INTERACTIONS OF ARBUSCULAR MYCORRHIZAL FUNGI WITH RHIZOSPHERE COMMUNITIES IN SALINE SOILS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2018

Major Subject: Soil Science

ABSTRACT

Soil salinity is a major constraint for crop production worldwide. Among many soil fertility issues associated with saline soils, phosphorus unavailability is most commonly noticed. Improving plant interactions with arbuscular mycorrhizal fungi (AMF) and other soil microbes have been widely shown to contribute to salinity tolerance and increase phosphorus (P) availability. However, there are many knowledge gaps to effectively improve AMF interactions in saline soils. Lower abundance of AMF is one of the limitations for increasing root colonization in saline soils. Supplementation of salt tolerant AMF spores is an emerging practice, however, identifying competent AMF species or combination of species beneficial at high salt concentration is a challenge. It is also not clear how introduced AMF species would interact with other native soil communities to influence phosphorus availability. Additionally, it is not clear whether improving soil conditions using a soil amendment can further enhance AMF interaction and colonization. In order to address these knowledge gaps, three experiments were conducted in a naturally saline soil to investigate: (1) the effects of AMF inoculation on plant growth and root colonization at different salinity levels, (2) the role of indigenous soil microbes with AMF inoculation to increase P availability to plants and (3) the combined effects of soil amendment and AMF inoculation on root colonization and P availability to plants. Microcosms were setup in growth chamber-based experiments to address above objectives separately. Results from the first experiment showed significantly different level of root colonization (p <0.05) among the three species of AMF inocula, and that colonization was generally higher with increasing salt concentrations, with some significant differences

between the inoculum species. Plant growth responses were significantly different between the AMF species as well. These results suggest that artificial inoculation can increase root colonization in saline soils, although at different levels by AMF species at different salinity levels. In the second experiment, soil sterilization significantly (p <0.05) reduced soil extractable P, ALP activity, *phoD* and fungal ITS gene abundances in the hyphosphere soil in both AMF-inoculated and uninoculated treatments. However, AMF inoculation in unsterilized soils resulted in a significantly (p <0.05) higher plant P uptake compared to uninoculated in both sterilized and unsterilized soils. These results indicate that AMF-hyphosphere microbial community interactions play a synergistic role in increasing P availability in salt-affected soils. Findings of the third experiment indicated that combined application of BC and AMF significantly improved (p <0.05) plant shoot and root growth, plant P uptake, extractable P in bulk soil, and microbial community abundance in the rhizosphere compared to control. However, addition of BC significantly (p <0.05) reduced AMF colonization in both rhizosphere and bulk soils. These results suggest that combined application of BC and AMF can significantly increase plant production and P availability in salt-affected soils.

DEDICATION

To whom Allah has enjoined upon me goodness to them.

To whom their prayers in secret and public were the best support for me. My parents and my wife, I dedicate this dissertation to all of you and to my lovely daughter Rima. May Allah reward you, bless you and elongate your lives.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my academic co-advisors, Dr. Terry Gentry and Dr. Anil Somenahally, whom great support, patience, motivation and guidance were a light on my path. Without their help, this work would not be possible.

I would also like to thank my committee members, Dr. Paul DeLaune, Dr. Elizabeth Pierson and Dr. Girisha Ganjegunte for their insightful comments and support throughout the course of this research.

I would also like to thank my sponsors, Jazan University in the Kingdom of Saudi Arabia and the Saudi Arabian Cultural Mission in Washington DC for their support and providing me unconditional educational funding.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

Finally, thanks to my parents and family for their encouragement and to my wife for her patience and love.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was co-supervised by Professor Terry Gentry and Assistant Professor Anil Somenahally, and dissertation committee members consisting of Associate Professor Paul DeLaune and Associate Professor Girisha Ganjegunte of the Department of Soil and Crop Sciences and Professor Elizabeth Pierson of the Department of Horticultural Sciences. All work for the dissertation was completed independently by the student in the laboratories of Professor Terry Gentry and Assistant Professor Anil Somenahally, and a part of Chapter IV analysis in the laboratory of Professor Elizabeth Pierson.

Funding Sources

My graduate study was supported by a scholarship from Jazan University in the Kingdom of Saudi Arabia, and a dissertation research funding from Professor Terry Gentry and Assistant Professor Anil Somenahally in the Department of Soil and Crop Sciences at Texas A&M University.

NOMENCLATURE

NaCl	Sodium chloride
Na^+	Sodium ion
Cl^-	Chloride ion
KOH	Potassium hydroxide
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
$CaCl_2$	Calcium chloride
NH_4NO_3	Ammonium nitrate
P	Phosphorus
iP	Inorganic phosphorus
oP	Organic phosphorus
N	Nitrogen
K	Potassium
AMF	Arbuscular Mycorrhizal Fungi
INVAM	International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi
Fm	*Funneliformis mosseae*
PGPR	Plant Growth Promoting Rhizobacteria
BC	Biochar
phoD	Alkaline phosphatase gene
ALP	Alkaline phosphatase enzyme

DNA	Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
qPCR	Quantitative Polymerase Chain Reaction
mg	Milligram
Kg	Kilogram
mm	Millimeter
mL	Milliliter
°C	Centigrade
TX	Texas
OR	Oregon
NY	New York
NH	New Hampshire
IA	Iowa
VT	Vermont
DE	Delaware
MO	Missouri
USA	United States of America

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Salinity as a global agricultural issue

Soil salinization is a persistent abiotic stress limiting agricultural productivity in cultivated areas around the world, especially in arid and semi-arid regions. It has been estimated that global human population will reach 8 billion by the year 2025. Thus, to meet growing food demand, salt-affected soils around the globe need to be improved to support agricultural production (Ladeiro, 2012). In terms of global distribution, nearly 10 % of total earth's land surface is covered with salt-affected soils (Szabolcs, 1994). Approximately 20% of total cultivated and 33% of irrigated agricultural lands are affected by higher salt concentrations. Moreover, salinization is rapidly increasing due to climate change effects such as prolonged droughts (Blaylock et al., 1994), usage of saline water for irrigation (Blanco et al., 2002), high surface evaporation due to lower ground cover (Carter 1975), in some areas weathering of native rocks (Salama et al., 1999), and intrusion of oceanic salts transferred by rain and winds (Parihar et al., 2015). The natural causes are referred to as primary salinity, while other causes induced by human activity are referred to as secondary salinity. By the year 2050, estimates indicate that more than 50% of arable land in the world will be salinized (electrical conductivity of saturated soil extract > 4 dS/m) (Jamil et al., 2011; Shrivastava and Kumar, 2015).

1.2 Classification of salt affected soils

Accumulation of soluble salts in soils or near root zone is known to negatively impact the growth of most plant species and crops. Generally, salt-affected soils are

classified to three types: saline soil, saline-sodic, and sodic soil. Saline soil is the soil that gives an electrical conductivity (EC) in a saturation extract greater than 4 *deci-Siemens per meter* (dS/m, approximately 40 mM NaCl), and have an *exchangeable sodium percentage* (ESP) less than 15 [or sodium adsorption ratio (SAR) less than 13]. Saline-sodic soils have both, soluble salts greater than 4 dS/m and high proportion of sodium ions (ESP greater than 15 or SAR greater than 13). Sodic soils have EC less than 4 dS/m but high levels of sodium ions (ESP greater than 15 or SAR greater than 13). Sodic soil is considered the most troublesome of salt-affected soils due to its high pH (above 8.5) and dispersion of soil particles damaging soil structure because of high Na^+ saturation (Brady and Weil, 2000).

Among soluble salts in soils, sodium chloride (NaCl) is the most abundant and soluble salt released from weathering of earth's parent material. Thus, NaCl is considered the most abundant salt in salt-affected soils. However, weathering of parent material releases other type of salts, such as chlorides of calcium, magnesium, and to a lesser extent, carbonates and sulfates (Szabolcs, 1989).

1.3 Salinity effects on plant

Salt stress is one of the major environmental factors limiting crop growth and production. High salt levels can negatively affect plants in two main ways. The first way is through the presence of high salt concentrations in soil disturbing the ability of roots to uptake and extract water from soil. The second way is through the presence of high salt concentrations within plant tissues, which can be toxic and inhibit different physiological and biochemical processes such as uptake and assimilation of plant nutrients (Hasegawa et

al., 2000; Munns, 2002). These two ways are explained in a two-phase model proposed by Munns (1995) as osmotic and ionic effects of salt stress.

1.3.1 Osmotic stress

The osmotic stress (the first phase) starts when salt concentration surrounding root surfaces increase to a level where roots start facing difficulties extracting water (approximately 40 mM NaCl for most plants), resulting in a significant shoot growth reduction (Carillo et al., 2011). The reduction in shoot growth in response to salinity is known to be expressed in plants by reduction in leaf area. In addition, stomatal closure and stunted shoots are also some of the symptoms due to salt stress. The negative impact of salt stress on leaf growth seems to be also a result of salt inhibition of symplastic xylem loading of Ca^{+2} in the roots (Läuchli and Epstein, 1990; Läuchli and Grattan, 2007). In addition, salt stress also inhibits the uptake of important mineral nutrients such as K^+ and Ca^{+2}, which in return negatively affect root cell growth and root tip expansion. However, shoot growth generally is more sensitive to salt-induced osmotic stress, which might be a result of leaf area reduction relative to root growth (Carillo et al., 2011).

1.3.2 Ionic stress

The ionic stress (the second phase) occur when ions (particularly Na^+) accumulate in the leaf blade after being transferred through the transpiration stream. Accumulation of Na^+ is toxic to plants, especially in old leaves due to their slower growth and dilution of new salt accumulation compared to young leaves. When the rate of old leaves dying increases more than the rate at which new leaves are produced, further reduction in growth rate will occur due to a reduction of photosynthetic capacity of the plant in supplying carbohydrates to young leaves (Munns and Tester, 2008). Moreover, the reduction of

photosynthesis related to salt stress in plants can also increase the production of reactive oxygen species (ROS), which will impair its removal by plants through antioxidative mechanisms (Foyer and Noctor, 2003).

1.4 Mechanisms of plant tolerance to salt stress

The mechanisms of salt tolerance in plants are still not fully understood. Plant tolerance to salt stress can vary depending on the plant species and/or environmental factors. Moreover, depending on the growth stage, plants response differently to salinity since some species are more sensitive to salts at germination, while others have increased sensitivity during reproduction (Howat, 2000). Plants have evolved different mechanisms to tolerate salt stress. These mechanisms can be generally grouped into three types: a) osmotic stress tolerance, b) Na^+ exclusion from leaf blades and c) tissue tolerance (Munns and Tester, 2008).

1.4.1 Osmotic stress tolerance

The most limiting growth factor of salt-stressed plants is osmotic effects of salinity, which result in reduction of growth rate and stomatal conductance. Thus, the osmotic tolerance mechanisms involve ability of plants to maintain leaf expansion and growth of new leaves by sustaining photosynthetic capacity to meet new cells' energy requirement, and stomatal conductance (Rajendran et al., 2009). Interestingly, some studies have shown a positive relationship between higher stomatal conductance in salt-stressed plants and higher CO_2 assimilation rate (James et al., 2008).

1.4.2 Na^+ exclusion

In most plant species stressed with higher salt levels, Na^+ usually reaches its toxic concentration before Cl^-. Hence, most studies focus on Na^+ exclusion and controlling its

transport within plant. Sodium exclusion mechanisms involve ability of plant to reduce ionic stress by reducing or minimizing Na^+ ions accumulating in the cytosols of transpiring leaf cells due to low net Na^+ uptake by root cortex and controlling net loading of the xylem by parenchyma cells in the stele. This mechanism (as well as in tissue tolerance) involves up- and down-regulation of the expression of specific ion channels and transporters (Munns and Tester, 2008).

1.4.3 Tissue tolerance

Tissue tolerance mechanisms have more ability to increase survival rates of older leaves in salt-stressed plants. This mechanism involves the compartmentalization of Na^+ and Cl^- at the cellular and intracellular levels to escape these ions from reaching toxic levels within the cytoplasm, especially mesophyll cells of leaves, as well as the production and accumulation of compatible solutes in the cytoplasm. These compatible solutes can regulate osmotolerance in many ways, stabilize membrane or macromolecules, protect enzymes from denaturation, or mediate osmotic adjustment (Ashraf and Foolad, 2007). Compatible solutes are small water-soluble molecules, comprised of nitrogen containing compounds such as amino acids (such as proline, glycine betaine, etc.) amines and betaines, as well as some organic acids, sugars and polyols (Mansour, 2000).

1.5 Alleviating salinity stress

Restriction of salinization can be obtained through different practices such as changing farm management, applying chemical amendments (such as Ca^{+2}-containing chemicals to replace Na^+ from exchange sites), leaching of salt from root zone, and the use of salt-tolerant plants. In irrigated agricultural fields, practices such as partial root zone drying method or micro-jet irrigation to optimize plant usage of water can be adopted

(Shrivastava and Kumar, 2015). Moreover, changing farming systems to incorporate perennial plants in rotation with annual crops (phase farming), in site-specific plantings (precision farming), or in mixed plantings (alley farming, intercropping) can also be adopted. (Munns et al., 2002). Even though using these approaches can alleviate yield reduction under salt stress, implementation of such approaches is often limited due to their cost and availability of good water quality or water resources. Developing low cost, highly efficient, and readily adaptable practices or methods for abiotic stress management is a major challenge in agricultural fields. Extensive research projects worldwide are being carried out to develop practices or strategies to alleviate salt stress effects in different ways, such as development of salt- and drought-tolerant plant varieties, resource management practices, shifting the crop calendars, etc. (Venkateswarlu and Shanker, 2009).

1.6 Application of organic amendments

Another way to alleviate salinity stress in soil is the application of organic amendments. Organic amendments can have diverse benefits to plant growth and soil physicochemical properties (Fan et al., 2016). Salt-affected soils generally have low organic matter content, which causes poor soil structural stability. Application of organic materials (such as compost, manures, etc.) has been shown to improve soil structure (Tejada et al., 2006; Oo et al., 2015). Organic matter amendments have been also found to accelerate Na^+ leaching, reduce EC, ESP, and increase water stable aggregates when applied to saline soils (Barzegar et al., 1997). In addition, organic amendments can also reduce the negative effects of salt on the soil microbial community and its mediated processes such as mineralization (Wichern et al., 2006). However, to have beneficial

impacts, organic amendments need to be applied at relatively high rates and multiple times, especially in arid and semi-arid regions where temperature causes high decomposition rates (Alessandro and Nyman et al., 2017). Consequently, high application rates can be costly and not eco-friendly (e.g., increases CO_2 emission) (Al-Wabel et al., 2017).

1.6.1 Biochar

Biochar has recently emerged in agriculture as a new soil amendment. Biochar is a solid carbon-rich residue (fine-grained charcoal), largely resistant to decomposition, and produced from pyrolysis (oxygen-free or oxygen-limited conditions at high temperature ranging from 300 – 1000 °C) of plant and waste feedstocks (Lehman, 2007; Hunt et al., 2010). The growing interest in biochar as a soil amendment is due to its diverse benefits to plants, soil, and the environment. Biochar benefits to salt-affected soils, and normal soils, include improving soil structure stabilization, increasing soil nutrients (especially cations such as Ca, K, Mg, etc.) and organic carbon content, increasing CEC, improving water retention and air porosity, and increasing Na^+ exchange by increasing Ca^{2+} in soil solution (Atkinson et al., 2010; Yue et al., 2016; Zheng et al., 2018). In addition, the high surface area of biochar can serve as habitat or shelter for many soil microorganisms that can alleviate salinity stress in salt-affected soils (Zheng et al., 2018; Dahlawi et al., 2018).

1.7 Microbial roles in alleviating salinity stress

Many recent studies have illustrated that adaptation of plants to their local environment is mostly driven by genetic differentiation in microorganisms closely associated with the plants (Rodriguez and Redman, 2008).

Microbial roles in promoting plant growth, controlling diseases, and managing nutrients is well known. These beneficial microbes colonize the rhizosphere, endosphere,

and phyllosphere of plants and promote plant growth through multiple direct and/indirect mechanisms (Saxena et al. 2005). A very well-known example is symbiotic nitrogen fixing microbes that convert atmospheric dinitrogen gas (N_2) into ammonia (NH_3), such as *Rhizobium* & *Frankia* spp. However, the roles of other plant-associative microbes in alleviating biotic and abiotic stresses are gaining much attention in recent years. Most attention is directed towards plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) as alternative strategies to improve crop tolerance to stresses such as salinity, as many of these microbes have been shown to improve a wide range of crops in salinized soils (Yang et al., 2009; Grover et al., 2011).

1.7.1 Role of PGPR in alleviating salinity stress

The term of PGPR was first introduced in 1978 (Kloepper and Schroth, 1978) describing rhizospheric bacterial populations that may colonize plant root and promote its growth. These bacteria can cause several physical and chemical changes in rhizosphere through different mechanisms that result in increasing plant tolerance to stresses and promote growth. These mechanisms can be divided into direct and indirect mechanisms. Direct mechanisms include nitrogen fixation, phosphate solubilization, potassium solubilization, siderophore production, and phytohormone production (such as indole-acetic acid (IAA), ethylene, cytokinins, gibberellins, etc.). On the other hand, indirect mechanisms include antibiotic production, hydrolytic enzymes production, siderophore production, induced systemic resistance (ISR), and exopolysaccharide (EPS) production (Gupta et al., 2015). The use of PGPR to alleviate salinity stress has been reviewed recently (Shrivastava and Kumar, 2015), and has been shown to improve plant growth

under saline conditions for different crops including pepper, lettuce, canola, tomato, and bean (Mayak et al., 2004; Yildirim and Taylor, 2005; Barassi et al., 2006).

1.7.1.1 Mechanisms of PGPR promotion to plant growth

Beside the well-known mechanisms of fixing atmospheric N to plant-available forms by N-fixing PGPR, other PGPR can solubilize phosphate to more available forms of phosphorus (P) through different strategies. These strategies include release of different complexed compounds (such as organic acids and protons), production of extracellular enzymes, and release of phosphate from substrate degradation (Sharma et al., 2013). Examples of such PGPR include: *Enterobacter, Pseudomonas, Rhizobium, Bacillus, Arthrobacter, Burkholderia, Beijerinckia, Flavobacterium, Microbacterium, Serratia, Rhodococcus*, and *Erwinia* spp. Such PGPR genera have grabbed attention in agricultural fields to be used as inocula or bio-fertilizers (Bhattacharyya and Jha, 2012).

Another example of PGPR mechanisms is the production of low molecular weight iron-chelating compounds (siderophores), which bind to ferric iron (Fe^{3+}) and sequester it in rhizosphere making Fe available to plants, but not to other organisms (Arora et al., 2013). PGPR are also known to produce many phytohormones. For instance, 80 % of rhizobacteria can produce IAA hormone (natural plant auxin), which is known for its important roles in plant and root growth and development (Miransari and Smith, 2014). Moreover, many PGPR produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can regulate ethylene levels in plants (under stress conditions, ethylene regulate plant homoeostasis, which reduces root and shoot growth) and reduce its deleterious effect, alleviate plant stress and promote its growth (Glick et al., 2007).

In term of indirect mechanisms, PGPR can benefit plants through the production of antibiotics and the competition with other microbes for nutrients. Antibiotic-producing PGPR are among the most studied biocontrol agents against phytopathogens in the last two decades (Shilev, 2013). Multiple antibiotics produced by PGPR have been identified such as amphisin, 2,4-diacetylphloroglucinol (DAPG), phenazine, oomycin A, pyrrolnitrin, pyoluteorin, tensin, tropolone, cyclic lipopeptides, etc. (Loper and Gross, 2007; Gupta et al., 2015).

1.7.2 Role of AMF symbiosis and its ecological importance

Association between plants and mycorrhizae is widely distributed in nature and possess a great ecological importance. Functions and structures of these mycorrhizas vary, but the most common plant-mycorrhizal association is that between arbuscular mycorrhizal fungi (AMF), belonging to the division of Glomeromycota, and the root of most terrestrial plants. It has been estimated that approximately 80 % of all terrestrial plants form association with AMF, including many agriculturally important crops (Smith and Read, 2010). Evidence based on the original descriptions of fossil records show that AMF are ancient fungi that have coevolved with plants for the last 400 million years (Pirozynski and Malloch, 1975; Krings et al., 2007).

Arbuscular mycorrhizal fungi are obligate biotrophic, asexual, multinucleate and unculturable eukaryotic microbes. Thus, species identification and recognition of this group of fungi remain open to discussion (Rosendahl, 2008). Nevertheless, advances in molecular technologies and analyses opened an access to their identification and enhanced knowledge to these fungi (Ligrone et al., 2007). In a recent review, Berruti et al. (2015) reported that introducing AMF inocula should be more adapted in sustainable agriculture

since natural level/richness of AMF can be disturbed by either anthropic inputs (such P fertilizers) and stresses such as salinity. In his review, he showed that studies that introduced AMF to fields had almost similar benefits on plants found with greenhouse experiments such as higher P uptake, higher colonization, and higher overall plant biomass. In general, colonization of root systems by AMF provide direct benefits to the growth and development of hosts plant by the acquisition of phosphate and other important nutrients and water from the soil through extensive hyphal network (extraradical hyphae) that extend and explore beyond plant root systems. In return, in this symbiosis relationship the fungus obtains carbohydrates required for the completion of its life cycle from plants. Moreover, these fungi can also enhance the plant's resistance to biotic and abiotic stresses (Reichenbach and Schönbeck, 1995). In general, the major effects of AMF symbiosis include: improving uptake of low mobile ions (mainly phosphate) and water, improving soil quality and structure (through glomalin produced by external hyphae), improving root and plant establishment, enhancing plant community diversity, improving soil nutrient cycling, and increasing plant tolerance to biotic and abiotic stresses (Smith and Read, 2010).

The process of AMF colonization of plant roots goes through different stages that involve multiple complex morphogenetic fungal changes. These stages include: germination of spore, differentiation of hyphae, appressorium formation, root penetration, intercellular growth, arbuscule formation, and transportation of nutrients (Harrier, 2001).

Although AMF are symbiotic microorganisms, they also carry structures within their hyphae called bacterium-like organisms (BLO), which have been detected in spores and symbiotic mycelia. The majority of these BLO belong to group II Pseudomonads

(*Burkholderia* spp.) based on sequence analysis of rRNA genes (Bianciotto et al., 1996). In addition, AMF also have associative bacterial community in their hyphosphere (zone surrounding individual fungal hyphae) aside from mycorrhizosphere communities (zone influenced by both, the root and the mycorrhizal fungus). However, the significance and the mechanisms by which these microbial communities interact with AMF are still poorly understood (Bianciotto et al., 2000; Johansson et al., 2004). Moreover, some of these associated bacterial species in mycorrhizosphere have been shown to promote mycorrhizal development and are called mycorrhizal helper bacteria (MHB) (Duponnois and Garbaye 1991).

1.7.2.1 Mechanisms of AMF in alleviating salinity stress

Many studies investigating the role of AMF (mainly Glomerales) in alleviating or protection plants against salt stress have shown that the symbiosis often results in increased nutrient uptake, increased photosynthetic rate and water use efficiency, and an accumulation of osmoregulator compounds, which suggest that alleviation of salt stress by AMF is a result of a combination of nutritional, biochemical, physiological, and molecular effects. However, depending on the AMF species involved, the positive effect on plant growth and development might vary (Marulanda et al. 2003, 2007; Wu et al. 2007; Evelin et al., 2009).

1.7.2.1.1 Nutritional effects and plant growth

AMF can enhance plant uptake of mineral nutrients (particularly low mobile ions such as phosphorus) and increase plant growth when grown under salt stress conditions (Al-Karaki and Clark, 1998). This effect is mainly regulated by improving the supply and uptake of nutrients and its transport to the root system through AMF. In addition,

increasing salt concentration has been shown to increase mycorrhizal dependency (Giri and Mukerji, 2004).

1.7.2.1.1.1 Phosphorus

Phosphorus (P) is one of the essential macronutrients required for plant growth. Phosphorus is a component of major molecules such as nucleic acids, ATP, and phospholipids; hence, a reliable supply of P is significant during plant growth. Yet, P is considered the second most limiting macronutrient after N for plant growth. Although its total amount in soil is high, P is mostly present in soil as unavailable forms. The available inorganic forms of P (iP) to plants ($H_2PO_4^-$ & HPO_4^{2-}) are scarce in soil solution pool due to its high reactivity and complexation with multiple soil compounds based on pH (**Fig.1**) (Schachtman et al., 1998).

Saline soils can significantly reduce P absorption (and other mineral nutrients) due to the precipitation of P ions with Ca^{2+}, Mg^{+2}, Zn^{+2} ions under elevated salt content, making P unavailable to plants (de Aguilar et al., 1979). AMF can greatly enhance P uptake through their extensive hyphae, which explore more soil volume than the root system (Ruiz-Lozano and Azcón, 2000). Based on estimations, AMF hyphae can deliver up to 80 % of a plant's P needs (Matamoros et al., 1999). Besides improving plant growth rate, improved P nutrition in mycorrhizal plants may also increase antioxidant production and reduce the negative effects of Na^+ and Cl^- ions through the maintenance of vacuolar membrane integrity, which will facilitate compartmentalization within vacuoles and selective ion intake. This will prevent ions from interfering in metabolic pathways of growth (Rinaldelli and Mancuso, 1996; Alguacil et al., 2003).

1.7.2.1.1.2 Na^+ and K^+ ions

Plants tend to take up more Na^+ under high salt content in soil, which decreases K^+ uptake since $Na^+$and K^+ uptake occurs simultaneously through the Na^+/K^+ symporter in plants (Munns and Tester, 2008). Potassium is an important nutrient for plant metabolism and is involved in multiple functions such as protein synthesis and stomatal movement. These functions cannot be replaced by Na^+ ions (Evelin et al., 2009). AMF in mycorrhizal plants can reverse the effects of salinity on Na^+ and K^+ nutrition by improving K^+ absorption and preventing Na^+ translocation to shoot tissues. Consequently, this will increase K^+: Na^+ ratio in root and shoot of plant hosts (Giri et al., 2007). Higher K^+: Na^+ ratio has multiple beneficial effects in plants in salt-stressed soil such as preventing the disruption of different K-mediated enzymatic processes and inhibition of protein synthesis. Furthermore, a higher K^+: Na^+ ratio is also beneficial by affecting ionic balance of the cytoplasm or Na^+ efflux from plants (Founoune et al., 2002; Colla et al., 2008).

1.7.2.1.2 Biochemical effects

When soil is getting dryer and the water potential starts to be more negative, plants must decrease their water potential to maintain a favorable gradient for water to flow from soil into roots. To achieve this, plants can use multiple mechanisms. The most important of these mechanisms is osmotic adjustment or osmoregulation through active accumulations of inorganic ions and compatible organic solutes such as proline, glycine betaine, and soluble sugars (Rabie and Almadini, 2005). Reports have shown increasing accumulations of compatible solutes such as proline in mycorrhizal plants when compared to non-mycorrhizal plants under salinity conditions (Jindal et al., 1993; Sharifi et al., 2007).

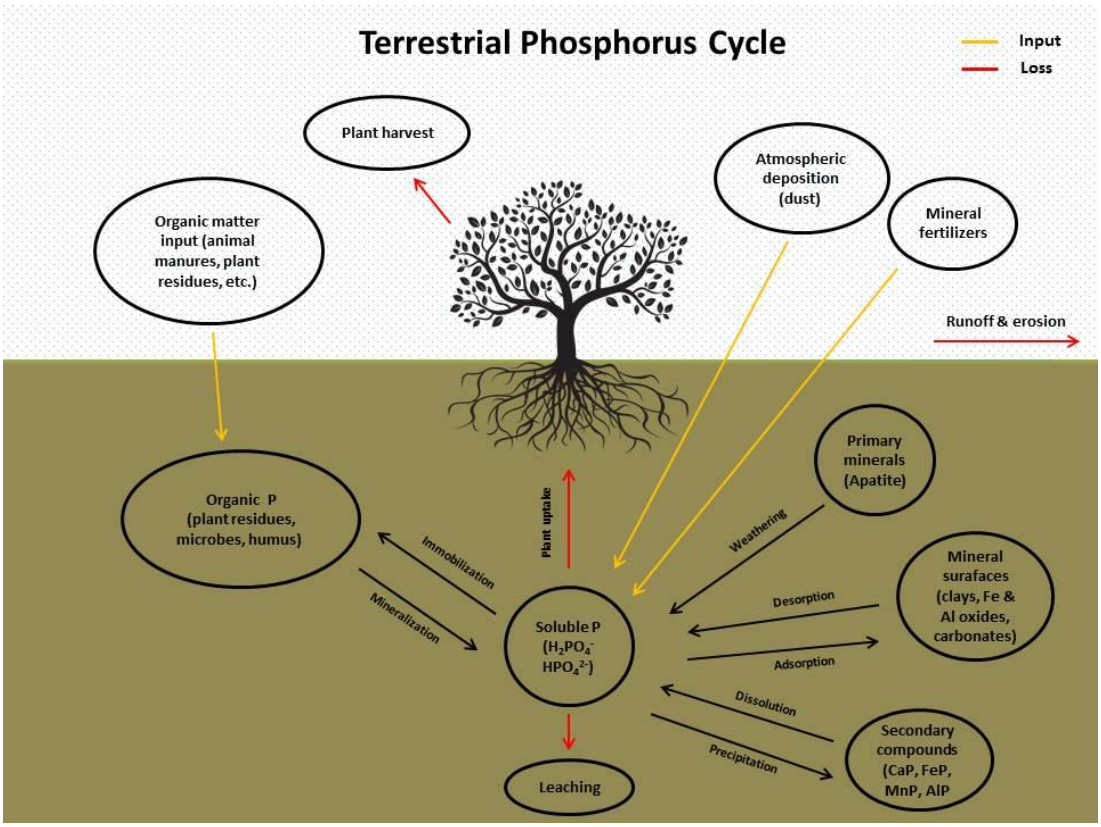

Figure 1.1. Terrestrial phosphorus cycle. Adapted from the Mississippi State University Extension (Mississippi State University Extension Service, 2017).

Mycorrhization also have been reported to change and increase abscisic acid (ABA) levels in plants grown in salt-stressed soils. ABS is a phytohormone that is known to regulate plant growth and development and to play an important role for plant responses to abiotic stresses such as salinity (Duan et al., 1996; Ludwig-Muller, 2000; Estrada-Luna and Davies, 2003). Moreover, studies have also suggested that AMF can help alleviating plant salt stress by enhancing the activities of antioxidant enzymes (Harisnaut et al., 2003; He et al., 2007). Antioxidant molecules and enzymes are involved in plants protective mechanisms to escape oxidative damage caused by ROS (such as damaging cell structure

and functions). Several plant species have been shown to have a correlation between tolerance to NaCl and antioxidant capacity (Zhang et al., 2001; Nunez et al., 2003).

1.7.2.1.3 Physiological and molecular effects

Elevated salt concentrations are known to affect several physiological processes in plants such as photosynthetic efficiency, gas exchange, membrane disruption, and water status. Some studies have demonstrated that AMF increases root hydraulic conductivities and prevented leaf dehydration caused by salinity (Aroca et al., 2007; Sheng et al., 2008). Inoculation with AMF has also been shown in some cases to change transpiration rate, and at the same time correlated with a change in ABA/cytokinins ratio (Goicoechea et al., 1997; Bolandnazar et al., 2007).

Studies focusing on the effects of AMF on plant molecular responses to salt stress are limited due to its complexity. By far, the few studies conducted in this area have focused on the expression of few proteins such as Δ1-pyrroline-5-carboxylate synthetase (*LsP5CS*) involved in proline biosynthesis, Na^+/H^+ antiporters, ABA (*Lsnced*), and late embryogenesis abundant protein (*LsLea*) (Jahromi et al., 2008; Evelin et al., 2009). In addition, a few studies have focused on the expression of aquaporin protein (an integral membrane *protein* facilitate water and some small solutes between cells across membranes) genes that are known to be induced under abiotic stresses such as drought and salinity (Ouziad et al., 2006; Aroca et al., 2007). However, some contradictions exist in these limited studies, indicating the need for further investigations to better understand the mechanisms by which AMF alleviates and affect plant responses to salt stress.

CHAPTER II

EFFECTS OF DIFFERENT ARBUSCULAR MYCORRHIZAL FUNGAL INOCULA ON COLONIZATION AND GROWTH OF BARLEY IN A NATURALLY SALINE CLAY SOIL

2.1 Synopsis

Soil salinity is a major constrain in agricultural production. Arbuscular mycorrhizal fungi (AMF) have been widely suggested as salt-stress ameliorators to plants. However, little is known about AMF colonization efficiency and plant responses to AMF in unsterile clay saline soils. In this study, root colonization and plant growth responses to 5 AMF inocula were examined in microcosms: non-inoculated as control, *Rhizophagus intraradices*, *Claroideoglomus etunicatum*, *Funneliformis mosseae,* and a mixed inoculum of all three AMF under three different salinity stresses: moderate salt level + gypsum, moderate salt level at 6.3 dS/m, and high salt level at 26 dS/m. Significant variations in percentage of colonization were found among different AMF inocula, and colonization was negatively correlated to salt level in the control and *C. etunicatum* inocula. However, depending on salt level and AMF species, some plants had either a significant increase or decrease in, root, total plant dry weights and shoot to total plant dry weight ratios. The results in this study suggest that depending on AMF species and the salt level, colonization and parasitic or beneficial effects of AMF on plant growth might vary when introduced to unsterilized naturally saline soils.

2.2 Introduction

Soil salinity is one of the most severe agricultural problems encountering the world. Most salinized areas are in arid and semi-arid regions, where organic matter is low, and alkalinity, calcareous and in many cases saline and/or sodic soils are present (Brady and Weil, 2010). Alleviating salinity stress and improving plant resistance by AMF have been reported with different plant hosts (Ruiz-Lozano and Azcón, 2000; Al-Karaki et al., 2001; Wu et al., 2007). Beside improving other nutritional and water uptake, these beneficial effects of AMF to plants are mostly attributed to increased P acquisition (Al-Karaki and Clark, 1998).

However, it seems that AMF colonization strategies vary considerably based on taxonomic variations, indicating possible different functions of AMF belonging to different taxa (Hart and Reader, 2002). Moreover, very little is known about the impacts of different environmental factors on AMF communities in nature (Xu et al., 2017). Most studies on the beneficial effects of AMF on plants under abiotic stresses such as salinity are conducted under sterilized soil conditions, and mostly on sandy soils (Chen et al., 1991). Soils under natural conditions and characteristics may behave differently when a microbial inoculum is introduced into it due to competition with natural microflora and other factors. Our knowledge about effects of AMF species/genotypes on plant growth is very shallow. Moreover, the current knowledge about AMF is not sufficient to allow us to categorize different AMF species based on a parasitic-mutualistic continuum since same AMF species can behave differently with different plants, as well as little is known about variations of introduced versus native AMF species effects on plants (Klironomos, 2003).

In this study, the goal was to evaluate plant growth and the percentage of colonization of different AMF inocula as single and mixed species to barley in a clay saline soil under its natural microflora. Therefore, it was hypothesized that different AMF inocula isolated from alkaline soils will vary in their ability to colonize barley and affect its growth in a clay saline soil with its natural microflora.

2.3 Material and Methods

2.3.1 Soil

Surface soil (top 0-30 cm) was collected near the Texas A&M AgriLife Research & Extension Center at Pecos in Reeves county, Texas, USA. The majority of soils found in this region are saline and moderately alkaline. The soil series in this region is Dalby clay and classified as Fine, smectitic, frigid Oxyaquic Vertic Hapludalfs (NRCS, USDA. Web Soil Survey). No vegetative cover or previous agricultural practices were present at the site where the soil was collected. Characteristics of the collected soil sample are listed in **Table 2.1**. The soil texture was determined by a hydrometer (Bouyoucos, 1962), and % organic matter content was determined by the wet oxidation method (Walkley and Black, 1934). Soil pH, EC (saturated paste extract), and soil P (Mehlich-3) were determined by the Soil, Water and Forage Testing Laboratory Department of Soil and Crop Sciences, Texas A&M University.

2.3.2 AMF inoculum and plant host

AMF species *Rhizophagus intraradices* (accession code UT118), *Claroideoglomus etunicatum* (accession code AZ414B), and *Funneliformis mosseae* (accession code UT101) isolated from alkaline soils were obtained from INVAM (International Vesicular Arbuscular Mycorrhizal collection facility, University of West Virginia) as whole

inoculum containing different AMF propagules (soil with spores, infected root pieces, and hyphae). Winter malt barley (Wintmalt, KWS Saat, Germany) was used as the plant host in this experiment since barley is one of the most salt-tolerant crops and one treatment contained a very high level of salts.

Table 2.1. Characteristics of the soil used in first experiment.

Parameter	Value
pH	8.5
EC (saturated extract, dS/m)	6.32
P (Mehlich-3, mg/kg)	45
Organic matter content (%)	0.34
Clay (%)	46.7
Silt (%)	20.7
Sand (%)	32.6

2.3.3 Experimental design and growth conditions

The experimental design used was a 3×5 factorial in a completely randomized design with 4 replicates. The treatment combinations consisted of 3 salinization levels [gypsum addition to lower the salinity stress by replacing Na^+ with Ca^{2+} at the exchange sites and improve soil texture, natural level of EC in the soil (6.32 dS/m) as a moderately saline level, and addition of NaCl to raise the soil EC to approximately 26 dS/m as a high salt level], and 5 treatments [3 single AMF treatments (*Rhizophagus intraradices*, *Claroideoglomus etunicatum*, or *Funneliformis mosseae*, control treatment (inoculation with the same media but free of AMF), and a mixture treatment of all 3 AMF species used, at 1/3 rate for each], giving a total of 60 samples. The addition of AMF inocula was to ensure that the different effects were relevant to their addition when compared to control soil with only native AMF. These inocula were selected because they were isolated from

alkaline soils and are like species used in research projects and commercial products. Small plant containers (Ray Leach Containers™, Stuewe & Sons, Inc., Tangent, OR, USA; 2.5-cm diameter, 12-cm length, 49-ml volume) were used to grow the plants. The soil was inoculated at two different depths (1 & 4 cm below seed) with 1 gm of inoculum at each depth. The plants were grown for 21 days after sowing in a growth chamber at 21 °C day/18 °C night, 16 h/8 h light/dark, 60 % humidity, & 500 µmol/m^2/s light intensity, and watered daily to 60% water (determined based on maximum water holding capacity) (Noggle and Wynd, 1941).

2.3.4 Plant growth parameters, root staining and AMF colonization

At harvest, plants were gently removed from the pots and shoots were separated from the root systems. Shoots were placed in an oven at 60 °C for 48 hours and then weighed to get the dry weight. Roots were gently removed from soil and washed under tap water. A subsample was separated and weighed before and after drying in an oven at 60 °C for 48 hours to obtain the moisture content which was used to estimate total root dry weight. Remaining roots were cleared (to remove cytoplasm content of cells) and stained with trypan blue using a modified procedure of Phillips and Hayman (1970). Briefly, roots were placed in tissue cassettes (Fischer Scientific Inc., Hampton, NH, USA) and submerged in pre-boiled 10 % KOH for 10 min to remove cytoplasmic content of root cells. Cassettes were then washed 5X with tap water and submerged in 2 % HCl for 30 min, followed by 5X washing with tap water. The cassettes were then submerged in pre-boiled 0.05 % trypan blue solution (water, glycerin, lactic acid in 1:1:1 (v/v/v)) for 5 min. The cassettes were then washed 5X with tap water and stored at 4 °C for 3-5 days immersed in distilled water to remove excess stain. The percentage of AMF colonization

was then determined using the gridline intersection method (Giovannetti and Mosse, 1980). Growth parameters of root dry weight, shoot dry weight, shoot to total dry weight and total dry weight were calculated.

2.3.5 Statistical analysis

Treatment effects were statistically analyzed using two-way ANOVA in SAS software (version 9.4), using PROC GLM procedure. Differences between treatments were obtained using Fisher's least-significant-difference (LSD) test at a *p*-value of <0.05.

2.4 Results

2.4.1 AMF root colonization

Mycorrhizal colonization results are shown in **Figure 2.1**. General comparisons between the different AMF inoculations showed that *F. mosseae* resulted in a significantly higher colonization (average of 21.6 %) compared to control (average of 12.3 %), *R. intraradices* (average of 15.2 %) and *C. etunicatum* (average of 14.8 %). Mixed AMF inoculum was significantly higher (average of 19 %) than only the control inoculum. Moreover, when looking to salt level effect on each AMF inoculum, control and *C. etunicatum* inocula had significantly higher colonization in the higher salt level compared to low and moderate ones. In contrast, *R. intraradices*, *F. mosseae* and mixed AMF inocula did not show significant colonization with salt level, although all trended higher at the highest salt level. *p*-values from ANOVA test of the different effects are listed in **Table 2.2.**

2.4.2 Plant growth parameters

Data in **Figures 2.2 – 2.5** illustrate the different barley growth parameters observed in this study. Total shoot dry weight showed no significant differences among the AMF

treatments in general (**Figures 2.2**). However, in the highest salt level (26 EC), all plants had significantly lower shoot dry weight for all AMF inocula compared to the low and moderate salt levels.

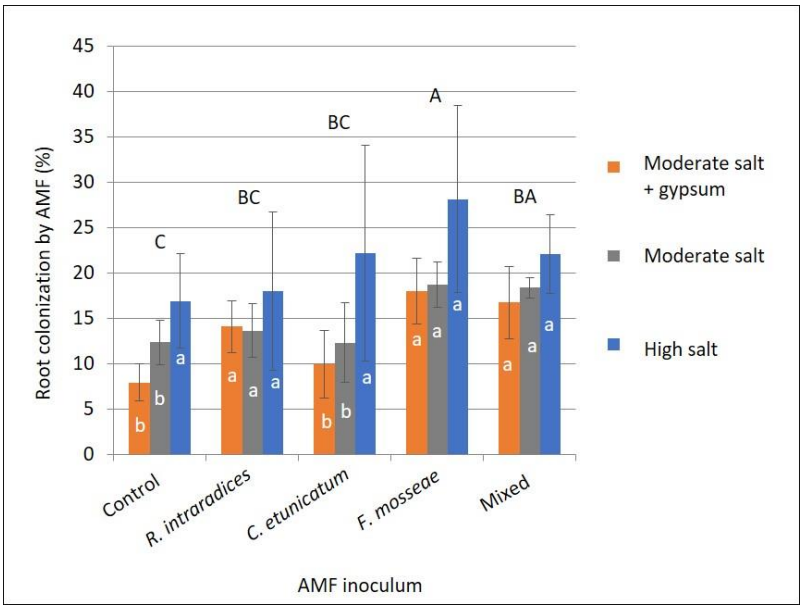

Figure 2.1. Percentage of root colonization by AMF. Moderate salt level + gypsum = soil with 6.3 dS/m + gypsum; moderate salt level = soil with 6.3 dS/m; high salt = soil with 26 dS/m. Data are mean ± s.d (n=4). Different upper-case letters above bars and lower-case letters within each inoculum bars indicate significant difference ($p < 0.05$).

Table 2.2. *p*-values of the different effects from analysis of variance (ANOVA) tests on % of colonization by AMF and plant growth parameters. AMF: effects caused by AMF inoculation factor. Salt: effects caused by salt factor. AMF× Salt: interaction effects of both AMF and salt. * = indicate significant difference ($p < 0.05$).

Effect	**ANOVA *p*-value**				
	% AMF colonization	**Shoot dry weight**	**Root dry weight**	**Total dry weight**	**Shoot to total dry weight**
AMF	0.0016*	0.6986	0.1651	0.1543	0.0220*
Salt	<.0001*	<.0001*	<.0001*	<.0001*	0.3173
AMF × Salt	0.8402	0.1630	0.0268*	0.0185*	0.0056*

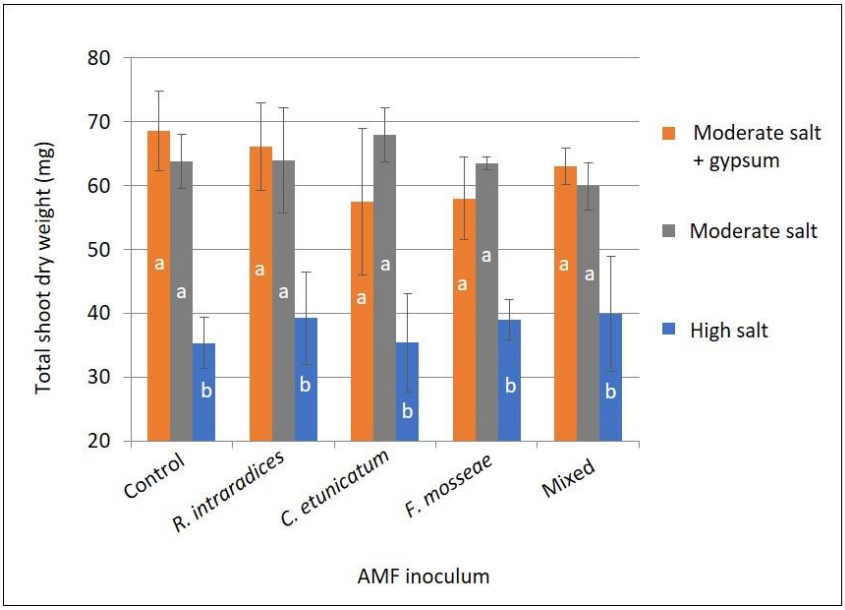

Figure 2.2. Total shoot dry weight. Moderate salt level + gypsum = soil with 6.3 dS/m + gypsum; moderate salt level = soil with 6.3 dS/m; high salt = soil with 26 dS/m. Data are mean ± s.d (n=4). Different letters within each inoculum bars indicate significant difference ($p < 0.05$).

Plant responses in term of root dry weight (**Figure 2.3**) had wider differences compared to shoot dry weight. No significant differences were found in overall root dry weight between the different AMF inocula. However, in term of salt level effect on plant root dry weight in each AMF inocula, *C. etunicatum* resulted in a significantly higher root dry weight in the moderate salt level compared to low and high salt levels (**Figure 2.3A**). In comparison, *F. mosseae* resulted in a significantly higher root dry weight in the low salt treatment compared to higher, but not moderate salt treatments (**Figure 2.3A**). All other AMF inocula did not show significant variations in term of root dry weight as impacted by salt level. In addition, within the moderate salt level (6.3 EC), inoculation with *C. etunicatum* species significantly increased root dry weight compared to the control and *F.*

mosseae inocula (**Figure 2.3B**). However, with the high salt level, *C. etunicatum* significantly lowered root dry weight comparing to all AMF inocula except *F. mosseae*.

The overall plant production in term of total plant dry weight (**Figure 2.4**) did not show significant differences between overall inocula (**Figure 2.4A**). However, in term of salt level effect on total plant dry weight in each AMF inocula, *C. etunicatum* inoculum resulted in significant variations in total plant dry weights between all salt levels, where the highest total plant dry weight was found in the moderate salt level followed by to low and high salt levels. On the other hand, control, *R. intraradices*, *F. mosseae* and mixed AMF inocula resulted in significantly lower total plant dry weight at the highe salt level compared to low and moderate ones. When looking to differences between AMF inocula within each salt level, *C. etunicatum* in the 6.3 EC salt treatment resulted in a significantly higher total plant dry weight compared to control and *F. mosseae*, but no difference was found when compared to *R. intraradices* and mixed inocula (**Figure 2.4B**). However, in the low salt level treatment, *C. etunicatum* resulted in a significantly less total plant dry weight compared to control and mixed inocula, but no difference was found when compared to *R. intraradices* and *F. mosseae*. Similarly, in the highest salt treatment (26 EC), *C. etunicatum* resulted in a significantly less total plant dry weight compared to *R. intraradices* and mixed inocula, but no difference was found when compared to control and *F. mosseae*.

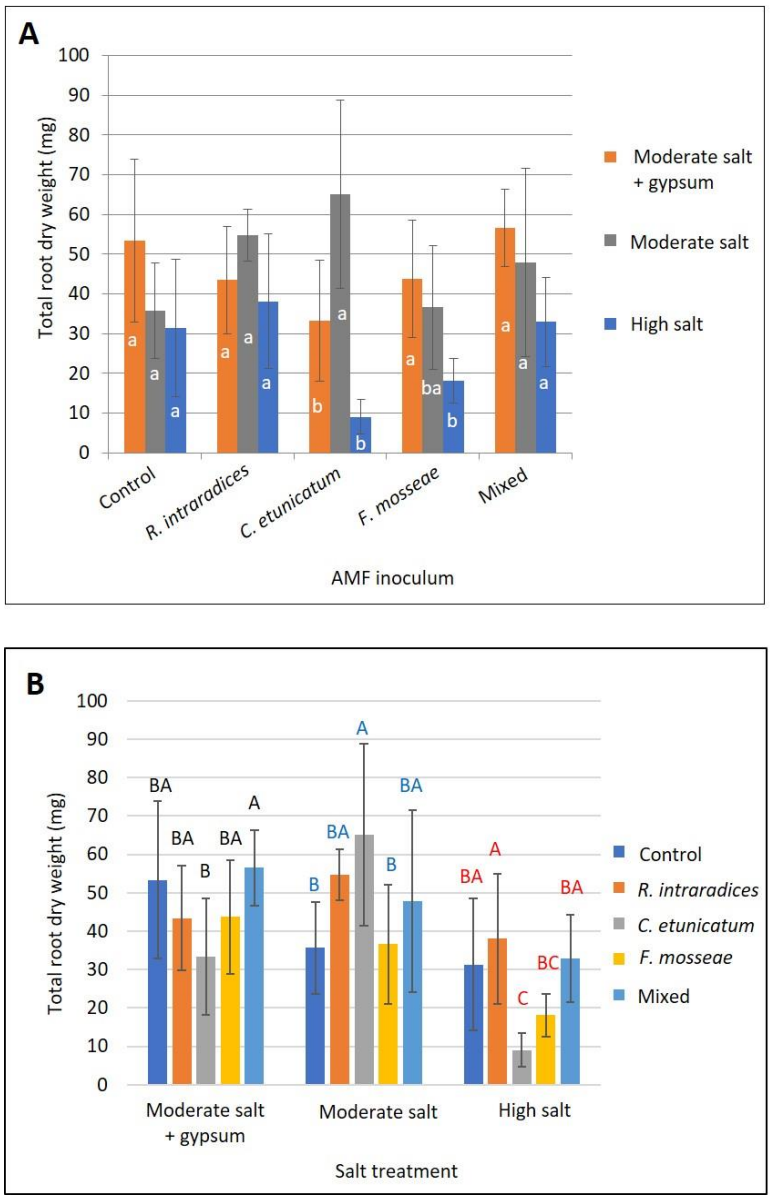

Figure 2.3. Total root dry weight. A: comparison of overall root dry weight between the different AMF inocula in general and as impacted by salt level; moderate salt level + gypsum = soil with 6.3 dS/m + gypsum; moderate salt level = soil with 6.3 dS/m; high salt = soil with 26 dS/m. B: comparison of shoot to total plant dry weight ratios in the different AMF inocula within each salt level. Data are mean ± s.d (n=4). Different lower-case letters within each inoculum bars (figure A only) and upper-case letters with the same color indicate significant difference ($p < 0.05$).

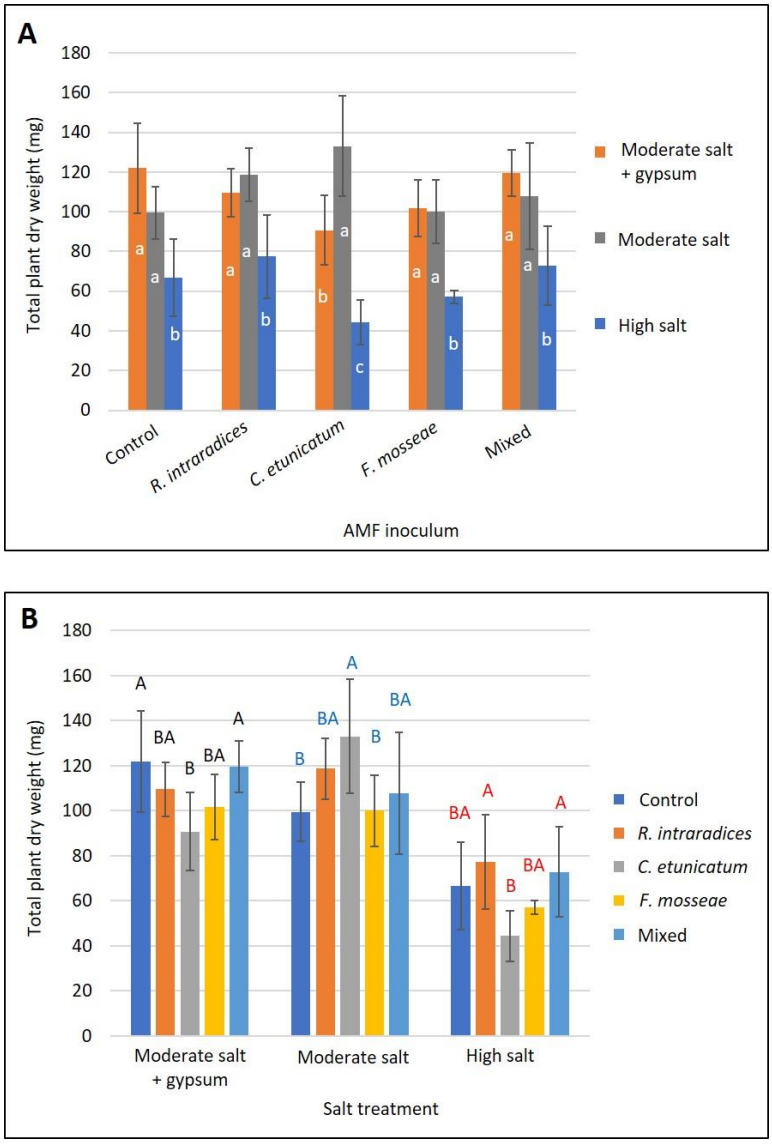

Figure 2.4. Total plant dry weight. A: comparison of overall total plant dry weight between the different AMF inocula in general and as impacted by salt level; moderate salt level + gypsum = soil with 6.3 dS/m + gypsum; moderate salt level = soil with 6.3 dS/m; high salt = soil with 26 dS/m. B: comparison of shoot to total plant dry weight ratios in the different AMF inocula within each salt level. Data are mean ± s.d (n=4). Different lower-case letters within each inoculum bars (figure A only) and upper-case letters with the same color indicate significant difference ($p < 0.05$).

To have a better assessment of the variation in plant responses to the different AMF inocula, I calculated the percentage of shoot dry weight to total plant dry weight ratios (**Figure 2.5**). No significant differences were found in the overall shoot to total plant dry weight ratio when comparing AMF species to the control inoculum. However, *C. etunicatum* and *F. mosseae* resulted in significantly higher shoot to total plant dry weight ratios compared to *R. intraradices* and mixed inocula (**Figure 2.5A**). In the comparison between AMF inocula within each salt level (**Figure 2.5B**), the only significant differences were found in the higher salt treatment (26 EC) where *C. etunicatum* and *F. mosseae* significantly resulted in higher shoot to total plant dry weight ratios compared to control, *R. intraradices* and mixed inocula. *p*-values from ANOVA tests of the different effects on all plant growth parameters are listed in **Table 2.2.**

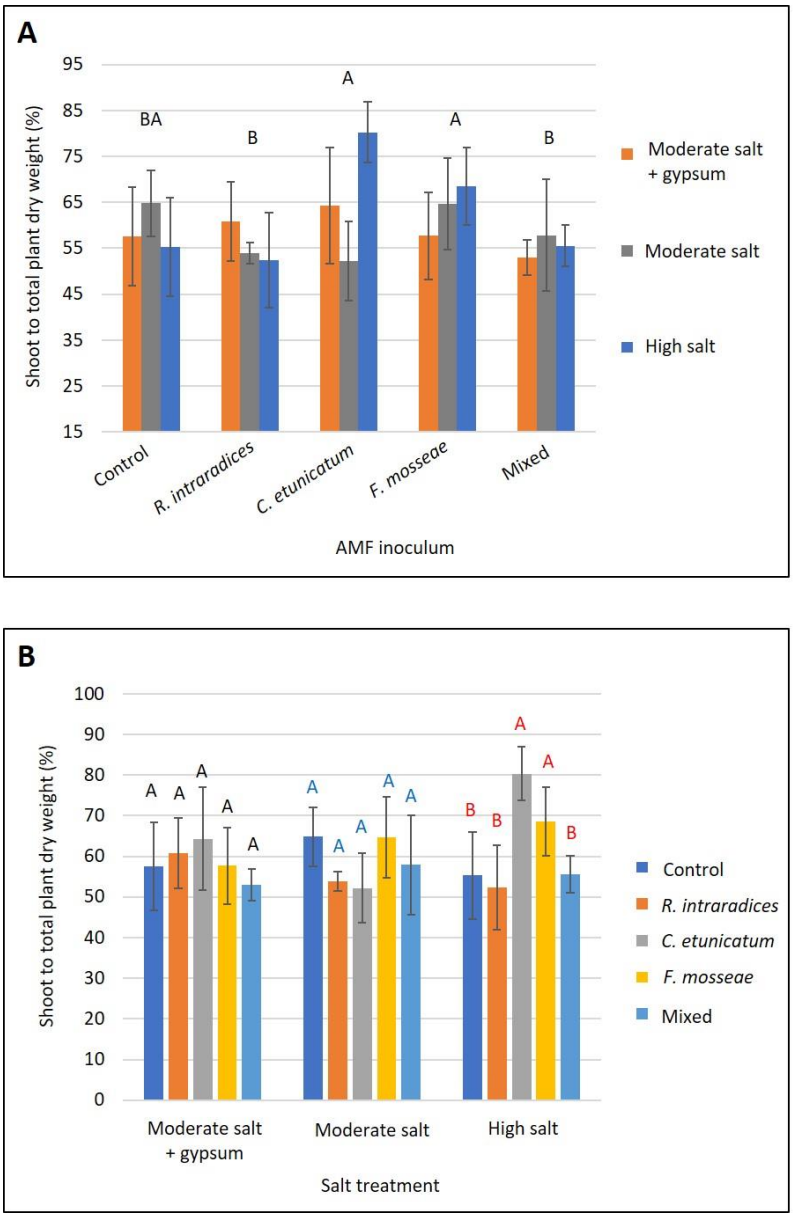

Figure 2.5. Shoot to total plant dry weight ratio as %. A: comparison of overall shoot to total plant dry weight ratios between the different AMF inocula in general and as impacted by salt level; moderate salt level + gypsum = soil with 6.3 dS/m + gypsum; moderate salt level = soil with 6.3 dS/m; high salt = soil with 26 dS/m. B: comparison of shoot to total plant dry weight ratios in the different AMF inocula within each salt level. Data are mean ± s.d (n=4). Different letters with the same color indicate significant difference ($p < 0.05$).

2.5 Discussion

In this study, overall plant growth in terms of shoot and total plant dry weights was significantly decreased in the highest salt level (26 EC) compared to low (moderate + gypsum) and moderate salt levels. On the other hand, AMF colonization significantly increased in the highest salt level (26 EC) compared to low and moderate salt levels with control and *C. etunicatum* inocula. AMF colonization also trended higher for the other inocula in the highest salt level treatment, but the increases were not significant. Since barley is a salt tolerant plant, that may explain the no significant differences in colonization between the low and the moderate salt level (6.3 EC) in this study. Although AMF abundance in control was lower (uninoculated) compared to other inoculated treatments (native AMF + introduced AMF), no significant differences were found in % colonization between control, *R. intraradices* and *C. etunicatum*, but *F. mosseae* and mixed inocula were significantly higher than control. These variations suggest that AMF abundance may not translate into higher % of colonization in roots. Similar differences between AMF abundance in soil and % colonization was reported by Aliasgharzadeh et al. (2001) who found higher spore number in soils with higher salt levels compared to lower salt levels. In addition, Juniper and Abbott (2006) reported that AMF spore germination can range from zero to maximum with increased NaCl levels depending on the AMF species. Therefore, these research evidences support the finding in this study that abundance of AMF may not translate into higher colonization, but rather the AMF species involved. In this study, the different AMF species, from alkaline soil sources, used in this study resulted in different colonization percentages. For example, the highest colonization was observed with *F. mosseae*, which was significantly higher than the control, *C. etunicatum*, and *R.*

intraradices treatments. Despite that the mixed AMF treatment was not significantly different from *F. mosseae*, both had the highest colonization percentages among the different salt levels used in this study. Although the control treatment in this study did not have introduced AMF, they had a similar overall colonization rate to *R. intraradices* and *F. mosseae* resulting from indigenous AMF in the soil. In a study conducted by Juniper and Abbott (2006), they found that different AMF isolates from both saline and non-saline soils had different germination and hyphal extension rate using sterilized soil under different salt concentrations. In their study, they found that increasing NaCl concentrations reduced the overall spore germination and hyphal extension. However, some AMF species isolated from non-saline soils reached the highest germination rates at the highest NaCl used in their study (300 mM), and other AMF species did not differ in hyphal extension rate with the different salt levels (Juniper and Abbott, 2006). In another study conducted by Muhammad and his co-workers (2003), a mixture of indigenous AMF species resulted in higher colonization to barley roots compared to introduced *Glomus intraradices* when plants were grown for 5 weeks in fumigated clay loam saline soils. Furthermore, the indigenous AMF mixture in their study resulted in a slightly higher colonization (49%) under salt stress of 16 dS/m compared to 6.2 dS/m (42.1%) (Muhammad et al, 2003). Although it has been generally suggested that AMF colonization is reduced as salt stress/concentrations increased (Evelin et al, 2009), some studies have shown increased AMF colonization (Aliasgharzadeh et al., 2001; Giri and Mukerji, 2004), or no effect (Hartmond et al, 1987) with high salt concentration. However, those studies that reported increased AMF colonization in high salt concentrations were based on indigenous AMF species. Therefore, these findings may explain the findings in the present study that

indigenous AMF or AMF isolated from similar soil conditions to native species (herein AMF had alkaline soil sources) might result in a higher colonization rate as salt level/concentration increased. Although AMF colonization in the present study was overall relatively low (highest colonization 28 % with *F. mosseae* in the 26 EC salt treatment), this is probably due to the short growth period of this study (21 days), since increased % root colonized by AMF has been strongly linked to late plant growth stages where plants rely more on AMF for nutrient uptake (Javaid and Riaz, 2008).

Despite that plant growth in this study generally was reduced under the highest salt level, parameters such as root and total plant growth indicated different plant responses depending on AMF species and salt level. For example, plants inoculated with *C. etunicatum* in the lower salt level were not significantly different from control in term of root dry weight, but they were significantly lower than mixed species. Yet, at the moderate salt level, plants inoculated with *C. etunicatum* had significantly higher root dry weight compared to both control and *F. mosseae*. In contrary, *C. etunicatum* resulted in a significantly lower root dry weights compared to control, *R. intraradices* and mixed inocula. Similar results were also found in the total plant dry weights. However, inoculation with *C. etunicatum* and *F. mosseae* resulted in a significantly higher shoot to total plant ratios compared to control, *R. intraradices* and mixed inocula only the highest salt level.

The overall variations among the different AMF were more apparent in the root growth, total plant yield, and shoot to total plant dry weight compared to shoot dry weight. A greenhouse study by Al-Karaki et al. (2001) also reported that salt-tolerant tomato cultivar had significantly higher colonization rates at 1.4 and 7.1 dS/m salt levels compared

to salt-sensitive tomato cultivar, although in both plants AMF colonization significantly decreased with increased salt levels. Yet, in their study (Al-Karaki et al, 2001) they reported that the salt-sensitive plants responded better to AMF colonization in term of growth enhancement compared to the salt-tolerant ones, despite that the later had significantly higher colonization percentages under all salt levels. Although their findings disagree with the findings in this study in term of increased colonization with increased salt level, but it may explain the findings in this study that barley as a salt-tolerant plant may had relied more on AMF colonization, which in return may had an adverse effect on its growth as salt level increased.

The results in this study also support the lack of knowledge that still exist in our understanding of how AMF can differ in affecting plant growth by causing either parasitic or beneficial effects (Klironomos, 2003). For example, in the current study, *C. etunicatum* had a significantly lower colonization rate at the low and moderate salt levels compared to the higher salt level. Yet, in contrast, *C. etunicatum* resulted in significantly higher root and total plant dry weights in the moderate salt level compared to both low and high salt levels. Thus, these differences suggest that *C. etunicatum* may had caused a parasitic effect on plants at the higher salt level, but beneficial effects at the moderate salt level in term of root and total plant dry weights. On the other hand, control plants had significantly higher colonization with the highest salt level compared to both lower and moderate salt levels but resulted in a significantly lower total plant dry mass at the highest salt level compared to low and moderate ones. Thus, in control plants, colonization by native AMF seemed to cause a parasitic effect on plants as salt level increased. These differences suggest that depending on salt level and the AMF species involved, AMF may cause either a parasitic

or a mutualistic effect on plant growth. As indicated above, the findings by Klironomos (2003) also support the findings in the present study that same AMF species can have a ranged of parasitic to mutualistic effects on plants. In addition, it is worth mentioning that different AMF species have been also shown to have different growth and colonization rates (Hart and Reader, 2002), which may also explain the differences between AMF species in impacting plant growth found in the present study.

It has been generally known that salt stress may reduce AMF growth through different mechanisms. Such mechanisms include inhibiting spore germination (Estaun, 1990), inhibiting hyphal growth or spreading in soil (McMillen et al., 1998), and reducing the number of AMF arbuscules (Pfetffer and Bloss, 1988). However, these studies were all conducted under sterilized conditions and may not necessarily represent AMF growth and subsequent effects on plants under field conditions. In addition to the above studies, most studies used sandy or sand rich media (Chen et al., 1991). Soil texture is relevant when comparing root colonization, as it has been suggested that AMF colonization maybe restricted in clayey soils compared to sandy soils (Carrenho et al, 2007).

It is not clear in this study whether the variations in AMF colonization and plant growth responses to different AMF species were caused by their different responses to the relatively high soil P concentration (45 mg/kg, Mehlich-3), soil texture, and/or the role of the native microbial community. However, some research has shown improved plant growth at even high P concentration when inoculated with AMF (Colla et al, 2008). Nevertheless, the microcosm size and short growth time might have reduced the resolution of variations observed in this study. A longer growth period as well as field studies may reveal different results. However, the discrepancy in results exist in AMF impacts on

plants grown in salt-affected soils invites more investigations to better understand factors regulating the parasitic or mutualistic effects of AMF in ecosystems.

As shown in this study, it seems apparent that AMF effects on plant growth might vary along a continuum of parasitism to mutualism (Klironomos, 2003), and that our understandings of such complexity are still not fully developed (Friede et al, 2016). Also, our knowledge about the complexity of AMF-soil organism interactions are very limited as these interactions may also have competition, inhibition, or stimulation effects (Fitter and Garbaye, 1994). A very recent study has shown that some soils can be suppressive while others can be conducive to AMF activity and subsequent plant uptake of nutrients such as P in soils (Svenningsen et al, 2018). In their study, Svenningsen et al. (2018) reported that certain microbial phyla can have roles in causing these conducive or suppression effects on AMF activity, which point out that the soil microbiome plays a strong role in AMF ecosystem services. Moreover, the discrepancy in results exist on the different studies of AMF impacts on plants grown in salt-affected soils highlights the needs for more studies to better understand factors regulating the parasitic-mutualistic continuum of AMF effects on plant in these ecosystems.

2.6 Conclusion

In this study, I found that different AMF species vary in colonization efficiency and effects on plants in a saline clay soil. Overall, significant variations were noticed in percentage of colonization between AMF inocula in general. In addition, percentage colonization significantly increased with increasing salt level in the control and *C. etunicatum* inocula treatments. *C. etunicatum* significantly improved root and total plant dry weights at the medium salt level while significantly decreasing them at the low and

high salt level, while others such as control significantly reduced total plant dry mass. These differences between AMF inocula in this study suggest that different AMF species can colonize plant roots and affect its growth differently under different salt levels. Therefore, depending on both, AMF species involved and the salt level, AMF may cause either parasitic or mutualistic effects on plant growth when grown in unsterilized naturally saline soils.

CHAPTER III

INTERACTIONS OF ARBUSCULAR MYCORRHIZAL FUNGI WITH HYPHOSPHERE MICROBIAL COMMUNITIES IN A SALINE SOIL: IMPLICATIONS ON PHOSPHORUS EXTRACTABILITY AND ALKALINE PHOSPHATASE GENE ABUNDANCE

3.1 Synopsis

Interactions between arbuscular mycorrhizal fungi (AMF) and soil microbes to solubilize organic and inorganic phosphorus (oP & iP) is not well understood, especially under stressed conditions such as salinity. I investigated the interaction effects of AMF (*Funneliformis mosseae*) and hyphosphere microbial communities (root-free soil) on P availability and uptake by plant (*Sorghum bicolor*), alkaline phosphatase (ALP) activity and its gene abundance (*phoD*) in a natural saline soil. The experiment was conducted using two-compartment microcosms (inner (I) as hyphosphere and outer (O) as rhizosphere compartments) separated with 25 µm nylon mesh. The soil in compartments had 4 sterilization treatments: both inner (hyphosphere) and outer (rhizosphere) compartments sterilized (IS-OS), inner sterilized and outer unsterilized (IS-OU), inner unsterilized and outer sterilized (IU-OS), and both unsterilized (IU-OU). The hyphosphere compartments were amended with 200 mg/kg Na-phytate (oP) and 200 mg/kg rock phosphate (iP). The rhizosphere compartments were inoculated with AMF *Funneliformis mosseae* (*Fm*) or uninoculated. Soil sterilization significantly reduced acid (Mehlich-3) extractable P ($p < 0.05$) in the hyphosphere compartments compared to unsterilized ones in both *Fm*-inoculated and uninoculated treatments. Inoculation with *Fm* significantly increased plant

P uptake (p <0.05) only in the treatments where outer compartments had unsterilized soils (IS-OU and IU-OU) compared to uninoculated ones. Similarly, sterilization significantly (p <0.05) reduced *phoD* gene abundance and ALP activity in the hyphosphere compartments compared to unsterilized ones in both *Fm*-inoculated and uninoculated treatments. Although ALP activities were significantly higher (p <0.05) in unsterilized soil treatments compared to sterilized ones, the presence of *Fm* hyphae reduced ALP activity in the hyphosphere compartments of *Fm*-inoculated comparing to uninoculated treatments. Overall, our results indicate that AMF-hyphosphere microbial community interactions play a significant role in increasing bioavailable P in saline soils, and that enriching AMF and other soil microbes (especially phosphate solubilizing microbial community) may be an option for improving P bioavailability in soils with sparingly soluble P minerals.

3.2 Introduction

Biological and chemical interactions occurring in the mycorrhizosphere are not well understood and need further exploration, especially in the level of mechanisms controlling microbial community structure and functions. The importance of processes driven by these microbial communities might depend on arbuscular mycorrhizal fungi (AMF) species, host plant species, as well as environmental variables (Bending et al., 2006). It is well known that AMF are obligate biotrophs; however, some studies have shown the ability of some bacteria (e.g., *Paenibacillus validus)* to support the growth of *Glomus intraradices* independently of the host plant (Hildebrandt et al., 2002). Production of raffinose and other unknown compounds by some *Paenibacillus* spp. might mimic the plant signaling molecules that may be important in the establishment of mycorrhiza (Bonfante and Anca, 2009). In addition, it seems that some diffusible compounds produced

by soil bacteria are important in spore germination and hyphal growth of AMF (Koltai and Kapulnik, 2010).

Furthermore, contradictions and poor understanding still exist regarding the ability of AMF to solubilize phosphate rock as well as organic P, and the synergistic interactions with the natural microflora that contribute to P acquisition by AMF and plants. Whether the bacteria in the mycorhizosphere or hyphosphere help increase or reduce P solubility might depend on the level of P available for both the fungus and the associated bacteria. Moreover, the current knowledge of phosphatase-encoding genes in microbes are mostly based on culture-dependent methods. This raises the need for culture-independent methods when trying to study phosphatase-encoding genes in environmental samples (Ragot et al., 2015). For example, alkaline phosphatase (ALP) activity has been shown to strongly correlate with *phoD* bacterial gene abundance in field agricultural soil (Fraser et al., 2015a). Furthermore, majority of studies on the beneficial effects of AMF on plants under abiotic stresses such as salinity are conducted under sterilized soil conditions, and mostly on sandy soils (Chen et al., 1991). Hence, our knowledge of AMF-soil microbial community interactions is very limited. Understanding such interaction might improve our knowledge of the ecological importance of AMF and its associated bacterial communities, especially under stressed environments.

Under salinity stress, both rhizosphere microbes and AMF have been shown to improve plant growth (Kumar et al., 2015; Shrivastava and Kumar). Such enhancement to plant growth have been attributed to many aspects such as production of phytohormones (Ahmad et al, 2013), N-fixation (Shukla et al, 2012) and P-solubilization (Tank and Saraf et al, 2010) by plant growth promoting rhizobacteria (PGPR), and improved soil structure

and water uptake (Kohler et al, 2010), P uptake (Giri and Mukerji, 2004), and increased K^+/Na^+ plant ratio (Giri and Mukerji, 2007) by AMF. However, our knowledge about interactive impacts of both groups of microbes on plant growth in salt-stressed soils is limited to studies with co-inoculation with single or a few species of PGPR and AMF, and mostly conducted in artificially saline soils (Rabie and Almadini, 2005; Kohler, 2009; Zhang et al, 2011). Aspects such as survival of introduced microbial species, competition with native microbes, and the need for re-application within same growing season might limit effectiveness of such approaches, especially in sustainable agriculture (Malusá et al, 2012). In addition, such studies conducted under salt stress usually evaluate the impact on plant growth and P uptake by plants rather than investigating interactions in soil and how it affects available P and microbial enzymes related to P availability such as ALP in soils, rather than within plant roots.

In this study, the goal was to assess the role of soil indigenous microbial community in enhancing AMF acquisition of P in a naturally saline soil. Therefore, I hypothesized that the indigenous saline soil microbial community would increase P access by AMF in hyphosphere and increase its uptake by plant shoots.

3.3 Material and methods

3.3.1 Soil

Surface soil (top 0-30 cm) was collected near the Texas A&M AgriLife Research & Extension Center at Pecos in Reeves county, Texas, USA. The majority of soils found in this region are saline and moderately alkaline. The soil series in this region is Dalby clay and classified as Fine, smectitic, frigid Oxyaquic Vertic Hapludalfs (NRCS, USDA. Web Soil Survey). No vegetative cover or previous agricultural practices were present at the site

where the soil was collected. Characteristics of the collected soil sample are listed in **Table 3.1**. The soil texture was determined by a hydrometer (Bouyoucos, 1962), and percent organic matter content was determined by the wet oxidation method (Walkley and Black, 1934). Soil pH, EC (saturated paste extract), and soil P (Mehlich-3) were determined by the Soil, Water and Forage Testing Laboratory Department of Soil and Crop Sciences, Texas A&M University.

Table 3.1. Characteristics of the soil used in second experiment.

Parameter	Value
pH	8.5
EC (saturated extract, dS/m)	6.32
P (Mehlich-3, mg/kg)	45
Organic matter content (%)	0.34
Clay (%)	46.7
Silt (%)	20.7
Sand (%)	32.6

3.3.2 AMF inoculum and plant host

The AMF species used in this experiment was *Funneliformis mosseae* (*Fm*), obtained from INVAM (International Vesicular Arbuscular Mycorrhizal collection facility, University of West Virginia, accession code UT101) as whole inoculum containing different AMF propagules (soil with spores, infected root pieces, and hyphae). This AMF inoculum was chosen since it colonized plant roots well in my previous experiment also performed in saline clay soil (**Figure 2.1**). The addition of AMF inoculum was to ensure

that the different effects such as plant P uptake were relevant to their addition when compared to uninoculated pots. *Sorghum bicolor* was used as the plant host in this experiment since it is a moderately salt tolerant plant, commonly used as a mycorrhizal host, and suitable for the EC level of this experimental soil.

3.3.3 Experimental design and growth conditions

The experimental design in this experiment was a 2×2×2 factorial completely randomized design with 3 replicates. This experiment was conducted using a two-compartment microcosm (inner (I) as hyphosphere and outer (O) as rhizosphere compartments) separated with 25 µm nylon mesh (LAB PACK, Sefar Inc., Buffalo, NY, USA) to allow hyphal penetration, but not roots (**Figure 3.1**). The hyphosphere compartment was a mini rectangular box (4.5-cm long, 2.5-cm wide, 1.5-cm height) (The Container Store Inc, Coppell, TX, USA) containing 12.5 gm soil/box (2 boxes/pot). The rhizosphere compartment was a small square nursery pot (6.5-cm diameter, 9-cm long, 280-ml volume) containing 235 gm soil. The soil in compartments had 4 sterilization treatments: both inner (hyphosphere) and outer (rhizosphere) compartments sterilized (IS-OS), inner sterilized and outer unsterilized (IS-OU), inner unsterilized and outer sterilized (IU-OS), and both unsterilized (IU-OU). Soils were sterilized by autoclaving for 1 hr at 121 °C three times, on three consecutive days. Soil in the rhizosphere (outer) compartment were amended with nitrogen at 50 mg N/kg soil as NH_4NO_3. The hyphosphere compartments were amended with 200 mg P/kg soil as Na-phytate (Santa Cruz Biotechnology, Santa Cruz, CA) as organic P (oP) and 200 mg P/kg soil rock phosphate as inorganic P (iP). Plant seeds were sterilized with 10 % sodium hypochlorite for 20 min and germinated in plug tray cells (cell size 7/8" deep and 9/16" wide, Harris Seeds Inc.,

Rochester, NY, USA) containing 2 gm inoculum (either *Fm* or no-*Fm* control inoculum) and 2 gm sterile low P sandy soil to promote AMF infection. This soil has 35 mg/kg P (Mehlich-3) and is a Darco loamy fine sandy soil collected from the Texas A&M Agrilife Research and Extension Center at Overton, TX, USA. After 12 days, seedlings with attached soils from the tray cells were transplanted to the designed pots of this experiment. Plants were grown for 42 days after transplanting in a growth chamber at 25 °C day/21 °C night, 16 h/8 h light/dark, 60 % humidity, and 500 µmol/m^2/s light intensity, and watered every other day to 85% water holding capacity (determined based on maximum water holding capacity) (Noggle and Wynd, 1941) using sterilized distilled water.

3.3.4 Plant growth parameters, root staining and AMF colonization

At the harvest time, plants were gently removed from the pots and shoot were separated from the root system. Shoots were placed in an oven at 60 °C for 48 hours and then weighed to get the dry weight. Roots were gently removed from soil and washed under tap water. A subsample was separated and weighed before and after drying in an oven at 60 °C for 48 hours to obtain the moisture content for calculating total root dry weight. The remaining roots were stained with trypan blue using a modified procedure of Phillips and Hayman (1970). Briefly, roots were placed in tissue cassettes (Fischer Scientific Inc., Hampton, NH, USA) and submerged in pre-boiled 10 % KOH for 10 min to remove cytoplasmic content of root cells. Cassettes were then washed 5X with tap water and submerged in 2 % HCl for 30 min, followed by 5X washing with tap water. The cassettes were then submerged in pre-boiled 0.05 % trypan blue solution (water, glycerin, lactic acid in 1:1:1 (v/v/v)) for 5 min. The cassettes were then washed 5X with tap water and stored at 4 °C for 3-5 days immersed in distilled water to remove excess stain. The

percentage of AMF colonization was then determined using the gridline intersection method (Giovannetti and Mosse, 1980). Growth parameters of root dry weight, shoot dry weight, shoot to total dry weight and total dry weight were then calculated.

3.3.5 Soil extractable P and plant shoot P content

The top surface layer (~2mm) of the hyphosphere compartments was removed and discarded to reduce biases and possible exchange of microbes and nutrients between the rhizosphere and hyphosphere compartments. The remaining soil from the hyphosphere compartments of each pot (two compartments) were then mixed to have a one homogenized hyphosphere soil sample/pot and stored at -80°C for later molecular and enzyme assays. Soil samples from the hyphosphere compartments (previously stored at -80 °C) and dried plant shoots were submitted to the Soil, Water and Forage Testing laboratory at Texas A&M University (College Station, TX, USA) to measure extractable P in soil (Mehlich-III) and determine P concentration in plant shoot tissue (ICP analysis).

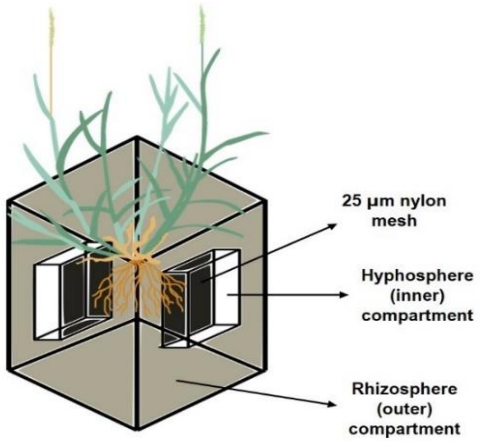

Figure 3.1. Diagram of the designed microcosm having rhizosphere and hyphosphere compartments separated by a nylon mesh.

3.3.6 DNA extraction

Soil DNA was extracted from 0.5 g of the frozen hyphosphere soil samples using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. After extraction, all DNA samples were quantified to detect DNA quality using a Nanodrop ND-1000 spectrophotometer (Thermo-Fisher Scientific Inc., Wilmington, DE, USA).

3.3.7 Quantitative PCR assays

Quantitative real-time PCR (qPCR) was used to quantify the abundances of microbial *phoD* (alkaline phosphatase), total bacterial 16S rRNA, total AMF 18S rRNA, and total fungal internal transcribed spacer (ITS) gene targets in both rhizosphere and bulk soil (root-free soil). For quality control, all qPCR runs included 5 different concentration of DNA standards (gBlock standards, Integrated DNA Technologies Inc.) for each target gene (for standard curve) (details on these standards are listed in **Table 3.2**), no-template control (NTC), positive control, negative control, and 2 spiked random samples from the study's DNA samples with one of the standards to test for possible qPCR inhibitors. Standards and NTC were run in triplicate, and the rest of controls and experiment samples were run in duplicate. Details on each target gene positive control and negative controls, R^2 value, and reaction efficiency of standard curves are listed in **Table 3.3**. Primers (obtained from Integrated DNA Technologies Inc.), qPCR conditions and references are outlined in **Table 3.4**. Amplifications of DNA was performed using Rotor-Gene SYBR® Green qPCR kit, with gene abundance measured using Rotor-Gene Q Software version 2.3.1.49 (QIAGEN, Hilden, Germany).

Table 3.2. Details of gBlock qPCR standards. Dilution range had 1 order of magnitude apart between each of 5 standards.

Target gene	Microbial source for sequence included in the gBlock standards	Dilution range of the standards having the targeted gene (copies/2 µl)
phoD	*Sinorhizobium meliloti*	$10^6 – 10^2$
16S rRNA	*Pseudomonas denitrificans*	$10^7 – 10^3$
AMF-18S rRNA	*Glomus intraradices*	$10^6 – 10^2$
ITS	*Rhizopus microsporus*	$10^7 – 10^3$

Table 3.3. Quality control details of the qPCR runs in second experiment.

Target microbial group	Positive control	Negative control	R^2 value of standard curve	Reaction efficiency
phoD-harboring microbes	*Sinorhizobium meliloti*	*Escherichia coli* K-12	0.99	1.07
16S rRNA	*Escherichia coli* K-12	*Methanospirillum hungatei*	0.99	1.03
AMF 18S rRNA	*Glomus intraradices*	*Escherichia coli* K-12	0.987	1.00
ITS	*Rhizopus microsporus*	*Escherichia coli* K-12	0.99	1.06

Table 3.4. Primers and conditions used for the qPCR assays in second experiment.

Target microbial group	**Primers and sequences**	**qPCR reaction mixture**	**Thermal profile**	**Reference**
phoD-harboring microbes	*phoD*-F733(5'-TGGGAYGATCAYGARGT-3')/ *phoD*-R1083 (5'-CTGSGCSAKSACRTTCCA-3')	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O.	5 min at 98°C for initial denaturation; 35 cycles of 30 s at 98°C, 30 s at 58°C, extension for 30 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 55-98°C (1° and 5 s/cycle melt).	Modified after Ragot et al (2015)
Total bacteria (16S rRNA)	341f-(5'-CCTACGGGAGGCAGCAG-3')/ 797r-(5'-GGACTACCAGGGTATCTAATCCTGTT-3')	7.5 µl SYBR Green (2x) Master Mix, 0.225 µl F primer (0.3 µM), 0.675 µl R primer (0.9 µM), 2 µl DNA template, 4.6 nuclease free H_2O.	3 min at 98°C for initial denaturation; 40 cycles of 30 s at 98°C, 30 s at 61.5°C, extension for 20 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50-99°C (1° and 5 s/cycle melt) after a pre-melt conditioning for 90 s at 50°C.	Modified after Harter et al (2014)

Table 3.4. Continued.

Target microbial group	Primers and sequences	qPCR reaction mixture	Thermal profile	Reference
Total AMF (18S rRNA)	GC-AMV4.5NF- (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G [GC clamp] AAG CTC GTA GTT GAA TTT CG-3′)/ AMDGR-(5′-CCC AAC TAT CCC TAT TAA TCA T-3′)	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O.	10 min at 98°C for initial denaturation; 35 cycles of 30 s at 98°C, 30 s at 55°C, extension for 45 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50-98°C (1˚ and 5 s/cycle melt).	Modified after Sato et al (2005)
Total fungi (ITS)	ITS1f-(5'-TCC GTA GGT GAA CCT GCG G-3')/5.8s-(5'-CGC TGC GTT CTT CAT CG-3')	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O.	10 min at 98°C for initial denaturation; 35 cycles of 60 s at 98°C, 30 s at 53°C, extension for 45 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 48-98°C (1˚ and 5 s/cycle melt).	Modified after Fierer et al (2005)

3.3.8 Alkaline phosphatase enzyme assay

Potential soil alkaline phosphatase (ALP) activity was measured from the frozen hyphosphere soil (-80°C) using a modified assay of Tabatabai and Bremner (1969). Briefly, 0.5 g soil in duplicate was incubated in 0.0625 M *p*-nitrophenyl phosphate substrate (Sigma-Aldrich, USA) along with modified universal buffer solution (pH 11) at 28°C in 2 mL deep-well plates. After 2 h, reactions were stopped with 2.5 M $CaCl_2$ and 2.5 M NaOH. Plates were then shaken for 5 min and centrifuged for 5 min at 500 rpm. Using 96-well plates, formation of *p*-nitrophenol was determined colorimetrically using a Biolog Microstation Elx808BLG (BIO-TEK Instruments Inc., Winooski, VT, USA) spectrophotometer at 405 nm.

3.3.9 Statistical analysis

All treatment effects were statistically analyzed using ANOVA in SAS software (version 9.4), using PROC GLM procedure. Differences between treatments were obtained using Fisher's least-significant-difference (LSD) test at a *p*-value of <0.05.

3.4 Results

3.4.1 Plant growth parameters and AMF colonization

Plant growth responses and AMF colonization percentages are shown in **Table 3.5**. The uninoculated IU-OS treatment significantly increased shoot dry weight compared to IS-OS, IS-OU, IU-OU in uninoculated, and IS-OS, IS-OU, and IU-OS in the *Fm*-inoculated pots. However, no significant difference was found in shoot dry weight when comparing uninoculated IU-OS to *Fm*-inoculated IU-OU. For root dry weight, no significant differences were found among the different soil sterilization treatments in both uninoculated and *Fm*-inoculated groups except with *Fm*-inoculated IU-OU which had

significantly higher root dry weight over *Fm*-inoculated IS-OU. However, within *Fm*-inoculated treatments, IS-OU resulted in a significantly higher shoot to total dry matter % compared to IU-OS and IU-OU, but not IS-OS. Percentages of AMF root colonization were significantly higher in all *Fm*-inoculated treatments compared to uninoculated IS-OS, IU-OS, and IU-OU, but not the IS-OU. However, within *Fm*-inoculated treatments, IS-OU and IU-OS treatments significantly reduced % colonization compared to the IU-OU treatment, but no significant difference was found when compared to the IS-OS treatment.

3.4.2 Extractable P in soil and its uptake by plants

Results of the extractable P in hyphosphere soils are shown in **Figure 3.2A**. Treatments of unsterilized soils in hyphosphere (IU-OS and IU-OU) had significantly higher extractable P compared to sterilized soils (IS-OU and IS-OS) in both *Fm*-inoculated and uninoculated pots. For example, in IS-OS treatments of both *Fm*-inoculated and uninoculated, extractable P in hyphosphere compartments was reduced by 20 % and 18.7 %, respectively compared to IU-OU. Similarly, in IS-OU treatments of both *Fm*-inoculated and uninoculated, extractable P in hyphosphere compartments was reduced by 11.8 % and 10 %, respectively compared to IU-OS. However, although not significant, *Fm* inoculation tended to result in less extractable P compared to uninoculated pots. On the other hand, inoculation with *Fm* significantly increased P concentrations in plant shoots compared to uninoculated ones in IS-OU and IU-OU treatments (**Figure 3.2B**). In contrast, inoculation with *Fm* did not significantly impact P uptake in IS-OS and IU-OS treatments compared to uninoculated ones. When looking to P content/plant shoot, no significant differences were found between uninoculated and *Fm*-inoculated, except with IU-OU treatment which significantly increased P content/plant shoot by 91 % over the uninoculated treatment

Table 3.5. Plant growth parameters and AMF percentage of colonization of the different compartment's soil treatment. IS-OS: soil in inner (hyphosphere) and outer (rhizosphere) compartments sterilized; IS-OU: inner sterilized and outer unsterilized; IU-OS: inner unsterilized and outer sterilized; IU-OU: both unsterilized. "None": control AMF inoculum. "*Fm*": inoculated with *Funneliformis mosseae*. Data are presented as the mean ± s.d (n=3). Different upper (only within uninoculated group) or lower-case letters (only within *Fm*-inoculated group) within same column indicate significant difference ($p < 0.05$).

AMF inoculum	Treatment	Shoot dry weight (mg)	Root dry weight (mg)	Shoot to total dry matter (%)	% root colonized by AMF
None	IS-OS	235.13±36.3 bc	440.31±145.2 BA	34.81±4 C	0±0 e
	IS-OU	274.03±67 bc	423.25±91.4 BA	39.3±7.6 BC	2.91±2.6 ecd
	IU-OS	455.06±97.5 a	474±84.5 A	48.98±3 BAC	0.05±0.08 e
	IU-OU	245.03±79.8 bc	402.21±150.3 BA	37.85±15 BC	1.95±1.5 ed
Fm	IS-OS	243.46±134.6 bc	276.28±209.4 ba	46.84±15.7 ba	25.9±15.1 ba
	IS-OU	155±139.5 c	178.46±162 b	46.48±2 a	15.30±6.2 bc
	IU-OS	223.3±115.8 bc	436.42±217.5 ba	33.84±7.3 c	14.47±7.6 bcd
	IU-OU	321.56±83.5 ba	485.20±105.4 a	39.85±2.2 bc	30.82±10.7 a

(**Figure 3.2C**). Within the uninoculated group of treatments, IU-OS had significantly higher P content/plant shoot (by 100 %) compared to IS-OS treatment. In contrast, within *Fm*-inoculated treatments, IU-OU had the highest P content/plant shoot compared to both IS-OU and IU-OS treatments, but not the IS-OS.

3.4.3 Quantitative PCR assays

The relative abundances of the targeted genes used in this study are shown in **Figure 3.3**. Alkaline phosphatase gene (*phoD*) abundances were significantly reduced by sterilization compared to unsterilized soils in both *Fm* and non-*Fm*-inoculated treatments (**Figure 3.3A**). In the *Fm*-inoculated treatment, sterilization reduced *phoD* gene abundance by 78.3 %, while in the uninoculated treatment the abundance was reduced by 77.7 %. Within unsterilized soils, *phoD* gene abundance was also significantly higher in the *Fm*-inoculated treatments compared to uninoculated treatments. No significant differences were found in the abundance of 16S rRNA and AMF 18S rRNA genes (**Figure 3.3B**, **Figure 3.3C**, respectively). However, fungal ITS abundance was significantly higher in unsterilized soils compared to sterilized ones (**Figure 3.3D**). Moreover, when comparing *phoD* relative proportion among the total microbial community abundance (total of 16S rRNA and fungal ITS gene abundances), *phoD* proportions ranged from 0.30 in the uninoculated IS-OS up to 0.71 in the *Fm*-inoculated IU-OU (**Figure 3.4**).

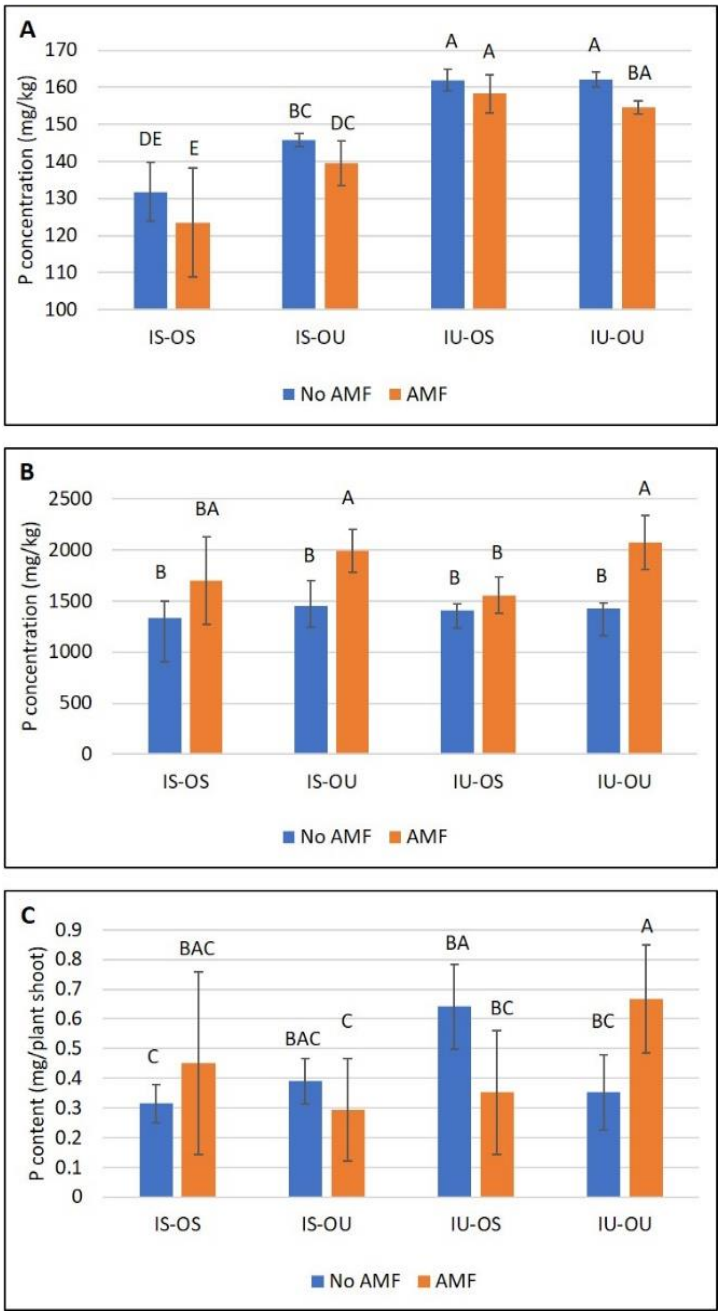

Figure 3.2. Extractable soil P and its uptake by plants in second experiment. A: Extractable P (Mehlich-3) in hyphosphere soil. B: P concentrations in plant shoot. C: P content/plant shoot. IS-OS: soil in inner (hyphosphere) and outer (rhizosphere) compartments sterilized; IS-OU: inner sterilized and outer unsterilized; IU-OS: inner unsterilized and outer sterilized; IU-OU: both unsterilized. Data are means ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

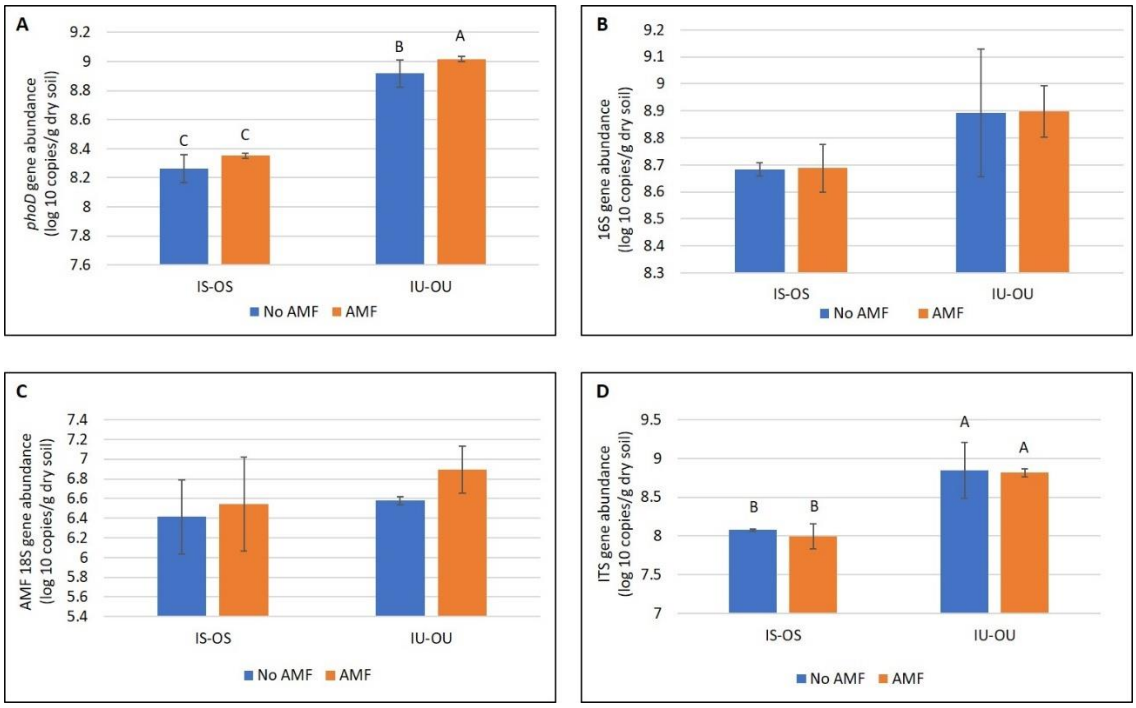

Figure 3.3. Abundances of targeted genes in hyphosphere soil. A: *phoD* gene. B:16S rRNA gene. C: AMF 18S rRNA. D: fungal ITS. IS-OS: soil in inner (hyphosphere) and outer (rhizosphere) compartments sterilized; IU-OU: both soils unsterilized. Data are means ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

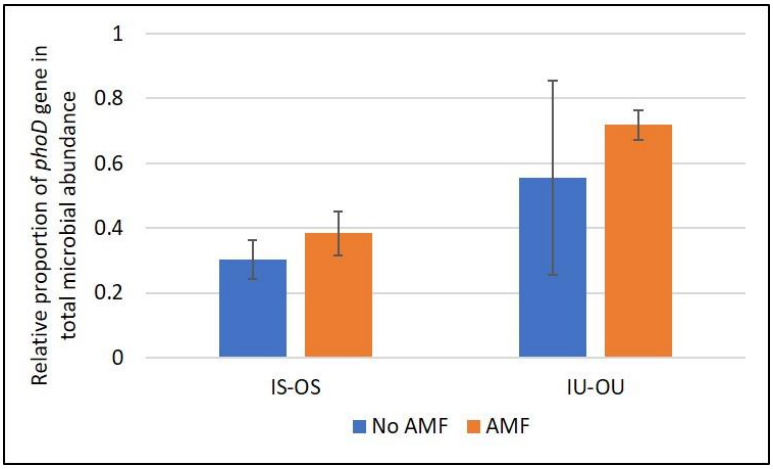

Figure 3.4. Relative proportion of *phoD* gene among the total microbial community abundances (as total 16S rRNA and ITS). 1 = 100%. IS-OS: soil in inner (hyphosphere) and outer (rhizosphere) compartments sterilized; IU-OU: both soils unsterilized. Data are means ± s.d (n=3).

3.4.4 Alkaline phosphatase enzyme assay

The potential activity of soil alkaline phosphatase (ALP) from the hyphosphere soils is shown in **Figure 3.5**. Activity of ALP showed significant differences between all treatments. Soil sterilization significantly reduced ALP activity compared to unsterilized soils in both *Fm*-inoculated (reduction by 78 %) and uninoculated (reduction by 70 %) treatments. Moreover, *Fm* inoculation resulted in significantly less ALP activity for both unsterile and sterile soils compared to uninoculated ones. In sterilized soils, *Fm* inoculation reduced ALP activity by 76 % compared to uninoculated treatment. Similarly, in unsterilized soils, *Fm* inoculation reduced ALP activity by 23.8 % compared to uninoculated treatment.

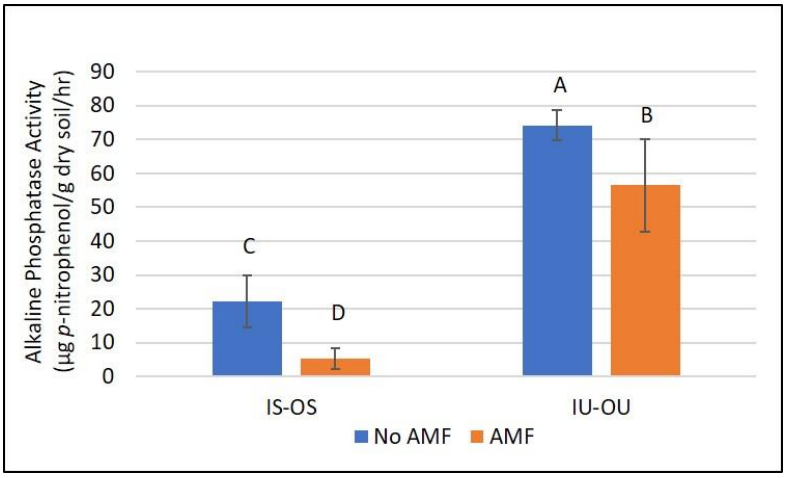

Figure 3.5. Potential soil alkaline phosphatase (ALP) activity in the hyphosphere soils. IS-OS: soil in inner (hyphosphere) and outer (rhizosphere) compartments sterilized; IU-OU: both soils unsterilized. Data are means ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

3.5 Discussion

3.5.1 Plant growth response and AMF colonization

Overall, soil sterilization did not have significant impacts on plant growth parameters in this study. Similarly, few significant differences were found in response to the AMF inoculation. However, some exceptions were found, where the uninoculated IU-OS had significantly higher shoot dry weight compared to IS-OS, IS-OU, IU-OU in uninoculated, and IS-OS, IS-OU, and IU-OS in the *Fm*-inoculated pots. Also, in *Fm*-inoculated IS-OS and IS-OU treatments, shoot to total dry matter % were significantly higher compared to IU-OS and to a lesser degree (significant only with IS-OU) to IU-OU treatments. Based on the results of this study, it is not clear why the above exceptions occurred. A possible explanation is that the root hairs of plants in the uninoculated IU-OS treatment may have accessed the unsterile hyphosphere compartments. Research have shown that the root hairs of plants may increase number and length in response to P demand, which may help to satisfy > 60 % of plant P needs (Gilroy and Jones, 2000). At the time of harvest of this study, roots were noticed growing along the outer surface (facing rhizosphere compartment) of the 25 µm nylon mesh, which may have allowed root hairs to access P sources that were applied to the hyphosphere compartments. Sorghum root hairs have an average diameter of ~ 10 µm (Morgenstern et al, 1987), which could easily enable them to penetrate the 25 µm nylon mesh used in this study. However, the less apparent growth differences in most of the other plant growth parameters may have been caused by the volume difference between the rhizosphere compartment (~245 cm^3, with total P mass of ~ 10.6 mg based on Mehlich-III prior conducting the experiment) compared to the hyphosphere compartments in each pot (~33 cm^3, with total P mass of ~ 11.1 mg

based on Mehlich-III + oP addition + iP addition prior conducting the experiment). In this study, N amendment was only applied to the rhizosphere compartment, while all P amendments (iP and oP) were only applied to the hyphosphere compartments. Therefore, it may not be applicable to rely on plant growth responses based on the experimental design of this study due to the volume and nutrient amendment differences between the compartments. Instead focus should be on the goal of this study, which was to measure P availability in hyphosphere soils (comparable to bulk soil) and assess the possible relationship between P availability and abundance of microbes and microbial functionalities affecting P availability (e.g., via microbial gene abundances), and determine how such relationships influence plant P uptake. In addition, the different sterilization treatments may potentially have changed root exudate and microbial composition in the rhizosphere compartments which have the larger volume (Chen et al., 1991; Jacoby et al, 2017), thus, causing changes in plant growth and uptake of other macronutrients not detected in this study (e.g., N and K).

Soil sterilization did not show an impact on native AMF colonization in the uninoculated treatments. However, when one compartment was sterilized and the other was not in the *Fm*-inoculated treatments (IS-OU and IU-OS), % colonization was significantly reduced compared to IU-OU, but no significant difference was found when compared to IS-OS treatment. It is not clear what are the causes of these low colonization rates under these situations where AMF are facing sterilized and unsterilized soils simultaneously (*Fm*-inoculated IS-OU and IU-OS treatments). However, our knowledge about AMF-soil microbes interactions are still poorly understood. For instance, a research study has shown that specific soil microbes such as mycorrhizal helper bacteria (MHB) can

promote hyphal growth and root colonization (Frey-Klett et al, 2007), and that suppression or stimulation of AMF growth and colonization is related to microbial composition in soils (Svenningsen et al, 2018). A recent report by Ordoñez et al. (2016) also found that some bacterial strains strongly affect AMF colonization inside roots and hyphae growth outside roots, and that soil microbial community might have a role in limiting or increasing this effect depending on the P-solubilizing microbial species (Ordoñez et al, 2016). These research evidences may explain the low % colonization in the *Fm*-inoculated IS-OU and IU-OS treatments as such situations would change hyphal associated microbial community composition when growing across two zones differ in microbial abundance and composition (sterilization difference). However, it is apparent that further research studies are needed to evaluate how changes in microbial abundances and/or composition in salt-affected soils could influence AMF colonization and their growth in such soils.

3.5.2 Soil extractable P and its uptake by plants

In the current study, sterilization significantly reduced extractable P in hyphosphere soils (IS-OS and IS-OU) compared to unsterilized ones (IU-OS and IU-OU) in both *Fm*-inoculated and uninoculated treatments. For instance, in IS-OS treatments of both *Fm*-inoculated and uninoculated, extractable P in hyphosphere compartments was reduced by 20 % and 18.7 %, respectively compared to IU-OU. Thus, this indicates a greater role in P solubilizing ability for native soil microbes comparing to AMF alone. The findings in this study suggest that AMF cooperation with native soil microbes is critical in solubilizing P in saline soils. Therefore, this suggestion supports the notion that role of AMF hyphae seems to be more efficient exploration of the soil volume and transfer of solubilized P to plants (Ruiz-Lozano and Azcón, 2000) than their ability to actively solubilize P

independently of interacting with native soil microbes in the hyphosphere. Research evidence has indicated the role of AMF in increasing plant P uptake in salt-stressed soils (Evelin et al, 2009; Zhang et al, 2017). Although the mechanisms behind AMF ability to solubilize soil P has not been fully explored (Antunes et al., 2007), some suggested mechanisms have been proposed including: (1) increased physical exploration by mycorrhizal root through extensive hyphal network, which reduces distance of diffused P while increases surface area of absorbing sites (Sanders and Tinker, 1973); (2) movement of P into roots due to high affinity for phosphate (Howeler et al, 1982) and through lower threshold concentration for P absorption (Mosse et al, 1973) in mycorrhizal roots compared to non-mycorrhizal roots; and (3) direct modification of rhizosphere zone through production of organic acids and phosphatase enzymes (Parfitt, 1979; Bolan, 1991), and/or indirect modification through the production of chelating agents such as siderophore by AMF or other soil microbes (Jayachandran et al, 1989). However, the ability of AMF to solubilize soil P independently of other soil microflora has been questioned, especially for less soluble inorganic P such as rock phosphate (Jayachandran et al., 1989) compared to organic P sources such as phytate (Koide and Kabir, 2000). Jayachandran et al. (1989) suggested that plant access to rock phosphate was the results of interactions between AMF and the native soil microorganisms.

In respect to salt-affected soils, studies evaluating AMF-microbial interactions roles in P availability are mostly conducted under artificial saline conditions with only a few AMF and other microbial PGPR inocula species such as P-solubilizers (Kohler, 2009; Zhang et al, 2011). Such studies only show the role of co-inoculation of AMF and P solubilizer microbe(s) in sterile soil under artificial salt condition. Therefore, the combined

AMF-native microbial community interactions have not been assessed in naturally salt-affected soil. For example, Zhang et al. (2011) studied the interaction effects of two AMF species (*Glomus aggregatum* and *Glomus mosseae*) and a fungal P-solubilizer (*Mortierella* sp.) isolated from saline soils of seashore on the growth of seashore mallow and activities of soil enzymes at different salinity levels. In their study, inoculation with either of the two AMF species alone resulted in significantly higher P availability (Olsen P) in bulk soil compared to inoculation with *Mortierella* sp. alone (almost by double) in a 0 mM NaCl treatment. However, under a 100 mM NaCl treatment, inoculation with *Mortierella* sp. alone significantly increased P availability in bulk soil by almost the double compared to AMF species each alone. Moreover, even when compared to inoculation with all species combined (the two AMF species + the P-solubilizer fungus) in their study, inoculation with *Mortierella* sp. alone had the highest available P in bulk soil in the 100 mM NaCl treatment, but not the 0 mM NaCl treatment. They suggested that due to adaptability of the fungal P-solubilizer to a saline soil, the fungus was not activated under the non-saline condition, but significantly flourished under the 100 mM NaCl treatment. This research supports the findings in my study that native soil microbes or P-solubilizers naturally occurring in salt-affected soils are more effective in increasing P availability or solubility than AMF alone. However, although in Zhang et al. (2011) study the fungal P-solubilizer resulted in higher P availability under salt stress, this impact was in sterile and artificial saline conditions. Such results may not be feasible under natural conditions where survival and competitions with native microbial community may take place (Malusá et al, 2012). Nonetheless, as has been suggested in literature, the role of AMF in soil seems to be more profound in exploring new soil volume rather than directly or actively solubilizing P

independently of interacting with other soil microbes, which may indicate that AMF act only as conduits for P solubilized by other soil microbes (Jayachandran et al, 1989; Bonfante and Anca, 2009; Hodge, 2017).

In the present study, inoculation with *Fm* significantly increased P concentration in plant shoots in the IS-OU and IU-OU treatments, where the rhizosphere (outer) compartment were unsterilized, compared to uninoculated ones. On the other hand, inoculation with *Fm* did not significantly impact P uptake in IS-OS and IU-OS treatments, which had sterilized soils in their outer compartments. These variations in P uptake between the above treatments support the earlier notation that the outer compartment had more volume (~245 cm^3, with total P mass of ~ 10.6 mg based on Mehlich-III prior conducting the experiment) compared to the hyphosphere compartments (~33 cm^3, with total P mass of ~ 11.1 mg based on Mehlich-III + oP addition + iP addition prior conducting the experiment) in the designed microcosm of this study. Therefore, plant roots and AMF in the outer compartment of unsterile soils may have had access to more native iP sources (since no P amendment were added to outer compartments) in soil due to the interactions with the native soil microbes, as illustrated in the extractable P results of unsterilized hyphosphere soils. However, in *Fm*-inoculated IS-OS treatment, shoot P concentration was not significantly different from all other treatments. Thus, the above explanations may explain literature reports that AMF might be only capable of hydrolyzing oP such as phytate (Koide and Kabir, 2000) rather than sparingly soluble iP (Jayachandran et al., 1989) such as Ca-phosphate in saline soils. Consequently, plants may have relied more on oP uptake by AMF, as the latter might have had more access to phytate in the hyphosphere compartment of IS-OS treatment due to the reduction in *phoD* harboring

microbial community caused by sterilization (compared to IU-OS treatment). Therefore, equalizing P demand in comparison to *Fm*-inoculated treatments where the outer compartments were unsterilized. The total P content in plant shoots aligns with the shoot P concentrations results in the IS-OS and IU-OU treatments, especially in the case of *Fm*-inoculated IU-OU where P was significantly higher in plant shoots compared to uninoculated treatment; thus, supporting the earlier explanation that there was less extractable P in the hyphosphere soil of *Fm*-inoculated IU-OU compared to uninoculated one because it was translocated to the plant. However, in the case of IS-OU and IU-OS, although P content in plant shoots was not significantly different between inoculated versus uninoculated treatments, it seemed to not match with the trend of shoot P concentrations results. This could be related to the differences in the shoot dry weights of these treatments, which were reflected in their total P content, as in the case of uninoculated IU-OS which had the highest shoot dry weight observed in this study. As illustrated earlier, this increase in shoot dry weight in the uninoculated IU-OS may have resulted from root hair growth and access to P pool in the unsterile hyphosphere compartments in demand for P since the outer compartment was sterilized (less available P) and did not have AMF inoculation.

3.5.3 Quantification of microbial genes

Solubilization and release of complexed P in soil are largely dependent on the functions of the microbial community in soils. These functions are the mineralization of oP through phosphatase enzymes (e.g., ALP) or solubilization of iP by low molecular weight organic acids (LMWOAs) as well as inorganic acids to release bioavailable orthophosphate (Sylvia et al, 2005). In this study, I only focused on the detection of potential ALP enzyme

activity and its gene (*phoD*) abundance as an indicator of organic P solubilization in soils since directly detecting LMWOAs as indicators of iP transformation is a challenge since they exist in low quantities (especially in a root-free soil as in hyphosphere) and are highly susceptible to degradation or utilization by microbes within short amount of time (Gunina et al, 2014). On the other hand, inorganic acids such as carbonic acids are very reactive and dissociate quickly in high soil pH (Qadir et al, 1996) as in this study (pH=8.5).

In this research study, the relative abundance of *phoD* was significantly reduced with sterilization (IS-OS) in both *Fm*-inoculated (by 78.3 %) and uninoculated (by 77.7 %) treatments compared to unsterilized soils (IU-OU). Also, within unsterilized treatments, inoculation with *Fm* lead to significantly higher *phoD* gene abundance compared to uninoculated. These results support my hypothesis that AMF and indigenous microbe interactions increase P availability and its plant uptake. These results suggest that the increased plant P uptake in this study in the *Fm*-inoculated IU-OU soil resulted partly from the increased abundance of *phoD* harboring microorganisms, as this treatment had the highest *phoD* abundance compared to all other treatments. The total bacterial (16S rRNA) and AMF (18S rRNA) gene abundances did not show any significant differences in the different treatments of this study. However, abundance of total fungi (ITS) was significantly reduced with sterilization. In this study, the *phoD* gene abundance was higher than the total 16S rRNA gene abundance, which was expected since other soil microbial community members (such as fungi and archaea) also possess this gene homologue (Ragot et al, 2015). Archaea are more likely to exist in a very low abundance in dry soils (Richter et al, 2014), such as the one I used in this study, collected from an area dominated by dry, saline, and high shrink-swell potential soils (Jaco, 1980). Therefore, the difference between

phoD and the 16S rRNA gene abundances were more likely to be caused by the fungi possessing the *phoD* gene. Supporting this view is the ITS abundance in this study, which was significantly higher in unsterile compared to sterile soils. Thus, in the *Fm*-inoculated IU-OU treatment, the significantly higher plant P uptake compared to uninoculated IU-OU probably resulted from the *phoD*-harboring microbial community (bacteria and fungi) mineralizing phytate, which was then taken-up by the AMF. This is more apparent when we look to the relative proportions of *phoD* gene among the total microbial community abundance (as 16S rRNA + ITS) which ranged from 0.30 in the uninoculated IS-OS up to 0.71 in the *Fm*-inoculated IU-OU.

3.5.4 Alkaline phosphatase activity

Soil sterilization significantly reduced ALP activity compared to unsterilized soils in both *Fm*-inoculated (reduction by 78 %) and uninoculated (reduction by 70 %) treatments. However, the inoculation with *Fm* significantly reduced ALP activity in the hyphosphere soils, as the highest ALP activity was found in the uninoculated treatments in both sterile and unsterile soils. It is not clear why AMF caused less ALP activity in this study. However, since extractable P was only measured at one time point post-harvest with no prior sampling points during the plant growth period, much of the P had possibly been solubilized by AMF-microbe cooperation and mobilized and translocated to the plant prior to harvesting. This is more apparent when knowing that autoclaving in this study did not significantly affects extractable P when comparing unsterilized to sterilized soils (46 and 47 mg P/kg soil, respectively) prior conducting the experiment. Therefore, when hyphosphere soil was tested post-harvest, soil P concentrations trended higher (but not significant) in all uninoculated pots compared to *Fm*-inoculated pots. Supporting this view

is the higher trend of P concentrations in plant shoots of all *Fm*-inoculated plants (only significant with IS-OU and IU-OU treatments) compared to uninoculated plants. Interestingly, the treatments with significantly higher plant P uptake with *Fm* inoculation compared to uninoculated ones (IS-OU and IU-OU) had unsterilized soil in their rhizosphere (outer) compartments (more volume). Hence, this may indicate higher access to native soil iP by AMF in the presence of indigenous soil microbes. Moreover, this may explain the low ALP activity found with *Fm*-inoculated pots compared to uninoculated ones. Literature reports have also shown positive correlations between ALP activity and soil P levels exist (Harrison, 1983; Garg and Bahl, 2008), which support the findings in the present study that uninoculated pots trended higher (but not significantly different) in extractable P concentrations at the sampling time compared to *Fm*-inoculated pots.

The above interpretations may also explain the different trends between *phoD* gene abundance and ALP activity observed in this study, where *Fm* inoculation has significantly reduced ALP activity while *phoD* abundance was significantly increased. This negative correlation probably was owing to the reduction of P solubility/availability caused by *Fm* inoculation as illustrated above. Research evidence has also shown similar trend where *phoD* gene abundance was significantly increased under low availability of soil P (Fraser et al, 2015b). Furthermore, it has been suggested that some microbial species have more inducible ALP genes that drive the enzyme production than other microbes (Fraser et al, 2015a). Also, there are multiple ALP encoded genes that have been identified in Pho regulon such as *phoD*, *phoA*, and *phoX*, and that 32 % of sequenced prokaryotic genomes contain at least one of these three genes (Zimmerman et al, 2013). However, *phoD* gene was identified as the key ALP encoded gene in soils (Tan et al, 2013). In their study, Tan

et al. (2013) has shown that high P availability lead to an increase in certain bacterial phyla (such as *Proteobacteria*) and a decrease in other phyla in term of abundance. Consequently, these research evidences support the notion that some microbial species with highly inducible *phoD* gene might flourish in the absence of AMF. Such ecological aspects might be related to the antagonistic interactions or/and competition on nutrients such as soluble/available P between both AMF and other soil microbes.

Very few studies on soil ALP activities as influenced by AMF under salt stress exist, which highlights research necessity to examine AMF roles in affecting soil enzyme activities in salinized soils. Most studies that have evaluated ALP activities in mycorrhizal plants under salt stress were mainly focused on root ALP rather than soil ALP activities (Grzybowska, 2004; Rabie and Almadini, 2005; Kohler, 2009). For example, Rabie and Almadini (2005) reported that root ALP activity decreased from 629 u/ml/min to 411 u/ml/min in mycorrhizal roots grown under soil salt stress of 1.5 dS/m and 6 dS/m, respectively. To the contrary, Grzybowska (2004) reported that ALP activity significantly increased by 45 % when salt level increased from 3.92 mS/m (0.03 dS/m) to 24.7 mS/m (0.24 dS/m) in plants inoculated with *G. geosporum* isolated from a saline soil, while no significant differences were found with inoculation with *G. intraradices* isolated from a non-saline soil. However, both studies did not report data from soil ALP activity.

In contrast to the present study and supporting the findings by Grzybowska (2004), a couple of studies have indicated increased soil ALP activity with co-inoculation of AMF and a fungal P-solubilizer in both sterile (Zhang et al, 2011) and unsterile (Zhang et al, 2014) saline soils. Thus, indicating a gap of knowledge in our understanding of the relationship between ALP activities and AMF in salt-affected soils.

In non-saline soils, studies on correlations between AMF and ALP activities are also not well understood as some contradictions exist. Some reports have shown a positive correlation (Tarafdar and Marschner, 1994), while others showed a negative one (Kunze et al, 2011), or no effect (Joner et al, 1995) of AMF on ALP activity. Similar contradictions also exist in the correlation between ALP activity and available P in soil. Some studies have found a positive correlation between the total available P in soil and ALP activity (Harrison, 1983; Garg and Bahl, 2008), while other studies suggested an inverse relationship (Tan et al, 2013).

Our knowledge about the AMF-soil microbial interactions driving the P cycle and their correlation to phosphatase encoded genes and P availability in salt-stressed soils is still limited. Future research will shed more light into the mechanisms driving AMF influence on P availability, ALP and its encoded microbial genes in salt-stressed soils. Such knowledge will enhance our ability for better management of P resources in salt-stressed soils.

3.6 Conclusion

In this study, I found a significant role of AMF-hyphosphere microbial community interactions in increasing extractable P as well as plant P uptake in saline soil. In all *Fm*-inoculated treatments, soil sterilization significantly reduced soil extractable P, *phoD* gene abundance and ALP activity compared to unsterilized soils. Thus, indicating the greater role of native microbial community in increasing P availability in salt-stressed soils, while AMF role seems to be less efficient in increasing P availability independently of interacting with these microbes.

This study along with the increased evidences in the scientific fields, highlight the role of AMF and its associated microflora in the hyphosphere in improving P uptake by crops. Thus, managing AMF/soil microbiome synergistic interactions seems to be a promising tool for the sustainable agriculture, especially under biotic or abiotic stresses such as salinity. Moreover, future studies should consider the contributions of other microbially regulated processes (release of low molecular weight organic acids and other phosphatase enzymes) and chemical processes (e.g., inorganic acids) driving P cycle in soils to have a comprehensive understanding of all aspects contributing to P availability in soils. Such tools and knowledge will enable us to better manage P resources in salt-stressed soils as well as in other agricultural lands with the increased food demand in our world.

CHAPTER IV

EFFECTS OF BIOCHAR ON ARBUSCULAR MYCORRHIZAL FUNGAL COLONIZATION AND PLANT PHOSPHORUS UPTAKE IN A SALINE CLAY SOIL

4.1 Synopsis

The use of biochar (BC) as a soil conditioner/amendment has attracted much attention in recent years due to its diverse beneficial effects on soil health. However, our knowledge about BC interactions with arbuscular mycorrhizal fungi (AMF) in the remediation of salt-affected soils are still limited. In this study, I investigated the role of BC (pine wood, 500 °C) and AMF (*Funneliformis mosseae*) as a joint management in affecting AMF colonization and plant P uptake (Winter Malt Barley) under salt stress in a growth chamber for 42 days. I also investigated their impact on extractable soil P (Mehlich-3), soil alkaline phosphatase (ALP) activity, and abundances of microbial alkaline phosphatase (*phoD*), bacterial 16S rRNA, AMF 18S rRNA, and fungal internal transcribed spacer (ITS) gene targets in rhizosphere and bulk (root-free) soils. The experiment was conducted using a two-compartment microcosm (root growth compartment and root-free compartment as bulk soil) separated with 25 µm nylon mesh. The soil had 4 treatments: control (no BC with control no-AMF inoculum), BC application at 3 % w/w (with no-AMF control inoculum), AMF as *Funneliformis mosseae* (*Fm*), and BC+AMF (*Fm*). All soils were amended with 200 mg/kg P in the form of Na-phytate as organic P (oP) and NaCl to raise soil EC to 16 dS/m. The combined treatment of BC+AMF

significantly increased plant shoot and root growth, plant P uptake (by 70.2 % over control), and extractable P in the bulk soil. For AMF colonization, BC alone significantly reduced AMF colonization by 56 % compared to control, possibly due to less reliance of the plant on AMF symbioses, but was not significantly different from AMF and BC+AMF. Similarly, BC+AMF significantly reduced % colonization by 69.6 % compared to control and by 51 % compared to AMF alone. The targeted microbial genes in this study significantly increased in all treatments over control in the rhizosphere soil, but not in the bulk soil. The effect of BC and *Fm* seems to be mainly through improving plant growth conditions and P uptake, respectively. Overall, the findings in this study suggest that the combined application of BC+AMF inocula can significantly improve plant production and P availability in salt-affected soils.

4.2 Introduction

Biochar has gained much attention in recent years due to its diverse benefits to plants, soil, and the environment. Some of these benefits include water and nutrient retention in soil, carbon sequestration (reduce greenhouse gas emissions), increased populations of beneficial soil microbes, increased soil fertility, etc. (Atkinson et al., 2010). In addition, the role of arbuscular mycorrhizal fungi (AMF) is well known in improving plant nutritional status (especially phosphorus) as well as water uptake. However, knowledge about AMF occurrence and development in salt-affected soils is scarce and contradictory. For example, relatively high AMF populations have been reported in some AMF studies in saline soils (Sengupta and Chaudhuri 1990; Bhaskaran and Selvaraj 1997), while other studies indicated small AMF populations (Kim and Weber, 1985; Barrow et al., 1997). However, it seems that AMF spore numbers do not correlate with soil salt levels

(Aliasgharzadeh et al., 2001), while the percentage of AMF colonization seems to generally have a negative correlation with the salt levels (Guo and Gong, 2014; Miransari, 2017), although some studies (including the study in Chapter II) have shown increased colonization with higher salt levels (Aliasgharzadeh et al., 2001; Giri and Mukerji, 2004). It is not clear how salt stress affects AMF occurrence in saline soils. It may be due to salt effects on the plant, the AM fungus, or both. Yet, there is evidence that increasing soil NaCl concentrations reduce root infection by AMF due to hyphal growth reduction or inhibition (McMillen et al., 1998). It is not clear whether this effect is due to specific ion toxicity or indirectly by osmotic stress in soil solution. On the other hand, the evidence that hyphal growth can occur under high salt concentration in soil has been linked to maintenance of turgor by mycorrhizal hyphae (Cooke and Whipps, 1993), which requires energy.

Many studies have suggested that addition of nutrients to salt-affected soils resulted in improved plant growth, possibly by compensating for the low plant uptake of nutrients resulting from osmotic stress of saline soils (Hu and Schmidhalter, 2005; Ahanger and Agarwal, 2017). Since biochar sources are organic wastes, they can provide a wide range of plant nutrients at different nutrient release rates. Thus, addition of biochar to salt-affected soils may improve soil fertility status as well as nutrient uptake by plants in these soils (Tagoe et al., 2008; Atkinson et al., 2010; Drake et al., 2016). Moreover, although some evidence indicates a role of biochar in changing or shifting rhizosphere microbial compositions (Grossman et al., 2010; Liang et al., 2010), the correlation between biochar and changing soil biota and its processes haven’t been yet fully understood and described (Lehmann et al., 2011).

Biochar has enormous surface area and different cation exchange capacities (CEC), which vary depending on the activation methods of biochar (Azargohar and Dalai, 2008). Consequently, its addition to soil will also increase soil CEC (Joseph et al., 2009). Biochar can also adsorb ions on its surfaces, and hence, prevent nutrient leaching (Yao et al., 2011). However, nutrients on biochar surfaces may not necessarily be available to plants, since most roots are unable to access the micro-habitat structures of the biochar due to their size, as the diameter of the finest roots of most plant species are of several hundred µm (Fitter, 2002). On the other hand, AMF hyphae are finer and much smaller in diameter, therefore, can capture adsorbed nutrients on biochar surfaces (Hammer et al., 2015). AMF are known to increase plant P uptake. (Evelin et al, 2009). Thus, since biochar has been shown in recent studies to adsorb and desorb phosphate on its surfaces (Yao et al., 2011; Trazzi et al., 2016), the presence of AMF may further increase or enhance plant P uptake through the access to biochar surfaces by its hyphal network. In addition, some literature reports have also indicated that both AMF and biochar can significantly enhance root system architecture (RSA) and change soil microbial community compositions (Wu et al, 2012; Xiang et al, 2017).

There are only a few studies that have evaluated the combined effects of biochar and AMF on plant growth, mycorrhizal colonization and P availability. Such studies have been mostly conducted in non-saline soils and show contradictory results (Warnock et al, 2010; Elmer and Pignatello, 2011; Yamato et al, 2006). For example, Warnock et al. (2010) reported that addition of different types of biochar did not affect plant biomass production and either decreased or did not affect AMF colonization, but soil P availability was increased. In contrast, Elmer and Pignatello (2011) found increased AMF colonization

with biochar addition, but no effects were found on plant P uptake. On the other hand, the studies that investigated combined effects of biochar and AMF under salt stress were conducted in sterilized and artificially saline soils (Hammer et al, 2015). Therefore, such studies don't consider soil microbial roles in interacting with biochar as well as with AMF, since the latest has been shown to increase P availability and uptake by plants (as illustrated in Chapter 3).

Hence, the goal of this study was to understand the effects of AMF and biochar in improving soil P extractability and its uptake by plant as well as increasing mycorrhizal colonization in a naturally unsterile saline soil. Therefore, I hypothesized that biochar amendment would increase soil extractability and plant uptake of phosphorus and improve AMF colonization in a native unsterile saline soil.

4.3 Material and methods

4.3.1 Soil

Surface soil (top 0-30 cm) was collected near the Texas A&M AgriLife Research & Extension Center at Pecos in Reeves county, Texas, USA. The majority of soils found in this region are saline and moderately alkaline. The soil series in this region is Dalby clay and classified as Fine, smectitic, frigid Oxyaquic Vertic Hapludalfs (NRCS, USDA. Web Soil Survey). No vegetative cover or previous agricultural practices were present at the site where the soil was collected. Characteristics of the collected soil sample are listed in **Table 4.1**. The soil texture was determined by a hydrometer (Bouyoucos, 1962), and % organic matter content was determined by the wet oxidation method (Walkley and Black, 1934). Soil pH, EC (saturated paste extract), and soil P (Mehlich-3) were determined by the Soil,

Water and Forage Testing Laboratory Department of Soil and Crop Sciences, Texas A&M University.

Table 4.1. Characteristics of the soil used in third experiment.

Parameter	Value
pH	8.5
EC (saturated extract)	6.32
P (Mehlich-3, mg/kg)	45
Organic matter content (%)	0.34
Clay (%)	46.7
Silt (%)	20.7
Sand (%)	32.6

4.3.2 AMF inoculum and plant host

The AMF species used in this experiment was *Funneliformis mosseae* (*Fm*), obtained from INVAM (International Vesicular Arbuscular Mycorrhizal collection facility, University of West Virginia, accession code UT101) as whole inoculum containing different AMF propagules (soil with spores, infected root pieces, and hyphae). This AMF inoculum was chosen since it colonized plant roots well in my previous experiment also performed in saline clay soil (**Figure 2.1**). The addition of AMF inoculum was to ensure that the different effects such as plant P uptake were relevant to their addition when compared to uninoculated pots. Winter Malt Barley (Wintmalt, KWS Saat, Germany) (salt tolerant) was used as the plant host in this experiment since barley is one of the most salt-tolerant crops and the soil in this study had additional added salt to increase its level.

4.3.3 Biochar

Biochar (BC) in this study was obtained from Wakefield Agricultural Carbon LLC, Columbia, MO, USA. The physical and chemical properties of BC are listed in **Table 4.2**,

as reported by the manufacturer, except for EC which was determined using an EC meter in a 1:10 w/v biochar-water suspension. Biochar was grounded to pass a 2 mm sieve prior to use in this experiment.

Table 4.2. Physical and chemical properties of biochar (as reported by the manufacturer, except for EC).

Property	Specification/Value
Pyrolysis temperature	500 °C
Feedstock material	Soft wood (Pine)
Bulk density	0.48 g/cm^3
Total organic matter	95.12 % total mass
Total carbon	88.01 % total mass
Total organic carbon	87.67 % total mass
Total inorganic carbon	0.34 % total mass
Total ash	4.88 % total mass
pH	7.4
EC	0.35 dS/m (in 1:10 (w/v))
Nitrogen (N)	0.59 % wt
Total phosphate	4.53 mg/kg
Potassium (K)	614 mg/kg
Sulfur	0.031 % wt
Hydrogen	0.40 % wt
Oxygen	6.09 % wt
Calcium	4128 mg/kg
Copper	5.38 mg/kg
Iron	595 mg/kg
Magnesium	1225 mg/kg
Manganese	234 mg/kg
Zinc	4.59 mg/kg
Surface area	365.69 m^2/dry g
Particle size: <0.5mm	22.4 %
Particle size: <1mm	70.1 %
Particle size: <2mm	93.9%

4.3.4 Experimental design and growth conditions

The experimental design in this experiment was a 2×2 factorial completely randomized design with 3 replicates. Treatment combinations were 2 biochar (BC)

conditions (with/without biochar) and 2 AMF treatments (mycorrhizal and non-mycorrhizal), giving 4 different treatments: control (no biochar with no-AMF control inoculum), biochar only (BC), AMF only (AMF), biochar + AMF (BC+AMF). Biochar was added at a 3 % rate (w/w) as an average rate observed from different biochar studies in the remediation of salt-affected soils (Dahlawi et al., 2018). Square plastic pots (Stuewe & Sons Inc., Tangent, OR, USA; 10.2 cm width × 14 cm height with 1.2-liter volume) were used to grow plants. Additionally, each pot had 2 root-free compartments (as bulk soil) as mini plastic boxes (The Container Store Inc, Coppell, TX, USA; 4.5-cm long, 2.5-cm wide, 1.5-cm height) wrapped with a 25 µm nylon mesh (LAB PACK, Sefar Inc., Buffalo, NY, USA) and buried in middle depth of each pot. All soils were amended with 200 mg/kg P of Na-phytate as organic P and 50 mg N/kg soil as NH_4NO_3, in addition to NaCl to raise EC of soil to ~ 16 dS/m as a moderate salt stress for barley. Seeds were sterilized with 10 % sodium hypochlorite for 20 min and germinated in a sterile soil (autoclaved for 1 hr at 121 °C three times, on three consecutive days) of the experiment without BC in square nursery pots (6.5-cm diameter, 9-cm long, 280-ml volume) for 10 days. Then, seedlings were transplanted to the designed pots with 10 gm of inoculum (either AMF or no-AMF control inocula) mixed with soil at the seedling zone. Plants were grown for 42 days (starting from emergence) in a growth chamber at 25 °C day/21 °C night, 16 h/8 h light/dark, 60 % humidity, & 500 µmol /m^2/s light intensity, and watered every other day to 50 % water holding capacity (determined based on maximum water holding capacity) (Noggle and Wynd, 1941) with distilled water.

4.3.5 Plant growth parameters, root staining and AMF colonization

At harvest, plants were gently removed from the pots and shoots were separated from the root system. Shoots were placed in an oven at 60 °C for 48 hours and then weighed to get the dry weight. Roots were gently removed from the soil and shaken to remove loose soil particles. Remaining soil adhering to the roots was then tapped gently to free it from roots and collected as rhizosphere soil and stored at -80 °C for later analysis. The soil from the root compartment was then sieved to manually collect remaining root pieces. All roots were then washed under tap water. A subsample of roots was then separated and weighed before and after drying in an oven at 60 °C for 48 hours to obtain the moisture content for estimating total root dry weight. The remaining root system was then washed and scanned with an Epson WinRHIZO scanner (Regent Instruments Inc., Quebec, Canada) to obtain measurements of root length, root surface area, root diameter, and root tip number using an installed Epson WinRHIZO software version 2017a. After scanning, roots were cleared (to remove cytoplasm content of cells) and stained with trypan blue using a modified procedure of Phillips and Hayman (1970). Briefly, roots were placed in tissue cassettes (Fischer Scientific Inc., Hampton, NH, USA) and submerged in pre-boiled 10 % KOH for 10 min to remove cytoplasmic content of root cells. Cassettes were then washed 5X with tap water and submerged in 2 % HCl for 30 min, followed by 5X washing with tap water. The cassettes were then submerged in pre-boiled 0.05 % trypan blue solution (water, glycerin, lactic acid in 1:1:1 (v/v/v)) for 5 min. The cassettes were then washed 5X with tap water and stored at 4 °C for 3-5 days immersed in distilled water to remove excess stain. Percentage of AMF colonization was determined using the gridline intersection method (Giovannetti and Mosse, 1980). Growth parameters of root

dry weight, shoot dry weight, shoot to total dry weight and total dry weight were then calculated.

4.3.6 Soil extractable P, plant shoot P content and salinity parameters

After plant harvest, approximately 300 gm of soil without roots from the root growth compartment were collected and stored at -80 °C for later analysis. The soils from the root-free compartment was then collected after removing and discarding the surface layer (~2mm) where there was possible exchange of microbes and nutrients between both compartments. Soils of both root-free compartments of each pot were then mixed to have a one homogenized root-free soil/pot and stored at -80 °C for later analysis. Soil samples from both compartments (previously stored at -80 °C), and the dried plant shoots were all submitted to the Soil, Water and Forage Testing laboratory at Texas A&M University (College Station, TX, USA) to measure extractable P in soil (Mehlich-III) (in root and roo-free compartments), soil pH (from saturated extract), sodium adsorption ratio (Na relative to Ca and Mg in a water extract from a saturated past using ICP analysis) and EC (based on a saturated paste extract) in root compartment soil only, and determine P, K and Na concentrations in plant shoots (ICP analysis). Shoot K/Na ratios were then calculated.

4.3.7 DNA extraction

Soil DNA was extracted from 0.5 g of soils (-80 °C) from the rhizosphere and the root-free compartments (as bulk soil) using DNeasy PowerSoil Pro DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. After extraction, all DNA samples were quantified to detect DNA quality using a Nanodrop ND-1000 spectrophotometer (Thermo-Fisher Scientific Inc., Wilmington, DE, USA).

4.3.8 Quantitative PCR assays

Quantitative real-time PCR (qPCR) was used to quantify the abundances of microbial *phoD* (alkaline phosphatase), total bacterial 16S rRNA, total AMF 18S rRNA, and total fungal internal transcribed spacer (ITS) gene targets in both rhizosphere and bulk soil (root-free soil). For quality control, all qPCR runs included 5 different concentrations of DNA standards (gBlock standards, Integrated DNA Technologies Inc.) for each target gene (for standard curve)(details on these standards are same as in **Table 3.2** in Chapter 3), no-template control (NTC), positive control, negative control, and 2 spiked random samples from the study's DNA samples with one of the standards to test for possible qPCR inhibitors. Standards and NTC were run in triplicate, and the rest of controls and experimental samples were run in duplicate. Details on each target gene positive and negative controls, R^2 value, and reaction efficiency of standard curves are listed in **Table 4.3**. Primers (obtained from Integrated DNA Technologies Inc.), qPCR conditions and references are outlined in **Table 4.4**. Amplifications of DNA was performed using Rotor-Gene SYBR® Green qPCR kit, with gene abundance measured using Rotor-Gene Q Software version 2.3.1.49 (QIAGEN, Hilden, Germany).

4.3.9 Alkaline phosphatase enzyme assay

Potential soil alkaline phosphatase (ALP) activity was measured from the frozen rhizosphere and bulk (root-free) soils (-80°C) using a modified assay of Tabatabai and Bremner (1969). In addition, ALP activity in soil with and without BC were measured using the same soil (as collected from field) to test possible interference of BC with the enzyme assay itself. For all enzyme assays, 0.5 g soil in duplicate was incubated in *p*-nitrophenyl phosphate substrate (Sigma-Aldrich, USA) along with modified universal

buffer solution (pH 11) at 28°C in 2 mL deep-well plate. After 2 h, reactions were stopped with 2.5 M $CaCl_2$ and 2.5 M NaOH. Plates were then shaken for 5 min and centrifuged for 5 min at 500g-force. Using a 96-well plate, formation of *p*-nitrophenol was determined colorimetrically using a Biolog Microstation Elx808BLG (BIO-TEK Instruments Inc., Winooski, VT, USA) spectrophotometer at 405 nm.

4.3.10 Statistical analysis

All treatment effects were statistically analyzed using ANOVA in a SAS software (version 9.4), using PROC GLM procedure. Differences between treatments were obtained using Fisher's least-significant-difference (LSD) test at a *p*-value of <0.05.

Table 4.3. Quality control details of the qPCR runs in third experiment.

Target microbial group	Positive control	Negative control	R^2 value of standard curve	Reaction efficiency
phoD-harboring microbes	*Sinorhizobium meliloti*	*Escherichia coli* K-12	0.99	0.90
16S rRNA	*Escherichia coli* K-12	*Methanospirillum hungatei*	0.99	0.94
AMF 18S rRNA	*Glomus intraradices*	*Escherichia coli* K-12	0.99	0.95
ITS	*Rhizopus microsporus*	*Escherichia coli* K-12	0.99	1.00

Table 4.4. Primers and conditions used for the qPCR assays in third experiment.

Target microbial group	Primers and sequences	qPCR reaction mixture	Thermal profile	Reference
phoD-harboring microbes	*phoD*-F733(5'-TGGGAYGATCAYGARGT-3')/ *phoD*-R1083 (5'-CTGSGCSAKSACRTTCCA-3')	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O.	5 min at 98°C for initial denaturation; 35 cycles of 30 s at 98°C, 30 s at 58°C, extension for 30 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 55-98°C (1° and 5 s/cycle melt).	Modified after Ragot et al (2015)
Total bacteria (16S rRNA)	341f-(5'-CCTACGGGAGGCAGCAG-3')/ 797r-(5'-GGACTACCAGGGTATCTAATCCTGTT-3')	7.5 µl SYBR Green (2x) Master Mix, 0.225 µl F primer (0.3 µM), 0.675 µl R primer (0.9 µM), 2 µl DNA template, 4.6 nuclease free H_2O.	3 min at 98°C for initial denaturation; 40 cycles of 30 s at 98°C, 30 s at 61.5°C, extension for 20 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50-99°C (1° and 5 s/cycle melt) after a pre-melt conditioning for 90 s at 50°C.	Modified after Harter et al (2014)

Table 4.4. Continued.

Target microbial group	Primers and sequences	qPCR reaction mixture	Thermal profile	Reference
Total AMF (18S rRNA)	GC-AMV4.5NF-(5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G [GC clamp] AAG CTC GTA GTT GAA TTT CG-3′)/ AMDGR-(5′-CCC AAC TAT CCC TAT TAA TCA T-3′)	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O.	10 min at 98°C for initial denaturation; 35 cycles of 30 s at 98°C, 30 s at 55°C, extension for 45 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50-98°C (1˚ and 5 s/cycle melt).	Modified after Sato et al (2005)
Total fungi (ITS)	ITS1f-(5'-TCC GTA GGT GAA CCT GCG G-3')/5.8s-(5'-CGC TGC GTT CTT CAT CG-3')	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O.	10 min at 98°C for initial denaturation; 35 cycles of 60 s at 98°C, 30 s at 53°C, extension for 45 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 48-98°C (1˚ and 5 s/cycle melt).	Modified after Fierer et al (2005)

4.4 Results

4.4.1 Plant growth responses and AMF colonization

The different growth responses of barley to BC and AMF are shown in **Figures 4.1 – 4.3**. Both biochar and AMF significantly increased plant height compared to the control (**Figure 4.1 A)**, with the highest height found in the BC+AMF treatment. However, compared to the control, biochar alone significantly increased plant growth in terms of shoot dry weight, root dry weight, and total plant dry matter (**Figure 4.1 B**, **C**, **and Figure 4.2 A**, respectively). Shoot:total plant dry matter did not show significant differences between all four treatments (**Figure 4.2 B**). For root length (**Figure 4.2 C**), AMF and AMF+BC were significantly higher than the control with 55.5 and 103.9 % increases, respectively, with BC+AMF being significantly higher than all other. Root length in BC treatment was higher than the control but was not significantly different. No significant difference was found in root length between the BC and AMF treatments. Similarly, root surface area (**Figure 4.3 A**) was significantly higher than the control with 61.4 and 84.6 % increases in the AMF and AMF+BC, respectively. Biochar also trended higher than control in root surface area but was not significantly different. However, root surface area in BC was significantly less than BC+AMF, but not significantly different from AMF treatment. For root diameter (**Figure 4.3 B**), BC and BC+AMF resulted in significantly smaller root diameter by 15.5 and 10.16 % compared to control, respectively. When compared to AMF, BC and BC+AMF treatments also resulted in significantly smaller root diameter by 18.15 and 12.9 %, respectively. No significant difference was found in root diameter when comparing control to AMF and BC to BC+AMF. For root tip number (**Figure 4.3 C**),

BC+AMF was significantly higher than the control by 141 %, while no significant differences were found between all other treatments. For AMF colonization (**Figure 4.3 D**), BC and BC+AMF significantly reduced % colonization by 56 and 69.6 % compared to the control. Inoculation with *Fm* significantly reduced % colonization by 38 % over control but was not significantly higher than BC+AMF treatment. No significant difference was found between BC and AMF treatments.

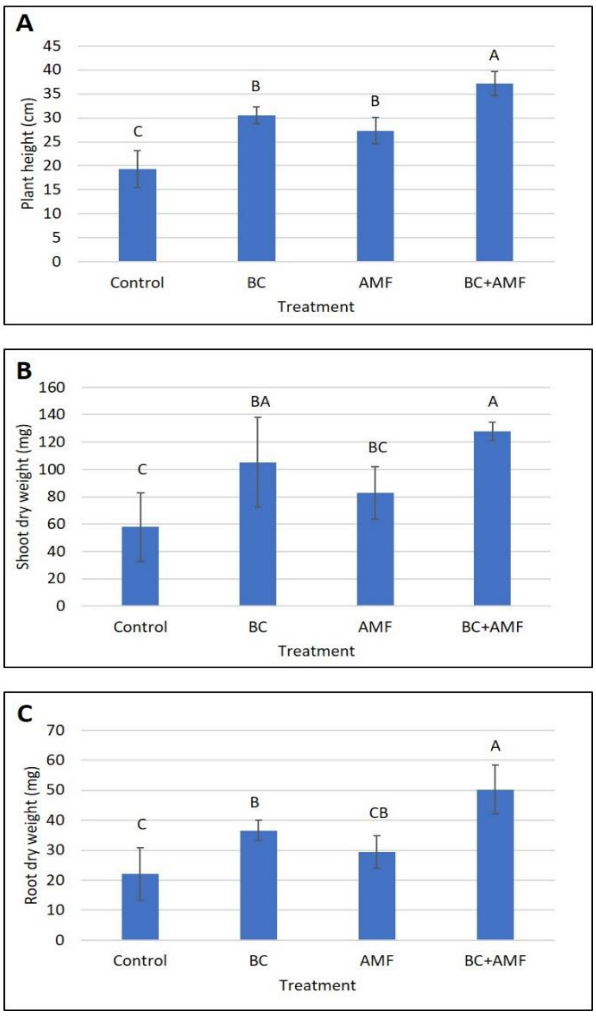

Figure 4.1. Plant and root growth parameters (part one). A: plant height. B: shoot dry weight. C: root dry weight. Control = saline soil with no biochar and no AMF. BC = saline soil with biochar. AMF: saline soil inoculated with *Fm* (*Funneliformis mosseae*). BC+AMF = saline soil with biochar and *Fm* inoculation. Data are means ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

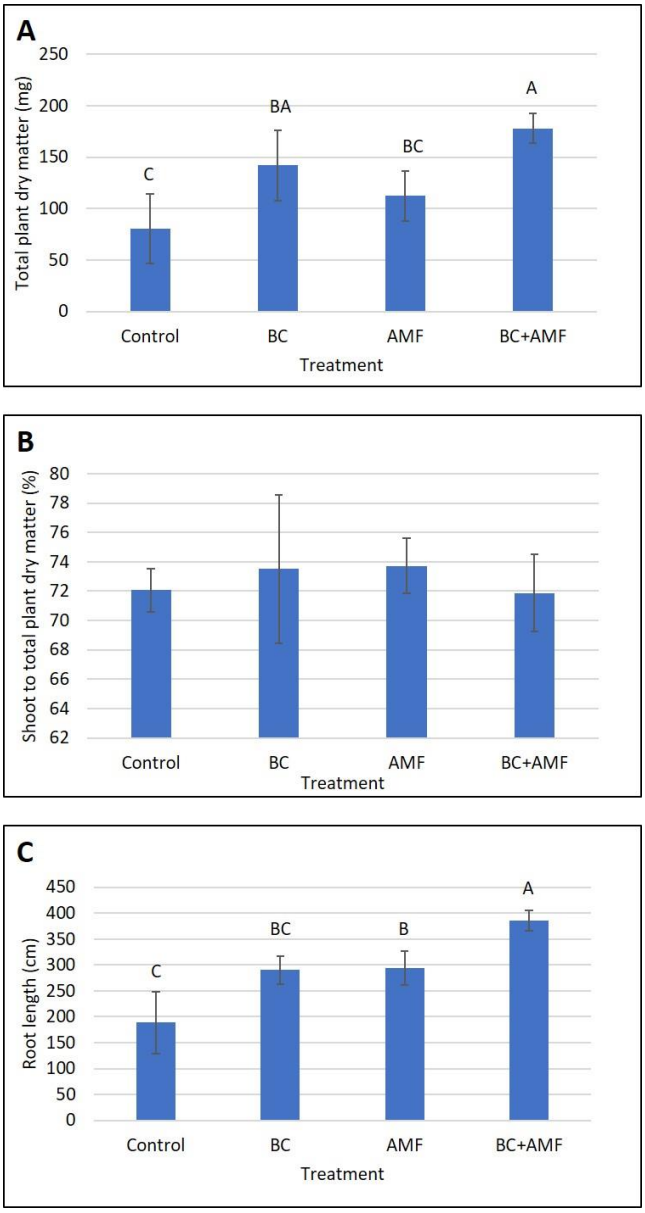

Figure 4.2. Plant and root growth parameters (part two). A: total plant dry matter. B: shoot to total plant dry matter. C: root length. Control = saline soil with no biochar and no AMF. BC = saline soil with biochar. AMF: saline soil inoculated with *Fm* (*Funneliformis mosseae*). BC+AMF = saline soil with biochar and *Fm* inoculation. Data are means ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

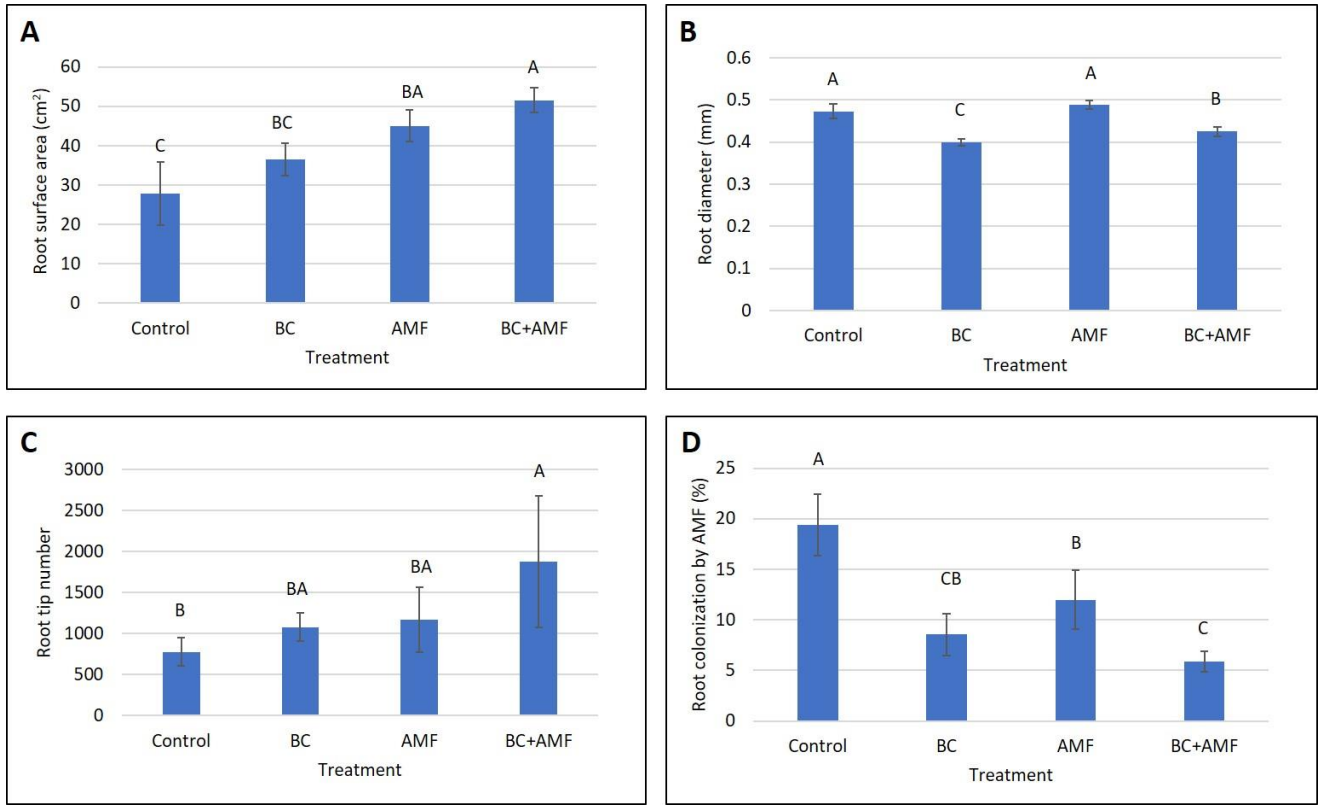

Figure 4.3. Root growth parameters and AMF colonization. A: root surface area. B: root diameter. C: root tip number. D: % root colonization by AMF. Control = saline soil with no biochar and no AMF. BC = saline soil with biochar. AMF: saline soil inoculated with *Fm* (*Funneliformis mosseae*). BC+AMF = saline soil with biochar and *Fm* inoculation. Data are means ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

4.4.2 Soil extractable P, plant shoot P content and salinity parameters

AMF and BC+AMF significantly increased plant P uptake in barley by 52.7 and 70.2 %, respectively (**Figure 4.4 A**). Biochar was not significantly different from control. Also, no significant differences were found between BC, AMF and BC+AMF in P uptake. For extractable P in soil (**Figure 4.4 B**), no significant differences were found among all treatments in the root compartment soils. However, BC and BC+AMF significantly reduced extractable soil P in the bulk soil (root-free) by 10.2 and 13.2 % compared to control, and by 10.6 and 13.6 % compared to AMF, respectively. No significant differences were found between BC and BC+AMF treatments or between control and AMF treatments in extractable P from bulk soil.

Post-harvest salinity parameters and shoot K:Na ratios are shown in **Table 4.5**. For shoot K:Na ratio (calculated from K and Na shoot concentrations obtained from the ICP analysis), treatments of BC, AMF and BC+AMF all resulted in significantly lower K:Na ratios, by 16.8, 25 and 23 %, compared to control, respectively. No significant differences were found in K:Na ratios between BC, AMF, and BC+AMF treatments. For soil pH, BC+AMF had significantly higher pH by 0.2 units compared to control. No significant differences were found in soil pH between BC, AMF, and BC+AMF. For soil EC, BC+AMF was significantly lower than AMF by 3.95 units, while no significant differences were found between control, BC and AMF treatments. Similarly, soil SAR was significantly lower by 5 units in BC+AMF compared to AMF, while no significant differences were found between control, BC and AMF treatments.

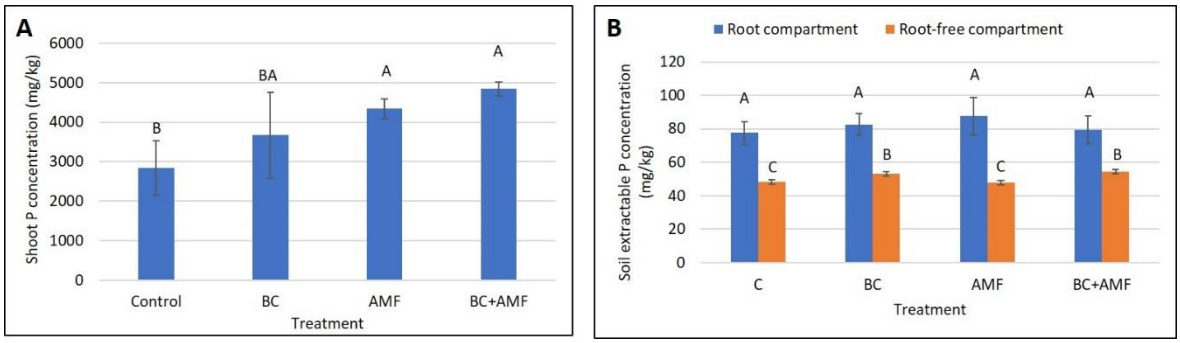

Figure 4.4. Extractable soil P and its uptake by plants in third experiment. A: shoot P concentration. Soil extractable P (Mehlich-3) from root and root-free (bulk) compartments. Control = saline soil with no biochar and no AMF. BC = saline soil with biochar. AMF: saline soil inoculated with *Fm* (*Funneliformis mosseae*). BC+AMF = saline soil with biochar and *Fm* inoculation. Data are means ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

Table 4.5. Plant shoot K:Na ratios and soil (from root compartment) post-harvest salinity parameters; soil pH, soil electrical conductivity (EC), and sodium adsorption ratio (SAR). Control = saline soil with no biochar and no AMF. BC = saline soil with biochar. AMF: saline soil inoculated with *Fm* (*Funneliformis mosseae*). BC+AMF = saline soil with biochar and *Fm* inoculation. Data are presented as the mean ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

Treatment	Shoot K:Na	Soil pH	Soil EC	SAR
C	1.20 ± 0.09 a	7.58 ± 0.1 b	7.57 ± 2.7 ba	18.14 ± 1.9 ba
BC	1.00 ± 0.09 b	7.63 ± 0.08 b	8.69 ± 1.9 ba	18.57 ± 1.4 ba
AMF	0.90 ± 0.14 b	7.64 ± 0.05 b	11.17 ± 1.3 a	21.42 ± 2.2 a
BC+AMF	0.93 ± 0.01 b	7.79 ± 0.04 a	7.22 ± 1.6 b	16.41 2.9 b

4.4.3 Alkaline phosphatase activity

Potential ALP activity of both rhizosphere and bulk soil is shown in **Figure 4.5 A**, expressed as µg *p*-nitrophenol/dry g soil/hr. In all treatments, activity of ALP was significantly higher in rhizosphere than bulk/root-free soil. No significant differences were found between the control and AMF in ALP activity in rhizosphere and bulk soils. Since

treatments with BC (BC & BC+AMF) was significantly low compared to control and AMF in rhizosphere and bulk soils, I further tested whether BC itself impacted ALP activity during the assay procedure. The data in **Figure 4.5 B** show that BC significantly interfered with ALP assay.

4.4.4 Quantitative PCR assays

Abundance of the all targeted genes are shown in **Figure 4.6**. In rhizosphere soil, *phoD* abundances (**Figure 4.6 A**) were significantly higher in BC, AMF and BC+AMF compared to the control. No significant differences were found in *phoD* abundances between the BC, AMF and BC+AMF treatments in rhizosphere soil. In the bulk soil, no significant differences were found between all four treatments in *phoD* abundances. Similarly, bacterial 16S rRNA gene abundance (**Figure 4.6 B**) was significantly higher in BC, AMF and BC+AMF compared to the control rhizosphere soil. No significant differences were found in 16S rRNA abundances between the BC, AMF and BC+AMF in rhizosphere soil. In the bulk soil, no significant differences were found between all four treatments in 16S rRNA gene abundance. Like *phoD* and 16S rRNA, AMF-18S rRNA gene abundance (**Figure 4.6 C**) was significantly higher in BC, AMF and BC+AMF compared to the control rhizosphere soil. No significant differences were found in AMF-18S rRNA abundances between the BC, AMF and BC+AMF rhizosphere soil. In the bulk soil, no significant differences were found between control, BC, and BC+AMF. However, AMF inoculation resulted in a significantly higher AMF-18S rRNA gene abundance than BC in the bulk soil. For ITS gene abundance (**Figure 4.6 D**), only BC+AMF resulted in a significantly higher gene abundance compared to control in the rhizosphere soil, while no

significant differences were found when compared to BC and AMF. In the bulk soil, no significant differences were found in ITS gene abundance between all four treatments.

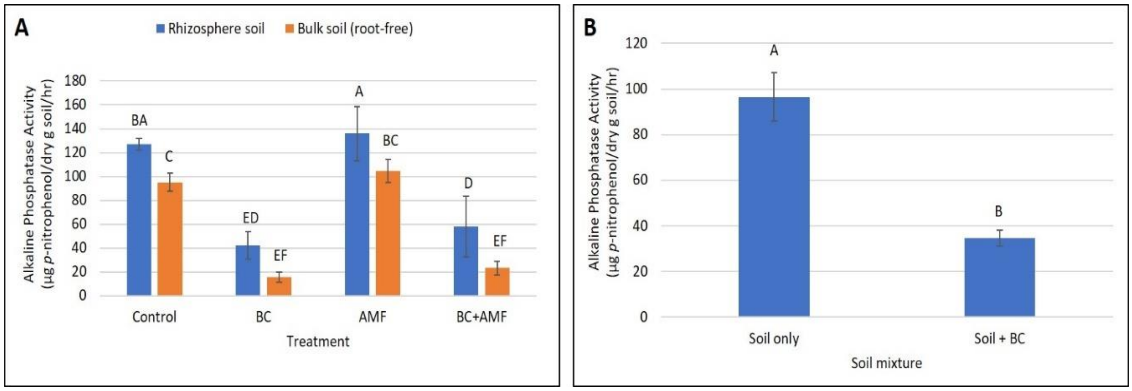

Figure 4.5. Potential soil alkaline phosphatase (ALP) activity. A: ALP activity in the rhizosphere and root-free soils. B: ALP activity in a field soil mixture with/without BC (data in figure B are presented as the mean ± s.d (n=8)). Control = saline soil with no biochar and no AMF. BC = saline soil with biochar. AMF: saline soil inoculated with *Fm* (*Funneliformis mosseae*). BC+AMF = saline soil with biochar and *Fm* inoculation. Data are presented as the mean ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

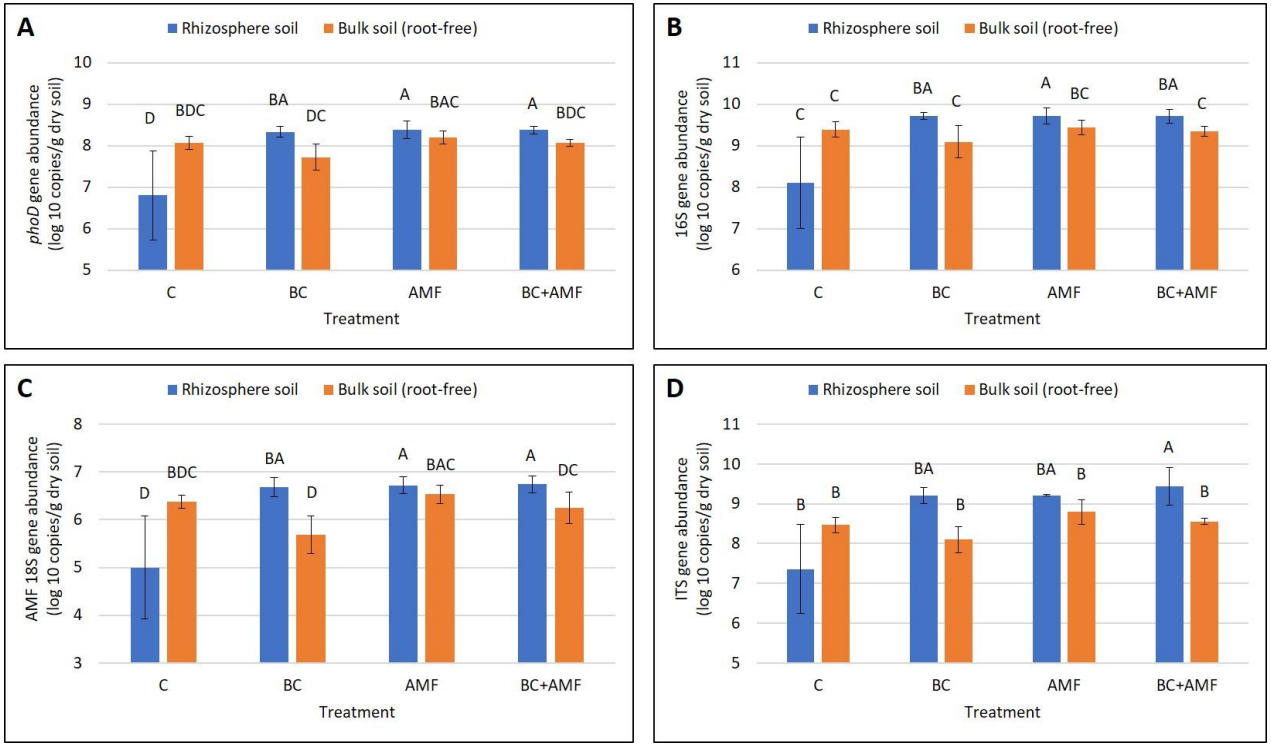

Figure 4.6. Abundances of the targeted genes in rhizosphere and bulk (root-free) soils. A: *phoD* gene. B:16S rRNA gene. C: AMF 18S rRNA. D: fungal ITS. Control = saline soil with no biochar and no AMF. BC = saline soil with biochar. AMF: saline soil inoculated with *Fm* (*Funneliformis mosseae*). BC+AMF = saline soil with biochar and *Fm* inoculation.Data are presented as the mean ± s.d (n=3). Different letters indicate significant difference ($p < 0.05$).

4.5 Discussion

4.5.1 Plant growth responses

Combined application of BC and AMF significantly increased all plant shoot and root growth parameters, except for shoot:total plant ratios, when compared to the control plants. These effects varied when considering each of BC and AMF alone. In terms of BC, its effects seemed to be significantly higher than AMF in improving shoot, root, and total plant dry weights when compared to the control. On the other hand, AMF was not significantly different from the control for these growth parameters (shoot, root, and total plant dry matter). However, AMF significantly increased root surface area compared to control. Inoculation with AMF (*Fm*) significantly increased root length and root surface area, while BC alone was not significant when compared to control. On the other hand, root diameter was significantly reduced in the treatments that had BC addition (BC and BC+AMF) compared to control and AMF. Root tip number was significantly higher only in BC+AMF compared to control. However, it trended higher in the order of control < BC < AMF < BC+AMF.

The beneficial effects of combining BC with AMF in this study were most likely caused by the physicochemical changes in soil through BC addition, and increased plant available/accessible P by AMF. Application of BC is known to enhance soil physicochemical properties such as improving soil CEC, improving water infiltration and air exchange (through improved porosity, aggregation and water retention), and nutrient availability (Atkinson et al., 2010; Dahlawi et al, 2018).

The role of BC in improving plant growth and its nutrient availability under salt stress has been reported in multiple recent laboratory and field studies (Wang and Xu,

2013; Thomas et al, 2013; Lin et al, 2015; Agbna et al, 2017). For example, in a glasshouse experiment conducted by Thomas et al. (2013), they reported that addition of sawdust beech wood BC at a rate of 50 t/ha rate (~ 2 % wt/wt) increased biomass production by ~50 % and survival by 100 % of *Prunella vulgaris* over control plants with the addition of 30 g/m^2 NaCl to a commercial potting soil. However, when combined with AMF, BC has been shown to additionally improve plant production and alleviate salt stress on plants. For example, Hammer et al. (2015) reported additive beneficial effects of BC on plant biomass, P and Mn uptake and reduced Na:K ratio when combined with AMF inoculation. AMF is well known to improve plant uptake of nutrients, especially P, in salt-affected soils (Evelin et al., 2009).

The improvement in root traits measured in this study, especially in the BC+AMF treatment, may also indicate better growth conditions (such as improved porosity and water retention) nutrient acquisition (such as extractable P in bulk soil and its plant shoot concentration in this study) caused by BC and AMF. It has been shown that under drought or salt stress, several root traits could be advantageous for enhanced plant uptake of both water and nutrients. Such traits include larger root length, greater root surface area, smaller root diameter and higher number of root tips which are the primary sources of water and nutrient uptake in plants (Comas et al, 2013). In this study for example, root diameter was significantly reduced in BC and BC+AMF treatments which is considered an advantage in plant growing under drought stress or salt stress, as this would improve total root surface area and root hydraulic conductivity (Comas et al, 2013).

Comparing to the study in Chapter II, the application of BC seemed more effective than amendment such as gypsum that is known to also improve physical properties of

saline and/or sodic soils (Amini et al, 2016). For example, in Chapter II, addition of gypsum did not significantly improve plant shoot and root dry weights (Figures 2.2 and 2.3) in the uninoculated control plant when comparing soil with and without gypsum under the same EC levels. In contrast, addition of BC in this experiment significantly increased shoot and root dry weights compared to control. Similar observations have also been reported where addition of BC was more effective, and a better strategy compared to gypsum addition alone under a stress such as drought (Batool et al, 2015). As illustrated above, the improved plant shoots and roots growth found with BC addition in this study were most likely related to improved physical (e.g., hydraulic conductivity and increased porosity) and chemical (e.g., P availability) properties.

4.5.2 AMF colonization

In the present study, unlike what I hypothesized, addition of BC significantly lowered AMF colonization in the BC+AMF treatment (5.8 %) compared to both, control (19.4 %) and AMF (12 %), but not to BC alone (8.5 %). On the other hand, control plants had the significantly highest colonization in this study (19.4 %) compared to all other treatments, which may reflect increased salt stress effects on these plants. Since the results in this study suggest that BC could have a role in reducing dependency of plants on AMF, in the control it seems that plants increased their reliance on the native soil AMF due to increased salt stress, as has been shown with control plants in the same soil in Chapter II (increased % colonization with the highest salt level). Thus, this possibly may have caused a higher colonization rate compared to other treatments where salt stress could have been ameliorated by the introduced AMF (*Fm*), BC, or both. Moreover, the higher colonization rate in control plants might have resulted in a higher K:Na ratio, since control plants are

the only treatment that had significantly higher K:Na ratio compared to all other treatments. Correlation between higher colonization and increasing plant K:Na ratio have been well documented in literature (Smith and Read, 2010; Hammer et al, 2011). However, overall colonization rate in this experiment was generally low as the highest rate was found with control plants (19.4 %). This was expected since salt stress is known to negatively impact AMF colonization (Guo and Gong, 2014; Miransari, 2017).

It is not clear why BC seems to have limited AMF colonization in this study. However, this negative effect of BC on AMF colonization might have been caused by changes in physicochemical properties and nutrient status of soil after addition of BC (Atkinson et al., 2010; Dahlawi et al, 2018), which may have resulted in less reliance of plants on AMF symbioses to reduce wasted energy. In addition, higher P availability or accessibility by plant roots could also have a role in reducing reliance of plants on AMF (Smith and Read, 2010). This was apparent in extractable P from bulk soil which was significantly higher in BC treatments (BC and BC+AMF compared to the control and AMF. However, these differences in extractable P were not observed in the rhizosphere soils (root compartment), which could be due to the presence of root pieces that would increase total extractable P as Mehlich-3 has been shown to extract organic P such as phytate (Cade-Menun et al, 2018). Another explanation of reduced colonization is that BC could have interfered with plant-AMF signaling interactions or acted as shelter for microbial grazers feeding on AMF hyphae (Warnock et al, 2007). Yet, the mechanisms on how BC affect soil biota are still not clearly understood (Lehmann et al, 2011). This lack of knowledge is related to multiple factors that can play roles in positively or negatively affection soil microbes such as nature and properties of BC, soil properties, presence of

biotic and abiotic factors (Thies et al, 2015). For example, Ogawa and Okimori (2009) related increased soil microbial population to BC micropores acting as microbial habitat. In the contrary, Dempster et al. (2012) found decreased microbial biomass following BC addition due to presence of toxic volatile organic compounds on BC surface.

Studies on the combined effects of BC and AMF in salt-affected soils are scarce. Yet, several studies have evaluated BC effects on AMF colonization in non-saline soils, but some contradictions still exist. For instance, Blackwell et al. (2010) reported high a colonization rate in a field study when soil collected during a dry season and low in P was amended with BC. He suggested that BC may have improved AMF access to water and nutrients, therefore, increasing the symbioses level with plant. Contrarily, Warnock et al. (2010) found that AMF root colonization was significantly reduced with addition of BC, even when different feedstocks and application rates were used. He suggested that both decreased and increased P availability along with changes in soil pH resulted in these significant reductions in AMF colonization. Therefore, such gaps on our knowledge of BC behavior in soil and its effects on AMF and plants should invite researchers to conduct more studies on these aspects, especially in salt-affected soils.

4.5.3 Plant P uptake and its extractability in soil

In this study, both AMF and BC+AMF treatments significantly increased P uptake in barley by 52.7 and 70.2 % compared to control plants, respectively. However, BC was not significantly different from all other treatments. These differences suggest that AMF (through transport soil P to plant) and BC (through physical changes such as porosity and water access as illustrated above) may have increased plant access to P in soil. Although no significant differences in extractable P in root compartment soils were found in this study,

BC significantly increased extractable P in bulk (root-free) soils compared to treatment without BC (control and AMF). It is possible that BC may have also increased P extractability (possibly through reducing PO_4^{3-} adsorption into clay surfaces) in the root growth compartment but, as illustrated above, the presence of some root pieces may have reduced resolution of variations in extractable P between the treatments since Mehlich-3 extraction method can extract organic P forms such as phytate found in plant roots (Cade-Menun et al, 2018). The significant increase of extractable P in bulk soil by BC may not be caused by possible release of P from BC surfaces, as the BC used in this study had only traceable amount of total phosphate (4.53 mg/kg) as reported by the manufacturer. Indeed, these results suggest that BC may have increased available P in soil. This increase in P availability might be caused by reduction in precipitation/complexation of P with Ca in soil or reduced P (e.g., PO_4^{3-}) adsorption into soil clay particles caused by the high anion exchange capacity (AEC) usually found in BC surfaces (Taghavimehr, 2015).

Generally, P availability is known as a limiting factor for plants growing under salt stress (Hu and Schmidhalter, 2005). There is increasing evidence that BC amendment increases P uptake and its availability in salt-affected soils through multiple ways including: (1) biochar acting as a source of P (may depend on release rate and feedstock type of BC) or improve soil conditions in a way that enhance plant nutrient uptake (Lashari et al, 2013). For example, Taghavimehr (2015) have shown that BC increased soil P availability through reducing phosphate adsorption into soil clay particles. (2) Biochar leading to increased relative abundances of phosphate solubilizing microbial community (Liu et al, 2017; Dahlawi et al. 2018). For example, Liu et al. (2017) reported that addition of BC to a saline soil tended to change structure of bacterial community compared to

unamended soil. The authors reported increased abundances of the well-known P-solubilizing bacterial genera *Thiobacillus, Bradyrhizobium* and *Pseudomonas* in the saline soil, making them similar to abundances observed in a non-saline soil used in their study that had natively high abundance of these microbes.

4.5.4 Post-harvest salinity parameters

In this study, only BC+AMF resulted in a significantly higher pH (7.79) compared to all other treatments. However, all treatments had lower pH than the soil pH value prior conducting the experiment (pH = 8.5). On the other hand, BC+AMF had significantly decreased soil EC than AMF alone by 3.95 units, while no significant differences were found between control, BC and AMF treatments. In addition, BC+AMF significantly decreased SAR by 5 units compared to AMF alone, while no significant differences were found between control, BC and AMF treatments. The reduced EC values in all treatments of this study compared to the original EC value of soil (16 dS/m) was expected since it has been shown that EC in soil is regulated by changes in nutrient concentrations and carbon sources in soil solution caused by plant root growth and microbial activity (Carmo et al, 2016). It is not clear why only the BC+AMF treatment resulted in a significantly higher pH compared to all other treatments, and significantly lower EC and SAR when compared to AMF alone, however, contradictory results still exist in studies using BC as a soil amendment. It has been widely suggested that properties of BC such as feedstock, physicochemical properties, pyrolysis temperature, aging and amount of BC added can control its ability to cause changes in salt-affected soil properties (Amini et al, 2016; Dahlawi et al. 2018). These aspects may explain the contradictions in some BC studies regarding impact on pH and salinity parameters such as EC and SAR. For example, Lin et

al. (2015) did not find a significant role of BC in changing pH in saline soil, but the soil and BC had similar pH (9 and 9.6, respectively) prior conducting their study. Contrarily, Sun et al. (2017) reported increased pH in saline soil amended with BC relative to control soil with no BC. Similarly, while some studies have shown BC to decrease SAR or exchangeable sodium percentage (ESP) (Amini et al, 2016) in saline-sodic/sodic soils, others have shown reduced ESP only because of increased supply of Ca, while no measurable effects were found of BC on CEC (Chaganti et al, 2015). Moreover, it is noteworthy here that BC studies in reclamation of salt-affected soils are still at infancy and mostly conducted in laboratories and for a short time (Dahlawi et al, 2018). Such studies are usually don't apply leaching to remove excessive Na out of soil profile would be the case for field conditions (Dahlawi et al, 2018).

4.5.5 Quantification of microbial genes

In this study, I found significant increases in the abundances of *phoD*, 16S rRNA, and AMF-18S rRNA in the rhizosphere soil of BC, AMF, and BC+AMF treatments compared to the control. On the other hand, ITS gene abundance was only significantly higher in BC+AMF treatment of rhizosphere soil compared to control. No significant differences were found between BC, AMF and BC+AMF in all target gene abundances in rhizosphere soil. Also, no significant differences were found between all treatment in bulk soil except with AMF-18S rRNA, which was significantly lower in the BC compared to AMF treatment. This higher AMF-18S rRNA abundance could be explained by the AMF inoculation which could have enrichened bulk/root-free soil with hyphae over BC treatment that was not inoculated. The higher abundance of microbes (especially *phoD*, 16S rRNA, and AMF 18S rRNA) in this study over the control treatment in the

rhizosphere soil could be attributed to multiple aspects. First, improved plant shoots and roots growth (especially in the combined treatment of BC+AMF) indicated an amelioration of salt stress through BC addition and AMF inoculation compared to the control plants. Consequently, this may have resulted in higher microbial abundances in rhizosphere as more C sources in root exudates would impact total microbial abundance as well as composition (Gul et al, 2015; Dahlawi et al, 2018). Second, increased root growth and enhanced traits could all translate into higher microbial abundances as more rhizosphere soil would be impacted by these roots as compared to control plant roots. The results of quantitative PCR in combination with plant growth responses indicate that BC and AMF both enhanced total microbial abundances in rhizosphere of salt-affected soils.

It is well known that soil microbial communities are vulnerable to multiple changes and sensitive to different management practices and organic amendments inputs (such as biochar) (Thies and Grossman, 2006). The effects of BC on plants may also be indirect through mediation by soil microorganisms as a response to BC (Thies et al, 2015). Both BC and AMF have been shown to inhibit or increase proliferation of some microbial taxa (Vestergård et al, 2008; Kodadad et al, 2011). However, in the case of BC, controversial reports on its impact on soil microbes still exist. For example, some studies have shown increased growth and development of soil microbes with BC application through higher water retention and nutrient release in salt-affected soils (Ajayi and Rainer, 2017). Similarly, increased microbial abundance also have been attributed to increase C sources such as those in root exudates (Gul et al, 2015; Dahlawi et al, 2018). In contrast, some research has reported decreased soil microbial biomass in a coarse textured soil (Dempster et al, 2012), while others reported no effect of BC on microbial growth when added to a

saline soil (Chaganti et al, 2015). It is highly apparent that additional research is needed to fully develop a comprehensive understanding of BC interaction with soil microbes.

4.5.6 Alkaline phosphatase activity

The potential activity of ALP in this study was significantly higher in all rhizosphere soils compared to bulk soils. However, ALP activity was reduced significantly in treatments with BC addition (BC and BC+AMF) compared to control and AMF treatments. On the other hand, I did not find differences between control and AMF or between BC and BC+AMF treatments. Different studies have reported increased ALP activities in saline soils. For example, Liu et al. (2017) has reported a significant increase in ALP activity and Olsen-P in soil amended with rice husk biochar produced at 400 °C. In another study, Du et al. (2014) also reported an increased ALP activity in surface soil with addition of corncob biochar produced at 360 °C. In both studies, ALP activity was determined using the method described by Tabatabai and Bremner (1969). In contrast, Paz-Ferreiro (2015) found that soil amended with poultry litter BC prepared at 400 °C had significantly low temperature sensitivity to phosphomonoesterase activities, indicating an underestimation of enzyme activities using current soil enzyme assays in BC-amended soils. Similarly, Jin (2010) found that ALP activities were underestimated up to 6-fold in soil slurries amended with BC, likely through the strong adsorption affinities of BC. In the present study, although extractable P increased with BC addition in bulk soil, activity of ALP was significantly reduced in both rhizosphere and bulk soil. As a result, I further tested whether BC had a role in interfering or impacting the colorimetric assay of ALP with the same soil used in this experiment. The results indicated that the presence of BC significantly reduced ALP activity compared to soil with no BC, which supports the notion

that BC can introduce biases in colorimetric assays of soil enzymes. Such biases might be a result of sorption/binding of assay substrate into BC or as illustrated above, a low temperature sensitivity of BC. Therefore, this bias of the ALP assay in this study explains the contradiction between *phoD* gene abundance and ALP activity when comparing BC treatment with control. Therefore, the results of ALP activity in this study may not be reliable to draw a conclusion or understand how it was impacted by BC due to the interference of BC with the enzyme assay. However, it is noteworthy here that increased ALP activities in BC-amended soils in some literature studies might be related to the feedstock and/or the pyrolysis temperature of BC. Such aspects have been increasingly suggested in the literature to control BC behavior and its interaction with soil physicochemical processes and soil microbes (Amini et al, 2016; Dahlawi et al. 2018).

4.6 Conclusion

The results of this study showed that combining BC with AMF inoculum significantly increased plant shoot and root growth, plant P uptake, and extractable P in bulk soil, but significantly reduced AMF colonization. Reduced AMF colonization in BC may be due to increased plant access to nutrients by higher root biomass, and less reliance on AMF symbioses. This assumption is also supported by higher colonization in control treatments with reduced root biomass. Positive effects of BC on root growth was probably due to improvements in soil physical characters such as porosity and water retention, that are more favorable for root expansion in clay soils, similar to the one used in this study. On the other hand, the role of AMF inoculum was evident in increasing plant P uptake compared to the control. BC and AMF treatments significantly increased rhizosphere *phoD*, 16S and ITS abundance compared to control. However, no significant differences

were observed in bulk soil microbial abundance. AMF inoculation did not alter the ALP activity compared to control. Since biochar appeared to have interfered with this colorimetry assay, its effect on ALP activity is considered invalid. Overall, results of this study suggested that combined application of BC and AMF inocula can significantly improve plant growth in saline soils. Therefore, soil amendments and artificial inoculation of AMF appear to be potential tools for sustainable improvement of saline soils.

CHAPTER V

SUMMARY

The first experiment in Chapter II was conducted to investigate the ability of different saline-adapted AMF species to colonize plant roots and promote plant growth under different salinity levels in a naturally saline clay soil. The results indicated that artificial inoculation of AMF species increased root colonization under saline conditions, although only significantly higher in some AMF treatments. Results also indicated that AMF inoculation influenced plants growth, but at different scales as salinity level increased. Symbiotic effect of AMF colonization was evident, but is dependent on AMF species, and that not all AMF species may exhibit mutualistic interactions as salinity increases. It is evident from this experiment that artificial inoculation of AMF is beneficial for saline soils when competent AMF species are used.

The aim in the second experiment in Chapter III was to evaluate tripartite interactions between plant roots, AMF and the native microbial community for their role on P solubility, accessibility and uptake by plants. The results indicated that the native community play a significant role in solubilizing mineral-P and organic-P, as soil sterilization significantly reduced extractable P concentrations in hyphosphere. Concurrently, ALP activity, *phoD* and fungal ITS gene abundances also significantly reduced in sterilized hyphosphere, regardless of AMF-inoculation. However, P uptake by plants was significantly higher only in AMF inoculated treatments, suggesting that AMF play a role in channeling solubilized P to roots. These results indicate that tripartite interactions are critical for supplying sparingly soluble-P to plants in saline soils.

The aim of the third experiment in Chapter IV was to evaluate the combined effects of amending soil with BC and AMF on root colonization, root biomass and P uptake by plants. The results demonstrated disparate role of BC compared to AMF, in enhancing P availability and plant growth. Results showed that BC significantly improved root biomass, but reduced AMF colonization, which suggest that BC may significantly improve physical attributes in clay soil, that are beneficial for root expansion. Microbial community abundance was also significantly influenced by both BC and AMF. These results suggest that applying BC with AMF to salt-affected soils could be a promising tool for improving P supplementation for crop production.

Overall, the results indicate the importance of AMF and native microbiome interactions in salt-affected soils in improving P bioavailability to plants. Either improving soil conditions using an amendment such as BC or supplying beneficial AMF through artificial inoculation are potential avenues to increase P supply in saline soils.

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