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INFLUENCE OF ARBUSCULAR MYCORRHIZAL FUNGI (AMF) ISOLATED FROM ARID ZONES ON PLANT GROWTH, PROTEIN CONCENTRATIONS AND RESPONSE TO SALINITY CONDITIONS IN ALFALFA (MEDICAGO SATIVA)

BY

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ABSTRACT

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Title: Influence of Arbuscular Mycorrhizal Fungi (AMF) isolated from arid zones on plant growth, protein concentrations and response to salinity conditions in Alfalfa (Medicago sativa)

Supervisor of Dissertation: Professor **Mohammed, H, Abu-Dieyeh.**.

The present study is aimed to isolate native effective species of arbuscular mycorrhizal fungi from the Qatari environment and to subject these isolates for evaluation to support the growth of *Medicago sativa* under saline environment, as well as to study the effect of the isolated AMF on Alfalfa wilt caused by *F. oxysporum*.

At the species level, 13 mycorrhizal fungi have been recorded. The AMF from different samples were identified based on the sequencing of the PCR product of the amplified conserved ITS region. The collected species belong to 16 genera, 3 different orders and 6 families. The results showed that *Tamarix aphylla* recorded to be with highest AMF infection rate (100%) while the lowest value (12%) was attributed to *Zygophyllum qatarense.*

The isolated AMF species were then used in two different research investigations. The first was to study the effect of AMF on alfalfa plant growth under salinity stress which was conducted in two conditions, greenhouse and field conditions. Saline irrigation reduced growth (biomass), chlorophyll and protein content significantly. Potassium, phosphorus, and magnesium uptake were also significantly reduced, whereas sodium uptake increased as compared to AMF-inoculated and control plants. In both experiments, the results of inoculation with AMF were better than non-inoculated in

terms of plant growth, chlorophyll content, enzyme activity, protein content, and nutrients uptake.

The second experiment looked into the effect of the AMF on Alfalfa wilt caused by *F. oxysporum*. Our findings showed that AMF can help to mitigate the negative impacts of *Fusarium oxysporum* stress on alfalfa by improving the plant's general health.

This thesis highlights, for the first time, the role of native isolated arbuscular mycorrhizae to enhance *Medicago sativa* plant under salinity and disease stress. Because all our isolates come from arid environments, these AMF could help conserve biodiversity in desert areas. Therefore, the knowledge about the community of AMF in Qatari rhizosphere is important for possible application of such mutualistic association as an alternative biological mechanism to mitigate the negative effects of abiotic stress and use them as biofertilizers and/or biopesticides in arid environments to enhance sustainable agriculture systems.

DEDICATION

I dedicate this work to my entire family for the unalloyed support throughout my study.

Also, to all, that instilled discipline in me. Thank you all.

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CHAPTER 1: INTRODUCTION

Salinization of the soil, whether natural or anthropogenic, is one of the major factors that influences the crops productivity and agriculture sustainability. Soil in arid and semi-arid areas of the world has high salinity, which affects the growth and development of plants. Saline soil occupies up to 7% of earth surface (Namdari, et al., 2018). Excessive salinity of soil results in severe effects on plants' growth. Living plant cells respond to the presence of excessive sodium and chloride ions, by disruption of cell organelles, nutrient imbalance and subsequent effect on the growth of plants (Ruiz-Lozano, 2001). Through scientific investigations, scientists have developed different solutions to deal with salinity and salt stress, among which the development of salt tolerant plants, leaching of excessive salt from the soil and desalinisation of sea water for irrigation are some of the methods that have employed with limited success (Wang, et al., 2003; Dar, et al., 2018). One of the far more successful methods is the use of microorganism that are symbiotic with plants in saline environment and that could be useful to help plant to overcome the salinity stress. Internal and external microbes invade plants in their natural habitat. (Wang, 2018). AMF establish symbiotic association with plants and are considered natural fertilizers because of their ability to provide nutrients, water, and pathogen protection to plants in exchange for photosynthetic products. The absence of these fungi can result in a less effective ecosystem functioning.

The AMF must have a host to complete its life cycle. This brings about its association with the plants, an association that has discovered to be mutually beneficial; therefore, the fungus helps the plants in nutrient uptake and protecting them against pathogens and the plant supplies needed carbon to the fungus (Berruti et al., 2016).

Arbuscular mycorrhizal fungi (AMF) are associated with many of the plants including xerophytes, halophytes and hydrophytes (Brundrett, 2017). The AMF have been shown to colonize halophytes and earliest studies have been done in this regard in early 1920's (Mason, 1928). Many of the studies done by various research groups have shown that AMF improves plant development in saline environment by improving nutrient uptake, improving soil conditions, protecting roots against pathogens, modifying biochemical and physiological properties of the host plant and producing plant growth hormones (Al-karaki,1997; Evelin, et al., 2009).

AMF enhances salinity tolerance via several mechanisms, which include enhancing nutrient acquisition, general improvement of soil conditions and by altering the physical and biochemical properties of the host. AMF has thus been successfully tested for bio-amelioration of saline soils for better growth of plants (Namdari, et al., 2018).

AMF are naturally present in several saline soils. The most common species of mycorrhizae observed in extreme saline soils belongs to the genus *Glomus*. *Glomus intraradices*, G. *versiform* and *G. eutunicatum* are the most commonly noticed species of genus *Glomus* in saline environment. The use of arbuscular mycorrhizae in amelioration of salt tolerance has been tested in variety of plants including *Trifolium* spp., *Cucurbita* spp., *Acacia* spp., *glycine max*, and *Musa* species (Alarcon, 2012). In most of these species, the use of the AMF shows most of the time an increase in plant yield and growth. Thus, there are evidences about the use of AMF for growth promoting of plants in saline soils.

This particular research will uncover the positive impacts of the isolated mycorrhizae fungi in alleviating the conditions of plant life in the semi-arid and arid regions, especially in Qatar. Mainly, it will delve into the details of isolating the native

ecologically adapted fungi and using them in the large-scale production of alfalfa crops (*Medicago Sativa*). All results were adapted to fit with the previous studies. The research will highlight the importance of the isolated mycorrhizae fungi in enhancing the plant growth under the saline condition and the role of these AMF in resisting plant fitness pathogen. The research is thus of particular importance to the Gulf region as it will provide potential solutions and applications for enhancing crop production in arid land environment.

1.2 Research rationale:

Drought-induced water and salt stress, as well as soil salinity, are two of the most challenging abiotic pressures to overcome, restricting the world's fundamental food production. Such factors are unavoidable; however, they can be managed and alleviated.

Due to the climate change and urbanization, the water security has been affected in most regions of the world where more arid areas have emerged (IPCC, 2007). Besides, in the next 50 years, the world's population is expected to double., thus greater amount of corps needs to planted and additional agricultural areas in order to secure adequate amounts of food. The increased amount of planted land requires additional amounts of water; the increasing consumption for water led to some critical shortages in the aquafer and this intensifies the salt concentration in the remaining water. In return, the salinity of the irrigated lands augmented and affected the plants especially in the arid areas.

The Gulf region is typically known for the overly dry conditions and highly saline soils and characterized by a desert environment that pose a challenge against the continuous efforts to develop the agricultural sector. Also, they are suffering a shortage of water

resources. Several researchers have conducted a series of experiments for many years to find out possible solution for remediation of environmental stressed damaged lands. One of these solutions is inoculating AMF with plant. Evidence from literature suggests that AMF can improve plant resistance to the salinity and diseases compare to non-AMF plant. The plant-symbiosis with AMF can significantly improve the overall growth as the lengths of the roots are considerably enhanced. The relationship is also known to increase leaf area, nutrient uptake as well as plant biomass in the plants under dry conditions (Mohammadi et al., 2011, Latef et al., 2016 and Heshamet al., 2018). This is due to the creation of large hyphal networks and glomalin secretion, both of which boost the roots' ability to absorb nutrients and water. This depicts that the adoption of AMF especially in the Gulf region can significantly increase the crop yields in the area despite the harsh environmental and soil conditions. AMF help to get better yield on the other countries. Though the studies have been done in many plants no much literature available on effects of AMF on growth of Medicago sativa. In this research we want to discover and investigate about AMF and then search for the potential ones that can help alfalfa plant to grow better in saline condition. In the same manner, due to the increase in crop yields imports from countries like the US can significantly be reduced and thus saving on costs due to the subsequent economy of scale.

Alfalfa is the most important livestock feed crop in the world and necessary for food security because it can be used to graze horses, sheep, goat, chicken and dairy cows because of its high protein content and easy to digest by animals. In addition, it is highly adapted to weather conditions. It can tolerate to drought, and it consider moderate sensitive to the salinity.

Based on Ministry of Development Planning and Statistics 2018 the population of Qatar increased from 25,000 in 1950 to 2,700,000 in 2018 and as mentioned before also the number of livestock increase therefore, there is an urgent need to grow animal feed especially alfalfa to achieve food security by finding natural ways to increase crop production.

1.3 Research Importance:

According to the researcher knowledge, no single publication is available about applications of mycorrhizae that have been isolated from Qatari plants on enhancing the growth of crop plants. Results from the current study is important to provide a baseline data about Qatari Mycorrhizae and their future applications.

Research on the importance of AMF inoculation on Alfalfa growth under saline conditions are limited especially in field experiment. Using this research, novel agricultural and forestall treatments can be envisaged especially in the Gulf region, whereas the quest for the corresponding receptors in plants can commence.

The areas where this particular research will embark on and depicting its importance are:

- The research highlights the importance of isolation of native AMF in the sustainable environment and agriculture.
- The significance of AMF in the plant production process of alfalfa (Medicago sativa).
- The crucial actions of AMF in aiding the plant host to resist stresses due to saline conditions.

- The importance of AMF as bio-fertilizer in the process of production
- The significance of AMF's capacities to aid the plant host in resisting pathogens.

1.4 Hypothesis:

Hypothesis-1. Qatari flora are rich in mycorrhizal interactions and can enhance the growth of important crops under stress saline treatment such as alfalfa.

Hypothesis-2. The isolated mycorrhizae can be propagated and applied as inoculum to infect root of *Medicago sativa.*

Hypothesis-3. The isolated mycorrhizae are effective against plant pathogen like *Fusarium oxysporum*

1.5 Objectives

The first aim of this study is to screen and isolate endogenous mycorrhizae from Qatari flora including representative from herbs, shrubs, trees.

The second aim is to propagate native AMF, which is isolated from different plant rhizosphere using *Z. mays* as the host plant and then this will be used as inoculum for experimental work.

The third aim is to evaluate the effectiveness of the prepared mycorrhizal inoculum to enhance the growth of alfalfa crop under both greenhouse and field conditions.

The fourth aim is to find out if the endogenous mycorrhizal inoculum can help alfalfa to overcome saline irrigation.

The fifth aim is to evaluate the efficacy of the isolated AMF as a biocontrol of *Fusarium* wilt disease in alfalfa,

CHAPTER 2: LITERATURE REVIEW

2.1 Saline soil as challenge to plant growth

Desert or arid soils are soils that have no water availability to plants for extended periods. Because of this, the plant is subjected to hyperionic and hyperosmotic stresses, which can significantly affect plants life (Ruiz-Lozano et al., 2012). Primary factors that influence plant development and formation are: daily variations in temperature, lack of water, wind deflations, and the survival of the associated microorganism (Verheye, 2009). Moreover, physical weathering is present in the arid zones and hyper-arid zones but is replaced gradually by chemical weathering and precipitation of solutions in the soil. These conditions increase the concentrations of salts in such soil and thus significantly increasing its levels of salinity (Giri, Kapoor, & Mukerji, 2007).

According to (Kalaji & Pietkiewicz, 1993), primary injuries by salts usually include specific toxic impacts either directly on the external plasma membrane or after the penetration via the membrane into the protoplast. The intake of salts usually increases the injuries. However, the toxic effects are due to the effects of anions (Cl^-) instead of cations (Na^+). This is approved by the (Brawley & Mathes, 1990) research on nitrate reductase. (Munns & Gilliham, 2015) signifies that the solubility of salts may affect the growth of plants in two ways. Initially, the availability of salts in the soil solutions minimizes the plant's ability to take up water, which causes the decline in the pace at which plants grow. It happens as a result of a mechanism known as the water deficit effect or the osmotic effect of salinity. Second, when too much salt enters the plant through the transpiration streams, it causes physical harm to the cells in the leaves. Many studies on different plants showed that older leaves of glycophytes grown at high salinity generally have higher Cl- concentrations than younger leaves, which may cause

a reduction in the growth. It is referred to, as ion excess effect or the salt-specific salinity effect (Greenway & Munns, 1980).

Salinity tolerance is defined as a proportion of biomass production in the salt accumulated soils in relation to plants existing in salineless conditions, after development over long periods. For long-lived, slow growing, and uncultivated species, it is usually difficult to evaluate the reduction in the production of biomass, percent survival will be often used. Because salinity is generally caused by the rise in the water levels, waterlogging is usually a common phenomenon (Gebrehiwot, 2018). Waterlogging in itself is commonly known to affect the plant's growth as it reduces the ability of plant roots to exclude the salts, therefore increasing the rates of uptakes of salts and then salt accumulation in shoots (Gebrehiwot, 2018).

Principally, the bearings of salinity occur inside, outside the roots, and within the undergrowth after absorption. The salt in the solutions minimizes the growth of leaves and to a lesser extent, the growth of roots. It also decreases the conductance of the stomata and consequently the reduction of the plant's photosynthetic activities. The metabolic and cellular activities that are involved in the drought-affected plants are significantly reduced.

2.1.1 Osmotic stress

Salt accumulation in the plant system affects the plant in three ways, stress due to high salt concentration, water stress, and ion imbalance stress. When water used by plant evaporates, it leaves behind a mass of salt that causes huge reduction in water potential that consequently leads to water stress. However, the access to adequate water by a plant is limited when there are excessive salt amounts dissolved in the soil

solution. An obvious way for the plant to respond to their reduced ability to access water is through the reduction of the osmotic potential.

An attempt to retain a consistent gradient which is the water potential between the soil and leaves, the cell sap has to vary its cell sap. Osmotic stress accredited to the build-up of osmotic pressure in the emerging cells that counter-act the increased osmotic pressure at the root zone and at the same time sustain root turgor results to a decline in the rate at which the plant grows and the overall development (Porcel, Aroca, & Ruiz-Lozano, 2012).

Plants utilize multifaceted oxidant mechanisms to protect against the harmful consequences of oxidative stress facilitated as a result of high concentration of salt. The complex system includes carotenoids, glutathione, and enzymes that scavenge on ROS. Guaiacol peroxides, superoxide dismutase, glutathione reductase is among the ROS scavenging enzymes. Superoxide, for instance, can convert gaseous oxygen to hydrogen peroxide, therefore, working as a scavenger on ROS (Yadav et al., 2017).

When a plant is experiencing abiotic stress, ROS is produced at the zones of electron transport, that is, the mitochondria and chloroplast, therefore accumulating ROS amounts to levels that are lethal (Tripathy & Oelmüller, 2012). Lipids, DNA, proteins and carbohydrate deprivations are caused by the highly reactive processes that cause ROS overproduction. Plants have very effectual non-enzymatic and enzymatic pathways of dealing with the increased ROS levels that aid in protecting cells from negative oxidative effects (Yadav et al., 2017).

2.1.2. Ion Toxicity

The damaging impact of salinity may be caused by the presence of a certain ion to a level that is toxic, a high alkalinity level or osmotic pressure that may hinder water availability or affect the physiological processes of the cells and metabolic reactions (HanumanthaRao, Nair, & Nayyar, 2016). The nutritional imbalance will occur in plants when excess sodium or chloride ions are absorbed relating to harmful levels of certain ions. According to (Teakle & Tyerman, 2010), sodium chloride salinity increases the accumulation of salts in aging leaves of rice plants that is facilitated by a particular ion being excluded from the xylem vessels of the young leaves. In normal salt stress conditions, the leaves of the plants tend to appear small, thick and dark green. When salt concentration increases, sodium is transported to aging leaves to ensure that emerging and vibrant leaves are protected (Orlovsky et al., 2016).

Ion homeostasis as a bioprocess in plants is disrupted due to stress caused by an increased amount of salt. It is therefore critical for the plants to regulate ion absorption and ensure they are compartmentalized to counteract the salt stress(Almeida, Oliveira, & Saibo, 2017). Plants may either limit salts from accessing the vacuole or have them compartmentalized in other tissues to enhance metabolism(Almeida et al., 2017). Three strategies may be adopted by the plant to minimize the significant negative effects of a reduced potassium-sodium ratio. The plant may decrease the quantity of sodium getting into the cells; it may get rid of sodium ions from the cells, or it may compartmentalize the ions in sites that cannot affect the normal functioning of the cells (the vacuole) (Yadav et al., 2017).

2.2 Plant-Mycorrhizal interaction

According to the Greek words, the term mycorrhiza is denoted as “root” and “fungus.” Mycorrhizal fungi is dominate and thrives within the root systems of plants where they established a symbiotic association called “mycorrhiza”. In many ecosystems, mycorrhizal fungi are an important constituent of soil. Interestingly, two important classes of mycorrhizal fungi are recognized based on morphological characteristics: ectomycorrhizal and endomycorrhizal fungi. However, not less than seven different types of mycorrhizal associations were reported (S. Smith & Read, 2008). The mycorrhizal fungi are uncharacteristically recognized for their aptitude to create an extensive hyphal network that will form the part of the soil, known as the wood-wide web that can link different plants communities thus offering the efficient horizontal nutrient and water transfer. The fungi develop areas that are specialized, known as symbiotic interfaces, which form the interaction platform with the plant host. Mycorrhiza can be divided into aseptate endophytes such as Glomeromycota, or septate Asco- and Basidiomycota. The Mycorrhizal fungi, like any other plants-interacting fungi, have their life cycle to depend on the absorption of organic carbon that is produced by the living plants so that the mutualistic interaction is usually based on nutrients exchange(Kumar et al., 2016) (Figure 1). The primary functions of mycorrhizal connections include improved plant establishment, increased water and nutrient intake, improved soil structure and protection from environmental challenges. The mycorrhizal fungi in return get their energy source as carbohydrate from the host plant partner.

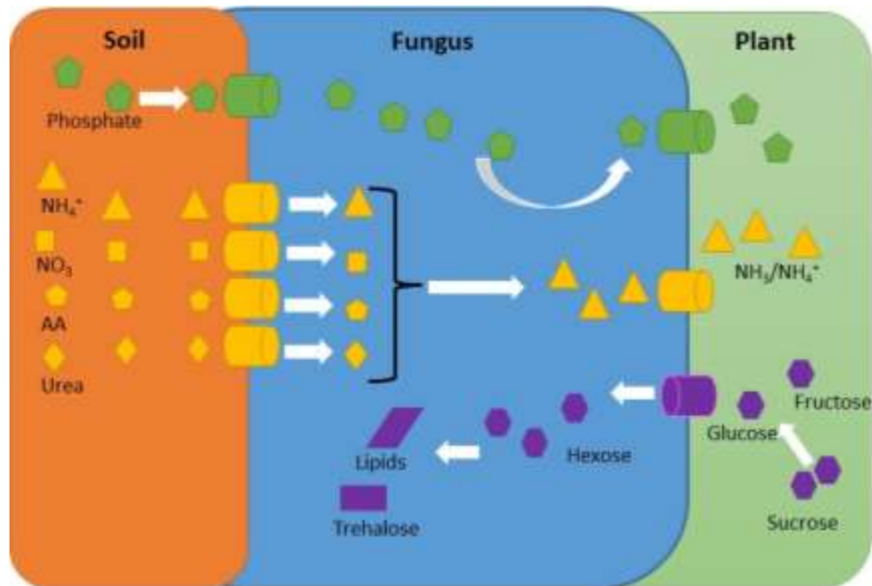


Figure 2. 3 Summary of the nutrient exchange in AM symbiosis (Modified from Kumar, et al., 2016).

Among the soil microbes, because of their involvement in stress reduction, AM fungi are regarded as a crucial component of desert plant-soil systems (Requena, Jeffries, & Barea, 1996) and can live in harsh and limited settings (Sylvia & Williams, 1992) .

AMF have symbiotic associations contributes immensely to the development and growth of plants under certain conditions through the modification of the root system as well as via the enhancement of mobilization and uptake of nutrients that are essential (Sylvia & Williams, 1992). They also stimulate the plants to be able to withstand and survive stress through the enhancement of enzymatic antioxidant as well as non-enzymatic systems of defence mechanism(Ahmad et al., 2016; Y. Wang et al., 2018) Phytohormone synthesis (Navarro-Ródenas et al., 2013), and the peroxidation of lipids (EF, Abeer, & AA, 2015). The symbiotic association is usually found among the entire early species thus affirmed the fact that the symbiotic association (symbiosis) is the

ancestral phenomenon common with mycorrhizal that played a significant role in the evolution of plants (EF et al., 2015). In contrast, the rhizobia bacteria's symbiotic relationship (nitrogen fixing) evolved later and is thus only restricted to a specific plant clade (EF et al., 2015). The fungi are typically septate hyphae or coenocytic where the spores have a high number of nuclei. These nuclei's polymorphic nature and the large genome has made the sequencing of genomes and annotation of this fungi group challenging. They are generally asexual, but their genetic material exchange between fungi that are closely related via anastomosis have been seen (Sanders, Clapp, & Wiemken, 1996).

On the surface of the host, the AM fungi form an appressorium called the hyphopodium. The hyphae that emerge from this hyphopodium enter the root via apparatus of penetration that guides the fungal hyphae via the root cells towards the cortex. However, within the cortex, the fungal hyphae penetrate the apoplast, and move straight through the axis of the root, thus penetrating the cortical cells of the inner root (Bonfante & Genre, 2010). The AM associations enter the fungus using small hyphal branches that develop intracellular hyphal coils, which have the characteristics of arbuscular branching (Bonfante & Genre, 2010). It does not enter the symplast of the plant and is not usually found in the cytoplasm's hosts using the periarbuscular membrane of the hosts (Bonfante & Genre, 2010). AMF penetrates cortical cells in plant roots, and that will lead to change root morphology, increasing its tolerance to disease, pests, and weeds (Schouteden et al., 2015). Inside the cortical cells, they establish arbuscules, which they use as sites for exchanging nutrients. Vesicles are also formed amidst the cortical cells to act as storage sites for the nutrients(Figure 2) (Yadav et al., 2017).

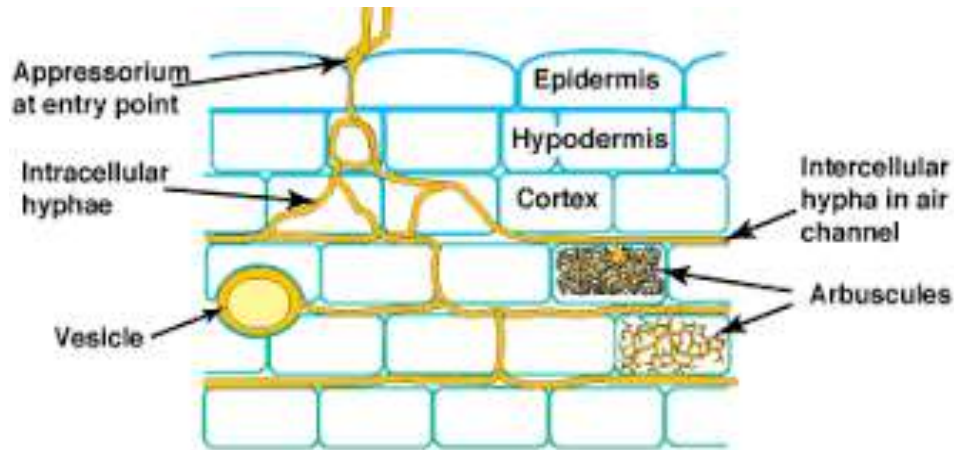


Figure 2.4 Diagrammatic representation of the cellular features of the arbuscular mycorrhizal fungi. (Source: https://mycorrhizas.info/vam.html)

Interestingly, it has been discovered that AM fungi are unculturable without a host. AMF lack the ability to store food. (carbohydrate) themselves except they are inside the plant cell, thus these organisms solely depend on their hosts for development and proliferation, which make them to be known as committed biotrophs. Looking from developmental and evolutionary perspective, the environmental success of AM fungi exhibits that the benefits of such a strict relationship with plants have beaten the risks emerging from the loss of saprotrophic abilities (Bonfante & Genre, 2010).

2.2.1 The role of AMF in inducing systemic resistance and inhibiting or minimizing the pathogenic of plant disease

AMF is the primary soil components that promote rhizosphere functioning. They are advantageous because they can guard the host counter to a wide variety of phytopathogenic infections through various approaches (Ciancio et al., 2017). These mechanisms include direct competition for the limited space and nutrients, enhanced root tolerance, altered rhizosphere interactions, and induced systematic resistance.

Therefore, it is through these mechanisms that the AMF can produce systematic resistance and in return inhibiting or eliminating the plant pathogens that lower quality and yield of the crops(Ciancio, Pieterse, & Mercado-Blanco, 2019). Moreover, AMF inoculation association does not only promote therapeutic plant growth, but also raises their productivity and the amount of chemicals (Song et al., 2015)**.**

2.2.2 The Importance of AMF in Increasing Crop Yield

The AMF- plant symbiotic relationship is significant in enhancing plant growth and yield and determine the success of plants. The mechanisms include enhanced water and nutrient uptake directly by the fungal partner. Many researchers have confirmed that AMF can indirectly or directly stimulate the generation of the secondary metabolism, resulting in vast plant alteration of their secondary metabolic amounts. For example, the AMF can stimulate the caffeic acid and rusmarenic acid generation in the medicinal plant, *O. basilicum*. Additionally, AMF inoculation does not only promote therapeutic plant growth, but also raises their productivity and amount of chemicals(Song et al., 2015). Therefore, results in an improvement in yield, more especially, of crops, leading to the eradication of hunger cases in various countries**.** Ref.

Despite the abilities of AMF to improve yield in crops, it is important to note that soil type can affect their association, and therefore, not always that the fungus will promote crop yield. Although AMF colonization is known to benefit plant growth in general, the physicochemical state of the soil may have a direct impact on the plant-fungus symbiotic interaction(S. J. Kim et al., 2017). That, therefore, should be an indispensable factor to evaluate when reviewing the impacts of AMF on crop development, growth as well as yield-augmentation.

2.2.3 Effect of salinity on mycorrhizae

AMF are found in numerous distressing conditions. Generally, saline environments have harbored large populations of AM spores as thrive well there(Sengupta & Chaudhuri, 1990; Aliasgharzadeh et al., 2001; F.-Y. Wang et al., 2004). Also, the outcome of glasshouse research have demonstrated that AM association have the potential to improve both plant resistance to salt stress and yield in saline conditions, yet salinity may negatively affect AM fungal development and hyphal augmentation (Juniper & Abbott, 1993; Peat & Fitter, 1993).

Studies done by different groups have different views on the effect of salinity on mycorrhizae development. Barrow et al., (1977) reported that sporulation and colonization of fungus is inversely related with salinity of the soil. The decreased colonization is mainly associated with high concentrations of sodium chloride (Barrow, 1997). Glomus species were found in very saline soils in the Tabriz plains, Turkey, according to Aliasgharzadeh et al., (2001). Based on the research, authors hypothesized that fungal spores germinate in high salt and thus salt acts as a stressor for seed germination. The amount of AMF spores exhibits negative relatinship with soil depth because as it decreases, the soil depth increase (Aliasgharzadeh, 2001). In as similar way, a negative correlation was reported between salinity and mycorrhizal infectivity by Saint-Etienne et al. 2006. An increase in salinity from 5% to 22% results in decrease in infectivity from 100% to 6% respectively. Thus, in overall a conflicting report was reported on the effects of high saline concentrations on fungus growth and germination(L Saint-Etienne et al., 2006). In another instance, different researchers discovered abnormal levels of AMF colonization presence in saline environments of a temperate grassland, that contents of water, sodicity, and salinity in soil were connected

positively with arbuscule and AM root colonization in *Lotus tenuis*, yet contrarily so in the grasses. The impacts of soil salinity on spore germination of AM growths and consequently hyphal generation is a standout amongst the most critical adverse impacts of salinity on mycorrhizal colonization(S. Juniper & L. Abbott, 2006). Therefore, the presence of AMF in salt soil could be determined by the type of host plant rather than environmental stress. (Nurbaity, 2014).

2.3 Mycorrhizae based plant growth under saline stress

Various investigations are reporting that mycorrhizal affiliations lead to the enhancement of crops like rate of development, biomass as well as mineral nutrient up-take under dry or saline conditions. Mycorrhizae have often demonstrated to exhibit a beneficial impact in deferring or adapting to severe impacts caused by the level of salt in the soil by keeping up a general physiological balance. AM organisms are frequently found in saline situations notwithstanding the fact that they exhibit a relatively minimal affinity with halophyte plants(Evelin, Kapoor, & Giri, 2009). In any case, halophytes could be rewarded to certain extent from AM advantageous interaction as mentioned in the case of *Phragmites australis* (Khare & Rai, 2012). Curiously, *Glomus* spp was among the most regularly observed AM in terms growth and development. Nonetheless, when contrasting a few *Glomus* spp., some researchers showed that each AM species has various efficiencies in reducing salt stresses on plants. (Khare & Rai, 2012). have explored the taxonomic variety of AMF growths in alkaline soils of upper Gangetic fields of Allahabad and bordering regions, and it was discovered that such soils detrimentally affect AMF spore population, dissemination and variety.

A thorough research into the function of AMF in security against salinity stress have exhibited the existence of symbiosis interaction which regularly results in the

enhancement of supplement up-take, increment photosynthetic rate, water utilization efficiency and gathering of osmo-regulator compounds, recommending that salt stress mitigation by arbuscular mycorrhizae fungi results from a mix of molecular, nutritional, physiological, and biochemical impacts. Knowing that the beneficial outcome of plant advancement depends on the AMF species that are involved. Therefore, AMF was found to build the wellness of the plants by upgrading its process of development(Adriana Marulanda, Azcon, & Ruiz-Lozano, 2003; A Marulanda et al., 2007; Wu et al., 2007). Interestingly, a few specialists have reported that AMF-inoculated plants exhibit more development when compared to the non-inoculated plants that is subjected to salt pressure. For example, (Hajiboland et al., 2010) in their study showed that high saltiness decreased dry matter biomass in two tomato cultivars, mycorrhizal plants outperformed non-mycorrhizal plants in each case. The mycorrhizal relationship is thought to aid the host's mineral acquisition, especially phosphorus(S. E. Smith & Read, 2010). The enhanced development of inoculated plants in salty environment is principally identified with the mycorrhiza-intervened improvement of host plant phosphorus sustenance.

The researchers conducted a meta-analysis(Chandrasekaran et al., 2014). to gain a better understanding of the importance of AMF's effect on plant growth and development in saline soils. A total of 530 studies were analyzed for the meta-analysis. *Glomus* species was the major inoculant. The metanalysis showed that inoculation with AMF increased the plant growth when compared to control plants. Also, AMF inoculation increased the total biomass of the plants. The increase in biomass was significantly different between roots and shoots. There was also difference in the extent of increase among various plant species and among different *Glomus* species used.

AMF inoculation for plants under salt stress had significant difference in uptake of phosphorous, nitrogen, potassium and sodium when compared to controls. AMF inoculation in plants showed increased content of enzymes that act as antioxidants, including catalase, superoxide dismutase and POD respectively (Chandrasekaran et al., 2014). AMF helps in maintaining the osmotic balance by promoting the production of various osmolytes such as betaine, proline and glycine by altering the sodium to potassium ratio (Borde, Dudhane, & Kulkarni, 2017). AMF helps plants in enhanced nutrient availability which allows better growth. It has been shown in date palm model that it is very effective in enhancing growth under saline conditions (Qaddoury, 2017).

2.4 Methods and techniques used to screen, inoculate and evaluate mycorrhizal interaction

2.4.1 Methodology of screening and isolation

The spores of the AMF were collected from the soil using a wet and sifting method described by (J. Gerdemann & T. H. Nicolson, 1963). and the sucrose gradient described by Smith and Skipper (1979). In an experiment by Wu et al. (2002), five different root and adhering rhizosphere samples of soil were taken, approximately every 3 months, from four sites that included peanut field, mixed plantation of forest, orange plantation, and tea plantation. In this manner, 200 samples of rhizosphere soils and roots were collected. The spores of the AM fungi were recovered from the soil by means of wet sieving and decanting as per Gerdemann and Nicolson (1963) experiment. The spores were examined under stereomicroscope while each morphotype was tallied. The spores of each morphotype were permanently mounted in Melzer's reagent and Polyvinyl-Lacto-Glecerol (PVLG) for identification. They identified the species as per the manual from Schenck and Perez (1988) and Morton (1996) INVAM identification manual. They then noted the frequency of the species in the sample.

2.4.2 Methodology of identification (morphology and DNA)

In an experiment, Weickel, Dodd, and Dehne (1997) used soil samples from two different sites and mixed it with autoclaved sand. The seedlings of the maize were used to induce sporulation of the indigenous AMF. After 2-4 months, viable spores were isolated for production of pure cultures with multispore or single inoculum for experimentations. The species screening of identification was based on the morphology of the spores where they were individually examined under a microscope on diagnostic slides. The primary parameters of taxonomy like the color of the spore, size of the spore, shape, spore walls number, and hyphal attachment were individually assessed utilizing the BEG technique (Bank of European Glomales) (Weickel, Dodd, and Dehne, 1997). Moreover, the biochemical properties of the isolates were individually analyzed. The other screening pattern utilized by this researcher was also spore isozyme patterns where the protein was extracted from spores and separated electrophoretically after which their malate dehydrogenase patterns were contrasted with those of Rosendahl and Sen (1992) described cultures. The variations in the banding patterns identified the differences between the AMF fungi.

In another experiment, Amutha and Shamini (2016), collected soil samples from the rhizosphere soil of the host plant roots. The spores were then extracted by using wet sieving and decanting method. The spores were then sorted via a dissecting microscope and confirmed their identities based on morphology features like shape, color, size, and the type of hyphal attachment. The spores were then multiplied using trap culture methodology just like Morton e. al., (1993). For the multiplication of the AMF spores, *Allium cepa* was utilized as a plant host. The DNA was isolated from the spores using the cetyltrimethylammonium bromide technique to acquire the AMF's molecular

characterisation. The root samples were treated with the CTAB extraction buffer. After an hour at 65°C, 4.5 milliliters of Isoamyl alcohol/Chloroform were added at a 1:24 ratio, and the mixture was gently rocked for about 10 minutes before being centrifuged at 10,000 revolutions per minute for ten minutes at room temperature. 0.6 volumes of isopropanol were added to the upper aqueous solution (Amutha & Shamini, 2016). The DNA that resulted from the precipitation process was It was then washed with 70% ethanol, dried at 65 degrees Celsius, and dissolved in 100 liters of buffer.

After this process, the researchers estimated the concentrations of the DNA at absorbance determination at 260 nm.

2.4.3 Methodology of inoculating

Wu, et al. (2002) conducted an experiment to check the effects of inoculation. Initially, they chose the most promising isolates and tested them based on the greenhouse experiment. Each treatment was repeated four times where 24 plots were arranged in a randomized order. Each plot had 36 planting holes that were 10 cm deep and wide and were 20 cm apart. They then applied superphosphate and other nutrients and put the inoculum in each hole in order to provide a number of propagules that was constant in each planting hole. *Trifolium repens*, *Sorgham vulgare* or mungbean were planted as trap plants into these pots. A soil layer was then added, and the seeds that they had initially treated with Rhizobium sp (to enhance the nodulation) were sown.

2.4.4 Methodology of propagation of arbuscular mycorrhizal fungi

In an experiment by Selvakumar, Krishnamoorthy, Kim, and Sa (2016), there major aim was to use a single spore inoculation technique to propagate AMF. They collected soil samples from Saemangeum that was affected by salinity. They then subjected the sample to soil analysis. Initially, in the single spore method of inoculation, *Sorghum*

bicolor L. was inoculated with one spore. In the 150 inoculants, six spores germinated in vitro. They were then moved to 1-Kilogram pots that had sterilized soil. After 4 months, they were then mixed and moved to 2.5-kilogram pots where they were maintained for another 4 months. They then checked the spore count after 8 months, and the colonization and spore count were checked. The spores that were propagated recognized using PCR and sequencing. This experiment was similar to that of Selvakumar, et al. (2018). In another experiment by Harikumar (2017), they conducted the experiment using oil cake that was entrapped with sesame seeds and AM fungus that was made through the mixing of coconut cake that was sterilized and neem cake. They also used surface sterilized sesame seeds and spore sieving of *Funneliformis dimorphicus* that was sterilized from a pot culture in a solution of polysaccharide gum which was obtained from *Strychnos potatorum* seeds. The whole mix was moulded in cubes that contained 20-30 seeds and spores that were dried before application. They were then broadcasted in inoculated treatments. On the other hand, the uninoculated used oil cake cubes that did not have the inoculums.

2.5 Mechanism of AMF on enhancing crop yield under salt stress

Salinity is a key abiotic factor that has linked to a) rate of leaf expansion, a reduction in growth rate, the leaf area index, and net absorption capacity (Hasanuzzaman et al. 2009, 2013), b) increase the reactive oxygen species generation and c) damage photosynthesis and other related variables (Latef e.t al, 2016). There are numerous reports on the application of AMF to improve plant development, particularly in saline environments. The different researchers attributed AMF mediated enhanced nutrients uptake, absorption of water and elevated rates of photosynthesis. The mycorrhizal fungi colonization was shown to enhance salinity exposed plant hosts to increase the

absorption of more water via the hyphal network and also increase the capacity for gaseous exchange (José Beltrano et al., 2013; Borde et al., 2017; Parvin et al., 2020). In plants that are thriving under conditions that are saline, the colonization can as well enhance the root's conductivity at low potentials of water, alter and stimulate the morphology of the root system, and increase the conductance of the stomata (Latef e.t al, 2016). They were also reported to have more chlorophyll contents, higher intake of Mg and N but reduced transport of Na under saline environments. Others reported an amended concentration of cytokinin and a higher photosynthates translocation in AMF inoculated plants under saline conditions (Latef et al., 2016). It was also reported to increase the PS I and PS II performance as well as enhance the carbonic and chlorophyll contents (Latef e.t al, 2016). The inoculation can as well reduce the stress via the decrease in membrane lipid peroxidation in the plants that are exposed to salinity, lipid peroxidation product, and fine-tuning of antioxidant systems of defense which are considered as low-stress markers in plants (Latef e.t al, 2016) .

Mycorrhization has been lauded for the ability to enhance the integrity of the plasma layer by bringing down the lipid peroxidation and minimizing the leakage of electrolytes. (Talaat & Shawky, 2014) revealed that AM plant life displayed lower malondialdehyde (MDA) and H2O substance when contrasted with non-AM plants, demonstrating less gathering of ROS and lower film destruction in the previous plants when compared to the latter. AMF treatment can likewise diminish oxidative stress via the decreasing of layer lipid peroxidation in plants that are found in saline environments. MDA amassing diminished in *Triticum aestivum* leaves through mycorrhiza under hyperosmotic conditions of salinity and might be because of the amassing of nitrogen containing mixes, for example, glycine betaine, as well as proline.

Colonization with arbuscular mycorrhizae fungi ensures plants against unsafe impacts of ROS by upgrading cell reinforcement compounds like, catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), and POD (peroxidase). AMF can likewise regulate plant status of osmoprotectants / osmolytes, (for example, sugars, glycine betaine, and proline) and that of natural acids (Latef & Chaoxing, 2014); (Evelin & Kapoor, 2014). AMF enhanced pool of glycine betaine and proline was contended to ensure thylakoidal films against the ROS harm. Amassing of sugars in plants treated with mycorrhiza has been accounted for as a protection system against salinity (Sheng et al., 2008). High accumulating of solvent sugar in mycorrhizal inoculated plants was presented because of mycorrhizal improved photosynthesis. Nonetheless, the mycorrhizal association and sugar aggregation in the inoculated plant during salinity may have deleterious consequences. Inoculation using *Glomus mossaea* elevated the levels of the protein that is soluble and aggregate amino acids of *Capsicum annuum* plants manifested to salt pressure (Sheng et al., 2008). Plants treated with AMF had a greater proline content than plants not treated with AMF have been stated by numerous scientists. Notwithstanding, a few scientists said that level of proline plants is diminished due to treatment with AMF meaning that the stress side effect in species that are salt sensitive or that this amassing it's possible that this is related to salt rather than mycorrhizal colonization. (Sheng et al., 2008).

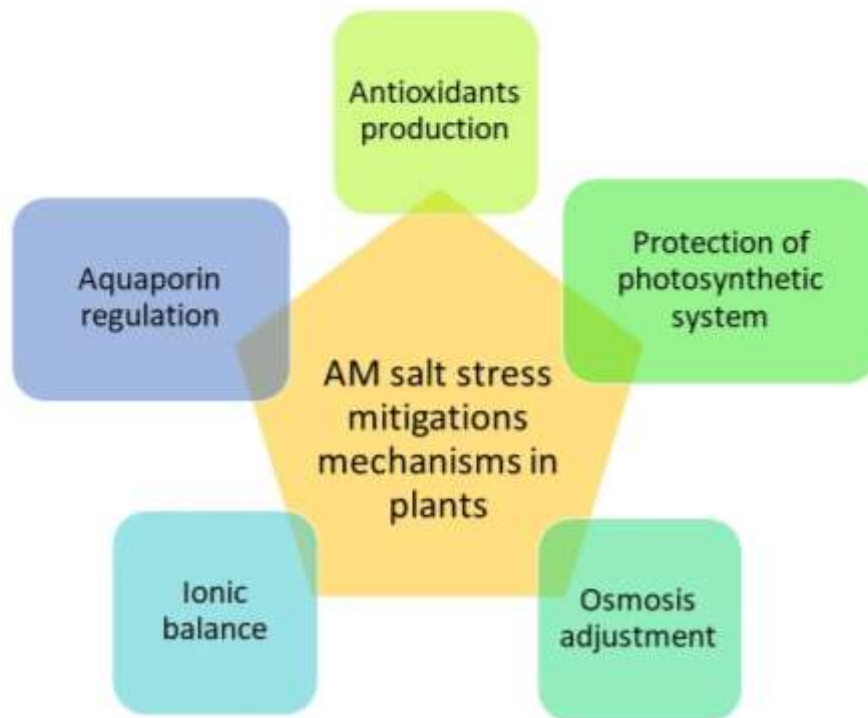


Figure 2.5. Mechanisms of AMF to alleviate saline stress. Modified from (Yadav et al., 2017).

AM-induced betaine synthesis was discovered to contribute to osmotic change and, as a result, to the productive photosynthetic process in plants growing in stressful environments like salt water (Sheng et al. 2008). While changes in polyamine pools may be one of the components employed by AMF to aid plant acclimatization in saline soils, natural/organic acids are also critical for changing cation abundance and maintaining pH homeostasis, in addition to osmotic change (Hatzig et al. 2010). Furthermore, an abundance of of organic/natural acids can boost sugar synthesis by making the CO2 transport to the Melvin Calvin cycle easier (Kapoor et al. 2013). Plant tolerance to saline environments may be influenced by the regulation of organic acids metabolism, where organic acids may behave as osmolytes in vacuoles of the plant cell, preventing damaging chloride aggregation in cell (Hajibaland 2013). Inoculation using

AMF was shown to encourage the collection of organic acids in maize that was exposed to salinity (Sheng et al. 2011). Specifically, the AM advantageous interaction (symbiotic) enhanced the amassing of natural acids, for example, fumaric acid, malic acid, acetic acid, oxalic acid and citrus extract; though the concentrations of succinic and formic acid diminished no noteworthy impact was discovered on concentration of lactic acid (Sheng et al. 2011). According to Zhang et al. (2005), AMF colonization regulated the organic acids concentration in root exudates. Additionally, the release of organic acids into the AM plants' rhizosphere, caused a decrease in organic carbon, the soil pH, and EC, and yet increments the availability of P, K, and N in the soil. Saltiness may irritate the stregolactone's biosynthesis, another category of plant hormone. An elevation in the levels of strigolactone in plants that were mycorrhizae treated were shown to overcome the impacts of salt in plants because of invigorated AMF symbiosis and development (Aroca et al. 2013).

2.6 Commercial importance of *Medicago sativa*

According to FAO, the Alfalfa plant is originally starting in the Mediterranean region (“Alfalfa,” 2018). It is mostly cultivated as forage crop to produce either hay or fresh product. It is usually grown under variable climatic conditions where the daily average temperature during the growing seasons is more than 5°C with an optimum growth temperature around 30 ^{o}C (Evenson, 1979). It is also depicted as having produced more under dry conditions as opposed to humid conditions. It can be used as a break crop during the growing season.

This crop is mostly perennial, and the highest yield is produced in the second year growing season. In areas with mild winters the crop is grown for about three to four years constantly, however, in the continental climatic region with freezing winters, is

typically grown for 6 - 9 years within the winter's dormant period (http://www.fao.org/land-water/databases-and-software/crop-information/alfalfa/en/).

According to FAO (2018), the production of good fields in the initial year are approximate 2-2.5 tons per ha per cut (with 10-15% moisture) of around 25-30 day intervals of cutting. For instance, in Hofuf, Saudi Arabia, the production was estimated as 28 ton/ha of hay in approximately 10 months that entails 12 cuts (FAO); In Davis, California (USA), 22 tons per hectare of hay were produced throughout a six-month growing period with seven cuts under experimental conditions. The efficiency of water utilization for the harvested yield of hay with 10-15% moisture is 1.5-2.0 Kg/m^3 after the initial year. The contents of moisture of green matter are approximately 80% while protein content in the dry mass is 18-20% ("Alfalfa," 2018).

The global alfalfa production was reported to be 210.9 million metric tons in the year 2017, and this amount is projected to escalate by 7.3% amid 2018 to 2023. North America is the largest producer of alfalfa while UAE, China, Japan and Saudi Arabia are among the major importers of alfalfa from U.S and Spain (Figure 4). Among the biggest states in the US that produce hay are California (5175 thousand metric ton), Minnesota (2610 thousand metric ton), Idaho (4400 thousand metric ton) and Montana (3150 thousand metric ton) (Dublin, 2018). They all account for more than 25% of the production of alfalfa in the US.

China is anticipated to be the major key market for the United States exports of hay in the future (Barker, 2017).

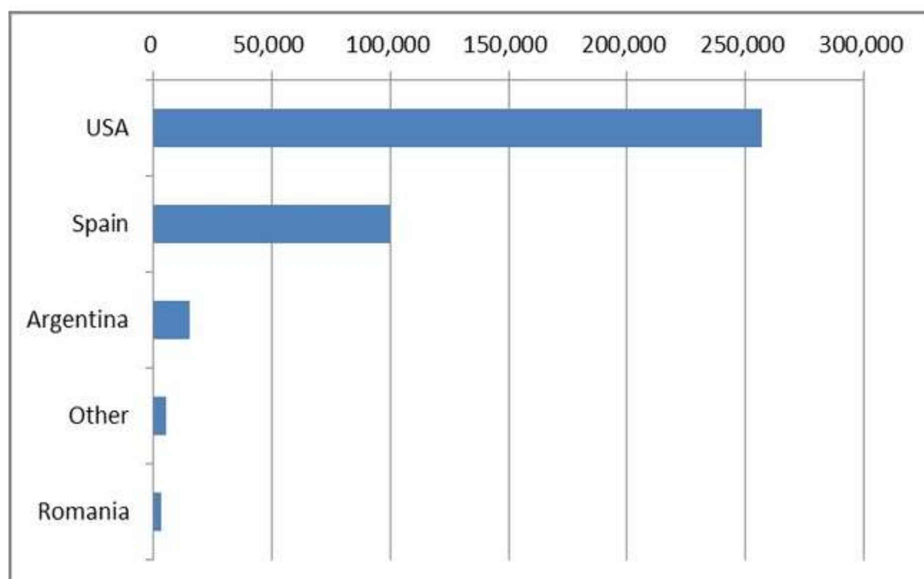


Figure 2.6.Imports of alfalfa hay (Thousand Metric Ton) by Saudi Arabia in 2016 (USDA, 2017)

2.6.1 Medicago sativa in Qatar

According to the agriculture statistic report in the state of Qatar 2018, the alfalfa production in Qatar went down from 147597.8 ton in 2015 to 133909 in 2017. Although the livestock count keeps increasing exponentially in Qatar, e.g., the number of Camels in the country was 68371 in 2013 whereas it increases to 105387 by 2017 in just four years. Similarly, the number of sheep more than doubled in four years, with 399776 in 2013 and 932472 in the year 2017. These animals need forage plants to survive hence their plantation is of significance for the agriculture in Qatar.

Nutritional content

Alfalfa is commonly used as a supplementary herb or in the form of alfalfa sprouts by humans. There is usually no standard form of nutritional information because the seeds and leaves are sold as herbal supplements. Despite this, they are high in vitamin K and

contain a variety of other minerals such as vitamin C, copper, folate, and manganese (Jones, 2016). The sprouts contain the same nutrients as well and are also extremely low in calories.

For instance, one cup of alfalfa sprouts have eight calories and contains the following :

Vitamin C: 5%- Vitamin K: 13% - Copper: 3% - Thiamin: 2% - Iron: 2% Magnesium: 2% of the RDI.

One cup also contains 1 gram of protein and 1 gram of carbohydrates. The plant is also known to have high bioactive compounds of plants, and they include saponins, coumarins, flavonoids, phytosterols, alkaloids, and phytoestrogens (Jones, 2016).

2.7 Water in Qatar:

Sustainable water resources are of great significance for life to exist on the planet. According to the Ministry of Development and Planning and Statistics Qatar's 2015 report, there has been a considerable decrease in the average recorded rainfall in the country. This is not a benefiting situation considering the rain and groundwater are the only natural freshwater resources in Qatar. Considering the fast-growing population and economy of Qatar, there is a dire need to create a balance in the availability and consumption of water in the country (Shomar, et al., 2014). The average annual water consumption rate in 2012 was as follows: 59 % for agriculture, 39 % for domestic uses and 2 % for industry (Shomar, et al., 2014). So, the agriculture sector consumed the large amount of water.

To summarize the entire situation Qatar faces immense stress in terms of its water resources due to the scarcity of its water resources both natural and renewable compared to the high demands. On top of that the unpredictable rain patterns make the said water

resource not so feasible for the agricultural practices. This and many other factors combined are the reason behind a dire need of taking actions to improve the situation of water sustainability in the country. For example, the country is seeking to use triple treated wastewater in the genre of feed cultivation only for now. Although there is some farm used treated wastewater for agriculture but until now There is no recorded data present regarding the use of treated of wastewater in agricultural system in Qatar. (Personal communication Dr. Mohammed Awaadh, Agriculture Affairs Department).

CHAPTER 3: BIODIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI IN PLANT ROOTS AND RHIZOSPHERE SOIL FROM DIFFERENT ARID LAND ENVIRONMENT OF QATAR

3.1 Abstract

Recently more attention or interest has been developed towards the role of Arbuscular Mycorrhizal Fungi (AMF) in plant growth. Qatar, which is a part of the Arabian Gulf region is mostly arid with hot and dry climatic condition. The goal of this study is to learn more about the occurrence, species composition, and abundance of AMF in Qatar. rhizosphere soil samples and roots of 16 plants belonging to 12 families from eight locations were collected. The AMF from different samples were identified based on the sequencing of the PCR product of the amplified conserved ITS region. The results showed that the AMF infection rate vary with location and plant species. *Tamarix aphylla* recorded the highest AMF infection rate (100%), followed by *Blepharis ciliaris* (98%) and *Sporobolus ioclados* (92%). AMF spore counts per 100g of soil ranged from 29.3 spores in *Blepharis ciliaris* to 643 spores /100g in *Fagonia indica*. The spore counts per location is variable and the range was 29.3 to 643/100g soil, however, no correlation has detected between root colonization rate and spore counts. While all AMF identified at species levels were reported in other regions this research will be the first to investigate the AMF biodiversity from Qatar. However, new species are still expected since some were identified only at higher taxonomic levels. *Claroideoglomus drummondii* and *Rhizophagus irregularis* were the most widespread species while *Claroideoglomus claroideum* and *Diversispora aurantia* were the less present. This study provides comprehensive biological data about taxonomy, distribution and

prevalence of AMF in Qatar soil, which opens new research towards developing its future applications for environmental conservation and sustainable agriculture.

Keywords: Arbuscular mycorrhizal fungi, Colonization, Biodiversity, Qatar.

3.2 Introduction

In many ecosystems, mycorrhizal fungi are necessary components of soil. There are at least seven main types of mycorrhizal connections known. These are: endomycorrhiza particularly arbuscular mycorrhiza, orchidaceous mycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, ect-endomycorrhiza, and ericoid mycorrhiza (Ajit Varma & Kharkwal, 2009). Survey study done by (B. Wang & Qiu, 2006) they found that mycorrhizal plants account for between 80 and 92% of all land plant species and families studied. In addition, they found among land plants, AMF is the most common and ancestral mycorrhiza type. AMF establish symbiotic association with plants. They are a general constituent of rhizosphere microflora and AMF is associated with over 75% of the world's plant, and the symbiosis can be found in nearly all terrestrial habitats (M. C. Brundrett, 2009). The morphological and anatomical characteristics of their spores, as well as other current approaches like molecular tools, have been used to classify AMF.

Recently, molecular methods, like as ribosomal DNA sequencing of chosen species, have revealed an entirely new picture of AMF systematics at the genus, family, and higher taxonomic levels. The molecular phylogenetic analysis based on the SSU rRNA sequences carried out by (Schüβler, Schwarzott, & Walker, 2001) resulted in considerable alterations in the classification of AMF. As a result, AMF were removed from the polyphyletic Zygomycota and assigned to the Glomeromycota, a newly formed monophyletic group (Stürmer & Siqueira, 2006) which has been published in

sections for many years (Schüßler & Walker, 2010). Currently, 342 AMF species have been identified in the phylum Glomeromycota ("AMF species list ") .

Several studies have found the presence of fungi that form mycorrhizae in desert plants, and their activities are crucial to the survival of these plants. (Al-whaibi, 2009). AM fungus associated with most plants are prevalent in the arid soil of India's Thar Desert, according to (Tarafdar, Pande, & Gupta, 1999). The geographic distribution of AMF species, which is impacted by edaphic variables, is critical for understanding fungus dynamics, quantification, and identification, as well as predicting levels of indigenous AMF populations.

Factors such as habitat and plant community type, soil temperature, soil nutrient & composition, root exudations, rainfall; pH and the competition associated with other microorganism that have interactions with them have been suggested to contribute to patterns of AMF distribution (Öpik et al., 2006; Yongjun Liu et al., 2009; Kivlin, Hawkes, & Treseder, 2011; Gong et al., 2012; J. Jefwa et al., 2012; Chandrasekaran et al., 2014; Mosbah, Philippe, & Mohamed, 2018). Although all these factors have been confirmed as essential factors determining AMF community, there is no one major factor affecting the community of mycorrhiza fungi (Gong et al., 2012; Chaudhary et al., 2014; Melo et al., 2017).

Diversity and distribution of arbuscular mycorrhizal fungi have remained an area of active interest across agriculturists, botanists, and environmental scientists. The interests in the mycorrhizal relationship are attributed to the benefits extended by the fungal partner in enriching the hosts with minerals and waters. In return, AMF derives their carbon skeleton from the hosts. It was revealed that AMF enters plant root cortical cells, which enables the root of morphology to alter and develop its tolerance against weeds, pests and diseases (Siddiqui & Futai, 2008). AMF also enhances the plants

capability to cope with salt pressure by developing the absorption of mineral nutrients, keeping ion, preserving the acts of enzyme, and facilitating water uptake, while the rise of salinity resistance in many plants including tomato and maize (G. N. Al-Karaki, Hammad, & Rusan, 2001). The plant-symbiosis with AMF can significantly improve the overall growth as the lengths of the roots are considerably enhanced. The relationship is also known to increase leaf area, plant biomass as well as nutrient uptake in the plants under dry conditions (Mohammadi et al., 2011; Latef et al., 2016).

Due to the importance and usefulness of these fungi, in certain countries the research is advanced to commercialize products of different species of AMF mainly produced for agricultural use. Currently products of Mycorrhizae fungi were produced by 28 factories around the world (Sulaimon Basiru, Hopkins P. Mwanza, & Mohamed Hijri, 2021a). The Glomeraceae account for 100% of the items, with three species dominating the Family: *R. irregularis* (39%), *F. mosseae* (21%), *C. etunicatum* (21%) and *R. clarus* is the least popular (16 %) (Basiru et al., 2021a). Despite the importance of arbuscular mycorrhizae fungi in improving plant growth and increasing yield, structural colonization studies and spore populations in various countries with desert plants, particularly the Arabian Gulf region, have very few studies about occurrence and diversity of arbuscular mycorrhizal fungi (Al-Qarawi, Mridha, & Alghamdi, 2012). We hypothesized that arid environment are rich source of potential arbuscular mycorrhizal associations that should be explored for uses in agriculture and conservation applications. Hence the importance of this study was to discover the types of arbuscular mycorrhizae fungi in the rhizosphere of several plant species in Qatar as an arid environment. Results from the current proposed research will be important to provide vital data about Qatari mycorrhizae and their future applications and management options. Considering that there are no many studies on mycorrhizal fungi in Qatar, the

current study is conducted to further establish the trending facts for a better understanding of the importance of AMF in crops agriculture. Thus, embarking on the screening for AMF from the rhizosphere of Qatari flora and collecting information about the prevalence of AMF in plant roots and the surrounding soil with a major goal to establish future research ideas about agricultural sustainability in the arid land.

3.3 Materials and methods

3.3.1 Study sites

Qatar (25°35’48’’ N and 51°18’39’’ E) is a small peninsula that is located in the Arabian Gulf with an approximate area of 11,000 Km^2 (Ben Hassen, El Bilali, & Al-Maadeed, 2020). It lies in the northern hemisphere desert. It can be described as a warm and semi desert ecosystem. Rainfall in this region is highly unpredictable and erratic both in space and time with an annual average of about 80 mm. Moreover, because of its variability and low intensity, it is and characterized by a high temperature of more than 40ºC in the summer with high rates of evaporation (Frenken, 2008).

During this study, plant roots and rhizosphere soil were collected from eight different locations in Qatar and 16 different plant species. Fig. 1 shows the distribution of the collection sites in Qatar.

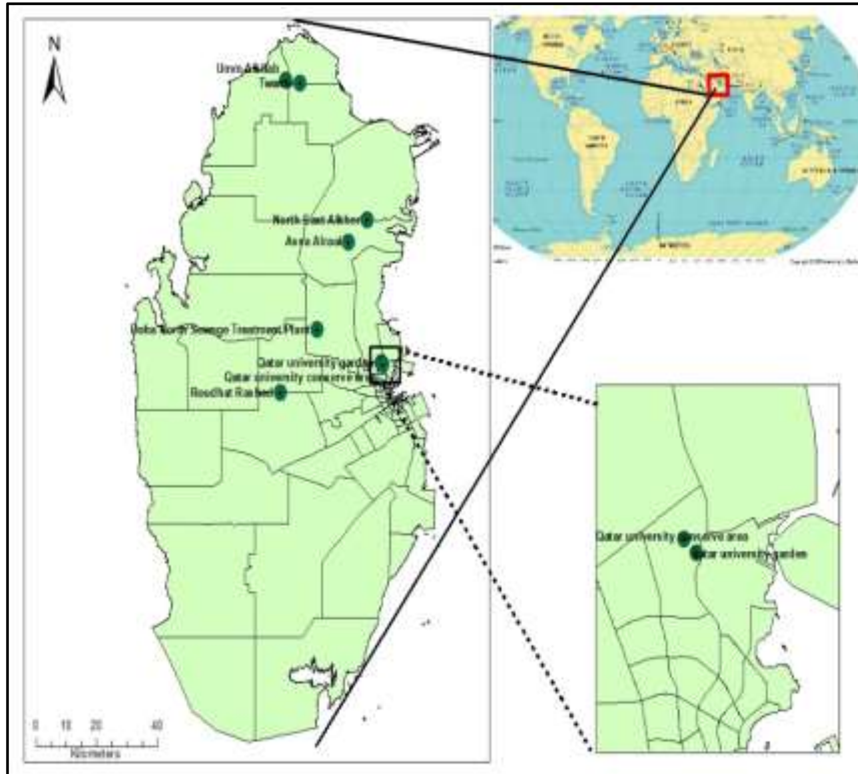


Figure 3.1 A map of State of Qatar showing the samples collection sites

Samples collection

Using a metal core borer of 10 x 20 cm (see appendix Fig. A1), soil and plant roots sample were collected from rhizosphere soil of eight different locations as listed in Table 3.1 (see appendix Fig. A2, A3 and A5). Soil sub-samples from various sites within each uniform sampling region were combined to generate a composite sample (a total of 16 samples per representative plant species). All samples were collected during the month of March 2020. Ten field trips were conducted to collect random samples of soil with a consideration to having representative samples from different plant families. The dominant species in each location was chosen. The samples were transferred into labeled polythene bags and kept at a temperature below 10ºC in an icebox until being arrived at the lab, then transferred to a refrigerator (4ºC) for later determination of the number of spores and respective identification for future analysis.

All plant samples were collected after proper permissions and All procedures were carried out in conformity with the applicable rules and regulations. Until processed, the root samples were stored in 95% ethyl alcohol. Soil samples were divided into two halves. The total number of spores in 100 g of dry soil was calculated using the first portion, while the second section examined the soil's physical and chemical qualities.

Table 3.3 Studied plants species and their families and location

Location	Host plant	family
Doha North Sewage Treatment Plant	*Zygophyllum qatarense*	Zygophyllaceae
	Tamarix aphylla	Tamaricaceae
	Launaea nudicaulis	Asteraceae
Assa Alraai	*Sclerocephalus arabicus*	Caryophyllaceae
	Fagonia indica	Zygophyllaceae
Umm Alkilab	*Spergula fallax*	Caryophyllaceae
	Cynodon sp.	Poaceae
Twame	*Plantago ovata*	Plantaginaceae
North East Alkhor	*Salvia aegyptiaca*	Lamiaceae
	Lycium shawii	Solanaceae
Roudhat Rashed	*Aizoon canariense*	Aizoaceae
	Pulicaria undulata	Compositae
Qatar university Campus	*Malva parviflora*	Malvaceae
	Paronychia arabica	Caryophyllaceae
Qatar university protected field	*Blepharis ciliaris*	Acanthaceae
	Sporobolus ioclados	Poaceae

3.3.2 Physical and Chemical Properties of Soil

Soil samples were tested for their pH (using ASTM 9045D method), salinity, total dissolved solids (TDS) (YSI EC 300 – conductivity meter), Total organic matters (TOM), granules' size by finding the percentages of clay, sand and silt, texture and for the available amounts of minerals such as calcium (Ca), phosphorus potassium (K), (P), magnesium (Mg), sodium (Na), Chloride (Cl) and calcium carbonate (CaCO3). Physicochemical characteristics and texture quality were examined using acid digestion method of soils by flame atomic absorption spectrometry (FLAA) for inorganic parameters and Manual of Oceanographic Observations and Pollutant Analyses Methods (MOOPAM) for organic parameters (Edgell, 1988) Kimbrough et al, 1989).

3.3.3 Extraction AMF spores from soil

One hundred grams of soil were kept in a 1 litre bucket and subsequently filled up to three quarters of tap water to complete wetting of the soil. The suspension was sieved by wet sieving and decanting method (Walker, Mize, & Jr, 1982). Three sieves of 500µm, 250µm and 32µm were used respectively for sequential soil sieving (see appendix Fig. A6). Post sieving the solution with spores were distributed equally into two tubes and for five minutes, the sample was centrifuged at 2000 rpm. The supernatant was discarded, and the tubes were filled with 50% sucrose. After that, the spores are mixed with sucrose solution and centrifuged for one minute at 2000 rpm. The supernatant was sieved through 38µm mesh and washed rapidly with water to remove residual sucrose adhering to the spores. The spores transferred from the sieve to the Petri dish. For each rhizosphere sample, in a 100 g soil sample, the total number of spores was counted. using a stereoscopic (LEICA 10450028) microscope at 2x to 10x magnifications., there are three replicates for each soil sample (see appendix Fig.

A7 and A8) (Gerdemann & Nicolson, 1963; Abrol, Yadav, & Massoud, 1988; Parial et al., 2014).

3.3.4 Assessment of AMF root colonization

Fresh plant roots from different Qatari native plants were separated from the soil and washed with water. The roots were cleaned with 10% KOH to remove root colors and cellular components. Trypan blue 0.05% was used to dye the roots. (a chitin specific stain that stains the cell wall of AM fungi) and stored in 50% glycerol to remove the excess stain. Roots were then investigating the colonization using a compound light microscope YMPUS BX43) at different magnifications. Percent root colonization was counted according to the following equation (M. Brundrett, Piche, & Peterson, 1984).

$$Percent\ Root\ colonization = \frac{Number\ of\ Root\ Segments\ Colonized}{Number\ of\ Root\ segments\ observed} X100$$

3.3.5 DNA Extraction of Rhizosphere Soil Samples

Soil samples (2 g/sample) were properly prepared by drying them at room temperature for a week and sifting them with a 500 µm sieve. The dried samples were packaged and sent to SYMPLANTA laboratory in Germany (the end of March 2020). The 16 soil samples were utilized to extract DNA for the metagenomic analysis, which was done using a customized FastDNA (Spin Kit for soil-MP Biomedicals, Heidelberg, Germany) and an MP FastPrep-24 5G machine. For each sample, 500mg of fine homogenous soil was transferred and repeated bead-beating was performed using ¼ inch ceramic beads in lysing matrix A to insure a better DNA yield. Samples were then treated as per the manufacturer's instruction except that 2X40s disruption was used 1X40s (Senés-Guerrero et al., 2014). Extracted DNA was then stored at -20°C for further analysis.

Polymerase Chain Reaction (PCR)

Nested PCR

Four sets of primers were used for a nested PCR that amplifies the large subunit (LSU) of the rDNA of AMF allowing species-specific identification. The PCR method used was described by Senés-Guerrero *et al*. 2020 and Kruger *et al*. 2009. DNA template used for the first PCR was between 1 and 5µl, and the first set of primers are SSUmAf and LSUmAr. As for the nested PCR, 0.5µl of the DNA template was used with the forward primer LSUD2Af and reverse primer (Table 2). The fragment amplified include a part of the small subunit of the rRNA gene (SSU), the ITS region (ITS1-5.8S-ITS2) and a part of the large subunit of the rRNA gene (SSU) (Krüger et al., 2009; C. Senés-Guerrero et al., 2020). Three PCR replicate were conducted for each of the extracted DNA sample of the 16 soil samples. The target specific nested PCR (TS-PCR) products were run on a 1% agarose gel (EvaGreen stained) to analyze the quality of the generated amplicons and to evaluate their sizes. TS-PCR nested PCR amplicons were purified individually utilizing SPRI paramagnetic bead-based technology (AMPure XP beads, Beckman Coulter) with a Bead to DNA (PCR-product) ratio of 0.8:1 (v/v) (see appendix Fig. A9).

Table 3.4 Polymerase chain reaction primers used for amplification of a species-specific DNA fragment of AMF (Krüger et al., 2009; C. Senés-Guerrero et al., 2020)

Primer	Nucleotide sequences (5’-3’)
mAf1SSU	TGG GTA ATC TTT TGA AAC TTY A
mAf2 SSU	TGG GTA ATC TTR TGA AAC TTC A
mAf SSU	Af1-2SSUm
mAr1 LSU	GCT CAC ACT CAA ATC TAT CAA A

Primer	Nucleotide sequences (5'-3')
mAr2 LSU	GCT CTA ACT CAA TTC TAT CGA T
mAr3 LSU	T GCT CTT ACT CAA ATC TAT CAA A
mAr4 LSU	GCT CTT ACT CAA ACC TAT CGA
mArLSU	LSUmAr1-4 (equimolar)
Af LSUD2	GTGAAATTGTTRAWARGGAAACG
Br1LSUm	DAA CAC TCG CAT ATA TGT TAG A
Br2LSUm	AA CAC TCG CAC ACA TGT TAG A
Br3LSUm	AA CAC TCG CAT ACA TGT TAG A
Br4LSUm	AAA CAC TCG CAC ATA TGT TAG A
Br5LSUm	AA CAC TCG CAT ATA TGC TAG A
LSUmBr	LSUmBr1-5 (equimolar)

Index PCR

The purpose of the index PCR is to tag the amplicon generated by the nested PCR to prepare for Illumina sequencing. Fig. 2 shows the details of the index PCR, the inner target-specific (TS) primer pairs used in the nested PCR had already a universal tag. Using 5l of each TS-PCR product as template, index PCR was performed directly on the tagged TS-PCR LSU amplicons to barcode all samples with distinct indices for pooling, with the index primer pair comprising a complementary tag, indices, and sequencing adapters. All PCR products generated can be pooled together and run in a single sequencing experiment by integrating sample-specific indices (Table 3).

The 50 µl index PCR reactions contained: 5 µl DNA, 5 µl Nextera XT Index Primer 1, 5 µl Nextera XT Index Primer 2, 25 µl Phusion Polymerase MasterMix, 10µl PCR

Grade water. PCR conditions were adopted to the Phusion® polymerase as follows: (98°C) for 3 min, 8 cycles (98°C) for 30 sec, (57°C0 for 30 sec, (72°C) for 20 sec), final elongation (72°C) for 5 minutes.

The index PCR amplicons were purified individually utilizing paramagnetic bead-based solid phase reversible immobilization (SPRI) technology (AMPure XP beads, Beckman Coulter) with a Bead:DNA (PCR-product) ratio of 1:1 (v/v).

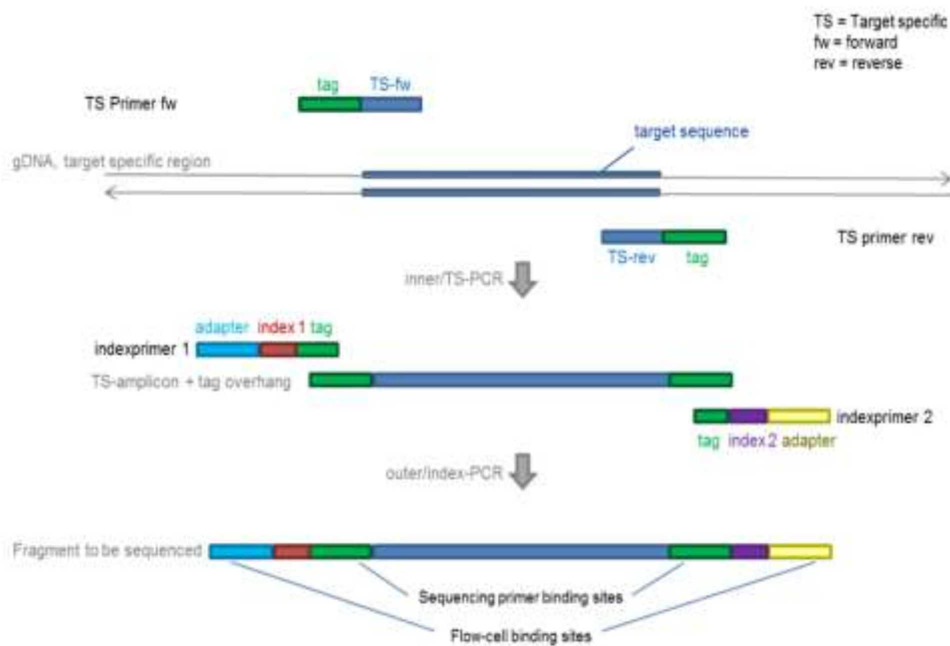


Figure 3.1 Scheme of the four-primer amplification strategy

Table 3.5 The sample-specific indices used in the index PCR.

Sample ID	I7-i5 index-combination	I7 index sequence	I5 index sequence
QAT01	N716-S510	ACTCGCTA	CGTCTAAT
QAT02	N718-S510	GGAGCTAC	CGTCTAAT

Sample ID	I7-i5 index-combination	I7 index sequence	I5 index sequence
QAT03	N719-S510	GCGTAGTA	CGTCTAAT
QAT04	N720-S510	CGGAGCCT	CGTCTAAT
QAT05	N721-S510	TACGCTGC	CGTCTAAT
QAT06	N722-S510	ATGCGCAG	CGTCTAAT
QAT07	N723-S510	TAGCGCTC	CGTCTAAT
QAT08	M724-S510	ACTGAGCG	CGTCTAAT
QAT09	N726-S510	CCTAAGAC	CGTCTAAT
QAT10	N727-S510	CGATCAGT	CGTCTAAT
QAT11	N728-S510	TGCAGCTA	CGTCTAAT
QAT12	N729-S510	TCGACGTC	CGTCTAAT
QAT13	N716-S511	ACTCGCTA	TCTCTCCG
QAT14	N718-S511	GGAGCTAC	TCTCTCCG
QAT15	N719-S511	GCGTAGTA	TCTCTCCG
QAT16	N720-S511	CGGAGCCT	TCTCTCCG

Pool preparation and library qualification

For the purified index-PCR products, one library pool was generated from all samples by mixing 5 µl of each of the purified amplicon samples. The library was stored at -20°C until sequencing. The High sensitivity DNA LabChip Kit (Agilent Technologies) was used on the 2100 Bioanalyzer (Agilent Technologies) to analyze the integrity and peak distribution of the library pool of the purified and pooled index-PCR amplicons.

The library pool was quantified using the Qubit® dsDNA HS Assay Kit, which uses highly sensitive fluorescent dyes (Invitrogen).

Sequencing

Library pool was subjected to a denaturation step using NaOH, to ensure the presence of single stranded DNA (ssDNA) fragments for cluster generation. Thus, the library consisted of ssDNA fragments with sequencing adapters and indices. The library pool was diluted and used for loading on the MiSeq® system for cluster generation and sequencing. Sequencing was performed at a final concentration of 16 pM and with a 20% genomic DNA control library spike-in. Cluster generation and sequencing was done using Miseq Reagent kit v3 600 cycles. Bidirectional sequencing was performed producing a maximum of 600 bases of sequence information in 2 x 300 bp paired-end (PE) reads.

Data processing

The Illumina software MiSeq® Reporter (MSR) on the MiSeq® system and the Illumina Sequence Analysis Viewer (SAV) were used for imaging, data processing and evaluation of the sequencing run. Raw sequences reads were analyzed using dada2 implemented in QIIME2 (Callahan et al., 2016). Data was then subjected to an evolutionary placement algorithm (EPA) analysis by RAxML EPA. The principle methods, including the MAFFT alignment of individual sequences to a reference alignment were performed as per. Identified species were given EP numbers. Evolutionary placement numbers (EP) are species-identifier numbers based on numbers previously used by SYMPLANTA. EP numbers were used because they do not change through process of taxonomic changes, and they allow comparison of species communities between studies.

Biodiversity indices calculation

Pielou's evenness (*E)* and Shannon-Wiener (*H*) diversity indices were calculated to assess diversity of samples collected from different locations. Indices were calculated using the following formulas (Suleiman et al., 2019).

H=-Σpi.log(pi), where pi is the proportion of frequency of number of reads of a certain identified representative sequence over the total numbers of reads of all sequences identified in a location.

E= H/log(S), where S is the total number of identified sequences in a location.

Statistical analysis

Spearman correlation test was conducted at a significance level of $P \leq 0.05$ to test for correlation between the number of spores per 100g of soil sample and each of the number of AMF identified per sample and Shannon biodiversity index.

3.4 Results

Soil analysis of the sampling sites

During the study soil analysis form 8 location. The findings revealed that the texture classes of soil samples were mostly silty sand with alkaline pH, ranging between 7.56–9.10. The soil is generally calcareous with total calcium carbonate ($CaCO_3$) values ranging between 302518.16 and 455782.04 ppm. The (EC) electrical conductivity on the rhizosphere soil samples ranges between (0.1 –3.5 ppt) indicating imply that the soil is appropriate for regular plant growth except the soil collected from Qatar university protected field which consider moderately saline (FAO, 2020). Alternatively, the soil is very poor in organic matter and a negligible proportion appeared in only one site (Table 4).

Table 3.6 Physical and Chemical Characteristics of soil samples in studied location.

Locations	Doha North Sewage Treatment Plant	Assa Alraai	Qatar university Protected field (Sabkha)	Northeast Alkhor	Umm Alkilab	Tweem	Qatar university Campus	Roudhat Rashed
pH	7.56	8.22	8.65	8.88	8.53	8.69	8.91	9.1
Salinity, ppt	1.2	0.2	3.5	0.1	0.1	0.1	0.1	0.1
TDS, g/L	1.816	0.283	5.03	0.1889	0.1748	0.1723	0.1809	0.1216
Ca (ppm)	131414	168704	168308	221277	112275	171339	144637	116951
K (ppm)	8654	11015	7882	8281	16547	9607	8188	8946
Mg (ppm)	17549	37696	23779	24755	48255	44078	10383	15891
Na (ppm)	9215	5772	10504	5792	4866	5338	9209	7881
P (ppm)	147	476	172	316	1028	400	423	177
% TOM	NIL	NIL	NIL	NIL	1.63	NIL	NIL	NIL
CaCO3 (%)	36.65	41.71	45.58	43.55	30.56	38.51	31.9	30.25
Cl (ppm)	2667.65	0.08	8111.4	999.83	1164.05	1493.65	2501.93	1820.6
Clay %	1.16	5.41	1.55	3.18	3.99	3.17	0	1.04
Silt %	15.61	47.29	21.42	36.64	34.36	37.71	7.46	10.58
Sand %	83.23	47.3	77.03	60.18	61.75	59.12	92.54	88.38
Texture	Silty Sand	Silty Sand	Silty Sand	Silty Sand	Silty Sand	Silty Sand	Sand	Silty Sand

Plant identification

The total number of plants obtained from the eight research zones was 16. Plant samples collected from each area were identified at the herbarium of Qatar University. The collected plant samples belong to 12 different plant families (see Table 1).

AMF colonization in roots

All segments of the roots examined were colonized by AMF with the presence of hyphae, vesicles and arbuscules, whether internally and/or in combination with the roots. The intensity of infection with arbuscules was less in comparison to mycelium and vesicles. Most of the samples were found to have no arbuscular presence in their root systems.

Table 5 shows the percentages of roots that are infected with mycorrhizal fungus. The colonization rate among plant species varied, the high percentage of AMF root colonization was found to be 100 % in *Tamarix aphylla*, and the lowest value was 12 % in *Zygophyllum qatarense* (Fig. 3).

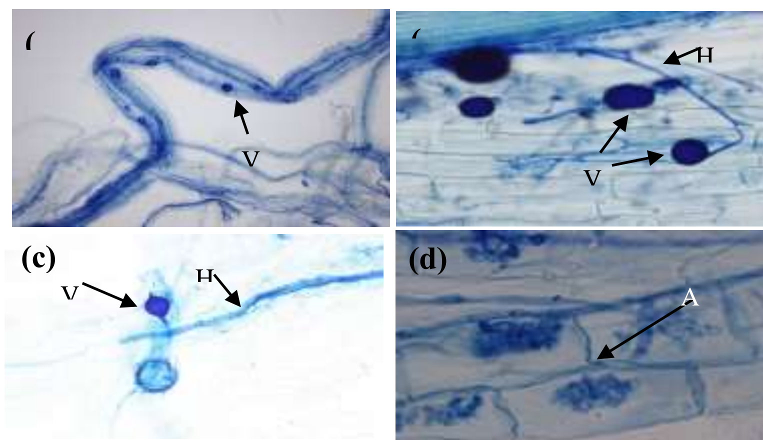


Figure 3. 2 Fungal structures of arbuscular mycorrhizae (AM): (a) Launaea nudicaulis roots (4x). (b) Tamarix aphylla roots (10x). (c) and (d) Pulicaria undulata roots (40x). V: vesicle. H: hypha. A: arbuscular

AMF spores in soil

Spore population varied from 29 to 643 spores /100g, the highest spore number was 643 spores /100g dry soil accompanied with *Fagonia indica* at Assa Alraai location followed by *salvia aegyptiaca* 581 at North East Alkhor, while the lowest value was 29 spores /100g accompanied with *Blepharis ciliaris* at Qatar university protected field.

the average number of spores per sample collection was calculated as spore count per 100g soil. The highest spore count was recorded in soil samples of the Twame area with 562.7, followed by Assa Alraai 515.2, and Northeast Alkhor 494 spore/ 100g soil (Fig. 5).

Table 3.7 Means of root colonization (±SD, *n*= 3) (%) of AMF and spore density (number / 100g soil) in different plants at different site locations.

Location	Geographic location Latitude(°N) / Longitude (°E)	Host plant	Spores/100g soil	AMF root coloniz-ation %
Doha North Sewage Treatment Plant	*25.454167/51.300564*	*Zygophyllum qatarense*	171.7±6.11	12%
		Tamarix aphylla	150.7±6.11	100%
		Launaea nudicaulis	443±6	63%
Assa Alraai	*25.662816/51.393684*	*Sclerocephalus arabicus*	387.3±252.42	32%
		Fagonia indica	643±6.08	32%
Umm Alkilab	*26.049472/51.208722*	*Spergula fallax*	318.3±23.63	10.9%
		Cynodon sp	570.6±88.94	49%
Twame	*26.041618/51.249680*	*Plantago ovata*	562.6±76.96	77.8%
North East Alkhor	*25.715596/51.449514*	*Salvia aegyptiaca*	581±243.91	78%
		Lycium shawii	407±151.92	50.8%
Roudhat Rashed	*25.303106/51.192371*	*Aizoon canariense*	66±6.24	32%
		Pulicaria undulata	39.7±4.93	76.6%
Qatar university Campus	*25.374962/51.490011*	*Malva parviflora*	129±36.43	33.9%
		Paronychia arabica	220.3±14.29	22%
Qatar university protected field (Sabkha)	*25.370381/51.495726*	*Blepharis ciliaris*	29.3±8.33	98.3%
		Sporobolus ioclados	86.3±8.5	92%

DNA integrity and concentration of the library

The integrity and purity of the AMPure XP beads purified library pool was checked using the High Sensitivity DNA LabChip Kit on the Bioanalyzer. The electropherogram of the purified amplicon library quality check is shown in Fig. 4.

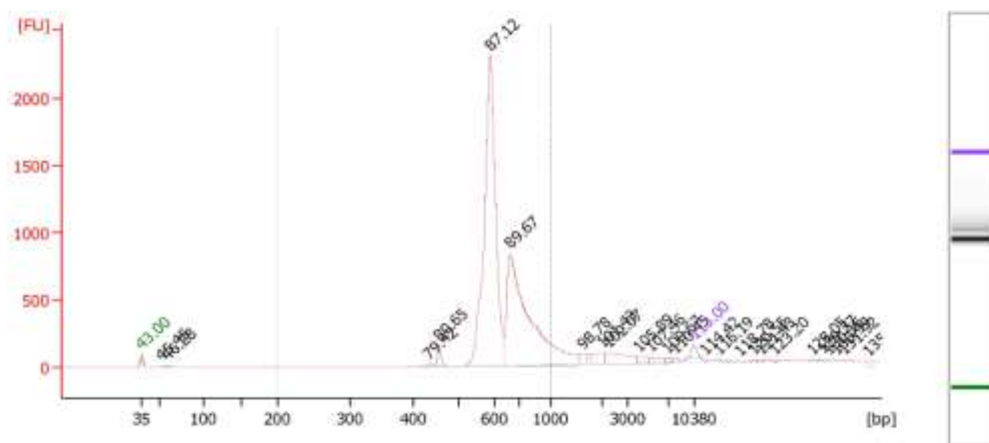


Figure 3.3 Integrity control of the pool library using the HighSensitivity LabChip Kit. The peak at 87.12 s migration time corresponds to c. 587 bp (this includes MiSeq adapter and index sequences). There is a second peak at 89.67 seconds, corresponding to 661 bp. 43 s is 35 bp marker, 113 s is 10380 bp marker.

The pool comprised a main amplicon peak at 587 bp and a second one with 661 bp, corresponding with the expected amplicon size and no signs for contamination with sequencing-compromising primer dimers in lower molecular ranges. Some weak higher molecular weight background signals are detected; however, this is of no concern as cluster generation highly favors lower molecular weight fragments.

The pool library was quantified using the highly sensitive fluorescent dye-based Qubit® dsDNA HS Assay Kit (Invitrogen) and tt showed a final DNA concentration of 219 ng/ul and a molar concentration 48 nM (calculated for a length of 600 bp).

Sequencing results' quality

Quality criteria according to Illumina Inc. were evaluated and are summarized in Table 6.

Table 3.8 Sequencing quality values, of the entire run.

Lane	PF clusters	% of the lane	% perfect barcode	% one mismatch barcode	Yield (M bases)	% PF clusters	% >= Q30 bases	Mean quality score
1	25,361,487	100.00	94.10	5.90	15,268	80.83	58.63	28.05

Read counts per sample showed high quality and enough read counts and are suitable for downstream in-depth data analysis. The filter-passed clusters led to an amount up to 242000 reads cumulated per custom sample, indicating the high variability of the samples. All samples reached the target number of approximately 10000 reads per sample.

Molecular characterization of the AMF communities in the soil samples

AMF diversity - In selected plant rhizosphere of this study, 13 mycorrhizal fungi species have been identified at species level. After identification of these AMF, it appeared that they belonged to 16 genera which belong to 3 different orders and (6) families, table 7 (see appendix Fig. A10-A13 and table A1).

Table 3.9 Number of genera, family and order of identified strains.

Genius	No. of species strains	Family	No. of species strains	Order	No. of species strains
Claroideoglomus	5	Claroideoglomeraceae	5	Diversisporales	9
Corymbiglomus	1	Diversisporaceae	7	Glomerales	28

Genius	No. of species strains	Family	No. of species strains	Order	No. of species strains
Desertispora	1	Gigasporaceae	1	Paraglomerales	1
Diversispora	4	Glomeraceae	23		
Dominikia	5	Paraglomeraceae	1		
Funneliformis	1	Sacculosporaceae	1		
Glomus	1				
Kamienskia	1				
Mikrokamienskia	1				
Nanoglomus	1				
Orientoglomus	1				
Praglomus	1				
Rhizophagus	8				
Sacculospora	1				
Sclerocystis	1				
Septoglomus	1				

DNA of the entire community extracted from rhizosphere samples was subjected to the evolutionary placement algorithm (EPA) analysis, MAFFT alignment permit the identification of 13 representative sequences at the species levels. The total abundance of each of the representative sequences in all samples was calculated, *Funneliformis coronatus* is the most abundant and it dominates samples 1, 2 and 3. *Rhizophagus arabicus* and *Claroideoglomus drummondii* are the second and third most abundant species dominating samples 13 to 16 and samples 1 and 6 respectively (Fig. 5).

Table 8 summarizes the overall identified sequences. Eleven representative sequences were identified to a basal to clade level (btc) which means that the sequences could belong to the species with the given EP number or they could belong to a sister species. Six representative sequences are originated from the node that is basal to the 3 or 4 species which EP numbers are listed. The remaining 9 representative sequences were identified at a broader level with seven of them affiliated to the large clade giving rise to the species listed in the table and the last two affiliated to the basal node of the family listed in the table.

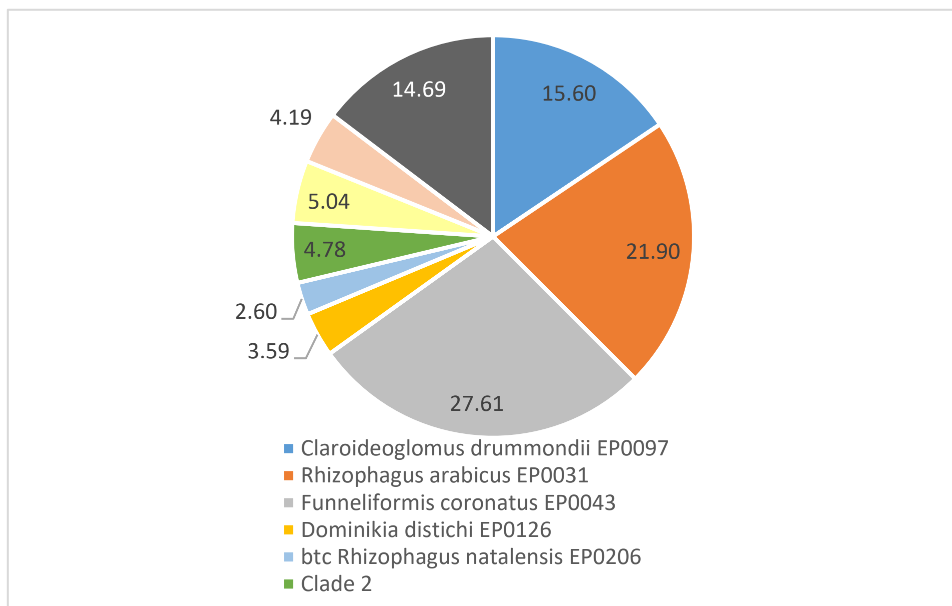


Figure 3.7 Identified AMF species abundance (percentage) in respect to overall results

Claroideoglomus drummondii is the most widespread (the only species detected in all samples) and the second most abundant after *Funneliformis coronatus* in terms of read numbers. Sample 16 showed the highest number of identified representative sequences with 26 AMF species detected, followed by samples 13 and 14 with 23 AMF species

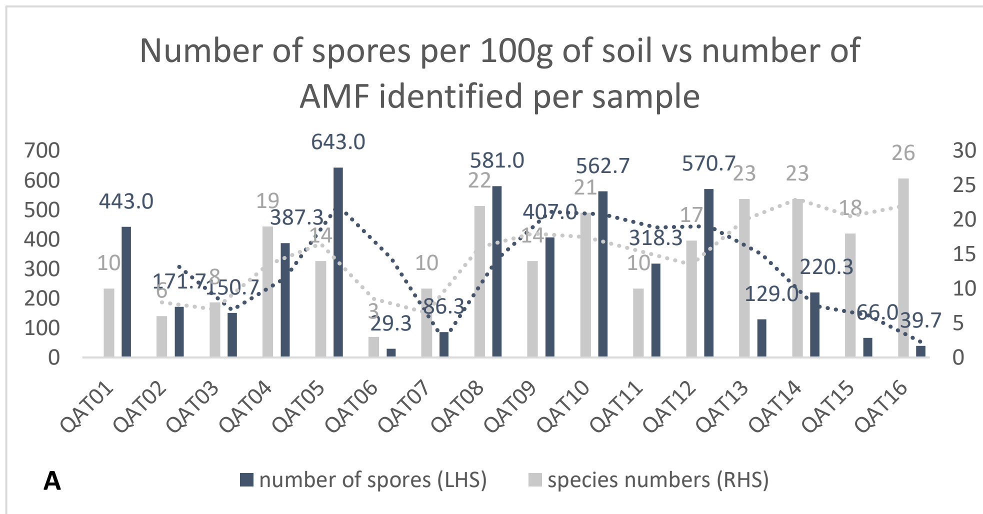
and samples 8 and 10 with 22 and 21 AMF species respectively. According to Shannon diversity index, samples 8 and 10 are the most diverse (Table 8). *Corymbiglomus pacificum* or a sister species is found with seven reads only in only one sample (sample 7), this is extremely low, but cannot be explained with any artifact, thus is interpreted as a real, but low abundance occurrence. Sample 6 shows the lowest diversity, with three species, all in significant abundance (*Claroideoglomus drummondii* EP0097 66%, btc *Rhizophagus natalensis* EP0206 10%, btc *Rhizophagus silesianum* EP0318 24%).

Table 3.10 Diversity indices and frequency of samples from different origins.

Rhizosphere sample	Total number of species	Shannon-Wiener index *H*	Pielou's eveness index E	*e.H.*
QAT01	10	0.76	0.33	2.14
QAT02	6	0.23	0.13	1.26
QAT03	8	1.21	0.58	3.34
QAT04	19	1.3	0.44	3.66
QAT05	14	1.57	0.59	4.8
QAT06	3	0.86	0.78	2.35
QAT07	10	1.22	0.53	3.39
QAT08	22	2.43	0.79	11.3
QAT09	14	1.91	0.72	6.77
QAT10	21	2.34	0.77	10.4
QAT11	10	1.98	0.86	7.21
QAT12	18	1.44	0.5	4.21

Rhizosphere sample	Total number of species	Shannon-Wiener index *H*	Pielou's eveness index E	*e.H.*
QAT13	23	0.96	0.31	2.61
QAT14	23	0.87	0.28	2.38
QAT15	18	0.71	0.25	2.03
QAT16	26	0.9	0.28	2.47

Figure 6 shows the correlation between the total number of spores isolated per 100g of a soil sample and the total number of identified AMF per sample in part a and the total number of spores isolated per 100g of a soil samples and Shannon biodiversity index in part B. As we can see, there is more harmony between trend-lines of part B of the figure, with the exception of the first sample compared to part A. When observing the number of spores identified per 100g of soil in comparison with the total number of identified AMF, the trend-lines did not show harmony in the first samples and in the last four samples. If we connect those results to the percentages of identified AMF in each soil sample represented in figure 7, we notice that those samples have a dominant species, which occupies around 80% of the sample, this is in harmony with their low eveness in those samples (Table 8). Spearman correlation analysis support the results by showing a significant correlation between the number of spores per 100g of soil and Shannon diversity index (P=0.017<0.05). Note that correlation between the number of spores per 100g of soil and the number of identified AMF per sample was not significant.



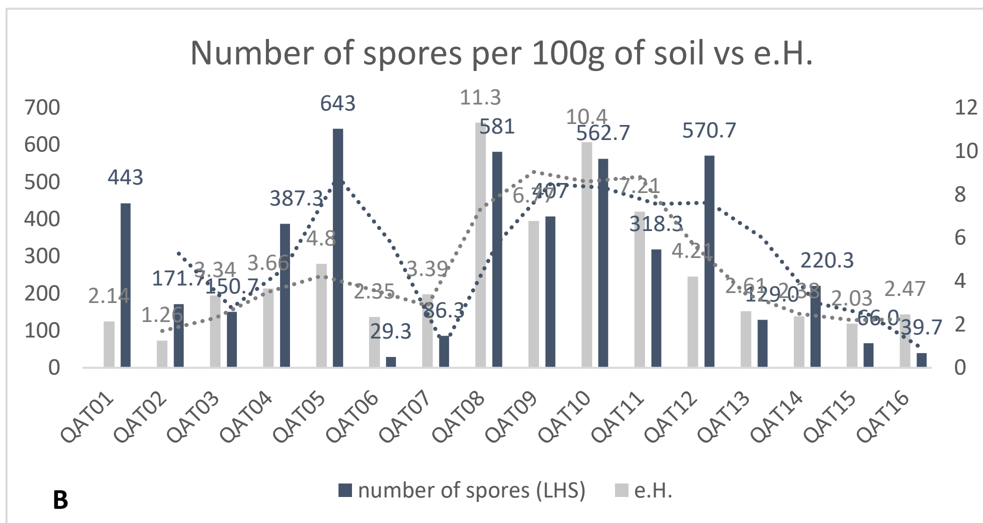


Figure 3.8 Correlation between the spores count per 100g of soil sample and the number of AMF identified per sample (A) compared to the correlation between the spores count per 100g of soil sample and exponential of Shannon diversity index (e.H.) (B) in the 16 studied samples.

Sample 1 is dominated by *Claroidoglomus drummodii* and *Funneliformis coronatus*, while the last four samples are dominated by a species related to *Septoglomus titan.* Considering that metagenomic analysis was used for AMF identification, the number of identified AMF does not necessary reflect active germinated spores (Figure 8), it

could be that the soil in locations 1, 13, 14, 15, and 16 are rich with dormant spores while the actual number of active mycorrhiza is low which explains the low spore count per 100g of soil. Figure 7 also shows that the samples with the most varied sequences are samples 8, 10 and 11. This is in great harmony with the Shannon indices calculated where sample 8 showed the highest diversity index (2.4) followed by samples 10 and 11 with diversity index of 2.3 and 2.0 respectively.

Figure 3.8 shows the distribution of the identified AMF according to plant species. The species *Claroidoglomus drummodii* was found in all studied host plants. Other species were occurred in 15 (out of 16) host plant species and few species were found only in one host plant (Figure 3.8).

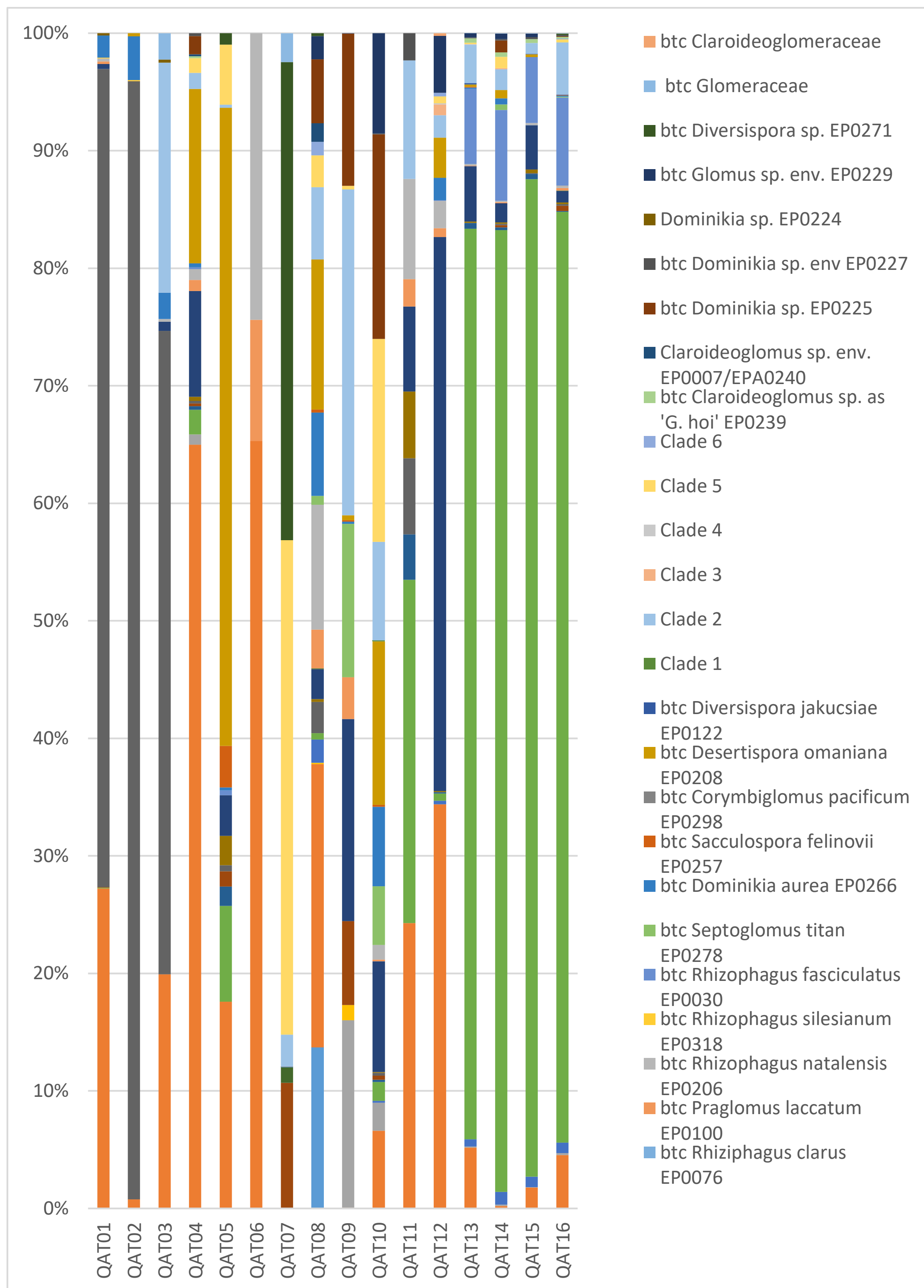


Figure 3.9 Percentages of identified AMF in each soil sample out of the total identified representative sequences per sample.

No.	I-no.	Annotation	Sample code/Plant species															
			QAT 01	QAT 02	QAT 03	QAT 04	QAT 05	QAT 06	QAT 07	QAT 08	QAT 09	QAT 10	QAT 11	QAT 12	QAT 13	QAT 14	QAT 15	QAT 16
			Launaea nudicaulis	*Zygophyllum qatarense*	*Tamarix aphylla*	*Sclerocephalus arabicus*	*Fagonia indica*	*Blepharis ciliaris*	*Sporobolus ioclados*	*salvia aegyptiaca*	*lycium shawii*	*Plantago ovata*	*Spergula fallax*	*Cynodon sp*	*Malva parviflora*	*Paronychia arabica*	*Aizoon canariense*	*Pulicaria undulata*
1	425	Claroideoglomus claroideum EP0039																
2	462	Claroideoglomus drummondii EP0097																
3	530	Rhizophagus invermaius EP 0035																
4	584	Rhizophagus intraradices EP0032																
5	674	Rhizophagus irregularis EP0009																
6	776	Rhizophagus arabicus EP0031																
7	840	Sclerocystis sinuosa EP0204																
8	851	Kamienskia bistrata EP0119																
9	1150	Funneliformis coronatus EP0043																
10	1245	Nanoglomus plukenetiae EP0323																
11	1262	Dominikia distichi EP0126																
12	1979	Diversispora peloponnesiaca EP0322																
13	2087	Diversispora aurantia EP0074																
14	736	btc Rhiziphagus clarus EP0076																
15	2719	btc Praglomus laccatum EP0100																
16	705	btc Rhizophagus natalensis EP0206																
17	716	btc Rhizophagus silesianum EP0318																
18	761	btc Rhizophagus fasciculatus EP0030																
19	1032	btc Septoglomus titan EP0278																
20	1345	btc Dominikia aurea EP0266																
21	1918	btc Sacculospora felinovii EP0257																
22	2197	btc Corymbiglomus pacificum EP0298																
23	2228	btc Desertispora omaniana EP0208																
24	2102	btc Diversispora jakucsiae EP0122																
25	928	btc Kamienskia divaricata EP0255 / Kamienskia perpusilla EP0120 / Mikrokamienskia peruviana EP0321																
26	981	btc Septoglomus turnaue EP0113 / jasnowskae EP0132 / xanthium EP0263 / fuscum EP0139																
27	1304	btc Dominikia duoreactiva EP0260 / D. achra EP0116 / D. lithuanica EP0259																
28	1438	btc Mikrodominikia litorea EP0304 / Orientoglomus emiratum EP0267 / Dominikia sp. EP0202																
29	1940	btc Diversispora varaderana EP0222 / insculpta EP0121 / sp. EP0242 / sp. env. EP0017																
30	2099	btc Diversispora jakucsiae EP0122 / arenaria EP0143 / slowinskiensis EP0217																
31	385	btc Claroideoglomus sp. as 'G. hoi' EP0239																
32	355	Claroideoglomus sp. env. EP0007/EPA0240																
33	1338	btc Dominikia sp. EP0225																
34	1373	btc Dominikia sp. env EP0227																
35	1388	Dominikia sp. EP0224																
36	1504	btc Glomus sp. env. EP0229																
37	2055	btc Diversispora sp. EP0271																
38	360	btc Claroideoglomeraceae																
39	485	btc Glomeraceae																

Colour	Meaning
(blue)	Spores‘ representative sequences allowed identification at the species level with matching between our DNA samples and previously sequenced environmental samples (EP number)
(pink)	Spores‘reresentative sequences allowed to relate the spores to the listed species in the table keeping a margin that it could also be a closely relate sister species (basal to clade)
(orange)	Spores‘reresentative sequences indicates that the spores are affiliated to the node giving rise to the 3 or 4 species listed in the table *
(green)	Spores‘reresentative sequences indicates that the spores are affiliated to the large clade giving rise to the species listed in the table *
(yellow)	Spores‘reresentative sequences indicates that the spores are affiliated to the basal node of the family listed in the table **

Figure 3.10 AMF distribution in various host plant collected from 16 study sites

* The EPA makes a maximum likelihood based computation of the representative sequence under consideration into the reference tree. It gives the likelihood value to where the sequence evolutionary belongs, it is based on real phylogenetic computation.

** It is usual that the representative sequence itself is unknown. In AMF maybe only 5-10% of species are known and even less are defined by DNA data. Therefore, unknown sequences are either new discovered species or just previously described species which have their DNA not sequenced yet.

3.4 Discussion

Several factors can affect the life cycle of AMF, among those temperature, anthropogenic disturbances, plant species dynamisms, light availability, rainfall, soil nutrient & composition, root exudations, as well as competition associated with other microorganism that have interactions with them (Y. Liu et al., 2009; Gong et al., 2012; J. M. Jefwa et al., 2012; Chaudhary et al., 2014) . Although significant correlation has been identified between the diversity index of each sample and the total number of spores per 100g of sample, indicating that soil with convenient conditions are likely to be diverse with different AMF species. Yet when Spearman test was conducted between the number of spores and the raw data of total number of identified AMF per soil sample, correlation was far from significance, further look at the data shows that many samples rich with spores had one or two dominant species, which lower their diversity. As a conclusion, convenient soil conditions might lead to the growth of various types of AMF, while specific conditions might also lead to the domination of particular species that leave the soil rich with a specific type of AMF and eliminate others per competition.

Although properties of soil have been confirmed as essential factors determining the AMF community, there is no one major factor affecting the community of mycorrhiza fungi (Gong et al., 2012; Chaudhary et al., 2014; Melo et al., 2017). In our study no specific correlation was found to be significant between each of the soil parameters and the number of spores neither the percentage of root colonization. These results agree with the study by Oehl *et al.* (2010).

The pH range among our study sites were very narrow from 7.56–9.10 so no significant correlation was found with soil spore count or root colonization, similarly a study carried out by Bainard *et al*. (2014) reached the same conclusion. This non-significant correlation with soil pH has been explained by the fact that optimum pH is variable among mycorrhizae species. The effect of pH may vary from one species to another, A study by Melo *et al*. (2017) found that members belonging to *Acaulosporaceae* expressed a negative correlation with pH, contrarily, the members belonging to Glomoid undetermined group expressed a positive correlation with pH. Other studies indicated that there is a positive relationship between the population of mycorrhizae and the pH, as the percentage of infection increases when pH rise (Gunasekaran et al., 1987; Gai & Liu, 2003; Postma, Olsson, & Falkengren-Grerup, 2007). , while others found some species are strictly reported in acidic soil (Bainard et al., 2014).

The pH of the soil was also found to significantly affect the diversity of AMF populations (Mosbah et al., 2018), however this is not the case in our investigation, possibly due to the narrow differences in pH among soil samples.

It can be noticed in the chemical analysis of the soil that the phosphorus amount has changed from one area to another. The highest amount of phosphorus encountered was in Umm Alkalib, which is linked to low roots colonization (10.9% and 49%), this makes sense as colonizing spores provide plants with the phosphorus from the soil and lack of colonization of AMF would lead to phosphorus accumulation in the soil.

In our study, the only soil sample that showed the highest salinity (3.5 ppt) was collected from a protected field at QU Campus where the number of fungal spores is the lowest compared to other studied sites. Salinity may negatively affect AM fungal development and hyphal augmentation (Gabchenko, 2008). Studies done by different

scientists have different views on the effect of salinity on mycorrhizae development. Barrow *et al.* (1977) reported that sporulation and colonization of fungus is inversely related to the salinity of the soil. The decreased colonization is mainly associated with high concentrations of sodium chloride (Barrow, Havstad, & McCaslin, 1997). Saint-Etienne *et al*. (2006) reported a negative correlation between salinity and mycorrhizal infectivity. An increase in salinity from 5% to 22% results in decrease in infectivity from 100% to 6% respectively. Thus, overall there are conflicting reports on the effects of high saline concentrations on fungus growth and germination (L. Saint-Etienne et al., 2006). The impacts of soil salinity on spore germination of AM growths and consequently hyphal generation is a standout amongst the most critical adverse effects of salinity on mycorrhizal colonization (S. Juniper & L. K. Abbott, 2006). Therefore, the presence of AMF on salt land may be influenced by the type of host plant rather than environmental stress. (Nurbaity, 2014).

The amount of AMF spores found in rhizosphere soils are different from one plant species to another despite the fact they are from the same habitat. This implies that the distribution of AMF does not influence by the zonation arrangement of vegetation instead its more linked with plant species. Therefore, differences in spore counts may be attributed to the variation in the behavior associated with each AMF species and its host, even in the same ecosystem (Klironomos et al., 2011).

The mycorrhizal colonization for selected plant species growing in Qatar was not studied before for their structural colonization with AMF. All the plants studied were infected with AMF but in different proportions. Biotic and abiotic factors affect the number and type of AMF, so the percentage of infection varies from plant to plant and from one region to another (Escudero & Mendoza, 2005; Moreira et al., 2006) . For example, our findings of the Zygophyllaceae family are against the finding of Varma

(1999) who mentioned that this family is mycorrhizae free. We found that the percentage of AMF infection of two plant species belong to this family, *Zygophyllum qatarense* and *Fagonia indica* was 14% and 32%, respectively.

Also a study by Shafiq et al. 2013 showed that the status of root colonization of arbuscular mycorrhiza with *Tamarix aphylla* was very variable even in the same rhizosphere, ranging from 46% -72% and the spore counts ranged between 60 to 246 spores /100g soil, while in our study the percentage of roots infection for the same species was 100% and the average spore count was 150.7 spores /100g soil. This discrepancy in the results confirms that the rate of the infection of the root with the number of AMF is affected by many interrelated factors like salinity, temperature, season, and other factors.

In previous literature, authors have been reported positive correlations between soil spore counts and AMF root colonization rate (Błaszkowski, 1994; Khakpour & Khara, 2012; Sivakumar, 2013), in contrary, others reported negative correlations (Louis & Lim, 1987). While others found both a positive and negative association between AMF colonization and spore population (Khanam et al., 2006). Our results imply no correlations between spore counts and root colonization, similarly as found by Diaz and Honrubia (1994). According to Hetrick and Bloom (1986), the number of AMF fungal spores in natural habitats is not always related to the amount of AMF colonization and The reason for this was due to the fact that AMF spores of certain species sometimes take a long time to germinate in addition to the importance of seasonality and variations in environmental factors among different seasons (McGee, 1989).

Annotations that are said to originate from a node basal to species or a large clade giving rise to some species or even a basal node of a family could be new discovered species.

However, they could be described species which are not defined by DNA sequences yet. This is not unexpected, as for fungi in general and AMF in particular only 5-10% of species are known, and even less are defined by DNA data. Therefore, for many AMF occurring in nature no sequence data are yet available. The EPA methods allows to recognize such species, not represented in the databases, to a certain level, whereas an EPA affiliation at a “basal” node (e.g. basal to a family) might be composed by more than one species, as branching at deep nodes are unknown.

According to Schüßler and Walker (2010), *Claroideoglomus drummondii, Rhizophagus irregularis* are the most widespread species. Our results indicated that *Claroideoglomus drummondii* was the only species detected in all study site samples, which indicates the ability of this species to withstand high temperatures and alkaline soil in addition to its efficiency in establishing symbiosis with roots of different host plants. Similarly the species *R. irregularis*, was found in all our soil samples except one, however many studies mentioned that this species is the same as *Glomus intraradices* (J. Beltrano et al., 2013; Berruti et al., 2016; Fileccia et al., 2017; Hashem et al., 2018). However a study was conducted by Stockinger *et al*. (2009) genetically separated the two types. Our personal communication with Professor Arthur Schüßler (Head of R&D Wilhelms GmbH, Managing Director & Owner Symplanta GmbH & Co. KG) about the two species, he mentioned to us “what formally was named "*intraradices*" is *irregularis*, in at least 99% of the cases”. Numerous studies have proven the effectiveness and usefulness of *G. intraradices* in agricultural fields, from the aspects of promoting plant growth as well as enhancing soil potassium content (Zhang et al., 2017); increasing crop yields (Ceballos et al., 2013); inducing growth promotion of cucumber (Ravnskov & Larsen, 2016) and alleviating the damage caused by salt stress (J. Beltrano et al., 2013).

Diversispora aurantia EP0074 we found in only one sample. This species has never been found in its northern portion, according to the 6,000 rhizosphere soils surveyed from around the world (Blanke, Renker, & Buscot, 2004; J. Błaszkowski, 2012) , possibly due to the inability of this species to withstand extreme temperatures.

In this research, some of the recorded fungal species were already investigated by other authors to improve plant growth and help plants to overcome stress for instance *rhizophagus irregularis* (previously known as *Glomus irregulare*) which is one of the most widespread species in our study, it was subjected as an inoculum in many studies to investigate plant growth promotion (Zhang et al., 2017), not only that but also it was formulated to become a commercial product with 39% production rate compared to other AMF. The results of the above-mentioned product were positive in several research applications for example: Potassium content and plant growth have both improved. (Zhang et al., 2017), increase cassava yields (Ceballos et al., 2013), caused growth promotion of cucumber (Ravnskov & Larsen, 2016) and alleviating the damage caused via salt stress (J. Beltrano et al., 2013). In addition to that *Rhizophagus intraradices* (formerly known as *G. intraradices* and *Rhizoglomus intraradices)* experiments have also proven their positive effect on enhancing plant growth such as increasing dry matter biomass and chlorophyll content (Hajiboland et al., 2010) and increased phosphate content (Bayani, Saateyi, & Faghani, 2015).

The discovery and identification of these fungi that help plants to overcome various environmental conditions and bear to improve plant growth in the arid Qatari environment is essential for paving the way for future potential research into environmental protection and sustainable agriculture applications.

3.6 Conclusion

Rhizosphere soil samples and roots of 16 plant species from eight locations were investigated. 12 representative sequences have been identified at the species levels based on DNA molecular markers. While 2 representative sequences were identified at the family level which means these two sequences are either newly discovered species or just previously described species which have their DNA not sequenced yet. Part of these species were already investigated and proved to be effective in sustainable agriculture applications. However, strains that have been discovered to be effective in one environment might be with no effect in another environment, particularly in arid lands so relying on native AMF species is a better choice. Results from such study, will open research for agricultural applications in greenhouse and field crops.

For the selected plant species growing in Qatar, mycorrhizal colonization with AMF was not studied before. The AMF species described in this study are the first to be discovered throughout the Qatar state which contributes to the literature about the number of species known to exist in this arid region. Because all our isolates come from arid environments, these AMF could help in conserving the biodiversity of desert ecosystems via biological mechanisms to mitigate the negative effects of abiotic stress. In addition, it might have beneficial applications, mainly in agriculture and food security.

CHAPTER 4: UTILIZING ENDOGENOUS ARBUSCULAR MYCORRHIZA ISOLATED FROM NATIVE DESERT ROOTS TO ALLEVIATE ADVERSE IMPACT OF SALINITY IN ALFALFA, *MEDICAGO SATIVA*

4.1 Abstract

The symbiotic relationship between plants and indigenous arbuscular mycorrhizal fungi (AMF) has been found to assist plants to cope with salt stress. Our knowledge of the mechanisms behind the reduction of salt stress by AMF symbiosis is still limited. Thus, pot and field experiments were done to examine the response of the *Medicago sativa* plant to salinity and examine the effect of arbuscular mycorrhizal fungi (AMF) on the growth, chlorophyll, Proline concentration, antioxidant enzyme activity (i.e. catalase CAT and glutathione reductase GR and protein content. Saline irrigation reduced growth (biomass), chlorophyll, and protein content significantly. Potassium, phosphorus, and magnesium uptake were also significantly reduced, whereas sodium uptake increased as compared to AMF-inoculated and control plants. Inoculation with AMF generally improved the above-mentioned growth parameter as well as alleviated salt stress to some extent. Apart from that, AMF boosted mineral element absorption, which has a direct impact on plant osmoregulation. The results of this study suggest that AMF has the ability to improve *Medicago sativa* salt tolerance.

Keywords: Arbuscular mycorrhizal fungi, *Medicago sativa*, salinity, Antioxidant enzymes

4.2 Introduction:

Salinization of the soil, whether natural or artificial, is one of the most important factors affecting crop productivity. The salinity of the soil in arid and semi-arid areas of the world has a considerable impact on plant growth and development. Saline soil occupies up to 7% of the earth's surface (Namdari, et al., 2018). Excessive salinity of soil results in severe effects on plant growth and causes physical drought to the plants. Living cells respond to the presence of excessive sodium and chloride ions, by disruption of cell organelles, nutrient imbalance, and the subsequent effect on the growth of plants (Ruiz-Lozano, 2001). Through scientific investigations, scientists have developed different solutions to deal with salinity and salt stress, among those: development of salt-tolerant plants, leaching of excess salt from the soil, or desalinization of seawater for irrigation are some of the methods that have been employed with limited success (Wang, et al., 2003; Dar, et al., 2018). One of the far more successful methods is the use of microorganisms that are symbiotic with plants in a saline environment and that could be useful to help the plant to overcome the salinity stress. Plants in their natural habitat are colonized by both epiphytic and endophytic microorganisms (Wang, et al., 2018). Arbuscular Mycorrhizal Fungi (AMF) establish a symbiotic association with plants and are considered natural fertilizers because of their ability to provide nutrients, water, and pathogen protection to plants in exchange for photosynthetic products. The absence of these fungi can result in a less effective ecosystem functioning.

The AMF must have a host to complete its life cycle. This brings about its association with the plants, an association that has been discovered to be mutually beneficial; therefore, the fungus helps the plants in nutrient uptake and protecting them against pathogens and the plant supplies needed carbon to the fungus (Berruti et al., 2016).

AMF are associated with many of the plants including xerophytes, halophytes, and hydrophytes (Brundrett, 2017). The AMF have been shown to colonize halophytes and earliest studies have been done in this regard in the early 1920s (Mason, 1928). Many of the studies done by various research groups have shown that AMF increases the plant growth in the saline environment (Al-karaki,1997) for example, enhancing nutrient uptake, improving soil conditions, protecting roots against pathogens, modifying biochemical and physiological properties of the host plant and producing plant growth hormones (Evelin, et al., 2009).

AMF enhance salinity tolerance via several mechanisms, which include enhancing nutrient acquisition, general improvement of soil conditions, and altering the physical and biochemical properties of the host. AMF has thus been successfully tested for bio amelioration of saline soils for better growth of plants (Namdari, et al., 2018).

Several saline soils have been discovered to contain AMF naturally. The most common species of mycorrhizae observed in extreme saline soils belong to the genus *Glomus*. *Glomus eutunicatum*, *Glomus versiform,* and *Glomus intraradices* are the most commonly noticed species of genus *Glomus* in a saline environment. The use of arbuscular mycorrhizae in amelioration of salt tolerance has been tested in a variety of plants including *Trifolium* spp., *Cucurbita* spp., *Acacia* spp., *glycine max*, and *Musa* species (Alarcon, 2012). In most of these species, the use of the AMF showed an increase in plant yield and growth with one or two exceptions, which showed decreased yield. Thus, there are evidences about the use of AMF for growth promoting of plants in saline soils.

This particular research will uncover the positive impacts of the isolated mycorrhizae fungi in alleviating the conditions of plant life in the semi-arid and arid regions,

particularly in Qatar. Mainly, it will delve into the details of isolating the native ecologically adapted fungi and using them in the large-scale production of alfalfa crops (*Medicago Sativa*). The research will highlight the importance of the isolated mycorrhizae fungi on enhancing plant growth under saline condition. Experiments will be carried out on fungi isolated from the rhizosphere of local desert plants in greenhouse and field conditions. The research is thus of particular importance to the Gulf region as it will provide potential solutions and applications for enhancing crop production in an arid land environment.

Alfalfa (*Medicago sativa)* is a popular leguminous crop and is cultivated for animal consumption. According to FAO, the Alfalfa plant is originally starting in the Mediterranean region ("Alfalfa," 2018). It is mostly cultivated as a forage crop to produce either hay or fresh product. In this research, we examined the effect of salinity stress on *M. sativa* development and essential physio-biochemical characteristics, as well as AMF's capacity to mitigate salt stress-induced negative changes.

4.3 Materials and methods

4.3.1 AMF inoculum

The *Zea mays* plant was chosen as the host plant (Trap plant, see appendix Fig. A4) for AM fungus inoculum production. The gathering of indigenous starter inoculum from the rhizosphere soil of naturally growing plants in Qatar's desert initiated the inoculum production method (see appendix Fig. A4). The mixed starter inoculum is made up of rhizosphere soil, native populations of AM spores and fungal hyphae, and chopped root pieces collected from the rhizosphere areas of various native plants in Qatar's desert and pooled for the starter inoculum. *Zea mays* L. was grown as a trap host plant in plastic pots filled with 1 L of 1:4:1 (v/v) mixture of clay, desert sand, and starting

inoculum for four months and during the growing season, the culture pots were irrigated as needed (Figure 1). The used rhizosphere soil was already analyzed for arbuscular mycorrhizal identification based on molecular markers and Table 1 shows the most common occurred mycorrhizal taxa.

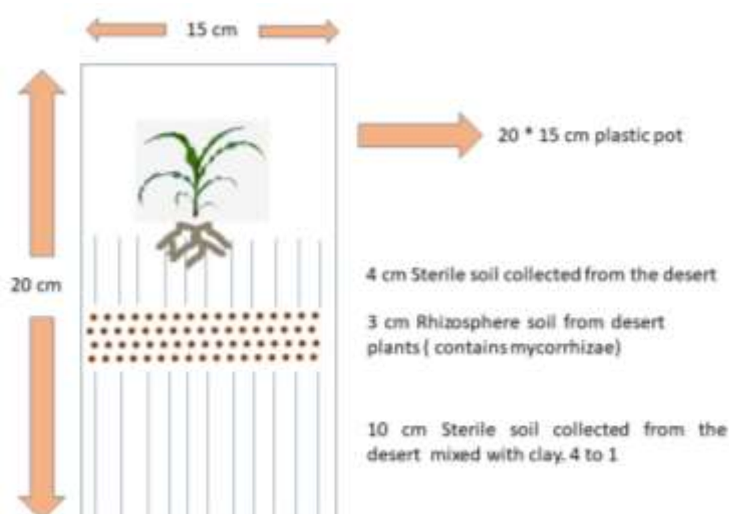


Figure 4.1 A sketch graph showing the start Mycorrhiza inoculum production

4.3.2 Estimation of AM Root Colonization and Spore Population

The 10% KOH was used to clear the roots from the root pigments and cellular contents and staining with trypan blue 0.05% (is a chitin specific stain that stains the cell wall of AM fungi). Then store the roots in 50% glycerol to remove the excess stain from the roots and transfer them onto slides to be viewed under the microscope to verify the existence of fungal structures within the roots for microscopic observations. The stained roots 4cm were placed on glass slides (3 root pieces on each slide) and examined under a light microscope to see the degrees of infection with symbiotic fungi, as seeing the fungus hyphae, vesicles, and arbuscular inside the root hairs means that there is

Infection with these fungi and the degrees of infection were estimated. Percent root colonization was counted according to the following equation (Brundrett et al. 1984).

$$\text{Percent Root Colonization} = \frac{\text{Number of Root Segments Colonized}}{\text{Number of Root Segments Observed}} \text{X100}$$

4.3.3 Experimental setup and growth conditions

Plant growth with salt and AMF treatments

In this study, two separate experiments were conducted. The first experiment was conducted in the greenhouse and the second experiment was conducted in an open field inside Qatar University Campus, Doha, Qatar (25°3748523 N and 51°4900238 E).

In the greenhouse experiment, a sandy loam soil was used with the following properties: organic carbon, 1.84%; total soluble salts, 1.703%; electrical conductivity (EC), 2116 µS/cm, and pH 7.98. fine sand and Dried soil (1:2; w/w) was autoclaved twice for 60 min at 120°C to completely remove the soil microbiota and then a 1:1 mixture of sand and regular soil was prepared and transferred to plastic pots (15 cm in diameter). The seeds of *M. sativa* (Certified Alfamaster 9 lucerne Australia) were surface sterilized with 2% v/v NaOCl, for 5 min then thoroughly washed three times with distilled water. In each pot, three seeds were sown. Ten days post sowing, the number of seedlings reduced to one per pot after choosing the healthiest seedling. The plants watered day after day to prevent drying of the seedlings, low phosphorous fertilizer (10-5-10) was given once for all treatments. Saline treatment is done by irrigation with the assigned NaCl solutions. The pots were divided into six groups with 6 different treatment levels: (1) control plants (no any treatment); (2) plants with regular tap water irrigation and inoculated with AMF; (3) plants stressed with 0.5% NaCl

irrigation regime without AMF; (4) plants inoculated with AMF and stressed with 0.5% NaCl; (5) plants stressed with 1% NaCl without AMF and (6) plants inoculated with AMF and stressed with 1% NaCl. The salt concentration was increased gradually to avoid osmotic shock and to acclimatize plants before salt treatment. With a completely random design, the investigation was done in six replicates. The mycorrhizal inoculum was supplied to the experimental soil as 10 g of trap soil culture (about 100 spores/g soil) per pot. The plants were grown in greenhouse conditions (28±2 ^{O}C) for 63 days. Before harvesting, plant height, leaf chlorophyll content, and the number of branches were measured then the plant samples have been dried in an oven at 75 ^{O}C for at least 72 hours and the dry weight was recorded. The root of the plant samples were collected to determine the rate of mycorrhizae infection. Alfalfa plant leaves were gathered and stored at -80 °C until they were needed for biochemical examination.

AMF colonization and spore counts were accomplished for each plant according to the above-mentioned method (4.3.2 section).

Field experiment

The experiment was carried out outdoor near the greenhouse in Qatar University Campus. The experiment was started on November 25 and ended on March 30-2021. The experiment was one way in a completely randomized design with five replications and with the following treatments: (1) Control, No AMF and irrigated with regular tap water, (2) AMF inoculated, irrigated with regular tap water, (3) No AMF, irrigated with saline water (0.5% NaCl), and (4) Inoculated with AMF, irrigated with saline water 0.5% NaCl).

The soil properties before planting were pH 7.98; salinity 1.1 ppt and composed of 97.83% sand, 2.17% silt; 1.84% organic matter.

The experimental area was prepared by dividing the land into 24 plots with dimensions 50*50 cm for each plot and adding 330 g of natural fertilizers in each plot. 10 grams Seeds of *Medicago sativa* were surface sterilized with Clorox at a final concentration of 2% for 5 minutes then rinsed 4 times with water and planted by hand in each plot. Weeds were weekly controlled by hand as required. Salinity treatment started after one month to ensure that there is no negative effect of the treatment on the seedlings established. Saline treatment was done by irrigation with NaCl solutions with one concentration of 0.5%. The saline irrigation has been started with 0.1% for a week, followed by 0.3%, and then continued with the assigned concentration of 0.5% up to harvesting time. The gradual increase in salt concentration is essential to avoid osmotic shock in alfalfa plants. Irrigation is done every day for each plot to prevent the drying of the seedlings. All plots were harvested at 4 months post after sowing. The chlorophyll content, biomass, and dry weight of various plant parts including aboveground parts (leaves and stems), Shoot length, number of branches, and Chlorophyll content were measured.

Biomass Production: The fresh weight (FW) of the aboveground foliage was measured immediately after harvesting, and the plant weight, height, and the number of branches were recorded. The dry weight (DW) of the plant samples was measured by drying them at 70°C for a minimum of 72 hours and then weighed.

Chlorophyll measurement: Before harvesting the plants, leaf chlorophyll content was recorded using SPAD-502 (Konica Minolta, Japan). In each plot. Chlorophyll contents of 10 different leaves were measured and then averaged to represent the growth of alfalfa.

Determination of proline:

The Bates et al. 1973 method was used to calculate proline content. Free proline content was determined from 0.2 mg frozen leaves (-80 °C). Using 3% of the sulfosalicylic acid aqueous solution, the extract was extracted and then reacted with glacial acetic acid and ninhydrin acid. Proline concentration was measured in a spectrophotometer (Jenway, 6715 UV/ Vis. Spectrophotometer, UK) at 520 nm absorbance. The amount of proline in each unit of fresh weight (FW) was computed using: the following formula:

$\mu mols\ proline\ g^{-1}\ FW = [(\mu g\ proline / mL \times mL\ toluene) / 115.5 \mu g / \mu mols] / (FW / 5)$

Assay of antioxidant enzymes

Preparation of enzyme extracts: An enzymatic extract was obtained by homogenization of 0.2 mg of frozen leaves (-80°C) of each replicate were homogenized in ice 1.5ml 50 mM potassium phosphate buffer (ppb) (pH 7.2). The homogenate extract was centrifuged at 14,000 rpm for 15 minutes at 4°C, with the supernatant used for CAT and GR enzyme activity tests. Bradford's method was used to calculate the amount of protein in the enzyme extract (1976).

The activity of CAT and GR enzymes was measured according to the manufacturer's instructions using Commercial Catalase Assay Kits (Catalog No. CAT100; Sigma-Aldrich, Inc.) and Commercial Glutathione Reductase Assay Kit (Catalog Number: GRSA, Sigma-Aldrich, Inc.), respectively.

Enzyme assays: Based on the amount of decomposed H_2O_2, the absorbance change at 240 nm was used to estimate the activity of CAT. The amount of enzyme catalyzing a breakdown of 1 µmol H_2O_2 per minute estimated from the extinction coefficient 45.2 $mM^{-1}\ cm^{-1}$ was defined as one unit of CAT activity. Whereas NADPH consumption was used to determine GR activity indirectly. At 340 nm using a spectrophotometer (Jenway, 6715 UV/ Vis. Spectrophotometer, UK), the reduced absorbance produced by

NADPH oxidation during GR reduction of oxidized GSH was observed. This allowed the enzyme's activity to be assessed by a drop in absorbance at 340 nm caused by NADPH consumption.

DNA Extraction of Rhizosphere Soil Samples

Soil samples (60 mg/sample) were properly prepared by drying them at 50°C for 48 h. The dried samples were packaged and sent to SYMPLANTA laboratory in Germany (April 2021). The DNA from the 10 root samples was extracted using a modified DNA extraction kit for soil samples and a MP FastPrep-24 5G machine. For each root sample, 60 mg were used for DNA extraction. Samples were then stored at -20°C upon further processing.

Data processing and Polymerase Chain Reaction (PCR)

We followed the same procedures in the previous methodology session (3.3.5).

7. Statistical analysis

If sets of data are normally distributed, then each was subjected to one-way ANOVA. p values ≤ 0.05 will be considered for statistical significance. Mean comparisons were made between control and treated or among all treatments using Tukey's test at $p \leq 0.05$. All data analysis will be carried out using Systat software (SigmaPlot 13 and Sigmastat 4).

4.4 Results

Greenhouse experiments results:

Root colonization:

Non-inoculated alfalfa plants did not show any colonization with AMF in roots. The colonization rate in inoculated roots increased at 1% NaCl by 72%, while in plants inoculated with AMF that were not treated with saline water, it was 67% (Table 2).

Table 4.11 Percentage of arbuscular mycorrhizal root colonization and above-ground foliage dry weight (g) in alfalfa plants. Plants were given NaCl concentrations of 0.5%, or 1%. At each salt level, different letters show significant differences ($p \leq 0.05$) among salt treatments. AMF refers to Arbuscular mycorrhizal fungi inoculation.

Treatment	NaCl (%)	AM root colonization (%)	aboveground foliage part Dry Weight (g)
No AMF (control)	0	0	7.652 ± 0. 87a
No AMF	0.5	0	3.333 ± 0.40c
No AMF	1	0	2.228 ± 0.17d
AMF	0	69 ± 2.5 ab	7.902 ± 0.34a
AMF	0.5	59 ± 2.8 b	5.225 ± 0.21b
AMF	1	74.4 ± 0.28 a	2.793 ± 0.35d

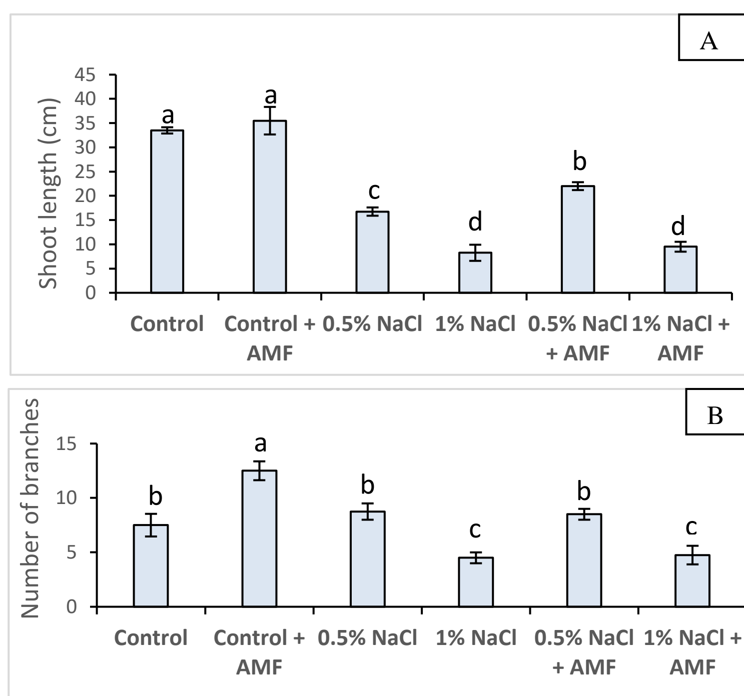
Effect of AMF inoculation and salinity on plants growth

Plant growth parameters: The results of all parameters showed declining with increasing concentrations of the salinity. The shoot length decreased to 50% and 75.4%

at 0.5% and 1% salinity respectively compared to control. However, the application of AMF under saline irrigation showed to have a positive role under salinity treatments, the shoot length increased by 31.34% compared to 0.5% NaCl treatment and by 15.15% compared to 1% NaCl. In the like manner, the number of branches decreases to 43.75% at 1% NaCl while the application of AMF showed a little increase of 5.55% with AMF compared to 1% NaCl without AMF (Figure 3, A-C).

Chlorophyll content: Chlorophyll content significantly decreased using 1% NaCl irrigation. Application of AMF with both saline irrigation treatments showed to have a positive role, compared to control (Figure 3, D).

Proline estimation: In the present study accumulation of proline in non-mycorrhizal and mycorrhizal alfalfa plants increased significantly by raising salinity. The proline content was significantly increased in the saline stressed of non-mycorrhizal and mycorrhizal plants. The greatest proline content was recorded at 1% salinity without AMF treatment (Figure 4.4).



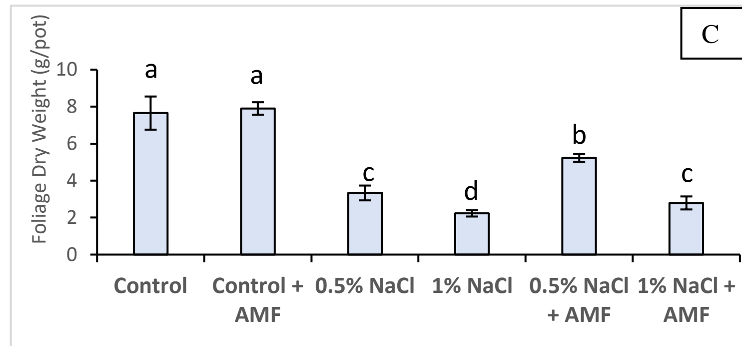


Figure 4.2 Effect of arbuscular mycorrhizae and salinity treatments on the growth parameters of *Medicago sativa* grown under Greenhouse conditions. A. Shoot length, B. number of branches, C. dry weight of shoot, and D. chlorophyll content. Within each graph, values with the common letter(s) are not significantly different according to Tukey's test at $p \leq 0.05$ (N= 5).

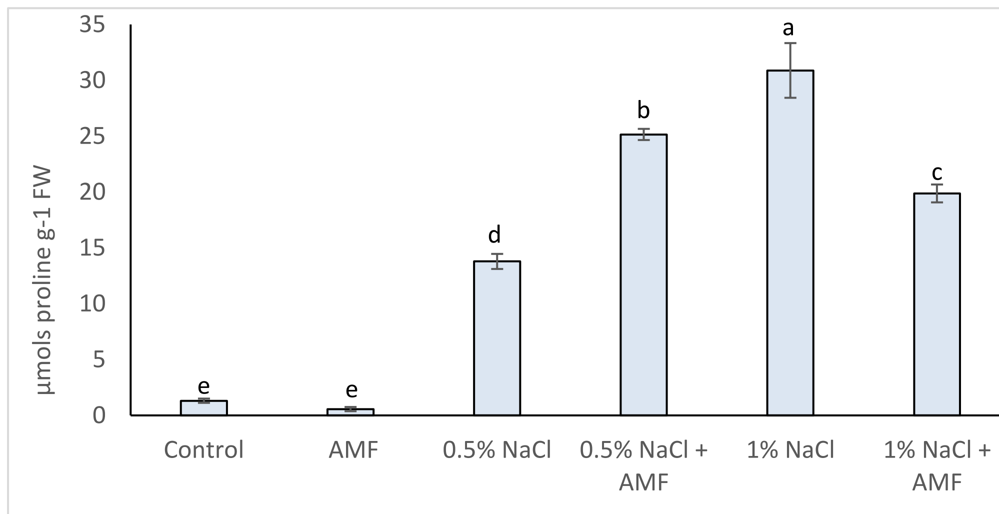


Figure 4.3 Proline concentrations in alfalfa plant leaves grown under greenhouse conditions as influenced by salinity treatment and arbuscular mycorrhizal fungi (AFM). Error bars refer to the standard error of the means. Values with the common letter(s) are not significantly different according to Tukey's test at $p \leq 0.05$ (N= 3).

Antioxidant enzymes:

The results pertaining to the effect of NaCl in the presence and absence of AMF on antioxidant enzymes are presented in Figures (4.5 & 4.6). In the absence of salinity factor, the CAT activity was significantly increased in AMF treatment. Under 5% saline treatment CAT also increased significantly in AMF treatment (Figure 4.5). The CAT activity increased to 36.8% at 0.5% NaCl in the presence of AMF as compared to control. No significant differences were obtained among treatments for the glutathione reductase activity (Figure 4.6).

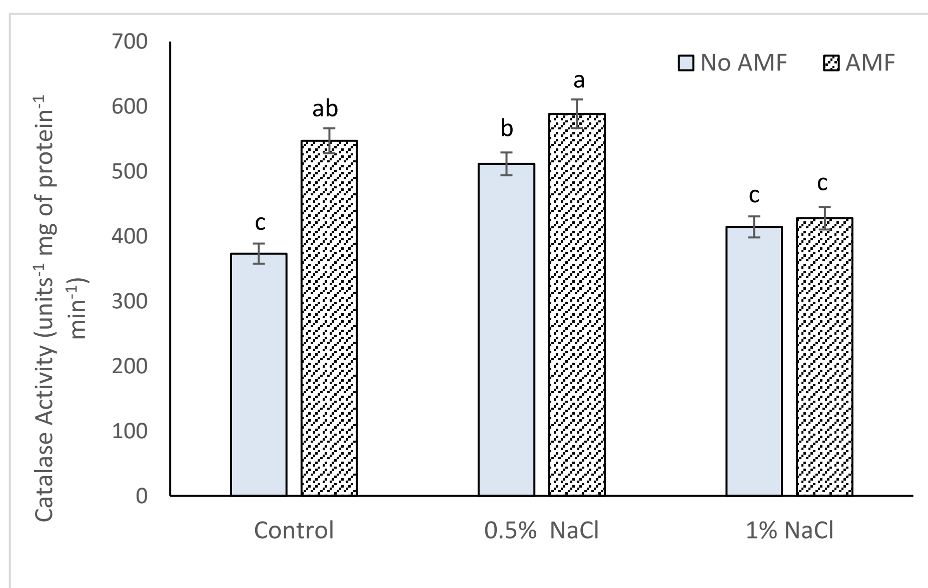


Figure 4.4 Catalase activity in alfalfa plant leaves grown under greenhouse conditions as influenced by salinity treatment and arbuscular mycorrhizal fungi. Error bars refer to the standard error of the means. Values with the common letter(s) are not significantly different according to Tukey's test at $p \leq 0.05$ (N= 3).

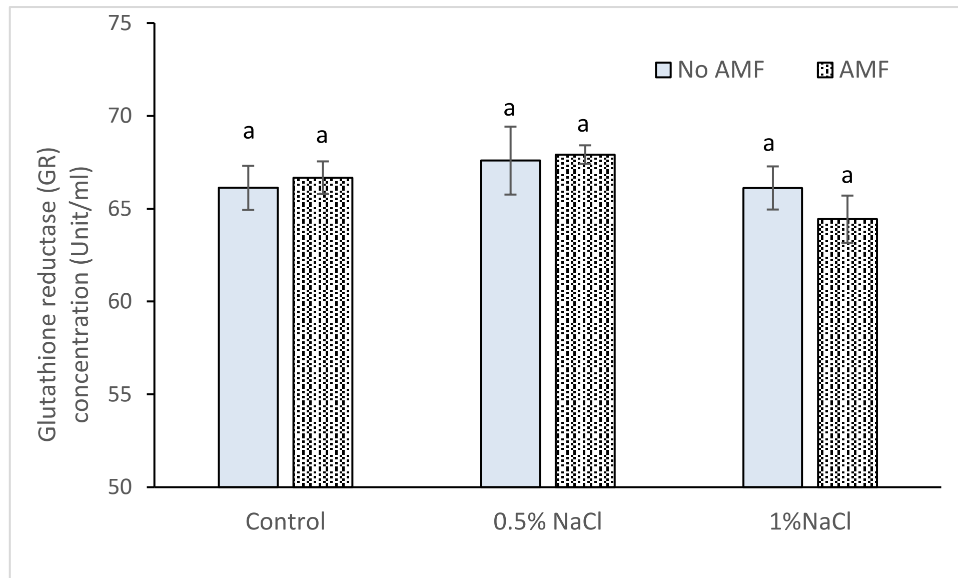


Figure 4.5 Glutathione reductase in alfalfa plant leaves grown under greenhouse conditions as influenced by salinity treatment and arbuscular mycorrhizal fungi (AFM). Error bars refer to the standard error of the means. Values with the common letter(s) are not significantly different according to Tukey's test at $p \leq 0.05$ (N= 3).

Protein content

The treatment of AMF inoculation under regular water irrigation had no significant effect on leaf protein content. Saline irrigation without AMF had negative effects on protein content, however, combining salinity with AMF led to a significant positive effect on protein content (Figure 4.7).

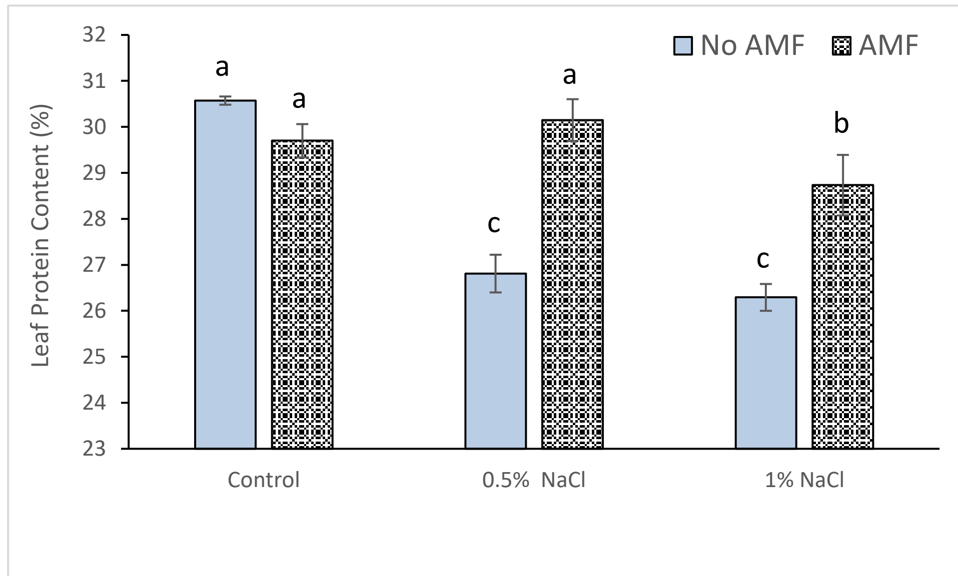


Figure 4.6 Protein content (%) in alfalfa plant leaves grown under greenhouse conditions as influenced by salinity treatment and arbuscular mycorrhizal fungi (AFM). Error bars refer to the standard error of the means. Values with the common letter(s) are not significantly different according to Tukey's test at $P \leq 0.05$ (N= 3).

Results of Field Experiment:

Table 4.2 shows the level of AM root colonization under field conditions. Under field conditions, colonization of AM can occur anytime from soil and our results indicated better colonization with regular water irrigation than using 5% saline water. However, after AMF treatment, our results indicated that the best colonization occurred in the presence of 5% saline watering (Table 4.2).

Table 4.1 Percentage of mycorrhizal root colonization in alfalfa plants as influenced by saline watering. Plants were given NaCl concentrations of 0, 0.5%. Values with a common letter(s) are not significantly different according to Tukey's test at $P \leq 0.05$ (N=4).

AMF treatment	NaCl (%)	AMF root colonization (%)
No AMF	0	43.5 ± 0.8b
No AMF	0.5	25.5 ± 0.8c
AMF	0	51.3 ± 1.4b
AMF	0.5	69 ± 0.3a

Effect of salinity and AMF inoculation on plants growth

In this experiment, all the growth parameters showed a significant reduction with 0.5% NaCl compared to control plants irrigated with regular water. The plant height showed a 21% decline at 0.5% NaCl. Contrary to that, with the application of AMF the height increased by 20.5% compared to the 5% saline water treatment without AMF (Figures 4.9 and 4.10 A). Similarly, a 70% increase in the number of branches in the presence of AMF treatment under regular water irrigation and around 100% increase occurred in the presence of AMF treatment under 5% NaCl water irrigation (Figure 4.10 B). Above-ground biomass was improved significantly with AMF inoculation under both conditions, the presence, and absence of saline irrigation (Figure 4.10 C). The chlorophyll content was decreased by 36% at 0.5% NaCl when compared with the control i.e. the treatment with AMF showed a tremendous increase of around 60% (Figure 4.10 D).

Figure 4.7 General view of the field experiment

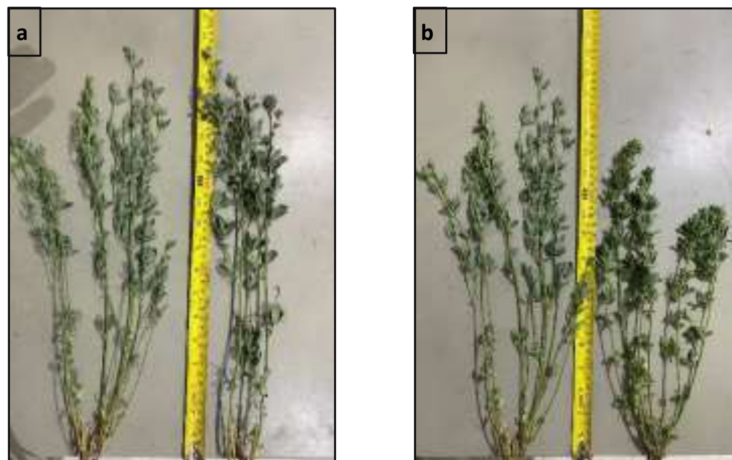
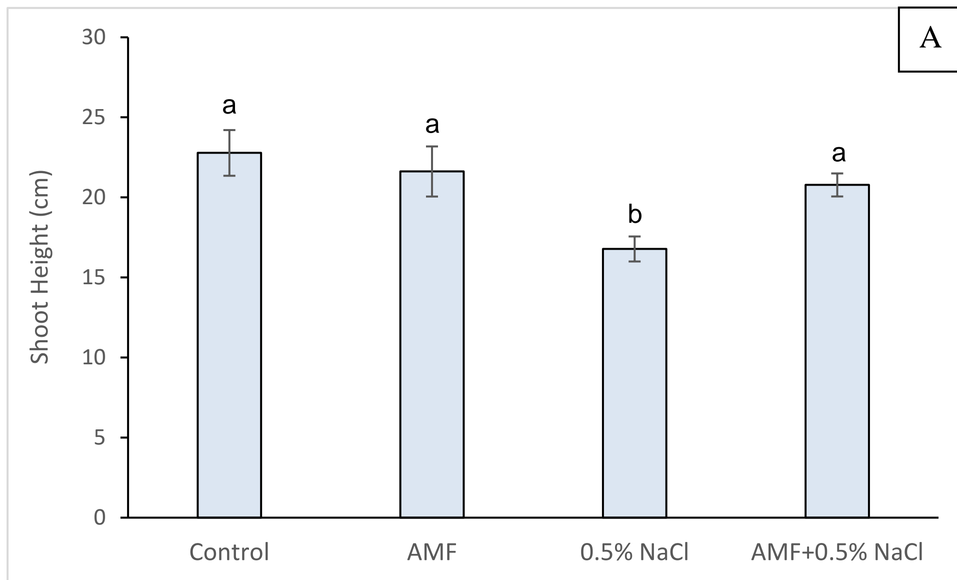
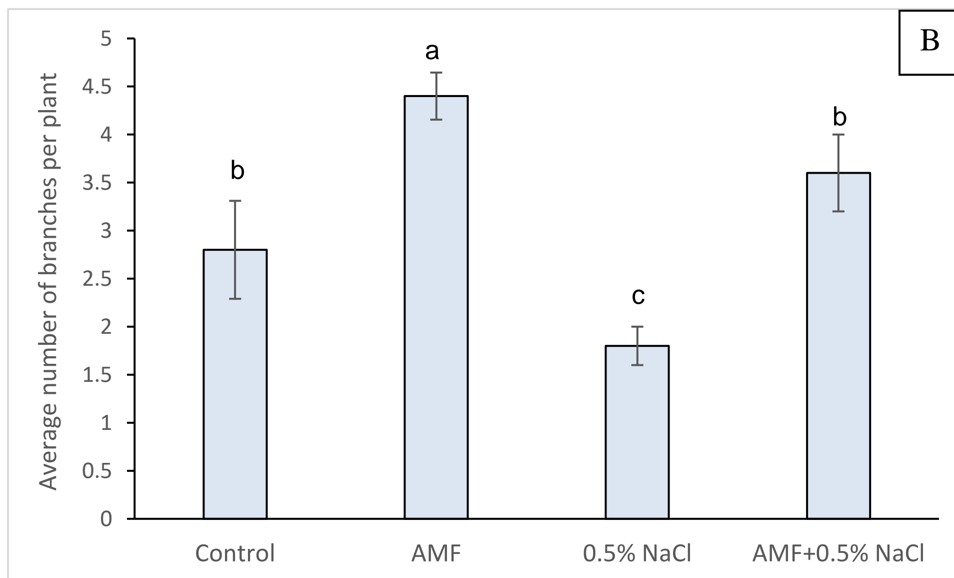
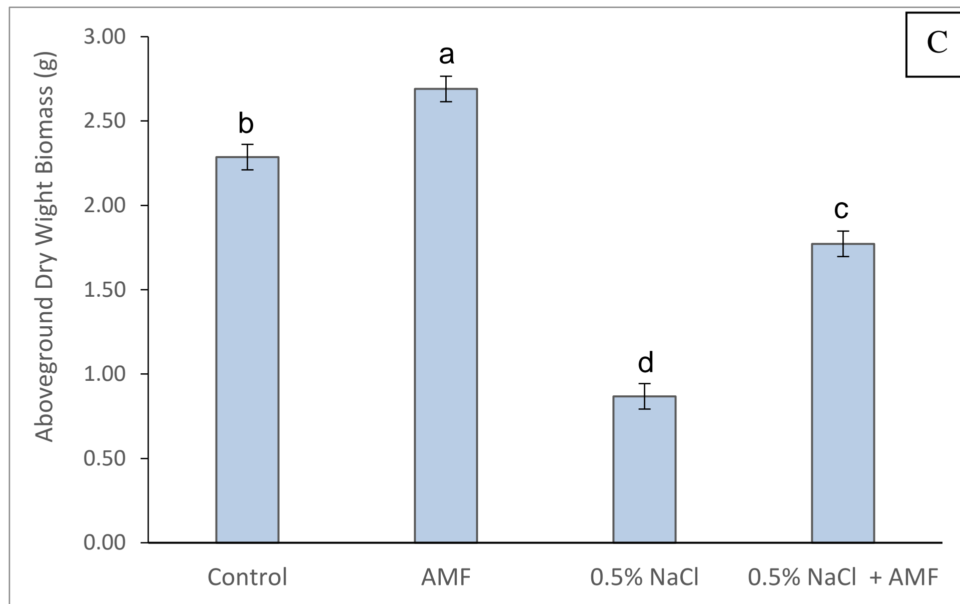


Figure 4.8 Seedlings of Medicago sativa at harvesting time. a) irrigated with regular water in the presence of (right) and in the absence of (left) AMF treatment. b) irrigated with 0.5% salt concentration in the presence of (right) and in the absence of (left) AMF treatment.

Chlorophyll content (SPAD value): The chlorophyll content was decreased by 36% at 0.5% NaCl when compared with the control i.e. the treatment with AMF, which showed a tremendous increase of 57%.







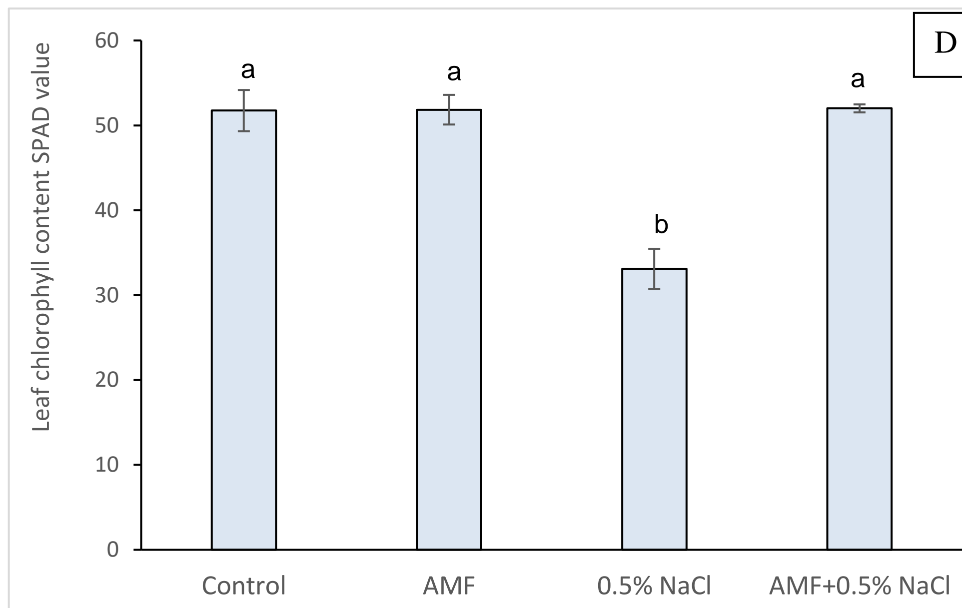


Figure 4.9. Effects of arbuscular mycorrhizae and salinity treatments on the growth parameters of *Medicago sativa* grown under field conditions. A- Shoot length, B- number of branches, C- Dry weight of shoot, and D- Leaf chlorophyll content. Error bars refer to the Standard error of the Means. Values with the common letter are not significantly different according to Tukey's test at $p \leq 0.05$ (N=5).

Proline estimation: In the present study accumulation of proline in the presence and absence of mycorrhizal treatments in alfalfa plants increased significantly by raising salinity. However, at field conditions, the presence of AMF inoculation significantly decreased proline concentration under 0.5% NaCl (Figure 4).

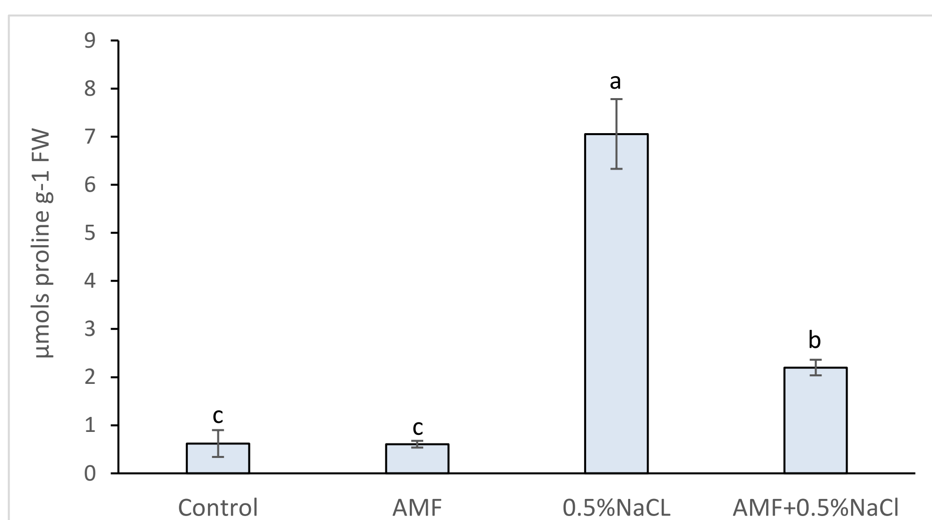


Figure 4.10 Proline concentrations in alfalfa plant leaves grown under greenhouse conditions as influenced by salinity treatment and arbuscular mycorrhizal fungi (AFM). Error bars refer to the standard error of the means. Values with the common letter (s) are not signifecantly different according to Tueky's test at $P \leq 0.05$ (N=3).

Antioxidant enzymes:

The results pertaining to the effect of NaCl in the presence and absence of AMF on antioxidant enzymes are presented in Fig (4.10). Under regular tap water irrigation, the CAT activity had about 2 folds increase in the presence of AMF compared to the control. In the treatment of 0.5%, NaCl decreased significantly CAT concentration, however plants under AFM inoculation treatment possessed a significant increase in

CAT (Figure 4.12). For the GR activity, there is no significant difference exhibited due to AMF inoculation under regular tap water irrigation, however, GR was significant higher in AMF inoculated plants and at 0.5% NaCl irrigation (Figure 4.13).

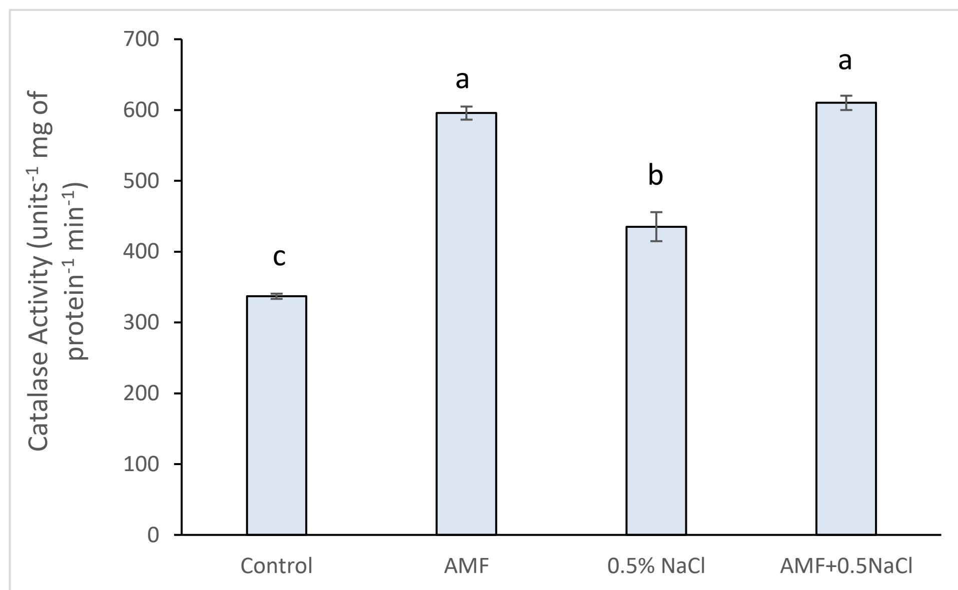


Figure 4.11 Catalase activity in alfalfa plant leaves grown under field conditions as influenced by salinity treatment and arbuscular mycorrhizal fungi. Error bars refer to the standard error of the means. Values with the common letter(s) are not significantly different according to Tueky's test at $P \leq 0.05$ (N=3).

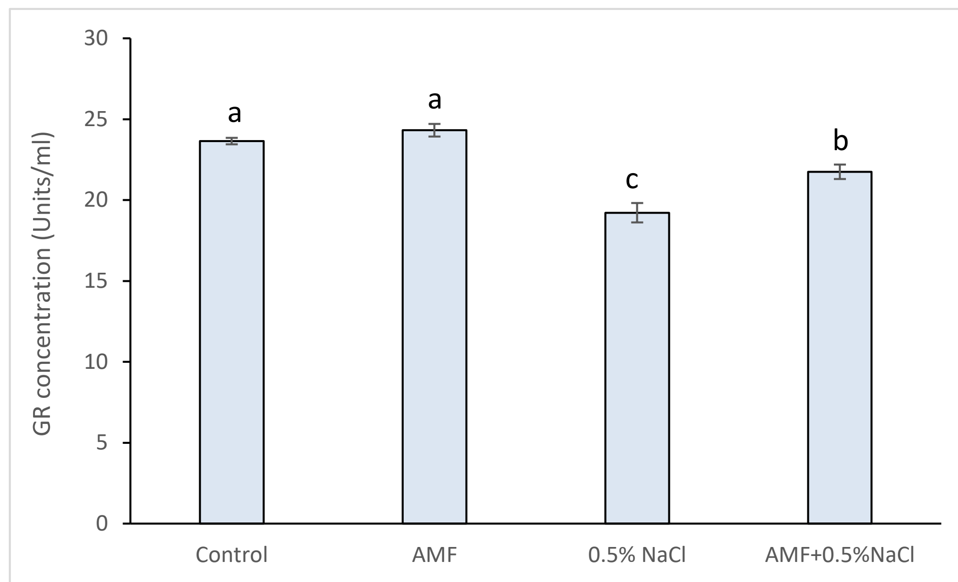


Figure 4.12 Glutathione reductase in alfalfa plant leaves grown under field conditions as influenced by salinity treatment and arbuscular mycorrhizal fungi (AMF). Error bars refer to the standard error of the means. Values with the common letter(s) are not significantly different according to Tueky's test at $P \leq 0.05$ (N=3).

Protein content. The effect of salinity on plant protein production was less than that of the greenhouse experiment. The plants inoculated with the fungi had significant greater protein content compared to the non-inoculated plants and under both kinds of irrigation (Figure 4.14).

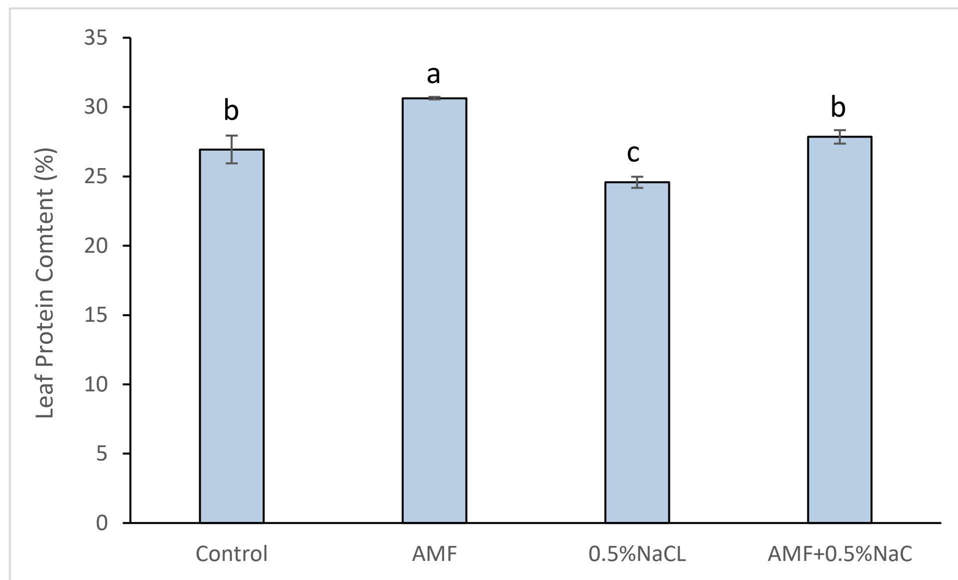


Figure 4.13 Protein content (%) in alfalfa plant leaves grown under field conditions as influenced by salinity treatment and arbuscular mycorrhizal fungi (AFM). Error bars refer to standard error of the means. Values with common letter(s) are not signifecantly different according to Tueky's test at $p \leq 0.05$ (N=3).

Molecular characterization of the AMF communities in the root samples

The total community DNA isolated from soil samples was subjected to the evolutionary placement algorithm (EPA) analysis, MAFFT alignment permit the identification of 12 representative sequences at the species levels. The total abundance of each of the representative sequences in all samples was calculated, *Rhizophagus intraradices* EP0032 and *Nanoglomus plukenetiae* EP0323 are the most abundant most widespread (detected in all root samples). *Rhizophagus* species dominate the reads, however, in the respect that these species form spores in roots and thus may represent a high DNA ratio in roots. *Rhizophagus intraradices, R. variabile,* a species basal to *Mikrokamienskia, Nanoglomus plukenetiae* and a species basal to it, a species basal to *Dominikia*

distichi/D. iranica, a species basal to *Corymbiglomus pacificum/globiferum*, and *Paraglomus laccatum* are highly abundant species represented.

At harvesting time, the species richness of AMF was generally higher under field conditions in the presence of 0.5% NaCl irrigation. Under field conditions, samples from plots exposed to 0.5% NaCl irrigation and inoculated with AMF showed 27 species compared to 10 species under 0.5% NaCl without AMF inoculation. Without inoculation with AMF, 0.5% NaCl irrigation reduced the species richness from 17 under regular water to 10 under saline irrigation (Figure 4.15).

For greenhouse samples, the used soil was sterilized so all non-AMF inoculated samples showed no AMF presence at harvesting time. However, more species richness of AMF had detected with the highest salinity treatment 1% NaCl (Figure 4.15).

No.	I-no.	Annotation	Sample code/Plant species						
			Field exp.				GH. Exp.		
			CON	AMF alone	AMF+0.5%	0.50% alone	AMF alone	0.5%+ AMF	1% + AMF
1	97	*Claroideoglomus hanlinii* EP0098	■	■	■	■			■
2	135	*Claroideoglomus drummondii* EP0097		■	■		■	■	■
3	864	*Rhizophagus natalensis* EP0206			■		■		
4	970	*Rhizophagus intraradices* EP0032	■	■	■	■	■	■	■
5	785	*Rhizophagus irregularis* EP0009	■	■	■	■			■
6	907	*Rhizophagus arabicus* EP0031b	■	■	■		■	■	■
7	1106	*Kamienskia bistrata* EP0119		■			■	■	■
8	435	*Nanoglomus plukenetiae* EP0323	■	■	■	■	■	■	■
9	2280	*Diversispora aurantia* EP0074	■						
10	1989	*Acaulospora herrerae* EP0295				■			
11	2762	*Paraglomus occidentale* EP0324	■	■	■				
12	2803	*Pervetustus simplex* EP0264		■	■				
13	2448	*Archaeospora schenckii* EP0213b			■				
14	875	btc. *Rhizophagus silesianum* EP0318	■				■		■
15	926	btc. *Rhizophagus variabile* EP0326			■		■	■	■
16	997	btc. *Sclerocystis sinuosa* EP0204		■	■		■	■	■
17	1085	btc. *Mikrokamienskia peruviana* EP0321	■	■	■				
18	360	btc. *Septoglomus titan* EP0278					■	■	■
19	433	btc. *Nanoglomus plukenetiae* EP0323	■	■	■	■	■	■	■
20	698	*btc. Halonatospora/Sclerocystis/Rhizophagus*	■	■	■		■	■	■
21	700	bbtc. *Rhizophagus melanus* EP0078/*invermaius* EP0035					■	■	■
22	732	btc. *Rhizophagus dalpeae* EP0316/*dunense* EP0269/*clarus* EP0076	■		■	■	■	■	■
23	365	btc. *Septoglomus sp./viscosum* EP0233b/0233	■	■	■	■	■	■	■
24	202	btc. *Funneliformis coronatus* EP0043/*pilosus* EP0327		■	■		■	■	■
25	429	btc.*Dominikia/Mikrodominikia/Orientoglomus/Nanoglomus*					■	■	■
26	502	btc. *Dominikia distichi* EP0126/*iranica* EP0136/sp. env. EP0008			■		■	■	■
27	523	btc. *Dominikia duoreactiva* EP0260/achra EP0116/*lithuanica* EP0259							
28	592	btc. *Dominikia glomerocarpica* EP0330/*sp.* EP0224/EP224b/*sp.* env. EP0227			■		■	■	■
29	2074	btc. *Corymbiglomus pacificum* EP0298/*globiferum* EP0311	■	■	■	■	■	■	■
30	2728	btc. *Paraglomus laccatum* EP0100/*occultum* EP0100b			■		■	■	■
31	57	*Claroideoglomus* sp. as *Glomus hoi* EP0239	■				■	■	■
32	1063	btc. *Microkamienskia*			■		■	■	■
33	255	btc. *Septoglomus*	■	■					■
34	644	btc: *Dominikia sp*. env. EP0227					■		■
35	634	*Dominikia sp*. EP0224		■	■				
36	2288	btc. *Diversispora sp.* EP0271		■	■	■	■	■	■

37	2787	btc. *Innospora*							
38	24	btc. *Claroideoglomeraceae*							
39	155	btc. *Glomeraceae*							
		Total	17	20	27	10	27	23	29

	Spores' representative sequences allowed identification at the species level with matching between our DNA samples and previously sequenced environmental samples (EP number)
	Spores' representative sequences allowed to relate the spores to the listed species in the table keeping a margin that it could also be a closely relate sister species (basal to clade)
	Spores' representative sequences indicates that the spores are affiliated to the node giving rise to the 3 or 4 species listed in the table *
	Spores' representative sequences indicates that the spores are affiliated to the large clade giving rise to the species listed in the table *
	Spores' representative sequences indicates that the spores are affiliated to the basal node of the family listed in the table **

Figure 4.11 AMF distribution in *Medicago sativa* roots in different treatments of both greenhouse (GH) and field experiments.

* The EPA makes a maximum likelihood based computation of the representative sequence under consideration into the reference tree. It gives the likelihood value to where the sequence evolutionary belongs, it is based on real phylogenetic computation.

** It is usual that the representative sequence itself is unknown. In AMF maybe only 5-10% of species are known and even less are defined by DNA data. Therefore, unknown sequences are either newly discovered species or just previously described species that have their DNA not sequenced yet.

4.5 Discussion

Perhaps AMF is the most earlier type of mutualism that exists between plants and microorganisms in a different environment (He et al., 2007). Under unfavorable and stressful environments, Mutualism between mycorrhizal fungi and plants could be beneficial to plant and soil health. It was revealed that AMF enters plant roots cortical cells which enables the root of morphology to alter and develop its tolerance against weeds, pests, and disease (Alderfasi, Al-Suhaibani, Selim, & Al-Hammad, 2016). AMF also enhances the plant's capability to cope with salt pressure by developing the absorption of mineral nutrients, keeping ions, preserving the acts of the enzyme, and facilitating water uptake, while the rise of salinity resistance in many plants including tomato and maize (Al-Karaki, 2001). AM mutualism can often enhance the resistance of the potential host to the likelihood of salinity stress although AMF growth can be influenced by salinity at various magnitudes depending on the salt levels (Giri & Mukerji, 2004).

However, the activities that influence AMF to enhance and improve salt tolerance remain ambiguous. Several studies conducted indicated that the improved salt resistance solely leads to mycorrhizal controlling the improvement of essential nutrients uptake, particularly of those that are characterized as sessile soil mineral nutrients such as phosphorus (P), copper (Cu), and zinc (Zn) (Marschner & Dell, 1994; G. Al-Karaki, 2000; G. N. Al-Karaki et al., 2001). (Marschner & Dell, 1994; G. Al-Karaki, 2000; G. N. Al-Karaki et al., 2001). Interestingly, several studies conducted recently have investigated this and put other likely mechanisms/activities like the greater osmotic amendment in plants with AMF, oxidative damage reduction (Ruiz-Lozano, 2003) as

well as over-expression of aquaporins induced by the AMF symbiosis (Krajinski et al., 2000).

Several studies examined the effect of AMF on alfalfa under the influence of salinity stress. These studies differed in the types of AMF inoculum used, for example, an inoculum consisting of several native isolated AMF combined with some types of mycorrhiza helper bacterium (Ashrafi, Zahedi, & Razmjoo, 2014; Ben Laouane et al., 2019) and other studies used an inoculum from one species (Campanelli et al., 2013), (Shi-Chu et al., 2019). The effects of native isolated Qatari indigenous consortium AMF under an abiotic stressful environment, have not been analyzed recently. Thus, in this research, we studied the impacts of indigenous AMF on alfalfa under salt stress.

AMF roots colonization

Regarding the effect of salinity on AMF colonization, studies conducted by different groups have different views on the effect of salinity on mycorrhizae development. Barrow et al., (1977) reported that sporulation and colonization of fungus are inversely related to the salinity of the soil. The decreased colonization is mainly associated with high concentrations of sodium chloride (Barrow, 1997). However, the presence of *Glomus* species in highly saline soils of Tabriz plains, Turkey led to AMF spores germination, and thus salt acts as a stressor for spore germination (Aliasgharzadeh et al., 2001).

In our study at the greenhouse experiment, the colonization rate in inoculated roots increased at 1% NaCl by 72%, While in plants inoculated with AMF that were not treated with saline water, it was 67%. While in field experiment the colonization rate in inoculated roots increased at 0.5 % NaCl by 69%, While in plants inoculated with

AMF that were not treated with saline water, it was 51%, which means that the rate of colonization on roots treated with salinity is greater than the roots which were irrigated with normal water. This is consistent with findings of (Estrada et al., 2013) where the root colonization with AMF at the salinity level of 100 mM was 84.3%, while in the control sample 67%. But other studies found an inverse relationship between the increase in salinity and the colonization of AMF in the roots (Miranda, Fischer, & Ulrichs, 2011; Hashem et al., 2015). (Campanelli et al., 2013), in their research of alfalfa, it was found that the root colonization decreased by increasing the salinity which was 47.7% at 150 mM NaCl comparing to control which was 69.7%. In addition, the mycorrhizal rate in inoculated alfalfa significantly decreases in the presence of NaCl. Also, it was reported by Saint-Etienne et al. (2006) that a negative correlation between mycorrhizal infectivity and salinity. An increase in salinity from 5% to 22% resulted in a decrease in infectivity from 6% to 100% respectively. Thus, overall, there are reports that contradict the impacts of high saline concentrations on fungus growth and germination (Saint-Etienne, 2006). In another instance, the studied conducted by several researcher revealed that in the temperate grassland environments, AMF colonization levels are abnormal, thus, the water contents, sodality, and soil salinity were positively connected with arbuscular as well as AMF root colonization in *Lotus tenuis*, yet contrarily as find in the grasses. Furthermore, the impacts of soil salinity on the germination of AMF spores, growths, and consequently hyphal generation is a standout amongst the critical adverse impacts of salinity on the colonization of mycorrhizal (Juniper and Abbott, 2006). Therefore, the root colonization of AMF in salt land may depend on the developmental level of the plants (Miranda et al., 2011), the type of host plant, and may not on the environmental stress (Nurbaity, 2014).

The level of root colonization is mostly not linked to the encouragement of plant development by AMF symbiosis; however, the research on AMF indicates that there is no root colonization benckmark value for improving plant growth and which varies depending on the plant and the involved fungal in the mutualism. Results from the present study, although the treatment of alfalfa with NaCl had a higher rate of colonization than the autochthonous AMF, no clear indication if it has higher symbiotic efficiency. Furthermore, the general assumption is that the enhancement of AMF impacts on the development of the plant is influenced by the higher rate of fungal root colonization.

Plant growth parameters:

Generally, salt stress may inhibit development in plants, accelerate their rate of growth, deteriorate their state, or even kill the plants if the exposure is for a long period. The major effect of salinity is the inhibition of growth in plants even though shocks from grave saline conditions may cause the automatic death of cells (Amirjani, 2010).

Salinity harms the performance of *M. sativa* plants, as shown in the present study and earlier investigations (Anand et al. 2000; Maggio et al. 2009 and hi-chu et al. 2019). Interestingly, because of the direct impacts of ion toxicity or indirect impacts of cation and anions in saline that create the imbalances in soil/plant osmotic (Abdel Latef, 2010), Thus, considering this study, the concentration of salt had a significant effect on the growth of alfalfa plant especially at 1% NaCl, salinity stress dramatically reduced all the parameters showed (shoot length, dry weight, and the number of branches) compared to the control treatment. High salinity hurts the vegetative growth of most plants. Several studies conducted previously on the effects of salinity on plant growth indicated that plant length and dry weight are the most affected, and this is mostly

because osmotic balance and ion uptake are compromised (Dastogeer, Tumpa, et al., 2020).

However arbuscular mycorrhizal fungi, as found in different plant species, serve to attenuate the impacts of salinity treatments (Sheng et al. 2008; Borde et al. 2011; Laouane et al. 2019; Parvin et al. 2020). Although decline all the growth parameters in *M. sativa* with increasing concentrations of NaCl, in the entire treatments, the mycorrhizal plants developed well, faster, and showed a slight decline in growth under salt stress conditions than non-mycorrhizal plants. For instance, the current study showed that colonization with AMF significantly enhanced the dry weight as well as shoot length in both greenhouse and field experiments at 0.5% NaCl irrigation, whereas at 1% NaCl level the improvement was not sometimes significant. This may be attributed to AMF propagules' delayed germination and reduced hyphal development. (S. Juniper & L. Abbott, 2006) or due to plant damage because of intolerance to 1% salinity.

Additionally, soil salinization limits plants' ability to absorb mineral nutrients, whereas the AMF symbiosis boosts nutrient uptake by the host plant's roots via increasing the area of root contact due to the extraradical mycelium (Evelin et al., 2009).

Proline and antioxidants enzymes

When exposed to environmental stress, the activities of several antioxidant enzymes are increased. Antioxidant enzymes help to scavenge hazardous ROS, lowering the damaging effects of oxidative stress on proteins, nucleic acids, and lipids. Salt stress may cause a combination of deleterious impacts on plants with high salt sensitivity viz. osmotic stress, oxidative stress as well as ion toxicity. Thus, the triggering of

antioxidant enzymes like CAT and GR and other enzymes can be seen as one of the ways plants response to mitigate salinity stress (Mo et al., 2016). Interestingly, these antioxidant enzymes have participated in the destruction of H_2O_2 from the root systems of salt-stressed plants (S.-Y. Kim et al., 2005). However, CAT (EC 1.11.1.6), which is situated in the membrane-bound organelle called peroxisomes, will reduce H_2O_2 to H_2O and O_2 without utilizing reductants and, therefore, may have enough capacity to supply enough energy to the plant cells for the removal of hydrogen peroxide (Scandalios, Guan, & Polidords, 1997).

Glutathione reductase is an antioxidant enzyme that belongs to the NADPH-dependent oxidoreductase family and is also referred to as GR or GSR (EC 1.8.1.7). Like many other antioxidant enzymes, it can be found in both prokaryotic and eukaryotic organisms within a given environment.GR is mostly found in chloroplast, cytosol, mitochondria of cells but over 80% of its enzymatic metabolism occurs in photosynthetic tissues which were described as chloroplastic isoform (Edwards, Rawsthorne, & Mullineaux, 1990; Ashraf, 2009). The importance of GR is manifested as a key player in cell defense to cub the deleterious activities of reactive oxygen species (ROS) by maintaining the reduction of the cellular GSH (Glutathione) pool via speeding/accelerating the reduction of GSSG (oxidized glutathione) to Glutathione so that the oxidation of NADPH is accompanied (Contour-Ansel et al., 2006; Anjum, Umar, & Chan, 2010). In our study, the framework, catabolism, and importance of enzyme GR in alfalfa under salt stress conditions are duly discussed with references to several literature. In the same vein, GR is an essential antioxidant that equally scavenges H_2O_2 and O_2^- (Noctor & Foyer, 1998)

Several antioxidant enzymes were examined to see if AMF had an antioxidant defense to scavenge ROS damage caused by salinity stress in different plant species. (Estrada et al., 2013; Mo et al., 2016; Pandey & Garg, 2017; Bijalwan et al., 2021).

There are few studies on AMF-induced alterations of antioxidative enzymes activity particularly CAT in alfalfa plants under salinity stress. Also, to the best of our knowledge, we could not find a previous study that specifically examined the activity of GR on inoculated alfalfa plants under salinity stress which might make our study the first to examine the effect of AMF on salt-stressed alfalfa at different concentrations.

Furthermore, different studies had reported that the content of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), oxygen (O_2^-) was elevated when the salinity is increased, as a result of the non-proportionality between the production as well as the destruction of ROS (Ghorbanli, Ebrahimzadeh, & Sharifi, 2004). Interestingly, the ROS activities and metabolism is influenced by different functionally interrelated antioxidant enzymes viz. catalase (CAT), SOD, POD, and APOX. Thus, various studies have shown that a strong correlation exists between antioxidant defense mechanisms snd the ability to mitigate in many plants (Benavídes et al., 2000; Garratt et al., 2002). The prevention of damages from ROS formation can be prevented when the antioxidant capacity is constitutively high (Harinasut et al., 2003).

Also, according to many studies, under abiotic stress especially salinity stress, plants ignite different antioxidants enzymes viz. APX. CAT, and SOD to mitigate the negative consequences of stresses on plants. From our study, it was shown that an increase in CAT activities in alfalfa plants subjected to salinity stress is shown in Figure 4.5 conform with this statement. Likewise, particular antioxidant genes were reported to have been triggered with the application of AMF (Hediye Sekmen, Türkan, & Takio,

2007; Ren et al., 2019; Ben-Laouane et al., 2020; Cortés-Estrada et al., 2020). Thus, the inclusion of AMF may likely contribute to the scavenging ROS species with the stressed condition of salinity. In a study conducted by Bose et al. (Bose, Rodrigo-Moreno, & Shabala, 2014) have pointed out that the H_2O_2 formed is broken down into water (H_2O) and Oxygen (O_2) by CAT. Our findings are in line with this report because the application of AMF to different concentrations of salinity treatments showed a significant increase in CAT activity compared to control.

Antioxidant enzymes regulate the scavenging of virulent reactive oxygen species and thus mitigate the potential negative effects of the oxidative stressed-induced on different fragile molecules viz proteins (amino acids), lipids, and nucleic acids (Hashem et al., 2015). Interestingly, our results have shown an increasing trend of CAT activity with all treatment at different salt concentrations which coincide with the findings from studies conducted on *Medicago sativa*, *Sesamum indicum*, *Brassica juncea,* and *C. arietium* respectively (Koca et al., 2007; Mittal, Kumari, & Sharma, 2012; Campanelli et al., 2013; Rasool et al., 2013). Furthermore, the improvement in antioxidant enzyme activity during the stressed conditions assists in the quick removal of ROS and keeping the levels within or below less harmful levels (Hashem et al., 2015). Also, in our findings, AMF inoculation enhanced the increment of the studied antioxidant enzyme, thus taking speedy scavenging of ROS to ensure that metabolic activities are not negatively affected. This finding is well supported by studies conducted by Ghorbanli et al. (2004) and Abdel Latef and Chaoxing (2011) for *Solanum lycopersicum* plant as the antioxidant enzymatic activities in AMF plants increased.

In our study, in the greenhouse environment, the activity of GR was not changed due to treatments of salinity while under field conditions with 0.5% NaCl irrigation, the GR

was reported to be significantly greater in the presence of AMF inoculation. The activity of GR may be linked to the strain of the fungi used, the environmental factors, and the level of stress due to salinity and other factors. However, in some situations, antioxidant activity in plants may not change as a result of AMF colonization in stressless or low salinity circumstances. which is enough evidence to justify that the low salinity and controlled environmental conditions in the greenhouse experiment in our study could be responsible for the non-significant effect of AMF on the stressed alfalfa as evident in a meta-analysis conducted by (Dastogeer, Zahan, et al., 2020).

Protein contents:

When a plant is exposed to any biotic or abiotic stressor, the first observable response is a drop in its usual metabolic activity, which leads to a decrease in plant growth. protein synthesis is one of the most significantly impacted anabolic activities (Bonjoch & Tamayo, 2001).

Indeed, the host plant tolerates osmotic stress caused by salinity via altering biochemical responses by increasing metabolite biosynthesis (proline), which acts as osmolytes, thereby maintaining water potential, hydration, and turgor levels, all of which are important for overall physiological activity in harsh environments. (Jogawat, 2019).

Increasing the production of a protein that lowers the negative effects of reactive oxygen species (ROS) and can assist sustain photosystem functionality in drought-stricken areas)

On all salt treatments, our data showed that salinity reduced the protein content of non-inoculated Alfalfa plants. Many researchers have previously similar reports of exhibition poor protein synthesis under salt stress conditions in other plant species, such

as wheat (Abdul-Kadir & Paulsen, 1982), *Vigna radiata* L. (Kabir, Karim, & Azad, 2004), soybean grains (Ghassemi-Golezani et al., 2010) and *Panicum turgidum* (Koyro et al., 2013), The reduction in protein content was explained by several metabolic mechanisms like a reduction in amino acid incorporation into protein (Pessarakli & Tucker, 1985) or a reduction in polyribosome levels (Morilla, Boyer, & Hageman, 1973) due to salt stress. This is most likely the reason for the decrease in protein levels in alfalfa plants under stress in the current study. The protein content ratios of plants under salt stress compared to plants under normal (non-stressed) conditions showed that plants under stress produced much less protein than plants under normal (non-stressed) conditions. Furthermore, compared to non-AMF plants, protein content in inoculated AMF plants exposed to salt excess was substantially higher, most likely due to the direct influence on K^+ accumulation, which may aid in maintaining a high K^+/Na^+ ratio, decrease protein synthesis and minimize enzymatic disruption. Our results agree with those of (Hajiboland et al., 2010), Ashrafi et al 2014 and Laouane et al (2020) who reported that the protein contents of alfalfa were increased with inoculated AMF alfalfa plants compared to non-AMF plants.

4.6 Conclusion

Alfalfa plant exposed to salinity stress showed a significant decrease in physiological and biochemical indices, especially at 1% NaCl. Our findings support the idea that the isolated native AMF can help protect plants from salt stress. As a result, the AMF plays an important role in assisting alfalfa plants by reducing the negative effects of salinity stress. AMF inoculations enhanced the growing biomass, CAT activity, and protein content. This is in line with prior research on mycorrhizal fungi's role in stimulating plant growth and reducing the detrimental effects of salt stress. ((Campanelli et al., 2013; Ashrafi et al., 2014; Namdari, Arani, & Moradi, 2017; Ben-Laouane et al., 2020).

By understanding more about the isolated native arbuscular mycorrhizal symbiosis with alfalfa plan it is hoped that using these isolates can aid in the protection of plants against salinity in the arid and semi-arid regions of the world where salt stress is a common feature. Thus, the present study indicates a possible correlation between increased the salt tolerance of *M. sativa* and the presence of AMF. Therefore, AMF offers an environmental and sustainable safe treatment to develop salinity tolerance in certain crops under both greenhouse and field agriculture.

CHAPTER 5: ARBUSCULAR MYCORRHIZAL FUNGI (AMF) AS BIOCONTROL AGENT TO THE PATHOGEN *F. OXYSPORUM* IN ALFALFA

5.1 Abstract

Over 75% of the world's plants create symbiotic relationships with soil-borne arbuscular mycorrhizal fungi (AMF) through their roots. According to numerous research, mycorrhizal colonization improves plant resistance to harmful fungi. In this research, we studied the effect of AMF on *Medicago sativa* growth, phosphorus and nitrogen uptake, and the efficacy of fusarium wilt caused by *Fusarium oxysporum*. We found that mycorrhizal inoculation alleviated the resistance of the *Medicago sativa* plant to *F. oxysporum*. The *F. oxysporum* infection caused 62% of alfalfa leaflets to turn yellowish. However, AMF inoculation of pathogen-treated plants significantly ($p \leq 0.05$) reduced plant leaflet yellowing by 33.26%. Mycorrhizal colonization enhanced plant height, foliage dry weight, and leaf chlorophyll content. The protein level of mycorrhizal inoculated plants was higher than control. The foliage protein level of pathogen-infected plants was much lower in the absence of AMF. In addition, the percentage of phosphate and potassium was significantly higher in AMF treated with or without the pathogen inoculation. Our findings demonstrate that AMF may mitigate the detrimental effects of *F. oxysporum* stress on alfalfa by enhancing the overall health of the plant, which sheds light on rhizosphere plant–microbiome interactions as well as the utilization of the microbiome in sustainable crops production.

Keywords: *Medicago sativa*, Arbuscular mycorrhizal fungi, *Fusarium oxysporum*, pathogen.

5.2 Introduction

In many areas around the world, including Qatar, alfalfa (*Medicago sativa*) is an important legume feed crop. According to FAO, the Alfalfa plant is originally starting in the Mediterranean region (“Alfalfa,” 2018). It is mostly cultivated as a forage crop to produce either hay or fresh product. Wilt and root rot diseases, caused by soil-borne pathogens, are key issues affecting alfalfa produce around the world (Li et al. 2019). Root rots caused by pathogens like Fusarium sp. are one of the diseases that can reduce yield starting when the crop cycle is attaining the second year of cultivation that can persist more than eight years (Wallenhammar et al. 2008). In addition to *Fusarium* sp. (Foley et al. 2013), *Verticillium* sp., *Phytophthora* sp., *Pythium* sp. are common pathogens to *M. Sativa* (Wiggins and Kinkel 2005, Ao et al. 2018).

F. oxysporum is a well-known plant disease that causes significant harm to a variety of crops in the field and during postharvest storage (Ulf Thrane, 2014). It is a fungus that causes vascular wilt in woody and herbaceous plants, especially in warm temperate and tropical climates (Jacobs, Wingfield, & Gibbs, 2004). The infection by *Fusarium oxysporum* reduces *M. Sativa* growth and caused yellowing of plant leaflets, showing that the fungus was extremely harmful to the *Medicago sativa* plant, which is consistent with earlier studies (Wang et al., 2020).

This disease is believed to be extremely devastating, typically destroying a considerable portion of the crop. In the field, affected regions typically begin as tiny spots that gradually develop in a more or less concentric fashion. A yellowing of the outer leaves of the infected plants is the first sign of the illness. This chlorotic disease worsens with time, until all of the leaves, including the stems, have lost their green colour. This is usually followed by wilting and the death of the plant (Weimer, 1928).

As a result of the environmental damage caused by fungicides to soil and groundwater, a lot of research has been directed to finding biological alternatives to control plant diseases, one of these options is the application of Arbuscular mycorrhizal fungi to help the plants in nutrient uptake and protecting them against (Berruti et al., 2016). AMF are advantageous to plant because they can guard the host counter a wide variety of phytopathogenic infections through various approaches. These mechanisms include direct competition for the limited space and nutrients, enhanced root tolerance, altered rhizosphere interactions, and induced systematic resistance (Song et al., 2015).

Therefore, it is through these mechanisms that the AMF can produce systematic resistance and in return inhibiting or eliminate the plant pathogens that lower the yield and quality of the crops. Moreover, the AMF inoculation association does not only promotes therapeutic plant growth but also raises their productivity (Whipps, 2004).

Arbuscular mycorrhizal fungi (AMF) can boost agricultural yields, but it is important to note that soil type can affect their association, and therefore, not always the fungus will promote crop yield. Kim et al., 2015 stated that "Generally, Arbuscular mycorrhizal fungi (AMF) colonization has been found to have a positive effect on plant growth, but the soil physiochemical components could directly impact the symbiotic association between the plant and the fungus (Whipps, 2004; Kim et al., 2015)". Therefore, this research will evaluate the effect of the isolated AMF on Alfalfa wilt caused by *F. oxysporum,* which is known to cause root rot (Pozo et al., 2009).

5.3 Materials and methods

The mixture of AMF species that isolated and propagated in previous experiments (see section …) will be used as an inoculum in this experiment. For each pot 100g of inoculum is used (contains at least 900 spores and *Zea mays* root fragments). The inoculum was placed directly under the seeds of the alfalfa plant.

Dried regular fine sand and soil (1:2; w/w) were mixed and then autoclaved twice for 60 min at 121°C to completely remove the soil microbiota. The used soil has the following properties: organic carbon 1.84%; total soluble salts 1.703%; electrical conductivity (EC), 2116 µS/cm, and pH 7.98. The soil was then transferred to plastic pots (15 cm in diameter). The seeds of *M. sativa* (Certified Alfamaster 9 lucerne Australia) were surface disinfected in 2% (v/v) NaOCl for 5 min followed by thorough washing with D.W 3 times (da Silva et al., 2015). 3 seeds were sown per pot. Ten days after the seedling's growth, the number of seedlings reduced to one seed per pot after the healthiest seedling have been chosen. The plants were watered day after day to prevent drying of the seedlings and low phosphorous fertilizer (10-5-10) was given once for all treatments After two weeks of germination.

The *Fusarium oxysporum* used in this study was provided by the Biological and Environmental department at Qatar University. The fungus *F. oxysporum* was cultivated on (PDA). The PDA culture began out white and soon turned purple-red. After one week of cultivation at 25°C, oval-shaped spores were detected under the microscope (see appendix figure A14). A hemocytometer was used to determine the pathogen inoculum concentration, which was adjusted to approximately 2 X 10^6 conidia per milliliter. Two weeks post seedling emergence, 10mL spore suspension was added to the soil of each inoculated treatment pot two weeks after seedling emergence. The

study was one-way ANOVA with 4 different treatments and five replications in a completely randomized design. Each pot contains one plant. The treatment levels were as follows: (1) control: without AMF, or *F. oxysporum*; (2) AMF inoculated: with AMF but without *F. oxysporum*; (3) pathogen inoculated with *F. oxysporum* only but without AMF and (4) AMF and pathogen *F. oxysorum* inoculated. The pots were placed randomly in the growth chamber with a constant temperature of 29° C, 16 hours of light, and humidity of 60- 65% day/ night throughout the growth period. Tap water was used to water the plants as needed.

Estimation of the severity of disease

To assess the severity of the disease for all treatments, the method outlined in Wang et al (2020) was followed. The degree of disease was estimated according to the appearance of the incidence based on the percentage of yellowing on the alfalfa following evidence: On a scale of zero to five, all of the seedlings' leaves in each pot were rated separately, table 1.

Table 5.1 The used disease symptoms scale (Wang et al., 2020)

Scale	Symptoms
0	Leaves that look healthy
1	<25% of leaf area showed yellowish coloration or stunted
2	<25-49% of leaf area showed yellowish coloration or stunted
3	<50%-74% of leaf area showed yellowish coloration or stunted
4	75-100% of leaf area showed yellowish coloration or stunted
5	Dead leaves

The following formula was used to compute the disease index (DI) and incidence rate (I) (McKinney, 1923; X. Wang et al., 2020):

$$I(\%) = (D/T) \times 100;$$
$$\mathrm{DI} = [\sum_{0}^{i}(\mathrm{Ln} \times i)/(T \times 5)] \times 100$$

D= Total No. of disease

T= Total No. of leaves

i= The scale of disease from 0 to 5

Ln= The total number of leaves in each disease severity category

All growth parameters were measured after two months, and the plants were harvested.

Plant growth measurements

All growth parameters were measured two months post treatment, at harvesting time. For each plant, the plant height, number of branches, and leaf chlorophyll contents were recorded before harvesting. The chlorophyll in the leave samples was determined using a Chlorophyll meter SPAD-502 Plus (Konica Minolta, Japan). Five random leaves per plant were measured using the portable chlorophyll meter SPAD meter, and the average value per plant was recorded (Yamamoto et al., 2002). For Biomass, the plants were carefully removed from the soil, washed with water to remove soil debris and all above-ground foliage was separated from roots. The above-ground foliage fresh weight (FW) of each plant was measured immediately after harvesting. The dry weight (DW) was obtained by oven drying the plant samples at 75 °C for a minimum of 72 hours and subsequently weighing them. Separately weighed oven-dried foliage and root materials were ground into a powder, which was then sieved via a 500-um sieve. Subsequently,

0.5 g of the powdered plant materials were sent to Central Lab Unit at Qatar University to determine total nitrogen, P, Na, K, and Mg concentrations.

Root colonization

The root samples were cleaned with tap water and sliced into 2 cm pieces to assess AMF colonization of the roots, then soaked in 10% KOH for one hour at 100 °C and staining with 0.05% trypan blue at 90 °C for 90 min. After, that the roots were placed in 50% glycerol to remove the excess stain from the roots. The treated roots were placed on the slides and viewed under the microscope to verify the existence of fungal structures within the roots under the microscope. The percentage of colonization was counted according to the following equation (Brundrett et al. 1984).

$$Percent\ Root\ colonization = \frac{Number\ of\ Root\ Segments\ Colonized}{Number\ of\ Root\ segments\ observed}\ X100$$

Statistical analysis

Variables used for different analyses were accordingly adjusted to fit with the Gaussian law of distribution. The data were subjected to one-way ANOVA. P values ≤ 0.05 were deemed statically significant. Comparisons were made between control and treated or among all treatments using Tukey's test at $P \leq 0.05$. All data analyses were carried out using Systat software (SigmaPlot 13 and Sigmastat 4).

5.4 Results

AMF colonization: At harvest, AMF colonization was established in all AMF inoculated plants. Non-inoculated alfalfa plants did not show any colonization with AMF in the roots. AMF colonization in *M. sativa* roots was significantly inhibited ($p \leq 0.05$) by *F. oxysporum* infection. The colonization rate in AMF inoculated plants was 63% but in pathogen & AMF treated plants was 39% (Table 1).

Disease incidence*:* as Alfalfa plants were inoculated with the AMF, disease incidence and severity were significantly reduced ($p \leq 0.05$) when compared to pathogen inoculated plants in the absence of mycorrhizal fungi (Table 1). *Fusarium oxysporum* infection caused 62% of alfalfa leaflets to turn yellowish. However, in comparison to the control plants, AMF inoculation of pathogen treated plants significantly ($P \leq 0.05$) reduced plant leaflet yellowing by 33.26% (Figure 5 & Table 1).

Figure 5.1 Alfalfa seedlings control (left), AMF+pathogen treated (middle), and pathogen treated (right).

Table 5.2 Rate of AMF colonization, disease incidences, and disease indices of Alfalfa plants.

Treatments	Root colonization %	Disease Incidence (%)	Disease Index (%)
control	0	0	0
AMF inoculated	63	0	0
Pathogen inoculated	0	32.29 a	11.64 a
AMF and pathogen inoculated	33.26	23.73 b	05.61 b

Plant growth

Fusarium oxysporum infection reduced *M. sativa* growth and caused yellowing of plant leaflets, showing that the fungus was extremely harmful to alfalfa. Independent of the presence of the pathogen, mycorrhizal colonization significantly raised plant height (Figure 5.2), number of branches (Figure 5.3), dry weight of aboveground-foliage biomass (Figure 5.4). In the presence of fungal infection, the plants that have been inoculated with AMF showed significantly better measures in all studied growth parameters (Figures 5.2 to 5.5), demonstrating that AMF may mitigate the detrimental effects of *F. oxysporum* stress on alfalfa by enhancing the overall health of the plant.

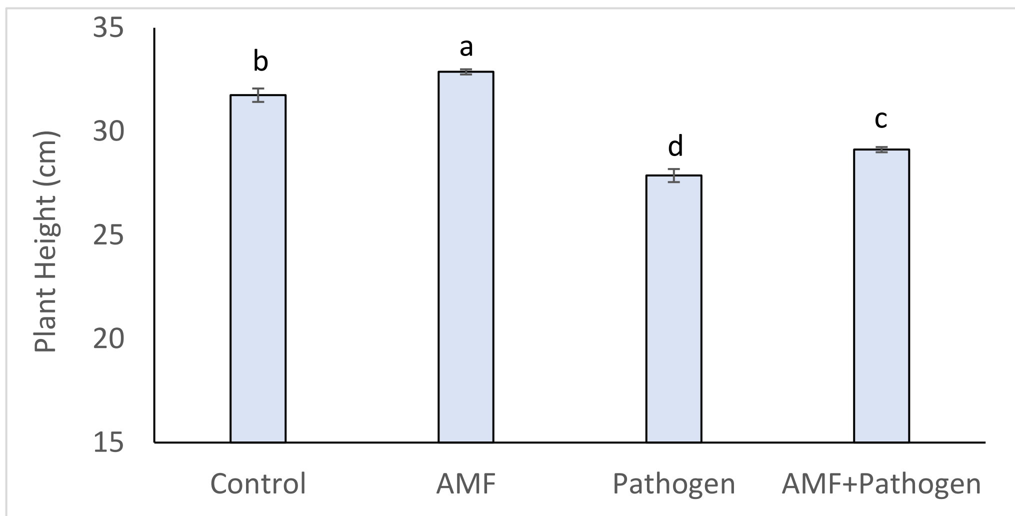


Figure 5.2 Effect of AMF inoculation on alfalfa plant height in the presence and absence of the pathogen, *F. oxysporum*. Error bars refer to standard errors of the means. According to Tueky's test at $p \leq 0.05$ (N=5), values with the same letter are not substantially different.

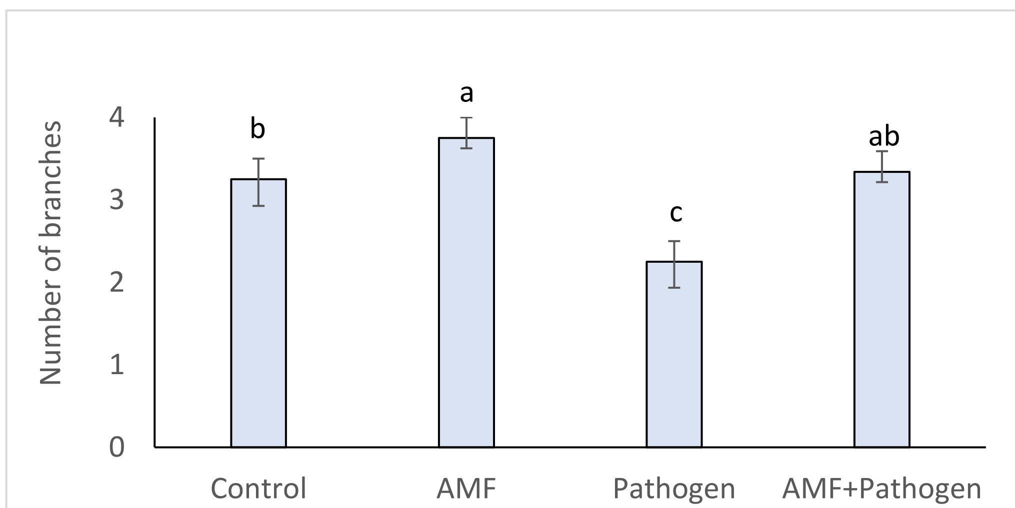


Figure 5.3 Effect of AMF inoculation on alfalfa shoot branching in the presence and absence of the pathogen, *F. oxysporum*. Error bars refer to standard errors of the means. Values with a common letter(s) are not significant at $p \leq 0.05$ according to Tukey's test. (N = 5).

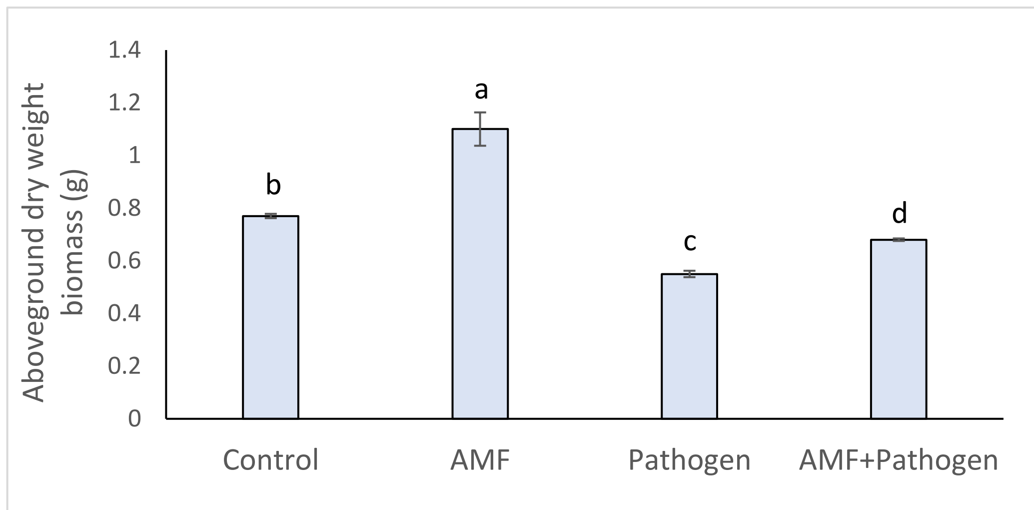


Figure 5.4. Effect of AMF inoculation on alfalfa aboveground biomass (dry weight) in the presence and absence of the pathogen, *F. oxysporum*. Error bars refer to standard errors of the means. Values with a common letter(s) are not significant at $p \leq 0.05$ according to Tukey's test (N = 5).

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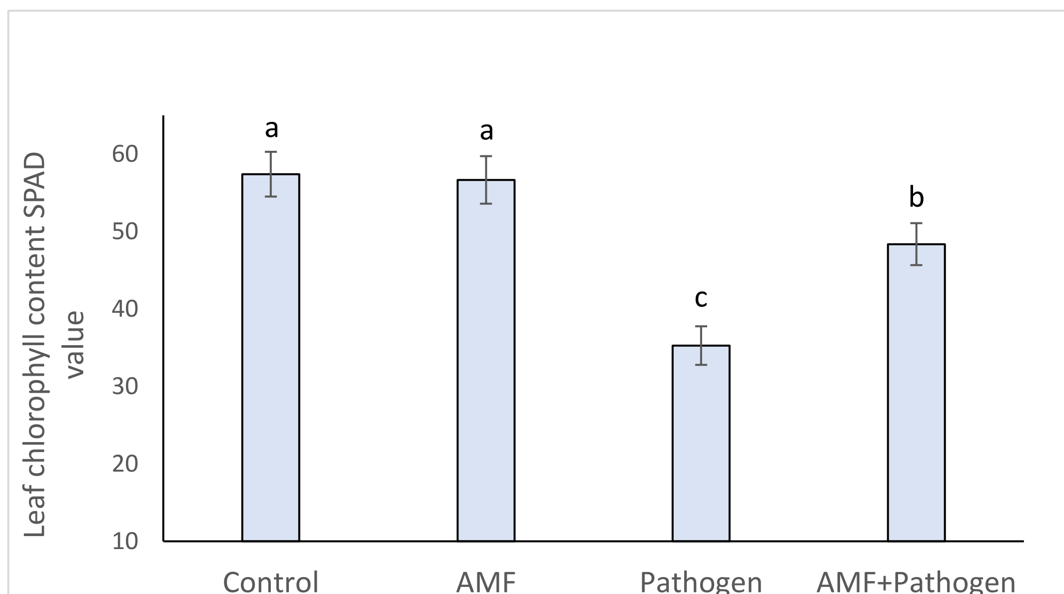

Figure 5.5 Effect of AMF inoculation on alfalfa leaf chlorophyll content in the presence and absence of the pathogen, *F. oxysporum*. Error bars refer to standard errors of the

means. Values with a common letter(s) are not significant at $p \leq 0.05$ according to Tukey's test. (N = 5).

Protein and minerals content

The leaf protein content of alfalfa plants was significantly increased in the presence of mycorrhizal inoculation under both treatments of presence or absence of *F. oxysporum* infection (Figure 5.6). In addition, in the absence of pathogen infection, AMF treated alfalfa plants showed a significant increase in phosphate and potassium, and sodium (Table 5.3). In the presence of pathogen infection, AMF treated plants showed a significant increase in phosphate and magnesium (Table 5.3).

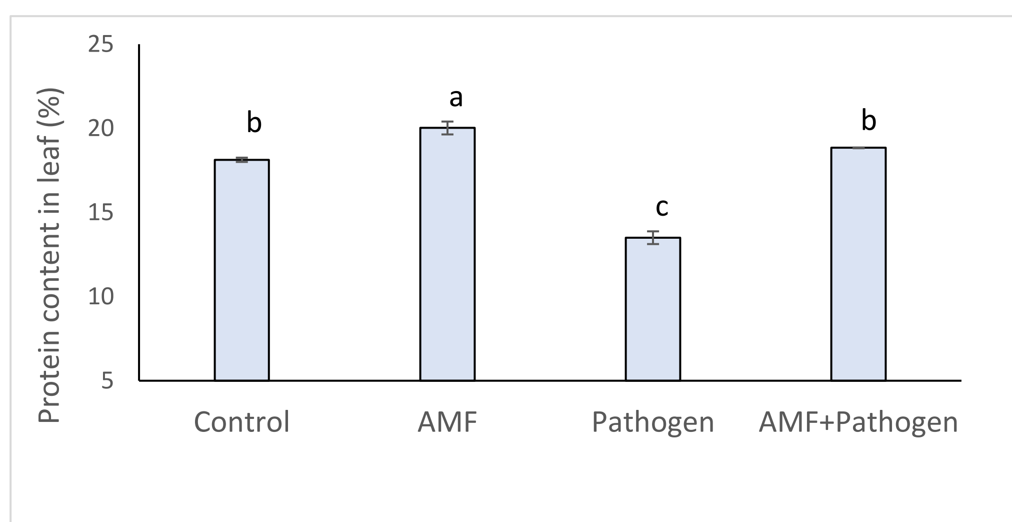

Figure 5.6. Effect of AMF inoculation on alfalfa leaf protein content in the presence and absence of the pathogen, *F. oxysporum*. Error bars refer to standard errors of the means. Values with a common letter(s) are not significant at $p \leq 0.05$ according to Tukey's test. (N = 5).

Table 5.3 Effect of arbuscular mycorrhizal inoculation on mineral contents in *M. sativa* leaves as influenced by *F. oxysporum*. In each column, values with a common letter(s) are not significant at $p \leq 0.05$ according to Tueky's test. (N = 5).

Treatments	Phosphate	Na	K	Mg
control	4.68 b	0.72 b	21.33 b	2.17 ab
AMF inoculated	6.6 a	1.20 a	22.35 a	2.33 a
Pathogen inoculated	3.8 c	1.00 ab	19.16 ab	2.13 b
AMF and pathogen inoculated	4.36 b	1.15 a	20.01 a	2.3 a

5.5 Discussion

In order to see if AMF influences alfalfa growth and resistance to *Fusarium oxysporum* wilt, researchers looked at the incidence of illness, biomass and chlorophyll content, protein and mineral content, and disease resistance to *Fusarium oxysporum* wilt in *Medicago sativa*. When their host plants were stressed by the pathogen *F. oxysporum*, the results revealed a complex interaction of AMF and their beneficial effects on the physiology and biochemistry of the host plant. This study's findings mostly confirmed the notion that inoculating alfalfa with AMF enhances overall health, plant growth, and disease resistance by enhancing and improving nutrient uptake and levels of other pathogen defense chemicals. This is in line with prior research findings by (X. Wang et al., 2020); Soil-borne arbuscular mycorrhizal fungi (AMF) are capable of forming a

stable and long-term mutualistic symbiosis with the roots of more than 75% of vascular plant species, including most crops. The AMF play the role of biocontrol agents (S. E. Smith & Read, 2010). Plants benefit from mycorrhizal associations not just for nutrient absorption, but also for resistance to abiotic stressors and soil-borne fungal diseases (Bi, Song, & Zeng, 2007).

The significant decrease in the dry weight and other growth parameters of alfalfa plants that have been subjected to *F. oxysporum* infection can be explained by the fact that this fungus secretes toxic substances that disturb the gas exchange function of stomata of the leaves. The pathogen infected plants possessed excess water in transpiration and higher gas exchange rate and thus increase energy consumption in addition to the pathogen is known to cause clogging of the xylem vessels and thus hinders the plant from absorbing nutrients and water (Al-Maghribi, Taweel, & Rizk, 2013; U. Thrane, 2014).

Our results indicated that the AMF inoculation significantly enhanced plant growth parameters including plant height, the number of branches, and plant biomass compared to plants treated with *F. oxysporum* but with no AMF inoculation. This is consistent with (Kapoor, 2008), which indicated that tomato plants treated with AMF and *F. oxysporum* had better growth when compared to plants treated with *F. oxysporum* only. This is because AMF increases the surface area absorption of the roots through the extension of the hypha and its spread in the soil which increases the uptake of water and mineral nutrients (Baum, El-Tohamy, & Gruda, 2015). It also preserves the functions of the root cells through its structure, thus increasing plant growth and helping to increase its resistance to diseases.

In addition, alfalfa plants treated with AMF and *F. oxysporum* had more dry weight biomass than plants treated with only *F. oxysporum*. This result is consistent with the study by (Manila & Nelson, 2013), who indicated an increase in the dry weight of tomato plants inoculated with Mycorrhizal fungi compared with no AMF inoculation.

In certain research, AMF's disease-fighting properties were connected to their beneficial impacts on plant nutrients, particularly phosphorus (Bolandnazar et al., 2014). Coinciding with other research; our results indicated higher concentrations of phosphate, sodium, potassium, and magnesium in plant tissues due to AMF inoculation. According to Bodker (1998), phosphate content alone is not enough to prevent disease progression. As a result, we hypothesized in this work that the disease inhibition of arbuscular mycorrhizal fungi may not be entirely due to an increase in phosphate content, even though phosphate contents and dry weights of plant biomass increased significantly. In addition to plant nutrient intake, activation of plant defense systems, alterations in the root system, the competition among soil microorganisms for space and nutrients, and the effect of rhizosphere environment, are all collectively assumed to be responsible for disease control by AMF inoculation, (Demir, 2005).

5.6 Conclusion

Alfalfa plants that had been pre-inoculated with arbuscular mycorrhizae fungi improved alfalfa resistance to *Fusarium* wilt. When AMF colonizes the roots, alfalfa possessed better growth parameters and less disease severity. Our findings imply that increasing nutrient levels in plant tissues, particularly phosphorus, is linked to mycorrhizal-induced disease resistance in Alfalfa plants. The applications of AMF fungi as a form of defense could be a feasible alternative for crop disease management in sustainable

agriculture, as well as can be a naturally based tool for conservation of wild plants via enhancing their defense against pathogens.

CHAPTER 6: GENERAL DISCUSSION, RECOMMENDATION, AND FUTURE WORK

In many regions of the world, soil salinization as a result of rising sea levels and groundwater irrigation has become a major agronomic issue. Salinity impacts plant growth in semi-arid and arid parts of the world. Saline soil occupies up to 7% of the earth's surface (Beltrano, et al,. 2013). One of the environmentally compatible solutions that have been proven by many studies to improve plant growth in salty environmental conditions is the use of mycorrhizal fungi. Under any unfavorable and pressured conditions, plant and soil health could benefit from mycorrhizal mutualism. It was discovered that AMF reaches the cortical cells of plant roots, allowing the root morphology to change and build a tolerance to weeds, pests, and disease (Alderfasi, Al-Suhaibani, Selim, & Al-Hammad, 2016). AMF also improves a plant's ability to cope with salt pressure by increasing mineral nutrient absorption, maintaining ions, conserving enzyme functions, and promoting water uptake, as well as increasing salinity resistance in a different crop, like maize and tomato (Al-Karaki, 2001).

The present study aimed to isolate endogenous native effective species of arbuscular mycorrhizal fungi from the Qatari environment and utilize these isolates to understand and to study the effect of isolated AMF on the growth of *Medicago sativa* under saline environment using greenhouse and field conditions. In addition, we aimed to study the effect of the native isolated arbuscular mycorrhizae fungi on Alfalfa wilt caused by *F. oxysporum*.

The AMF from different rhizosphere plant samples were identified based on the sequencing of the PCR product of the amplified conserved ITS region. AMF diversity in the selected plant rhizosphere of this study, 13 mycorrhizal fungi species have been

identified at the species level. After identification of these AMF, it appeared that they belonged to 16 genera, 3 different orders, and 6 families. However, new species from the current study are still expected since some were identified only at higher taxonomic levels. We found that the highest rate of root colonization by AMF was reported in the roots of the Tarfa (*Tamarix aphylla*) plant in addition to the high ability of this plant to tolerate salinity therefore, the plant might be a promising candidate for the propagation of AMF inoculum. Our results indicated that *Claroideoglomus drummondii* was the only species detected in all study site samples, which indicates the ability of this species to withstand high temperatures and alkaline soil (due to salinity) in addition to its efficiency in establishing symbiosis with roots of different host plants. Similarly, the species *R. irregularis*, was found in all our soil samples except one, however many studies mentioned that this species is the same as *Glomus intraradices.* This species was widely used in various experiments to study its effect on plant growth under different environmental conditions, and the results of its use as a biofertilizer were positive in helping the plant grow better than control samples (Berruti et al., 2016; Igiehon & Babalola, 2017).

The results from this part of the research provide comprehensive biological data about taxonomy, distribution, and prevalence of AMF in Qatar soil which opens new research towards developing its future applications for environmental conservation and sustainable agriculture. As a result of researching plant species and new habitat types, particularly unique and uncommon plant species, there is a chance of discovering an excess of novel AM fungal species in the Qatari rhizosphere. In addition, the results from the salinity and disease resistance experiments showed the ability of these isolated fungi to improve plant growth. AMF's effect on alfalfa under salt stress has been studied severally by different researchers. The types of AMF inoculum employed in these

experiments varied, including an inoculum containing numerous native isolated AMF mixed with several types of imported mycorrhiza or helper bacteria, as well as an inoculum containing only one species. There hasn't been any research done on the impact of native isolated Qatari indigenous consortium arbuscular-mycorrhizal fungus (AM) under abiotic stress. The results of the studies discussed in chapter four of this thesis demonstrate that the presence of AMF does alleviate some of the negative effects of salt. The ability of AM fungus to improve salt tolerance has major ecological implications. Many experiments have proven the ability of these fungi to help plants tolerate salinity, although so far, there are no experiments in our region for the propagation of local AMF fungi and their exploitation as a vital fertilizer in the agricultural field, whether at the level of scientific or commercial research.

Generally speaking, this research has shown that mycorrhizal fungi can help to mitigate at least part of the negative impacts of salt exposure and the pathogen effect. However, the magnitude of these advantages will be determined by several circumstances. As a result, more research is still needed to look at the effects of various environmental conditions on mycorrhizal function.

AMF Biodiversity studies

Despite the importance of arbuscular mycorrhizal fungi, the study of its diversity in our region is very few. The number of studies until writing this research did not exceed 13 studies to explore and identify these fungi (Khazna and Abu-Dieyeh, 2021 unpublished). According to Öpik et al. similar situation applies at the global level as the diversity of AMF were not accurately estimated (Öpik et al., 2006; Öpik et al., 2013). Furthermore, our efforts in extracting, propagating, and characterizing AMF from Qatar led to the discovery of the number of AMF species based on DNA molecular markers, although some of them could not be identified at the species level and might

contain new species. So unknown sequences are either newly discovered species or just previously described species that have their DNA not sequenced yet. Based on this, we suggest that there should be a future comprehensive study to identify these fungi morphologically, and there is a need to establish a database for native AMF isolated from Qatari soil, which can help future studies in AMF taxonomy and identification.

In addition, there is a lack of studies that investigate the interaction between the host plant and the mycorrhizal fungi. We found that the AMF infection rate varies with location and host plant. Therefore, it is necessary to focus on the plants in which the colonization of the AMF was high to understand the relationship and the environmental conditions that contributed to this high colonization. Interestingly, we found that the highest rate of root colonization of AMF in the roots of the Tarfa plant (*Tamarix aphylla*) plant, which may imply the possibility of exploiting this plant for the propagation of AMF and to be used as inoculum in further applications. Further investigations in this area may give a better understanding of the direct effects of and abiotic biotic factors as well as the interactions between the host plant and AMF that is responsible for this variation. Additionally, when studying the benefits of AMF colonization, there is a need to cross examine the activity of the mycorrhizae itself and the extent to which it is influenced by a variety of factors like the type of host plant, temperature, pH, salinity...etc. to obtain a better knowledge of how mycorrhizas work naturally.

Using native AM fungi as a biotechnological tool

AMF have lots of potential as a biotechnological tool in horticulture, agriculture, and revegetation operations. The number of research demonstrating enhanced plant growth, nutrient uptake, and soil amendments as a result of inoculation is continuously

increasing. We were able to demonstrate that inoculating *Medicago sativa* plants with natural AM fungus improved their development under salt stress conditions.

The widespread use of AMF in agricultural applications necessitates large quantities of fungal inocula, which must be produced in large quantities. As a result of the importance and usefulness of these fungi, in certain countries, the research is advanced to commercialize products of different species of AMF mainly produced for agricultural use. A variety of commercial AMF inocula began to be produced and sold by several companies. Currently, products of Mycorrhizae fungi were produced by 28 factories around the world. The Glomeraceae account for 100% of the items. The percentage of production of *Rhizophagus irregularis* (39%), and *Rhizophagus clarus* is the least popular (16 %) of these products (Basiru et al., 2021). Our study has proven the presence of these two fungi in the Qatari environment, and we may suggest that further studies on these adaptable fungi to our local environment will improve their propagation which could be used as biofertilizers. The findings from our study as related to the physical and biochemical parameters may add to the existing literature when it comes to the composition of an effective inoculum combination of AM fungus which best suits various cultivation systems by improving agricultural operations.

In addition to the aforementioned, future research should concentrate on the following:

1- to find out how the different AMF strains can help plants to flourish,

2- The elevated synthesis of antioxidant enzymes in mycorrhizal plants has to be investigated further in order to reveal the ultrastructure properties of AM fungus.

3- Future research could provide predictive knowledge on AMF features involved in symbiotic efficiency, which is needed to increase the successful use of bioenhancer inoculants in sustainable agriculture.

4- More research is needed on the physiological, genetics, and molecular mechanisms involved in symbiosis establishment and fine signaling between the host and the symbiont.

5- At the transcriptional, proteomic, and hormone omics levels, more study is needed to uncover possible stress.

Application of native AMF as a biocontrol agent

There are more than 80 disease biocontrol products that are available in the marketplace worldwide; however, none of those contains mycorrhizal fungi. This is notwithstanding adequate proof that arbuscular mycorrhizal fungi can manage some plant diseases (Whipps, 2004; Sulaimon Basiru, Hopkins Pachalo Mwanza, & Mohamed Hijri, 2021b). From our results, we found that the AMF antagonists the fungus *F. oxysporum,* which was positively reflected on the plant growth, consequently, more comprehensive investigation of the association in different pathosystems as well as the relationship between the AMF and the hosting plants are required to improve the biocontrol that will eliminate the prevailing diseases, also, these isolates should examine under field conditions and using other plant crop models.

LIST OF ABBREVIATIONS

AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of Variance
^{o}C	Degrees Celsius
DNA	Deoxyribonucleic acid
PhD	Doctor of Philosophy
g	Gram
h	Hour
L	Litre
Mg	Magnesium
µmol	Micromole
mL	Millilitre
N	Nitrogen
%	Percent
P	Phosphorus
K	Potassium
KOH	Potassium hydroxide
PCR	Polymerase chain reaction
pH	Power of hydrogen
w/v	Weight/Volume Percent

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APPENDIX

Figure A. 1 a metal core borer of 10 x 20 cm that used to collect rhizosphere sample.

Figure A. 2 Roots of some plant samples collected in this study and the low density of the root system

Figure A. 3 The shape of the soil after sifting and the rocky nature of the ground, as it was difficult to extract the roots from it.

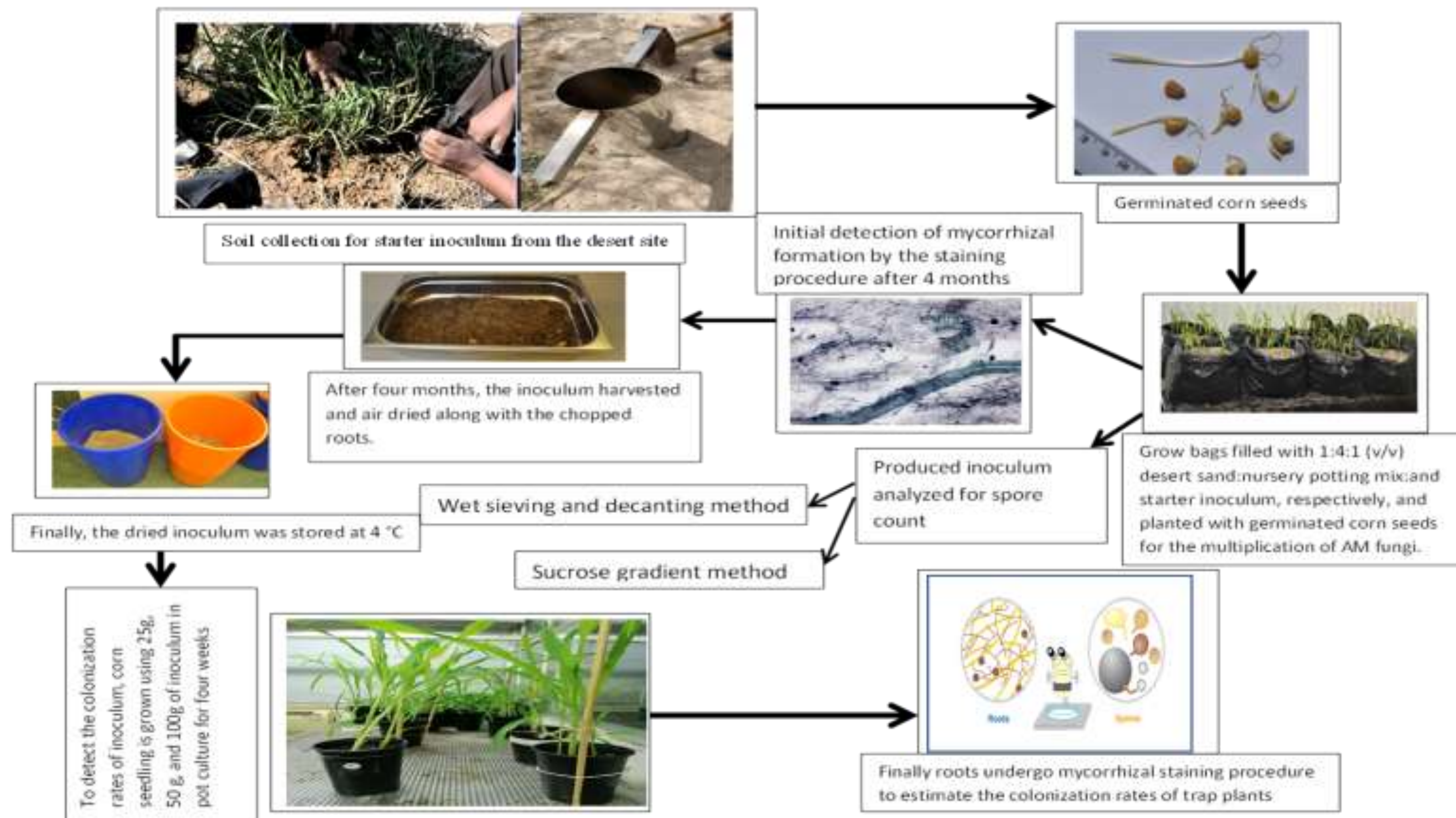

Figure A4 . Flow chart showing the step-by-step protocol for collecting, isolating, and propagating of AMF (modefied from Quoreshi, 2017) .

Figure A. 5 Some of studied plant samples.

Figure A. 6 Spore extraction protocol Modified from Daniels and Skipper

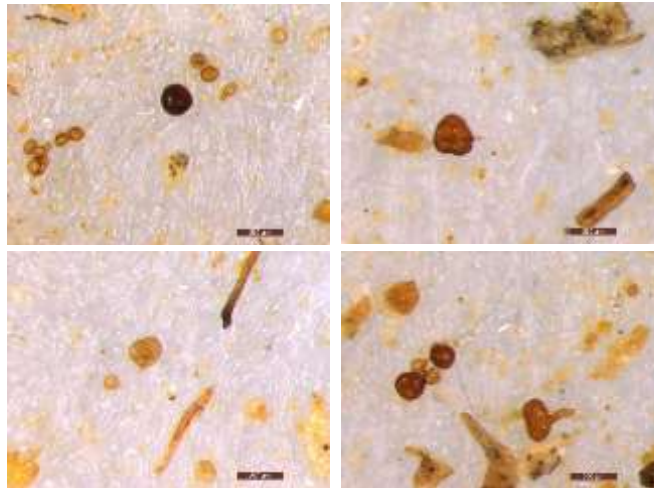

Figure A7 . Spores extract from field soil from different location following the wet sieving procedure and sucrose method.

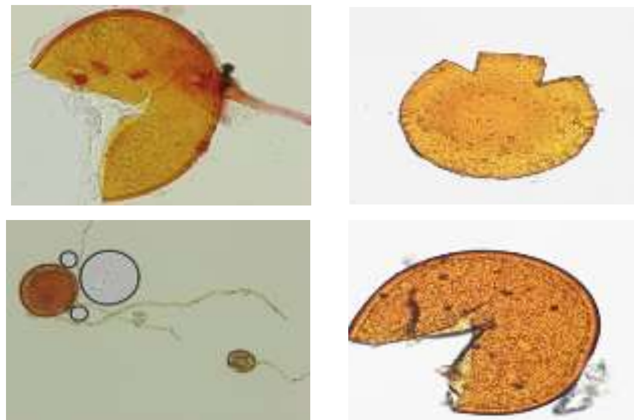

Figure A. 8 Photos of the spore of the AMF species under microscope after melzer's reagent.

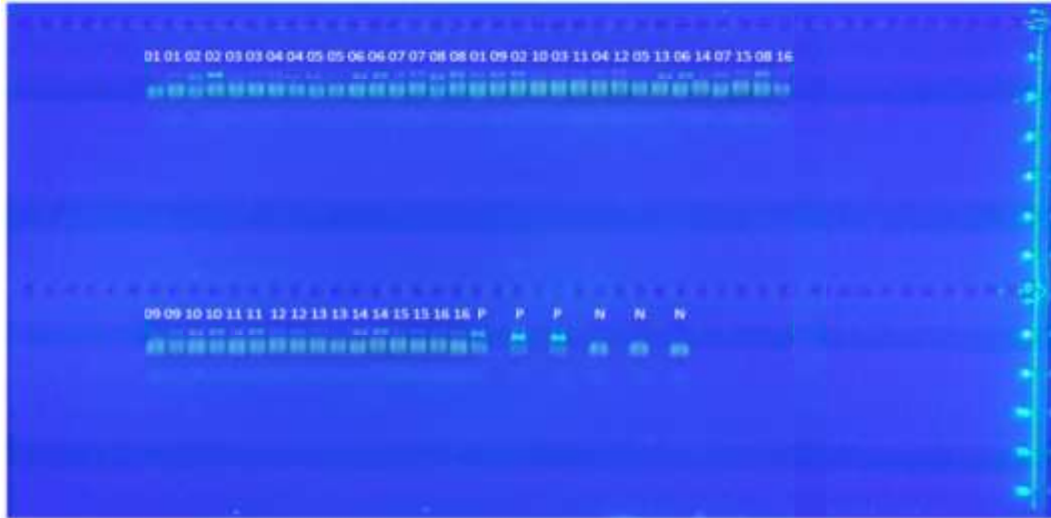

Figure A9 . 1% agarose gel to analyze the quality of the generated amplicons and to evaluate its expected size.

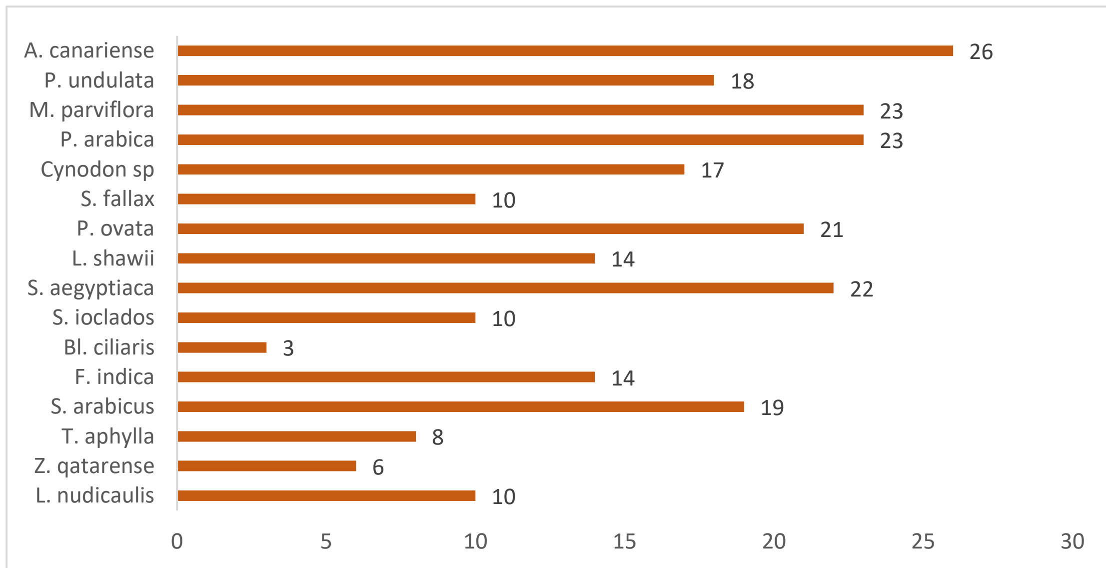

Figure A10 . Number of identified selected sequences (identified AMF) in each rhizosphere sample

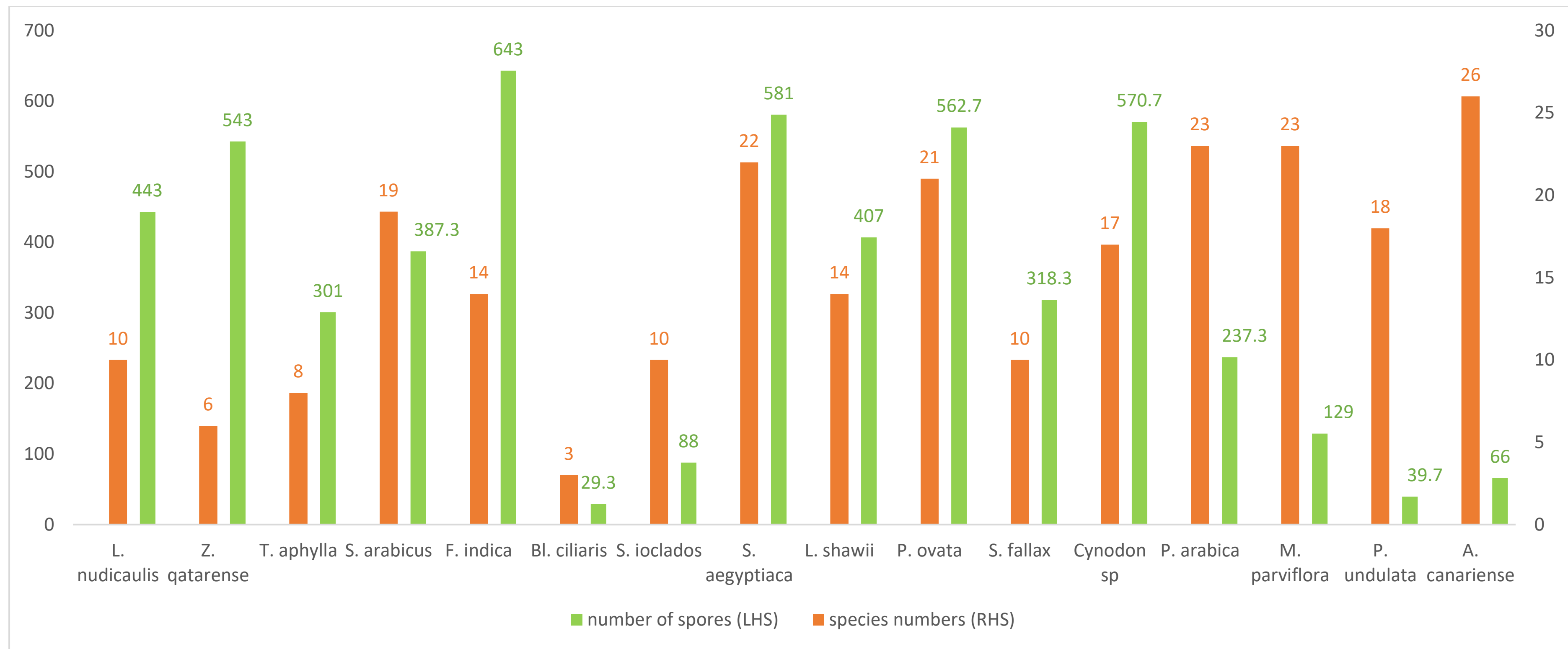

Figure A11 . Number of spores per 100g of soil vs number of AMF identified per sample

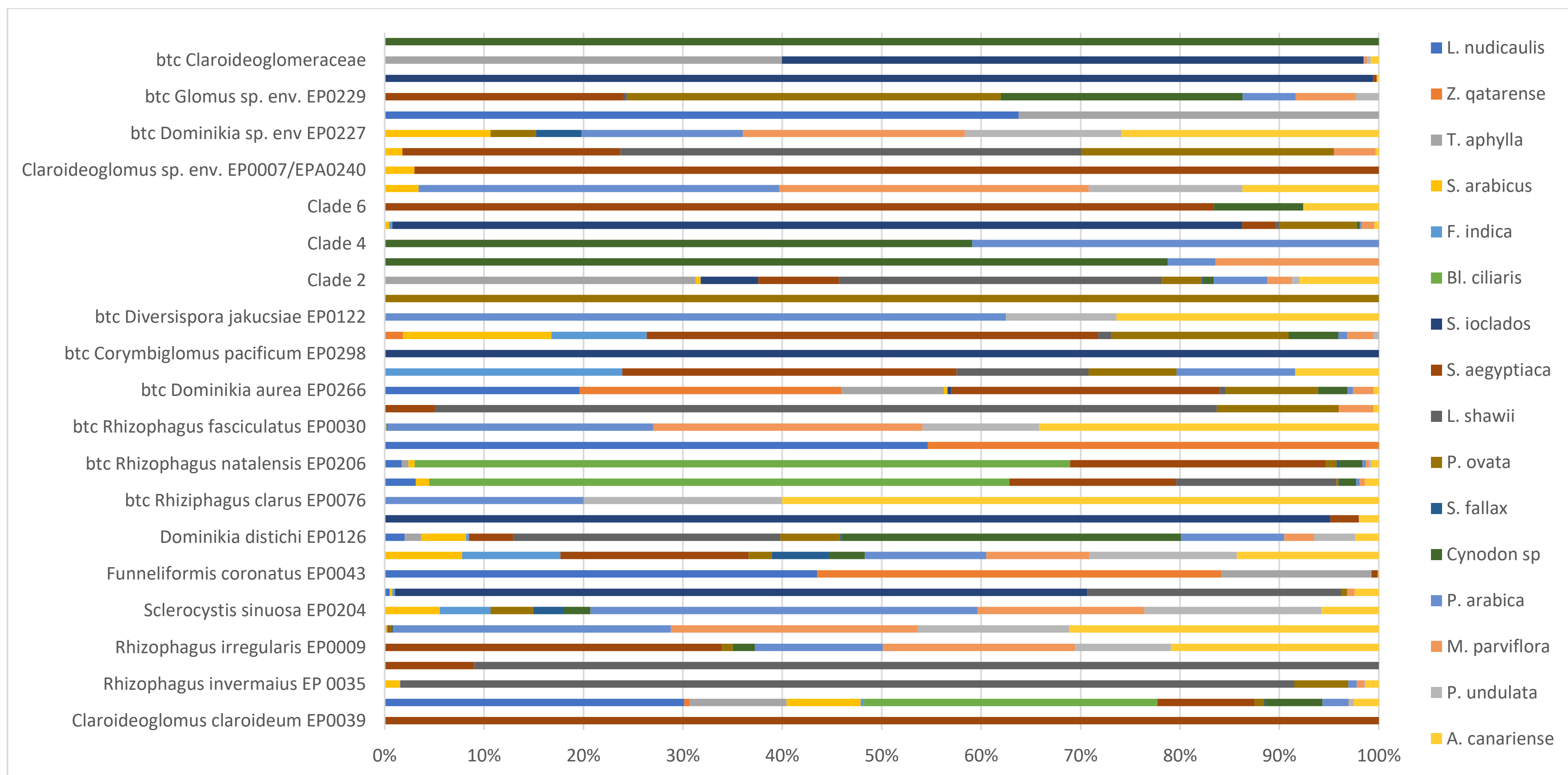

Figure A12 . Percent contribution of each solid sample to the total amount of sequences of every identifier AMF.

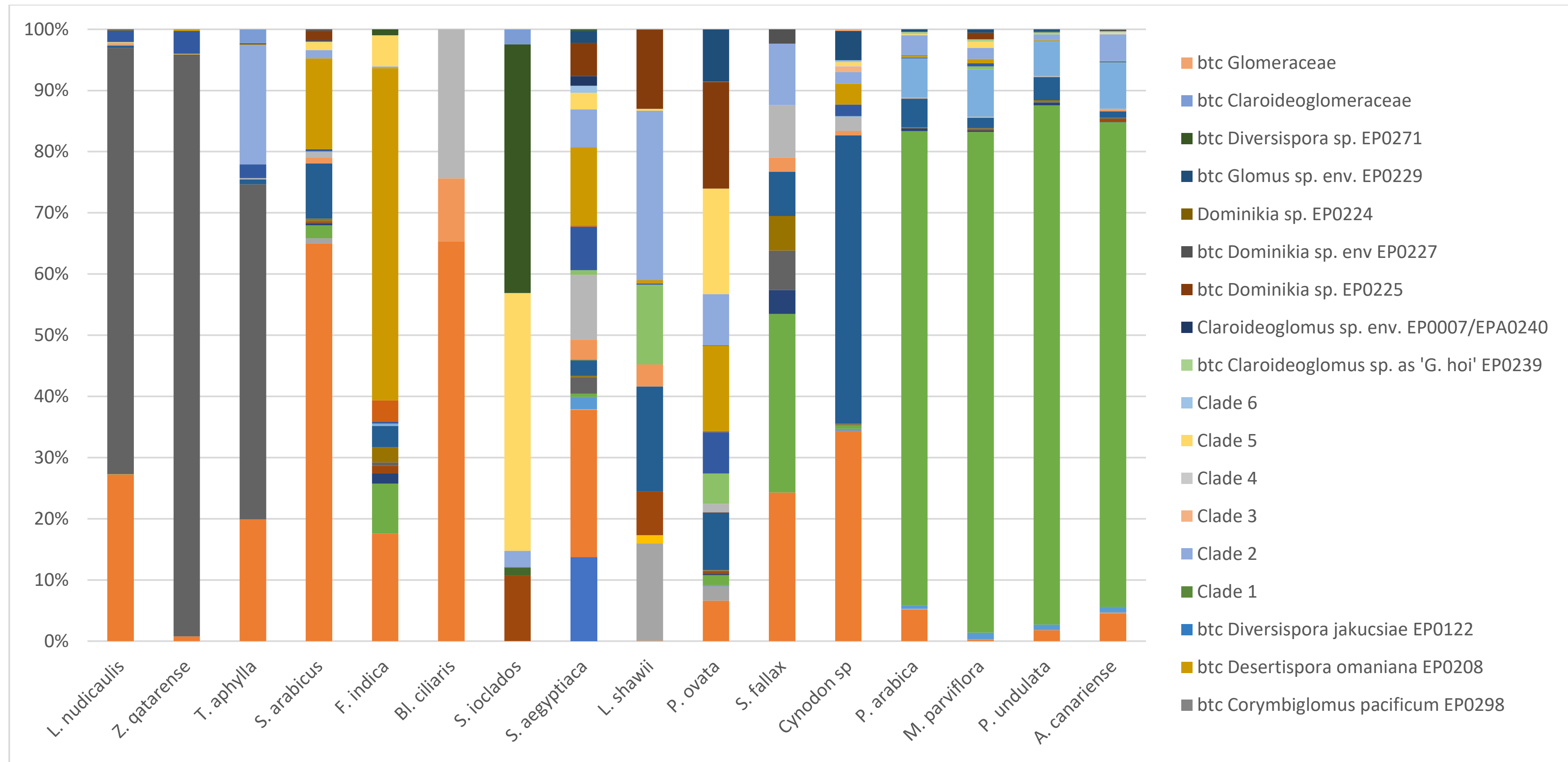

Figure A13 . Percentages of identified AMF in each soil sample (plant rhizosphere) out of the total identified representative sequences per sample

Table. A1 Genius, family and order of identified strains.

No.	Annotation	Genus	Basionym	Synonyms	Family	Order
1	*Claroideoglomus claroideum* EP0039	Claroideoglomus	*Glomus claroideum*		Claroideoglomeraceae	Glomerales
2	*Claroideoglomus drummondii* EP0097	Claroideoglomus	*Glomus drummondii*	*Albahypha drummondii*	Claroideoglomeraceae	Glomerales
3	*Rhizophagus invermaius* EP 0035	Rhizophagus	*Glomus invermaium*	*Rhizoglomus invermaium*	Glomeraceae	Glomerales
4	*Rhizophagus intraradices* EP0032	Rhizophagus	*Glomus intraradices*	*Rhizoglomus intraradices*	Glomeraceae	Glomerales
5	*Rhizophagus irregularis* EP0009	Rhizophagus	*Glomus irregulare*	*Rhizoglomus irregulare*	Glomeraceae	Glomerales
6	*Rhizophagus arabicus* EP0031	Rhizophagus		*Rhizoglomus arabicum*	Glomeraceae	Glomerales
7	*Sclerocystis sinuosa* EP0204	Sclerocystis		*Glomus sinuosum*	Glomeraceae	Glomerales
8	*Kamienskia bistrata* EP0119	Kamienskia	*Glomus bistratum*		Glomeraceae	Glomerales
9	*Funneliformis coronatus* EP0043	Funneliformis	*Glomus coronatum*		Glomeraceae	Glomerales
10	*Nanoglomus plukenetiae* EP0323	Nanoglomus			Glomeraceae	Glomerales
11	*Dominikia distichi* EP0126	Dominikia			Glomeraceae	Glomerales
12	*Diversispora aurantia* EP0074	Diversispora	*Glomus aurantium*		Diversisporaceae	Diversispora les

13	btc *Rhizophagus clarus* EP0076	Rhizophagus	*Glomus clarum*	*Rhizoglomus clarum*	Glomeraceae	Glomerales
14	btc *Praglomus laccatum* EP0100	Praglomus	*Glomus laccatum*		Paraglomeraceae	Paraglomerales
15	btc *Rhizophagus natalensis* EP0206	Rhizophagus	*Rhizoglomus natalense*		Glomeraceae	Glomerales
16	btc *Rhizophagus silesianum* EP0318	Rhizophagus			Glomeraceae	Glomerales
17	btc *Rhizophagus fasciculatus* EP0030	Rhizophagus	*Endogone fasciculata*	*Glomus fasciculatum* *Rhizoglomus fasciculatum*	Glomeraceae	Glomerales
18	btc *Septoglomus titan* EP0278	Septoglomus			Glomeraceae	Glomerales
19	btc *Dominikia aurea* EP0266	Dominikia			Gigasporaceae	Diversisporales
20	btc *Sacculospora felinovii* EP0257	Sacculospora			Sacculosporaceae	Diversisporales
21	btc *Corymbiglomus pacificum* EP0298	Corymbiglomus			Diversisporaceae	Diversisporales
22	btc *Desertispora omaniana* EP0208	Desertispora	*Diversispora omanana*		Diversisporaceae	Diversisporales
23	btc *Diversispora jakucsiae* EP0122	Diversispora			Diversisporaceae	Diversisporales
24	btc *Kamienskia divaricata* EP0255 /	Kamienskia	*Kamienskia divaricata*		Glomeraceae	Glomerales
	Kamienskia perpusilla EP0120 /	Kamienskia	*Glomus perpusillum*	Kamienskia perpusilla		

	Mikrokamienskia peruviana EP0321	Mikrokamienskia				
25	btc Septoglomus turnauae EP0113	Septoglomus			Glomeraceae	Glomerales
	jasnowskae EP0132	Septoglomus				
	xanthium EP0263	Septoglomus	Glomus xanthium	Funneliformis xanthium		
	fuscum EP0139	Septoglomus				
26	btc Dominikia duoreactiva EP0260 /	Dominikia	Glomus achrum		Glomeraceae	Glomerales
	D. achra EP0116 /	Dominikia				
	D. lithuanica EP0259	Dominikia				
27	btc Mikrodominikia litorea EP0304 /	Mikrodominikia	Dominikia litorea		Glomeraceae	Glomerales
	Orientoglomus emiratium EP0267 /	Orientoglomus				
	Dominikia sp. EP0202	Dominikia	Dominikia emiratia			
28	btc Diversispora varaderana EP0222	Diversispora			Diversisporaceae	Diversispora les
	insculpta EP0121		Glomus insculptum			
	sp. EP0242					
	sp. env. EP0017					
29	btc Diversispora jakucsiae EP0122	Diversispora			Diversisporaceae	Diversispora les
	arenaria EP0143		Glomus arenarium			
	slowinskiensis EP0217					
30	btc Claroideoglomus sp. as 'G. hoi' EP0239	Claroideoglomus			Claroideoglomeraceae	Glomerales

31	Claroideoglomus sp. env. EP0007/EPA0240	Claroideoglomus			Claroideoglomeraceae	Glomerales
32	btc Dominikia sp. EP0225	Dominikia			Glomeraceae	Glomerales
33	btc Dominikia sp. env EP0227	Dominikia			Glomeraceae	Glomerales
34	Dominikia sp. EP0224	Dominikia			Glomeraceae	Glomerales
35	btc Glomus sp. env. EP0229	Glomus			Glomeraceae	Glomerales
36	btc Diversispora sp. EP0271	Diversispora			Diversisporaceae	Diversispora les
37	btc Claroideoglomeraceae				Claroideoglomeraceae	Glomerales
38	btc Glomeraceae				Glomeraceae	Glomerales
Total		16			6	3

Figure A14. Macroconidia and microconidia of *fusarium oxyspurum* under microscop X20.