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ARBUSCULAR MYCORRHIZAL FUNGAL COMMUNITIES FROM THE ATACAMA DESERT AND THEIR EFFECT ON THE IMPROVEMENT OF SALINITY STRESS TOLERANCE OF *LACTUCA SATIVA* PLANTS

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"ARBUSCULAR MYCORRHIZAL FUNGAL COMMUNITIES FROM THE ATACAMA DESERT AND THEIR EFFECT ON THE IMPROVEMENT OF SALINITY STRESS TOLERANCE OF LACTUCA SATIVA PLANTS"

Esta tesis fue realizada bajo la supervisión del Director de tesis, Dr. Pablo Enrique Cornejo Rivas perteneciente al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera y ha sido aprobada por los miembros de la comisión examinadora.

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Dedico esta tesis a mi Familia

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Summary and thesis outline

Soil salinization is a serious problem for agricultural productivity and sustainability. Salinity causes water stress, ion toxicity, nutritional disorders, alteration of metabolic processes, membrane disorganization, reduction of cell division, and DNA damage. The plant response to salinity consists of numerous mechanisms that must function coordinately to alleviate both drought stress and ion imbalance. The mechanisms for salt tolerance are classified into three main categories. The first is the osmotic stress tolerance, which is involves biosynthesis and accumulation of compatible solutes to maintain water uptake. The second mechanism is ion exclusion, in which Na^+ transporters reduce the accumulation of toxic Na^+ within roots and leaves. The third mechanism is tissue tolerance, in which high Na^+ concentration is found in leaves, but it is compartmentalized at the cellular and intracellular level reducing the deleterious effect of Na^+ in the cytosol (Munns & Tester, 2008; Peleg et al., 2011).

As biological strategy, plants form a symbiosis between arbuscular mycorrhizal fungi (AMF) and their roots. Arbuscular mycorrhizal (AM) symbiosis is the most widespread in terrestrial ecosystems, and it is formed by 80% of all terrestrial plants. AM fungi are recognized for their ability to improve plant tolerance to saline stress by producing a root-soil interface that enhances the absorption of water and nutrients. These improvements will vary for each AM fungal species based mainly where were isolated, as well their root colonization capacity, hyphal production, innate stress metabolic systems, and production of exudates. Several studies have showed that AM fungi isolated from limiting ecosystems may have a higher ability to promote plant growth under stress conditions, compared to other fungi isolated from non-limiting ecosystems. Therefore, obtaining AM fungal species adapted to abiotic stresses as salinity, it able to be used in

application as bio-inoculants in different types of crops, likely to result in considerable economic and ecological benefits.

In this Doctoral Thesis we aimed to study the role of AM fungal communities isolated from arid ecosystems in the improvement of the mechanisms associated with salinity stress tolerance in *Lactuca sativa* L. plants. In chapter I, we present a general introduction of this Doctoral Thesis, showing the hypothesis and aims of this study. In chapter II is deeply reviewed the contribution of AM fungi on plant tolerance to osmotic stress. We describe the principal direct and indirect mechanisms by which AM symbiosis modify plant responses to osmotic stress, highlighting the role in photosynthetic activity, water use efficiency, osmoprotectant production, antioxidant activities, and gene expressions. We also discuss the potential for using AM fungi to improve plant performance under osmotic stress conditions, and the lines of research needed to optimize AM use in plant production. In chapter III, we report for the first time the presence of the AM symbiosis in representative plant species growing in different elevation belts of the Atacama Desert (Pampa del Tamarugal Desert, pre-Puna and Puna), as a way to highlight the importance of this association in the establishment and functioning of plant ecosystems in one of the most extreme environments in the world (see specific objective 1). The results showed that all plants presented AM colonization and fungal propagules in rhizosphere. A wide distribution of the AM symbiosis through the three elevation belts of the Atacama Desert was found, suggesting a high dependence of these plants by AM symbiosis. Noticeably, the higher values of electrical conductivity and soluble cations were highly related with both spore density and hyphal length. According to this, AM symbiosis play a role helping plants to cope with the stress conditions, which is of importance in limiting conditions like present in arid and semi-arid zones. Thereby, two native consortia of AM fungi were selected as bio-inoculants, and then used in to achieve

the following specific objectives: i) the first AM native consortium was isolated from the rhizosphere of *Werneria pinnatifida* Remy (Asteraceae) plants in the Salar del Huasco National Park (Puna elevation belt); the second AM native consortium was isolated from the rhizosphere of *Baccharis scandens* (Ruiz & Pav.) (Asteraceae) plants in the Camiña Valley (pre-Puna elevation belt). Both AM native consortia are representing to AM fungi presumably well-adapted to salt stress.

The chapter IV is associated to specific objective 2, where we evaluated the functional contribution of two AM fungi strains in the growth, proline production, nutrition, and ionic balance of lettuce plants under increasing salt stress conditions. *Claroideoglomus claroideum* (N.C. Schenck & G.S. Sm.) as reference fungus, isolated from non-saline soil from the Araucanía Region (Chile) associated with the rhizosphere of wheat plants, and a native AM fungi consortium isolated from saline soils in the Salar del Huasco National Park were used. The results showed functional differences between AM fungal inocula, where the best performance was reached with *C. claroideum* fungus, and the results were directly related to higher biomass production, increased synthesis of proline, increased N uptake and noticeable changes in ionic relations, based in a diminish of Na^+, compared to non-mycorrhizal plants. The results suggest that AM fungi improve plant growth by means of a filtering effect of AM fungal structures both in the soil and in the root that prevents the entry of toxic Na^+ ions. Therefore, the directed inoculation with efficient AM fungi strains can benefits the lettuce growth in saline soils, as vast soil surfaces worldwide. The chapter V was linked also to specific objective 2. To accomplish this study, two cultivars of lettuce were selected according their stress tolerance responses, where Grand Rapids (GR) shown a higher biomass production than Lollo Bionda (LB), under saline stress (Supplementary data 1). Also, we used another native consortium of AM fungi isolated from saline soils of Camiña Valley (pre-Puna), also

comparing it with *C. claroideum* as reference fungus isolated from non-saline soil. The two AM fungal inocula were evaluated on the growth and both enzymatic and non-enzymatic antioxidant responses of two cultivars of lettuce, GR and LB, at increasing salt stress conditions. The results showed both inocula had capacity to colonize the roots in the two lettuce cvs.; however, both cultivars responded differently to each AM inoculum. Detailing, both AM inocula enhanced the salinity tolerance of lettuce, triggering increase of the antioxidant enzyme activities, a high proline production, and a low lipid peroxidation levels, which was correlated with reduced oxidative stress and increased growth in colonized plants. On the contrary, the increased of non-enzymatic antioxidant compounds (mainly hydroxycinnamic acids and flavonols) was higher in non-inoculated plants. This reflects the high degree of metabolic disruption induced by salinity in non-mycorrhizal plants, which was related with a high oxidative stress, and low fresh biomass production. Our results provide new support regarding the beneficial role of the AM symbiosis in mitigating the negative effects of salinity stress in plants. Despite the strong differences of environments from which the two AM fungi were isolated, both inocula had similar behavior in conferring tolerance to salinity in lettuce.

The chapter VI was linked to specific objective 3. Here, we evaluated the effects of AM symbiosis on the expression pattern of genes associated with K^+ and Na^+ uptake and translocation, and the K^+/Na^+ homeostasis, as well as the effect on the relative abundance of PIP aquaporins in relation with water status. To accomplish this study, two AMF species, *Funneliformis mosseae* (T.H. Nicolson & Gerd.) and *Claroideoglomus lamellosum* (Dalpé, Koske & Tews), were inoculated to two lettuce cultivar (Grand Rapids and Lollo Bionda) growing under saline conditions. The AM species were isolated from the rhizosphere of *Baccharis scandens* (Ruiz & Pav.) from saline soils of Camiña Valley, and multiplied using monosporic mass crops with *Bidens pilosa* L. as host. The

results showed that salinity increased the AM colonization, as well as in lettuce plants inoculated with *F. mosseae* or *C. lamellosum*. On the contrary, salinity affected adversely plant growth, being higher in non-inoculated plants. AM symbiosis improved plant growth, enhancing photosynthetic capacity and water status. This beneficial effect could be linked with the regulation in the expression of several genes encoding cation-proton antiporter (*LsaNHX2*, *LsaNHX4* and *LsaNHX6*) involved in K^+/Na^+ homeostasis, and also an increased abundance of PIP2 and PIP2-phosphorylated, involved in plant water relations. This results give new evidences about the role of AM symbiosis improving salinity stress tolerance in lettuce plants, being the first research studying together genes involved in K^+/Na^+ homeostasis and abundance of aquaporins as a consequence of the AM symbiosis. All the results here obtained are discussed in a comprehensive manner in the chapter VII of this thesis.

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CHAPTER I

General Introduction

1.1 General Introduction

Saline soils occupy nearly 8% of the earth land surface, being more common in arid and semi-arid regions where leaching of salt is poor due to low rainfall (Pitman & Läuchli, 2002). The 23% of the arable lands worldwide are affected by salinity. Nowadays, approximately 95 Mha are under primary salinization (salt accumulation through natural processes), whereas 77 Mha suffer secondary salinization, as a result of human activities (Amini et al., 2016). Soil salinization is a major factor contributing to the loss of arable soils, limiting crop production. The soil solution of saline soils is composed of a range of dissolved salts, but NaCl is the most prevalent (Rengasamy, 2010).

Salt stress affects various physiological and metabolic processes, and may eventually impede crop production depending on the extent and severity (Gupta & Huang, 2014). In the early stages, salinity causes physiological drought due to high concentrations of solutes present in the soil, which reduces the capacity of root systems to absorb water and, meanwhile, accelerates the loss of water from the leaves. Also, salinity produces ion-specific stresses, where overaccumulation of Na^+ causes toxicity, ionic imbalance and K^+ deficiency (Munns & Tester, 2008). Subsequently, it increases the production of reactive oxygen species (ROS), producing oxidative damage, and leading to serious disturbances in the structure of enzymes and other macromolecules, damaging cellular tissues, altering photosynthesis, respiration, and protein synthesis (Zheng et al., 2008). Throughout evolution, plants have developed different responses and adaptation mechanisms that allow them to cope with the multiple stress conditions (Simontacchi et al., 2015). Tolerance to salinity by means of the activation of complex mechanisms include developmental, morphological, physiological and biochemical strategies (Gupta & Huang, 2014), addressing ion homeostasis, osmolyte biosynthesis, compartmentation of toxic ions, and ROS scavenging systems (Colmer & Flowers, 2008).

The AM fungi are known for their ability to form a non-specific symbiotic association with most of the terrestrial plants (Azcón & Barea, 2010). AM symbiosis also occurs in saline soils (Aliasgharzadeh et al., 2001), being a strategic adaptation that acquires greater importance in arid and semiarid ecosystems. According to Sylvia and Williams (1992), the native ecotypes of AM fungi are the result of their continuous adaptation to the extreme conditions of the soils from they were isolated. In this way, several studies have showed that AM fungi communities isolated from ecosystems affected by salinity stress have a greater ability to enhance plant growth under this condition, compared to AM fungi isolated from non-stress conditions (Copeman et al., 1996; Estrada et al., 2013). AM symbiosis can ameliorate several mechanisms in plant to manage salt stress, among them: (i) improve nutrient acquisition and maintain ionic homeostasis; (ii) improve water uptake and maintain osmotic equilibrium in plants; (iii) induce antioxidant system to prevent damage by ROS, and (iv) protect photosynthetic apparatus and enhance photosynthetic efficiency (Ruiz-Lozano et al., 2012; Evelin et al., 2019).

Lettuce (*Lactuca sativa* L.) is the most widely grown leafy vegetable in Chile (and probably worldwide), with a total of 6,673 ha yr^{-1}, representing 9.6% of all vegetables cultivated in the country (ODEPA, 2015). It is mainly cultivated in the regions of Coquimbo, Valparaiso, Maule and Metropolitana, but also it is cultivated in little plantations from region of Arica y Parinacota to region of Magallanes. At present, lettuce production have showed a decrease (INIA-INDAP, 2017), which could be related to lack of water, constant salinization of soils, and also to climate changes. In this way, Lettuce is a glycophyte plant with a threshold of soil electrical conductivity of 1.3 dS m^{-1}, being sensitive to saline stress (Shannon & Grieve, 1998). Lettuce plants are well-colonized by

AM fungi, usually improving biomass production, photosynthesis, and nutrient acquisition even under saline stress (Aroca et al., 2013; Jahromi et al., 2008).

It is well known that the Atacama Desert (Northern Chile) is the driest desert worldwide (Azua-Bustos et al., 2012), and represents one of the most extreme conditions to life develop. Several studies have showed AM symbiosis in native plants in different arid and semi-arid ecosystems (Azcón-Aguilar et al., 2003; Cavagnaro et al., 2017; Liu et al., 2017), but the presence of AM symbiosis in native plants of north of Chile has not been studied so far. Due to lack of information about AM fungi living in the limiting conditions of Atacama Desert, together with the lack of studies on the bioinoculation with these native AM fungi in crop that grow under saline soils in Chile, this study represents a pioneer research line in Chile with a potential high impact. This is of great importance considering that zones affected by drought and salinity in Chile are expanding due to climate change, directly affecting crop production. The search of technologies to address this problem becomes necessary, especially if are environmentally-friendly.

According to the antecedents previously mentioned, we proposed the following hypotheses and objectives.

1.2 Hypotheses

- Arbuscular mycorrhizal fungi isolated from the rhizosphere of plants growing under salinity stress conditions in the Atacama Desert, have adaptations allowing them to develop functional symbiosis under these conditions in comparison with other AM isolated from non-stressed environments.
- The AM symbiosis between fungi isolated from saline soil and *Lactuca sativa* improve the mechanisms of saline stress tolerance in plants, through changes on water relations and ionic homeostasis, which produce a decrease of the oxidative stress.

1.3 General objective:

To study the role of arbuscular mycorrhizal fungal communities isolated from arid ecosystems in the improvement of the mechanisms associated with salinity stress tolerance in *Lactuca sativa* plants.

1.4 Specific objectives:

1) To characterize the AM fungal status of representative species of native flora in different bioclimatic areas of the Atacama Desert, Tarapacá Region.

2) To evaluate the effect of selected AM fungal community on the growth, nutrition and the antioxidant response of lettuce plants under salinity stress conditions.

3) To determine the effect of selected AM fungi strains on the expression pattern of genes associated with ionic homeostasis, and on the relative abundance of proteins related with hydric parameters in lettuce plants subjected to salinity stress.

CHAPTER II

"Arbuscular mycorrhiza effects on plant performance under osmotic stress"

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Arbuscular mycorrhiza effects on plant performance under osmotic stress

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Abstract

At present, drought and soil salinity are among the most severe environmental stresses that affect the growth of plants through marked reduction of water uptake which lowers water potential, leading to osmotic stress. In general, osmotic stress causes a series of morphological, physiological, biochemical, and molecular changes that affect plant performance. Several studies have found that diverse types of soil microorganisms improve plant growth, especially when plants are under stressful conditions. Most important are the arbuscular mycorrhizal fungi (AMF) which form arbuscular mycorrhizas (AM) with approximately 80% of plant species and are present in almost all terrestrial ecosystems. Beyond the well-known role of AM in improving plant nutrient uptake, the contributions of AM to plants coping with osmotic stress merit analysis. With this review, we describe the principal direct and indirect mechanisms by which AM modify plant responses to osmotic stress, highlighting the role of AM in photosynthetic activity, water use efficiency, osmoprotectant production, antioxidant activities, and gene expression. We also discuss the potential for using AMF to improve plant performance under osmotic stress conditions and the lines of research needed to optimize AM use in plant production.

Keywords AM inoculants. Drought stress. Osmotic stress. Rhizosphere. Salinity.

2.1 Introduction

When a plant is exposed to conditions significantly different from those optimal for growth and development, it is subjected to stress (Sade et al. 2013). Plants constantly are exposed to different types of stress such as lack of water or water excess, extreme temperatures and pH, low nutrient availability and soil salinity, all potentially reducing plant growth (Sánchez-Rodríguez et al. 2012; Atkinson and Urwin 2012). Globally, drought and soil salinity represent the environmental abiotic stresses that most limit plant growth at present (Kramer and Boyer 1997; Cattivelli et al. 2008; Lambers et al. 2008; Trenberth et al. 2014), with greatest effects in arid and semiarid areas (Knapp et al. 2001; Seki et al. 2003; Fischlin et al. 2007). These stresses are produced by water imbalance in ecosystems caused by both decreasing rainfall and rising average temperatures that elevate evapotranspiration rates, thus reducing water storage in soils (Wery et al. 1994; Rapti-Caputo 2010). Moreover, the use of irrigation water with high ion content, excessive application of chemical fertilizers, and overuse of water resources are the main factors contributing to soil salinization (Cantrell and Linderman 2001; Al-Karaki 2006) which produces salinity stress in plants. This stress is globally present in about 20% of cultivated land and 50% of irrigated systems (Kapoor et al. 2008; Abdel-Latef and Chaoxing 2014). Many of the areas affected by salinity remain unproductive, mainly because of reduced plant establishment (Asghari 2008).

Drought and salinity reduce water absorption by roots causing tissue dehydration, resulting in osmotic stress (Zhu et al. 1997; Seki et al. 2003; Aroca et al. 2012). Osmotic stress caused by drought interferes with normal growth and development of plants by altering physiological and biochemical processes, thereby affecting their survival and productivity (Kramer and Boyer 1997; Bray 2004). The osmotic stress caused by drought negatively affects water relations, gas exchange, photosynthesis,

nutrient absorption, and metabolism of carbohydrates, proteins, amino acids, and other organic compounds (Sircelj et al. 2005; Ashraf and Foolad 2007; Anjum et al. 2011). The same effects are produced by salt stress, adding the toxic effect of Na^+ and Cl^- ions which produce homeostatic imbalances and denaturation of proteins and other organic molecules (Adiku et al. 2001; Evelin et al. 2009). Additionally, in many regions with arid and semi-arid climates, Gypsum soils are present. They have stressful chemical properties for plant growth related to the nutritional impoverishment of the soil caused by the exchange of calcium (Ca^{2+}) for other ions retained in the soil complex and by a high concentration of sulphate ions, which can be toxic to plants (Palacio et al. 2007). Moreover, high temperatures increase the water demand of plants causing leaf water deficits, which produce osmotic stress manifested as denaturation and aggregation of proteins, hyper fluidity of membrane lipids, loss of membrane permeability, and direct chemical breakdown by the accumulation of toxic elements and ion efflux (Levitt 1980; Gong et al. 1998).

Different studies have reported that beneficial soil microorganisms improve plant tolerance to abiotic stresses by producing a root-soil interface that enhances the absorption of water and nutrients. These microorganisms most importantly include the symbiotics such as nitrogen-fixing bacteria and arbuscular mycorrhizal fungi (AMF) (Barea et al. 2005; Hidri et al. 2016). The AMF generate symbioses called arbuscular mycorrhizas (AM), which protect plants against the vast majority of abiotic stresses by different mechanisms such as increased mineral nutrient absorption, enhanced photosynthetic activity, accumulation of osmoprotectant solutes, production of antioxidant enzymes, and modification of the rhizosphere environment (Bárzana et al. 2015; Calvo-Polanco et al. 2016a; Yin et al. 2016).

Given the importance of AM for the establishment and growth of plants under stress conditions, aspects that allow for efficient use of this symbiosis to ameliorate limiting conditions require additional study. This review provides an update on the role of AM in coping with osmotic stress, one of the main constraints on plant performance worldwide.

2.2 General characteristics of the arbuscular mycorrhizal symbiosis

Mycorrhizas are symbiotic relationships that occur between plant roots and several different types of fungi, in which the fungus obtains its sugars from the plant, while the plant benefits from efficient uptake of mineral nutrients and water by the fungus (Smith and Read 2008). Arbuscular mycorrhizas are the most widely distributed of all mycorrhizal associations (Smith and Read 2008) and are formed by more than 80% of vascular plants (Jeffries et al. 2003), including about 90% of plants of agricultural interest (Finlay 2008; Smith and Read 2008). According to molecular evidence, the AMF belong to the subphyllum Glomeromycotina, which is included within the phylum Mucoromycota (Spatafora et al. 2016). Four classes have been identified in the subphylum: Paraglomerales, Archaeosporales, Diversisporales, and Glomerales, which include 25 genera (Redecker et al. 2013; updated January 16th, 2017; see http://www.amf-phylogeny.com), being the most widely accepted taxonomy for this fungal group at present.

The arbuscular mycorrhizal symbiosis began approximately 460 million years ago, in the Devonian period. The association allowed aquatic plants to colonize the land, producing adaptations that favoured the early transition of plants from aquatic to terrestrial habitats (Simon et al. 1993; Schüβler 2000; Redecker et al. 2000; Heckman et al. 2001). The symbiosis occurs when fungus spores in the soil germinate to form a germ

tube, a stage that depends on physical factors such as temperature and humidity (Giovannetti et al. 1993). Recognition between the fungus and the plant is influenced by interacting molecular signals (Hause and Schaarschmidt 2009). Strigolactones exuded by host plant roots are considered a significant component in the initiation and establishment of the association, stimulating branching and presymbiotic hypha metabolism by increasing ATP production and mitochondrial division (López-Ráez et al. 2011; Kohlen et al. 2012; Bonfante and Genre 2015). According to Aroca et al. (2013), rice plants inoculated with *Claroideoglomus etunicatum* under saline conditions showed increased root colonization related to enhanced strigolactone production by plants subjected to salt stress.

Several studies have shown that other plant hormones also promote root colonization, growth of extraradical and intraradical mycelium, and production of spores and arbuscules (Etemadi et al. 2014; Nagata et al. 2016; Martín- Rodríguez et al. 2016; Cosme et al. 2016). Gibberellic acids (GAs) play an important role affecting root colonization. Genetic studies using the model AM fungus *Rhizophagus irregularis* have indicated that AM fungi lack exoenzymes able to degrade cell walls necessary for root penetration, suggesting that the plant itself causes the distension of intercellular spaces allowing hyphal penetration, which can be regulated by GAs (Takeda et al. 2015). Moreover, Takeda et al. (2016) found that GAs are needed for AM development in *Lotus japonicus*. In that case, GA signalling interacts with symbiosis signalling pathways, directing AM fungal colonization in host roots. The same authors found that root colonization is inhibited by excess GAs. Conversely, suppression of GA biosynthesis causes irregular morphology of AM colonization, suggesting that proper regulation of GAs is essential for AM development. Likewise, jasmonic acid (JA) has various effects on colonization. Low levels of JA promoted AM colonization, while high levels reduced

it (Regvar et al. 1996; Ludwig-Muller et al. 2002). Tejeda-Sartorius et al. (2008) found that AM colonization was severely reduced in tomato plants with impeded synthesis of JA (spr2 mutants), but was restored by methyl jasmonate application. Those authors suggested that the pathway by which JA might modulate the AM colonization process could be related to carbon partitioning in the plant. Similarly, several studies have found a direct role of abscisic acid (ABA) in AM development. For example, Charpentier et al. (2014) found that ABA promotes AM colonization in *Medicago truncatula* plants at low concentrations and inhibits it at high concentrations. Similarly, Martín-Rodríguez et al. (2010) showed that this hormone plays an important role in the development and functionality of arbuscules by using an ABA-deficient mutant tomato (sitiens). In addition, ABA deficiency might induce ethylene production, which adversely affects mycorrhizal colonization intensity.

Subsequently, the mycelium penetrates the root inter- and intracellularly, but without invading conducting vessels. Within roots, hyphal spread follows either of two different morphological growth patterns: the Arum and Paris types. The Arum type is characterized by intercellular growth of hyphae with penetration of cortical cells to form arbuscules. In contrast, the Paris type is characterized by intracellular growth of hyphae, during which coils are formed (Smith and Read 2008). Arbuscules and coils are responsible for the exchange of mineral nutrients to the plant and simple carbohydrates to the fungus (Cavagnaro et al. 2001, Dickson et al. 2007). In parallel, the extraradical mycelium provides a large contact surface between the plant and soil, acting as a complementary ‘root system’ that increases the absorption of mineral nutrients and water beyond the root depletion zone (Peterson et al. 2004; Smith and Read 2008; Mikkelsen et al. 2008). Hart and Reader (2002) found taxonomic differences among AMF families in mycelium structure. Glomeraceae and Acaulosporaceae tend to have sparse mycelium

with densities ranging from 1 to 2 m cm^{-3}, whereas Gigasporaceae tend to have robust, densely aggregated mycelium with densities ranging between 6 and 9 m cm^{-3}. Additionally, some AMF species develop structures called vesicles, which appear at late stages of colonization within and between root cells. These structures contain copious lipids and function as carbon (C) storage organs and propagules (Smith and Read 2008). A portion of the C fixed by photosynthesis is transported to the fungus, representing 4 to 20% of total photosynthate (Pearson and Jakobsen 1993). The C is transported as hexose sugars and is stored by the fungi as triacylglycerols and glycogen (Solaiman and Saito 1997; Bago et al. 2002; Morgan et al. 2005; Sjöberg 2005; Xie et al. 2014). Also have demonstrated lipids are transferred from plants to fungi in addition to sugars, and that AMF are dependent on this lipid supply because they lack genes encoding fatty acid synthase I subunits.

2.3 The role of AM in the soil-plant continuum

The first study of the effects of AMF improving plant growth was not performed until the mid-twentieth century (Mosse and Hepper 1975). It demonstrated that mycorrhizal apple plants had improved growth and elevated concentrations of iron (Fe) and copper (Cu) when grown in soils deficient in these nutrients. AM are especially important for the absorption of immobile soil nutrients, such as phosphorus (P), zinc, Cu, manganese, potassium (K), calcium (Ca), magnesium (Mg), and Fe (Sjöberg 2005; Smith and Read 2008; Cornejo et al. 2008a, 2008b, 2009; Kaya et al. 2009; Campanelli et al. 2012; Evelin et al. 2012; Garg and Bhandari 2016). AM also have an ability to improve nitrogen (N) nutrition (Hodge et al. 2001; Cornejo et al. 2007, 2008b, 2009). Moreover, AM improve soil structure through the formation of stable aggregates because the mycelia form networks in the soil, preventing surface erosion and improving nutritional and water status

(Barea et al. 2005; Curaqueo et al. 2010; Mardhiah et al. 2016). These fungal networks can range from a few centimeters to several meters and can account for up to half of the microbial biomass in grassland soils (Olsson et al. 1995; Soka and Ritchie 2014). Also, AMF produce recalcitrant forms of C such as chitin and glomalin (Zhu and Miller 2003). The latter is a hydrophobic glycoprotein that participates in the formation and stabilization of soil aggregates which can be affected by different tillage systems and soil management (Miller and Jastrow 2000; Cornejo et al. 2008a, 2017; Curaqueo et al. 2011). Several studies have shown the ability of some AM fungi to increase glomalin production under osmotic stress conditions, e.g., *Glomus versiforme*, *Glomus mosseae*, and *Glomus diaphanum* associated with *Poncirus trifoliata* under drought (Wu et al. 2008), *Glomus mosseae* associated with *Lactuca sativa* under salinity (Kohler et al. 2010), *Glomus etunicatum* and *Glomus mosseae* associated with *Citrus tangerine* under drought (Zou et al. 2014), and *Rhizophagus intraradices* and *Funneliformis mosseae* associated with *Pelargonium graveolens* also under drought (Amiri et al. 2016).

Furthermore, AM cause changes in root exudates, selectively affecting communities of microorganisms in the rhizosphere (Marschner and Timonen 2005). For example, inoculation with *Diversispora versiformis* resulted in an abundance of ammonifying bacteria, nitrobacters, denitrifying bacteria, and phosphobacteria, which improve urease and alkaline phosphatase activity in soils cultivated with *Lolium multiflorum* (Ye et al. 2015). An increase in plant growth promoting rhizobacteria (PGPR) in the presence of AMF stimulates the absorption of P by host plants (Fernández-Bidondo et al. 2012). Additionally, some PGPR species like *Paenibacillus favisporus* and *P. rhizosphaerae* produce auxins that promote the development of the AM mycelium (Fernández-Bidondo et al. 2011). Another effect of AMF in the soil is that the extrarradical mycelium can produce enzymes that contribute to the solubilization of

mineral nutrients. For example, Sato et al. (2015) detected a 187-KDa acid phosphatase in a monoxenic culture of *Rhizophagus clarus*. Thus, AM have prime importance in the rhizosphere that deserves to be extensively studied.

2.4 Mechanisms of arbuscular mycorrhizal symbiosis that contribute to osmotic stress tolerance by plants

Benefits provided to plants by AM are not limited to improvements in nutritional status and soil structure, but also are key components of plant adaptations to osmotic stress (water and salinity stress) through diverse mechanisms, which may be direct and/or indirect.

2.4.1 Direct mechanisms

Water transport

AMF may be able to affect plant-water relations in several different ways including hormonal changes, effective absorption of soil water, enhanced soil-root contact, amelioration of gas exchange, regulation of osmotic adjustment, and direct water uptake from the soil through the mycelium and transport to the plant (Augé 2001). The AM mycelium has the ability to penetrate soil pores inaccessible to root hairs and might be able to transport water both internally and on external hyphal surfaces that is not available directly to roots (Allen 1982; Hardie 1985; Marulanda et al. 2003). Rates of internal water transport by extraradical mycelium to the root of 0.1 $\mu L\ h^{-1}$ per hyphal entry point (Allen 1982) and up to 0.76 $\mu L\ h^{-1}$ per hypha (Faber et al. 1991) have been found. AM plants of *Lactuca sativa* inoculated with *Glomus coronatum*, *G. intraradices*, *G. claroideum*, and *G. mosseae* have received a flow of 3 to 4.75 ml $H_2O\ plant^{-1}\ day^{-1}$ greater than non-colonized plants, with the amount related to the production of extraradical mycelium and

root colonization frequency although the AMF differed in their ability to enhance water uptake (Marulanda et al. 2003). These levels of water flow are sufficient to change water relations in plants. Ruiz-Lozano and Azcón (1995) reported considerable water uptake by AM colonized *Lactuca sativa* plants, but could not quantify it. The latter authors observed that mycelia of *Glomus deserticola* and *Glomus fasciculatum* differed in their influence on water uptake, despite similar mycelium length.

Khalvati et al. (2005) designed a split-root system experiment to quantify the contribution of the AM mycelium to plant water uptake in AM mycorrhizal barley plants grown under well-watered and drought conditions. Compared with the non-mycorrhizal treatment, only 4% of total water absorbed by plant was transferred from the hyphal compartment to the root compartment through the AM hyphae under drought conditions. In the latter experiment it is important to highlight the presence of a 5-mm separation between both compartments that ensured the absence of a mass flux of water. Moreover, Ruth et al. (2011) measured the hyphal contribution to total plant water uptake by means of high- resolution on-line monitoring of soil water content, showing that the hyphal contribution to total plant water uptake was at least 20%, which is considerably higher than the 4% previously estimated by Khalvati et al. (2005). The latter study estimated a flux density for water in the hyphae of 22.8 μl cm^{-2} day^{-1}. In spite of the great differences found between the above studies and the large evidence about the AM hyphae contribution to water uptake by plant, it is still necessary to elucidate the role of this fungal structure in the transport of water to clarify if it is a direct effect by absorption, or an indirect effect in which the hyphae maintain a flow of water due to forces of cohesion and adhesion in its surface.

Under osmotic stress, aquaporins (AQPs) are important in regulating plant water flow. These proteins belong to a multifunctional family that branches from the

superfamily of major intrinsic proteins which act as water channels and are important in osmoregulation (Maurel et al. 2008). AQPs facilitate passive transport of water following a potential gradient (Kruse et al. 2006). Additionally, they have the ability to transport low molecular weight molecules such as glycerol, ammonium, and CO_2 (Kruse et al. 2006; Maurel et al. 2009). Aroca et al. (2009) cloned the first aquaporin from an AM fungus (*GintAQP1*). Although the functionality of this aquaporin could not be demonstrated, the authors found evidence supporting that fungal AQPs could compensate for the down regulation of host plant AQPs caused by drought. Also, they found that *GintAQP1* expression was upregulated in parts of the mycelium that were not osmotically stressed by NaCl while other parts of the mycelium were stressed. This suggests possible communication between non-stressed and stressed mycelium. There also are two characterized functional genes, *GintAQPF1* and *GintAQPF2*, that encode for AQPs present in the AM fungus *Rhizophagus intraradices* (Li et al. 2013a), which are overexpressed under osmotic stress conditions, helping the fungus to tolerate stress and potentially increasing the water supply to a host plant under these conditions (Li et al. 2013b).

Absorption of nutrients and ion balance

Another direct mechanism that AM colonized plants experience under osmotic stress is an increased absorption of mineral nutrients, especially in soils where drought affects the diffusion of nutrients (Marschner et al. 1997). Safir et al. (1971, 1972) carried out the first studies showing that AM improves nutritional status and generate tolerance in plants under osmotic stress conditions. Just as the extraradical mycelium transports water to the plant, mineral nutrients also are transported across the root-soil interface (Smith and Smith 2011). Several studies indicate improved nutritional status of AM plants under

osmotic stress (Augé 2001; Augé et al. 2014; Lehmann et al. 2014; Lehmann and Rillig 2015).

One of the mineral nutrients that has been most studied in AM is phosphorus (P) because this element, although rapidly absorbed by plants, has slow diffusion in the soil solution generating a depletion zone around roots (Smith and Read 2008). When AM are formed, plant P absorption under limiting conditions is improved (Smith et al. 2003; Garg and Manchanda 2008; Shokri and Maadi 2009; Bowles et al. 2016). Several studies have found that AM colonization can reduce the P direct uptake pathway of roots in some plant species and suppress it completely in other species (Liu et al. 1998; Smith et al. 2004). According to Smith et al. (2004), 100% of P in flax and tomato plants may be absorbed via AM. Phosphate is absorbed by the extraradical mycelium and is polymerized to polyphosphate which is accumulated in the vacuoles of the extraradical mycelium (Viereck et al. 2004). Two phosphate transporters of high affinity from *Rhizophagus intraradices* and *Diversispora versiformis* expressed exclusively in extraradical mycelium have been cloned (Maldonado-Mendoza et al. 2001). Moreover, recent evidence has suggested that the translocation of polyphosphate through fungal hyphae is mediated by the activity of AM fungal aquaporins (Kikuchi et al. 2016).

In contrast to immobile P, osmotic stress conditions reduce the high mobility of NO_3^- and NH_4^+. Under such conditions, AM increase the absorption of NO_3^-, NH_4^+, and some N organic sources (Hodge et al. 2001; Govindarajulu et al. 2005; López-Pedrosa et al. 2006; Hodge et al. 2010). Bago et al. (2001) proposed that for the transfer of N and subsequent absorption by the plant, a process associated with the urea cycle and transport of polyphosphates occurs. This process was confirmed by Govindarajulu et al. (2005). According to Bago et al. (1996), NO_3^- is absorbed by the extraradical mycelium with its uptake achieved by proton symport. Subsequently, assimilation of NO_3^- in the AM

mycelium involves conversion to NH_4^+ by nitrate reductase and inclusion in the glutamine synthetase cycle (GS) followed by glutamate synthase (GOGAT) being transformed into arginine and transported to intraradical mycelium associated with the transport of polyphosphates (Subramanian and Charest 1998; Bago et al. 2001; Govindarajulu et al. 2005). The same cycle occurs when NH_4^+ is absorbed by extraradical hyphae. AMF preferentially take up NH_4^+ over NO_3^-, because the uptake of NH_4^+ is energetically more efficient than the uptake of NO_3^- (Fellbaum et al. 2012). Several NH_4^+ and NO_3^- transporters have been identified in extraradical or intraradical mycelium of AM fungi (Tisserant et al. 2012). In *Rhizophagus irregularis*, three sequences have been elucidated as NH_4^+ transporters while only one NO_3^- transporter has been identified (López-Pedrosa et al. 2006). The increase of plant N concentration under limiting conditions improves plant metabolism by promoting protein synthesis and increasing concentrations of compatible osmolytes (Ruiz-Lozano 2003; Bhoopander and Mukerji 2004). Several studies have found that AM are responsible for a significant amount of total N uptake (from 21 to 75%) (Govindarajulu et al. 2005; Tanaka and Yano 2005; Smith and Read 2008; Kobae et al. 2010).

Mainly in saline soils, osmotic stress contributes to mineral nutrient deficiency and decreased concentrations of K^+, $Mg2^+$, and Ca^{2+} in plant tissues, causing ion imbalance because of antagonism of Na^+ towards K^+, Mg^{2+}, and Ca^{2+}, (Adiku et al. 2001; Hu and Schimdhalter 2005). The Na^+ produces cytotoxic effects by cellular organ damage, altering the structure of enzymes and other macromolecules, changing photosynthesis and respiration, and inhibiting protein synthesis (Juniper and Abbott 1993; Niu et al. 1995; Ramoliya et al. 2004). Several studies have found that AMF alleviate the toxic effect caused by salinity, improving plant growth under saline conditions (Ezz and Nawar 1994; Cantrell and Linderman 2001, Evelin et al. 2012; Zarei and Paymaneh 2014;

Abeer et al. 2015; Porcel et al. 2015; Garg and Bhandari 2016; Pedranzani et al. 2016; Elhindi et al. 2017). AMF act as a primary barrier, selecting ions, and thereby improving the absorption and selective transport of mineral nutrients (Daei et al. 2009). AM reduce the translocation of Na^+ ions to plant tissues preventing its concentration reaching toxic levels. This is because of the ability to retain these ions in structures such as the intraradical mycelium and vesicles by accumulating ions in their vacuoles (Allen 1982; Augé 2001; Al-Karaki 2006; Mardukhi et al. 2011). Additionally, studies with *Rhizophagus intraradices* showed its ability to selectively absorb mineral nutrients such as K^+, Mg^{2+}, and Ca^{2+} while preventing the entry of Na^+ to the mycorrhizal structures, thereby maintaining high K^+:Na^+, Ca^{2+}:Na^+ and Mg^{2+}:Na^+ ratios (Hammer et al. 2011). Lower concentrations of leaf Na^+ were found in mycorrhizal than in non-colonized plants, such as *Olea europea* (Porras-Soriano et al. 2009), *Acacia nilotica* (Giri et al. 2007), and *Triticum aestivum* (Talaat and Shawky 2013) growing under saline conditions. Four gene sequences of *Rhizophagus irregularis* that encode for K^+ transporter systems have been identified; three encode SKC (small conductance calcium-activated potassium channels) ion channels, and one encodes transporter family type KT/KUP/HAK (Casieri et al. 2013) that selectively can modify K^+:Na^+ ratios.

2.4.2 Indirect mechanisms

The improvement of soil characteristics by AMF, which represents indirect mechanisms that reduce osmotic stress, may increase soil water retention capacity through physical and biochemical effects (Augé 2004; Martin et al. 2012). The physical effect mainly is produced by the extraradical mycelium interacting with soil particles to form stable aggregates that contribute to the protection of soil organic matter and help retain soil water (Mardhiah et al. 2016). The ability to produce mycelium differs among species of

AMF with lengths ranging from 1 to 40 m per gramme of soil in experimental trials (Smith and Smith 2011). In a natural ecosystem, 111 m of hyphae per gramme of grassland soil have been found (Rillig et al. 2001). Biochemical effects are caused by the ability of AMF to release organic products to soil such as glomalin (operationally measured as "glomalin-related soil protein" GRSP), polysaccharides, mucilage, and hydrophobins (Singh et al. 2012). Such compounds participate in the formation of stable aggregates and C sequestration (Tisdall and Oades 1982). These stable aggregates improve infiltration and water retention, retarding drought effects on the rhizosphere (Rillig and Mummey 2006; Manoharan et al. 2010; Audet 2012). A recent study by Zhang et al. (2016) reported that in rhizosphere of *Poncirus trifoliate* plants inoculated with *Diversispora versiformis* and growing under salinity (irrigated with 100 mM NaCl) higher amount of GRSP than non-colonized plants was quantified, which was also associated with an increased percentage of water-stable aggregates. Additionally, Zou et al. (2014) found a high correlation between water stable aggregates and GRSP, as well as between GRSP and the amount of available water, which highlights the important role of this substance to cope with soil osmotic stress conditions. Additionally, according to Verbruggen et al. (2016), AMF also can stabilize organic C by slowing mineralization of soil organic matter (SOM). For an arbuscular mycorrhizal system, the latter authors detected the "Gadgil effect" which occurs when a mycorrhizal mycelium quickly depletes available P and N in competition with saprotrophic microbes, thereby retarding SOM mineralization.

2.5 AM and plant responses to osmotic stress

2.5.1 Growth promotion and development

According to Janos (2007), plant growth responses to AM colonization are represented by the difference in growth between plants of the same species with and without mycorrhizae. Klironomos (2003) has shown that plant growth responses to AM inoculation can range greatly, from negative to positive, with responses depending on the combination of plant and fungus species. For example, different responses by two genotypes of tomato plants (LA1709 and large cherry) to AM colonization during early vegetative growth were found (Bryla and Koide 1998). AM increased dry mass, root length, and P content of the LA1709 genotype while it had less effect on these characteristics of the large cherry genotype. Graham and Abbott (2000) found negative responses to AM by wheat. All the previous results were found under non-osmotic stress conditions, but several studies have found that AM can improve the production of shoot and root biomass under osmotic stress (see Table 1). Chitarra et al. (2016) have shown that AM mitigate osmotic stress by altering hormonal profiles, thus affecting physiology and development of the host plant. Similarly, Ruiz-Lozano et al. (2015) found increased dry weight of *Lactuca sativa* and *Lycopersicon esculentum* inoculated with *Rhizophagus irregularis* and subjected to mild and severe water stress (75 and 55% field capacity, respectively). Sheng et al. (2008) found that AM improve water use efficiency (WUE) from 61 to 206% versus non-colonized maize plants. In the latter study, WUE was measured for leaves as the ratio of net photosynthetic rate to transpiration rate. Inoculation of *Helianthus annuus* with *Glomus mosseae* and *Glomus hoi* under water stress increased dry weight, relative water content (RWC), oil content, and seed production (Gholamhoseini et al. 2013). For *Citrullus lanatus*, AM increased the RWC by 23% and fruit production by 19% (Omirou et al. 2013). Ortiz et al. (2015), applying irrigation at 50% of field capacity, found greater RWC and root growth of AM maize versus non-colonized plants. Moreover, higher values of RWC for AM than for non-colonized plants

were found for *Citrullus lanatus* (Kaya et al. 2003), *Lactuca sativa* cv. Roman (Ruiz-Lozano et al. 1995) and *Citrus tangerine* (Wu and Xia 2006).

Salinity in soil decreases growth of *Jatropha sp*., but inoculation with a consortium of AMF increased length and diameter of stems and root dry weight. Inoculation also increased tap root length, which was related to higher RWC in leaves (Kumar et al. 2015). *Glomus viscosum* decreased Na^+ and Cl^- content and increased K^+ content in all organs of *Medicago sativa* var. icon irrigated with a solution of 150 mM NaCl, as well as increasing dry weight, plant height, leaf area, root density, and RWC versus non-colonized plants (Campanelli et al. 2012). Similarly, *Funneliformis mosseae* improved shoot and root dry weight, stem diameter, leaf area, and fruit production of *Solanum lycopersicum* irrigated with 50 and 100 mM NaCl. The improvements were related to increased foliar P and K^+ concentrations and decreased Na^+ concentration in plants with AM versus non-colonized plants (Abdel-Latef and Chaoxing 2011). In *Triticum aestivum*, AM significantly increased yield per plant by 75, 85, and 96% in cv. Sids and in 47, 58, and 73% in cv. Giza, at soil salinity levels of 0.1, 4.7, and 9.4 dS m^{-1}, respectively, in addition to increased concentrations of P, N, and K^+, and a decreased concentration of Na^+ in leaves and grains (Talaat and Shawky 2013).

2.5.2 Increased photosynthetic activity

Osmotic stress reduces photosynthesis by diminishing photosynthetically active leaf area and inducing premature leaf senescence. Osmotic stress results in deterioration of the photosynthetic machinery and disruption of electron transport in thylakoids, declining uptake of CO_2 by stomatal closure and reduced C flux (Allen and Ort 2001; Wahid and Rasul 2005; Anjum et al. 2011). Several authors have shown that AM increase photosynthesis under stress (Sheng et al. 2008; Hajiboland et al. 2010; Ruiz-Lozano et al.

2015). Moreover, osmotic stress causes alteration of the pigments involved in photosynthesis and causes damage to chloroplasts (Behera et al. 2010). Several studies have reported that versus non-colonized plants, AM increase chlorophyll concentrations under saline conditions (Kumar et al. 2011; Campanelli et al. 2012; Evelin et al. 2012; Abdel-Latef and Chaoxing 2014), and improve the rate of photosynthesis and C fixation (Wright et al. 1998). AM *Solanum lycopersicum* showed higher concentrations of chlorophyll a and b and total carotenoid pigments versus non-inoculated plants growing at 150 mM NaCl (Abeer et al. 2015). *Claroideoglomus etunicatum* inoculated maize growing under water stress had concentrations of chlorophyll a and b and total chlorophyll increased by 18.6, 27.5, and 20.5%, respectively (Zhu et al. 2012). Likewise, Zuccarini (2007) found higher concentrations of chlorophylls in leaves of lettuce inoculated with a mix of *Funneliformis mosseae*, *Rhizophagus intraradices* and *Funneliformis coronatum* under saline irrigation (0; 1.5 and 3 g NaCl L^{-1}). AMF increase chlorophyll synthesis as a result of increased absorption of N and Mg^{2+}, both needed for chlorophyll biosynthesis and structural functions (Kaya et al. 2009; Abdel-Latef and Chaoxing 2011).

Chlorophyll fluorescence is a key index for evaluation of the photosynthetic efficiency of plants under environmental stresses (Zhu et al. 2012). *Oryza sativa* inoculated with *Rhizophagus intraradices* showed greater photosynthetic efficiency under water stress than non-inoculated plants (Ruiz-Sanchez et al. 2010). *Oryza sativa* inoculated with *Claroideoglomus etunicatum* (EEZ isolate 163) also showed higher PSII photochemical quantum yield by 25 and 34% versus non-AM plants under saline conditions at 75 and 150 mM NaCl, respectively. In contrast, the quantum yield of non-photochemical quenching (ΦNPQ) was between 30 and 40% lower in AM plants than non-inoculated plants, because AM colonized plants had a more efficient and robust PSII (Porcel et al. 2015). The ΦNPQ is a photoprotective mechanism that plants employ to

dissipate excess light energy as heat, thus preventing photooxidative damage of PSII (Inderjit 2003; Sheng et al. 2008). AM rice maintained a higher net photosynthetic rate, stomatal conductance, and transpiration rate than non-AM plants (Porcel et al. 2015).

The first response of plants to osmotic stress is not only stomatal closure, preventing water loss through transpiration, but also reducing the flow of CO_2 and C assimilation and favouring photorespiration (Mansfield and Atkinson 1990; Yokota et al. 2002; Samarah et al. 2009). Mycorrhizal plants show elevated rates of net photosynthesis under stress conditions. This occurs by increased stomatal conductance (gs) (Augé 2001), indicating prolonged open stomata, favouring gas exchange and photosynthesis (Subramanian and Charest 1995). Inoculation with AMF improved gs in plants of *Rosmarinus officinalis* and *Citrus tangerine* under water stress conditions (Sánchez-Blanco et al. 2004; Wu et al. 2006). *Oryza sativa* inoculated with *Cloroideoglomus etunicatum* (EEZ Isolate 163) showed greater gs, by 43% at 75 mM NaCl and by 32% at 150 mM NaCl than non-colonized plants (Porcel et al. 2015). The increase of gs mediated by AM has been related positively to changes of host plant hormone levels and to enhanced uptake and translocation of water (Ruiz-Lozano and Aroca 2010).

Stomatal closure reduces gas exchange, decreasing CO_2 at the cellular level. Low levels of CO_2 in cells decrease the activity and efficiency of carboxylation of the enzyme ribulose-1,5-bisphosphate (RUBISCO) (Allen et al. 2000), leading to overproduction of reactive oxygen species (ROS) (Saibo et al. 2009). In AM *Oryza sativa*, however, RUBISCO activity was not reduced by salinity, instead the opposite, increased activity was observed (ranging 76 to 196% increases at 75 and 150 mM NaCl; Porcel et al. 2015).

A recent study by Mo et al. (2016), found that *gs* and transpiration rate were not different between mycorrhizal and non-colonized watermelon plants under drought stress. Nevertheless, they also found higher photochemical efficiency of PSII, electron transport

rate, and photochemical quenching in mycorrhizal plants, versus non-colonized ones. There was a greater loss of photosynthetic efficiency caused mainly by chloroplast ultrastructural integrity loss, decreased leaf chlorophyll content, desactivation of photosynthesis related enzymes, inhibition of the functional activity of PSII, and impairment of the photosynthetic apparatus in non AM plants versus mycorrhizal ones (Mo et al. 2016). Thus, AM favouring chloroplast integrity under osmotic stress seems to be more important than effects on stomatal responses.

Table 1: Main beneficial responses observed on plant performance as a consequence of the use of natural consortia or inocula of arbuscular mycorrhizal fungi (AMF) under diverse osmotic stress conditions (drought and salinity).

Stress conditions	**AMF species**	**Plant species**	**Observed responses**	**Reference**
Drought (irrigation to 75, 50, and 25% of WHC[a])	*Septoglomus constrictum*	*Tagetes erecta*	+Dry weight shoot	Abdul-Wasea and Khalid (2011)
Drought (irrigation to 40 and 60% of WHC)	*Rhizophagus intraradices* strain BGCBJ09	*Zea mays*	+Uptake in shoot P, N, K, Mg + Development root + Water use efficient	Zhao et al. (2015)
Drought (irrigation to 75% of FC[b])	*Rhizophagus intraradices*, *Funneliformis mosseae*	*Pelargonium graveolens*	+Total phenol + Activity APX, SOD, GPX − Lipid peroxidation and H_2O_2	Amiri et al. (2015)
Drought (irrigation to 50% of WHC)	Natural consortia: *Septoglomus constrictum* *Diversispora aunantia* *Archaesopora trappei* *Diversispora versiformis* *Paraglomus occultum*	*Zea mays*	+ Root hydraulic conductivity + Uptake P + Expression of genes Aquaporin in shoot and root − Leaf electrolyte leakage − Lipid peroxidation, proline and H_2O_2 in root	Armada et al. (2015)
Drought (irrigation to 70 and 60% of ETp[c])	*Funneliformis mosseae* *Funneliformis geosporus*	*Fragaria x ananassa*	+ Water use efficiency (WUE) + Shoot fresh + Total fruit weight	Boyer et al. (2015)
Drought (irrigation to 50% of WHC)	Natural consortia: *Septoglomus constrictum* *Diversispora aunantia* *Archaespora trappei* *Diversispora versiformis* *Paraglomus ocultum*	*Trifolium repens*	+ Shoot and root dry weight + P, K, Ca, Mg and B + Relative water content − Proline root	Ortiz et al. (2015)
Drought (irrigation to 50% of WHC)	*Rhizophagus intraradices* strain EEZ 195	*Trifolium repens*	+ Shoot and root dry weight + P, K, Ca, Mg, Zn and B + Relative water content + Activity APX root + Glutathione reductase − Leaf electrolyte leakage	Ortiz et al. (2015)
Drought (irrigation to 60% of FC)	*Rhizophagus irregularis* strain EEZ58	*Digitaria eriantha*	+Shoot Dry matter + Stomatal conductance + Activity CAT − APX + Lipid peroxidation and	Pedranzani et al. (2016)

			H_2O_2 in shoot − H_2O_2 in root	
Drought (irrigation to 55 and 75% of FC	*Rhizophagus irregularis* strain EEZ58	*Lactuca sativa*	+ Dry weight shoot + Stomatal conductance + Photosystem II efficiency + ABA content in root	Ruiz-Lozano et al. (2015)
Drought (irrigation to 55 and 75% of FC)	*Rhizophagus irregularis* strain EEZ58	*Lycopersicum esculentum*	+ Dry weight shoot + Photosystem II efficiency + Strigolactone production	Ruiz-Lozano et al. (2015)
Salinity (watered with 75−150 mM NaCl)	*Claroideoglomus etunicatum* strain EEZ 163	*Oryza sativa*	+ Shoot fresh weight + Shoot dry weight + Net photosynthetic rate + Stomatal conductance + Transpiration rate + PSII photochemistry + Total Rubisco activity − Non-photochemical quenching	Porcel et al. (2015)
Salinity (watered with 66 and 100 mM NaCl)	*Rhizophagus intraradices* strain EEZ 58 de Gata	*Zea mays*	− Stomatal conductance − Shoot dry weight	Estrada et al. (2013a)
Salinity (watered with 66 and 100 mM NaCl)	*Claroideoglomus etunicatum* native isolate from Cabo	*Zea mays*	+ Dry weight shoot + Stomatal conductance + Photosynthetic efficiency − Lipid peroxidation shoot and root − H_2O_2 shoot and root − Leaf electrolyte leakage + Activity CAT	Estrada et al. (2013b)
Salinity (watered with 66 and 100 mM NaCl)	*Septoglomus constrictum* native strain from Cabo de Gata	*Zea mays*	+ Dry weight shoot + Stomatal conductance + Photosynthetic efficiency − Lipid peroxidation shoot and root − H_2O_2 shoot and root − Leaf electrolyte leakage	Estrada et al. (2013b)
Salinity (watered with 66 and 100 mM NaCl)	*Rhizophagus intraradices* strain EEZ 58	*Zea mays*	+ Mycorrhization − Dry weight shoot − Stomatal conductance − Leaf electrolyte leakage − H_2O_2 shoot and root + Photosynthetic efficiency + Lipid peroxidation shoot and root	Estrada et al. (2013b)
Salinity (watered with 66 and 100 mM NaCl)	*Rhizophagus intraradices* strain EEZ 58	*Zea mays*	+ Mycorrhization − Dry weight shoot − Stomatal conductance − Leaf electrolyte leakage − H_2O_2 shoot and root + Photosynthetic efficiency + Lipid peroxidation shoot and root	Estrada et al. (2013b)
Salinity (watered with 200 mM NaCl)	*Rhizophagus irregularis* strain EEZ58	*Digitaria eriantha*	+ Stomatal conductance + Activity SOD, CAT and APX in shoot + Activity APX in root + Jasmonic acid + Lipid peroxidation in shoot − H_2O_2 in shoot − H_2O_2 in root	Pedranzani et al. (2016)
Salinity (watered with 0, 100, and 150 mM NaCl)	*Septoglomus viscosum*	*Medicago sativa* var. Icon	+ Dry weight + Stomatal conductance + Chlorophyll a, b and total	Campanelli et al. (2012)

			+ Relative water content + Proline content + K concentration in root − Na and Cl concentration in root and shoot	
Salinity (watered with 0, 100, 150, and 200 mM NaCl)	*Rhizoglomus intraradices* strain CMCC Wep 319	*Trigonella foenum-graecum*	+ Glycine Betaine + Total sugar + α - Tocopherols − Proline content	Evelin et al. (2013)
Salinity (watered with 150 mM NaCl)	*Claroideoglomus etunicatum*	*Zea mays*	+ Dry weight total + Stomatal conductance + Photosystem II efficiency + P and K shoot and root − Na root	Porcel et al. (2016)
Salinity (4.7 and 9.4 dS m^{-1} in soil)	Consortia *Glomus sp.*	*Triticum aestivum* cv. Sids 1 and Giza 168	+ Number of grains plant + Grain yield plant + N, P and K content in shoot and grain − Na content in shoot and grain	Talaat and Shawky (2013)
Salinity (0.9, 1.9 and 3.6 dS m^{-1} in soil)	*Funneliformis mosseae*	*Zea mays*	+ Dry weight shoot + Soluble sugars + Reducing sugars + Total organic acid + Oxalic acid, Acetic acid and Fumaric acid − Proline content	Sheng et al. (2011)

[a]WHC (water holding capacity) refers to a soil's ability to hold water

[b]FC (field capacity) is the amount of water held in soil after all freely-draining water no longer is retained

[c]ETp (potential evapotranspiration) is the maximum amount of water that could be lost from soil through evaporation from the soil surface together with plant transpiration

2.5.3 Compatible solutes

Most plant species accumulate certain organic solutes compatible with response to osmotic stress, such as amino acids (proline), quaternary and other amines (glycine betaine and polyamines), or a variety of sugars and organic acids (oxalate, malate, etc.) (Valliyodan and Nguyen 2006). They are known as compatible solutes or osmoprotectants, because they do not produce any damaging effects on membranes, enzymes, or macromolecules, even at very high concentrations (Kiani et al. 2007; Farooq et al. 2008). Compatible solutes reduce water potential within the cell, preventing water loss and improving osmotic adjustment (Delauney and Verma 1993), maintaining physiological activity during periods of stress and enabling water flow from the soil towards plants (Kramer and Boyer 1997). Proline is one of the most studied free amino

acids that acts as an osmoprotectant, because it is highly accumulated in plants when subjected to abiotic stress, especially osmotic stress. It is synthesized in the cytoplasm and chloroplasts and accumulated in the vacuoles. Under normal growing conditions, it is synthesized from ornithine and, under stress, from glutamate (Perez-Perez et al. 2009; Lehmann et al. 2010; Kishor and Sreenivasulu 2014). The effects of AM on proline accumulation are contradictory. Several authors have found that proline production is lower in mycorrhizal plants versus non-colonized plants under stress conditions (Rabie and Almadini 2005; Yooyongwech et al. 2013). Evelin et al. (2013) found increased production of proline by non-AM *Trigonella foenum-graecum* under saline conditions, suggesting that proline is a stress indicator; therefore, a diminished concentration of proline in AM plants indicates that mycorrhizas decrease stress. Estrada et al. (2013a) similarly found increased production of proline by non-colonized maize plants versus mycorrhizal plants. These results suggest that proline accumulation is a consequence of salinity, not of mycorrhizal colonization, and depend on the plant species and symbiotic efficiencies shown by specific plant-fungus combinations (Ruiz-Lozano et al. 1995; Ruiz-Lozano et al. 2012). In contrast, several authors have found that AM stimulate plant production of proline (Sharifi et al. 2007; Garg and Manchanda 2009; Mo et al. 2016; Chitarra et al. 2016). For example, proline production was higher in maize colonized by *Funneliformis mosseae* under saline conditions of 0.5 and 1 g NaCl kg^{-1} soil than in non-colonized plants (Sheng et al. 2011). Ruiz-Lozano et al. (2011) found higher proline accumulation in mycorrhizal lettuce than in non-colonized plants under drought stress. This suggests that mycorrhizal plants had the highest osmotic adjustment capacity, because plants with AM accumulated the most content of proline in root tissues, allowing them to cope with the low water potential of soil and to maintain a water potential gradient favourable for water entrance into roots. Accordingly, an increase in the proline content

of mycorrhized plants under low water potentials improve the absorption of water by plant roots and enhances osmotic balance and root hydraulic conductivity (Evelin et al. 2009).

Sugars play an important role under stress conditions because like proline, they also act as osmoprotectors, contributing up to 50% of a plant's osmotic potential, strongly helping with osmotic adjustment and also serving as a C source (Abdel-Latef and Chaoxing 2014; Sheng et al. 2011). Mycorrhizal colonization increases the accumulation of total soluble sugars as a defense mechanism in plants under osmotic stress (Porcel and Ruiz-Lozano 2004; Talaat and Shawky 2011; Mo et al. 2016). Sheng et al. (2011) found that concentrations of soluble and reduced sugars were significantly higher in mycorrhizal maize than non-colonized plants. Similar results were found for AM *Trifolium alexandrinum* (Khaled et al. 2003). In lettuce watered at 2/3 field capacity, AM increased the concentration of soluble sugars in cv. Rubia Munguia in contrast to cv. Summer Wonder, for which the concentration was reduced (Baslam and Goicoechea 2012). This is important because it demonstrates that the interaction between a specific AMF isolate and a host can differ even among plant genotypes. The accumulation of sugars in mycorrhizal plants is caused by increased photosynthetic capacity under osmotic stress (Sheng et al. 2008; Wu et al. 2010). Al-Garni (2006) found that there is a positive relationship between root colonization and the accumulation of sugars in plants. AM induce a positive stress response, avoiding membrane protein structural damage and maintaining the osmotic balance in cells as a result of accumulation of soluble sugars (Abd-El Baki et al. 2000). In this sense, Yooyongwech et al. (2016) found increased soluble sugars in mycorrhizal sweet potato, with a greater accumulation in roots than shoots. Those authors showed an inverse correlation between osmotic potential and soluble sugar accumulation in plants.

Additionally, under unfavourable conditions, organic acids play important roles as active metabolites involved in osmotic adjustment in plant vacuoles, as cation counter-ions that avoid toxic accumulation of Cl^- in cells, and as cytosolic pH regulators (Yang et al. 2007; Hasegawa et al. 2000). Under saline conditions, the concentration of total organic acids in mycorrhizal maize was 31, 24, and 8% higher than in non-colonized plants at 0, 0.5 and 1 g NaCl kg^{-1} soil, respectively (Sheng et al. 2011). The plants differed strongly in oxalic and succinic acid contents, especially under saline conditions where the mycorrhizal plants showed increased accumulation of oxalic acid while the non-colonized plants accumulated succinic acid. At all salinity levels, concentrations of oxalic, fumaric, acetic, malic, and citric acids were highest in mycorrhizal plants (Sheng et al. 2011). Rozpadek et al. (2016) found a significant decrease in the concentration of tartaric acid and an increase in the concentrations of malic, propionic, valeric, and citric acids in onion seedlings inoculated with *Rhizophagus irregularis*. Regardless of sometimes contradictory results, the relationship between AM and production (and root exudation) of organic acids requires further investigation because organic acids not only are important osmoprotectors but also confer tolerance to additional environmental stresses as well as solubilizing mineral nutrients in soil, principally P.

2.5.4 Enzymatic and non-enzymatic antioxidants

One of the first responses of plants confronting stressful conditions is overproduction of reactive oxygen species (ROS). The production of ROS, such as superoxide radicals (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), alkoxy group (RO^-), and hydroxyl radicals (°OH), mainly occurs in chloroplasts in PSII and PSI complexes, as well as in mitochondria. Overproduction of ROS induces an imbalance, causing oxidative stress, impairing the metabolism of plants through damage to membrane lipids, proteins, and

nucleic acids (Apel and Hirt 2004; Reddy et al. 2004; Ozkur et al. 2009; Gill and Tuteja 2010). Several studies have shown that AM protect plants by reducing ROS production, maintaining the integrity of membranes and stabilizing proteins and enzymes (Wu et al. 2010, 2014; Abeer et al. 2015).

Plant cells have physiological mechanisms that cope with oxidative damage by inducing the production of antioxidant enzymes (Ozkur et al. 2009; Caverzan et al. 2012). AM improve the production of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPOD) under conditions of osmotic stress (Huang et al. 2010; Kumar et al. 2015), decreasing production of H2O2 and membrane lipid peroxidation (Zhi et al. 2010; Amiri et al. 2015). Estrada et al. (2013b) found improved cell membrane stability, reduced lipid peroxidation in roots, and diminished accumulation of H_2O_2 in maize plants inoculated with native AMF strains obtained from Cabo de Gata (southeast Spain) and grown under saline conditions (66 and 100 mM NaCl). These results were related to a better PSII efficiency, lower production of ROS, and greater activity of SOD and CAT in AM plants than in non-colonized plants.

ROS removal from plant cells, in addition to being influenced by enzyme systems, in many cases, occurs in coordination with non-enzymatic antioxidant systems. Non-enzymatic antioxidants act as potent free radical scavengers and reducing agents to protect plants from oxidative damage (Rice-Evans et al. 1996; Wu et al. 2014). Non-enzymatic compounds include ascorbic acid, glutathione (Foyer et al. 1994), and phenolic compounds, which include simple phenols (phenolic acids and derivatives) and polyphenols (flavonoids and polymer compounds) (Sreenivasulu et al. 2000; eff et al. 2007). Phenolic compounds are secondary metabolites that help detoxify ROS, neutralizing the radicals before cell damage takes place (Løvdal et al. 2010). Under stress,

AM improve plant responses to oxidative damage through increased production of phenolic compounds, as reported for *Zinnia elegans* (Heidari and Nazari Deljou 2014), *Rehmannia glutinosa* (Chung et al. 2006), Viola tricolor (Zubek et al. 2015), *Cynara scolymus* (Ceccarelli et al. 2010), and *Vitis vinifera* (Eftekhari et al. 2012).

Chen et al. (2013) found increased production of phenols and flavonoids by *Cucumis sativus* cv. Jincun inoculated with *Funneliformis mosseae* which showed a significant increase in the concentrations of cinnamic acid (76%), p-coumaric acid (78%), caffeic acid (69%), and ferulic acid (72%) in leaves versus those of non-colonized plants. Catford et al. (2006) found increased flavonoids in mycorrhizal alfalfa. Similarly, AM induce a significant increase in enzyme activity associated with the production of secondary metabolites (Ceccarelli et al. 2010). AM enhanced anthocyanins and carotenoids in two cultivars of lettuce, Maravilla Blonde and Summer Munguia, when the plants were subjected to water restriction (Baslam and Goicoechea 2012). The accumulation of antioxidants in lettuce leaves (mainly carotenoids, anthocyanins, and other phenolic compounds) has a potential to affect human health by increasing the nutritional status of leaves (Baslam and Goicoechea 2012). Such an effect of AM also might improve nutraceutical crop quality and could be of agronomic importance as an aspect of management.

2.5.5 Changes in the expression of functional genes

Aquaporin genes and root hydraulic conductivity

AM may induce changes in gene expression under osmotic stress. As already mentioned, AM can alter the hydraulic properties of roots, increasing water flow. Greater hydraulic conductivity in roots of mycorrhizal plants has been found (Robert et al. 2008; Smith et al. 2010). For example, hydraulic conductivity 192% higher in mycorrhizal *Trifolium*

repens than in non-colonized plants has been found (Ortiz et al. 2015). This is regulated by expression of genes encoding AQPs which selectively allow passage of water and other compounds (Hill et al. 2004). Bárzana et al. (2014) proposed that besides the direct activity of AM fungal hyphae taking up water and nutrients for the host plant, AM act on host AQPs such that the plant's water relations and physiology cope well with stressful environmental conditions.

Under saline conditions, an overexpression of genes encoding AQPs in mycorrhizal *Solanum lycopersicum* (Ouziad et al. 2006), *Phaseolus vulgaris* (Aroca et al. 2007), and lettuce (Jahromi et al. 2008) has been found. Under stress, increased expression of genes encoding AQPs (*ZmPIP1;2*, *ZmPIP2;1*, *ZmPIP2;2*, *ZmPIP1;6*, *ZmPIP2;5*, and *ZmPIP2;6* in leaf tissue, and *ZmPIP2;3* and *ZmPIP2;4* in root tissue) was found in maize inoculated with a native AMF consortium (Armada et al. 2015). This was associated with increased root hydraulic conductivity and lower levels of plant stress. In addition, the entire aquaporin gene family was studied in maize (33 genes) and AM were found to regulate expression of many AQP genes comprising members of the different aquaporin subfamilies (Bárzana et al. 2014).

Under conditions of water stress, *Robinia pseudoacacia* plants inoculated with *Rhizophagus irregularis* had greater expression of AQP genes *RpTIP2;1* and *RpPIP2;1* in roots, stem, and leaves, improving water flow to the plant tissues (He et al. 2015). Similarly, Bárzana et al. (2015) found the highest leaf water potential (-0.75 MPa) in maize plants inoculated with *Rhizophagus intraradices*, while the lowest potential (-1.06 MPa) was in non-colonized plants. Those authors suggest that AMF could regulate PIP (plasma membrane intrinsic protein) aquaporin genes, maintaining water movement in mycorrhizal roots and favouring improved water status of mycorrhizal plants. PIPs can play a principal role in the control of stomatal movements and in mesophyll conductance

because they transport water and CO2, leading to subsequent effects on photosynthesis (Lopez et al. 2013). Calvo-Polanco et al. (2016b) found that root hydraulic conductivity of olive trees was correlated positively with AQP genes *OePIP1;2* and *OeTIP1;2* and negatively correlated with *OePIP1;3*, *OePIP2;4*, and *OeTIP1;3* under drought. At the same time, hyphal length and root colonization were closely related to root hydraulic conductivity (Calvo-Polanco et al. 2016b). Thus, greater aquaporin PIP gene expression in mycorrhizal versus non-colonized plants could increase water-use efficiency, reduce ROS accumulation, and diminish oxidative damage (Liu et al. 2016). On the contrary, Quiroga et al., (2017) found the PIPs and TIPs genes were down-regulated by the AM symbiosis in the drought-sensitive maize. In the same way, AM symbiosis down-regulated *LsPIP1* and *LsPIP2* in lettuce plants subjected to water stress. This down-regulation of PIP genes is likely to be a mechanism to decrease membrane water permeability and to allow cellular water conservantion (Porcel et al., 2006).

Ionic-balance genes

Under salt stress conditions, Estrada et al. (2013a) found a higher concentration of K^+ and a reduction in the accumulation of Na^+ in mycorrhizal maize compared to non-colonized plants, with an increase in the K^+/Na^+ ratio. This was associated with the regulation of the K^+ transporter genes *ZmAKT2*, *ZmSKOR3*, and *ZmSOS1*. AM help in the detoxification of Na^+ ions influencing the expression of specific Na^+ related genes in plants. For example, AM can affect salt overly sensitive (SOS) genes that regulate the excretion of excess Na^+ ions from cells by a Na^+/H^+ plasma membrane antiporter, thereby improving ion homeostasis (Zhu et al. 1998; Ruiz-Lozano et al. 2012). Porcel et al. (2016) showed that AM of *Oryza sativa* regulate the expression of several genes which encode transporters involved in ion homeostasis such as *OsSOS1*, *OsNHX3*, *OsHKT2;1*, and

OsHKT1;5 that are highly expressed in leaf tissue. This increased gene expression in leaves results because AM favour Na^+ extrusion from the cytoplasm, its sequestration into the vacuole, as well as the unloading of Na^+ from the xylem and its recirculation from photosynthetic organs to roots. As a result, there is a decrease of Na^+ root-to-shoot distribution and an increase of Na^+ accumulation in roots, which seems to enhance plant tolerance to salinity and allows mycorrhizal *Oryza sativa* to maintain growth under salt stress.

2.6 Concluding remarks

As is widely recognized, environmental conditions that generate osmotic stress strongly affect agricultural production as well as the stability of natural ecosystems. Nevertheless, osmotic stress also imposes selection pressure on native AMF to cope with the limiting conditions of the soil in which they occur (Sylvia and William 1992). Attesting to such selection, the effect of AMF on plant responses to osmotic stress depends in large part on the origin of the fungi. Those isolates obtained from osmotic stress conditions often have the greatest ability to promote plant growth under water and salt stress (Copeman et al. 1996; Daei et al. 2009; Wu et al. 2010; Estrada et al. 2013a, 2013b). Because a particular species of AMF may be able to associate with many plant species, and a single plant can be colonized by several different species of AMF (Smith and Read 2008); however, plant growth, physiology, and biochemistry may respond differently to AM under osmotic stress. As a result, reported data may be contradictory with respect to the effects of AM, which suggests different degrees of functional compatibility between specific AMF strains and plant species. Indeed, such differences in functional compatibility even may be found among plant genotypes (Baslam and Goicoechea 2012; Aguilera et al. 2015). In consequence of the differences among AMF, at least two areas of research must be

addressed in order to maximize the benefits to be obtained from AM. First is to deeply understand the plant biochemical and physiological mechanisms that are modified by AM. Second is to focus on technological development of AMF-based bioproducts that facilitate establishment of highly effective combinations of plants and fungi. In the first case, further studies are needed to elucidate the specific functions of genes regulated by AM, especially at the transcriptomic level which at present has barely been investigated in relation to osmotic stress. That could reveal the exact mechanisms by which AM alter plant adaptation under drought. In the second case, the corroboration of functional compatibility between AMF and plants is a key component in the process of formulating bioproducts that enhance the performance of plants under the stresses we have reviewed.

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CHAPTER III

"Presence and significance of arbuscular mycorrhizal fungi in the elevations belts of the hyper-arid Atacama Desert"

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Presence and significance of arbuscular mycorrhizal fungi in the elevations belts of the hyper-arid Atacama Desert

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Abstract

When plants from desert ecosystems form the AM symbiosis increase its survival due to the enhancement of nutrient and water acquisitions. Here, we characterize the AM presence and densities of fungal structures associated to native plants species growing in different elevation belts in the Atacama Desert (Northern Chile). Rhizosphere soil and roots were sampled in plants from: i) the hyper-arid desert elevation belt, Pampa del Tamarugal; ii) the Camiña and Huatacondo Valleys, pre-Puna elevation belt , and iii) five high-Andean basins, Puna elevation belt. pH, electrical conductivity (EC), soil organic matter and soluble cations were analyzed in rizosphere. The AM colonization, spore density, hyphae length and Total Glomalin-Related Soil Protein (T-GRSP) were also determined. All plants showed AM colonization and fungal propagules in rhizosphere. The rates of AM root colonization ranged from 3.5 to 87%, meanwhile hyphae showed densities from 0.13 up to 204 m g^{-1}, and spore densities between 20 and 45,500 per 100 g of soil. T-GRSP contents fluctuating between 0.13 and 7.2 mg g^{-1}. The highest spore density and length of mycelium in rhizosphere soils were found in *Prosopis tamarugo*, *Baccharis scandens*, *Werneria pinnatifida*, *Deyeuxia curvula* and *Festuca deserticola*. Interestingly, the EC and soluble cations were positively related with both spore density and hyphae. A wide distribution of the AM symbiosis through all the elevation belts of the Atacama Desert is reported, suggesting a high AM dependence to cope with the extreme conditions of aridity and salinity as the main constraints to plant establishment and growth. As main finding, AM structures seem to be playing a role to face with the salt stress, represented by the accumulation of toxic ions in spores, which could affects the ionic homeostasis in the fungus-plant continuum.

k**eywords:** arbuscular mycorrhizal fungi, hyper-arid ecosystems, salinity, zonal and azonal vegetation.

3.1 Introduction

Desert ecosystems cover approximately 40% of land surfaces, being characterized by several extreme environmental conditions such as intense solar radiation, high temperatures, and a large diurnal temperature interval (Peel, Finlayson, & McMahon, 2007). A desert is defined as a region that receives extremely low rainfall, far less than the amount required to support the growth of most plants, having an average annual rainfall of less than 400 mm $year^{-1}$ (Azua-Bustos, Urrejola, & Vicuña, 2012). Desert ecosystems exist where potential evapotranspiration exceeds precipitation, with drought and salinity as the most important limiting factors (Jafari, Tavili, Panahi, Zandi Esfahan, & Ghorbani, 2018). In arid regions, annual rainfall is less than 250 mm per year, but a single rainfall event may be of high intensity. This presence of long dry periods and short high-intensity rainfall has a marked effect on soil, landscape, and vegetation in desert ecosystems (Miranda et al. 2011). Moreover, high evapotranspiration levels and low rainfalls produce massive salt accumulation in upper soil layers (Power & Prasad, 2010).

Ecosystem functioning depends mainly on the activities performed by soil microorganisms, because they carry out most of the biogeochemical cycles, influencing productivity, diversity, and plant community structure (Jacoby, Peukert, Succurro, Koprivova, & Kopriva, 2017). Among the most influential soil microorganisms, we highlight arbuscular mycorrhizal (AM) fungi, which establish symbiosis with plant roots (Smith & Read 2008). AM symbiosis is formed by more than 80% of vascular plants (Jeffries, Gianinazzi, Perotto, Turnau, & Barea, 2003). Moreover, it is well known that AM fungi protect plants against the vast majority of abiotic stresses, especially increasing water and mineral nutrient absorption under drought and salinity conditions (Evelin, Giri, & Kapoor, 2012), which facilitates plant survival (Liu, Zheng, Bai, Bai, & Wang, 2017; Martínez-garcía & Pugnaire, 2011).

Plants growing in desert ecosystems must face extensive drought periods between precipitation events, which may last months or years (Zhang, Shan, & Li, 2018). Therefore, plants must cope with several stresses, such as the lack of water, nutrient deficiencies, presence of high levels of toxic ions such as Na^+ and Cl^- in soil, intense solar radiation, high temperatures, among others (Jafari et al., 2018). Moreover, AM fungi improve soil structure through the formation of stable aggregates, because mycelia form networks in the soil, preventing surface erosion and improving nutritional and water status (Mardhiah, Caruso, Gurnell, & Rillig, 2016).

Several studies have shown the presence of AM symbiosis in native plants in different arid and semiarid ecosystems (Azcón-Aguilar et al., 2003; Cavagnaro, Ripoll, Godeas, Oesterheld, & Grimoldi, 2017; Liu et al., 2017), but little is known about the most severe desert ecosystem worldwide. In this sense, it is well known that the Atacama Desert, located between 17° and 27° S latitude in northern Chile (Azua-Bustos et al., 2012). The Atacama Desert is the oldest and driest desert on Earth, having evolved over 100 My of aridity and 10-15 My of hyper-aridity. The low availability of liquid water and high solar irradiance are generally acknowledged to be the major environmental factors controlling microbial colonization. Coupled with very low concentrations of organic carbon, very sparse or zero microbial population densities and a high oxidizing capacity, these properties have promoted the Atacama Desert as an accurate analogue of Martian soils (Bull & Asenjo, 2013). Despite the extreme conditions and the existence of previous ecological studies covering plants and other microorganisms, as far as we know, there are no other related studies about AM symbiosis in such an interesting ecosystem. Therefore, the aim of this study was to report for the first time the presence of AM symbiosis in representative plant species growing in different elevation belts of the Atacama Desert, as a way to highlight the importance of this association in the establishment and

functioning of plant ecosystems in one of the most extreme environments in the world. This study also represents an excellent opportunity to find adapted AM fungal ecotypes able to be used in further application as bioinoculants oriented to improve the agricultural plant production in vast arid or saline areas worldwide.

3.2 Material and methods

3.2.1 Sampling sites

Plant and soil sampling was carried out in several areas throughout the majority of the geographical distribution of the Tarapacá Region (northern Chile), considering three elevation belts (Figure 1; Table 1): i) the desert elevation belt of "Pampa del Tamarugal", which is a plain between the "Cordillera de la Costa" (coastal mountain range) and the Andes piedmont (between 700-2,000 masl), characterized by the almost total absence of precipitation and extremely low relative humidity (commonly considered the driest area worldwide). This sector has little plant cover and shows extensive areas without the presence of vascular plants. In this elevation belt, the *Prosopis tamarugo* is the most representative endemic plant species in this ecosystem (Luebert & Pliscoff, 2008); ii) The pre-Puna elevation belt, which is located at the eastern edge of Pampa del Tamarugal, beginning at the piedmont and extending up to 3,100 masl The transverse valleys of "Camiña" and "Huatacondo" are located in this elevation belt; both valleys were sampled. In the pre-Puna zone, some characteristic native plants are *Lycopersicon chilense* and *Baccharis scandens* (Trivelli & Valdivia, 2009; Villagrán, Kalin Arroyo, & Marticorena, 1983); iii) The Puna elevation belt is approximately located from 3,000 to 4,500 masl, where it is possible to differentiate two kinds of vegetation formations: iiia) Azonal vegetation associated with water courses, also called "bofedales", and iiib) zonal

vegetation that is dependent on rain, also called "tolares" or communities of tropical Andean shrubs (Trivelli & Valdivia, 2009). In detail, saline bofedales are formations of high Andean tundra, which represent an important forage resource for local fauna. Bofedal soils have high levels of organic matter, and salt outcrops cover more than 5% of the soil (Faundez & Ahúmada, 2009). In the bofedales, the characteristic native plant species are *Deyeuxia curvula*, *Festuca deserticola*, *Werneria pinnatifida*, *W. poposa* and other taxa. Furthermore, tolares are characterized by the dominant presence of *Adesmia sp.*, *Baccharis sp.*, *Fabiana sp.*, and *Parastrephia sp.*, also included in our study (Trivelli & Valdivia, 2009; Villagrán et al., 1983). Five basins were sampled in the Puna elevation belt: high Andean river of Lirima, Salar de Lagunillas, Salar de Huasco, Salar de Coposa and Salar de Michincha.

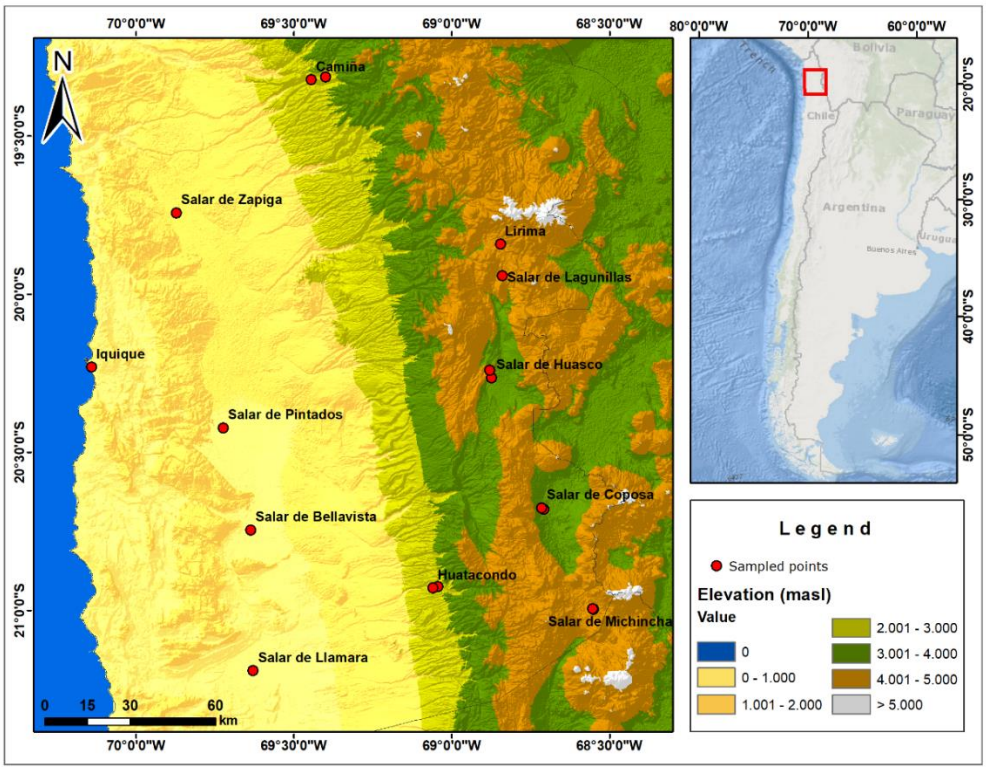

Figure 1. Study area showing sampled points in different elevation belts from Atacama Desert, Northern Chile, Tarapacá region. Circles show the 15 sampled point. Source: Base map from Natural Earth.

Table 1: Geographical characteristics of study sectors in different elevation belts from Atacama Desert of Northern Chile, Tarapacá region.

Elevation belt	Sector	Climate	Annual precipitation (mm year $^{-1}$)	Elevation (masl)	Geographical coordinate
Pampa del Tamarugal	Salar de Zapiga	Hyper-arid	<10	1,144	19°44'32.91"S; 69°52'23.76"W
	Salar de Pintados	Hyper-arid	<10	1,001	20°25'20.19"S; 69°43'30.07"W
	Salar de Bellavista	Hyper-arid	<10	964	20°44'42.30"S; 69°38'16.10"W
	Salar de Llamara	Hyper-arid	<10	763	21°11'22.42"S; 69°37'47.54"W
pre-Puna	Camiña	Marginal desert of height	<100	2,281	19°19'17.56"S, 69°26'46.38"W
				2,571	19°18'47.93"S; 69°24'1.82"W
	Huatacondo	Marginal desert of height	<100	2,256	20°55'40.47"S; 69° 3'37.26"W
			<100	2,348	20°55'28.86"S; 69° 2'43.23"W
Puna	Lirima	Cold desert of height	<250	4,102	19°50'30.57"S; 68°50'48.71"W
	Salar de Lagunillas	Cold desert of height	<250	4,025	19°56'31.89"S; 68°50'28.18"W
	Salar de Huasco	Cold desert of height	<250	3,797	20°15'49.30"S; 68°52'28.60"W
				4,083	20°14'54.92"S; 68°52'47.82"W
	Salar de Coposa	Cold desert of height	<250	3,757	20°40'45.53"S; 68°42'31.99"W
				3,802	20°40'29.94"S; 68°42'59.25"W
	Salar de Michincha	Cold desert of height	<250	4,131	20°59'43.82"S; 68°33'9.38"W
				4,132	20°59'40.73"S; 68°33'18.39"W

Source: Trivelli & Valdivia (2009).

3.2.2 Plant and soil sampling

Sampling was carried out in a total of five botanical families: Asteraceae (*Baccharis tola* Phil., *B. scandens* Ruiz & Pav., *Parastrephia quadrangularis* Meyen., *Parastrephia lucida* Meyen., *Senecio nutans* Sch.Bip., *Werneria poposa* Phil., and *Werneria pinnatifida* Remy.), Fabaceae (*Adesmia melanthes* Phil. and *Prosopis tamarugo* Phil.), Gramineae (*Deyeuxia curvula* Wedd., and *Festuca deserticola* Phil.), Solanaceae (*Fabiana squamata* Phil. and *Lycopersicon chilense* Dunal.), and Verbenaceae (*Lampaya medicinalis* Phil.). Three samples for each plant species in each sampling site were

collected, and the number of collected samples was based on the number of dominant plants for each study sector. One hundred-eight plants with respective rhizosphere soil were sampled at 20 cm depth, using a spade to collect approximately 1 kg of soil. Root samples were placed in a plastic bag and refrigerated at 4°C until analysis. Soil samples were air dried at 25°C to determine AM fungal structures, total glomalin-related soil protein (T-GRSP), and soil chemical characteristics.

3.2.3 Chemical characteristics of the rhizosphere soils

Soil samples were sieved at 2 mm before laboratory analysis. The pH was determined with potentiometry using a soil:water 1:2.5 (w/v) mixture; electrical conductivity (EC) was determined in a soil:water (1:5 w/v) extract (Sadzawka et al., 2006). Sodium, potassium, magnesium, and calcium content were determined by means of extraction with ammonium acetate ($AcNH_4$) 1 M at pH 7.0 in an atomic absorption spectrophotometer (Unicam SOLAAR, mod. 969, England). The soil organic matter (SOM) content was determined according to the Walkley-Black method (Walkley & Black, 1934). The available P was determined by spectrophotometry according to (Olsen SR, Cole CV, Watanebe FS, 1954) at an absorbance of 880 nm.

3.2.4 Determination of AM fungal structures and T-GRSP

Arbuscular mycorrhizal root colonization was quantified using a dissection microscope (20-40X) after cleaning a portion of roots in 10% KOH (w/v) and staining with 0.05% trypan blue in lactic acid (w/v) (Phillips & Hayman, 1970). The gridline intersection method (Giovannetti & Mosse 1980) was used to determine the proportion of AM colonized root. The AM fungal spores were separated from soil by wet sieving and

decanting in a 70% (w/v) sucrose solution (Gerdemann & Nicolson, 1963) and quantified using a dissecting microscope. The total extraradical AM hyphae were determined by the method described in (Borie, Rubio, Morales, & Castillo, 2000). To quantify the total hyphal density, we used the Newman's line intersect method (Newman, 1966). T-GRSP was extracted from rhizosphere soil and determined according to (Wright & Upadhyaya, 1998).

3.2.5 Statistical analyses

For all the studied variables, ANOVA was performed in each elevation belt with descriptive purposes. The data that did not meet normality and homoscedasticity assumptions were transformed, but the results are presented in their original measurement scale. For the variables with significant differences, means were compared by the Tukey multiple range test. All data sets were subjected to factorial analysis with Principal Component (PC) extraction for each elevation belt, together with a grouping procedure based on hierarchical cluster analysis by the Ward method. The software SPSS 22.0 (IBM™) was used for all the analyses. The level of statistical significance was established at $P < 0.05$.

3.3 Results

3.3.1 Chemical characteristics of rhizosphere soils

The chemical characteristics of rhizospheric soils of *P. tamarugo* were different between each sector sampled in the Pampa del Tamarugal (Table 1). The highest EC values were found in Salar de Zapiga (11.4 mS cm^{-1}) and Salar de Bellavista (8.4 mS cm^{-1}). On the contrary, the lowest EC values were found in Salar de Pintados (2.8 mS cm^{-1}). Moreover,

Salar de Zapiga and Bellavista showed a higher SOM content, 6.4 and 9.5% respectively. All soils were characterized as alkaline soil, showing pH values above 8.0, with Salar de Zapiga soils showing the highest value (8.7). Phosphorous concentrations were similar between Salar de Zapiga, Bellavista and Pintados (30, 34 and 34 mg kg^{-1}, respectively), but different with respect to Llamara (20 mg mg^{-1}). Regarding exchangeable cations, the highest values of Ca^{2+} and Na^{+} were found in Salar de Zapiga and Bellavista, K^{+} levels were higher in Salar de Zapiga and Pintados and Mg^{+} levels were higher in Salar de Bellavista.

Table 2: Chemical characteristics of rhizosphere soil of *Prosopis tamarugo* plants growing in elevation belt of the Pampa del Tamarugal, Atacama Desert, Tarapacá Region, Northern Chile.

Sector and plant species	E.C. dS cm^{-1}	pH	SOM %	P mg Kg^{-1}	Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}
					cmol kg^{-1}			
P. tamarugo								
Salar de Zapiga	11.4 ± 0,6a	8.7 ± 0.6a	6.4 ± 0.4a	30 ± 3a	10,699 ± 825ab	3,349 ± 40b	94 ± 7b	1,235 ± 98b
Salar de Bellavista	8.9 ± 0.5a	8.0 ± 0.5b	9.5 ± 0.8a	34 ± 3a	16,521 ± 780a	1,500 ± 71c	149 ± 5a	4,528 ± 117a
Salar de Pintados	2.8 ± 0.3b	8.2 ± 0.4ab	0.85 ± 0.3c	34 ± 4a	2,262 ± 250ab	5,317 ± 144a	85 ± 5b	204 ± 18b
Salar de Llamara	4.5 ± 0,2b	8.4 ± 0,2ab	0.7 ± 0.2c	20 ± 6a	5,872 ± 310b	911 ± 89c	91 ± 11b	526 ± 46b
p-value	**<0,0001**	**<0,0001**	**<0,0001**	**n.s.**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**

Data presented are the mean ± S.E. (n=3). Different letter indicates significant differences between treatments ($p < 0.05$) based on Tukey's test.

Rhizosphere soil characteristics in the pre-Puna elevation belt are shown in Table 2. The highest values of EC, Ca^{2+}, K^{+}, Mg^{2+} and Na^{+} were obtained in the rhizosphere soil of *B. scandens* (5.38 mS cm^{-1}; in cmol(+) kg^{-1} 440 Ca^{2+}; 373 K^{+}; 112 Mg^{+} and 617 Na^{+}) in Camiña Valley, in comparison with the same plant species in Huatacondo Valley (1.3 mS cm^{-1}; in cmol(+) kg^{-1} 285.86 Ca^{2+}; 5.4 K^{+}; 15 Mg^{+} and 90 Na^{+}). On the contrary, the lowest values of EC, Ca^{2+} and Na^{+} were found in the rhizosphere soil of *L. chilense* in Camiña Valley. Both sectors of the pre-Puna elevation belt showed a lower percentage of SOM, ranging from 0.24 to 0.7% in Camiña Valley, and 0.74 to 0.9% in Huatacondo

Valley. The pH levels and P concentration were similar in the rhizosphere soil of *B. scandens* and *L. chilense* in both sectors.

Table 3: Chemical characteristics of rhizosphere soils of plant species growing in the pre-Puna elevation belt, Atacama Desert, Tarapacá Region, Northern Chile.

Sector and plant species	E.C. dS cm^{-1}	pH	SOM %	P mg Kg^{-1}	Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}
					cmol kg^{-1}			
Camina								
B. scandens	5.38 ± 0.2a	7.6 ± 0.4a	0.24 ± 0.01b	50 ± 4a	440 ± 33a	373 ± 52a	122 ± 25a	617 ± 83a
L. chilense	0.45 ± 0.01c	7.6 ± 0.1a	0.70 ± 0.1a	33 ± 6a	58 ± 5c	22 ± 2b	18 ± 2b	6,7 ± 0.5b
Huatacondo								
B. scandens	1.3 ± 0.1b	8.3 ± 0.2a	0.90 ± 0.1a	50 ± 4a	285 ± 21b	5.4 ± 0.4b	15 ± 1b	90 ± 5b
L. chilense	1.1 ± 0.02b	8.3 ± 0.1a	0.74 ± 0.02a	33± 5a	190 ± 9b	3.5 ± 0.9b	13 ± 2b	31 ± 3b
p-value	**<0,0001**	**n.s.**	**<0,0001**	**n.s.**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**

Data presented are the mean ± S.E. (n=3). Different letter indicates significant differences between treatments ($p < 0.05$) based on Tukey's test.

Plants present in the Puna elevation belt are differentiated in two type of vegetation: Azonal vegetation or bofedales and zonal vegetation or tolares. Rhizosphere soil characteristics in the Puna elevation belt are shown in Table 3. The rhizosphere soil of azonal plant species living in bofedales associated with saline lakes showed the highest EC values compared to zonal vegetation. In this sense, rhizosphere soils in Salar de Coposa showed values ranging from 10.9 to 9.3 mS m^{-1} (*D. curvula* and *F. orthophylla*, respectively), in Salar de Michincha ranging from 9.3 to 5.4 mS m^{-1} (*D. curvula* and *F. orthophylla*, respectively), in Salar de Huasco ranging from 5.8 to 5.7 mS m^{-1} (*D. curvula* and *W. pinnatifida*, respectively), and in Salar de Lagunillas ranging from 5.1 to 3.7 mS m^{-1} (*P. lucida* and *D. curvula*, respectively). Likewise, the highest values of SOM and soluble cations were found in the above-mentioned plant species from the same sectors. In relation to pH levels, Salar de Huasco soils showed the highest levels associated with *W. pinnatifida* (9.9), *D. curvula* (9.2), *F. deserticola* (9.10) and *W. poposa* (9.3), and the

lowest pH values were found in *P. lucida* (7.2). The levels of available P were different in all soil samples, with concentrations that varied from 58 mg kg^{-1} in the rhizosphere soil of *D. curvula* (Salar del Coposa) to 16 mg kg^{-1} for rhizosphere soil of *F. deserticola* (Salar de Huasco).

The rhizosphere soils from the Lirima river sector were associated mainly with riparian flora, equally classified as azonal vegetation. EC values of rhizospheric soil were much lower in this sector, ranging from 0.9 to 1.7 mS cm^{-1}. Similarly, pH levels in the rhizosphere soil of *F. deserticola* (6.4), *P. lucida* (6.8) and *D. deserticola* (7.2) showed lower values compared to all the other sectors. The results showed homogenous values between soluble cation levels, with the exception of rhizosphere soil of *D. curvula*, which showed a high Ca^{2+} level related to high EC and pH levels. Similar results were found for soil available P concentrations.

On the other hand, the rhizosphere soils of zonal vegetation in the Puna elevation belt (*A. melanthes*, *B. tola*, *Fa. squamata*, *La. medicinalis*, *P. quadrangularis* and *S. nutans*) showed lower SOM content than rhizosphere soils of azonal vegetation (values ranging from 0.30 to 1.9%), mainly in Salar de Huasco (Table 3). The pH levels were much higher in Salar de Coposa soils (8.1 to 8.4), whereas the lowest pH levels were registered in a soil from Salar de Huasco (5.8, associated with *L. medicinalis*). Likewise, EC values were lower compared to levels determined in azonal vegetation, which was associated with lower soluble cation levels.

Table 4: Chemical characteristics of rhizosphere soils of plant species growing in the Puna elevation belt, Atacama Desert, Tarapacá Region, Northern Chile.

Sector and plant species	E.C. dS cm^{-1}	pH	SOM %	P mg Kg^{-1}	Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}
					cmol kg^{-1}			
Coposa			**Azonal vegetation**					
F. deserticola	9.3 ± 0.5a	8.2 ± 0.1cdef	6.7 ± 1.0a	28 ± 1cdefg	10,229 ± 462b	336 ± 8c	104 ± 4b	788 ± 140d
D. curvula	10.9 ± 0.1a	8.4 ± 0.2cdef	4.2 ± 1.5abc	58 ± 7a	12,583 ± 1263a	2,380 ± 80a	203 ± 45a	692 ± 42d
P. lucida	1.1 ± 0.1ef	8.1 ± 0.1efgh	2.4 ± 0.1bcdef	30 ± 1cdefg	107.99 ± 5f	12 ± 0.3d	18 ± 3de	11 ± 2e
				Zonal vegetation				
L. medicinalis	1.02 ± 0.1ef	8.1 ± 0.1defgh	1.1 ± 0.1defg	25 ± 3cdefg	128 ± 6f	9.1 ± 0.3d	12 ± 0.3e	9.1 ± 0.3e
B. tola	0.8 ± 0.01f	8.2 ± 0.2fgh	1.9 ± 0.1defg	24 ± 1cdefg	199 ± 5f	11.1 ± 0.6d	19 ± 1de	11 ± 0.9e
S. nutans	0.43 ± 0.05f	8.45 ± 0.9 cde	2.29 ± 0.2bcdef	31 ± 4bcdef	167 ± 9f	8.3 ± 0.4d	6.17 ± 0.9e	4.8 ± 0.4e
P. quadrangularis	0.6 ± 0.03f	8.5 ± 0.1cd	1.8 ± 0.2bcdef	15 ± 1gh	162 ± 1f	12.2 ± 0.9d	19 ± 2de	9.3 ± 0.4e
Huasco				**Azonal vegetation**				
D. curvula	5.8 ± 0.2b	9.2 ± 0.4b	4.3 ± 0.5abc	29 ± 5cdefg	7,460 ± 261c	1,729 ± 46b	91 ± 7b	1,517 ± 201ab
F. deserticola	4.9 ± 0.5bc	9.1 ± 0.6b	4.1 ± 0.6abc	16 ± 3efgh	5,992 ± 301d	17 ± 3d	65 ± 3bcd	1,460 ± 20ab
P. lucida	4.3 ± 0.1bc	7.4 ± 0.2jk	4.0 ± 0.2ab	18 ± 5fgh	4,161 ± 194e	7.4 ± 0.2d	32 ± 2de	1,383 ± 166ab
W. pinnatifida	5.7 ± 0.1b	9.9 ± 0,7a	4.7 ± 0.1bcd	31 ± 2bcdef	6,240 ± 155cd	54 ± 2d	29 ± 1de	1,670 ± 143a
W. poposa	2.3 ± 0.2ef	9.3 ± 0.2b	1,2 ± 0.1defg	22 ± 1cdefg	205 ± 3f	57 ± 1d	49 ± 0.6e	35 ± 2e
				Zonal vegetation				
P. quadrangularis	0.54 ± 0.05f	7.2 ± 0.1jk	0.6 ± 0.1fg	24 ± 1cdefg	11 ± 1f	10 ± 1d	6.6 ± 0.2e	4.6 ± 0.1e
L. medicinalis	1.11 ± 0.01ef	5.8 ± 0.2n	0.7 ± 0.1efg	36 ± 2bc	13 ± 1f	11 ± 2d	8.1 ± 0.3e	4.5 ± 0.2e
A. melanthes	0.67 ± 0.09f	7.1 ± 0.1kl	0.6 ± 0.1efg	33 ± 3bcd	16 ± 3f	12 ± 1d	6.1 ± 0.2e	4.7 ± 0.2e
F. squamata	0.49 ± 0.01f	7.0 ± 0.1kl	1.0 ± 0.1defg	32 ± 4bcde	25 ± 2f	11 ± 3d	6.4 ± 0.4e	5.1 ± 0.3e
B. tola	0.76 ± 0.04f	7.8± 0.1hi	0.9 ± 0.2efg	32 ± 5bcde	21 ± 3f	7.4 ± 0.1d	12 ± 1e	15 ± 2e
S. nutans	0.54 ± 0.02f	7.5 ± 0.3ij	0.3 ± 0.1g	17 ± 1efgh	20 ± 2f	8.1 ± 0.5d	6.1 ± 0.2e	5.6 ± 0.5e
Michincha				**Azonal vegetation**				
D. curvula	9.3 ± 0.6a	8.3 ± 0.2cdef	4.2 ± 0.7abc	32 ± 5bcde	1, 333 ± 18f	45 ± 7d	31 ± 3de	1,285 ± 15bc
F. deserticola	5.4 ± 1bc	8.1 ± 0.4efgh	6.7 ± 0.6a	38 ± 3bc	1,243 ± 46f	56 ± 2d	82 ± 3bc	982 ± 24cd
P. lucida	2.3 ± 0.2ef	8.5 ± 0.2c	1.5 ± 0.1cdefg	23 ± 4cdefg	807 ± 26f	10 ± 1d	11 ± 2e	26 ± 1e
W. poposa	3.6 ± 0.3de	8.3 ± 0.3cdefg	2.6 ± 0.1bcdef	28 ± 2bcdef	1,170 ± 19f	24 ± 4d	29 ± 4de	25 ± 3e
				Zonal vegetation				
S. nutans	0.72 ± 0.3f	7.3 ± 0.1jk	0.35 ± 0.1g	5.6 ± 0.2h	41 ± 3f	5.3 ± 0.4d	13 ± 0.3e	6.3 ± 0.5e
Lagunillas				**Azonal vegetation**				
P. lucida	5.1 ± 0,3bc	7,93 ± 0.5fg	0.80 ± 0.1efg	39 ± 3b	1258 ± 33f	32 ± 3d	36 ± 4cde	14 ± 3e
D. curvula	3.7 ± 0.2cd	8,07 ± 0.2efg	0.70 ± 0.1efg	35 ± 4bcd	859 ± 21f	29 ± 4d	8.3 ± 0.5e	12 ± 3e
F. deserticola	0.6 ± 0.1f	9,07 ± 0.4b	0.38 ± 0.1g	21 ± 2defgh	48 ± 8f	25 ± 3d	9.4 ± 0.3e	10 ± 1e
Lirima				**Zonal vegetation**				
D. curvula	3.7 ± 0.8cd	7.2 ± 0,3efgh	1.8 ± 0.1bcdef	37 ± 4bc	86 ± 7f	23 ± 3d	8.0 ± 0,6e	15 ± 5e
F. deserticola	1.0 ± 0.2ef	6.4 ± 0.4m	2.5 ± 0.2bcdef	33 ± 5bcd	44 ± 5f	22 ± 1d	13 ± 1e	4,9 ± 0.3e
P. lucida	0.91 ± 0.1f	6.8 ± 0.6l	3.4 ± 0.3bcde	36 ± 4bc	50 ± 4f	25 ± 4d	16 ± 2e	4.6 ± 0.2e
p-value	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**	**<0,0001**

Data presented are the mean ± S.E. (n=3). Different letter indicates significant differences between treatments ($p < 0.05$) based on Tukey's test.

AM fungal structures and T-GRSP production

Spore density, mycelium length, AM colonization and T-GRSP production varied significantly among plant species and among elevation belts, indicating that AM presence is strongly affected by soil and environmental characteristics.

Rhizospheric soils of *P. tamarugo* from Pampa del Tamarugal (hyperarid conditions) showed marked differences in AM characteristics for each sector (Figure 2). The Salar de Bellavista and the Salar de Zapiga showed greater spore density (2,760 and 642 spores in 20 g of soil, respectively) and hyphal length (204 and 62 m g^{-1}) compared to the other two sectors. Additionally, the Salar de Bellavista had high values of root colonization (28.3%) and T-GRSP production (3.3 mg g^{-1}). However, the lowest spore density was associated with the Salar de Pintados (4 spores in 20 g of soil); these plants also showed high values of root colonization (23.6%), being similar to *P. tamarugo* roots in the Salar of Bellavista. Moreover, the principal component analysis (PCA) showed homogeneous groups of experimental variables (Figure 5A), where PC1 explained 58.3% of the total experimental variance and was positively correlated to spore density, hyphal length, T-GRSP production, root colonization, and EC as well as SOM and Ca^{2+}, Mg^{2+} and Na^{+} content. On the contrary, PC1 was negatively correlated with pH. Cluster analysis allowed the differentiation of three well-defined groups (Figure 5B). Group 1 was represented by *P. tamarugo* plants from Salar de Bellavista growing in soils with high EC, SOM and Na^{+} and Ca^{2+} content, which was related to higher values of AM characteristics. Group 2 mainly included *P. tamarugo* plants growing in Salar de Pintados and was related to lower spore density, hyphal length, and T-GRSP, and also to lower values of EC, SOM and Ca^{2+} content. Group 3 included *P. tamarugo* plants growing in Salar de Zapiga and Salar de Pintados, which showed intermediate values between the above-mentioned groups.

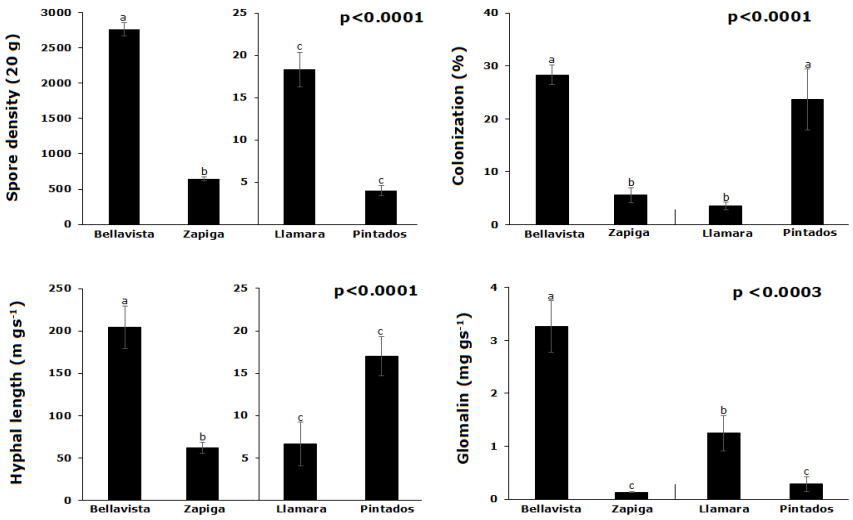

Figure 2: Arbuscular mycorrhizal colonization and fungal propagule densities in roots and rhizosphere soils of *Prosopis tamarugo* plants growing in different sectors of the Pampa del Tamarugal, Atacama Desert, Tarapacá Region, Northern Chile. Data presented are the mean (n=3). Different letters indicate significant difference ($p<0.05$), according to Tukey multiple range test.

The AM presence in plants growing in the pre-Puna elevation belt is shown in Figure 3. The highest spore density and hyphal length were found in *B. scandens*, which had values ranging between 2,782 and 395 spores in 20 g of soil and between 2 and 1.6 m g^{-1} of hyphal length (Camiña and Huatacondo Valleys, respectively). AM root colonization of *L. chilense* was higher in Camiña Valley compared to *L. chilense* in Huatacondo Valley and *B. scandens* from both valleys. The production of T-GRSP was not different between rhizosphere soils for all plant species and sectors. Additionally, the first two PCs accounted for a total of 80.4% of the total variance, also generating highly homogeneous groups of experimental variables (Fig. 5). PC1 (54.4%) was positively associated with spore density and T-GRSP production, and also with EC and soluble

cation content (Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}), and was negatively related to SOM. PC2 (26%) was positively related to hyphal length, pH and P content, although was negatively related to T-GRSP production. The PCA together with cluster analysis allowed the differentiation of two well-defined groups. Noticeably, group 1 only included *B. scandens* plants from Camiña Valley; this plant species showed a higher density of AM spores, concomitantly with high EC and cation levels. Group 2 was composed of *B. scandens* and *L. chilense* plants that grew under low salinity conditions (Figure 5D).

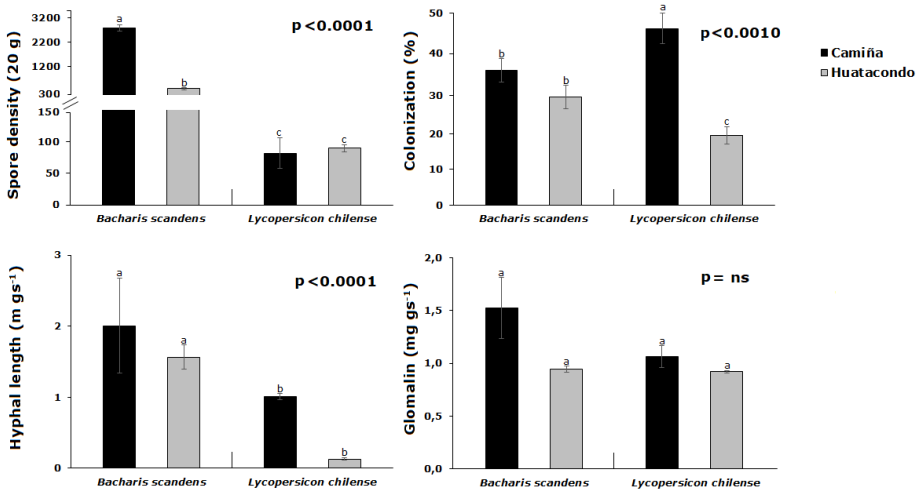

Figure 3: Arbuscular mycorrhizal colonization and fungal propagule densities in roots and rhizosphere soils of plant species growing in the pre-Puna elevation belt in the Atacama Desert, Tarapacá Region, Northern Chile. Data presented are the mean (n=3). Different letters indicate significant difference ($p<0.05$), according to Tukey multiple range test.

The AM presence in plants growing in the Puna elevation belt is shown in Figure 4. According to our results, the azonal vegetation grew mainly under highly saline conditions, and this phenomenon was associated with greater spore densities and mycelium lengths. Accordingly, the highest spore density was found in the rhizosphere soil of *D. curvula* with 8,500 spores in 20 g of soil, *F. deserticola* with 3,079 spores in 20

g of soil (Salar de Coposa), and *W. pinnatifida* with 3,015 spores in 20 g of soil (Salar del Huasco). Moreover, *D. curvula* plants showed higher spore densities in all sampled sectors compared to the other plant species. In contrast, *F. deserticola* in Salar de Lagunillas, *P. lucida* in Salar de Coposa, and *W. poposa* in Salar de Huasco showed the lowest spore densities under saline conditions, with 55, 146, and 243 spores in 20 g of soil, respectively. Similarly, the plants growing in saline soils showed the highest AM mycelium length, highlighting *F. deserticola* and *D. curvula* with lengths of 20.1 and 11.8 m g^{-1}, respectively (Salar de Coposa) and *D. curvula* with a length of 5.8 m g^{-1} (Salar de Huasco). On the contrary, the zonal vegetation was associated mainly with non-saline soil, and it is well known that this plant community grows under drought stress. In these conditions, spore density and mycelium length were lower compared to azonal vegetation. In detail, *P. quadrangularis, La. medicinalis,* and *S. nutans* from Salar de Coposa showed high spore density with values of 493, 233, and 216 spores in 20 g of soil, respectively. In the same way, the highest AM mycelium length was found in *La. medicinalis* (4.14 m g^{-1}) and *B. tola* (3.94 m g^{-1}), also sampled from Salar de Coposa. In relation to T-GRSP production, rhizosphere soils of *D. curvula* and *P. lucida* sampled from Salar de Michincha and *P. lucida* sampled from Salar de Huasco showed higher values compared to the other rhizosphere soils, with values of 5.8, 7.22, and 6.6 m g^{-1}, respectively. On the other hand, all analysed plant species presented mycorrhizal colonization; the highest value was found in *La. medicinalis* at 86.7% (Salar de Coposa). Other samples reached the following high values: *P. lucida* 81.8% and 68.9%, *P. quadrangularis* 70.3 and 75.2% (Salar de Huasco and Salar de Coposa, respectively), *W. pinnatifida* 68% (Salar de Huasco) and *S. nutans* 65.2% (Salar de Huasco). The PCA reflects the formation of highly homogeneous groups of experimental variables (Figure 5E), where PC1 explains 48.8% and PC2 explains 13.7% of the total experimental

variance. The PCA together with cluster analysis allowed the differentiation of three well-defined groups. Noticeably, groups 1 and 2 included only plants associated with azonal vegetation, which reached high spore densities and hyphal length, which are also characterized by growing in soils with high EC cation content. Group 2 was represented by zonal vegetation plants, which showed low values of AM presence and also low soluble cation concentrations in soil (Figure 5F).

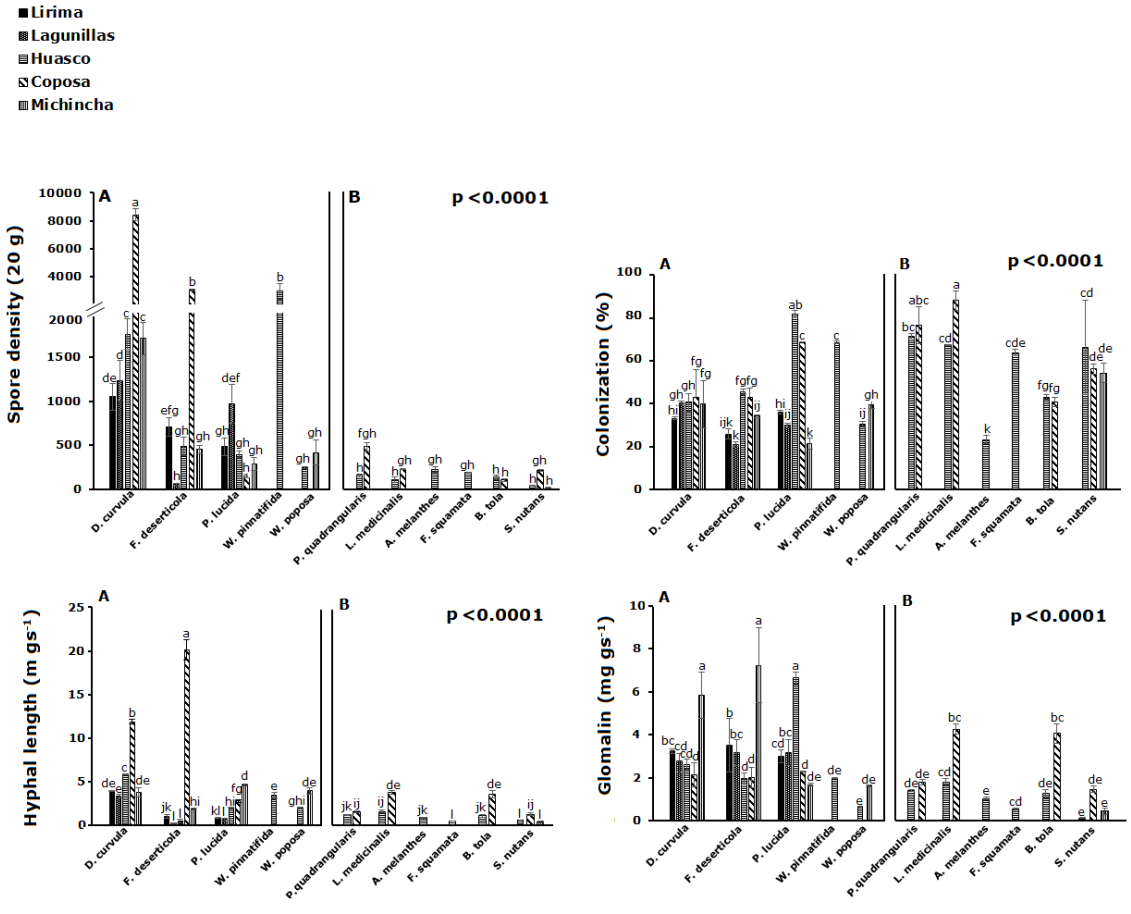

Figure 4: Arbuscular mycorrhizal colonization and fungal propagule densities in roots and rhizosphere soils of different sector in the Puna elevation belt in the Atacama Desert, Tarapacá Region, Northern Chile. **A:** azonal vegetation. **B:** zonal vegetation. Data presented are the mean (n=3). Different letters indicate significant difference ($p<0.05$), according to Tukey multiple range test.

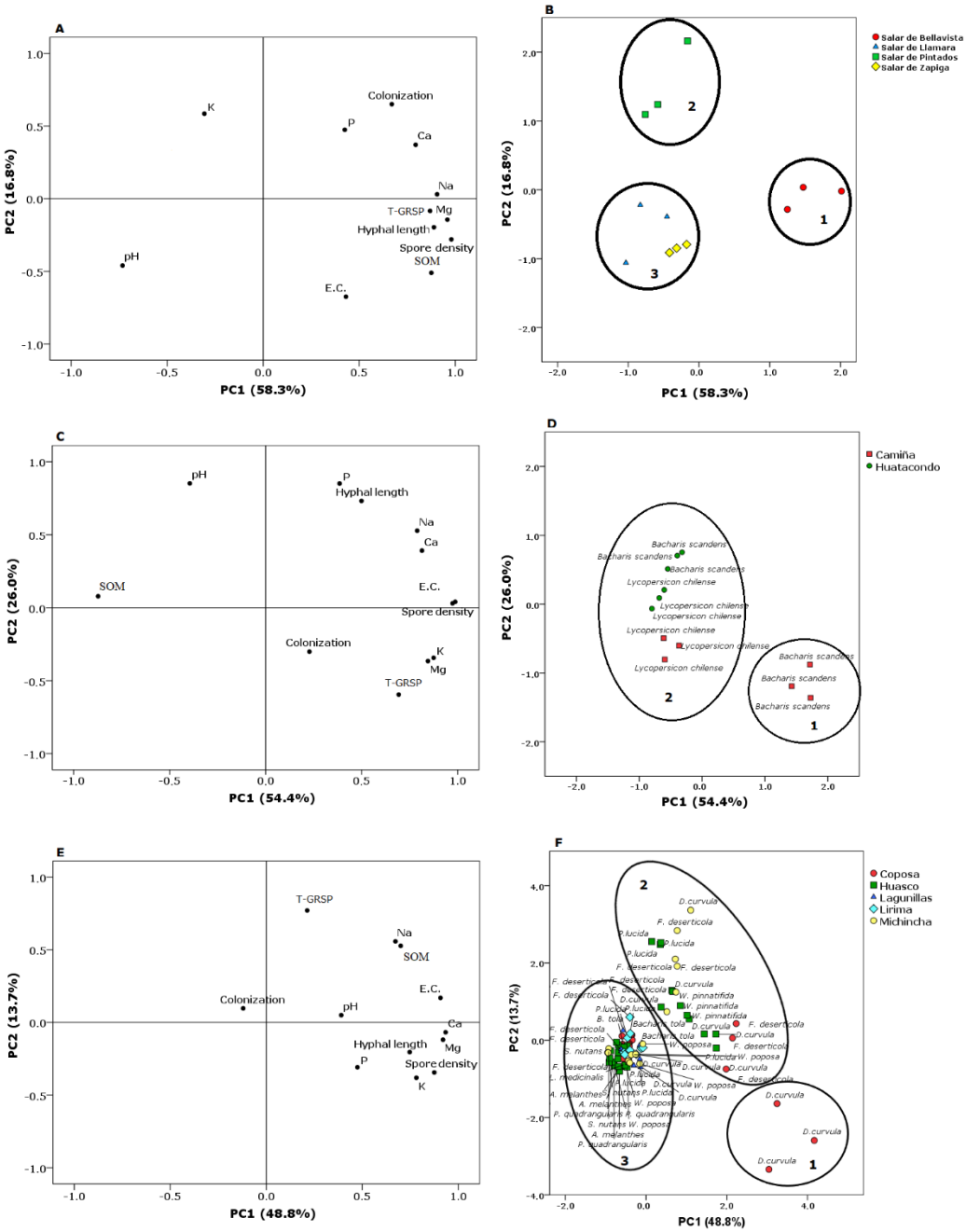

Figure 5: Principal component analysis for studied parameters in different elevation belts and cluster analysis for the samples: Pampa del Tamarugal (A, B), pre-Puna elevation belt (C, D), and Puna elevation belt (E, F). The mean value was used in each situation. Percentage values in parentheses indicate the variation explained by each PC. The circles comprise individuals with similar characteristics according to the analysis and should be understood as a visual aid for discrimination of groups.

3.4 Discussion

Vegetation exposed to extreme environmental conditions has developed different evolutionary strategies to overcome prevailing stressful conditions, such as the establishment of AM symbiosis (Rodriguez, Redman, & Henson, 2004). AM symbiosis also plays a very important role in ecosystem vegetation succession, species diversification and productivity, and an appropriate and stable population of AM fungal species is an essential component of a healthy ecosystem (Klironomos, McCune, Hart, & Neville, 2000; Yang, Chen, & Li, 2008). Our results have shown that all sampled plant species are able to establish AM symbiosis, in accordance with several studies that have found that several native or endemic plants in arid and semiarid ecosystems produce AM symbiosis (Barea et al., 2011; Estrada et al., 2013; Silvani et al., 2017). However, the scale both in geographical distribution and numbers of plant species is noticeable, here included. It is well known that under the extreme conditions of desert ecosystems, AM fungi can play an important role helping plant growth through increased water and nutrient uptake (Amiri et al. 2015; Pedranzani et al. 2016), but this improvement in tolerance to drought and salinity stress can also occur through the means of direct mechanisms (Calvo-Polanco et al. 2016), which deserve in-depth investigation to elucidate the functional contribution of AM fungi plant establishment in the most extreme arid conditions worldwide.

A difference in AM fungal propagule densities was demonstrated between plants associated with water stress (zonal vegetation) and those associated with saline stress (azonal vegetation), being many times higher in saline soils. Under drought stress, spore germination, hyphal growth, and root colonization are inhibited (Estaun, 1989; Jacobson, 1997). In arid environments, a reduction in soil moisture availability was shown to significantly reduce AM fungal populations and root colonization levels (Mohammad,

Hamad, & Malkawi, 2003). Contrarily, high soil cation content increased the water holding capacity, facilitating high soil moisture conditions for the AM fungal population (Rathore & Singh, 1995).

In this research, under the most extreme saline stress conditions reported to date, the highest spore density and AM mycelium length were found in *F. deserticola* and *D. curvula* in the Salar de Coposa sector. Similar trends in increased mycorrhizal structure densities have been reported in saline soils (Mohammad et al., 2003). High spore and propagule density in plants growing in arid and semiarid habitats might be demonstrating that these plants are more dependent on AM symbiosis, compared with plants that grow under no limiting conditions (Cuenca & Lovera, 1992; Tao, Jianping, & Zhiwei, 2004). Therefore, under the extreme conditions of desert ecosystems, AM fungi are believed to play an important role for prospering vegetation. Indeed, under stress conditions, plants need AM associations for both acclimatization and allowing nutrient uptake during all growth stages (Giri & Mukerji, 2004).

Seasonal conditions, edaphic factors, host dependence, host plant age, soil conditions and climatic conditions affect AM fungal distribution in soil differently (Mohammad et al. 2003). Here, a possible explanation of this behaviour is that high densities of AM structures could be related to AM fungal responses to cope with salinity as a plant protection method, even accumulating toxic ions in its spores and external hyphae to avoid the entry of toxic ions beyond the plant roots (Hammer, Nasr, Pallon, Olsson, & Wallander, 2011). On the other hand, high soil salinity can produce AM structure accumulation due to a reduction in spores and mycelium degradation, producing and accumulating non-viable propagules in soil. Similarly, higher spore accumulation in soil could occur because salinity affects AM fungal spore germination and the spores tend to constantly accumulate on the soil (Aliasgharzadeh, Saleh Rastin, Towfighi, &

Alizadeh, 2001). Juniper and Abbott (2006), determined a negative correlation between spore germination and levels of NaCl. In this way, salinity can affect AM fungal spore germination as the viability loss of these propagules is a critical factor in the survival of AM fungal populations (Campagnac & Khasa, 2014; Peng, Guo, & Liu, 2013). On the contrary, in the presence of NaCl, spore germination is delayed rather than prevented (Cantrell & Linderman, 2001; Juniper & Abbott, 2006).

Finally, our results noticeably showed that all plant species analysed here presented AM colonization independent of the wide range of intensity or densities of fungal structures. No relation was found among colonization levels and edaphic and environmental factors. This finding is in agreement with (Füzy, Biró, Tóth, Hildebrandt, & Bothe, 2008), who have found that colonization results obtained from field sampling should be carefully interpreted because during the annual cycle, the number of fungal structures, particularly arbuscules (with a short half-life), can vary significantly. Additionally, AM fungi produce recalcitrant forms of C such as glomalin (GRSP), with hydrophobic characteristics that participate in the formation and stabilization of soil aggregates, contributing to erosion prevention in desert ecosystems (Rillig & Mummey, 2006). In this study, we found a strong relationship between the levels of SOM and T-GRSP, especially in soil samples from Pampa del Tamarugal, which represent an important fraction of the total soil organic components. Moreover, AM fungal hyphae form networks in the soil, improving soil structure through the formation of stable aggregates and also preventing surface erosion and improving nutritional and water status (Santander et al., 2017).

3.5 Conclusion

This study focused on AM fungal populations of some representative plants growing under extremely arid and saline conditions in the Atacama Desert, northern Chile. The obtained results showed that all plants growing in this particular desert ecosystem presented AM fungal structures and glomalin accumulation. Similarly, plants living in desert ecosystems require mechanisms allowing adaptation to limiting conditions; our results then evidenced that AM association could play a role in plant colonization of these ecosystems. Moreover, in this study, the highest spore density and mycelium length in rhizosphere soils were shown in association with *B. scandens*, *W. pinnatifida*, *D. curvula* and F. *deserticola*, precisely the plants associated mainly with high saline accumulation, which supports the search for effective ecotypes in such conditions to be used in bioinoculant formulation oriented to agricultural production, and to the programs of recovery of threatened plant species in vast areas with increasing aridity and salinity worldwide.

Acknowledgments

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CHAPTER IV

"Arbuscular mycorrhizal colonization promotes the tolerance to salt stress in lettuce plants through an efficient modification of ionic balance"

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Arbuscular mycorrhizal colonization promotes the tolerance to salt stress in lettuce plants through an efficient modification of ionic balance

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Abstract

Soil salinity is the biggest problem which hinder the productivity of agricultural crops, causing adverse effects on plant growth and development. In this regard, it has been shown that the arbuscular mycorrhizal fungi (AMF) can establish a symbiosis with most agricultural plants and are capable of improving water and nutrient absorption under salinity stress conditions. In this study, the functional contribution of AMF strains (*Claroideoglomus claroideum* -Cc- and a consortium of AMF isolated from saline soils -HM-) on the growth and nutrition of lettuce plants (*Lactuca sativa* var. *longifolia*), was evaluated under increasing salt stress conditions (0, 40 and 80 mM NaCl). At 60 days of growth, the biomass production, nutrient content (N, P) and other ions (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), chlorophyll and proline content, and densities of AMF propagules were evaluated. The highest growth was observed in plants inoculated with Cc, which produced a higher percentage of root colonization and hyphal length at all levels of salinity, compared to plants inoculated with HM or non-inoculated plants. These results were directly related to higher biomass production, increased synthesis of proline, increased N uptake and noticeable changes in ionic relations, based in a diminish of Na^{+}, compared to non-mycorrhizal plants. Our results suggest that this improved ionic balance is due to a filtering effect of AMF structures both in the soil and in the root that prevents the entry of toxic Na^{+} ions, which is important due to the level of lettuce production on saline soils and the possibility to improve the crop by means of directed inoculation with efficient AMF strains.

Keywords: AMF strains, fungal propagules, ionic balance, osmotic stress, salinity.

4.1 Introduction

Soil salinity is one of the most important environmental stresses which affects plant growth and agricultural production globally (Torbaghan, Lakzian, Astaraei, Fotovat, & Besharati, 2017). Salt stress is present in approximately 20% of cultivated land and 50% of irrigation systems (Abdel Latef & He, 2011). Saline soils have electrical conductivities in excess of 4 dS/m (Richards, 1954), which is equivalent to approximately 40 mM NaCl and an osmotic pressure of -0.2 MPa.

Plants subjected to salinity stress can manifest a wide range of physiological defects related to metabolism, water relations and mineral nutrition. These defects may include: i) chlorine (Cl^-) and sodium (Na^+) at toxic concentrations, which can alter the structure of enzymes and other macromolecules, damaging cellular tissues, altering photosynthesis, respiration and protein synthesis (Zheng et al., 2008); ii) physiological drought in plants due to the reduced osmotic potential, which prevents water moving from the roots to the soil; and iii) nutritional imbalances caused by a decline in mineral absorption and a decrease in transport of these nutrients to the shoot (Evelin, Kapoor, & Giri, 2009).

In order to improve agricultural crop responses to salinity stress, different approaches are currently being used, such as the application of osmo-protectant compatible solutes and growth regulators, the cultivation of salt stress resistant cultivars, and the inoculation of plants with plant growth promoting microorganisms (PGPM). Within the applications of PGPM, the use of arbuscular mycorrhizal fungi (AMF) is of particular importance to crop plants, because AMF are symbiotically associated with most terrestrial plants, of which more than 80-90% of plants are of agricultural interest (Azcón & Barea, 2010).

The role of AMF in promoting the growth of host plants during salinity stress is well known. It has been shown that arbuscular mycorrhizal (AM) symbiosis can promote tolerance to salinity by various mechanisms, such as improved nutrient absorption, increased photosynthetic activity and increased water use efficiency (Santander et al., 2017). At the metabolic level, the AM symbiosis can also promote the synthesis of osmo-protectant compatible solutes, affect the control of reactive oxygen species (ROS) and stimulate the production of antioxidant enzymes by the host plant (Evelin, Giri, & Kapoor, 2013). As a result, horticultural crops inoculated with AMF under saline conditions showed an increase in biomass production (Zhao et al., 2015). This was particularly prominent when the AMF inoculants were native ecotypes, isolated from saline environments (Campagnac & Khasa, 2014; Chandrasekaran, Boughattas, Hu, Oh, & Sa, 2014). These findings highlight the importance of not only the use AMF inoculants during salt stress, but moreover the benefits of some specific highly efficient AMF ecotypes from saline environments.

Since the functional responses of mycorrhizal plants vary with the origins of AMF isolates, this presents an interesting question about the plasticity in the functional efficiencies of AMF isolates in comparison with native AMF isolates from very specific soils, such as in saline areas, which can be of an appreciable interest to crop production in areas worldwide, subjected to saline stress. Therefore, the objective of this study was to evaluate the physiological effect of two AMF isolates (indigenous of contrasting geographical areas) on the growth of *Lactuca sativa* plants under saline conditions, by comparing the functional efficiencies of a native AMF consortium from the Salar del Huasco (Atacama desert steppe, Tarapacá, Chile) with a reference AMF strain isolated from soils without saline restrictions. This would enable the elucidation of functional differences between native strains, based on soil origin and to establish parameters that

could serve as a screen for efficient AMF ecotypes, potentially employed as inoculants in crop production under saline conditions.

4.2 Material and Methods

4.2.1 Experimental design

A completely randomized factorial 3 x 3 design was used, which included three levels of inoculation with AMF: i) non-mycorrhizal plants (-M); ii) plants inoculated with a native consortium of AMF (HM) isolated from a saline soil in the high desert steppe, Atacama Desert (one of the driest environment worldwide), Tarapacá Region, Chile; and iii) plants inoculated with the fungus of reference *Claroideoglomus claroideum* (Cc) isolated from an agricultural soil in the Araucanía Region (Chile). In addition, three salinity levels were included, according with previous studies in similar conditions (Aroca et al., 2013): i) plants irrigated with 0 mM NaCl, ii) 40 mM NaCl, and iii) 80 mM NaCl. Five repetitions for each of the treatment combinations (n = 45) were used. The plant growth analyses were carried out at 45 days post-transplantation after maintaining them for 15 days in seed trays.

4.2.2 Soil and biological material

The soil used was obtained from the Camiña Valley, Region of Tarapacá, Chile (19° 18'59, 53 "S and 69° 26'14, 98" O, 2300 and 2600 m a.s.l.), collected between 0-25 cm deep, air-dried, sieved at 2 mm, diluted with sand (1:1 soil: sand, v/v) and subsequently sterilized by autoclaving at 121°C for 30 min on 3 consecutive days. The soil had a pH 7.7 (1:2.5; H_2O), electrical conductivity 270 (1:5; mS cm^{-1}), 2.2% organic matter and nutrient concentrations (mg Kg^{-1}): N, 27; P olsen, 13; K, 911; Ca, 150; Na, 135.

Lettuce seeds (*Lactuca sativa* var. *longifolia* cv. White Paris) were sterilized with 5% sodium hypochlorite solution for 5 minutes and then sown on polystyrene trays

with 80 mL capacity for each hole. The substrate for planting was as follows: i) a sterile soil mixture and vermiculite 1:1 (v/v) with a soil filtrate (<20 μm) to reconstitute the microbial population free of AMF propagules; ii) a sterile soil mixture and vermiculite 1:1 (v/v) with a soil sieving sample collected from trap pots on a mesh of 53 μM containing propagules of AMF native from saline soil (approximately 1600 spores per plant, see details below); and iii) a sterile soil mixture and vermiculite 1:1 (v/v) with a soil sieving sample collected on a mesh of 53 μM containing propagules of *C. claroideum* (1400 spores per plant). The consortium of native AMF was isolated from saline soils in the Salar del Huasco National Park and was associated with *Werneria pinnatifida* Remy (Asteraceae: Asterales). The fungus *C. claroideum* was isolated from agricultural soils from the Araucanía Region (Chile) and was associated with the rhizosphere of wheat plants. The reproduction of both inocula was performed in trap pots using a mix of plants consisting in *Zea mays*, *Plantago major* and *Melilotus indicus* as hosts.

4.2.3 Growth conditions

Once germinated, the seedlings were maintained for 15 days in the trays in the above-mentioned substrate. Subsequently, they were transferred to 1 L pots with a mixture of sterile soil and sand 1:1 (v/v) and were grown for 45 days under greenhouse conditions (25/21°C; 50/60% relative humidity, 14/10 hr day/night photoperiod). Salinity treatments were applied post-transplantion via irrigation with saline and control solutions. They were irrigated every two days with 100 mL of solutions with concentrations of 0, 40 and 80 mM NaCl. The final electrical conductivity in the different substrates was as follows: i) 1.53±0.14 dS m^{-1} at 0 mM NaCl, ii) 4.86±0.11 dS m^{-1} at 40 mM NaCl, and iii) 8.86±0.55 dS m^{-1} at 80 mM NaCl. The same amount of tap water was applied on alternate days to maintain humidity and to prevent excessive salt accumulation.

4.2.4 Measurements

At harvest, roots were separated from shoots, and subsamples (1 g) of fresh material from each organ were preserved for biochemical analysis and mycorrhizal colonization; the remainder of the material was dried at 70°C for 48 h in a forced-air oven to calculate biomass. Subsequently, the dried material was pulverized, incinerated at 550°C, and finally digested in an acid mixture of H_2O/HCl/HNO_3 (8/1/1, v/v/v). The digests were used for the spectrophotometric determination of P using the blue-molybdate method and determinations of K, Ca, Mg and Na by atomic absorption spectroscopy (Unicam SOLAAR, mod. 969). Nitrogen was determined by elemental analysis. The relative chlorophyll concentration was determined using a computer SPAD 502 (Konica Minolta®, Osaka, Japan) before harvesting, using the standardized, youngest, and fully expanded leaves of each plant. The free proline concentration was determined using 0.5 g of fresh tissue and spectrophotometrically assayed at 530 nm according to the method of Bates, Waldren, & Teare, (1973).

Separation of AMF spores was performed by wet sieving and decanting in a sucrose solution at 70% w/v and then quantitated under a stereomicroscope (30-90x magnification). Mycorrhizal colonization was determined by the gridline intercept method (Giovannetti & Mosse, 1980) using a stereomicroscope (40 - 60x magnification), after clearing the roots in 10% (w/v) KOH and staining with 0.05% (w/v) trypan blue in lactic acid. The length of AMF mycelium was determined by the method described by Borie, Rubio, Morales, & Castillo (2000).

4.2.5 Statistical analysis

The main effects for type of AMF inoculum, salinity level and their interaction were analyzed by using a factorial analysis of variance (ANOVA). Data were transformed

where it did not meet the requirements for normality and homoscedasticity. Nonetheless, the results are presented in their original numeral scale. Where the ANOVA revealed significant differences among the factors, the *post-hoc* Tukey's multiple range test were used to compare the differences between all the treatments. Subsequently, data sets were also subjected to principal component (PC) and correlation analyses, in order to establish the relationships among different variables of response and the PCs. In addition, the factors obtained were subjected to non-hierarchical cluster analysis using the farthest neighbor method, in order to determine the similarity among the different experimental units and to perform the conglomerates. The software SPSS 22.0 (IBM™) was used for all the analyses. The level of statistical significance was set at $P < 0.05$.

4.3 Results

4.3.1 Response of AMF structures to salt stress

The inoculant type, salinity level and their interaction, had a strong influence on almost all mycorrhizal parameters analyzed (Table 1). Colonization did not present significant differences between the HM consortium and Cc strain. Furthermore, salinity caused a colonization decrease of between 20 to 26% in both inocula when the NaCl concentration was increased. The Cc inoculum had a higher mycelial density under all salinity levels, increasing by 70, 45 and 42%, relative to the HM inoculum at 0, 40 and 80 mM NaCl, respectively. The highest spore density was found for Cc at 0 mM NaCl and decreased with increased salinity, being 75% lower at 40 mM NaCl and 85% lower at 80 mM NaCl (Figure 1). In non-mycorrhizal control plants, there were no AMF structures found.

Table 1. F-values and probabilities of significance for the main effects and interaction for the variables measured and analyzed by means of a two-way ANOVA.

Variable	Main effects		Interaction
	Salinity	AM	(Salinity x AM)
Colonization	***9.5***	***80.4***	***2.9****
Hyphal lenght	***22.6***	***169.7***	***17.1***
AMF spores	***14.8***	***20.6***	***6.8***
Shoot biomass	***52.2***	***73.4***	***10.5***
Root biomass	***39.3***	***21.3***	***6.6***
Nitrogen shoot	***73.3***	***45.9***	***3.4****
Phosphorus shoot	***4.3***	***6.5***	***1.2ns***
Phosphorus root	***13.9***	***5.8***	***5.7***
SPAD value	***23.8***	***37.8***	***9.3***
Proline	***58.6***	***13.7***	***4.6***
Potassium shoot	***2.4ns***	***0.9ns***	***1.9ns***
Potassium root	***1.6ns***	***12.5***	***1.1ns***
Sodium shoot	***129.3***	***17.0****	***0.2ns***
Sodium root	***95.7***	***2.8ns***	***5.6****
Calcium shoot	***31.9***	***2.4ns***	***3.4****
Calcium root	***5.6***	***8.6***	***4.1***
Magnesium shoot	***51.5***	***10.6***	***5.2***
Magnesium root	***10.2***	***10.2***	***26.6***

*ns, not significant;*P < 0.05; ** P <0.01.*

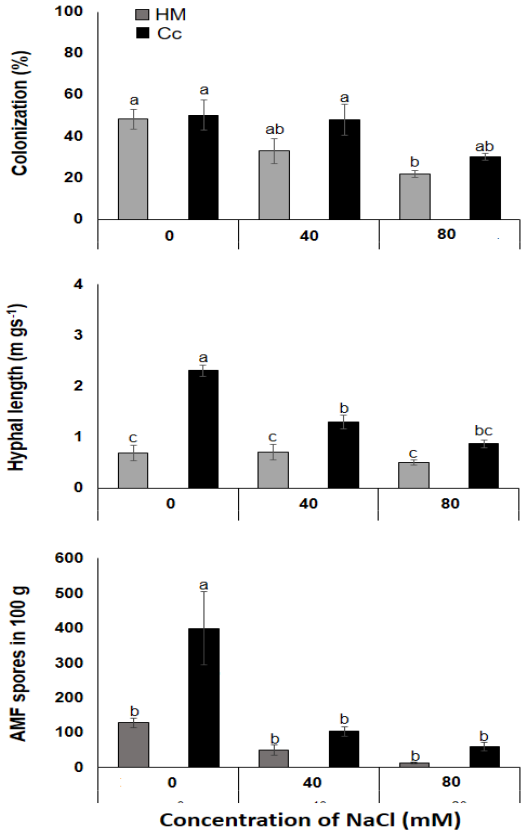

Figure 1. Mycorrhizal root colonization, hyphal density and AMF spores in *Lactuca sativa* plants grown at increasing NaCl levels using two different AMF inocula (-M: non-mycorrhizal; HM: native arbuscular mycorrhizal fungal; Cc: *Claroideoglomus claroideum*). Data presented are the mean ± S.E. (n=5). Different letters indicate significant differences ($p<0.05$) according to Tukey's multiple range test.

4.3.2. Lettuce plant biomass production

Both experimental factors and their interactions strongly influenced the shoot and root biomass (Table 1). Biomass production was strongly reduced at increasing salinity levels in all treatments. The -M control plants had the lowest biomass production at 0, 40 and 80 mM NaCl. In contrast, the AMF inoculated hosts produced higher dry biomasses at the three levels of salinity studied. Particularly, Cc inoculated lettuce plants had the highest biomass production (3.1 g shoot and 1.8 g root) at 0 mM NaCl (Figure 2).

However, relative to the non-mycorrhizal plants, the Cc inoculated host plants had an increased biomass production of between 125 and 159% in shoots and between 207 and 57% in roots at 40 and 80 mM NaCl, respectively. The effects of the native consortium of AMF were intermediate between the extremes described above (Figure 2).

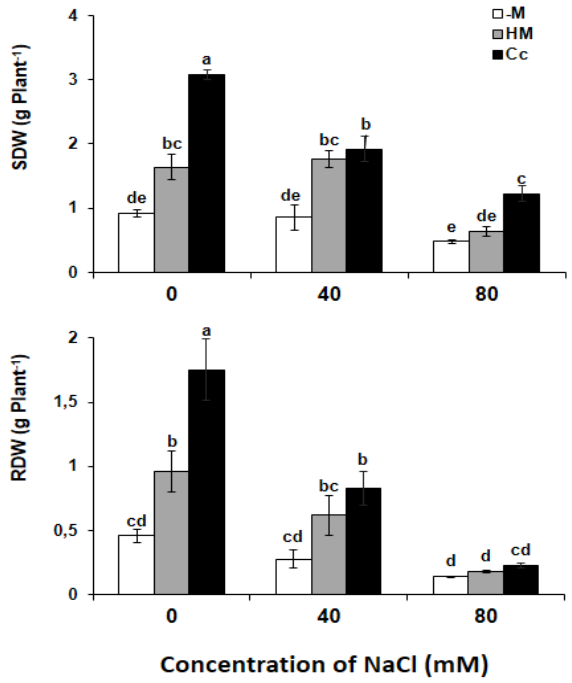

Figure 2. Shoot dry weight (SDW) and root dry weight (RDW) in *Lactuca sativa* plants grown at increasing NaCl levels using two different AMF inocula (-M: non-mycorrhizal; HM: native arbuscular mycorrhizal fungal; Cc: *Claroideoglomus claroideum*). Data presented are the mean ± S.E. (n = 5). Different letters indicate significant differences ($p<0.05$) according to Tukey's multiple range test.

4.3.3 Nitrogen and phosphorus concentration in lettuce

The root phosphorous (P) concentration was affected by the type of inoculation and the level of salt stress, as well as the interactions of these factors (Table1). Shoot P concentration was affected only by the separate factors, but not by their interactions. The increase in NaCl supply caused a decrease in shoot P concentrations; however, the root P

concentration increased with an increase in salinity. In all cases, inoculated plants showed higher concentrations of shoot and root P at all salinity concentrations compared to -M, especially when Cc was used as the AMF inoculum (Figure 3).

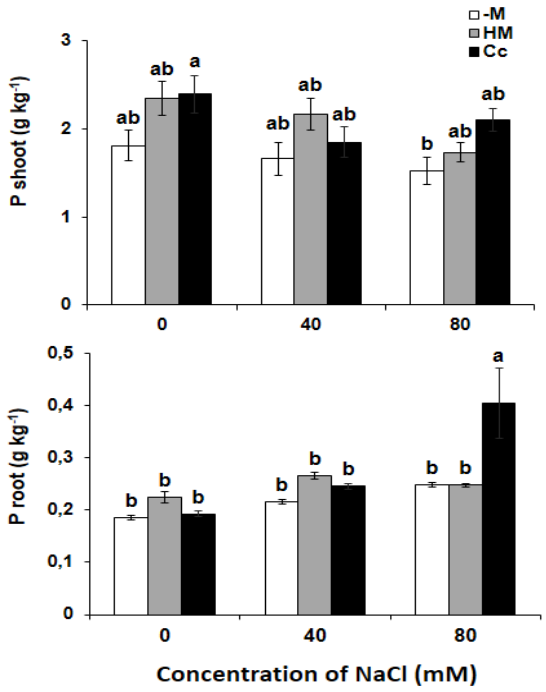

Figure 3. Phosphorus concentration in shoot and root in *Lactuca sativa* plants grown at increasing NaCl levels using two different AMF inocula (-M: non-mycorrhizal; HM: native arbuscular mycorrhizal fungal; Cc: *Claroideoglomus claroideum*). Data presented are the mean ± S.E. (n = 5). Different letters indicate significant differences ($p<0.05$) according to Tukey's multiple range test.

Inoculation with AMF, salinity and their interactions significantly affected the content of N in shoots (Table 1). Inoculation caused an increase in N absorption with both AMF inocula. The HM inoculum increased N uptake by 51, 59 and 19% at 0, 40 and 80 mM, respectively, compared to -M control plants. Inoculation with Cc showed the same trend as HM in N concentrations for 0 and 40 mM NaCl. However, at 80 mM NaCl, the

Cc inoculum caused an increase in N uptake of 98% and 137% compared to HM and -M, respectively (Figure 4).

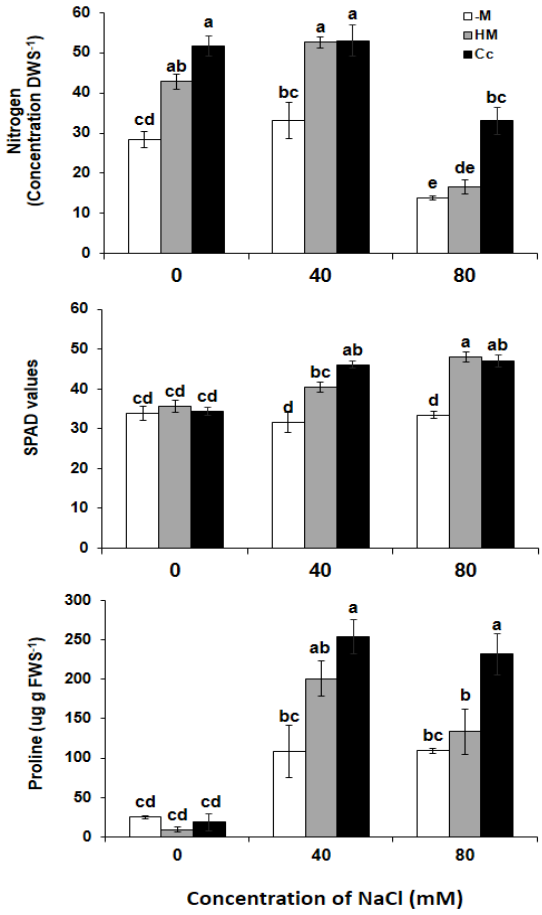

Figure 4. Nitrogen concentration, relative content of chlorophyll (SPAD) and proline production in shoot of *Lactuca sativa* plants grown at increasing NaCl levels using two different AMF inocula (-M: non-mycorrhizal; HM: native arbuscular mycorrhizal fungal; Cc: *Claroideoglomus claroideum*). Data presented are the mean ± S.E. (n = 5).

4.3.4 Relative chlorophyll and proline content in lettuce

The relative chlorophyll concentration (SPAD value) and proline accumulation were affected by AMF inoculation, salinity and their interactions (Table 1). Inoculated plants exhibited the highest SPAD values under salt stress (40 and 80 mM), being 27-45% higher than non-mycorrhizal plants. Proline concentrations increased by approximately 300% in

shoots at 40 and 80 mM NaCl exposure. Under these conditions, Cc produced an increase between 133 and 111% and HM produced an increase between 84 and 22%, relative to the –M controls. At 0 NaCl mM, there were no significant differences between the inoculated and non-mycorrhizal plants (Figure 4).

4.3.5 Absorption of cations and ionic balances

Although the lettuce plants accumulated more Na^+ in the shoots than in roots under all salt concentrations, this accumulation was differentially affected by the various factors (Table 1). Both HM and Cc AMF inoculants caused host plant to accumulate a lower concentration of Na^+ in shoots compared to non-mycorrhizal control plants. Relative to the non-mycorrhizal plants, Cc caused a decrease in Na^+ concentrations of between 170, 54 and 23% at the levels of 0, 40 and 80 mM NaCl. However, compared to the native consortium (HM), Cc caused a decline in Na^+ accumulation of between 89, 21 and 10% at 0, 40 and 80 mM NaCl levels. Significant differences between two inocula were found in Na^+ in roots at 40 mM, but with the HM inoculated plants showing a higher concentration of Na^+ in comparison to the Cc inoculum (Figure 5)

The K^+ root concentration was also affected by the type of inoculum (Table 1), and was lower in plants inoculated with Cc. In contrast, plants inoculated with HM reached the highest values of root K^+ concentration at all levels of salinity. In shoots, no differences in K^+ concentration were observed (Figure 5). The root Ca^{2+} concentration was affected by both factors and their interactions (Table 1), where HM-inoculated host plants had a higher Ca^{2+} concentration, as evidenced by the 63 to 199% increases at 0 mM and 23 to 50% enhancements at 40 mM, compared to the -M control and Cc-inoculated plants, respectively. In the shoot, non-inoculated plants had the highest concentration of Ca^{2+} at the 0 mM NaCl treatment. Plants increased the concentrations of

Ca^{2+} at the 40 and 80 mM NaCl levels, but this was not related to the effect of mycorrhizal colonization (Figure 5). The concentration of Mg^{2+} was significantly affected by the type of inoculum, salinity levels and the interaction of both factors in the roots and shoots (Table 1). In non-mycorrhizal plants, a higher concentration of Mg^{2+} was observed in shoots under saline conditions of 40 mM NaCl exposure. Moreover, the HM-inoculated plants showed a greater root Mg^{2+} concentration, of between 65 to 198% at the 0 mM NaCl level and a 28 to 29% at the 40 mM NaCl exposure, relative to the -M and Cc treatments. At 80 mM NaCl, both inocula caused host plants to accumulate a higher Mg^{2+} concentration compared to non-mycorrhizal plants (Figure 5). The K^+/Na^+ ratio strongly decreased in leaf tissues as with increasing salinity exposure, displaying differences from one- to two-fold, between saline and non-saline treatments (Figure 6A). The highest K^+/Na^+ ratio was obtained in the shoots of plants inoculated with Cc at 40 and 80 mM NaCl supply. At the root level, the K^+/Na^+ ratio decreased 1- to 1.5-fold when the plants were subjected to high salt concentrations compared to 0 mM NaCl (Figure 6B). Under saline conditions, there were no effects of the AMF inoculation on the K^+/Na^+ ratio in roots. Salinity strongly decreased the Ca^{2+}/Na^+ ratio mainly in the root. Under all saline conditions studied, Cc inoculation caused the highest Ca^{2+}/Na^+ ratios in leaf tissue of host plants, compared with non-mycorrhizal plants (Figure 6C). Although inoculation with AMF had no effect on the Ca^{2+}/Na^+ ratio in shoot and root at 0 and 40 mM NaCl levels, the HM and Cc inoculated plants showed a greater ratio compared to non-mycorrhizal plants at 80 mM NaCl (Figure 6D).

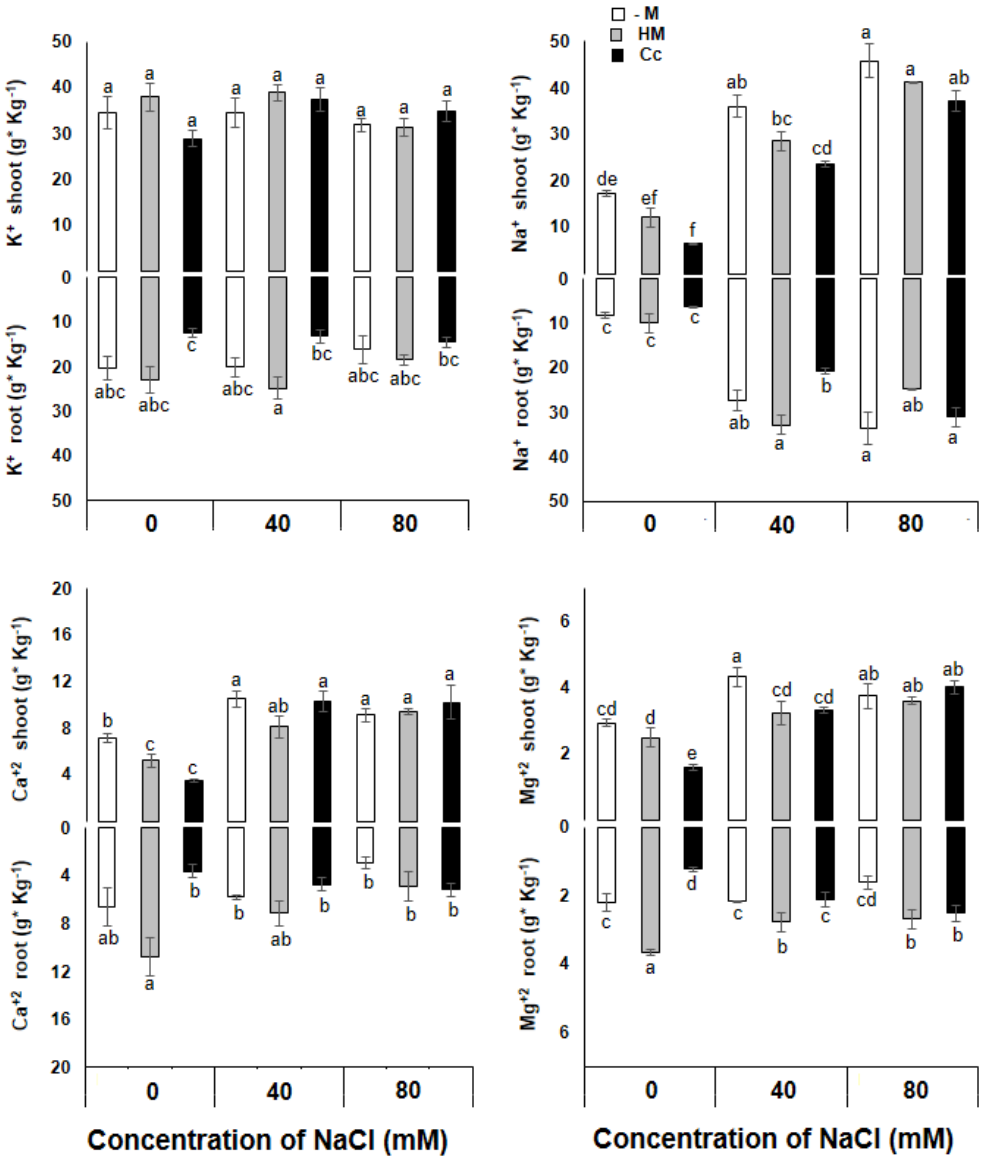

Figure 5. Effect of inoculation with different AMF on the uptake of K^+, Ca^{2+}, Mg^{2+} and Na^+ in *Lactuca sativa* plants grown at increasing NaCl levels. -M: non-mycorrhizal; HM: native arbuscular mycorrhizal fungal; Cc: *Claroideoglomus claroideum*. Data presented are the mean ± S.E. (n = 5). Different letters indicate significant differences ($p<0.05$) according to Tukey's multiple range test.

The root Mg^{2+}/Na^+ ratio decreased strongly with increasing salinity. Similarly, in shoots the Mg^{2+}/Na^+ ratio also decreased 1-2-fold with increasing salinity. AMF inoculation had significant effects on the Mg^{2+}/Na^+ ratio in shoots under 0 and 80 mM NaCl (Figure 6E). The HM consortium caused a higher Mg^{2+}/Na^+ ratio in host roots under

non-saline conditions (0 mM NaCl), with higher values of 2-4-fold compared to non-mycorrhizal plants and plants inoculated with Cc. At 40 mM NaCl, inoculation had no effect on the Mg^{2+}/Na^{+} ratio. In contrast, at a salinity of 80 mM NaCl, both AMF inocula produced higher values compared to non-inoculated treatments (Figure 6F).

4.3.6 Multivariate relations

The principal components (PC) analysis reflects the formation of highly homogeneous groups of experimental variables (Figure 7A), where PC1 explains 43.1% and PC2 explains 16.8% of the total experimental variance. PC1 was positively related to shoot and root biomass production as well as mycorrhizal variables, such as the length of mycelium and the density of spores. PC1 was also positively associated with the N-P concentration and ionic relations in the shoots and roots, such as K^{+}/ Na^{+}, Ca^{2+}/Na^{+} and Mg^{2+}/Na^{+}, and negatively associated with Na^{+} concentration in the shoots and roots (Figure 4A). Additionally, PC2 was positively related to mycelium length, proline production and SPAD value (Figure 7A). The PCA together with cluster analysis allowed the differentiation of five well-defined groups (Figure 7B). Groups 1 and 2 mainly include plants grown under conditions of 0 mM NaCl exposure and inoculated with Cc and HM AMF isolates, representing the experimental units with higher biomass production, densities of mycorrhizal propagules, root colonization and N-P concentrations. Group 3 includes non-mycorrhizal plants, mainly at 40 and 80 mM NaCl, and includes plants with lower growth and a high Na+ concentration. Finally, groups 4 and 5 were formed by plants inoculated with Cc and HM under conditions of 40 and 80 mM NaCl, which represent plants with intermediate growth, a higher production of proline and a higher concentration of P in roots.

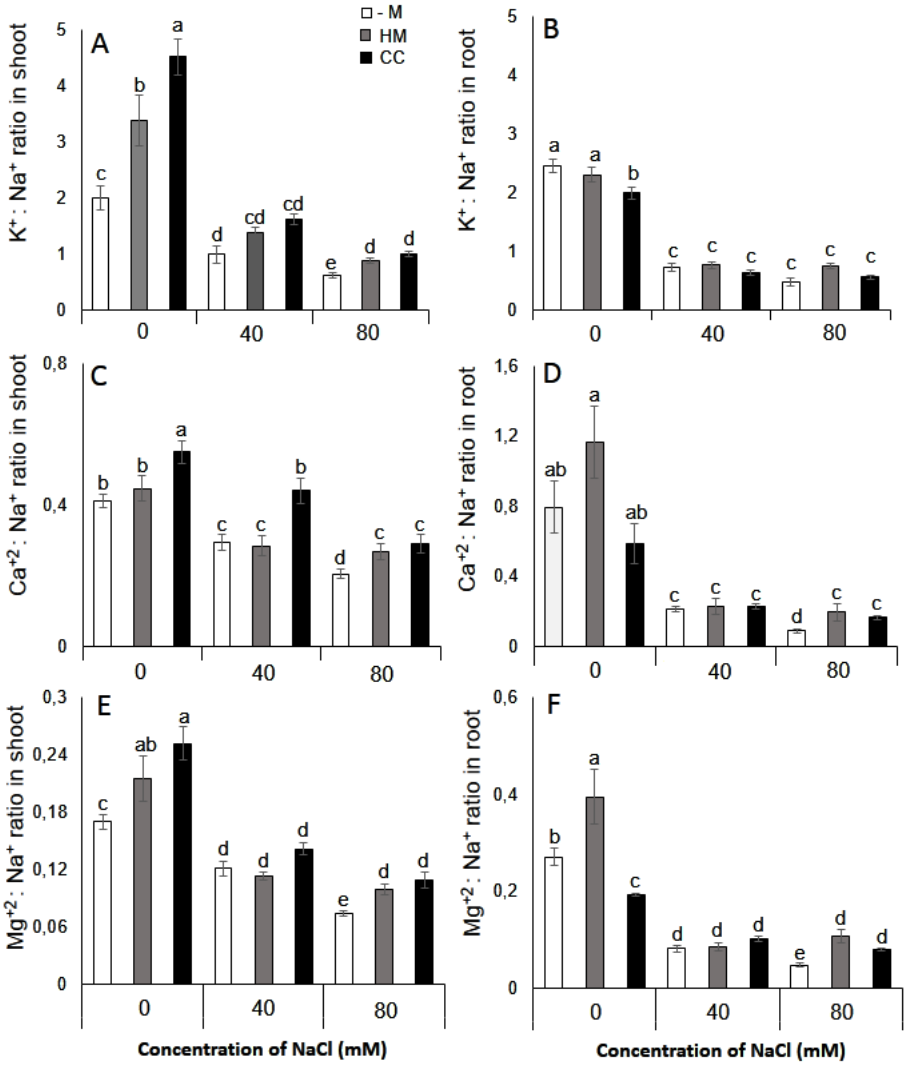

Figure 6. Effects of NaCl and AMF inoculation on the K^+/Na^+, Ca^{2+}/Na^+ and Mg^{2+}/Na^+ ratios in shoots and roots in *Lactuca sativa* plants grown at increasing NaCl levels. -M: non-mycorrhizal; HM: native arbuscular mycorrhizal fungal; Cc: *Claroideoglomus claroideum*. Data presented are the mean ± S.E. (n = 5). Different letters indicate significant differences ($p<0.05$) according to Tukey's multiple range test.

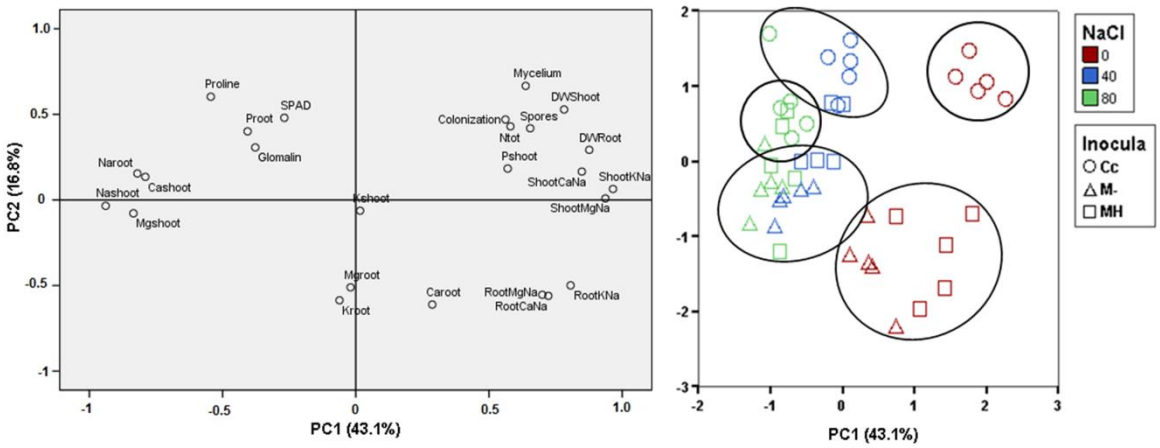

Figure 7. A) PCA scores for the respective combinations between inoculum type and NaCl levels added. The mean value was used in each situation. Percentage values in parentheses indicate the variation explained by each PC. **B)** Cluster analysis for the experiment. The circles comprise individuals with similar characteristics according to the analysis and should be understood as a visual aid for discrimination of groups.

4.4 Discussion

Although AMF can overcome the limitations of salt stress to host plants, the present study found that the AMF isolates from saline soils are not necessarily the more efficient symbiotic partners for host plants grown under salt stress conditions. These findings raise important issues for both our fundamental understanding of mycorrhizal function, as well as the practical applications of inocula for the alleviation of environmental stress in crop production. The unraveling of these underlying mechanisms require further investigation. It is well known that salinity decreases plant production (Evelin, Giri, & Kapoor, 2012), which is in accordance with the findings of the present study. Furthermore, it has been widely reported that arbuscular mycorrhizal colonization can significantly protect host plants from the deleterious effects of salinity (Aroca et al., 2013). In this regard, the current study found that AMF inoculation improved the tolerance of lettuce host plants to

saline stress and is in agreement with the other studies. Similarly, in this study, salinity affected nutrient uptake (N-P), and concurs with Shokri & Maadi (2009), who reported that the salinity-induced increase of Na^+ and Cl^- ion absorption, which can compete with the acquisition of other minerals. AMF can improve the mineral nutrition uptake of the host through their extensive extraradical mycelium network (Zhao et al., 2015). This increase in nutrient uptake under high salinity conditions can improve the host plant's metabolism, by enhancing the synthesis of proteins and compatible osmolytes, associated with salt stress adaptation (Garg & Manchanda, 2008; Shokri & Maadi, 2009).

With respect to AMF traits, salinity strongly affected plant root colonization, the hyphal length of extraradical mycelium and spore density, which is consistent with numerous previous observations (Estrada et al., 2013; Evelin et al., 2012). Na^+ produces a direct toxic effect on AMF, decreasing colonization (Sheng et al., 2008). Moreover, Hammer et al., (2011), found a decrease in the biomass of AMF at high NaCl concentrations. In contrast, other studies have reported increased sporulation and colonization in plants growing under saline conditions (Aliasgharzadeh, Saleh Rastin, Towfighi, & Alizadeh, 2001). With respect to the adaptations of AMF obtained from saline conditions, Juniper & Abbott (2006), determined that isolated AMF propagules from non-saline and saline soils differ in their capacity to germinate and grow under saline conditions.

Copeman, Martin, & Stutz (1996), suggested that the differential efficiency and behavior of AMF depend on the source of their isolation. Thus, for example, Yamato, Ikeda, & Iwase (2008), reported higher plant growth when plants were inoculated with AMF isolated from saline conditions. In the present study, the HM consortium had a lower symbiotic efficiency, percentage of colonization, number of spores and mycelial length compared to the isolate of Cc, which has been obtained under non-saline

conditions. Similar results were reported by Estrada et al., (2013), who compared an isolate of *Rhizophagus intraradices* reference (Ri collect, isolate EEZ 58) with native isolates growing under salt stress conditions. In this case the isolate of reference was the one that showed positive effects on plant growth. This may be due to intrinsic characteristics of the isolates used, in which the association with the host is more compatible and therefore the benefits obtained are greater. This aspect must be further investigated because the possibility of using efficient AMF ecotypes under saline conditions, is emerging as an advanced biotechnological tool to cope with the limitations in plant production in vast areas of the world.

It is noteworthy that in situations of abiotic stress, the isolated *Claroideoglomus claroideum* has previously been shown to be highly efficient in increasing plant biomass growing in contaminated soils, related to a high colonization and densities of propagules (Meier et al., 2015), an increased production of glomalin and also accumulating high amounts of Cu in spores (Cornejo et al., 2017), which together may act as a symbiotic rhizosphere barrier to the entry of phytotoxic ions to host roots, Na^+ in this case. This aspect has been previously studied, suggesting that the AMF acts as a primary barrier to Na^+ ions when they reach toxic levels, by increasing the concentration in the root and decreasing its translocation towards the shoot. This protective mechanism supports the strong relationships found in shoots for K^+/Na^+, Ca^{2+}/Na^+ and Mg^{2+}/Na^+ ratio (Elhindi, El-Din, & Elgorban, 2017; Hammer & Rillig, 2011). Lower concentrations of foliar Na^+ were determined in mycorrhizal plants (Santander et al., 2017), in congruence with the results reported here. It should be noted that in the case of K^+ in our study, there was no increase in the foliar concentration due to mycorrhization, as has been observed in other studies (Evelin et al., 2012; Garg & Bhandari, 2016). However, since the foliar K^+/Na^+

ratio was higher in the inoculated plants, this is indicative of the barrier effect against the Na^+ ion.

Benito et al., (2014), proposed that the molecular similarity of the Na^+ and K^+ ions can account for their competition for the binding sites on the plasma membrane. Therefore, when the K^+/Na^+ ratio is decreased, the metabolic processes which exclusively depend on K^+ thereby are inhibited. Furthermore, according to Evelin et al. (2012), a high K^+/Na^+ plasma ratio is important to increase the plant tolerance to salinity, and also to enhance enzymatic processes. This improved cellular homeostasis can therefore promote the metabolism associated with alleviation of salt stress in a variety of physiological mechanisms, which include chlorophyll and compatible solute synthesis. The enhanced synthesis of chlorophyll is an important adaptive mechanism under salt stress, because it can lead to increases in photosynthetic efficiency (Elhindi et al., 2017). Since salinity can induce the suppression of enzymes involved in chlorophyll synthesis, this NaCl toxicity can thereby decreases photosynthetic capacity of plants (Tsunekawa et al., 2009). In the present study, mycorrhizal colonization increased the relative chlorophyll concentration (SPAD), which concurs with several studies which have shown that mycorrhizal colonization can enhance chlorophyll synthesis in host plants under stress, thereby increasing the potential capacity of carbon fixation (Porcel et al., 2015; Zhu, Song, Liu & Liu, 2012).

The synthesis of compatible solutes is an important adaptation during salt stress. In this regard, the known physiological oslmolyte, proline is an amino acid which plays a highly beneficial role in plants exposed to salinity stress conditions. Proline accumulation can help to maintain the osmotic equilibrium in plants under stressed conditions, by increasing its concentrations in leaves and roots (Ashraf & Foolad, 2007). Although the current results show an increase in the concentration of proline due to the

increase in salinity, this effect was greater in mycorrhizal plants just as the studies from Tuo, Li, Wu, & Zou (2015). In contrast, several authors have determined that under stress conditions, proline production is lower in mycorrhizal plants compared to non-mycorrhizal plants (Krishnamoorthy et al., 2016; Yooyongwech, Samphumphuang, Tisarum, Theerawitaya, & Cha-um, 2016). There are several possible explanations for these inconsistencies. Firstly, these results suggest that proline accumulation is primarily a result of salinity and not necessarily of mycorrhizal colonization (Ruiz-Lozano, Porcel, Azcón, & Aroca, 2012). Secondly, proline accumulation is not the only compatible solute that plants can synthesize during salt stress and other well-known osmolytes include sugars, alcohols (Sorbitol), quaternary ammonium compounds (Glycine betaine) and tertiary sulphonium compounds (3-Dimethylsulphoniumpropionate) (Ashraf & Foolad, 2007). Nonetheless, in the present study the accumulation of proline should be taken into account as an indicator of AMF efficiency, because it may be the specific metabolic response of lettuce and it is one of the main compatible solutes in plants growing under salinity. However, other plant biochemical indicators can be also taken into account as indicator of AMF efficiency under salt stress, mainly related to oxidative stress (Santander et al., 2017), which is also of agronomical importance if a correct choice of AMF based inoculant is carried out to improve crop production in saline soils.

4.5 Conclusion

Our results show that salinity has a clear detrimental effect on the growth and development of lettuce plants, affecting biomass production, nutrient absorption and ionic homeostasis. Under these conditions, AMF inoculation is able to act as a symbiotic rhizosphere barrier, by reducing the deleterious effects of salinity stress. The mechanisms of action for the AMF appear to be related to maintaining cellular homeostasis, via AMF-

induced alterations in cation balances. Although both AMF inocula assisted the host plant via this mechanism, the degree of symbiotic efficiency differed with the type of AMF. Rather surprisingly, the best performing inoculum for host resistance to salt stress was *C. claroideum*, isolated from non-saline soils. The capacity of this fungal species to out-perform the AMF isolates from native saline soils, suggests that the energy and carbon cost efficiencies of host-symbiont functional relationship, may be of greater importance under salt stress, than the evolutionary origin of the AMF symbiont. Therefore, the consideration of AMF strains as inoculants for host plants under salt stress conditions should be based on an analysis of the host-fungus symbiotic and functional compatibility. These findings potentially reveal important new avenues of investigation, in which more in-depth plant-fungus interactions must be elucidated at the metabolic, transcriptomic and proteomic levels.

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CHAPTER V

"Efficiency of two arbuscular mycorrhizal fungal inocula selected to improve saline stress tolerance in lettuce plants by changes of antioxidant defense mechanisms"

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Efficiency of two arbuscular mycorrhizal fungal inocula to improve saline stress tolerance in lettuce plants by changes of antioxidant defense mechanisms

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Abstract

Arbuscular mycorrhizal (AM) fungi establish symbioses with most agricultural plants and improve growth under soil stress conditions. The objective of the present study was to evaluate the functional contribution of two AM inocula (a native consortium isolated from saline soils of the Atacama Desert, 'HMC', and a reference inoculum *Claroideoglomus claroideum*, 'Cc') on the growth, antioxidant compounds enzyme activity of two cultivars of lettuce (*Lactuca sativa* cvs. 'Grand Rapids' and 'Lollo Bionda') at increasing salt stress conditions (0, 40, and 80 mM NaCl). At 60 days of plant growth, the symbiotic development, biomass production, lipid peroxidation, proline content, antioxidant enzymes, phenolic compound profiles, and antioxidant activity were evaluated. The two AM inocula differentially colonized the roots of Grand Rapids and Lollo Bionda lettuce plants. The AM symbioses increased proline synthesis and superoxide dismutase, catalase, and ascorbate peroxidase activities and diminished phenolic compound synthesis and oxidative damage in lettuce, which was related positively with higher growth of inoculated plants under salt exposure. The higher concentration of phenolic compounds induced by salinity in non-inoculated plants was associated with high oxidative stress and low fresh biomass production. Modulation of salinity stress in lettuce by AM fungi is due to changes of antioxidant enzymatic systems that reduce oxidative damage and sustain growth. The application of AM fungi to improve crop production by means of directed inoculation with efficient AMF strains may enhance lettuce production on soils plagued with salinity worldwide.

Keywords: enzymatic activity; phenolic compounds; salt stress; indigenous AMF strains.

5.1 Introduction

Soil salinity is a significant constraint to the production of agricultural crops due to its adverse effects on plant growth and development (Jamil, Riaz, Ashraf, & Foolad, 2011). More than 45 million ha of irrigated land have been damaged by salt, with 1.5 million ha becoming unproductive each year as a result of high salinity levels in soils (Munns & Tester, 2008). Salinity mainly affects soils in arid and semiarid regions and affects commercial horticulture developed in these regions (Colla, Rouphael, Leonardi, & Bie, 2010). Salinity is a soil condition characterized by high concentrations of soluble salts. Sodium chloride (NaCl) is the main compound present in such soils (50 to 80% of affected soils), but these soils may also contain significant concentrations of Ca^{2+}, Mg^{2+}, SO_4^{2-}, and CO_3^{2-} (Rengasamy, 2010), and these ions affect plants at the physiological and biochemical levels (Ahmad & Prasad, 2011).

Deleterious effects of sodium (Na^+) on plants are associated with (i) Na^+ over-accumulation in the root zone affecting the osmotic potential of soils (Ashraf, 2004; Chowdhury, Marschner, & Burns, 2011), and (ii) ion imbalances induced by excess Na^+, which affects nutrient, mainly potassium (K^+), uptake by plants (Zhu, 2002). Overabundance of Na^+ in cytoplasm causes replacement of K^+ by Na^+ in biochemical reactions, altered levels of growth regulators, enzymatic inhibition, decreases of protein synthesis, and impairment of photosynthetic processes (Tsunekawa et al., 2009; Zhu, 2002). Excessive exposure of plants to salt also increases the production of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2), which results in lipid peroxidation and oxidative damage to proteins and nucleic acids (Alqarawi, Abd Allah, & Hashem, 2014; Bose, Rodrigo-Moreno, & Shabala, 2014). ROS in plants are produced continuously by imbalances in the flow of electrons through the electron transport chains in the

chloroplast, mitochondria, and plasmatic membrane, and may also be produced by various cellular metabolic pathways (Foyer, 2018). While the control of ROS within strict bounds is fundamental to cell homeostasis, the loss of this balance occurs in plants growing in saline soils (Miller, Suzuki, Ciftci-Yilmaz, & Mittler, 2010).

Plants have enzymatic and non-enzymatic antioxidant systems for scavenging ROS to protect them from destructive oxidative reactions and enhance the plant tolerance to salinity stress (Das & Roychoudhury, 2014). The enzymatic ROS-scavenging systems are located in different subcellular compartments and include superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) (Mittler, 2002). The most important non-enzymatic ROS-scavenging compounds are ascorbate, glutathione, carotenoids, phenols, and amino-acids such as proline. Several of these compounds also play roles in osmotic adjustment and support plant growth and development on saline soils (Fini et al., 2011).

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil microorganisms that form mutualistic associations with plant roots, including about 90% of plants with agricultural importance (Smith & Read, 2008). The AM symbiosis plays an important role improving plant growth under salinity conditions (Santander et al., 2017), and several studies have showed that AM fungal inoculation improves water absorption and nutrient uptake under saline stress, resulting in greater photosynthetic efficiency, maintenance of nutrient balance, and up-regulation of the antioxidant systems (Estrada et al., 2013; Porcel et al., 2016; Ruiz-Lozano et al., 2012). Most species of AM fungi may associate with many plant species, and a single plant may be colonized by several different species of AMF (Smith & Read, 2008). Nevertheless, the responses of plants and their AMF may be different under saline stress conditions, suggesting different degrees of functional compatibility between specific AM fungal strains and plant species (Santander et al.,

2017). Also, AM fungi isolated from saline soils may have a higher ability to promote plant growth under saline stress compared to other fungi isolated from non-saline soils (Estrada, Barea, Aroca, & Ruiz-Lozano, 2013).

Lettuce (*Lactuca sativa* L) is a widely-consumed fresh vegetable that is sensitive to salinity, with a threshold electrical conductivity of 1.3 dS m^{-1} (Shannon & Grieve, 1998). In arid areas, lettuce production is restricted to those locations where salinity is low or can be modified (Costigan, 1986). AM fungi have been shown to establish symbiosis with lettuce and alleviate saline stress (Aroca et al., 2013; Jahromi, Aroca, Porcel, & Ruiz-Lozano, 2008; J. M. Ruiz-Lozano, Azcon, & Gomez, 1996); however, little is known about the mechanisms of increased salinity tolerance induced by AM symbioses under salinity conditions, especially how the enzymatic and non-enzymatic mechanisms may be regulated in lettuce plants. Therefore, the aim of this study was to compare the efficiency of two AM fungal inocula in improving saline stress tolerance in lettuce plants through modulation of antioxidant defense mechanisms of lettuce exposed to salt. One inoculum was isolated from saline soils in the Atacama Desert, the most arid ecosystem worldwide, and the other inoculum was a reference strain isolated from a non-saline agricultural soil, which will allow us to evaluate the existence of possible environmental adaptations based on the conditions of indigenous soils where the AM fungi were obtained.

5.2 Material and methods

5.2.1 Experimental design

A completely randomized factorial 3 × 3 × 2 design included three AMF inoculants, three salinity treatments, and two cultivars of lettuce. The AMF inoculations were: (1) non-

inoculated plants (NI); (2) plants inoculated with a consortium containing indigenous ecotypes of AM fungi from Camiña valley (HMC); and (3) plants inoculated with the reference AMF strain *C. claroideum* (Cc). For each level of AM fungal inoculation, we used three irrigation saline levels: (i) non-saline irrigation; (ii) plants irrigated with a 40 mM NaCl solution; and (iii) plants irrigated with an 80 mM NaCl solution, according to previous studies in similar conditions (Santander et al., 2019). Two cultivars of lettuce were cropped crossed with the AMF and salinity treatments. Thereby, the different combinations of these factors gave a total of nine treatments for each cultivar, and five replicates were used for each treatment per each lettuce cultivar. Plants were harvested after 60 days of cultivation.

5.2.2 Soil and biological materials

A clay-loam Andisol was collected from Mahuidache (Araucanía Region, Chile) at a 0-20 cm depth. The soil had a pH of 6.0 (measured in water, 2:5 w/v), 14% organic matter, and nutrient concentrations of (mg kg^{-1}): N, 18; P (molybdenum blue method), 10; and K, 235. The soil was sieved (2 mm), diluted with sand (<2 mm) (1:1 soil/sand, v/v), and sterilized by autoclave (121°C for 30 min on 3 consecutive days). Lettuce (*Lactuca sativa* L.) seeds of cv. 'Grand Rapids' (GR) and cv. 'Lollo Bionda' (LB) were sown on polystyrene trays with a mix of sterilized sand/vermiculite (1:1, v/v) and then transferred to pots filled with 1,500 g of the soil/sand mixture described above. The AM native consortium was isolated from the rhizosphere of *Baccharis scandens* plants in the Camiña Valley (Tarapacá Region, Chile, 19°18'38.20"S 69°25'35.24"W, placed into the Atacama Desert), representing an AM fungi consortium presumably well-adapted to salt stress. The electrical conductivity in the rhizospheric soil varied with soil depth, ranging from 4.5 dS m^{-1} at the surface to 7.4 dS m^{-1} at deeper rhizospheric soil layers. The model AM fungus,

C. claroideum, was isolated from the rhizosphere of wheat plants growing in a volcanic soil (La Araucanía Region, central-south Chile) and represents an AM strain presumably non-adapted to salt stress. This strain was produced at the germplasm collection of Centro de Investigación en Micorrizas y Sustentabilidad Agroambiental (CIMYSA). Both mycorrhizal inocula were bulked in an open-pot culture of *Zea mays* L., *Lactuca sativa* L., and *Bidens pilosa* L. and consisted in a mixture of soil, spores and mycelia. Ten grams of each inoculum, containing about 200 spores per gram were added to the corresponding treatment at sowing time. Control plants (non-inoculated) received the same amount of autoclaved mycorrhizal inoculum together with a 10 mL aliquot of a filtrate (in Whatman N°1 paper) of both AM inocula in order to supply the general microbial population free of AM propagules. Also, in each inoculated plant was added plus 10 ml filtrate from the other AM inoculum to provide the same soil conditions.

5.2.3 Growth conditions

The experiment was carried out under greenhouse conditions with temperatures of 18/26°C night/day, 50/60% relative humidity, and 16/8 h day/night photoperiod. Once germinated, the seedlings were maintained for 15 days in the trays, and then were transferred to pots and grown for 45 days. Salinity treatments were applied post-transplantation via irrigation with the respective saline and control solutions. They were irrigated every two days with 100 mL of solutions with concentrations of 0, 40, and 80 mM NaCl that maintained an electrical conductivity of 0.0004, 4, and 8 dS m^{-1}, respectively. The same amount of tap water was applied on alternate days to maintain soil moisture near to field capacity and to prevent excessive salt accumulation.

5.2.4 Biomass production and AM symbiosis

At harvest, shoot and root systems were weighted, separated, and subsamples (5 g) of fresh material from shoot were preserved for biochemical analyses. The percentage of AM colonization was determined by visual observation of root pieces (1 cm) after clearing and staining according to Phillips & Hayman (1970). The percentage of AM colonization was calculated according to the gridline intersect method (Giovannetti & Mosse, 1980), using a stereomicroscope (40–60 x magnification).

5.2.5 Oxidative damage to lipids and proline content

Lipid peroxides were extracted by grinding 500 mg of fresh leaf tissues with an ice-cold mortar and 3 ml of 0.2% trichloroacetic acid (TCA). Homogenates were placed in microtubes (2 ml) and centrifuged at 17,000×g for 10 min at 4°C. The chromogen was formed by mixing 300 µL of supernatants with 1.2 mL of a mixture containing 20% (w/v) TCA and 0.5% 2-thiobarbituric acid (TBA), and finally incubating the mixture at 95°C for 30 minutes. After, the tubes were cooled rapidly in an ice bath, and then were centrifuged at 12,000×g for 10 minutes at 4°C. The supernatants were used for spectrophotometric readings at 440, 532, and 600 nm in a Synergy H1 Hybrid Multi-Mode microplate spectrophotometer (BioTek Instruments, Inc., USA). The malondialdehyde (MDA) contents were calculated according to method of Du & Bramlage (1992). The free proline concentration was determined using 0.5 g of fresh tissue and spectrophotometrically assayed at 530 nm according to the method of Bates, Waldren, & Teare (1973).

5.2.6 Antioxidant enzymes

For enzyme extracts and assays, 1-g samples of fresh leaves were frozen in liquid nitrogen and then ground in 3 mL of solution containing 0.1 M phosphate buffer (pH 7.0) and 2.5% (w/v) polyvinylpyrrolidone (PVPP). The homogenate was centrifuged at 13,000×g for 15 min at 4°C, and the supernatant was collected for enzyme assays. Protein in the enzyme extract was estimated according to Bradford (1976). Protein content and enzymatic assays were measured by multi-mode reader. Briefly, the superoxide dismutase (SOD) activity assay was based on the method of Beyer & Fridovich (1987) with slight modifications. This activity is based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. The absorbance was measured at 540 nm. The activity of SOD was expressed as enzyme unit (EU) mg^{-1} protein. One unit of SOD was defined as the amount of protein causing 50% decrease of the SOD-inhibitable NBT-reduction. Catalase (CAT) activity assay was carried out according to method of Aebi (1984). The reaction mixture contained 20 mM H_2O_2 in 0.1 M phosphate buffer (pH 7.0). CAT activity was determined as a decrease in absorbance at 240 nm for 3 min following the decomposition of H_2O_2 at 25°C. Ascorbate peroxidase (APX) activity assay was measured according the method described in Nakano & Asada (1981), following the oxidation of ascorbate to dehydroascorbate at 290 nm at 25°C. The reaction mixture contained 20 mM H_2O_2, 0.1 M phosphate buffer (pH 7.0), and 20 mM ascorbic acid.

5.2.7 Identification and quantification of phenolic compounds and antioxidant capacity

Phenolic compounds were extracted according to Llorach, Martínez-Sánchez, Tomás-Barberán, Gil, & Ferreres (2008), with some modifications. One-gram samples of fresh leaves were pulverized in liquid nitrogen, mixed with 8 mL of a mixture of MeOH:water:formic acid (25:24:3, v/v/v) followed by 1 min of ultrasonication at 80% amplitude, shaken for two hours, and centrifuged at 4,000×g for 20 min. HPLC-DAD analyses were carried out using a Shimadzu HPLC system (Tokyo, Japan) equipped with a quaternary LC-20AT pump with a DGU-20A5R degassing unit, a CTO-20A oven, a SIL-20a autosampler and an UV-vis diode array spectrophotometer (SPD-M20A). Instrument control and data collection were carried out using Lab Solutions (Shimadzu, Duisburg, Germany). Identity assignments were performed on an Agilent 1100 Series system (Agilent, Germany), equipped with DAD (G1315B) and LC/MSD Trap VL (G2445C VL) electrospray ionization mass spectrometry (ESI-MSn) system, and coupled to an Agilent Chem Station (version B.01.03) data-processing station. The chromatographic separation method (HPLC-DAD) for the determination of phenolic compounds, mainly hydroxycinnamic acids and flavonols, was developed based on the procedure reported by Parada et al. (2018) through the use of a Kromasil ClassicShell-2.5-C_{18} (4.6 x 100 mm, 2.5 µm) column and a C_{18} precolumn (Novapak, Waters, 22 x 3.9 mm, 4 µm). The samples were injected using water:acetonitrile:formic acid (92:3:5 v/v/v) and water:acetonitrile:formic acid (45:50:5 v/v/v) as A and B mobile phases, respectively, with an elution gradient between 6 to 50% B over 30 minutes at 0.55 mL min^{-1} and 40°C.

Identities were assigned by comparison of the MS/MS spectra with those from the literature data and by comparison with commercial standards. MS/MS conditions were as follows: capillary temperature of 450°C, an auxiliary flow rate of 15 arbitrary units and −4,000 V ionization voltage (Ruiz et al., 2018). Flavonols and chicoric acid were quantified at 360 nm and chlorogenic acids at 320 nm, using rutin (R5143), chicoric

acid (C7243), and 5-caffeoylquinic acid (C3878) (Sigma-Aldrich, Steinheim, Germany), respectively, as external calibration standards. Total phenol concentrations were determined by the method of Alonso, Guillén, Barroso, Puertas, & García (2002) adapted to a microplate reader as described in Parada et al. (2018). The absorbance was read at 750 nm, and gallic acid was used as a standard. Results were expressed as milligrams of gallic acid per gram of fresh weight (FW). The $ABTS^{\bullet+}$ assay was used to determine the antioxidant capacity according to the method of Espín et al., (2000) with some modifications and adaptation to a microplate reader. In a 96-well microplate, 245 uL of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$ radical) were added and the first read was made (A1). Then, 5 uL of Trolox curve (0 to 0.5 mM) or 10 uL extract was added, and incubated for 30 min at 30°C in the dark to finally make a second reading (A2). The measurements were carried out at 734 nm.

5.2.8 Statistical analysis

For all the studied variables, factorial three-way ANOVAs with AM inoculum, NaCl levels, lettuce cultivars, and all the interactions as the sources of variation were performed. The significance level was established at $P < 0.05$. Data sets not meeting the assumptions for ANOVA were transformed as required, but the results are here presented in their original scale of measurement. In case of significant interaction between factors, the means of all treatments were compared with others by means of the Tukey´s multiple range test. When there was not significant interaction between factors, inoculum effects under all saline conditions were evaluated, with separate analyses for GR and LB cultivars by ANOVA, followed by Tukey´s multiple range tests. Data sets were also subjected to principal component (PC) and correlation analyses in order to establish the relationships among different variables of response and the PCs. In addition, the factors obtained were

subjected to non-hierarchical cluster analysis using the farthest neighbor method in order to determine the similarity among the different experimental conditions. Statistical analyses were performed using IBM SPSS Statistics software v. 23 (IBM Corp.).

5.3 Results

5.3.1 AM root colonization

There was a significant interaction between cultivar, salinity level and AM inoculum sources on mycorrhization (Fig. 1). Mycorrhization in GR did not present significant differences between HMC and Cc strains at any NaCl level. The highest level of root colonization occurred in the GR cultivar. In GR inoculated with HMC, the percentage of mycorrhization ranged from 65 to 67%, independent of salinity level. In contrast, salinity enhanced colonization by Cc between 0 (61%) and 40 or 80 mM NaCl (72%, for both). In LB, inoculation with HMC increased colonization by 24% at 40 mM compared to 0 mM NaCl. In contrast, AM colonization declined by 30 and 44% at 80 mM NaCl in comparison to 0 and 40 mM NaCl, respectively. There were no AM fungal structures in non-inoculated plants.

5.3.2 Biomass production

A significant interaction between cultivar, salinity level, and inoculum on shoot fresh weight (SFW) also occurred (Fig. 1). SFW declined with increasing salinity levels. The AM-colonized plants with either inoculum source exhibited improved SFW compared to non-inoculated plants under all saline conditions. In the GR and LB cultivars, the highest SFW was obtained when plants were inoculated with HMC under non-saline conditions. When subjected to saline stress, GR-inoculated plants produced 87% (HMC) and 60% (Cc) more SFW than non-inoculated plants at 40 mM NaCl.

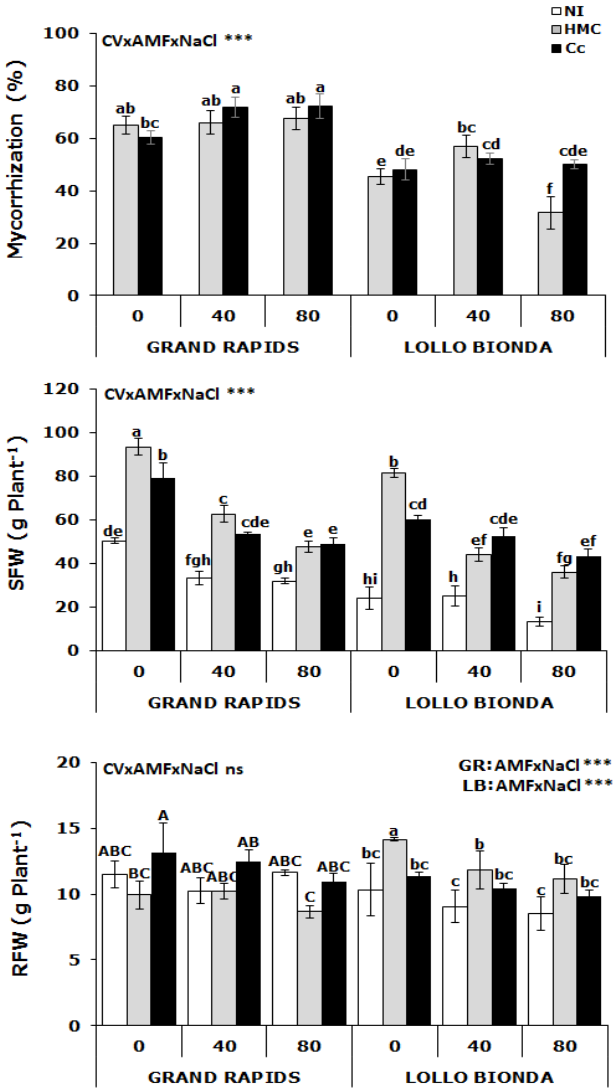

Fig. 1. **Effects of AMF inoculation on plant biomass and colonization under saline conditions.** The data includes means ± SE (n = 5). The data was analyzed through a three-way ANOVA conducted with Cultivar, AMF, and NaCl as sources of variation. The significance of the sources of interaction (CV x AMF x NaCl) was determined through the P-values: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. When no significant interaction between factors was observed, inocula effects at 0, 40 and 80 mM NaCl (AMF x NaCl) were evaluated through separate analyses by each cultivar with ANOVA. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

At 80 mM NaCl, GR plants inoculated with either HMC or Cc also exhibited higher SFW (48 and 52%, respectively) than non-inoculated plants. In the LB cultivar, both inocula showed similar increases in SFW compared with non-inoculated plants at 40 and 80 mM NaCl, ranging from 74 to 170% for HMC and 109 to 224% for the Cc strain. Root fresh weight (RFW) was not affected by the multiple interactions of cultivar, salinity level, and type of inoculum, but it was affected by interaction of salinity and AM inoculum when was analyzed per each cultivar (Fig. 1). RFW declined slightly in both cultivars at increasing NaCl and RFW tended to be greater in plants colonized by HMC in the LB cultivar (Fig. 1).

5.3.3 Lipid peroxidation and proline accumulation

Lipid peroxidation was affected significantly by cultivar, salinity level, and source of inoculum (Fig. 2). The highest values of lipid peroxides were in GR non-inoculated plants. In contrast, GR plants inoculated with HMC or Cc exhibited significantly lower lipid peroxidation compared to non-inoculated plants at 40 and 80 mM NaCl, ranging from 74 to 69% for HMC and 68 to 43 % for the Cc strain (Fig. 2). Lipid peroxidation was lower in the LB than GR cultivar in non-inoculated plants at 40 mM NaCl. Inoculation did not affect the MDA concentration at 0 or 40 mM NaCl. In contrast, inoculation with HMC and the Cc strain reduced lipid peroxidation damage compared with non-inoculated plants at 80 mM NaCl (Fig. 2).

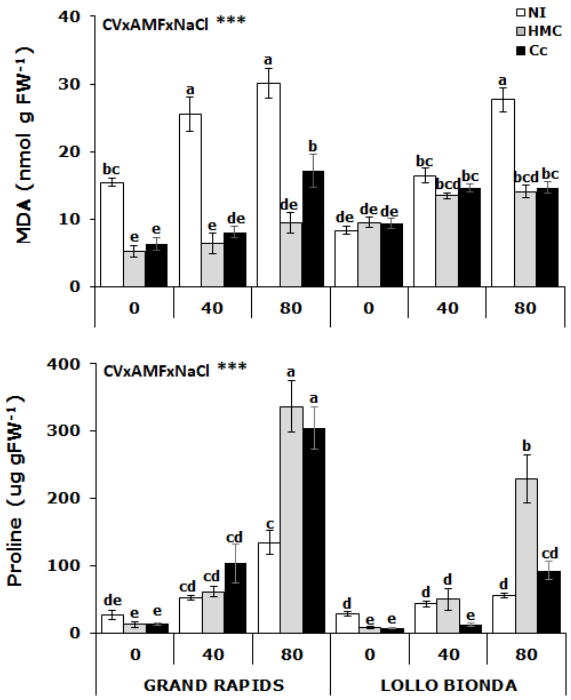

Fig. 2. Effects of AMF inoculation on lipid peroxidation (MDA) and proline content under saline conditions. The data includes means ± SE (n = 5). The data was analyzed through a three-way ANOVA conducted with Cultivar, AMF, and NaCl as sources of variation. The significance of the sources of interaction (CV x AMF x NaCl) was determined through the P-values: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

The proline content was affected significantly by cultivar, salinity, and source of inoculum (Fig. 2). Proline increased considerably when the plants were grown under high saline condition, but this increase depended on mycorrhizal source. In GR plants, the accumulation of proline was not significant between AM plants and non-inoculated plants at 40 mM NaCl. In contrast, the GR cultivar inoculated with HMC and Cc strains exhibited increased accumulation of proline at 80 mM NaCl by 128 and 152%, respectively, compared to non-inoculated plants. Similarly, the inoculation of the LB

cultivar with either HMC or the Cc strain increased the accumulation of proline by 314% and 67%, respectively (Fig. 2).

5.3.4 Antioxidant enzyme activity

Catalase (CAT) and ascorbate peroxidase (APX) activities were significantly affected by cultivar, salinity, and AM inoculum; superoxide dismutase (SOD) activity was not affected by multiple interactions, but it was affected by interaction of salinity and AM inoculum when was analyzed per each cultivar (Fig. 3). For SOD, GR plants inoculated with HMC exhibited 1.2- and 1.7-fold increases compared to non-inoculated plants or plants inoculated with the Cc strain at 80 mM NaCl. LB plants inoculated with Cc showed 1.5- and 1.3-fold elevated SOD activity at 40 mM NaCl and a 2.2- and 1.2-fold elevated SOD at 80 mM NaCl compared to non-inoculated and HMC-inoculated plants, respectively (Fig. 3). The lowest APX activity was noted in non-inoculated plants of the GR cultivar inoculation with HMC and Cc increased APX activity 39- and 101-fold at 40 mM NaCl and 24- and 49-fold at 80 mM NaCl (Fig. 3). Likewise, LB plants inoculated with HMC and Cc sources exhibited increased APX activity, ranging from 3.3- to 3.7-fold higher at 40 mM NaCl and 2.6- to 3.7-fold higher at 80 mM NaCl (Fig. 3). CAT activity was higher in plants inoculated by HMC and Cc in the GR cultivar, ranging from 4.8- to 5.8-fold higher at 40 mM NaCl and 4.9- to 4.6-fold higher in plants growing at 80 mM NaCl. Likewise, HMC inoculation in LB plants increased CAT activity under all saline conditions, being significantly greater than non-inoculated plants and plants inoculated with Cc (Fig. 3).

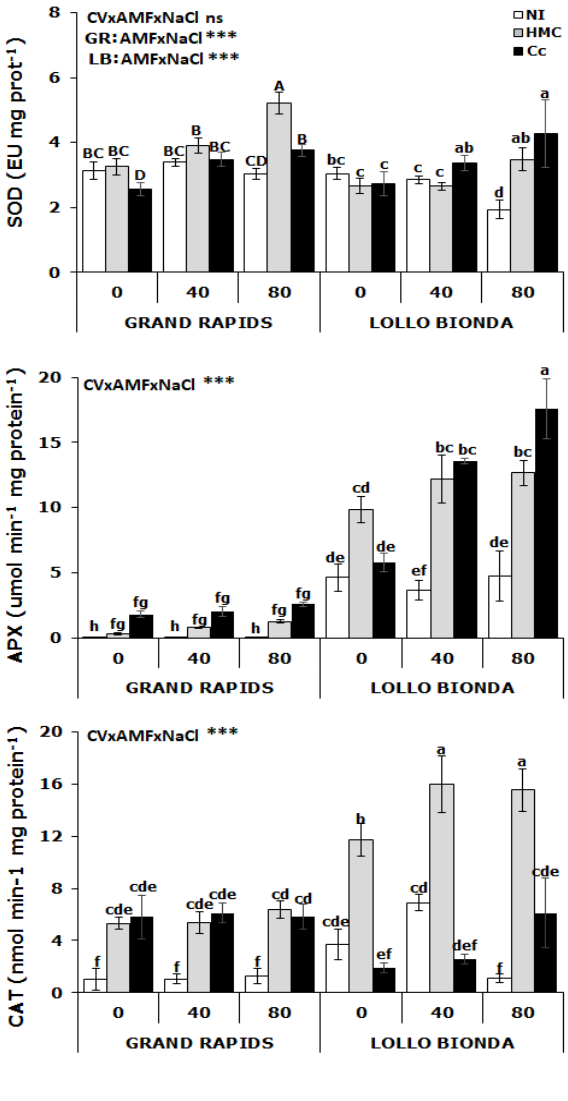

Fig. 3. Effects of AMF inoculation on superoxide dismutase activity (SOD), ascorbate peroxidase activity (APX) and catalase activity (CAT) under saline conditions. The data includes means ± SE (n = 5). The data was analyzed through a three-way ANOVA conducted with Cultivar, AMF, and NaCl as sources of variation. The significance of the sources of interaction (CV x AMF x NaCl) was determined through the P-values: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. When no significant interaction between factors was observed, inocula effects at 0, 40 and 80 mM NaCl (AMF x NaCl) were evaluated through separate analyses by each cultivar with ANOVA. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

5.3.5 Phenolic compounds profiles and antioxidant activity

Nine phenolic compounds belonging to hydroxycinnamic acids and flavonols were detected in lettuce leaves based on their UV-vis and MS/MS spectra, which were similar in both lettuce cultivars (Table 1; Fig. 4). Identification of 5-caffeoylquinic acid (peak 3) was confirmed by comparison with retention time of a commercial standard; however, the identity of caffeoylquinic acid isomer (peak 2) was not confirmed due to lack of coincidence of the retention time of the peak with the available standards 3-caffeoylquinic and 4-caffeoylquinic acid. Identification of the quercetin-hexoside (peak 5) isomer was also not confirmed due to lack of coincidence with the retention time of the commercial standard quercetin-3-glucoside, in contrast to reports by Romani *et al*. (2002) and Llorach *et al*. (2008) in lettuce; for this reason, this compound may possibly correspond to quercetin-5-glucoside or quercetin-7-glucoside, all with similar fragmentation patterns characterized by a pseudomolecular ion with m/z 463 and a product ion of m/z 301 in the negative ion mode. It is important to consider that glucoside and galactoside derivatives present similar fragmentation patterns by MS, characterized by a neutral loss to m/z 162; however, 3-galactoside was discarded due to the elution order, because galactoside derivatives must eluted at lower retention times than glucoside derivatives. Nevertheless, only three hydroxycinnamic acids and two flavonols were quantifiable by HPLC-DAD by external calibrations (Fig. 4).

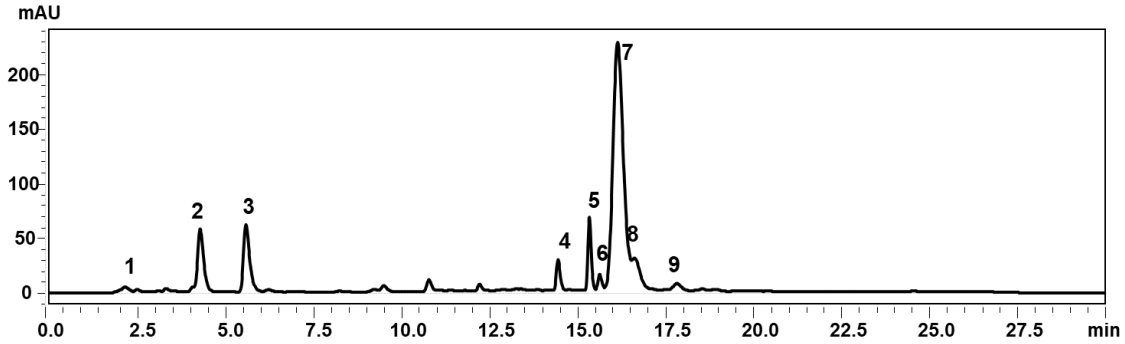

Fig. 4. HPLC-DAD chromatogram (320 nm) of phenolic compounds as flavonols and phenolic acids in lettuce leaves growing under saline conditions. Identifications according Table 1.

Table 1. Identification of phenolic compounds in lettuce leaves growing under saline conditions, by HPLC-DAD-ESI-MS/MS. Identifications according figure 5.

Peak	**t_R (min)**	**Identity assignment**	**λ_{max} (nm)**	**$[M - H]^-$ *(m/z)***	**Product ions *(m/z)***
1	2.0	caftaric acid isomer	-	311.7	178.6; 148.7
2	4.3	caffeoylquinic acid isomer	328	353.5	190.6
3	5.5	5- caffeoylquinic acid	325	353.5	190.7
4	14.4	quercetin-3-glucuronide	352	477.3	300.6
5	15.2	quercetin-hexoside	353	463.0	300.6
6	15.6	caftaric acid isomer	325	311.7	148.6; 178.7
7	16.2	chicoric acid	329	473.8	310.7; 178.9
8	16.7	dicaffeoylquinic acid isomer	329	515.6	352.7; 190.8
9	17.9	quercetin-acetylhexoside	351	505.3	462.7; 300.6

Phenolic compound concentrations were significantly affected by cultivar, salinity, and AM inoculum source (Fig. 5). In non-inoculated GR, phenolic compounds increased strongly from 0 to 80 mM NaCl, but these responses were mitigated by either HMC or Cc. Likewise, phenolic compounds increased strongly in the non-inoculated LB cultivar from 0 to 40 mM NaCl, but diminished from 40 to 80 mM NaCl. As with GR, these changes in phenolic compounds were reduced by mycorrhizal fungi, especially Cc in this cultivar (Fig. 5). Chicoric acid was the most abundant hydroxycinnamic acid, with 5-caffeoylquinic and caffeoylquinic acid isomer also contributing substantially. Additionally, two flavonols were quantified, quercetin-3-glucuronide and quercetin-hexoside, and the concentrations of these were higher in non-inoculated plants than in AM-inoculated plants under all saline conditions (Fig. 5).

Total leaf antioxidant activity (ABTS) suggested that non-inoculated plants under salt exposure were under greater oxidative stress than plants colonized by HMC or Cc (Fig. 6). ATBS activity increased in non-inoculated plants of both cultivars when exposed to 40 or 80 mM NaCl. In plants colonized by HMC, ABTS activity was not responsive to NaCl in either cultivar; ABTS activity increased at 40 mM NaCl in GR inoculated with Cc, but in LB, ABTS was low and did not respond to salt exposure (Fig. 6).

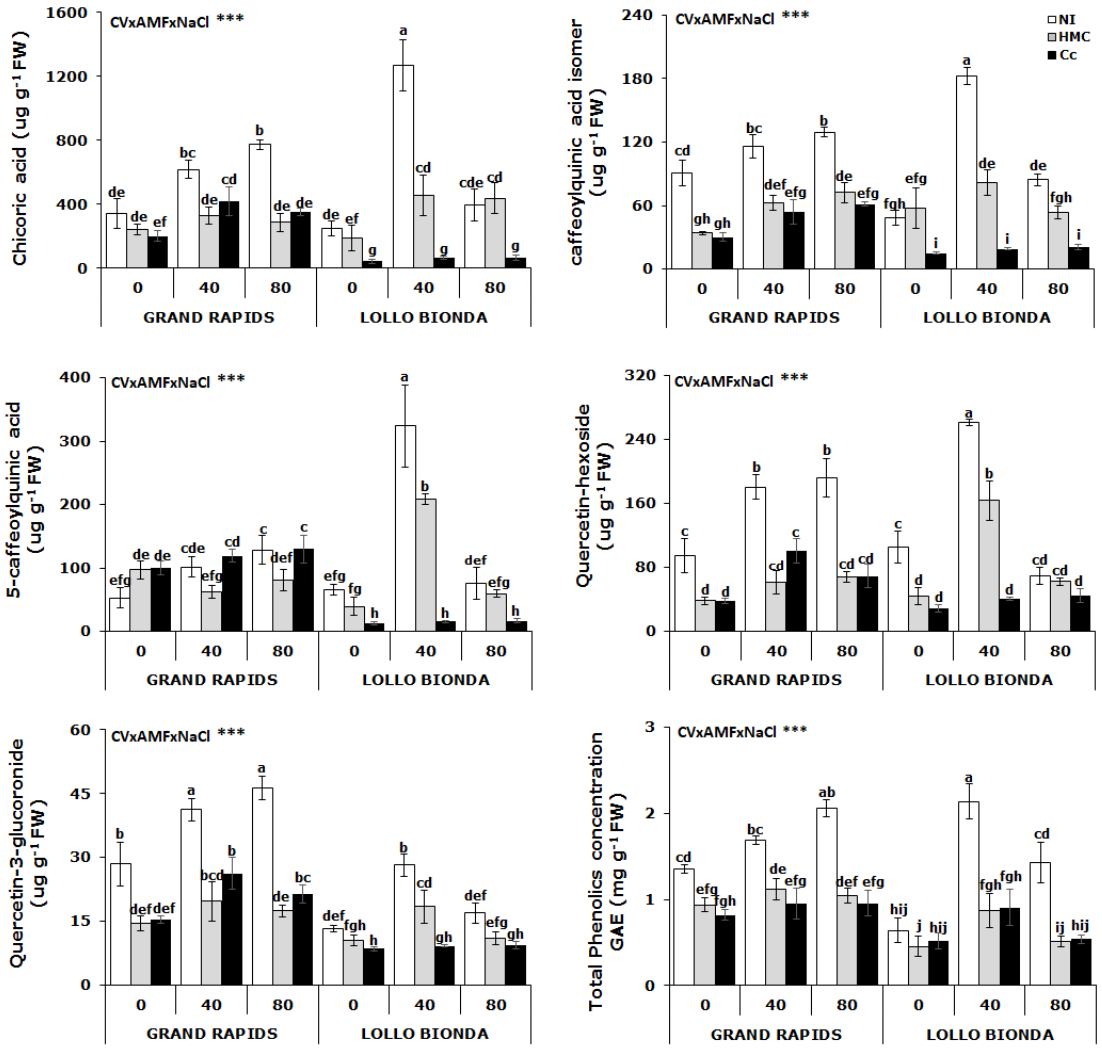

Fig. 5. **Effects of AMF inoculation on content of chicoric acid, caffeoylquinic acid isomer, 5-caffeoylquinic acid, quercetin-3-glucoronide and quercetin-hexoside, under saline conditions**. The data includes means ± SE (n = 5). The data was analyzed through a three-way ANOVA conducted with Cultivar, AMF, and NaCl as sources of variation. The significance of the sources of interaction (CV x AMF x NaCl) was determined through the P-values: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

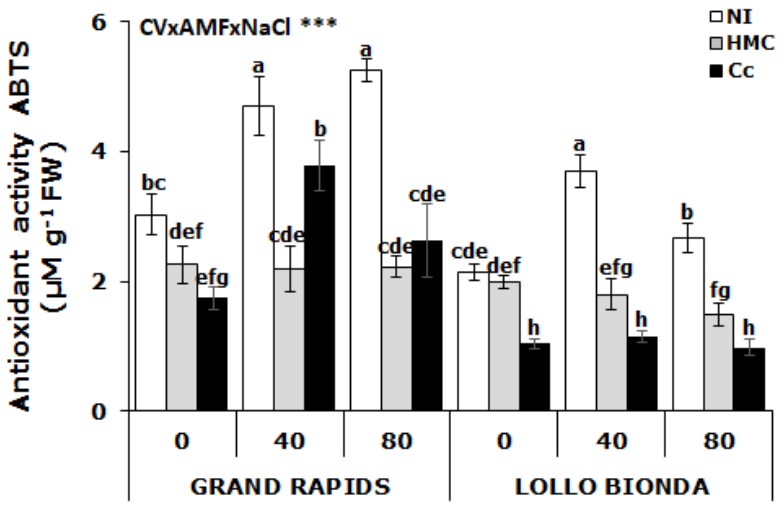

Fig. 6. **Effects of AMF inoculation on antioxidant activity (ABTS) under saline conditions**. The data includes means ± SE (n = 5). The data was analyzed through a three-way ANOVA conducted with Cultivar, AMF, and NaCl as sources of variation. The significance of the sources of interaction (CV x AMF x NaCl) was determined through the P-values: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

5.3.6 Multivariate associations

The principal components (PC) analysis reflected the formation of highly homogeneous groups of experimental variables (Fig. 7a), where PC1 explains 38.5% and PC2 explains 17% of the total experimental variance. PC1 was positively influenced by lipid peroxidation, content of chicoric acid, 5-caffeoylquinic acid, caffeoylquinic acid isomer, quercetin-3-glucoronide, and quercetin-hexoside, as well as total phenol concentration and ABTS. PC2 was positively related to shoot biomass production and mycorrhizal colonization, as well as proline content and SOD, CAT, and APX activity. The PCA, together with cluster analysis, differentiated eight well-defined groups (Fig. 7b). Noticeably, group 1 included LB plants inoculated either with HMC or Cc grown at 0 mM NaCl and LB plants inoculated by Cc at 40 mM NaCl. Group 2 included LB plants

inoculated either with HMC or Cc and grown at 80 mM NaCl. Group 4 included LB plants inoculated by HMC at 40 mM NaCl. These groups are represented by LB plants that reached high biomass production, and exhibited high antioxidant activity and low oxidative stress. In contrast, group 3 included non-inoculated LB plants at 0 and 80 mM NaCl, and group 6 include non-inoculated LB plants grown at 40 mM NaCl. These two groups were formed by LB plants with the lowest growth, the highest oxidative damage, and the highest concentrations of phenolic compounds. Groups 5 and 6 included non-inoculated GR plants growing under all saline conditions and represented GR plants with lower biomass production, and higher oxidative damage, phenolic contents, and ABTS. Finally, groups 7 and 8 were formed by GR plants inoculated with either HMC or Cc at all saline conditions, which exhibited high biomass production and high antioxidant activity concomitantly with low oxidative stress for each salinity level.

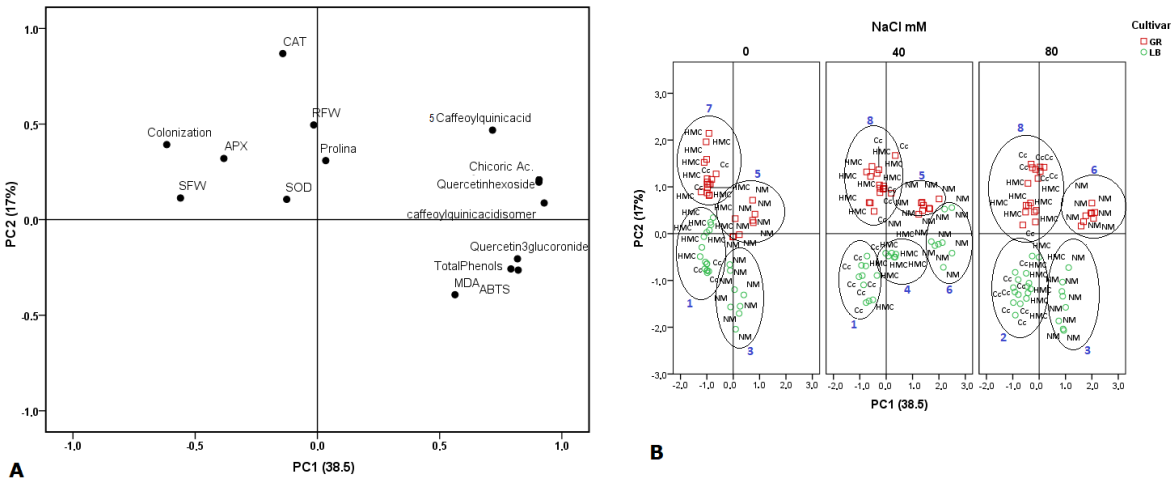

Fig. 7. A) PCA scores for the respective combinations between cultivars, inoculum type and NaCl levels added. The mean value was used in each situation. Percentage values in parentheses indicate the variation explained by each PC. B) Cluster analysis for the experiment. The circles comprise individuals with similar characteristics according to the analysis and should be understood as a visual aid for discrimination of groups.

5.4 Discussion

AM fungi are recognized for their ability to improve plant tolerance to saline stress by producing a root-soil interface that enhances the absorption of water and nutrients (Santander et al., 2017). These improvements will vary for each AM fungal isolate based on their root colonization capacity, hyphal production, innate stress metabolic systems, and production of exudates that modify the rhizosphere according to the ecosystems from which they were isolated (Juniper & Abbott, 2006). In our study, both AM inocula colonized the roots of GR and LB lettuce plants to a high degree. However, the Cc isolate, a fungus commonly used as reference inoculum by our research group, and HMC, a native inoculum consortium isolated from saline soils from a natural ecosystem in the Atacama Desert, showed differential colonization capacity for each cultivar. As reported by Estrada, Aroca, et al. (2013). AM associations are considered non-specific symbioses and each AM fungal species could have different colonization capacity and symbiotic efficiency that could vary under different salinity levels. Here, the Cc fungus increased colonization in the GR cultivar with increasing salinity levels, whereas the levels of salt did not affect colonization in LB. In contrast, colonization by HMC diminished at high salinity levels in LB plants, consistent with other previous studies in lettuce (Aroca et al., 2013; Jahromi et al., 2008). According to Juniper & Abbott (2006) salinity decreases the colonization capacity of AM fungi by suppressing hyphal growth, sporulation, and spore germination. The reason why Cc, an AM fungus presumably not adapted to saline stress, promotes high rates of root colonization is unknown and difficult to explain under *in vivo* conditions, but is a topic that deserves attention due to the possibility of expressing functional responses in plants by specific AM fungal symbionts.

Plants subjected to salinity stress exhibit reduced growth mainly due to the negative effects of high osmotic potential of saline soils, which produce lesions related to

metabolism, water relations, and mineral nutrition (Santander et al., 2017). Plant biomass production is also the most obvious trait reflecting symbiosis-mediated plant performance under abiotic stress (Chen, Zhang, Zhang, & Tang, 2017). Several studies have found that AM fungi alleviate the toxic effects caused by salinity and improve plant growth under saline conditions (Pedranzani et al., 2016; Porcel et al., 2015; Juan Manuel Ruiz-Lozano et al., 2016; Santander et al., 2019). In the present study, although shoot biomass decreased as salt concentrations increased in the soil, AM-colonized plants exhibited higher biomass than non-mycorrhizal plants at any salinity levels, reflecting alleviation of salt stress in lettuce plants and also suggesting a high symbiotic efficiency of both AM inocula. It is assumed that these AM symbioses improve plant growth under saline stress by different mechanisms, such as increased water uptake, high mineral nutrient absorption, maintenance of ionic balance, accumulation of osmoprotectant solutes, prevention of ultrastructural damage, and, ultimately, enhanced photosynthetic activity (Calvo-Polanco et al., 2016; J. Chen et al., 2017; Zhang, Zhu, Zhao, & Yao, 2013).

In addition to osmotic challenges, high salt concentrations reduce plant growth due to an increase in the production of ROS (Evelin & Kapoor, 2014). Under saline stress, plants close their stomata in order to limit water loss, concomitantly limiting supply of CO_2 to chloroplasts. This uncouples the light from the dark reactions of photosynthesis and limits electron acceptors, causing the electron transport chain to be excessively reduced and ROS production to increase (Pérez-López et al., 2009). Overproduction of ROS results in oxidative stress, which triggers peroxidation of essential lipids in the membranes of the cell and intracellular organelles (Pedranzani et al., 2016). Highly efficient antioxidant systems, including both enzymatic and non-enzymatic antioxidants, are necessary to alleviate the oxidative stress and maintain normal plant metabolism (Gill & Tuteja, 2010). MDA is a product of membrane lipid peroxidation as a consequence of

oxidative stress caused by abiotic stress (Paradiso et al., 2008). Here, the maintenance of biomass production by plants colonized by both HMC and Cc was related with lower lipid peroxidation and MDA production compared to non-inoculated plants at all saline conditions. Hence, these AM symbioses protected plants by reducing the pathways for ROS production, maintaining the integrity of membranes, and stabilizing proteins and enzymes (Wu, Zou, & Fathi Abd-Allah, 2014).

All of the measured antioxidant enzyme activities were positively regulated by AM inoculation in both lettuce cultivars, and this stimulation was also associated with a lower lipid peroxidation indicating lower oxidative damage. The SOD activity in AM-inoculated plants increased significantly at high-salinity levels. These results agree with the findings of previous studies by Ruiz-Lozano, Azcón, & Palma (1996) and Mo et al. (2016). This increase in SOD would catalyze dismutation of superoxide to H_2O_2, subsequently preventing H_2O_2 rise by higher activities of CAT and APX (Liu et al., 2016). SOD acts as first line of defense to cope with ROS production, catalyzing the conversion of superoxide radical ($O_2^{\bullet-}$) or singlet oxygen ($^1O_2$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Mittler, 2002). H_2O_2 is a potentially dangerous subproduct of oxygen metabolism because it is highly reactive with molecules containing Fe^{2+} or other transition metals through Fenton reactions, which results in homolysis of H_2O_2 to two harmful °OH radicals (Sharma, 2013). However, H_2O_2 is removed of cell compartments through the enzymatic action of CAT and peroxidases (Mittler, 2002). Under both saline conditions, CAT and APX activities were strongly increased by AM symbiosis in both lettuce cultivars. CAT has a key role degrading H_2O_2 into water and oxygen, being localized in all differentiated peroxisomes and in mitochondria (Anjum et al., 2016). Similarly, APX catalyzes the transformation of H_2O_2 in H_2O using ascorbate (ASC) as a hydrogen donor and produces monodehydroascorbate. Plant APX is found in several

cellular compartments including chloroplasts, cytosol, mitochondria, peroxisomes, and microbodies (Asada, Allen, Foyer, & Matthijs, 2000). APX has higher affinity for H_2O_2 than CAT, tightly regulating ROS levels (Anjum et al., 2016). In the current study, APX showed higher enzyme activity than CAT, and the two AM inocula greatly increased APX activity in both cultivars as NaCl increased, whereas APX did not change in non-mycorrhizal plants. APX is part of ascorbate-glutathione cycle and it is known that the AM symbiosis regulates this cycle, inducing stress tolerance associated with efficient neutralization of H_2O_2, which has been analyzed through the expression levels of APX genes in AM plants compared with non-inoculated plants (Liu et al., 2016).

In our study, proline production was stimulated by symbiosis with both AM sources, exhibiting higher values at 80 mm NaCl. Several studies have shown similar results (Chitarra et al., 2016; Mo et al., 2016). In contrast, other studies have indicated that proline production is lower in AM plants versus mycorrhizal plants under stress conditions (Santander et al., 2017), which may reflect amelioration of the stress (e.g., maintenance of K^+/Na^+ ratios) upstream of proline synthesis (Garg & Manchanda, 2009). High proline content may be related to a reduction of oxidative damage in AM colonized plants in our study. It is well known that proline acts as a compatible solute in osmotic adjustment and also acts as a free radical scavenger, activating detoxification pathways and stabilizing subcellular structures and membranes, reducing ROS damage as shown by our results. Also, proline reduces Na^+-induced K^+ efflux, which could increase concentration of K^+ within plant cells (Anwar Hossain, Hoque, Burritt, & Fujita, 2014).

The main phenolic compounds determined in this study were phenolic acids (derivatives of caffeic acid, 70% of total) and flavonols (30%), which is in agreement with other reports for lettuce by Llorach et al. (2008). These same authors reported that phenolic acids represent 70–94% of the total phenolic content in green lettuce. Phenolic

compounds are an important group of plant secondary metabolites and, under salt stress, increasing their content may contribute to ROS protection, osmoregulation, or the general defense systems of plants (Alqarawi et al., 2014). The lowest concentrations of phenolic compounds was obtained in AM inoculated plants under all saline conditions, plants also with the least lipid peroxidation and greater growth. Several studies have shown contradictory results on phenolic compound accumulation in plants under stress. Our results are agree with Geneva, Stancheva, Boychinova, Mincheva, & Yonova (2010), who noted decreased concentrations of phenolic compounds in leaves of *Salvia officinalis* in symbiosis with *G. intraradices.* In contrast, several studies have showed significant increases in the concentrations of phenolic compounds in plants inoculated with AM fungi (Chen et al., 2013; Zhang et al., 2013). In the current research, non-mycorrhizal plants increased total phenolic compounds and antioxidant activity under saline stress conditions. However, this response was not effective at detoxifying ROS and non-mycorrhizal plants exhibited high lipid peroxidation and low biomass production. The lower production of phenolic compounds by AM-colonized plants may again reflect alleviation of NaCl stress upstream of glycoside pathways. The accumulation of phenolic compounds is also related with tissue browning in plants under stress (López-Gálvez, Saltveit, & Cantwell, 1996) and once the plant has accumulated Na^+ in the shoot and suffers from the toxic effects of Na^+, the most visible symptom is yellowing, then browning, of leaves, due to leaf senescence and death(Negrão, Schmöckel, & Tester, 2017). Considering the fresh consumption of lettuce, a reduction in visible symptoms of Na^+ toxicity and oxidative damage strongly supports the application of efficient AM strains for use in lettuce crop in saline environments.

5.5 Conclusion

Our results provide new support regarding the beneficial role of the AM symbiosis in mitigating the negative effects of salinity stress in plants. Despite the strong differences of environments from which the two AM fungi were isolated, both inocula had similar behavior in conferring salinity tolerance to lettuce, an important horticultural crop. Interestingly, the two lettuce cultivars responded differently to each AM inoculum, with Grand Rapids having a higher AM colonization and perhaps AM dependency than Lollo Bionda at increasing salinity conditions. Nevertheless, both AM inocula enhanced the salinity tolerance, triggering overmodulation of the antioxidant enzyme activities compared to non-mycorrhizal plants, which was correlated with reduced oxidative stress and increased growth in AM plants. The AM symbiosis also changed the profiles of phenolic compounds produced in lettuce leaves, reducing their synthesis under saline conditions in comparison to non-mycorrhizal plants. The production of these phenolic compounds reflects the high degree of metabolic disruption induced by salinity in non-mycorrhizal plants and may be associated with browning and senescence of leaves. This modulation of the salinity stress response in lettuce by AM fungi through antioxidant enzyme pathways suggests that the use of efficient AM fungal strains can improve horticultural production in vast areas worldwide subjected to salinization.

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Chapter VI

"Arbuscular mycorrhizal symbiosis alleviates salt stress in lettuce plants regulating aquaporins activity and gene expression of cation transporters"

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Arbuscular mycorrhizal symbiosis alleviates salt stress in lettuce plants regulating aquaporins activity and gene expression of cation transporters

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Abstract

Salinity is a major constraint for agricultural crops with Na^+ as the major toxic ion due to competition with K^+, and inhibition of physiological and metabolic processes such photosynthesis and enzymatic activity. Arbuscular mycorrhizal (AM) fungi establish a symbiosis with most agricultural plants, helping to alleviate the impact of salinity by several responses in the host plant. The aim of this study was to determine the effects of AM symbiosis on the expression pattern of genes associated with K^+ and Na^+ uptake and translocation, and the K^+/Na^+ homeostasis in lettuce cultivars, as well as the effect on the relative abundance of plant AQPs (PIP1 and PIP2) in relation with water parameters. Two AM fungi species (*Funneliformis mosseae* and *Claroideoglomus lamellosum*) isolated from the Atacama Desert (northern Chile) were inoculated to two lettuce cultivars (Grand Rapids and Lollo Bionda) growing under saline conditions. Results showed that salinity increased the AM colonization by both inocula. AM plants showed an improved growth, efficiency of photosystem II, water status and upregulated *LsaHKT1;1*, *LsaHKT1;6*, *LsaNHX2*, *LsaNHX4*, *LsaNHX6* and *LsaNHX8* genes encoding for plant transporters involved in ion homeostasis, and also an increased PIP2s abundance involved in plant water relations. Our results are contributing with new evidences about the role of AM symbiosis to cope with the negative effects of salinity stress in lettuce plants, being the first research assessing together *LsaHKTs*, *LsaNHXs* genes and PIPs abundance, and how they are modulated by the AM symbiosis.

Keywords: Aquaporins; Na^+/H^+ antiporter, High-affinity potassium transporters; salt stress; indigenous AMF strains.

6.1 Introduction

Salinity is one of the major constraints to plant growth and productivity (Wang et al. 2003), currently progressing in most cropped lands (Munns and Tester 2008). The salinity affects morphological, physiological and molecular processes (Hasegawa et al. 2000) by two ways: i) osmotic stress caused by high salt concentrations that produce a negative osmotic potential of soil solution, decreasing plant water uptake generating a reduction in leaf expansion and a loss of turgor (Hasegawa et al. 2000; Tester and Davenport 2003; Ali et al. 2019), and ii) ion imbalance caused by excessive accumulation of Na^+, producing an ionic stress due to their toxicity (Zhu, 2002).

At molecular level, many ion channels and transporters play an important role in maintaining the ion homeostasis in plants under saline conditions (Yamaguchi et al. 2013), highlight the plant high-affinity potassium transporters (HKTs) that belong to the superfamily SKT of K^+ transporters (Levin and Zhou 2015). They are separated into two groups: i) HKT1, described as Na^+ uniporters found in both monocotyledons and dicotyledons, and ii) Na^+-K^+ co-transporter found in monocotyledons (Keisham et al. 2018). In the same way, NHXs transporters belong to the family of monovalent cation/proton antiporters 1 (CPA 1) (Saier et al. 2014), which transport Na^+ out of the cell or into the vacuole (Jia et al. 2018). NHXs antiporter family is categorized into two main groups: i) intracellular NHXs are localized on the tonoplast (NHX1 to NHX4), and two isoforms (NHX5 and NHX6) are localized in the Golgi, *trans*-Golgi network (TGN), and pre-vacuolar compartment (PVC) (Reguera et al. 2015); ii) plasma membrane-bound (NHX7/SOS1 and NHX8) reside on the plasma membrane (Ma et al. 2017). Additionally, under salinity stress plants also must face the problem of water uptake. In this sense, aquaporin proteins (AQPs) regulate water balance (Sade et al. 2010), being key for whole plant water transport (Bárzana et al. 2015). AQPs belong to a highly conserved super

family of membrane proteins known as major intrinsic protein (MIP) (Afzal et al. 2016). PIPs and TIPs families are the most abundant in membrane of plants, and work as the primary pathway for transcellular and intracellular water movement (Maurel et al. 2008).

The majority of plant species establish the arbuscular mycorrhiza (AM) symbiosis, which is widely found in roots of plants growing in saline soils (Smith et al. 2010; Estrada et al. 2013b). In plants, several mechanisms improved by AM fungi under saline stress have been reported, as the nutrient and water uptake, maintenance of ionic homeostasis, osmotic equilibrium, induction of antioxidant systems, protection of photosynthetic apparatus and enhancement of photosynthetic efficiency (Santander et al. 2017; Evelin et al. 2019). Lettuce plants are well-colonized by AM fungi, usually improving photosynthesis and nutrient uptake, even under saline stress (Jahromi et al. 2008; Aroca et al. 2013; Santander et al. 2019). However, to date little is known about the role of AM symbiosis in the molecular responses associated to water transport and regulation of Na^+ and K^+ homeostasis in lettuce plants, as well as their redistribution within the whole plant and among the cellular compartments under saline stress conditions.

Based on the previous, our aim was to determine the effects of AM symbiosis on the expression pattern of genes associated with K^+ and Na^+ compartmentalization and translocation, and the K^+/Na^+ homeostasis in lettuce cultivars, as well as the effect on the relative abundance of plant AQPs in relation with water parameters. The use of efficient salt-tolerant AM fungi can be a proper biotechnological tool for plant production in vast areas worldwide.

6.2 Material and methods

6.2.1 Identification of the AM fungal strains isolated from Atacama Desert

The AM fungi were isolated from the rhizosphere of *Baccharis scandens* (Ruiz & Pav.) plants in the Camiña Valley, Atacama Desert (Tarapacá Region, Chile, 19°18'38.20"S 69°25'35.24"W), and multiplied using monosporic mass crops with *Bidens pilosa* L. plants as host for six months. The spores of AM fungi were used for DNA isolation. The PCR was performed for the partial small subunit (SSU) ribosomal RNA gene, using primers NS31 (Simon et al. 1992) and AML2 (Lee et al. 2008). The reactions were performed according to Morgan and Egerton-Warburton (2017), with some modifications. PCR products were sequenced on an automated DNA sequencer ABI PRISM 3500xL (Applied Biosystems, CA, USA), by the sequencing service of Pontificia Universidad Católica de Chile, Santiago, Chile (CONICYT-FONDEQUIP EQM150077). Sequence data were compared to gene libraries (GenBank and MaarjAM database) using BLAST program (Altschul et al. 1990). Both isolated were identified and included to NCBI databases, *Funneliformis mosseae* with sequence accession number MN264635, and *Claroideoglomus lamellosum* with sequence accession number MN263071.

6.2.2 Experimental design

A completely randomized factorial 3 x 2 x 2 design was used, with three levels of AM fungal inoculation: (1) non-inoculated, (2) inoculated with *Claroideoglomus lamellosum* and (3) inoculated with *Funneliformis mosseae*. For each level of inoculation we used two irrigation saline levels: (i) non-saline conditions, and (ii) plants irrigated with a solution of 60 mM NaCl. Moreover, two cultivars of lettuce, Lollo Bionda (LB) and Grand Rapids (GR), were grown (12 treatments and 8 replicates, N = 96).

6.2.3 Soil and biological material

A loamy soil was collected at IFAPA (Granada, Spain), sieved to 2 mm, diluted with sand (1:1 soil/sand, v/v), and autoclave-sterilized (121 °C for 20 min on 3 consecutive days). The soil presented pH 8.1 (in water, 2:5 w/v); 0.85% organic matter, nutrient concentrations of (mg kg^{-1}): N 1; P 10; and K 110, and electrical conductivity (EC) of µS cm^{-1} (1:5, w/v). Surface-sterilized (chloramine-T 2% w/v during 5 min) seeds of LB and GR were sown on polystyrene trays with a mix of sterilized sand/vermiculite (1:1, v/v) and then transferred to pots filled with 1000 g of the soil/sand mixture described above. Ten grams of each inoculum, containing about 200 spores per gram were added per plant to the corresponding treatment at sowing time. Non-mycorrhizal control plants received the same amount of auto- claved mycorrhizal inocula together with a 10 ml aliquot of a filtrate (<20 µm) of the AM inocula in order to provide a general microbial population free of AM propagules. Also, in each inoculated plant was added plus 10 ml filtrate from the other AM inoculum to provide the same soil conditions.

6.2.4 Growth conditions

The experiment was carried out under greenhouse conditions with temperatures of 20/25°C night/day; 50-70% relative humidity and 16/8 h light/dark photoperiod (Estación Experimental del Zaidín, Granada, Spain). Once germinated, the seedlings were maintained for 15 days in the trays, and then transferred to pots where grown for 45 days. Salinity treatments were applied via irrigation with the respective saline and control solutions. They were irrigated every two days with 100 mL of solutions with concentrations of 0 and 60 mM NaCl. The same amount of tap water was applied on alternate days to maintain humidity near to field capacity and to prevent excessive salt

accumulation. During the growth period, plants received each week 10 mL per pot of Hoagland nutrient solution (Hoagland and Arnon 1950). At harvest, the EC in the growing substrate were 0.9 and 5.8 dS m^{-1} for 0 and 60 mM NaCl, respectively. Harvest was carried out after 8 weeks growing.

6.2.5 Biomass production, nutrients and AM symbiosis

At harvest, shoot and root systems were weighted, and subsamples (6 g) of fresh material were stored at -80 °C for molecular analyses. The residual material was dried (70°C, 48 h) in a forced-air oven for chemical analysis. P, K^+ and Na^+ were extracted from 100 mg of ground shoot and root dry material. Analyses were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES; Varian ICP 720-ES). The AM colonization was estimated by visual observation after clearing and staining with trypan blue according to Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse 1980).

6.2.6 Relative water content, osmotic potential, photosynthetic efficiency and stomatal conductance

Leaf relative water content (RWC) was measured according to Aroca et al. (2003). Leaf osmotic potential at full turgor (Ψ_π^{100}) was calculated as described by Aroca et al. (2006a). The osmotic potential of the collected sap was measured with a cryoscopic osmometer (Osmomat 030, GonotecGmbh, Berlin, Germany), using the second youngest leaf. The efficiency of photosystem II (Φ PSII) was measured *in vivo* with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic). Stomatal conductance (*gs*) was measured

2 h after the onset of photoperiod with a porometer system (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK), using the second youngest leaf.

6.2.7 Gene expression analysis

At harvest, total RNA was extracted from three biological replicates of roots and shoots and conserved at -80 °C. RNA extraction was carried out by phenol-chloroform method followed by precipitation with LiCl (Chang et al. 1993). The integrity of RNA was checked electrophoretically and quality was spectrometrically measured using NanoDrop (Thermo Scientific TM; NanoDrop 1000). The RNA was subjected to DNase treatment and reverse-transcription using the iScript^{TM} gDNA Clear cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions.

Table 1: Primer sequences used in the real time qPCR analysis.

Gene	ID	Primers (5´-3´)	Annealing temperature (°C)	Reference
LsaNHX2	XM_023872896	CTGGGTTTGTTGTTTTATAGGG	58	NCBI
		TCAAACCATCACTCACTTCAC		
LsaNHX3	XM_023888553	ACTTCAAATGGTCCGTTGTT	58	NCBI
		GTTCCCACCTCCAAACTAAAT		
LsaNHX4	XM_023888910	GACAGGACAAACTTTCTAGGG	57	NCBI
		AAGACCAAGACTCCCGTAAAT		
LsaNHX6	XM_023882462	CGAAATCTCAAAGTGTTAGGG	57	NCBI
		AGCATCTCAACTCCACTTGTT		
LsaNHX8	XM_023900505	GGTTTGGTGTCTTATTTGTGG	57	NCBI
		AAGCAAGGTAGCTAACAGCAA		
LsaHKT1;1	XM_023881311	GGTCAGTTGTGACTTCATGC	57	NCBI
		CACCATTACACACAATGCATAC		
LsaHKT1;6	XM_023884696	GTTTGTTGAAATCCGTTGTG	57	NCBI
		AATGGGTGGAAATACTCAGGT		
LsBtub3	AB232706	CAGGATCAGGAATGGGAACTC	57	Aroca et al. (2013)
		CCTTGGGAGAAGGGAATACAG		

The primer sets for each gene were designed with Primer3 tool, according to the genomic sequence deposited in GenBank, plus the other one described in other studies (Table 1). The gene expression analyses were carried out by qRT-PCR using 1 μL of diluted cDNA

(1:9) with PowerUp™ SYBR® Green Master Mix in a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) for 40 cycles (Table 1). The relative abundance of transcripts was calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Expression values were normalized using the housekeeping gene *LsBtub3*, encoding a beta-tubulin 3 (Aroca et al. 2013).

6.2.8 PIP abundance and PIP phosphorylation status

Microsomal fractionation was performed according to Hachez et al. (2006). Briefly, one gram of root and leaf were grinded with 6 mL of protein extraction buffer containing 250 mM Sorbitol, 50 mMTris-HCl (pH 8), 2 mM EDTA and protease inhibitors (1 µg mL^{-1} of Leupeptin, Aprotinin, Antipain, Chymostaton and Pepstatin). All subsequent steps were performed at 4 °C. The homogenate was centrifuged for 20 min at 10,000*g*. The supernatant was centrifuged for 2 h at 100,000*g* and the resulting pellet (microsomal fraction) was resuspended in 30 µL of suspension buffer (5 mM KH_2PO_4, 330 mM sucrose, 3 mM KCl, pH 7.8). For ELISA analysis, two micrograms of the protein extracts were processed. We used two antibodies that recognize several PIP1s and PIP2s (at a dilution 1:1000), and three antibodies that recognize the phosphorylation of PIP2 in the C-terminal region (at a dilution 1:1000): PIP2A (Ser-280), PIP2 B (Ser-283) and PIP2C (Ser-280/Ser-283) (Calvo-Polanco et al. 2014). In addition, one anti-NHX polyclonal antibody (Agrisera, AS09 484) that recognize cation/proton antiporter protein was used (at a dilution 1:8000). Goat anti-rabbit immunoglobulin g coupled to horseradish peroxidase (Sigma-Aldrich Co.) was used as a secondary antibody at 1:20000 for PIPs and 1:12000 for NHXs protein.

6.2.9 Statistical analysis

For all the studied variables, full-factorial three-way ANOVAs (lettuce cv., AM inoculum, NaCl treatment and interactions) were performed. Significance level was established at $P \leq 0.05$. The means were compared by the Tukey´s multiple range test. Data setsr were subjected to principal component (PC) and correlation analyses in order to establish the relationships among the different variables. In addition, the factors obtained were subjected to non-hierarchical cluster analysis using the farthest neighbor method to determine the similarity among the different experimental conditions. Statistical analyses were performed using IBM SPSS Statistics software v. 23 (IBM Corp.).

6.3 RESULTS

6.3.1 AM root colonization, biomass production and mineral concentrations

No significant interactions between cultivar, salinity level and AM inoculum on mycorrhization were found (Fig. 1A). Root colonization in GR inoculated by *C. lamellosum* did not show differences at all salinity levels. However, salinity increased from 17 to 37% the root colonization in GR inoculated by *F. mosseae*. The highest root colonization in LB was found at 60 mM NaCl, increasing from 12.6 to 23.3% and from 9.6 to 25.6% for *C. lamellosum* and *F. mosseae,* respectively.

Shoot and root biomass production responded to the interaction of cultivar, salinity level and type of inoculum (Fig. 1 B, C), being negatively affected the shoot fresh weight (SFW) in both cultivars. Under saline condition, the highest SFW production was found in inoculated plants. In this sense, an increase in SFW ranging from 20 to 14% (GR plants) and from 28 to 23% (LB plants) was observed at 60 mM NaCl in plants inoculated

by *C. lamellosum* and *F. mosseae*, respectively. When it compared both cultivars, the highest SFW was found in LB plants under saline condition (Fig. 1 B).

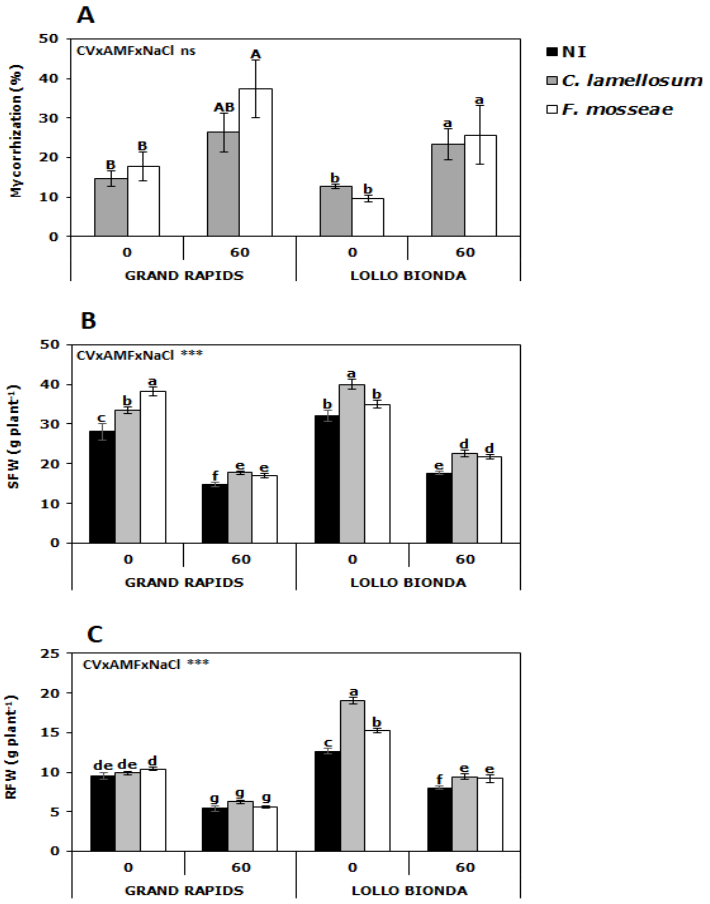

Fig. 1. **Percentage of colonization, shoot fresh weight (SFW) and root fresh weight (RFW) in lettuce plants non-inoculated (NI), inoculated by *Claroideoglomus lamellosum* and inoculated by *Funneliformis mosseae* subjected to two salinity levels (0 and 60 mM NaCl**). The data includes means ± SE (n = 8). The data was analyzed through a three-way ANOVA conducted with Cultivar, AMF, and NaCl as sources of variation. The significance of the sources of interaction (CVxAMFxNaCl) was determined through the P-values: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. When no significant interaction between factors was observed, inocula effects at 0 and 60 mM NaCl were evaluated through separate analyses by each cultivar with ANOVA. Different letters indicate significant differences ($P \leq 0.05$) according to Tukey's multiple range test. Grand Rapids (uppercase) and Lollo Bionda (lowercase) plants.

Moreover, root fresh weight (RFW) was significantly reduced by saline stress in both cultivars (Fig. 1 C). AM symbiosis did not have effect on the RFW improvement in GR plants at 0 and 60 mM NaCl. In contrast, both AM fungi improved RFW production in LB plants, ranging from 51 to 20% for plants inoculated with *C. lamellosum*, and from 17 to 20% for plants inoculated with *F. mosseae,* at 0 and 60 mM NaCl, respectively.

In GR plants, P shoot and root concentrations were increases by the AM symbiosis only in non-saline conditions. Contrarily, LB plants AM inoculated improved the shoot and root P concentration at all salinity concentrations compared to non-inoculated ones (Fig. 2 A, B). Shoot K^+ concentration in GR plants was only increased by *F. mosseae* under saline conditions compared to non-inoculated ones. In contrast, shoots of LB plants showed higher K^+ concentrations when were inoculated by both inoculants (Fig. 2C). The salinity increased the concentration of K^+ in roots of GR plants, specially plants inoculated with *F. mosseae*. In LB plants, both AM fungi produced a higher concentration of K^+ in roots compared to non-mycorrhizal plants at 0 mM NaCl, which was not observed at 60 mM NaCl (Fig. 2 D). The concentration of Na^+ in shoots and roots increased significantly at 60 mM NaCl (Fig. 2 E, F). In this condition, GR and LB inoculated plants did not exhibit a significantly diminishing of shoot Na^+ concentrations. However, both inoculants generated a lower concentration of Na^+ in roots compared to non-mycorrhizal plants. With respect to non-mycorrhizal plants, *C. lamellosum* decreased Na^+ concentrations between 30.3 and 29.3%, and *F. mosseae* reduced Na^+ concentrations between 20.6 and 27.8% in GR and LB cultivar, respectively. The shoot K^+/Na^+ ratio was affected by the interaction of cultivar, salinity and AM inoculum. In shoots and roots of both cultivars, the K^+/Na^+ ratio was negatively affected by salinity. The effect was more significant in shoots, where the differences between saline and non-saline treatments were highly significant (Fig. 3 A, B). AM inoculation did not improve K^+/Na^+ ratio in shoots

of GR and LB plants in neither of the saline conditions. Contrariwise, both AM inoculants had an improvement in value of K^+/Na^+ ratio in roots of GR and LB plants.

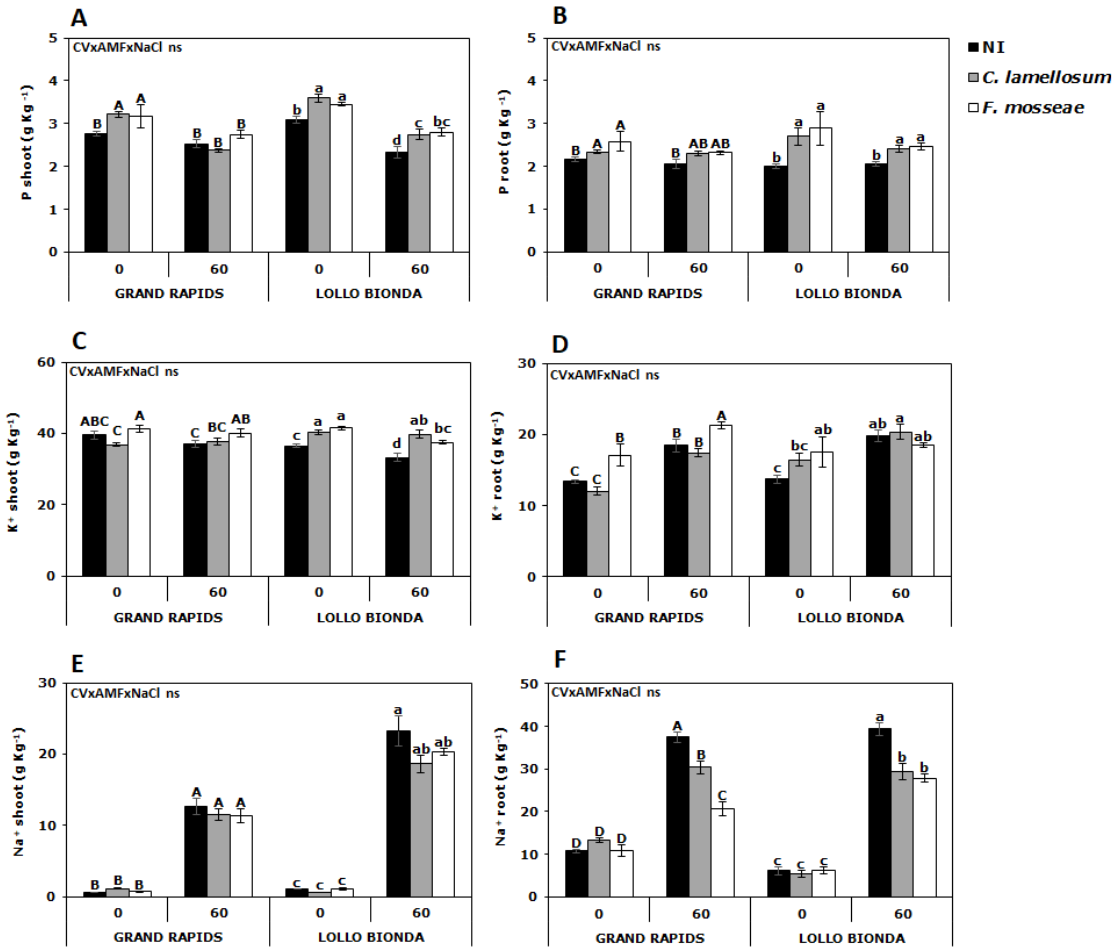

Figure 2: Concentration of P, Na^+ and K^+ (g Kg^{-1}) in shoots and roots of lettuce plants non-inoculated (NI), inoculated by *Claroideoglomus lamellosum* and inoculated by *Funneliformis mosseae* subjected to two salinity levels (0 and 60 mM NaCl). The data includes means ± SE (n = 5). See legend for Fig. 1.

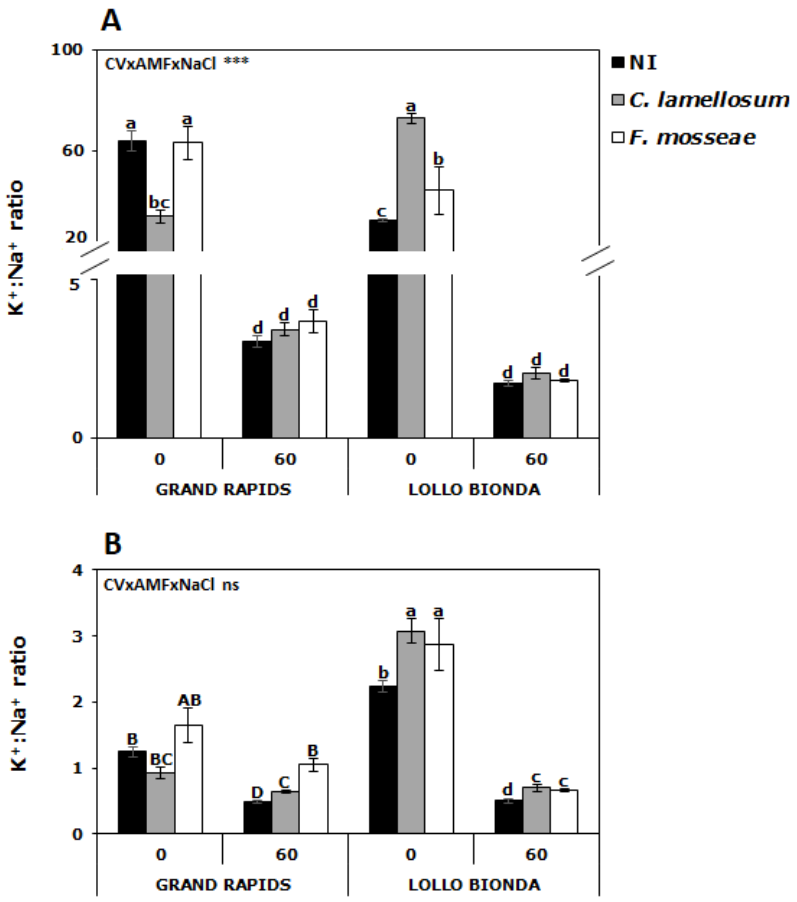

Figure 3: K^+/Na^+ ratio in shoots (A) and roots (B) of lettuce plants non-inoculated (NI), inoculated by *Claroideoglomus lamellosum* and inoculated by *Funneliformis mosseae* subjected to two salinity levels (0 and 60 mM NaCl). The data includes means ± SE (n = 5). See legend for Fig. 1.

6.3.2 Effect of AM symbiosis on water status

The RWC was not affected by the multiple interaction (Fig. 4 A). Instead, the leaf osmotic potential was affected by the interaction of cultivar, salinity and AM inoculum (Fig. 4 B). The saline stress decreased the RWC in both cultivars, being the highest RWC found in inoculated plants. In inoculated GR plants such increases were 7 to 10% for *C.*

lamellosum, and 5 to 9% for *F. mosseae*, compared to non-inoculated plants. Similarly, inoculated LB plants increased RWC from 5 to 9% compared to non-inoculated plants, for *C. lamellosum* and *F. mosseae* respectively (Fig. 4 A). The highest leaf value of osmotic potential were found in plants no subjected to saline stress, reaching values of -0.35 MPa, while saline stress reduced this variable in both cultivars. AM symbiosis did not affect this parameter in any saline condition (Fig. 4 B).

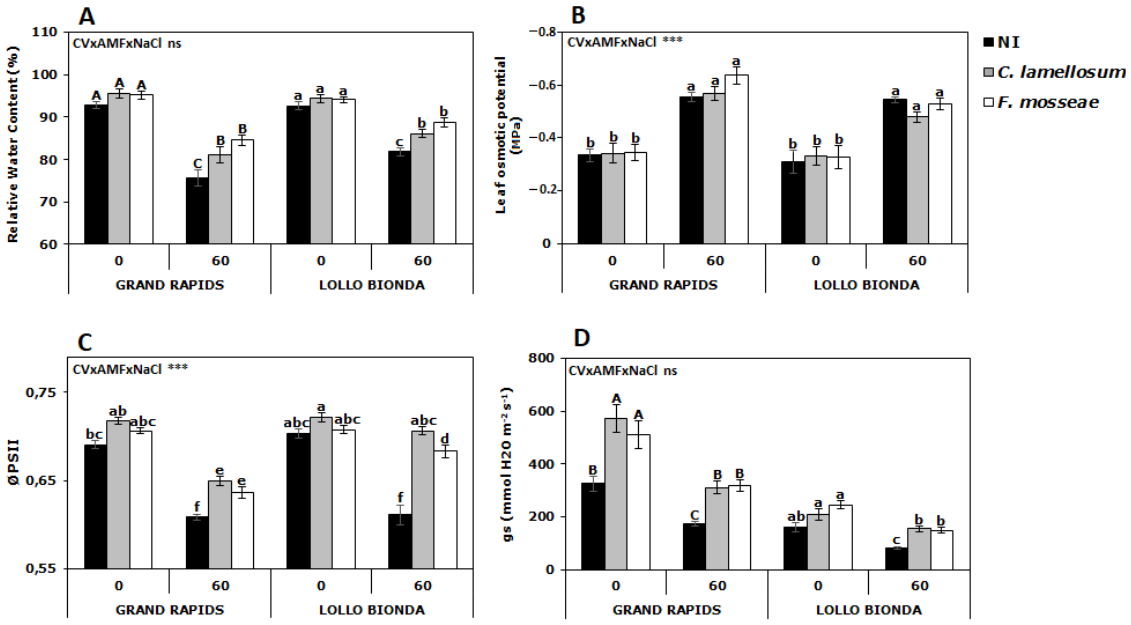

Figure 4: Relative water content, leaf osmotic potential, efficiency of photosystem II (ΦPSII) and stomatal conductance (gs) in leaves of lettuce plants non-inoculated (NI), inoculated by *Claroideoglomus lamellosum* and inoculated by *Funneliformis mosseae* subjected to two salinity levels (0 and 60 mM NaCl). The data includes means ± SE (n = 8). See legend for Fig. 1.

6.3.3 Efficiency of photosystem II and stomatal conductance

The ΦPSII was affected by the interaction of cultivar, salinity and AM inoculum; nevertheless, *gs* was not significant affected by the multiple interaction (Fig. 4 C, D).

ΦPSII was negatively affected by saline stress, mainly in GR cultivar, decreasing in colonized and non-colonized plants compared to non-saline conditions. Nevertheless, at 60 mM NaCl the ΦPSII was higher in inoculated GR plants compared to non-inoculated ones, with values ranging from 6 to 5% at 60 mM NaCl for *C. lamellosum* and *F. mosseae*, respectively (Fig. 4 C). LB cultivar at 60 mM NaCl showed the higher activity of ΦPSII in inoculated plants, compared to inoculated GR plants and non-inoculated LB plants (Fig. 4 C). The *gs* was enhanced by AM symbiosis in both cultivars under non-saline and saline conditions. At 60 mM NaCl, AM symbiosis increased the *gs* values in GR plants 79% (*C. lamellosum*) to 83% (*F. mosseae*). Similar behavior was found in LB plants, since both inocula improved the *gs* values near to 80% compared to non-inoculated plants (Fig. 4 D).

6.3.4 Gene expression of LsaHKT1;1 and LsaHKT1;6

The expression of *LsaHKT1;1* in shoots and roots was affected by the interaction of cultivar, salinity and AM inoculum (Fig. 5 A, B), being higher in roots than shoots in both cultivars. AM symbiosis diminished the expression of *LsaHKT1;1* in GR cultivar in shoot and root under non-saline conditions. At 60 mM NaCl, GR plants inoculated with *F. mosseae* showed higher gene expression, with values of relative expression (RE) ranging from 10 to 5-fold in shoots, and from 1.8 to 5.7-fold in roots, compared to non-inoculated plants and inoculated with *C. lamellosum*, respectively. Regarding LB cultivar, the RE of *LsaHKT1;1* only was affected by salinity, decreasing when the salinity increased. LB inoculated plants showed unaltered RE of *LsaHKT1;1* under saline and non-saline conditions.

A significant interaction between AM inoculum and salinity level on the RE of *LsaHKT1;6* was found in roots and shoots (Fig. 5 C, D). At 60 mM NaCl, GR plants inoculated with *F. mosseae* had the highest RE of *LsaHKT1;6* in shoots and roots, with a 4.1 and 6.6-fold increase, respectively. *LsaHKT1;6* was not detected in shoots and roots of LB plants.

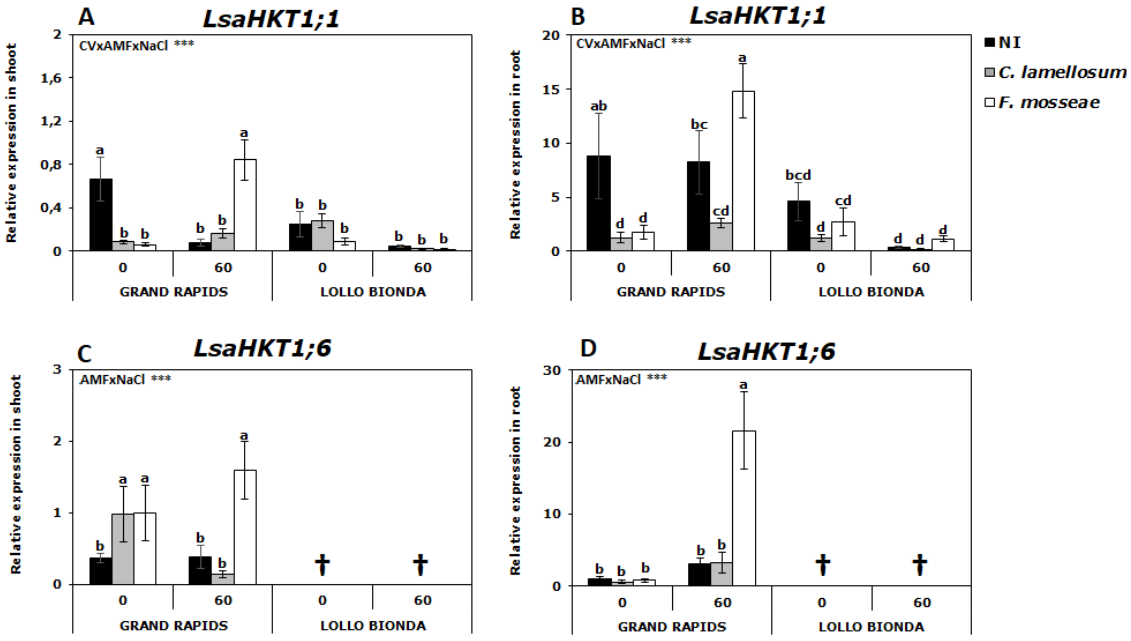

Figure 5: Analysis of *LsaHKT1;1* and *LsaHKT1;6* gene expression by RT-qPCR in shoots and roots normalized to *LsBtub3* gene of lettuce plants non-inoculated (NI), inoculated by *Claroideoglomus lamellosum* and inoculated by *Funneliformis mosseae* subjected to two salinity levels (0 and 60 mM NaCl). The data includes means ± SE (n = 3). In relation to *LsaHKT1;1*, the data was analyzed through a three-way ANOVA conducted with Cultivar, AMF, and NaCl as sources of variation. In relation to *LsaHKT1;6*, the data was analyzed through a two-way ANOVA conducted with AMF and NaCl as sources of variation. The significance of the sources of interaction was determined through the P-values: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Different letters indicate significant differences ($P \leq 0.05$) according to Tukey's multiple range test. †gene is not present.

6.3.5 Gene expression and protein abundance of NHXs family

The RE of *LsaNHX2* was not affected by the interaction of cultivar, salinity and inoculum (Fig. 6 A, B). In shoots of GR, the RE of *LsaNHX2* was up-regulated by *C. lamellosum* (6.5-fold) and *F. mosseae* (11.5-fold) at 60 mM NaCl, compared to non-inoculated plants. In shoots of LB plants the AM symbiosis did not produce changes of *LsaNHX2* RE under saline conditions (Fig. 6 A). In roots, only plants inoculated with *F. mosseae* showed higher RE of *LsaNHX2* at 60 mM NaCl, with changes of 4.2-fold in GR and 22-fold in LB plants, compared to non-inoculated plants. *LsaNHX3* was not detected in any lettuce cultivars likely because of its low expression level.

The RE of *LsaNHX4* in shoots was affected by the interaction of cultivar, salinity and type of inoculum, but in roots it was not affected (Fig. 6 C, D). In shoots, the highest RE of *LsaNHX4* was found in GR plants inoculated, either by *C. lamellosum* or *F. mosseae*. In the case of LB plants, AM symbiosis did not up-regulate *LsaNHX4* RE in shoots at 0 or 60 mM NaCl (Fig. 6C). In AM colonized GR plants growing under salinity, the RE of *LsaNHX4* in roots was higher than non-inoculated ones. For LB cultivar, the RE of *LsaNHX4* in roots was up-regulated only by *C. lamellosum* (Fig. 6 D). The RE of *LsaNHX6* in shoots and roots was affected by the interaction of cultivar, salinity and type of inoculum (Fig. 6 E, F). An increase of *LsaNHX6* RE was found in GR plants inoculated with *F. mosseae* in shoots and roots under saline conditions. No changes in the *LsaNHX6* RE in LB shoots was found; by the contrary, the RE of this gene was significantly higher in root of LB plants inoculated at 60 mM NaCl. The RE of *LsaNHX8* in shoots and roots was affected by the interaction of cultivar, salinity and type of inoculum (Fig. 6 G, H). In shoots, the highest up-regulation of *LsaNHX8* RE was found in LB plants inoculated by *C. lamellosum* at 0 mM NaCl (Fig. 6G). At 60 mM NaCl, both AM fungi strains increased RE of *LsaNHX8* in roots of both lettuce cultivars. Furthermore, it is remarkable that in

roots of non-inoculated LB plants was not detected *LsaNHX8* at either non-saline or saline conditions (Fig. 6H).

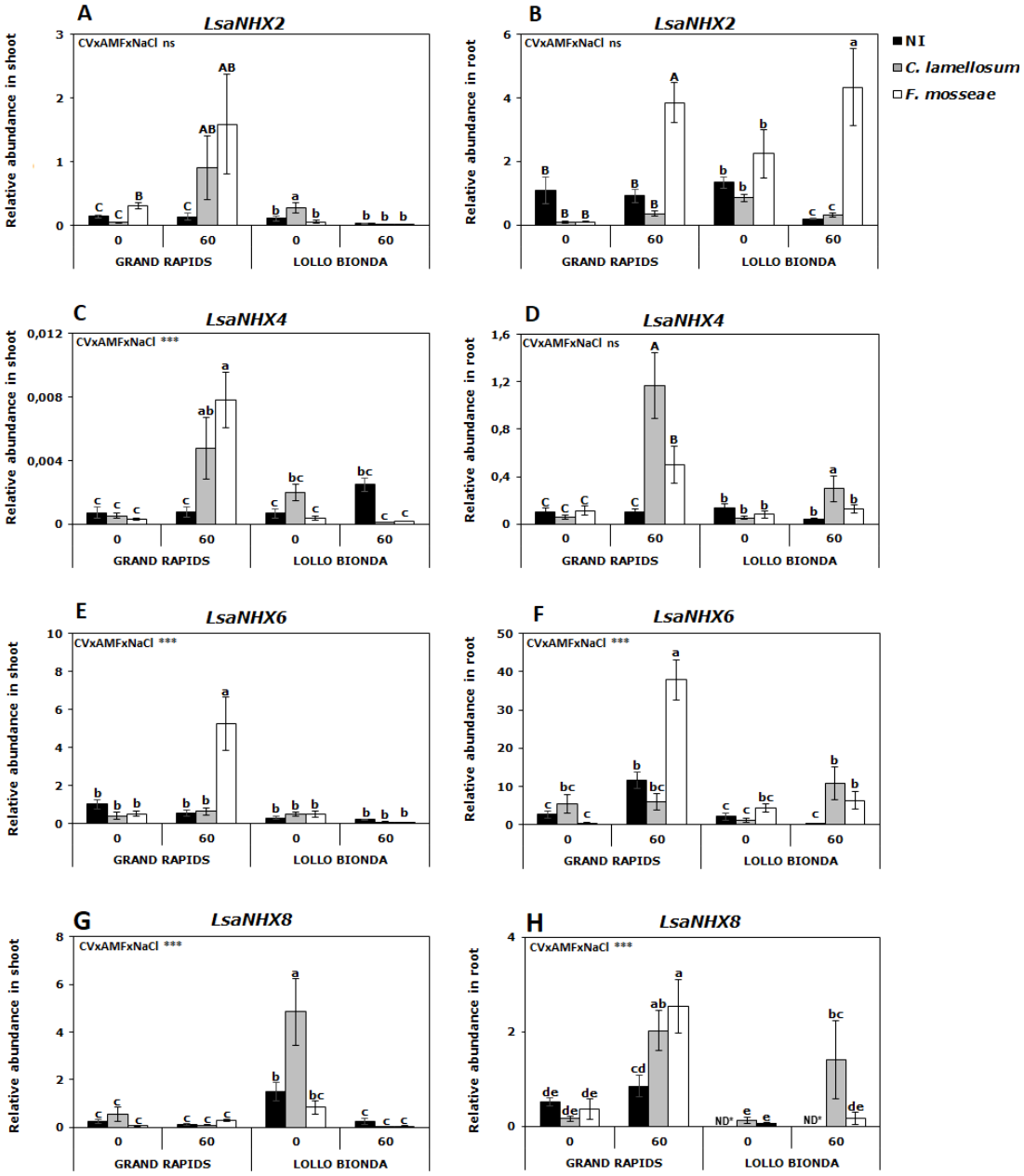

Figure 6: Analysis of *LsaNHX2*, *LsaNHX4*,*LsaNHX6*, and *LsaNHX8* gene expression by RT-qPCR in shoots and roots normalized to *LsBtub3* gene of lettuce plants non-inoculated (NI), inoculated by *Claroideoglomus lamellosum* and inoculated by *Funneliformis mosseae* subjected to two salinity levels (0 and 60 mM NaCl). The data includes means ± SE (n = 3). ND*, not detected. See legend for Fig.

The higher abundance of NHX protein was detected in roots compared to shoots. The interaction of cultivar, salinity and type of inoculum did not affect the NHX protein abundance in both the root and shoot (Fig. 7A, B); but in both, shoots and roots of GR and LB the NHX protein abundance was increased by inoculation with *C. lamellosum* and *F. mosseae*, especially under saline condition.

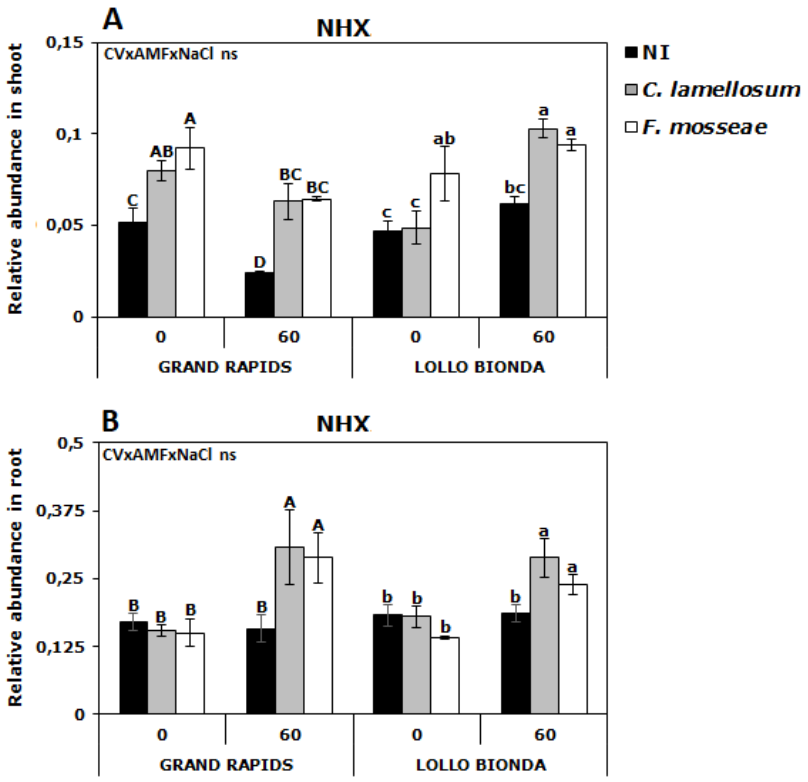

Figure 7: Relative abundance of NHX proteins in shoots and roots of lettuce plants non-inoculated (NI), inoculated by *Claroideoglomus lamellosum* and inoculated by *Funneliformis mosseae* subjected to two salinity levels (0 and 60 mM NaCl). The data includes means ± SE (n = 3). See legend for Fig. 1.

6.3.6 PIPs abundance and phosphorylation status

Under both saline conditions, PIP1 was not increased by AM symbiosis (Fig. 8). In LB plants inoculated with *F. mosseae* or *C. lamellosum*, PIP1 in shoots presented a significantly lower amount compared to non-inoculated plants at 60 mM NaCl. Moreover, *F. mosseae* decreased PIP1 abundance in roots at 0 and 60 mM NaCl. Regarding the PIP2 abundance, the multiple interaction had a strong effect mainly in roots (Fig. 8 C, D). At 60 mM NaCl, GR plants inoculated with *F. mosseae* or *C. lamellosum* showed an increase of PIP2 in shoots compared to non-inoculated plants, and in roots by the inoculation of *C. lamellosum*. PIP2 in shoots and roots was not affected by AM symbiosis in LB plants.

The plants exhibited higher accumulation of PIP2A, PIP2B and PIP2C in roots than shoots (Fig. 8 E, G, I). PIP2A and PIP2C abundance were affected by the interaction of all factors in roots (Fig, 8 F, J). The highest PIP2A, PIP2B and PIP2C level were found in roots of GR plants inoculated by *C. lamellosum* (8 F, H, J), and in shoots of GR plants inoculated by *F. mosseae*, at 60 mM NaCl (8 E, G, I). In the case of LB cultivar, the abundance of PIP2 phosphorylated was affected differentially. PIP2A abundance was increased by AM symbiosis in shoots and roots at 60 mM NaCl (Fig. 8 E, F). On the contrary, PIP2B was not affected by AM symbiosis in shoots and roots (Fig. 8 G, H). PIP2C was increased by *F. mosseae* in shoots and by *C. lamellosum* in roots at 60 mM NaCl (Fig. 8 I, J).

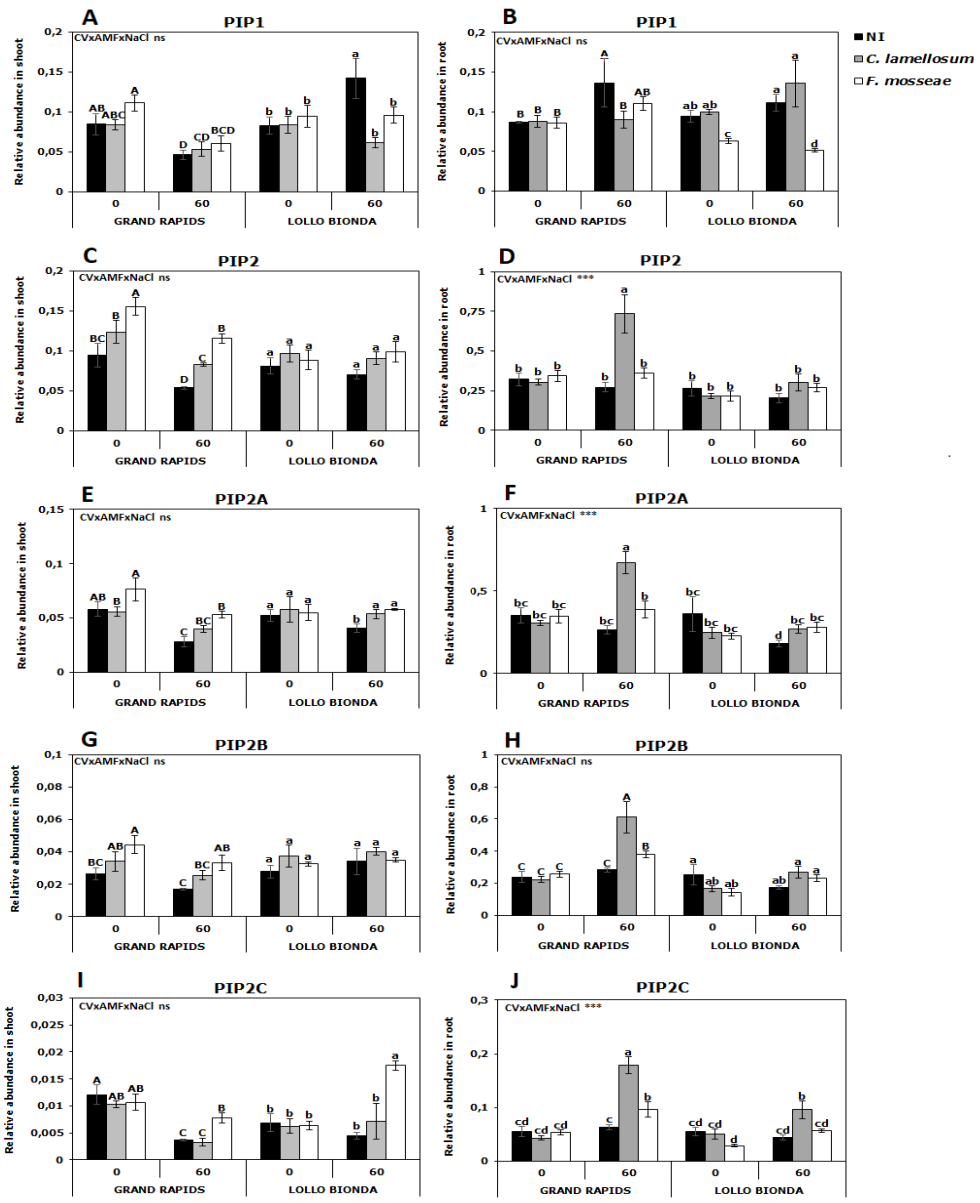

Figure 8: Relative abundance of PIP1, PIP2, PIP2A (Ph Ser280), PIP2B (Ph Ser283) and PIP2C (Ph Ser280/Ser283) in shoots and roots of lettuce plants non-inoculated (NI), inoculated by *Claroideoglomus lamellosum* and inoculated by *Funneliformis mosseae* subjected to two salinity levels (0 and 60 mM NaCl). The data includes means ± SE (n = 3). See legend for Fig. 1.

6.3.7 Multivariate associations

The PC analysis reflected the formation of highly homogeneous groups of experimental variables (Fig. 9 A, C), being observed a differential response to AM symbiosis and salinity. In GR cultivar, PC1 explains 41.7% and PC2 explains 17.1% of the total experimental variance. PC1 was positively associated to root colonization, concentration of K^+ in roots, concentration of Na^+ in shoots and roots, leaf osmotic potential (MPa), relative gene expression of *LsaNHX4*, *LsaNHX6* and *LsaNHX8* in roots, and relative gene expression of *LsaNHX4* in shoots, as well as by the protein abundance of NHX, PIP1, PIP2, PIP2A, PIP2B and PIP2C in roots. Likewise, PC1 was negatively related to biomass production (shoots and roots), shoot P concentration, K^+/Na^+ ratio in shoots and roots, photosynthetic parameters (ΦPSII and *gs*), RWC, and also to NHX, PIP1, PIP2, PIP2A, PIP2B and PIP2C in shoots. PC2 was positively related to shoot K^+ concentration, *LsaHKT1;1*, *LsaHKT1;6*, *LsaNHX2* and *LsaNHX6* in shoots, NHX abundance in shoots, and *LsaHKT1;1*, *LsaHKT1;6*, and *LsaNHX2* in roots. The cluster analysis differentiated three well-defined groups (Fig. 9B). Group 1 included GR plants inoculated and non-inoculated, and grown at 0 mM NaCl. Group 2 included GR plants inoculated with *F. mosseae* growing at 60 mM NaCl. Group 3 included GR plants inoculated with *C. lamellosum* and non-inoculated growing at 60 mM NaCl, being related to positive values of PC1.

In relation to LB cultivar, PC1 explained 35.5% and PC2 17.3% of the experimental variance (Fig. 9C). PC1 was positively correlated with shoot and root biomass, concentration of K^+ in shoots, and P concentration in shoots and roots, RWC, ΦPSII, *gs*, shoot and root K^+/Na^+ ratio, *LsaNHX2*, *LsaNHX6*, *LsaNHX8* in shoot and *LsaHKT1;1* in shoots and roots. PC1 was negatively correlated with Na^+ in shoots and roots, K^+ in roots, and leaf osmotic potential. PC2 was positively correlated to root

colonization, *LsaNHX4*, *LsaNHX6*, *LsaNHX8* in roots, NHX in shoots and roots, and PIP2, PIP2A, PIP2B and PIP2C in shoots and roots. Likewise, PC2 was negatively correlated with *LsaNHX4* in shoots, and PIP1 in shoots. The cluster analysis differentiated three well-defined groups (Fig. 9D). Group 1 included LB plants inoculated and non-inoculated growing at 0 mM NaCl, positively related to PC1. Group 2 included LB plants inoculated with *F. mosseae* and *C. lamellosum* growing at 60 mM NaCl, being related to positive values of PC2. Finally, group 3 included LB plants non-inoculated growing at 60 mM NaCl, being related negatively with PC1 and PC2.

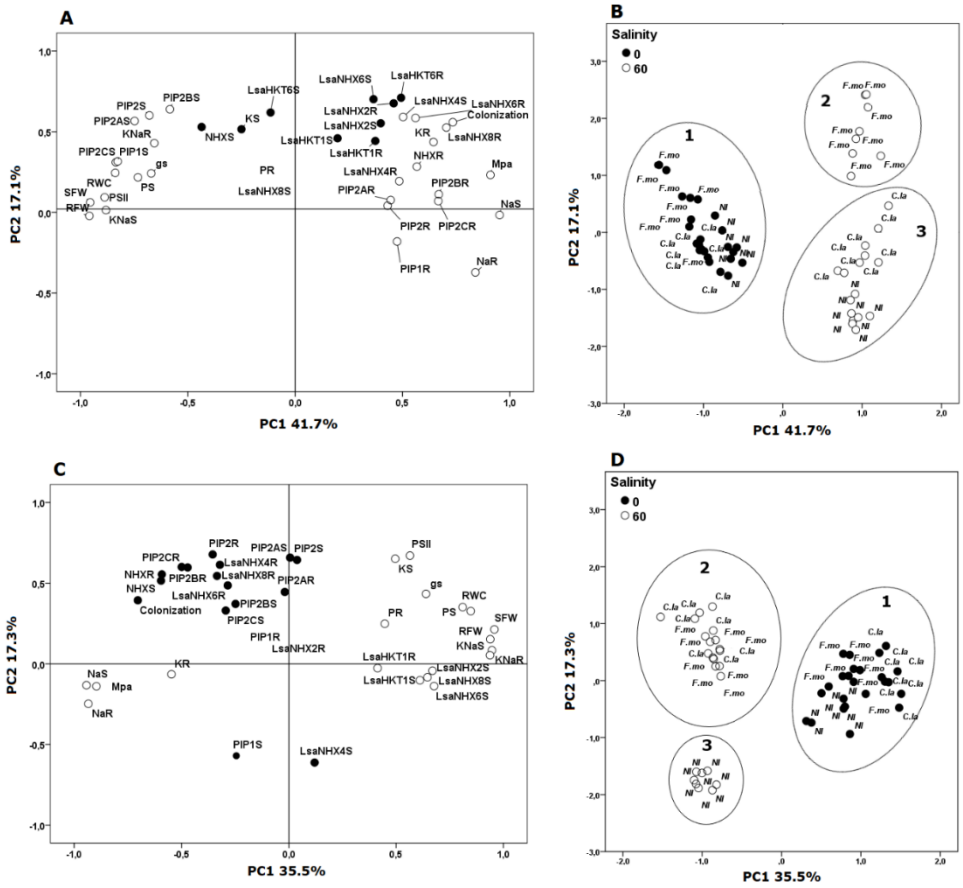

Figure 9: PCA scores for two lettuce cultivars, Grand Rapids (A) and Lollo Bionda (C). The mean value was used in each situation. Percentage values in parentheses indicate the variation explained by each PC. Cluster analysis for the experiment (B: Grand Rapids; D: Lollo Bionda). The circles comprise individuals with similar characteristics according to the analysis and should be understood as a visual aid for discrimination of groups.

6.4 Discussion

6.4.1 Mycorrhizal colonization and plant biomass

Our results regarding AM colonization in saline soil are contradictory. Several reports suggest that salinity decreases the AM root colonization by suppressing hyphal growth, sporulation, and spore germination (Jahromi et al. 2008; Aroca et al. 2013; Evelin and Kapoor 2014). Nevertheless, Santander et al. (2019) did not find changes of AM colonization in lettuce plants inoculated with *Claroideoglomus claroideum.* Here, salinity produced an increase in root colonization, especially in GR inoculated with *F. mosseae* and LB inoculated with both *C. lamellosum* and *F. mosseae*. Similar results were reported by Copeman et al. (1996) in AM inoculated tomato plants growing under saline conditions.

Salinity affects plant growth and development by osmotic stress, ionic toxicity and nutritional imbalance (Wang et al. 2003). Under this conditions, the AM symbiosis highlight as one of the most important biotic components to cope with the adverse environmental conditions (Liu et al. 2017). Plant biomass production is the main trait reflecting plant performance and the AM symbiotic efficiency under abiotic stress (Estrada et al. 2013b; Chen et al. 2017). Our study showed that salinity affected plant performance, being the salt effect higher in GR cultivar. Several studies have showed that AM symbiosis improved plant growth under salinity conditions through the increase of photosynthetic efficiency, maintenance of nutrient balance and up-regulation of the antioxidant systems (Santander et al. 2017). Here, the salinity reduced plant biomass production in both inoculated and non-inoculated plants, but fresh biomass production was higher in AM plant. This finding suggest that both AM inoculants alleviated salt stress regulating the water content of fresh organs, which are in accordance with previous

results in lettuce plants (Jahromi et al. 2008; Aroca et al. 2013). Moreover, the growth of lettuce plants was strongly dependent on AM symbiosis under both saline and non-saline conditions.

6.4.2 P, K, and Na concentrations

It is well known that AM symbiosis improve the plant mineral nutrition through an extensive extraradical mycelium network that forms an additional uptake system (Smith and Smith 1997). Here, the salinity decreased the shoot P concentration in both cultivars, but in LB plants a higher P concentration in shoots and roots of LB plants was found with both inoculants. Salinity affects P uptake by ionic strength on the activity of different phosphate species It is known that P demand of plants may increase under salinity stress, and an adequate level of P mitigates salt stress (Awad et al. 1990). AM colonization significantly improve P acquisition, increasing growth and development of host plant under salinity stress (Hammer and Rillig 2011). In this regard, increased P uptake by AM symbiosis helps to maintain the integrity of cell membranes, allowing an effective ion compartmentalization in vacuoles and the selective ion uptake (Cantrell and Linderman 2001).

Several physiological processes depend on K^+, as enzyme activation, osmotic adjustment, turgor, stomatal aperture and acquisition of other nutrients (Barragan et al. 2012). Our results showed an increase of K^+ concentration in roots of GR plants inoculated with *F. mosseae* under salinity, which could favor a more negative osmotic potential in roots, which reduce root water potential thus favoring water uptake (Yang *et al.*, 2009). Plants have developed efficient strategies to deal with limited K^+ availability, highlighting the AM symbiosis. Our results showed that under salinity *F. mosseae* and *C.*

lamellosum significantly increased K^+ concentration in shoots of LB plants, and *F. mosseae* significantly increased K^+ concentration in shoots of GR plants. Similar effects in the K^+ uptake have been demonstrated in maize (Estrada et al. 2013b; Porcel et al. 2016) and wheat (Talaat and Shawky 2011). According to Sannazzaro et al. (2006), AM symbiosis improve K^+ concentration in plants subjected to salinity as an alleviation mechanism. Thus, a high cytosolic K^+ concentration is vital for plants in saline conditions to maintaining a wide range of physiological processes (Velarde-Buendía et al. 2012; Benito et al. 2014).

During salt stress, plants take up more Na^+ than K^+ (Rus et al. 2001), in concordance with our results, especially in roots of non-inoculated plants. The chemical similarity between Na^+ and K^+ ions can account their competition for the binding sites on the plasma membrane (Benito et al. 2014). Therefore, it has been suggested that plants in saline conditions require a high cytosolic K^+/Na^+ ratio (Munns and Tester 2008). Na^+ inhibit many physiological and metabolic processes such photosynthesis, enzyme activity, production of carbohydrate, among other (Sairam and Tyagi 2004). Our study showed that shoot Na^+ concentration was similar between inoculated and non-inoculated plants, but inoculated plants accumulated less Na^+ in roots compared with non-inoculated ones, in agreement with previous reports (Evelin et al. 2012; Talaat and Shawky 2012; Porcel et al. 2015; Garcia et al. 2017). According to Hammer et al. (2011), AM fungi can select ion uptake, which AM fungi can store in their structures, thus reducing transfer of Na^+ toward plants and retaining into the vacuoles (Cantrell and Linderman 2001), maintaining the ionic balance and processes that exclusively depend on K^+ (Borde et al. 2011; Estrada et al. 2013a). Here, the AM symbiosis improved K^+/Na^+ homeostasis of roots, in agreement with many studies where colonized plants show a higher K^+/Na^+ ratio (Chen et al. 2017; Amanifar et al. 2019).

6.4.3 Plant physiological status

Photosynthetic efficiency is crucial for biomass productivity, which is strongly affected by salinity (Chaves et al. 2009) by two ways: i) at early stages of salt stress it is produced a reduction of water uptake that reduces cell and leaf expansion, coupled with a decrease in stomatal and mesophyll conductance limiting CO_2 assimilation (Rahnama et al. 2010), and ii) the ionic stress produces biochemical by an excessive cytosolic Na^+ accumulation and K^+ deficiency, increasing ROS that inhibit photosystems (Bose et al. 2017). Saline stress decreases the ΦPSII (Hidri et al. 2016), which agrees with results found in GR plants and LB non-inoculated plants. Here, AM symbiosis improved ΦPSII, being this increase higher in LB plants, which agree with several reports (Sheng et al. 2008; Hajiboland et al. 2010; Porcel et al. 2015; Hidri et al. 2016; Chen et al. 2017). A better performance of PSII indicates a better tolerance to stress (Loggini et al. 1999). Besides, the salinity reduces net photosynthetic rate, *gs*, and transpiration in plants (Chen et al. 2014), which result in low CO_2 supply to Rubisco (Porcel et al. 2015). We found a reduction of *gs* in both non-inoculated and inoculated lettuce plants under saline stress. Similar reductions on ΦPSII and *gs* were found in two lettuce cultivars (Vicente-Sanchez et al. 2014). In presence of AM symbiosis, GR and LB plants reached higher *gs* compared with non-inoculated plants, coinciding with similar results in lucerne (Campanelli et al. 2012), maize (Estrada et al. 2013b), rice (Porcel et al. 2016) and black locust (Chen et al. 2017). AM plants showed elevated rates of ΦPSII under stress conditions concomitant with an increased *gs* (Augé 2001), which favor gas exchange and photosynthesis (Subramanian and Charest 1995). In this sense, Ruiz-Lozano and Aroca (2010) observed that the increase of *gs* in AM plants is positively related to changes of hormone levels and an enhanced water uptake and translocation. Additionally, AM plants maintain a high

K^+/Na^+ ratio in photosynthetic organs, which determines high ΦPSII and plant production under saline conditions (Wu et al. 2013).

The loss of intracellular water and osmotic damage are the main consequences of salinity that generate a plant growth reduction (Munns and Tester 2008). RWC in leaf is commonly used to describe plant water status (Tanentzap et al. 2015). Saline stress induce the reduction of RWC, producing a decrease of plant turgor (Campanelli et al. 2012). In the present study, RWC diminished significantly due to salinity in the two cultivars, inoculated or non-inoculated. Nevertheless, AM symbiosis help the plants to maintain higher RWC under saline conditions. According to Chen et al. (2017), AM symbiosis improve water status in plants. Besides, a higher RWC in AM plants growing under salinity correlated with a higher *gs* in these plants. Likewise, Sheng et al. (2008) attributed the influence of the AM symbiosis on photosynthetic capacity to enhancement of plant water status.

At increasing saline conditions, in plants occur a decrease in osmotic potential (Jogaiah et al. 2014) called osmotic adjustment (OA), which involve a series of biochemical mechanisms that plants implement to cope with saline stress (Blum 2017). It is well known that OA allows to plants maintain turgor potential under saline conditions (Munns and Tester 2008), which is associated with maintenance of photosynthetic activity and growth under stress conditions (Heuer and Nadler 1998). In the present study, the osmotic potential was more negative when salinity stress increased, but there were not differences between AM and non-inoculated plants, maybe due to an increase in osmotic potential related to high Na^+ and k^+ accumulation in leaves. As example, Ottow (2005) emphasize that OA is obtained by means of increasing organic and inorganic solute concentrations, such as K^+, Na^+, sugars and proline, among others. A high OA

maintain physiological activity during periods of stress and enables water flow from the soil towards plants (Kramer and Boyer 1995).

6.4.4 NHXs and *HKTs* expression and PIPs abundance

Different studies have demonstrated the role of AM symbiosis improving the uptake of nutrients, photosynthesis capacity and water status in lettuce plants subjected to salinity stress (Ruiz-Lozano et al. 1996; Cantrell and Linderman 2001; Jahromi et al. 2008; Aroca et al. 2013; Santander et al. 2019); however, the molecular bases of the protective mechanisms that involve ionic homeostasis responses and water transport have not been studied so far. The ion exclusion is a plant mechanism to tolerate salt stress (Munns and Tester 2008; Almeida et al. 2017). HKT1 family take part in this mechanism, controlling Na^+ transport to long distance by means of Na^+ reabsorption from xylem sap and transport towards root cells, avoiding its transport to the shoots (Ali et al. 2019). In our study, it is remarkable that RE of *LsaHKT1;1* and *LsaHKT1;6* in shoots and roots was higher in GR plants inoculated by *F. mosseae*, but the upregulation of these genes did not contribute to reduce Na^+ accumulation in the leaves. Our results disagree with Deinlein et al. (2014), who proposed that the HKT1 transporters exclude Na^+ from leaves by unloading Na^+ to xylem and then to the roots, thus improving salt tolerance. On the contrary, here the K^+/Na^+ ratio in shoot did not improve by upregulated RE of genes *LsaHKT1;1* and *LsaHKT1;6.* Similar results were observed in AM plants of rice (Porcel et al. 2016) and black locust (Chen et al. 2017) under saline conditions. In LB the gene expression of *LsaHKT1;1* was unregulated by AM symbiosis, and the *LsaHKT1;6* transporter was not detected. Therefore, our results suggest that the HKT1 transporters here studied are not the main mechanisms of plants to cope with the salt stress, because they cannot diminish Na^+ concentration in leaf tissues of both cultivars.

The ion transporters that regulate K^+/Na^+ homeostasis maybe play a synergistic role in response to salt stress (Jia et al. 2018). Thus, we also studied the NHXs transporters that take part in the tolerance of plant tissues by maintaining Na^+ and K^+ homeostasis (Barragan et al. 2012). Our results evidenced a differential RE of NHXs genes. In detail, in GR plants the RE for *LsaNHX2* and *LsaNHX4* in shoots and *LsaNHX4* in roots was increased by *F. mosseae* and *C. lamellosum* under salinity. In the same way, *F. mosseae* upregulated *LsaNHX2* RE in roots of both cultivars, and upregulated RE of *LsaNHX6* in shoots. Likewise, both AM fungi strains up-regulated *LsaNHX8* gene in roots of both cultivars. Porcel et al. (2016), found that the expression of *OsNHX3* was upregulated in AM rice plants subjected to salt stress. Contrarily, it was found that AM symbiosis had not effect on the expression of NHX genes in black locust (Chen et al. 2017) and tomato (Ouziad et al. 2006; He and Huang 2013). The isoforms *LsaNHX2* and *LsaNHX4* are located in tonoplast (Jia et al. 2018), where compartmentalize Na^+ into the vacuoles (Roy et al. 2014). Despite in our study there were no significant differences on Na^+ concentration in shoots between inoculated and non-inoculated plants, a high RE of *LsaNHX2* and *LsaNHX4* and a higher NHXs protein abundance was found. This finding could be related with a higher Na^+ accumulation into shoot vacuoles, resulting in an improvement of photosynthesis and water status in AM plants. Compartmentalization of Na^+ into the vacuoles of tissues to reduce the toxic concentration of Na^+ in the cytosol is a critical strategy in plants to cope with salt stress (Fan et al. 2015). A decreased Na^+ accumulation in the chloroplasts protects photosynthetic organs against the toxic effects (Assaha et al. 2017), and Na^+ accumulation into vacuole maintain an osmotic potential that drives water into the cells (Kronzucker and Britto 2011). Different studies have shown that NHX 1-4 isoforms in some plants can also mediate vacuolar accumulation of K^+ (Rodríguez-Rosales et al. 2008; Porcel et al. 2016), thus affecting transpiration rate

via regulation of stomatal function (Andres et al. 2014). The *LsaNHX8* gene was upregulated by AM symbiosis in root of GR and LB plants subjected to salinity stress. The NHX8 isoform is located in the plasma membrane, alike to NHX7/SOS1 isoform. It is known that NHX7/SOS1 play a role in Na^+ extrusion from the cytosol, including the expulsion of Na^+ from the root, but the function of NHX8 antiporter in response to salinity is still uncertain (Jia et al. 2018), which deserves to be extensively studied. Here, high RE of *LsaNHX8* appear to be related to a low Na^+ concentration in roots; therefore, high RE might be helping to Na^+ extrusion from the cytosol and to improve K^+/Na^+ ratio in roots.

Salinity impairs plant growth by inducing osmotic stress caused by reduced water availability in the soil (Munns and Tester 2008). In this context the aquaporins, play a pivotal role in passive movement of water following a water potential gradient, thus helping to maintain water equilibrium and improving water use efficiency (Sade et al. 2010; Ruiz-Lozano et al. 2012). It has been showed that AM mycelium can transport water to the host plant, improving water content in plants under stress conditions (Augé, 2001; Li et al. 2013; Santander et al. 2017). Furthermore, AM symbiosis regulate the expression of aquaporinss and the accumulation of PIPs in plants (Aroca et al. 2006b; Calvo-Polanco et al. 2016; Liu et al. 2016; Quiroga et al. 2017). In the present study, we analyzed the isoforms PIP1 and PIP2, which differ in their capacity of water transport, being higher in PIP2 (Kapilan et al. 2018). The effect of AM symbiosis on PIP1 abundance under saline condition was from zero in shoots and roots GR plants, to a decrease in LB plants by both inocula in shoot, and a decrease by *F. mosseae* in root. Similar results were found by Ouziad et al. (2006), who showed that AM colonization drastically decreased the transcript levels of PIP1 in roots of tomato. According to Porcel et al. (2006), the reduction of PIP1 is hypothesized as a mechanism for allowing the conservation of water in plant tissues under drought. PIP1 are considered to have low

water permeability, and some must form heterotetramers with the PIP2 monomers to be able to facilitate water permeability (Kapilan et al. 2018). Contrariwise, we observed an increase of PIP2 levels in the membranes of GR inoculated plants compared to non-inoculated ones under salinity stress. In other studies under similar conditions the regulation of plant aquaporins by AM symbiosis has also been observed (Ouziad et al. 2006; Jahromi et al. 2008; Chen et al. 2017).

Plants must cope with frequent environmental changes, and aquaporins activity must be regulated by mechanisms that allow a fast response to these changes (Quiroga et al. 2018). One of the most simple post-translational mechanism that proteins undergo is phosphorylation/dephosphorylation via kinases and phosphatases (Maathuis 2008). Here, the abundance of phosphorylated PIP2A, PIP2B and PIP2C showed similar patterns, both in shoots and roots. When plants were subjected to salinity the AM symbiosis increased PIP2 phosphorylated levels compared to non-inoculated ones, which could be related with an improvement of RWC, *gs*, and photosynthesis capacity. It is well known that AM symbiosis improves the water uptake in plants exposed to salt stress (Aroca et al. 2006b), maintaining water homeostasis in cells with high salinity levels (Jang et al. 2004), being this improvement related to changes in the expression of aquaporin genes (Ruiz-Lozano et al. 2012). As mentioned above, the salinity restrict CO_2 diffusion through the stomata and mesophyll, and also obstruct the photosynthetic electron flow through photosystem (Chaves et al. 2009). It have showed that aquaporins can also transport CO_2 and they are involved in CO_2 diffusion across the plasma membrane of mesophyll cells (Yang and Cui 2009), which improves photosynthesis activity and plant growth.

6.5 Conclusion

It is noticeable that *F. mosseae* and *C. lamellosum* had a similar capacity to colonize plants roots, especially under saline stress condition. Thereby, the results evidenced that tolerance to salinity in lettuce plants was improved by both AM inoculant isolated from Atacama Desert. The improved growth was closely related with a higher water status given by an increased PIP2 abundance and activity, as well as to an upregulation of *LsaNHX* and *LsaHKT1* genes that improve plant nutrition and maintenance of K^+/Na^+ homeostasis. These changes on molecular mechanisms can allow the plants to continue with their photosynthetic processes under saline stress. It is also remarkable that AM symbiosis did not diminish Na^+ concentration in shoot. Thus, we propose that overexpression of *LsaHKT1* genes in AM plants could increase Na^+ transport from roots to shoots, reaching toxic levels, but concomitantly AM symbiosis could increase overexpression of *LsaNHXs* genes in shoot, favoring Na^+ sequestration into vacuoles also improving K^+/Na^+ ratio in cytoplasm and chloroplasts. This mechanism would explain the reduction of Na^+ concentration in roots. Moreover, the AM symbiosis increased the overexpression of *LsaNHX8* gene, and possibly NHX8 isoform might facilitate Na^+ extrusion from roots into soil improving K^+/Na^+ ratio in root. Our results showed that AM symbiosis is an important sustainable practical to be used in agriculture in saline soils. However, it is necessary deeply understand the mechanisms that confer saline stress tolerance to plants, by means of exclusion, transport and compartmentalization of Na^+, and how they are modulated by the AM symbiosis.

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Chapter VII

General discussion and concluding remarks

7.1 General discussion

7.1.1 AM fungi in saline soils

Arbuscular mycorrhizal fungi are well known to bring multiple benefits for plant growth and health, above all in stressful environments (Smith & Read, 2008). This is particularly important when the AM inoculants are native ecotypes, isolated from limiting environments (Campagnac & Khasa, 2014; Chandrasekaran et al., 2014). Thereby, native AM fungal communities inhabiting soils of desert ecosystems, such as the Atacama Desert, must be "stress-adapted" to withstand these environmental conditions. In this framework, this Doctoral Thesis displays a full circle of isolation of AM fungi from Atacama Desert, knowing the mycorrhizal status of native plants, propagation of these AM fungi, and the obtaining AM fungal species likely adapted to saline stress to their subsequent application in experiments under saline conditions. Several studies have showed adaptations of AM fungal species to saline environmental conditions (Evelin et al., 2012). Thereby, our study of AM status showed that all native plants in the Atacama Desert presented AM colonization and presence of fungal propagules in the rhizosphere. Our results are in accordance with several studies that reported the AM symbiosis in native or endemic plants of arid and semi-arid ecosystems (Azcón-Aguilar et al., 2003; Barea et al., 2011; Cavagnaro et al., 2017; Estrada et al., 2013; Liu et al., 2017; Silvani et al., 2017). Also, in this research the electrical conductivity and soluble cations were positively related with both spore density and hyphal length, which is accordance to Mohammad et al. (2003) and Wang et al. (2003), who found an increase of AM structures in saline soils. These findings results of importance for selecting effective AM fungi strains to be used as bio-inoculants during salt stress.

According to our results, we selected two native AM fungal consortia, which were compared with a reference AM fungus strain likely non-adapted to salt stress. All of them showed differential responses of root colonization. In the first study, the root colonization of lettuce plants cv. White Paris (WP) was affected negatively by salinity. According to Juniper & Abbott (2006) and Sheng et al., (2008), root colonization is reduced in the presence of NaCl. Contrarily, in the second study the root colonization was affected differently in Grand Rapids (GR) and Lollo Bionda (LB) cultivars. In the third study, *Funneliformis mosseae* and *Claroideoglomus lamellosum*, both isolated from Camiña Valley, showed an increase of root colonization capacity in GR and LB cultivars, when salinity was increased. As reported by Klironomos et al. (1993), the varying levels of AM colonization can also be related to the different behaviour of each AM fungus species, even in similar environment conditions, which suggests different degrees of compatibility between fungi and host.

7.1.2 AM symbiosis improves plant responses to saline stress

Improvement of plant growth

In our studies, all AM fungi improved biomass production of lettuce plants subjected to saline stress, isolated from saline and non-saline soils. According to Estrada et al. (2013), plant biomass production is an integrative measurement of plant performance under many types of abiotic stress conditions, and the symbiotic efficiency of AM fungi has been classically measured in terms of plant growth improvement. AM fungi in plants improve nutrient acquisition and water uptake, induce antioxidant system to prevent damage by ROS, protect photosynthetic apparatus enhancing photosynthetic efficiency, among others (Ruiz-Lozano et al., 2012; Evelin et al., 2019).

In our first study we found that WP lettuce cultivar reached the higher biomass production when inoculated with *C. claroideum* reference strain, compared to plants inoculated with Salar del Huasco native consortium (HM). This result disagrees with reported by Estrada et al. (2013), who showed that AM fungal communities isolated from ecosystems affected by salinity have a greater ability to increase biomass production of maize under this condition. On the contrary, in our second study we showed that both *Claroideoglomus claroideum*, and Camiña Valley native consortium (HMC) similarly improved biomass production of GR and LB cultivars under saline stress.

In our third study, similar results were found in GR and LB cultivars inoculated either with *F. mosseae* or *C. lamellosum*, both strains isolated from saline soils from Camiña valley. In this way, mycorrhizal development mostly depend on the compatibility of both AM fungus and host plants (Chandrasekaran et al., 2016), and the functional responses of AM plants vary with the origins of AM fungal isolate (Estrada et al., 2013a).

Acquisition of N and P by AM fungi

Phosphorus nutrition is probably the most widely known benefit of AM fungi for plants (Helgason & Fitter, 2005). In our studies, the P concentration was differentially affected by type of inoculant. In the first study, AM symbiosis did not increase P concentration in shoot, but it was increased in root by *C. claroideum*. Conversely, in the third study we found that *F. mosseae* and *C. lamellosum* improve P concentration in shoot in GR and LB cultivars; nonetheless, root P concentration was only affected in LB cultivar by both inoculants. AM symbiosis enhance P acquisition by throughout an increased P availability due to secretion of acid phosphatases by hyphae, and maintenance of intrinsic P

concentration by forming polyphosphates inside the hyphae (Selvaraj & Chellappan, 2006; Abdel-Fattah & Asrar, 2012).

Salinity conditions interfere with N uptake (Miransari, 2011), reducing flux from soil to roots (Hoff et al., 1992). Our results showed that N concentration was improved by AM symbiosis in WP cultivar subjected at increasing concentrations of salinity. In this way, it is remarkable that several studies have reported that AM colonization helps in increasing N uptake under stress condition (Garg & Bhandari, 2016; Wu et al., 2010; Zhang et al., 2014). According to Talaat & Shawky (2013), improved N uptake in AM plants is attributed to AM fungal-facilitated maintenance of membrane stability, and increased NR activity.

Maintenance of photosynthetic capacity

Plants' growth rates and productivity depend on photosynthetic efficiency, being the primary component of biomass productivity, and it is among the principal processes to be affected by salinity (Gupta & Huang, 2014). AM colonized plants are able to fix more CO_2 than non-inoculated ones, and hence their growth is improved (Porcel et al., 2012). This agrees with our study, where at 60 mM NaCl the stomatal conductance (gs) was increased significantly in GR and LB cultivars inoculated either with *F. mosseae* or *C. lamellosum* compared to non-inoculated plants. Similar results were found by Aroca et al., (2013) in lettuce plants inoculated by *Rhizophagus irregularis*. Additionally, AM symbiosis improves photosynthesis activity via the osmotic adjustment (OA), accumulating compatible solutes like proline to maintain a favorable gradient for water flow from soil into the roots (Chen et al., 2017; Ruiz-Lozano et al., 2012; Wu et al., 2016). Proline content was increased by AM symbiosis in WP, GR and LB cultivars more than

in non-inoculated plants, under saline conditions. Moreover, we found higher relative water content (RWC) values in GR and LB inoculated plants either with *F. mosseae* or *C. lamellosum*. This agree with Chen et al. (2017), who reported a higher RWC in inoculated *Robinia pseudoacacia* plants under salt stress, compared to non-inoculated ones. In this regard, the photosynthetic activity of plants subjected to salinity increases as their RWC increases (Hasanuzzaman et al., 2014). In the same way, the improvement of water status in AM plants is related to changes in the expression of aquaporin genes (Ruiz-Lozano et al., 2012). It is remarkable, because our study is the first that evaluated PIP protein abundance in phosphorylated (PIP2A, PIP2B, and PIP2C), and non-phosphorylated (PIP2) state. The relative protein abundance was higher in root than shoot under salinity stress. In this regard, AM symbiosis increased abundance of PIP2, and PIP2-phosphorylated in lettuce plants. On the contrary, PIP1 abundance did not increased, and even was down-regulated by AM symbiosis in LB plants at 60 mM NaCl.

In addition, photosynthetic tissues with higher levels of Na^+ exhibit reduction in chlorophyll content, changes in ultrastructure of chloroplasts, inhibition of photosystem II (PSII), and significant decrease in chloroplast K^+ contents (Murata et al., 2007; Suleyman et al., 2009). In this research, AM symbiosis enhanced the photosynthesis activity under saline conditions by significantly improving relative chlorophyll concentration, and efficiency of photosystem II. This agrees with Talaat & Shawky (2014), who demonstrated that AM symbiosis helps plants to alleviate salt stress by enhancing its photosynthetic capacity, thus improving its ability to generate further growth of harvestable biomass.

Regulation of the ionic homeostasis

AM symbiosis can improve K^+ absorption under saline conditions, while preventing Na^+ uptake and avoiding translocation to shoot tissues (Sharifi et al., 2007; Zuccarini & Okurowska, 2008). Some studies have explained the molecular basis of K^+/Na^+ ratio changes by AM symbiosis (Chen et al., 2017; Porcel et al., 2016). Here, we observed that mycorrhizal colonization increased the expression of *LsaHKT1;1*, *LsaHKT1;6* in shoots and roots of GR plants. Also, we found an increased expression of *LsaNHX2*, *LsaNHX4*, *LsaNHX6* and *LsaNHX8* genes in shoots and roots of GR and LB, as well an increase of NHX protein abundance. However it is noticeable that *F. mosseae* upregulated *LsaHKT1;1* and *LsaHKT1;6* genes in shoot of GR cultivar, but neither decreased Na^+ concentration nor increased K^+/Na^+ ratio in leaves. Our results disagree with Porcel et al., (2016), who found a reduction of Na^+ distribution from roots to shoots in colonized rice plants, and this was related to upregulated *OsHKT1;5* gene expression.

The NHX transporters are associated with prevent accumulation of Na^+ in the cytosol, through Na^+ compartmentalization into vacuoles (NHX1-4), and endosomes (NHX5-6), and efflux of Na^+ into soil and apoplast (NHX7/SOS1-NHX8) (Kumari et al., 2017). We did not found differences in Na^+ concentration in shoot between inoculated and non-inoculated plants, but we observed that AM symbiosis upregulated *LsaNHX2* and *LsaNHX4* genes in shoot. Overexpression of these genes improve salt tolerance by sequestering Na^+ into the vacuole (Kronzucker & Britto, 2011), increasing K^+/Na^+ ratio and improving plant growth. Also, we found an increase of NHX protein in shoots and roots, which confirms the above mentioned. Likewise, NHX6 is associated to salt tolerance (Bassil et al., 2018), and was upregulated by AM symbiosis in lettuce plants. In addition, *LsaNHX8* was upregulated by AM symbiosis in root. However, the role of NHX8 protein in plant abiotic stress response is still poorly understood. Thus, we suggest

that how it is a plasma membrane protein may promotes Na^+ export back to the soil or to the apoplastic spaces, and decrease Na^+ concentration in roots, restricting the toxicity of Na^+.

Antioxidant defense system

Salinity stress increases the production of ROS, producing oxidative damages in plants through lipid peroxidation (Mittler, 2002). As a consequence of the oxidative stress, photosynthetic pigments, proteins and nucleic acids are damaged, leading to reduction of plant growth or death (Bose et al., 2014). According to our results in GR and LB cultivars subjected to salinity a high lipid peroxidation rates was exhibited, being showed as high malondialdehyde (MDA) values, mainly in non-mycorrhizal plants. MDA is a product of membrane lipid peroxidation as a consequence of oxidative stress (Paradiso et al., 2008). Additionally, AM symbiosis strengthen salt tolerance in plants via attenuating membrane lipid peroxidation and plasma membrane permeability, and increasing osmolyte accumulation and antioxidant enzyme activities to detoxify and eliminate the generated ROS (Abd-Allah et al., 2015; Evelin & Kapoor, 2014). In this way, GR and LB cultivar AM inoculated had the higher capacity to reduce oxidative damage under salinity conditions. This was reflected in a reduced lipid peroxidation, and in an increased of superoxide dismutase, catalase, and ascorbate peroxidase enzyme activities. Our results agree with others found in lettuce (Kohler, Hernandez, Caravaca, & Roldan, 2009), tomato (Abdel Latef & He, 2011), pigeon pea (Pandey & Garg, 2017), and cucumber (Hashem et al., 2018).

Proline is also involved in ROS quenching, maintenance of membrane integrity, and enzyme as well as protein stabilization, hence is also known as osmoprotectant that

reduce damage produced by Na^+ (Ashraf & Foolad, 2007). Our studies showed higher proline content in lettuce AM plants compared to non-mycorrhizal plants under saline stress, and this trend was associated to a higher biomass production. Likewise, phenolic compounds are well-known like non-enzymatic antioxidants (Lim et al., 2012). In this regard, we identified nine phenolic compounds in leaves of GR and LB cultivars, belonging to hydroxycinnamic acids and flavonols, and six of them were over-accumulated under saline stress. However, we expected that AM symbiosis induce a significant increase of phenolic compounds in lettuce; contrarily, the increase of these secondary metabolites was found in non-AM plants, being related to high oxidative stress levels and low biomass production.

7.2 Concluding remarks

In this Doctoral Thesis the mycorrhizal status of native flora from Atacama Desert has been studied for the first time, as far as we known. All plants species studied form AM symbiosis as strategy for survive to extreme environmental conditions where they are established. Although, we found AM presence in soils affected by either salinity or drought, the highest densities of fungal structures was associated to saline soils, in the three elevation belts studied in the Atacama Desert. These results support the search for effective ecotypes of AMF to be used as inoculants oriented to agricultural that developes under limiting conditions (salinity, drought, cold environments, etc.), as well as to the programs of recovery of threatened plant species in vast areas with increasing aridity and salinity worldwide.

Despite, AM reference fungus was not isolated from saline soil, showing high adaptation to salinity under greenhouse conditions where these studies were developed. For that reason, the fisrt hypothesis was rejected due to that the reference AM fungus *Claroideoglomus claroideum* showed to have greater capacity for improving lettuce growth under saline conditions than native AM consortium isolated from Salar del Huasco. Likewise, *C. claroideum* showed similar capacity to improving lettuce growth under saline conditions that the native AM consortium isolate from Camiña Valley.

Nevertheless, the second hypothesis was accepted, despite of an absence of a clear trend about the behavior depending of the origin where were obtained the AM isolates, the symbiosis increased the salt tolerance of a glycophyte plant of agronomic interest, such as lettuce. This improvement was about two principal mechanisms: AM fungi improved osmotic adjustment, which is the first plant response mechanism to salinity, involving a high biosynthesis and accumulation of proline to maintain water uptake. This

effect could be related with the increase of relative abundance of PIP2, PIP2A, PIP2B and PIP2C in shoots and roots, and a higher relative water content in shoot. Also, AM fungi improved the third plant response mechanism to salinity, increasing tissue tolerance through high Na^+ compartmentalization at the cellular and intracellular level, reducing the deleterious effect of Na^+ in the cytosol, and improving K^+/Na^+ homeostasis. This effect could be related with a significant increase of *LsaNHX2*, *LsaNHX4*, *LsaNHX6*, and *LsaNHX8* gene expressions, as well NHX protein abundance in shoots and roots.

According to our experience, a specific AM fungus may be effective for one crop, while the same AM fungus specie may not produce the desired effect on other crop. The variability of the effects of AM fungi on their hosts can indicate that certain combinations are beneficial for the plant, whereas others are neutral or even negative. In this way, the selection of AM fungal "super-strain" it is necessary for better effect as bio-inoculant, mainly though the improvent on the production and quality of crops that are produces in the lands affected by salinity or drought. Also, it necesary under field conditions confirm the results obtained in greenhouse and laboratory, due to the variability in the expected results, which is of high impact in the crop production.

Arbuscular mycorrhizal symbiosis is immense promise for the development of more sustainable agricultural systems, mainly because AM symbiosis could help to reduce the use of chemical fertilizers, and preventing the salinization of agriculture soils, as well as could increase water use efficiency in agriculture. This is really importance to face the climate change at the present time. Also, AM symbiosis have a great effect on plant metabolism, increasing the production of specific plant secondary metabolism products (e.g., fatty acids, terpenoids, organic acids, among others), which has a positive effects improving the yield crops and the nutritional quality of foods. Therefore, these benefits of arbuscular mycorrhizae deserves to be deeply studied.

Graphical abstract

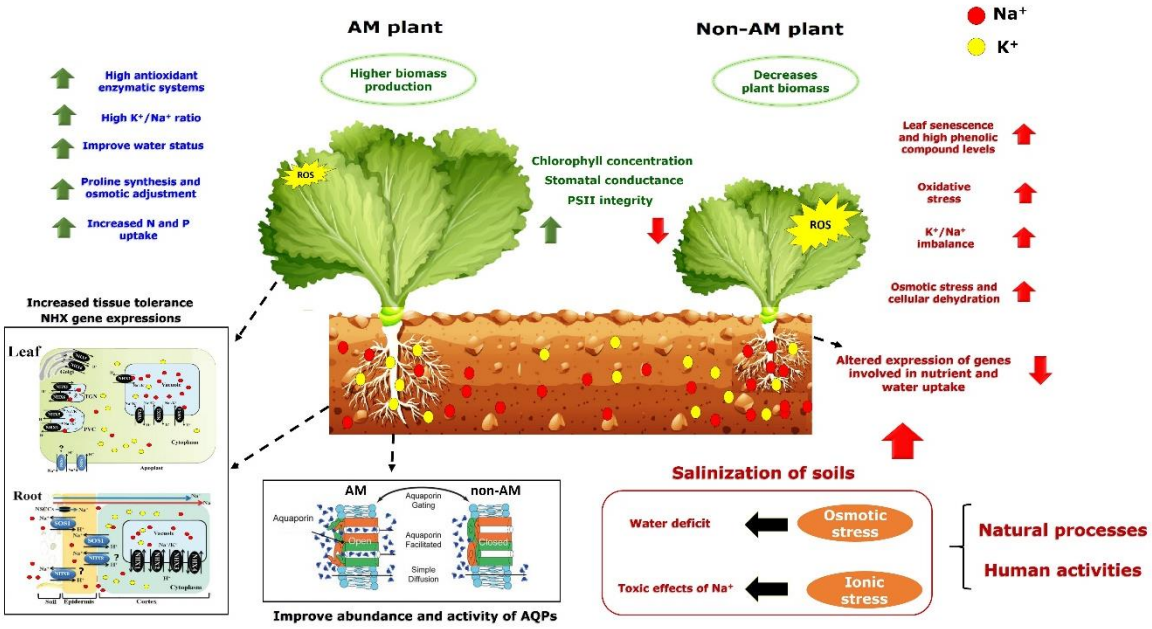

Fig 1. Overview of this Doctoral Thesis, which was developed with the aim to deeply understand the role of arbuscular mycorrhizal fungi in the tolerance of lettuce plants to saline stress. Here, the main effects of AM symbiosis are summarized as well, comparing between mycorrhizal, and non-mycorrhizal lettuce plants.

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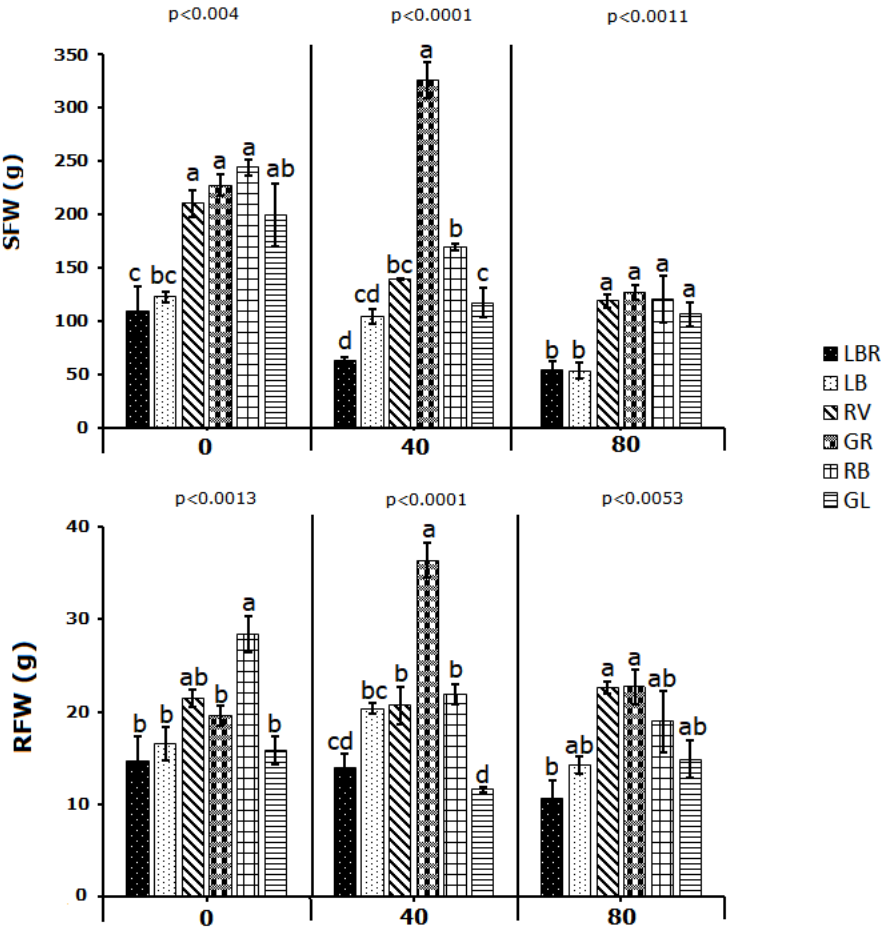
Supplementary data



SD1: Screening of lettuce cultivars growing under saline stress conditions. Shoot dry weight (SDW) and root dry weight (RDW). LBR: Lollo Bionda Rosso; LB: Lollo Bionda; RV: Romana Verte; GR: Grand Rapids; RB: Romana Bionda; GL: Great Lakes. The data includes means ± SE (n = 3). The data was analyzed through an ANOVA conducted by each salinity level. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test**.**