# PLANT-GROWTH PROMOTING RHIZOBACTERIA INCREASE SOIL WATER RETENTION BY CHANGING SOIL PHYSICAL AND HYDRAULIC PROPERTIES

by

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#### ABSTRACT

The growing global population and food consumption is challenging agriculture for higher productivity. Water is a key factor limiting crop yield in (semi-) arid regions, in this case, increasing water use efficiency is of great importance. The plant growth promoting rhizobacteria (PGPR) could potentially increase agricultural productivity in (semi-) arid regions as its beneficial effects on enhancing plant drought stress tolerance, which has been increasingly documented in the literature. However, most of previous researches have focused on PGPR-root interactions, less is known about PGPR's effects on physiochemical and hydrological properties in rhizospheric soil that may also contribute to plant drought stress tolerance. This study aimed to investigate changes in soil physical and hydraulic properties induced by *Bacillus* subtilis FB17, a generalist PGPR that has been commercialized (named as UD1022) for its ability to benefit plant growth and disease protection. In this study, soil water retention curves (SWRC) and water evaporation in soils with various textures (i.e., pure sand, sandy soil, and clay) as influenced by UD1022 were measured using HYPROP. In addition, X-ray and neutron radiography/tomography, an in-situ, nondestructive imaging technique were used to image water movement in UD1022-treated and control soil samples during evaporation. Results from both HYPROP and radiography imaging experiments showed that all UD1022-treated soils held more water and had reduced conductivity and cumulative evaporation compared to their corresponding controls. Analyses the HYPROP results combined with neutron radiography and SEM imaging revealed two potential mechanisms responsible for the

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changes in hydraulic properties and soil evaporation upon UD1022 treatment: (1) EPS alter the structure of soil matrix and connectivity of pore spaces and (2) EPS modify the physicochemical properties of water (surface tension and viscosity). These physicochemical and structural changes can lead to reduced evaporated and increased water retention.

To further understand how EPS mediate changes in water retention and evaporation, neutron and X-ray tomography were used to obtain 3D structures of UD1022 treated pure sand sample and its control. The estimated water content in the UD1022-treated sand column from neutron tomography images was higher and the water was more heterogeneously distributed compared to its control. With similar water content of both treatments based on weight measurement, the higher estimated water content in UD1022 treated sand column could come from artifacts in phase identification in image processing. In neutron tomography images, the histograms showed higher portions of pixels with greater grey scale value in the UD1022 treated sample, indicating that water was more concentrated as "clusters" that were more easily identified as water phase leading to the higher estimated water content in UD1022 treated sample than its control. There are two possible reasons that may be responsible for the changes in water distribution upon UD1022 treatment: 1) the presence of *B. subtilis* FB17 reduces the surface tension of liquid phase thus increases water retention in soil pore space sand 2) bacteria distribution is heterogeneous in soil pore space. The findings from this study suggest the potential effectiveness of PGPR in modulate changes in soil physiochemical and hydraulic changes that favor increased water retention, which may translate to enhanced plant stress relief during a drought

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event by increasing the time available for plants to make metabolic adjustment and improve plant drought tolerance.

# Chapter 1

# **INTRODUCTION AND LITERATURE REVIEW**

### **1.1 Problem Statement**

Increasing global population and food consumption are challenging agriculture for higher productivity. Even with the recent increase in productivity, one in seven people in the world is still starving or chronically malnourished. This situation may worsen in the future, as agricultural production needs to be doubled to keep pace with the increasing demands for food from the growing population (Foley et al., 2011). This increase of food production has to mainly come from increase in agricultural yield/hectare, as the land conversion to crop land is nearing its planetary limit. The expansion of agriculture into natural ecosystems has harmful effects such as decreasing biodiversity and carbon storage (Sposito, 2013).

Many new technologies have emerged in the effort to increase crop production to meet the increasing consumption for foods since the "Green Revolution", including application of chemical fertilizer to increase nutrients availability to plants, plant breeding and trans-genetic techniques to select plant with specific traits, i.e. stresstolerance and disease resistance. These methods had increased crop yield/hectare significantly, but all of them have shortcomings. For example, lack of management of fertilizer application can result in soil compaction and contamination of soil / water bodies; the period of plant breeding is long and costly; and genetic modification of plants is still controversial. One of the alternative solutions is using plant-growth promoting rhizobacteria (PGPR) as amendments, which are known as "biofertilizers".

Rhizosphere, where PGPR reside, is a small volume of soil surrounding plants' root where resides large and diverse communities of bacteria. It functions as the interface supporting the exchange of water and nutrients between plants and their soil environment (Berg et al., 2013). Rhizosphere is very complex and dynamic, understanding its ecology and evolution can lead to management strategies that increase crop productivity and ecosystem functioning.

PGPR could benefit plant growth in many aspects, such as increasing nutrient/water uptake, competitive exclusion of pathogens and so on. Literature about the enhancement of plant drought tolerance by PGPR have been increasing in recent years. However, most of the reported works have focused on microbe-root/plant interactions (Yang et al., 2009), less is known about the physical and hydraulic changes induced by bacteria in rhizospheric soil. A University of Delaware research team led by Dr. Harsh Bais found that UD1022, a specific strain of *Bacillus subtilis* could increase plant drought tolerance. They attributed the observation to UD1022's ability to control the opening and closing of stomata on plants' leaves, which could reduce water loss through transpiration and thus increase drought tolerance. However, whether UD1022 could also induce changes in soil physical and hydraulic properties corresponding to increase in drought tolerance of plants remains unknown.

Extracellular polymeric substances (EPS), excreted by most PGPRs, have large water holding capacity and are generally hydrophobic. Previous studies indicated that EPS could influence soil water retention characteristics and hydraulic properties (Roberson and Firestone, 1992). It is thus possible that EPS produced by UD1022 could plays an important role in increasing plant drought tolerance.

The main goal of this research is to quantify the changes of soil physical and hydraulic properties mediated by a representative PGPR (UD1022). Specific objectives are to figure out what soil physical and hydraulic properties are changed by PGPR, and to understand the mechanism behind those changes. The knowledge gained from this study may provide the scientific basis for exploring the application potential of PGPRs as a resource to promote plant growth under water restricted conditions to help alleviate food shortage we will face in the near future.

# **1.2 Literature Review**

#### **1.2.1 PGPR**

The utilization of bacteria to stimulate plant growth can be traced back to ancient times. For example, Theophrastus (372-287 BC) suggested the mixing of different soil samples for remedying defects and adding "heart" to soil (Tisdale and Nelson, 1975). Whipps (2001) suggested three basic categories of interactions (neutral, negative and positive) between rhizobacteria and plants. Most rhizobacteria are neutral (commensal), i.e., the bacteria inoculate the host plants but do not exhibit visible effects on growth and overall physiology of the host (Beattie, 2006). In negative interactions, phytopathogenic rhizobacteria produce phytotoxic substances such as hydrogen cyanide or ethylene, thus, negatively influence plant growth and physiology. Contrary to these deleterious bacteria, some rhizobacteria can benefit plant growth by direct mechanisms, such as nutrients solubilization, nitrogen fixation, growth regulators production; or by indirect mechanisms such as stimulation of mycorrhizae development, competitive exclusion of pathogens or removal of phytotoxic substances (Bashan and de-Bashan, 2010). Kloepper and Schroth (1981) termed these beneficial rhizobacteria as plant-growth promoting rhizobacteria (PGPR). PGPR are regarded as an indispensable part of rhizosphere biota that can stimulate the growth of host plants. They can easily establish a soil ecosystem due to their high adaptability in a wide variety of environments, fast growth rate and biochemical versatility to metabolize a wide range of natural and xenobiotic compounds.

Current understanding on PGPR is advancing at cellular, genomic and proteomic levels. Large numbers of PGPR strains of different bacterial classes and genera with multifunctional traits have been described for their potential application in boosting plant activities in modern agriculture. The very first report on PGPR-induced drought stress tolerance was published by Timmusk and Wagner (1999). The exact mechanisms of plant drought stress tolerance enhancement by rhizobacteria remain largely speculative, but possible explanations include: (1) production of hormones like abscisic acid, gibberellic acid, cytokinins and auxin; (2) production of essential enzymes, 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in the root of developing plants; (3) induced systemic resistance by bacterially-produced compounds; (4) formation of bacterial biofilm i.e. extracellular matrix (Yang et al., 1999; Kim et al., 2013; Dimkpa, Weinand and Asch, 2009; Conrath et al., 2006; Timmusk and Nevo, 2011). Biofilms contain sugars and oligoand polysaccharides that can play various roles in bacteria-plant interactions, such as improving water availability in root medium. This is because water retention capacity of some polysaccharides can exceed several-fold of their mass (Timmusk and Nevo, 2011). In fact, it has been demonstrated that even a small polysaccharide alginate content in the biofilm facilitates maintenance of hydrated microenvironment (Chang et al., 2007).

Previous investigation on drought stress tolerance enhancement to date has mostly focused on the biological or chemical interactions between microbe and plant/soil. Less is known about how these bacteria could modify soil physical and hydraulic properties, which could also be an important part of PGPR effects on PGPRinduced drought stress tolerance.

#### **1.2.2** Soil Physical and Hydraulic Properties

Water is a key factor that limits agricultural production in many places. Thus, it is important to know how water behaves in soil that is pertinent to plant growth. Soil physical and hydraulic properties, such as porosity, water retention characteristics and water conductivity, have great influences on soil water behavior. Understanding these properties is necessary to guide agricultural activities to increase crop production.

Soil water content ( $\theta$ ) and water potential ( $\psi$ ) are critical factors of soil physical and hydraulic properties. Soil water content is defined in two ways: volumetric water content  $\theta_v$  (volume of liquid water per volume of soil) and the gravimetric water content  $\theta_g$  (mass of water per mass of dry soil). Soil water potential ( $\psi$ ) describes the energy state of water in soil. Soil water retention curve (SWRC) is the relationship between  $\theta_v$  and  $\psi_m$ , where  $\psi_m$  is matric potential that is primarily determined by capillary actions. A typical SWRC plots  $\theta_v$  against  $\psi_m$  as shown in Figure 1.1.



Figure 1.1 Schematic of a typical soil water retention curve.

At high matric potentials (less negative) when soil is close to fully saturated, water is held in the soil primarily by capillary forces. As  $\theta_v$  decreases, bonding of the water becomes stronger, and at small potentials (more negative, approaching wilting point) water is strongly bond in the smallest of pores, at contact points between grains and as thin films due to adsorptive forces around particles. This curve is characteristic of different types of soil, and used to predict the soil water storage, water supply to plants (field capacity) and soil aggregate stability.

Soil hydraulic conductivity is another important property that describes the ease with which a fluid (usually water) can move through pore spaces or fractures. It depends on the intrinsic permeability of the soil, the degree of saturation, and on the density and viscosity of the fluid.

Soil structure is a key factor in the functioning of soil. It can support plant and animal life, moderate environmental quality, i.e. soil carbon sequestration and water quality. Aggregation can result from the rearrangement of particles, flocculation and cementation (Duiker et al., 2003). The stability of soil aggregate is used as an indicator of soil structure (Six, Elloitt and Paustian, 2000) and can be mediated by soil organic carbon (SOC), biota, ionic bridging, clay and carbonates. Soil structure and texture influence soil hydraulic properties such as water flow, availability and storage (Pachepsky and Rawls, 2003). Aggregation and interconnected pores can increase bypass flow in soil. This can result in increased infiltration and reduced runoff, resulting in water movement deeper into soil profile hence increased leaching (Franzluebbers, 2002; Nissen and Wander, 2003). Reduced matrix flow can lead to water stress in arid conditions.

#### **1.2.3** Soil Water Evaporation

Solar energy is consumed by evaporation, and 60% of terrestrial precipitation returns to atmosphere through evapotranspiration (20% through direct soil water evaporation and 40% through plant transpiration) (Or et al., 2013). Soil water evaporation involves interactions between soil and the ambient environment, such as energy input, water phase change, mass transfer through the boundary layer, as well as processes and factors that control water transport within the soil profile. The factors dominating soil water evaporation change during the evaporation process, which can be separated into two stages. Initially, when a soil surface is wet, evaporation rate is limited by the amount of energy available at the soil surface (Penman, 1948) and evaporation proceeds at the maximum rate. This period is called the first stage or constant-rate stage of drying. During the first stage, the soil surface is wet, and the upward flow of water within in the soil is assumed to be high enough to match the external evaporation rate. Gradually, drainage and evaporation will deplete water in the soil surface layer, which will trigger the second stage of drying. During the second

stage, the upward flow rate of water cannot supply enough water to sustain the maximum and constant rate of evaporation at the soil surface thus the evaporation rate becomes limited by the rate of water movement to the surface (Philip, 1957). More detailed discussions about soil water evaporation process, theoretical models and factors that influence evaporation rate are included in a review paper of Or et al (2013).

Sposito (2013) suggested that to ensure global food security, crop production must outpace human population growth significantly during the next 40 years. This challenge can be met by optimizing the management of "green water". "Green water" refers to water in soil that remains potentially available to plant roots and soil biota after precipitation losses to runoff and deep percolation have occurred (Rockstrom et al., 2009). Mekonnen and Hoekstra (2011) performed an exhaustive green water footprint of croplands, and their data indicate that nearly 90% of the water consumed by croplands worldwide is green water. In agriculture, soil water evaporation is always considered a loss of water because this part of water is not used by plants for growth (a loss of green water). Thus, reducing soil water evaporation is typically considered as a management strategy to increase water use efficiency. In this case, how PGPR can modify soil properties and how these changes of soil properties can affect soil water evaporation is of great importance.

# 1.2.4 Neutron and X-ray Imaging Technology

The emergence of new experimental imaging technologies in recent years has made direct visualization of many processes and reactions possible. As a result, previous assumptions, theories and models can be examined or verified, and research has been expanded to new fields. Among these novel technologies, X-ray tomography and neutron radiography/tomography are methods that allow in-situ imaging with no disturbance and damage of samples. High-resolution X-ray Computed Tomography (HRXCT) or micro-CT is a frequently used and well-developed non-destructive 3D imaging and analysis technique to investigate internal structure of various objects. This technique has been widely used in different fields, such as medical science, geoscience, and others.

Neutron imaging (radiography and tomography) is the process of producing a neutron image at a high resolution. Neutron imaging provides images similar to X-ray imaging. The difference is neutrons interact with the nuclei of atoms while X-ray interacts with electrons. X-ray attenuation depends on atomic number, Z, of the element whereas neutron attenuation coefficient is independent of Z and only a few elements such as hydrogen could strongly attenuate neutrons. Therefore, H-rich organic materials and water are visible in neutron radiographs, while many soil components and structural materials such as Si, Ca and Al are nearly transparent. With better sensitivity in certain components than X-ray, neutron imaging has been applied in many research fields where X-ray imaging has limited imaging quality, including material science, engineering, and environmental science. Early applications of neutron imaging included imaging plants and their roots (Willatt et al., 1978; Willat and Struss, 1979; Couchat et al., 1980) and examining water flow in soil samples (Brenizer and Gilpin, 1987). Improvement of neutron imaging facilities allowed better-quality imaging by the end of the 1990s (Fujine et al., 1999; Lehmann et al., 1999; Schillinger et al., 1999). Since then many more publications have appeared, addressing environmental science problems such as metal accumulation in plant leaves (Korosi, Balasko and Svab, 1999; Loria et al., 1999), soil compaction (Lopes et al., 1999), tomography of rock samples (Winkler et al., 2002) and glass bead-filled

columns (Lehmann et al., 2006). Neutron imaging applications on detecting and quantifying water in pedological and geological materials also increase in recent years, include imaging of wetting front profiles (Deinert et al., 2004), water gradients close to the main root of a bean plant (Nakanishi et al., 2005), imbibition in porous rock (Hassanein et al., 2006), water dynamics in a heterogeneous sand column (Kaestner et al., 2007), and water flow through soil aggregates (Carminati et al., 2007).

#### Chapter 2

# PLANT GROWTH -PROMOTING RHIZOBACTERIA (PGPR) REDUCE EVAPORATION AND INCREASE SOIL WATER RETENTION

#### 2.1 Introduction

Increasing global population and food consumption are challenging agriculture for higher productivity. Even with the recent increase in crop yield, one in seven people in the world is still starving or chronically malnourished. This situation may worsen in the future, as agricultural production needs to be doubled to keep pace with the increasing demands for food from the growing population (Foley et al., 2011). Water is a limiting factor in agricultural activities. A study of Glassman (2016) indicates that Sub-Saharan Africa, South and Southeast Asia are suffering the most from climate change, and these areas are also expecting high population increase by 2050. Water shortage is also a severe problem in the United States. California has recently encountered its worst drought in 500 years, and the government had spent over \$2.2 billion on it. Water shortage is a global concern challenging food production. Therefore, developing novel solution for plant growth under arid and semi-arid environment is of great significance.

Sposito (2013) proposed the concept "Green Water", which provides new perspectives on possible strategies to alleviate water shortage. Green water refers to the water in soil that remains potentially available to plant roots and the soil biota after precipitation losses to runoff and deep percolation have occurred (Rockstrom et al., 2009), and is the hydrologic complement of "Blue Water" (water flows in streams and

rivers, stored in lakes and reservoirs, or pumped from aquifers). Most of the water used by crop land worldwide (up to 90%) is green water (Mekonnen and Hoekstra, 2011). The green water loss from cropland includes evaporation and transpiration, whereas only transpiration is considered as productive green water flow. Rockstrom et al. (2007) illustrate that if the ratio of transpired green water can increase from 30% to 85%, the yield of rainfed maize (*Zea mays L.*) could triple. The percentage of green water flow depends on the efficacy of its rhizosphere in promoting transpiration over evaporation, along with intrinsically characteristics of the crop grown (Sposito, 2013).

Rhizosphere is a small volume of soil surrounding plants' root where resides large and diverse communities of bacteria. It functions as the interface supporting the exchange of water and nutrients between plants and their soil environment (Berg et al., 2013). Plant-growth promoting rhizobacteria (PGPR) is a group of bacteria that could benefit plant growth in many aspects, such as increase nutrient/water uptake, competitive exclusion of pathogens, and so on. Literature about the enhancement of plant drought tolerance by PGPR have been increasing in recent years. Timmusk and Wagner (1999) published the first paper on plant drought tolerance enhancement by PGPR. They found that Arabidopsis thaliana inoculated with PGPR Paenibacillus *polymyxa* B2 could survive longer than untreated samples under drought condition. Subsequently, other reports indicate that drought tolerance increased by PGPR is also found in other crops, including common bean (Phaseolus vulgaris L.) (Figueiredo et al., 2008), tomatoes and peppers (Mayak et al., 2004), in wheat (Timmusk et al., 2014, 2015). However, most of the reported work have focused on microbe-root/plant interactions (Yang et al., 2009), less is known about the physical and hydraulic changes induced by bacteria in rhizospheric soil.

Extracellular polymeric substances (EPS), excreted by most of PGPRs, have large water holding capacity and are generally hydrophobic. Previous studies indicated that EPS could influence soil water retention characteristics and hydraulic properties (Roberson and Firestone, 1992), such as increase soil water content (Roberson and Firestone, 1992; Kroener et al., 2014; Volk et al., 2016) and reduce hydraulic conductivity (Bozorg et al., 2015; Volk et al., 2016). In this case, we can confer that EPS produced by rhizobacteria could potentially increase green water availability and productive flow. However, the underlying mechanisms of PGPR's effects on modifying soil's hydraulic properties remain poorly understood. In addition, how soil evaporation, as one of the two components in the evapotranspiration process and a key factor in determining the magnitude of productive green water flow, is affected by the presence of PGPR remains unknown.

Neutron radiography is the process of producing a neutron image at a high resolution. In neutron imaging, H-rich organic materials and water are visible, while many soil components and structural materials such as Si, Ca and Al are nearly transparent. Early applications of neutron imaging included imaging plants and their roots (Willatt et al., 1978; Willat and Struss, 1979; Couchat et al., 1980), as well as examining water flow in soil samples (Brenizer and Gilpin, 1987). Improvement of neutron imaging facilities allowed better-quality imaging by the end of the 1990s (Fujine et al., 1999; Lehmann et al., 1999; Schillinger et al., 1999). Since then many more publications of neutron imaging applications on detecting and quantifying water in pedological and geological materials have appeared, include imaging of wetting front profiles (Deinert et al., 2004), water gradients close to the main root of a bean plant (Nakanishi et al., 2005), imbibition in porous rock (Hassanein et al., 2006), water

dynamics in a heterogeneous sand column (Kaestner et al., 2007), and water flow through soil aggregates (Carminati et al., 2007). The neutron radiography imaging is now a promising tool for understanding water movement in soil profiles and plant water uptake.

In this study, *Bacillus subtilis* strain FB17 (*B. subtilis* FB17) was used as a model rhizobacteria. This specific strain has been commercialized (named as UD1022) for its beneficial functions in promoting growth and disease protection in multiple staple crop species. HYPROP system is based on a simplified evaporation method (Peters and Durner, 2008) and was used in this study to measure soil water retention and hydraulic conductivity as affected by *B. subtilis* FB17. The simultaneously measured evaporation data were also analyzed for evaporation rate profiles and cumulative evaporation amount. In addition, water distribution of sand columns in the presence and absence of *B. subtilis* FB17 during evaporation was directly observed by neutron radiography imaging. The goal of this study was to advance mechanistic understanding of PGPR-mediated biophysical changes in the rhizosphere that may increase plant drought tolerance.

## 2.2 Materials and Methods

#### 2.2.1 Soil Samples and UD1022 Strains

In this study, three types of soil were used: pure sand, sandy soil and clay. The pure sand is a commercial product (Accusand, 40/50-sieve size; Unimin Corp., Le Sueur, MN); the sandy soil is a field soil collected from a field experimental station located in Georgetown, DE; the clay soil was collected from an agricultural farm on University of Delaware campus. Pure sand samples were acid-washed, and

agricultural soil samples were air dried and sieved through a 2-mm sieve before further treatment. All pre-prepared samples were autoclaved at 121°C for two 30-min cycle following a 24 h period at ambient room temperature. After 24 h cooling down, samples were inoculated with selected bacteria. Soil texture properties were determined using a laser light-scattering particle size analyzer (LSTM 13 320 Series, Beckman Coulter, Miami, FL, USA). The physical properties of the soils are listed in Table 2.1.

Table 2.1Physical properties of tested soil samples: particle size fraction and<br/>porosity. C and T refers to control (without bacteria inoculation) and<br/>treated (without bacteria inoculation) sample respectively.

		Pure sand (PS)		Sandy soil (SS)		Clay soil (CS)	
Particle	< 0.002 mm	0.297-0.420 mm		1.4		67.3	
fraction	< 0.05 mm			9.4		20.6	
(%)	< 2 mm			89.2		12.1	
		PS-C	PS-T	SS-C	SS-T	CS-C	CS-T
Porosity (%)		37.7±1.9	37.3±1.5	40.7±2.8	41.8±2.4	47.9±0.7	47.9±0.4

The selected bacteria in this study was UD1022 (*B. Subtilis* FB17) (Kumar et al., 2012), 3610 (the wild type of UD1022, can produce EPS) and DS646 (mutant of 3610 that cannot produce EPS). Both the commercial product of UD1022 (powder) and freshly grown liquid culture were used. The commercial UD1022 powder is a mixture of *B. Subtilis* FB17 spores and growth media, with a concentration of 2.8 x  $10^{10}$  CFU/g (colony-forming unit). Sand or soil samples were sterilized, weighed and mixed with 0.25% (w/w%) UD1022 powder (treatment) or without UD1022 addition (control), and then 5% (w/w%) sterilized DI water was added to each sample and

thoroughly mixed. The liquid cultured bacteria were incubated in LB solution at 37°C for 24 h from their fresh plates, respectively. For sample preparation using the liquid culture, the concentration of liquid bacteria culture was adjusted to  $1.4 \times 10^9$  CFU/ml, then mixed with sand/soil samples. Sterilized samples (pure sand or soil) were weighed and mixed with 5% (w/w%) sterilized DI water (control) or bacteria solution (treatment).

The above preparations resulted in an initial concentration of bacteria of  $7 \times 10^7$  CFU/g for all samples. Both control and treated samples were incubated overnight at ambient room temperature after thoroughly mixed with sterilized DI water or bacteria solution to allow water redistribution to reach an equilibrium, as well as bacteria growth.

# 2.2.2 Soil Water Retention Curve Measurement

Soil water retention curve (SWRC) is the relationship between water content,  $\theta$ , and soil water potential,  $\psi_m$ . This curve is characteristic of different types of soil and is also called soil moisture characteristic function. HYPROP (UMS, Munich, Germany) is a fully automated measuring and evaluation system that is used to determine the hydraulic properties of soil samples. It consists of two shafts with ceramic tips of different length and were connected to a sensor base. The soil sample was packed in the sample ring and attached to the sensor base. This system can precisely measure the water potential of the soil at two depths and record data continuously. The sensor base with soil sample is placed on a balance and the weight changes during evaporation are recorded continuously as well. The water content thus can be calculated and plotted with matric potential data to obtain SWRC. Figure 2.1 presents a schematic diagram of a HYPROP unit.



Figure 2.1 Schematic diagram of HYPROP for measuring soil water retention curve and unsaturated hydraulic conductivity (adapted from Decagon, Inc., HYPROP operation manual).

The three types of soil (pure sand, sandy soil, and clay), as described above, were used in HYPROP measurement. For both treatments (with/without UD1022 inoculation), soils were pack into sample ring of HYPROP, and the weight of moistened soil samples were recorded. After packing, sample rings were saturated overnight with DI water to reach maximum saturation. Then fully saturated samples were attached to sensor base to measure SWRC. After data collection, soil samples were oven dried at 104 °C and dry weight of soil samples were recorded.

### 2.2.3 Scanning Electron Microscope (SEM) Imaging

To verify UD1022's production of EPS and their presence on the surface of sand particles as well as possible resulted changes in soil physical properties, images of pure sand samples were taken using SEM (Hitachi, S-4700, Japan). At the end of HYPROP measurements, ~1 g pure sand was collected from each treatment (control and treated) and dried at ambient room conditions for 24 h. The samples were attached onto a piece of carbon tape for SEM visualization.

### 2.2.4 Neutron Radiography Imaging

Neutron radiography (NR) is one of the few non-destructive techniques available for imaging dynamic soil water distribution and plant roots *in situ*. By NR, visible and direct evidence of water movement and distribution during evaporation can be obtained. Images were acquired using the Neutron and X-ray Tomography (NeXT) system in the Center for Neutron Research (NCNR) BT-2 imaging beam line in National Institute of Standard and Technology (NIST). The NeXT system operates by orienting a microfocus X-ray tube perpendicular to the neutron beam. Figure 2.2 shows the sketch of NeXT system. The detector used for this study was an Andor NEO scientific complementary metal oxide semiconductor (sCMOS) camera that views a gadolinium oxysulfide scintillator (Lexel Imaging P-43 phosphor) (LaManna et al., 2017). Neutron radiography imaging can be operated alone with NeXT system.



Figure 2.2 Schematic of BT-2 imaging beam line facility in NIST. (a) shows the general layout of the whole facility; (b) shows the imaging site of the NeXT system with the neutron beam (red) and the X-ray beam (blue); (c) shows a sample column in a rotating sample holder and a humidity-controlled gas inlet.

For samples used in NR imaging, soils were prepared following the same procedures as described in Section 2.2.1. Columns were packed with control and incubated sandy soil samples with powder- and liquid-cultured bacteria, respectively, and saturated in a water bath from bottom to top. The three saturated soil columns (control, incubated with powder-bacteria, incubated with liquid-cultured bacteria) were placed in a row and exposed to the neutron beam for radiography. All samples were imaged simultaneously to ensure the same environmental conditions. The field of view was 6.4 cm by 7.6 cm, with a resolution of 60  $\mu$ m. Exposure time of 22 s yielded an optimal grayscale range in the radiograph. Images were captured continuously for 12 h as the samples dried.

## 2.3 Results and Discussions

#### **2.3.1 HYPROP Results**

Figures 2.3 to 2.5 show the results from HYPROP. Figure 2.3 presents the relationship between degree of saturation and evaporation rate of three types of soil. Figure 2.4 presents water retention curve of soil samples and Figure 2.5 shows the temperature variation of each sample during the experiments. The SWRC figures above (Figure 2.4) show that the UD1022-treated soil samples have higher initial water content than their respective controls, and at the same time have lower evaporation rates. From Figure 2.3 and 2.4, we can conclude that the bacteria effect is more pronounced at the drier end of SWRCs. In Figure 2.3, the evaporation rate of UD1022-treated pure sand and sandy soil samples is significantly lower at low saturation than that of the control samples. In Figure 2.4, the water content of UD1022-treated samples is higher than control samples at a given water potential at low potential end, especially between -103 to -102 hPa. The above effects are more significant in coarser textured samples. In Figure 2-3 and 2-4, pure sand samples show the greatest differences between control and UD1022 treated samples, followed by sandy soil samples and then clay samples. It is reasonable to conclude that UD1022 effects are more pronounced in coarser textured samples. Sandy soil samples treated with UD1022 have minor but observable shrinkage during evaporation, and the occurrence of shrinking could change soil pore structure, which can affect soil water matric potential measurement. The abnormal fluctuation on the SWRC of the treated sandy soil sample in Figure 2-4 may be explained by inaccurate measurements of soil water matric potential due to soil shrinkage, which could lead to failed contact between soil sample and tensiometers of HYPROP.



Figure 2.3 Evaporation rate versus saturation of three types of soil samples.



Figure 2.4 Soil water retention curve of three types of soil samples from HYPROP measurement.



Figure 2.5 Soil temperature of three different types of soil during evaporation.

# 2.3.2 SEM Results

Figure 2.6 shows SEM images of pure sand samples with different treatments at different magnification. Figures 2.6 (a) and (d) show the overall structure of control (sand particles) and treated sample (aggregated particles). The aggregation is likely caused by EPS excreted by UD1022 that 'glue' sand particles together. Figures (b) and (e) are detailed images of sand particle surfaces of different treatments. UD1022 treated samples have smoother surface than control sample (b), as the rough sand surface in treated sample is coated by EPS excreted by UD1022. And (c) and (f) is particle surface with higher magnification. The SEM images reveal that UD1022 could change soil structure and surface property by excreting EPS. EPS could also change soil pore structure by clogging small pores in soil sample, or connecting large pores into smaller ones, and increase the pore space, thus increase the porosity of soil.



Figure 2.6 SEM images of pure sand samples with different treatments. (a), (b) and (c) are control sand samples, (d), (e) and (f) are UD1022 treated sample. The short rods in (e) and (f) are UD1022 bacteria. The films that connect bacteria in (f) is EPS.

#### 2.3.3 Neutron Radiography Results

Time-lapsed images of evaporation of soil samples with different treatments were imaged using neutron radiography. In general, the neutron radiography results are consistent with HYPROP results, i.e., the treated samples have lower evaporation rates than the controls. Figure 2.7 shows the Time-lapsed images of evaporation from pure sand samples with different treatments. In this figure, the mutant DS646 treated sample, which is expected to have higher evaporation rate than UD1022 or 3610 treated sample during the evaporation but showed similar evaporation rate as UD1022 and 3610. The reasons for the similar evaporation rate of DS646 to other treatments remains unknown, possible explanations include: 1) all UD1022, 3610 and DS646 strains could have other mechanisms that reduce soil water evaporation; 2) DS646 could have other mechanisms other than EPS production that can reduce soil water evaporation rate. The control of sandy soil samples showed lower water content than UD1022 treated sandy soil sample after same elapsed time, which is opposite to my expectation. But this is due to different water pond above different samples. The control of sandy soil sample had more ponding water on its surface than that of treated sample. This error can be eliminated by comparing evaporation rate of different samples after over-saturated water had evaporated. Figures 2.8 and 2.9 shows the neutron radiography images of evaporation after ponding water is corrected. After correction of ponding water, the results are consistence with previous HYPROP results.

In Figure 2.8, pure sand samples inoculated with UD1022 culture solution, inoculated with UD1022 powder, and control sample are placed from top to bottom. In general, the control sample has more red (moderate dry) and yellow (very dry) areas than UD1022 inoculated samples. This is consistent with the HYPROP results, the
presence of UD1022 could reduce soil water evaporation rate. By comparing UD1022 culture solution inoculated sample (solution treatment) and UD1022 powder inoculated sample (powder treatment), solution treatment shows more red and yellow areas, while powder treatment shows more yellow areas. This indicates that water evaporation is more uniform in solution treatment and can be explained by more homogeneous bacteria distribution in solution treatment than powder treatment, as bacteria solution is easier to mix uniformly than bacteria powder. Despite this difference, solution treatment and powder treatment show similar remaining water content, which means UD1022 spore powder and freshly grown UD1022 culture solution have comparable effects on soil water evaporation.

In Figure 2.9, both UD1022 inoculated samples show lower evaporation rate than their controls, which is consistent with previous HYPROP results. By comparing images of sandy soil and clay samples, the clay sample always shows greater differences in colors than sandy soil sample, indicating higher evaporation rate, which is not consistent with HYPROP evaporation results. This difference is probably caused by shrinkage of soil samples. The swell/shrink effect is more obvious in clay soil, this could result in more obvious gap between soil body and aluminum cell wall. The existence of the gap will increase the surface area for evaporation, thus result in higher evaporation rate in the clay sample. In addition, the water content of clay sample changes faster from the column during neutron imaging than from the HYPROP experiment, which may also be explained by the increased evaporation surface area at the gap between soil and Al cell wall. Although this shrinking effect is likely existed in both HYPROP experiment and column experiment, the sample ring of HYPROP

imaging. As a result, the contribution of increased evaporation from increased surface area along the wall to the total evaporation is much smaller in HYPROP experiment compared to the small column used in neutron experiments.



Figure 2.7 Time-lapsed images of evaporation from sandy soil samples with different treatments after ponding water had evaporated. From top to bottom are: treated with UD1022 powder, UD1022 liquid solution, wild type 3610, mutant DS646 and control (without treatment); for each treatment, from left to right are: 0 h, 1 h, 3 h 5 h and 7 h. Water content decreases from blue to red to yellow.



Figure 2.8 Time-lapsed images of evaporation from pure sand samples with different treatments after ponding water had evaporated. From top to bottom are: pure sand inoculated with freshly grown UD1022 culture solution, with freshly grown wild type 3610 culture solution, with freshly grown mutant DS646, with UD1022 powder (commercial product) and control; for each treatment, from left to right: 0 h, 3 h, 6 h and 7.7 h.



Figure 2.9 Time-lapsed images of evaporation from sandy soil and clay samples with different treatment. From top to bottom: sandy soil inoculated with UD1022 powder (commercial product), sandy soil control (without treatment), clay treated with UD1022 powder, clay control. For each treatment, from left to right: 0 h, 3 h, 6 h and 9 h.

#### 2.3.4 Possible Mechanisms

# The role of EPS in increased soil water retention and decreased soil water evaporation

Both the quantitative results from HYPROP measurement and direct visualization from neutron radiography imaging show that UD1022 treatment can increase soil water retention and reduce soil water evaporation. The SEM images could be helpful to understand the mechanisms of how UD1022 modify soil properties in treated sample that led to increased soil water retention and decreased soil water evaporation. Figure 2.6 (b) and (c) shows that the sand particles of control sample have clean but rough surfaces, while Figure 2.6 (e) and (f) shows rod-shaped UD1022 cells on UD1022 treated samples, with a layer of biofilm connecting all those cells and covering the sand particle surfaces. In a previous work of Bridier et al. (2013), biofilm produced by *B. subtilis* was observed under hydrated and dehydrated conditions using Environmental SEM. They found that bacterial cells of B. subtilis were embedded in mucoid-like structures in hydrated matrix and were connected by dense and oriented network of fibers during dehydration, similar to the structures shown in Figure 2.6(e) and (f). The similar fibrous skeleton was also observed in Branda et al. (2006) and Kumer et al. (2012) and was identified as EPS. By comparing Figure 2.6 (a) and (d), it is easy to conclude that EPS caused the aggregation of sand particles after inoculation of UD1022. Also, Figure 2.7 (f) shows hollow spaces created by EPS between sand particles which could potentially responsible for modified pore structure of treated soil samples. The ability of EPS to increase soil water retention had been documented in other papers. For example, Roberson and Firestone (1992) found that *Pseudomonas* can also produce EPS and increase soil water retention. Volk et al. (2016) also

reported both saturated and unsaturated hydraulic conductivity decreases after inoculation of *Pseudomonas*.

In this case, although the neutron radiography result (Figure 2.7) shows that DS646, the mutant of 3610 that cannot produce EPS, also can reduce soil water evaporation, it is still reasonable to conclude that EPS produced by UD1022 played an important role in increased soil water retention and decreased soil water evaporation rate.

#### Soil Physical and Hydraulic Properties Modified by EPS

As described in Figure 2.6, the presence of EPS could modify soil pore structure. EPS, on the one hand, can increase soil aggregation by connecting small particles together and forming large pores, thus could increase soil pore size distribution. On the other hand, the fibrous network skeleton formed by EPS, as shown in Figure 2.6 (f), can also separate large pores into smaller ones, which can narrow the soil pore size distribution. Volk et al. (2016) also reported that EPS can clog small pores which would decrease soil pore size distribution. The overall effect on soil pore size distribution of UD1022 had been reported by Zheng et al. (2018). In that work, they found that the parameter n in fitted Van Genuchten model decreased after inoculation of UD1022, which means narrower pore size distribution. Zheng et al. (2018) also reported decreased hydraulic conductivity of UD1022 treated sample, which could be a consequence of modified soil pore structure by EPS.

EPS can also change the surface wettability and the surface tension of a water air interface. Read et al. (2003) found that EPS could reduce surface tension, which can be attributed to the phospholipids in EPS. Epstein et al. (2011) reported that biofilms tend to display persistent resistance to liquid wetting. Therefore, EPS can

generally increase the hydrophobicity of soil particles. The effects of biofilms on soil water retention is complex. On the one hand, based on Young-Laplace equation (Equation 2.1),

$$h_{cap} = -\sigma \cos \alpha \,/\, r \tag{2.1}$$

where  $h_{cap}$  (N/m<sup>2</sup>) is the capillary pressure needed to drain a cylindrical pore,  $\sigma$  (N/m) is the surface tension of the liquid-air interface,  $\alpha$  is the contact angle between liquid phase and particle surface, r is the radii of the pore. The UD1022 treated sample has lower  $\sigma$  and smaller  $cos\alpha$ , indicates that less work is required to drain a cylindrical pore. On the other hand, the lower surface tension will increase the amount of water held in pore corners based on a triangular pore space model developed by Tuller and Or (2001).

Both mechanisms described above could influence soil water retention characteristics, the dominant mechanism of these two depends on soil texture and degree of saturation. Coarse texture soils have narrower pore size distribution and more uniform particle/pore shape, thus the shape of SWRC of coarse texture soil is less significantly influenced induced by the presence of EPS. However, the drainage of water in large pores occurs earlier in UD1022 treated samples at lower surface tension with the presence of EPS, which explains the difference in water retention curve between the treatments in pure sand (Figure 2.4).

# 2.4 Conclusions

In this research, we studied the effects *B. subtilis* FB17 (UD1022) on soil hydraulic properties and evaporation for different textured soils, including pure sand, sandy soil, and clay. The EPS produced by *B. subtilis* FB17 helps treated soil sample retains more water, lower the unsaturated hydraulic conductivity and water

evaporation rate compared to the controls. Our analyses indicate that EPS mediate changes in soil water-holding capacity and evaporation characteristics via three potential mechanisms: (1) the hygroscopic EPS retains large quantities of water, (2) EPS modify soil pore-size distribution, and (3) EPS modify soil water properties (i.e., decrease surface tension and increase viscosity).

The increased water retention and decelerated evaporation point to the potential effectiveness of using PGPR to help relieve the stress plants experience during drought. Under drought conditions, the very limited amount of water is likely to cause hydraulic failures either because roots shrink, or the soil's conductivity cannot sustain the transpiration demand (Carminati et al., 2017). By retaining more water in the soil and for a longer period of time, the treatment can increase plant tolerance to drought by (1) directly providing more water to plants thus increase transpiration and (2) increasing the time available for metabolic adjustment for plants to better adapt to drier conditions. Rhizobacteria, when used to treat soil, may have a larger sphere of EPS influence compare to root mucilage as they are more mobile than plant roots, although B. subtilis cells were found to be unlikely to transport over long distances in sandy soils (Kinoshita et al., 1993). In addition, the shift of water consumption from soil evaporation to plant transpiration increases green water (i.e., the water stored in soil) availability and use efficiency. Along with B. subtilis FB17's ability to fix nitrogen and increase phosphorus solubility (Lakshmanan et al., 2012), the treatment can trigger positive soil-water-plant feedbacks including increase in crop biomass or canopy size that leads to shading effects of the canopy, which then further decreases soil evaporation and induces a "vapor shift" from soil evaporation to transpiration, and ultimately to increased crop production. Therefore, application of

PGPR represents a potentially viable technology and a soil-based, sustainable solution that can contribute to food security by providing increased crop yield for the growing population and maintain soil health under the changing climate.

#### Chapter 3

# STUDYING RHIZOBACTERIA EFFECTS ON SOIL PHYSICAL PROPERTIES USING NEUTRON AND X-RAY TOMOGRAPHY

#### 3.1 Introduction

Water is a key factor that limits agricultural production in many regions of the world. Increasing global population and food consumption are challenging agriculture for higher productivity. Therefore, developing novel solutions for plant growth under arid and semi-arid environments and increase plant water use efficiency are of great significance.

Sposito (2013) proposed the concept "Green Water", which provides new perspectives to alleviate water shortage. Green water refers to the water in soil that remains potentially available to plant roots and the soil biota after precipitation losses to runoff and deep percolation have occurred (Rockstrom et al., 2009), and is the hydrologic complement of "Blue water", i.e., the water that flows in streams and rivers, is stored in lakes and reservoirs, or pumped from aquifers. Most of the water used by crop land worldwide (up to 90%) is green water (Mekonnen and Hoekstra, 2011). Green water loss from cropland includes evaporation and transpiration, whereas only transpiration is considered as productive green water flow. Rockstrom et al. (2007) proposed that if the ratio of transpired green water could increase from 30% to 85%, the yield of rainfed maize (*Zea mays L.*) could triple. The percentage of green water flow depends on the efficacy of its rhizosphere in promoting transpiration over evaporation, along with the intrinsic characteristics of the crop grown (Sposito, 2013).

Rhizosphere is a small volume of soil surrounding plants' root where resides large and diverse communities of bacteria. It functions as the interface supporting the exchange of water and nutrients between plants and their soil environment (Berg et al., 2013). Plant-growth promoting rhizobacteria (PGPR) is a group of bacteria that could benefit plant growth in many aspects, such as increase nutrient/water uptake, competitive exclusion of pathogens, and so on. Timmusk and Wagner (1999) published the first paper on plant drought tolerance enhancement by PGPR. They found that Arabidopsis thaliana inoculated with PGPR Paenibacillus polymyxa B2 could survive longer than untreated samples under drought condition. Subsequently, additional research has demonstrated that drought tolerance increased by PGPR was also found in other crops, including common bean (Phaseolus vulgaris L.) (Figueiredo et al., 2008), tomatoes and peppers (Mayak et al., 2004), in wheat (Timmusk et al., 2014, 2015). However, most of the reported work have focused on microbe-root/plant interactions (Yang et al., 2009), less is known about the physical and hydraulic changes induced by bacteria in rhizospheric soil. Recently, Zheng et al. (2018) found that the extracellular polymeric substances (EPS) produced due to PGPR could enhance plant drought tolerance by its large water holding capacity, the ability to alter the structure of soil matrix and connectivity of pore space, which could in turn influence soil hydraulic properties. They also pointed out that EPS can modify the physicochemical properties of water such as surface tension and viscosity.

The emergence of new imaging technologies in recent years has made direct visualization of many processes and reactions possible. In soil science, X-ray computed tomography (CT) is an optimal method to study soil structure, which refers to the spatial arrangement of pore and particle networks in soil. Previous applications

of X-ray CT in soil science include investigating soil-pore networks and its characteristics such as porosity, connectivity, pore diameter and pore shape to assess soil structure. These characteristics, in turn, have been used to study and predict flow and transport processes in soil (Luo et al., 2010; Helliwell et al., 2013; Larsbo et al., 2014). Despite its wide use for studying soil structure, only few X-ray CT studies focused on partially saturated conditions. This is due to the difficulty in differentiating liquid and gaseous phases in unsaturated soil samples.

To identify liquid and gaseous phases in unsaturated porous media, Wilson et al. (2012) used synchrotron-based X-ray CT to obtain tomography images of unsaturated sand samples. Synchrotron radiation has higher photon flux and higher degree of collimation compared with traditional X-ray source, as well as well-defined time structure and the ability to tune the photon energy over a wide range using an appropriate monochromator for obtaining element- or compound- specific measurements. These features can be extremely helpful for differentiating fluid phases. But synchrotron X-ray source has a major constrain: the dimensions of typical porous media samples are very limited (<1 cm) because synchrotron X-ray source has lower energy (typically <50KeV) compared with conventional X-ray CT system (over 400KeV). In a previous study, Higo et al. (2014) developed an image processing algorithm named trinarization to identify the three phases in X-ray CT images of a sand sample. Their method provided reasonable results for CT images at a high pore saturation regime, while at a low pore saturation regime, overestimated the local void ratio. What's more, the images were obtained by X-ray source, which means their results in liquid/gaseous identification were limited by the properties of X-ray.

Neutron imaging (radiography and tomography) is the process of producing a neutron image at a high resolution. The difference between neutron and X-ray imaging is neutrons interact with the nuclei of atoms while X-ray interacts with electrons, respectively. In neutron imaging, H-rich organic materials and water are visible, while many soil components and structural materials such as Si, Ca and Al are nearly transparent. With better sensitivity in H-rich components than X-ray, neutron imaging has been applied in many research fields where X-ray imaging has limited imaging quality, including material science, engineering, and environmental science. Early applications of neutron imaging that is related to this study included imaging plants and their roots (Willatt et al., 1978; Willat and Struss, 1979; Couchat et al., 1980) and examining water flow in soil samples (Brenizer and Gilpin, 1987). Improvement of neutron imaging facilities allowed better quality imaging by the end of the 1990s (Fujine et al., 1999; Lehmann et al., 1999; Schillinger et al., 1999). Since then many more publications of neutron imaging applications on detecting and quantifying water in pedological and geological materials have appeared, include imaging of wetting front profiles (Deinert et al., 2004), water gradients close to the main root of a bean plant (Nakanishi et al., 2005), imbibition in porous rock (Hassanein et al., 2006), water dynamics in a heterogeneous sand column (Kaestner et al., 2007), and water flow through soil aggregates (Carminati et al., 2007). Based on the different properties of X-ray and neutron beams, it is possible that we could obtain images with better phase identification by combining X-ray and neutron images of the same sample. The coupled neutron and X-ray tomography (NeXT) system, located in National Institute of Standard and Technology (NIST), allows simultaneous neutron and X-ray imaging

of the same sample by orienting a microfocus X-ray tube perpendicular to the neutron beam.

In this study, we incubated pure sand samples with *Bacillus subtilis* FB17, a PGPR strain that has been commercialized (named as UD1022<sup>\*</sup>), as a model rhizobacteria. *B. subtilis* FB17 is an EPS-forming bacterium found in soil (Kumar et al., 2012) that has been shown to influence soil physical and hydraulic properties (Zheng et al., 2018). Using the NeXT system, it is possible to obtain 3D structure of pure sand columns and 3D water distribution of the same sample simultaneously. By analyzing the tomography data, along with our previous knowledge about *B. subtilis* FB17 effects on soil water retention and hydraulic conductivity (Zheng et al., 2018), we hope to provide mechanistic understanding on PGPR's ability to increase water retention and reduce evaporation.

#### **3.2 Materials and Methods**

#### 3.2.1 Sand

The pure sand used in this research is Accusand (40/50-sieve; Unimin Corp., Le Sueur, MN). Sand samples were acid-washed by immersing in 1 mol/L HNO<sub>3</sub> overnight and rinsing with DI water to adjust pH to neutral, then autoclaved twice at 121°C for 30 min each time. The washed sand was stored under room temperature overnight before inoculation with bacteria.

# 3.2.2 Bacteria Strains

In this study, we used UD1022 as a model PGPR strain (Kumar et al., 2012). The bacterium was plated on Lysogeny broth (LB) agar plates and incubated at 37°C overnight. The colonies on LB agar plates were transferred into 200 ml flasks with 50 ml pre-autoclaved LB solution (at 121°C for 15 min), and incubated in a shaker at  $37^{\circ}$ C, 120 rpm for 24 h. The bacteria in the flasks were transferred into 50 ml centrifuge tubes and centrifuged at 4°C, with 5000 rpm (revolution per minute, equivalent to 3214 rcf, relative centrifugal force) for 10 min. The bacteria pellets were rinsed with 1× PBS (Phosphate-Buffered Saline) solution twice and re-suspended in autoclaved DI water (at 121°C for 15 min) to reach a concentration of  $1.4 \times 10^9$  CFU/g (colony forming unit) for later use.

# **3.2.3** Sample Preparation

For the treated sample, 100 g of sterilized sand sample was thoroughly mixed with 5 ml bacteria solution (in DI water) manually in a sterilized beaker. Correspondingly, the control sample was prepared by mixing 100 g sterilized sand with 5 ml autoclaved DI water, following the same procedures as the treated sample. The initial bacteria concentration was  $7 \times 10^7$  CFU/g sand in the treated sample. The beakers were covered with aluminum foil to avoid potential pollution and stored in an oven at  $37^{\circ}$ C overnight. This allows bacteria growth and water in the samples to equilibrate with the sand. The control and treated samples were then respectively packed into aluminum columns, which are 1 cm in diameter and 2.5 cm in depth, following a standardized procedure (Klute and Dirksen, 1986). After packing, the columns were saturated in separate water reservoirs with sterilized and degassed DI water overnight before transporting to imaging site.

#### **3.2.4** Neutron and X-Ray Tomography

Images were acquired using the Neutron and X-ray Tomography (NeXT) system at the Center for Neutron Research (NCNR) BT-2 imaging beam line in

National Institute of Standard and Technology (NIST). The NeXT system operates by orienting a microfocus X-ray tube perpendicular to the neutron beam that allows simultaneous imaging using both beams. The detector used for this study was an Andor NEO scientific complementary metal oxide semiconductor (sCMOS) camera that views a gadolinium oxysulfide scintillator (Lexel Imaging P-43 phosphor) (LaManna et al., 2017). A schematic description of the NeXT system is shown in Figure 2.2.

For tomography imaging, only one sand column can be scanned at a time. Each run represents a 12 h simultaneous high-resolution neutron and X-ray tomography scanning with high relative humidity in flow chamber to hinder evaporation. The exposure time was 10 seconds; 3 images per angle and 1200 projection angles from 0-360 degree were captured. The field of view was set by the camera chip size at 2160 × 2560 times pixel dimension (or 6.5  $\mu$ m) times the reproduction ratio of lens (1.38), which gave a field of view of 19.5 mm × 23 mm.

# 3.2.5 Reconstruction and Combination of Neutron and X-Ray Tomography Images

The captured images were 2D front-views of the scanned columns. Reconstruction of these 2D slices was performed to obtain 3D intersections with the inner structures of the sand columns. The software Octopus (Octopus Imaging Software, Gent, BE) was used to process X-ray tomgoraphy images; ReconstructCT along with algorithm SIRT (Simultaneous Iterative Reconstruction Technique) were used (van Aarle et al., 2015, 2016; Palenstijn et al., 2011) for processing neutron tomography images. SIRT provides better quality of reconstructed slices but reconstruction takes longer compared to using algorithm FBP (filter backprojection), which is more commonly used for the purpose. Figure 3.1 shows the reconstructed slices of neutron and X-ray images. Attenuation of particles increases from black to white in both X-ray and neutron images. In X-ray images, elements with higher atomic number attenuate more X-ray beam, which means that white color indicates aluminum column, sand and metallic inclusions in sand particles (if there is any), light grey indicates water and dark grey/black indicates air. In neutron images, both air and sand are nearly transparent to neutron beam, while water is more visible. Thus, in the reconstructed neutron slices, black indicates sand particle/air whereas the color of water transitions from dark grey to light grey to white with increasing water content in the pixel that increases with neutron attenuation. It should be noted that dark grey in neutron images could be also sand with, for example, iron inclusions that could show up brighter in neutron images. The resolution of reconstructed X-ray and neutron slices is 18 µm.



Figure 3.1 Sample images of reconstructed X-ray and neutron tomography. The attenuation of beam increases from black to white in both images. In the X-ray images, attenuation increases from air to liquid to solid phases; in the neutron images, attenuation increases with water content.

In previous studies, when combining neutron and X-ray imaging for a sample, they were used sequentially with one technique followed by the other. If observing time-dependent processes, the sample will not be identical for each imaging mode. With the NeXT system, X-ray and neutron images are taken simultaneously, which improves phase identification of samples compared with the traditional sequenced imaging. In this research, X-ray and neutron datasets provide more complete information on solid and liquid phase with better quality than each equipment used alone. By combining X-ray and neutron tomography results, accurate 3D structure and phase distribution in the sand columns could be obtained.

For image analysis, corresponding slices from one dataset to the other need to be matched first. This can be achieved by using the Volume Registration tool from NIST. Two volumes (X-ray and neutron) are defined as moving or reference volume separately. This tool could register the moving volume and remapping its grid to the reference volume using the Mattes Mutual Information method in Matlab. The registration corrects for any translational, rotational or scaling misalignment between the volumes. By manually adjust the scale, x/y/z axis positions and rotation of moving volume, two volumes can get close to each other as shown in Figure 3.2. After manual alignments, the program iteratively aligns the volumes and calculates the transformation required to alter the moving volume, and the moving volume is transformed through a process called image warping. The newly transformed volume is then written to a disk as an image stack that could be further analyzed.



Figure 3.2 Screenshots of Volume Registration tool. Red indicates reference volume (X-ray data) and light blue color represents moving volume (neutron data). Picture (a) shows the position of X-ray and neutron volumes without adjustment; (b) shows the position of the two volumes after manual adjustment but before the iterative adjustment.

With X-ray and neutron volumes are at the correct scale and position, we can then combine them together to get better phase identification. The tool used here is Phase Segmentation from NIST. The screenshots of Phase Segmentation tool, shown in Figure 3.3, outlines how this tool works. By importing X-ray and neutron datasets together and combining them, the Phase Segmentation tool provides a clear visualization of different phases in the sand column, as shown in Figure 3.3 (a). A sample tomography slice, after selecting specific regions, defining regions as different phases and assigning them a color, is shown in Figure 3.3 (b).



Figure 3.3 Screenshots of Phase Segmentation tool. Picture (a) represents a sample slice of combined reconstructed neutron and X-ray slice without phase identification; (b) shows a sample slice of combined reconstructed neutron and X-ray slices after phase segmentation.

The program plots histograms of two datasets in a x-y coordination, which allows more precise phase segmentation by selecting specific regions in x-y coordination and define them as the same phase. In this experiment, solid and liquid phase can be easily differentiated through X-ray and neutron images, respectively. After identification of solid and liquid phases, all remaining pixels were defined as the gaseous phase. A sample slice after phase identification is shown in Figure 3-3 (b). When all phases are defined, images with colorized phases can be exported for future 3D analysis. The 3D rendering software used in this research is Drishti (Limaye, 2012).

# **3.2.6** Surface Tension Measurement

Surface tension measurements were made to exam how UD1022 treatment could influence the properties of the liquid phase in UD1022-treated. UD1022 was freshly grown in LB solution for 24 h at 37°C. Then 50 ml of bacteria suspension was harvested by centrifuging at 5000 rpm (revolution per minute, equivalent to 3214 rcf, relative centrifugal force) for 10 min. The collected bacteria pellets were washed using  $1 \times PBS$  solution twice by vortex and centrifugation, then the pellets were resuspended in 50 ml sterilized DI water. The concentration of bacteria suspension (in DI water) is  $\sim 1.5 \times 10^9$  CFU/ml for 3 replicates. The surface tension of *B. subtilis* FB17 suspension was measured using KSV Sigma 700 (KSV. Ltd).

### 3.2.7 Contact Angle Measurement

To measure the contact angle of water on control and *B. subtilis* FB17 treated sand surface, 25g sand for each sample (control and treated) was prepared as described previously in "Sample preparation". Instead of being stored in an oven overnight, sand samples for contact angle measurement were incubated for 5 days, to allow bacteria

growth and dry out of sand. The sand layer was prepared following procedures described in Bachmann et al. (2000). Briefly, dry sand from different treatments was sprinkled on a  $2 \times 3$  cm double-sided adhesive tape that can be fixed to a microscope glass slide. Then sand particles were placed under pressure equivalent to 100g weight for 3-5 seconds. Extra, loose sand was removed by gentle shake. Then the glass slide was placed on VCA optima (AST Products, Inc.) for contact angle measurement. A 0.05-ml droplet was dropped on the sand surface using a syringe, and images were captured at three different times: at initial contact of droplet and sand surface, 7s, 15s after initial contact. The contact angles were measured using DropSnake (Stalder et el., 2010; 2006), a plugin for ImageJ.

# 3.3 **Results and Discussions**

#### **3.3.1 3D** Structure and Phase Distribution in the Sand Columns

Figures 3.4 (a) and (c) show rendered 3D structure and phase distribution of control and treated samples. The inner structure can be viewed by observing intersections inside the columns, as shown in Figure 3.4 (b) and (d). Both 3D images of samples and their 2D intersections show higher water content in the treated sample than in the control, especially at the bottom part of the columns. This indicates that the upward flow rate of water in UD1022-treated sample could not supply enough water to sustain evaporation at the soil surface. The lower evaporation rate found in UD1022 treated sample than in control sample is consistent with the previous finding reported by Zheng et al. (2018).

# Treated sample



3D Column

**Central Intersections** 

Figure 3.4 3D images and 2D intersections of pure sand samples. In this figure, brown indicates sand particles, blue indicates water and yellow represents air.

#### 3.3.2 Quantitative Analysis of Water Content in Sample Columns

The water content of sand columns after evaporation was determined by weight measurement to be 25.3% for treated sample and 25.7% for control sample, respectively. The water content was also estimated from neutron tomography images using ImageJ, by calculating the average percentage of liquid area of the whole dataset. In phase identification of neutron images, the threshold value for water was arbitrarily set at 2.15, which means all pixels with gray scale value greater than 2.15 is considered as water. Using this threshold value, the water content of treated column estimated from neutron tomography was 22.5%, a reasonable value compared with the water content calculated from weight measurement (25.3%). With the same threshold value, however, the estimated water content of control sample was 16.9%, significantly lower than the water content from weight measurement (25.7%). The differences in water content of control sample between weight measurements and neutron tomography estimated results come from inaccurate liquid phase identification in neutron tomography images. It should be noted that different threshold value would result in different estimated water content of both samples from neutron tomography images, but the treated sample always have higher estimated water content than control sample.

In the reconstructed neutron images, the gray scale value of each pixel is determined by average neutron attenuation. Neutron attenuation is positively correlated with water content thus neutron attenuation is stronger at higher water content, which results in brighter color in the reconstructed neutron tomography images. In previous neutron image processing, phase identification was based on a gray scale value cutoff value of 2.15, i.e., pixels with grey scale values > 2.15 were considered to contain only water whereas those with < 2.15 grey scale values

contained only solid or gaseous phase. This operation would cause overestimation of water content in pixels with grey scale values >2.15 and underestimation in pixels with < 2.15 grey scale values. The errors stated above result in inaccurate estimated water content from neutron tomography images. The histogram of pixel value distribution in control and treated sample, shown in Figure 3.5, indicates that control sample has higher portion of pixels with low gray scale value (< 2.15), in which the water content is underestimated in these pixels. Moreover, higher portion of pixels with high gray scale value (> 2.15) in treated sample, which has overestimated water content, make the difference of water content between the two samples more pronounced.



Figure 3.5 The histogram of control (orange) and treated (blue) neutron tomography images. Pixels with value > 5 are shown together, due to their small individual percentage.

The higher portion of pixels with darker color in neutron tomography images of control sample indicates more water is "mixed" with other phases, which means more water in the control sample existed at solid-liquid interfaces and would most likely to be as thin water films. In this case, it is reasonable to conclude that water distribution is more uniform in the control sample and more heterogeneous in the treated sample. As reported in Tuller and Or (2001), the typical values for water film thickness close to saturation with  $\mu = -0.01$  J/kg (-10 Pa) and for very dry conditions, i.e.,  $\mu = -100$  kJ/kg (-10,000kPa) are  $7 \times 10^{-8}$  m and  $3 \times 10^{-10}$  m, respectively. Thus the water films cannot be resolved at the image resolution of 18µm, and would have very limited influence on pixel value in the reconstructed neutron slice. This explains why in neutron images of the control sample, portions of pixels with value 0 are significantly higher than that of treated sample.

# 3.3.3 Possible Mechanisms

The heterogeneous water distribution in the UD1022-treated sample was a result from bacteria activities. There are three possible mechanisms, which are discussed below.

# EPS reduces liquid phase surface tension in treated sample and affects water retention in pore space

Previous studies have shown that *B. subtilis* can produce extracellular polymeric substance (EPS), which can reduce surface tension (Read et al., 2003). The measured average surface tension value of UD1022 suspensions is 42.1 ( $\pm$ 0.1) mN/m, decreased by 42% compared with DI water's surface tension value of 72.75 ( $\pm$ 0.02) mN/m. The variation in surface tension measurements among the replicates could come from minor differences in UD1022 concentration, as well as temperature. Tuller and Or (2001) developed a triangular pore space model to describe water retention, film and corner flow in porous media. Their model and previous surface tension measurements can help explain the mechanisms of heterogeneous water distribution in the UD1022-treated sample. Figure 3.6 shows the conceptual schematic of water flow in triangular pore space during drainage or evaporation.



Figure 3.6 Conceptual schematic of water distribution in triangular pore space during soil water evaporation.

In the model, the soil particles and pores are idealized as sphere particles and triangles, respectively. When soil is fully saturated, all pores are filled with water. During drainage/evaporation, water will drain from the center of the pore first, then drained area gradually move to corner as evaporation continues. The radius of air space in the pore during evaporation can be expressed by Equation 3.1:

$$r(\mu) = -\frac{\sigma}{\rho\mu} \tag{3.1}$$

Where *r* (m) is radius of the air space,  $\sigma$  (N/m) is surface tension,  $\mu$  (Pa) is matric potential and  $\rho$  (kg/m<sup>3</sup>) is density of water. Lower surface tension (I.e., a smaller  $\sigma$  value) leads to a smaller *r*, which means more water will remain in the corners at the same value of  $\mu$ . The reduced surface tension upon UD1022 treatment thus could lead

to more water retention in the pore corners at a given matric potential value as compared to the control sample what pore space was filled with water with much higher surface tension. Zheng et al. (2018) measured the SWRC of UD1022 treated sample and its control and found that, at the same low water content, UD1022 treated sample had a higher matric potential than that of control sample. Increasing matric potential (less negative) would decrease r, thus more water could be held in corners of pore spaces. This means that, at the same water content, water distribution is more heterogeneous in B. subtilis FB17 treated sample compared to the control, as higher portions of water are concentrated in the pore corners while in control sample more water is uniformly distributed as thin films.

#### Bacteria have preferred habitats in soil pores

Bacteria have preferred habitats in soil pores. In a review paper, Or et al. (2006) pointed out that corners of pores, as shown in Figure 3.7 (a), are preferred bacteria habitats. Figures 3.7 (b), (c) and (d) refer to the water retained at particle contacts, by capillary forces in crevices and adsorbed water film, respectively. Regions shown in Figures 3.7 (b) and 3.7 (c) could be potential habitats for bacteria during the initial stage of evaporation but would shrink during evaporation and become too small to support full immersion or movement of bacteria cells. Or et al. (2006) indicated that for mildly unsaturated conditions (> -30 J/kg), water retained at particle contacts becomes smaller than a typical bacteria cell size. As for the water film in Figure 3.7 (d), the film thickness is always thinner than the bacteria size and is not optimal for microbial life.



Figure 3.7 A schematic illustrating preferred habitats of microbes in soil pore spaces concentrated in corners and crevices where water is comparatively abundant.

As corners of soil pore spaces are preferred habitats for microbes, more microbial products, including EPS, will accumulate in these areas. As discussed in the context of the triangular pore model, it was pointed out that EPS could reduce surface tension of pore water and lead to more water retention in corners of pore spaces. These corners are also preferred habitats of B. subtilis FB17 and would accumulate more EPS due to microbial activities, which in turn could further decrease surface tension and thus accentuate the surface tension effect. The two factors/processes both contribute to the heterogeneity of water distribution; this conclusion is supported by previous neutron tomography results.

Previous studies indicate that soil pore structure can be modified by EPS (Zheng et al., 2018; Volk et al., 2016). EPS can clog small pores or connecting large pores into smaller ones or form porous network. The newly formed pores can provide additional corners for water to be retained as water 'clusters', which further increase the heterogeneity of water distribution.

#### Plant drought stress tolerance enhanced by B. subtilis FB17

PGPR could enhance plant drought tolerance and various mechanisms have been proposed that focused mainly on microbe-root/plant interactions (Figueiredo et al., 2008; Mayak et al., 2004; Timmusk et al., 2014, 2015). Zheng et al. (2018) investigated changes in soil physical and hydraulic properties after UD1022 inoculation and found that UD1022 can produce EPS, which helps soil to retain more water. Also, UD1022 treated samples have lower unsaturated hydraulic conductivity and reduced soil water evaporation rate. They proposed three potential mechanisms by which EPS modulate soil physiochemical properties, including hygroscopic EPS could retain large quantities of water, modify soil pore-size distribution and soil water properties (i.e., decrease surface tension and increase viscosity). These changes can potentially provide more water to plants by reducing soil water loss from evaporation, as well as by retaining soil water for longer periods of time to allow metabolic adjustment of plants to better adapt to drier conditions.

Based on the observed heterogeneous water distribution upon *B. subtilis* FB17 treatment from this research, I propose two potential mechanisms that could be responsible for enhancing plant drought tolerance.

### Break liquid phase continuity and reduce soil water evaporation rate.

Soil water evaporation typically happens in two stages, Stage I and II. During Stage I, water evaporation rate is controlled by ambient conditions whereas soil water evaporation rate is controlled by intrinsic soil properties during Stage II. More detailed discussions about soil water evaporation process, theoretical models and factors that influence evaporation rate are included in a review paper of Or et al (2013). Briefly, toward the end of stage I evaporation, only a few liquid menisci remain pinned to the evaporating surface. The disruption of capillary flow results in interfacial instability and subsequent detachment from the surface. This breakage of liquid connections will result in rapid drop in soil water evaporation rate, marking the end of Stage I evaporation.

The presence of bacteria and EPS can increase the hydrophobicity of sand particles. The measured contact angle values of water on treated and control sand samples at initial contact time and 7 s after initial contact were 29.8° ( $\pm 3.3^{\circ}$ ) and <10° for the control and 40.1° ( $\pm 4.7^{\circ}$ ) and 20.3° ( $\pm 2.8^{\circ}$ ) for the treated sample, respectively. The greater contact value indicates that the hydrophobicity of sand increases with the presence of *B. subtilis* FB17.

With similar  $\theta_{v}$  in control and treated sample based on weight measurement, higher portion of water in UD1022 treated sample was concentrated in the corners of soil pore spaces as water "clusters", which in turn means less water exists as thin water films. In treated sample, with greater contact angle of liquid/air interface, along with heterogeneous water distribution described above, the liquid phase continuity would to be disrupted easier than that of control sample, indicating treated sample would enter stage II evaporation earlier than control sample, and thus decrease soil water evaporation rate. In previous work, Zheng et al (2018) measured evaporation rates of UD1022 treated sample and its control, their result showed that the evaporation rate of soil samples treated with UD1022 decrease earlier than the control, which is consistent with previous discussion.

# Heterogeneous water distribution increase water availability to plants.

Plants absorb water through their entire root surfaces, but the majority of water is absorbed by root hairs. The water is absorbed into root hairs through permeable cell wall, driven by osmotic pressure. The osmotic pressure required to pump water into root cells is related to the energy state of water. Based on the energy required to remove water from soil and soil water distribution, the overall soil water retention curve can be separated into several sections, including "capillary" regime, "adsorbed film" regime and "tightly adsorbed" regime, as depicted in Figure 3.8.



Figure 3.8 Schematic of different regimes in a typical soil water retention curve.

The soil suction of "capillary" regime is always in the lower range compared with "adsorbed film" or "tightly adsorbed" regime, which means less energy is required to remove soil water retained by capillary force than from adsorbed or tightly adsorbed water films. In the aspect of water uptake by plants, soil water retained by capillary force requires lower osmotic pressure to be "pumped" into root cells and are more available for plants. Water in UD1022 treated soil sample is concentrated in
corners of soil pore spaces and retained by capillary force, while in its control, more water is adsorbed as water film. This difference in water distribution will result in higher water availability for plants' roots in UD1022 treated soil than non-treated soil in all scenario, which can enhance plant drought stress tolerance.

# 3.4 Conclusions

In this study, I used B. subtilis FB17 (UD1022) as a model PGPR and investigated its effect on soil water distribution using a combined neutron and X-ray tomography (NeXT) system. Although the volumetric water content of UD1022 treated and non-treated sample were similar after evaporation, the estimated volumetric water content from tomography images shows that UD1022 treated sample contained more water than its control. This difference in water content can be explained by more water in treated sample are concentrated as water "clusters" that have higher gray scale value of pixels in reconstructed neutron slices, which is defined as water phase, than control. This also indicates that water distribution is more heterogenous in UD1022 treated sample. There are two potential mechanisms responsible for heterogeneous water distribution in UD1022 treated sample: 1) the presence of UD1022 can reduce the surface tension of liquid phase and result in more water concentrated in corners of soil pore spaces, which can be explained by the triangular pore space model; 2) corners of soil pore spaces are preferred microbial habitats and have greater amount of bacteria metabolites like EPS, which can help soil to retain more water in these areas.

The heterogeneous water distribution in UD1022 treated samples can enhance plants tolerance to drought stress better due to two possible mechanisms: 1) by decreasing soil water evaporation rate and 2) by increasing soil water availability to

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plants. The differences in pixel value distribution in reconstructed neutron slices of control and treated sample indicates more water in concentrated in corners of soil pore spaces and less in water films in treated sample. Along with increased hydrophobicity, the liquid phase continuity in UD1022 treated sample will decrease, which can further explain the mechanisms behind the reduced soil water evaporation rate. Also, more water in UD1022 treated sample are concentrated as water "clusters" in corners of soil pore spaces. The water "clusters" are retained in soil by capillary force instead of tightly adsorbed water films at soil particle surfaces, which require lower osmotic pressure for plants to uptake and thus are more available for plants.

By retaining more water in the soil and for a longer period of time and increasing water availability for plants, UD1022 can increase water availability to plant thus their tolerance to drought. The results from this study suggest that application of PGPR could provide a potentially viable and sustainable solution to reduce plant drought tolerance and contribute to solving food security issues and providing more foods for the growing population in the changing climate.

# Chapter 4

# Conclusions

Water is a key factor that limits agricultural production in many places. Increasing global population and food consumption are challenging agriculture for higher productivity. Therefore, developing novel solutions for plant growth under arid and semi-arid environment and increase water use efficiency of plants are of great significance.

The enhancement of plant drought tolerance by PGPR had been increasingly documented, but most of these works focused on plant/microbe interactions. Despite the ability of PGPR to modify plant activities corresponding to enhancement of plant drought tolerance, the model PGPR (UD1022) used in this research could decrease soil water evaporation by modifying soil physical and hydraulic properties, thus can increase plant drought tolerance and water use efficiency.

In Chapter 2, the neutron radiography experiment provided visualization of reduced soil water evaporation rate after inoculation of UD1022. The HYPROP experiment pointed out soil properties changed by the presence of UD1022, and SEM images illustrated the change of soil structure by UD1022. Based on these experiments and published works, it was concluded that EPS plays an important role in the enhancement of plant drought tolerance. Several possible mechanisms were discussed in this chapter including (1) EPS modifies soil pore structure; (2) EPS decreases surface tension of water and (3) EPS increase hydrophobicity of soil particles.

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In Chapter 3, to further investigated the possible mechanisms of changes in soil physical and hydraulic properties, NeXT system was used to analyze water distribution in pure sand samples. The surface tension of UD1022 suspension and contact angle of water and sand surface (UD1022 treated and control sand) was measured to verify pre-assumptions of possible mechanisms in Chapter 2. The neutron tomography results revealed that at similar water content of control and UD1022 treated sand sample, the water distribution of UD1022 treated sand sample is more heterogeneous, as higher portion of water concentrated in corners of pore spaces and less exist as thin water film. This heterogeneous water distribution could break liquid phase continuity and reduce soil water evaporation, as well as increase water availability to plants.

Future work may include experiments with DS646, the mutant of 3610 that cannot produce EPS, to figure out the mechanisms of reduced soil water evaporation other than the production of EPS. Quantitative analysis of water availability to plants in UD1022 treated sample may also be included, as competition of water between plants and PGPR may occur when water is limited. Root mucilage also have similar effects as EPS, so identify the contributions of root mucilage exerted by plant roots and EPS produced by PGPR on enhanced plant drought tolerance would also be very meaningful.

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