plant-stress tolerance-promoting phenazine-producers from dryland field soils? Results for Chapter III are summarized below:

- Colonization of indigenous phenazine-producing bacteria was found to be influenced by plant genotype, soil history, and water regime. Populations of phenazine-producing bacteria were not only influenced by the irrigation regime (as observed previously by Mavrodi et al. 2012a) but were also affected by the plant cultivar in a stress dependent manner. Texas A&M winter wheat cultivars were colonized by phenazine-producing *Pseudomonas*. The frequencies and densities of phenazine-producing bacteria were generally higher in the rhizospheres of the drought tolerant cultivars (TAM 111 and TAM 112) compared to TAM 304. Phenazine-producing bacterial populations were especially high in TAM 111, suggesting that this cultivar selects for this functionality more than the other two cultivars.

These results suggest that wheat cultivars differ in their ability to interact with indigenous phenazine-producing bacteria that may have the capacity to increase water stress tolerance. The development of rhizosphere microbiomes should be thought of as a dynamic process influenced by the plant genotype and environmental conditions ($G \times E$), wherein the environmental component must take into account land use history as well as ongoing conditions. The dynamic rhizosphere interactions between phenazine-producing bacteria and wheat are not well understood. The increased population sizes of phenazine-producing microorganisms may be a result of **recruitment** by the plant (in response to water stress) or increased **survival** of phenazine-producers in the rhizosphere of plants exposed to water deficit. Mavrodi et al. (2012b) suggest that both mechanisms (recruitment and survival) may result in increased populations of phenazine-producing bacteria.

Mechanisms

- **Recruitment**

In response to water stress plants have been shown to increase rhizodeposition or carbon investment, and the greater availability of these nutrients to the bacteria may in turn increase phenazine-producing microorganisms.

- **Survival**

The production of phenazines in the rhizosphere can also increase the fitness and survival of producers because of the important role phenazines play in biofilm development and in competition with other rhizosphere bacteria (Mazzola et al., 1992; Mavrodi et al. 2012). The phenazines producers may have enhanced capacity to withstand water deficit due to biofilm formation or capacity to inhibit other rhizosphere bacteria.

Given the increased colonization of water-stressed drought tolerant wheat cultivars by phenazine-producing pseudomonads, I propose that rhizodeposition by these plants is an important mechanism that favors colonization by phenazine-producers. I hypothesize that both recruitment and prevalence/survival of phenazine-producers, favors symbiosis under water-stressed conditions.

In summary, phenazine-producing bacteria provided a functional service to wheat by increasing water stress tolerance and resilience. Given the potential for phenazine-producers to enhance plant adaptation to water stress and the dynamic nature of colonization, it may be possible to breed for wheat cultivars that recruit phenazine-producing bacteria naturally occurring in the soil, thus taking advantage the genetic capacity of the soil microbiome to increase plant productivity without the need for application of microbial inoculum.

FUTURE WORK

The conclusions from my work led me to propose several new research questions for future research:

- Phenazine-producing bacteria alter water stress avoidance mechanisms, specifically root morphology (root area, root length, and number of root tip formation), and water stress recovery for seedling plants. Do phenazine-producers also improve the dehydration tolerance of plants, and if so, what physiological attributes may be effected? Traits such as hydraulic conductance, internal root morphology (changes in suberization and cortical cell death), and stomata conductance are of interest for future studies.
- Are phenazine-producing bacteria capable of altering the water-use efficiency of wheat? Agriculture is the dominant user of global fresh water and thus it is important to maximize production per unit water input. Water-use overtime of these plants should also be measured to gain insight into the onset of stress response, and mechanism for increased water stress resilience.
- The enhanced phenazine-producer increased water stress recovery and health after water stress largely altering plant allometry. The mechanisms contributing to this phenomenon should be explored along with *monitoring phenazine production in the rhizosphere under field conditions*.
- Phenazines can donate electrons to oxygen leading to the formation of ROS. This suggests that phenazine producers may be altering the abundance of plant-produced ROS and thus affecting ROS signaling and antioxidant defense mechanisms. It is important to explore the role of ROS signaling and peroxidase activity on the altered root morphology and plant health response conferred by phenazine producing bacteria. The induction of ROS-scavenging enzymes should also be investigated.
- As stated above, selection of phenazine-producing bacteria by drought tolerant cultivars may be an important trait to increase water stress resilience. The

processes leading to increased colonization of these organisms is of interest. Does selection by the plant or rhizosphere survival (or a combination of both) drive the increased populations of phenazine-producing bacteria in the rhizosphere?

- Most importantly, breeding for lines capable of selecting indigenous phenazine-producing bacteria may increase drought tolerance by enabling plants to derive the benefits of this functional capacity *from rhizosphere-dwelling organisms*. Using existing wheat mapping population of parents that are distinct in the recruitment of phenazine-producing rhizobacteria, I propose correlating recruitment and water stress response in the presence and absence of phenazine-producers to genetic loci. These experiments would allow us to answer questions such as: Can specific wheat QTLs be correlated with the ability to recruit phenazine producers? Does the selection of lines capable of recruiting important microbial functions such as phenazine-production (i.e., microbial functions, rather than taxa) lead to enhanced water stress tolerance, water use efficiency, favorable root phenotypes, or production under *field conditions*?

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