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DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI AND THEIR INCIDENCE ON ALUMINUM TOLERANCE OF *TRITICUM AESTIVUM* L. GROWING IN ACIDIC SOILS WITH PHYTOTOXIC ALUMINUM LEVELS

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PAULA ALEJANDRA AGUILERA ÑONQUEPAN

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“Diversity of arbuscular mycorrhizal fungi and their incidence on aluminum tolerance of *triticum aestivum* l. growing in acid soils with phytotoxic aluminum levels”

Esta tesis fue realizada bajo la supervisión del Director de tesis, Dr. FERNANDO BORIE BORIE del Departamento de Ciencias Químicas y ha sido aprobada por los miembros de la comisión examinadora.

PAULA ALEJANDRA AGUILERA ÑONQUEPAN

…….

DIRECTOR DEL PROGRAMA DE DOCTORADO EN CIENCIAS DE RECURSOS NATURALES

……

Dr. FERNANDO BORIE BORIE

…….

Dr. PABLO CORNEJO RIVAS

…….

Dr. CESAR ARRIAGADA

……

Dr. ROBERTO GODOY

……

Dr. JORGE MENDOZA

.....

DIRECCION DE POSTGRADO

UNIVERSIDAD DE LA FRONTERA

Dedico esta tesis a mi familia, mi esposo Gabriel, mis hijas Fernanda y Francisca, y mis padres.

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Summary and outline of this theses

Acid soils have some edaphic disadvantages to crop production, such as high P adsorption capacity and high exchangeable aluminum (Al) contents. These soil conditions results in a decrease on plant growth, mainly due to Al^{3+} phytotoxicity reducing significantly water and nutrient acquisition.

Cereals production is concentrated in South-Central Chile being wheat (*Triticum aestivum* L.) established predominantly in volcanic soils. Wheat is the most important cereal produced in Chile, mainly due to the economic value of their production, acreage and mass consumption. To cope negative effects of these soils on plant growth, several management practices have been implemented, among which are: a) liming materials application, b) P fertilization, and c) the use of Al-tolerant wheat plants are the most important. In this sense, the enhancement of arbuscular mycorrhizal (AM) fungal activity through management practices emerges as an interesting alternative.

Several agricultural practices can affect AM fungal diversity especially management practices that contribute to soil acidification process increasing phytotoxic Al chemical forms. On the other hand, some studies have demonstrated that AM symbiosis can protect plants against stress produced by high levels of toxic elements such as heavy metals and Al. Recent evidence in our research group showed a strong relationship between soil exchangeable Al levels and glomalin-related soil protein (GRSP), a glycoprotein produced by AM fungi. Besides, the AM association allows plant improve its ability to acquire water and nutrients, principally P, which is antagonistic to Al injury. Some studies have reported that certain AM fungal species associated with specific hosts could be more effective promoting plant growth, and

improving water and nutrient acquisition. Thus, the beneficial aspects that are provided by AM fungi are related to genetic variation and functional diversity.

The outline of this Thesis begins with a general introduction. In Chapter I, we address the general objectives regarding to AM fungal diversity and its contribution to Al tolerance of *Triticum aestivum* L. plants growing in acidic soils with phytotoxic Al levels.

In Chapter II we performed a review focused on the AM fungal diversity present in acid soils as well as related to their presence with Al phytotoxicity alleviation. This review performs a compilation of AM fungi species that have been worldwide studied in acid soils with high levels of Al focusing the main contributions of AM fungi to plants growing in soils like Andosols with high levels of Al phytotoxic. Among the species found in acid soils *Glomus, Acaulospora, Gigaspora* and *Scutellospora*, those showing differences in the alleviation of Al phytotoxicity evaluated in terms of biomass production, organic acid exudation and nutrient acquisition. Data provided in this review emphasizes the contribution of AM fungi to plants growing in acid soils mainly based on increased nutrient acquisition, contributing to plant resistance, decrease in Al and Mn translocation into the shoot and possible Al immobilization/sequestration by AM fungal structures and GRSP.

The above mentioned aspects have been target of studies focused mainly on technological applications. However, inocula formulation for use in biofertilization requires a better understanding of physiological, molecular and ecological bases regulating AM fungi diversity and its contribution to plants growing in soils with phytotoxic Al levels.

Biofertilization with AM fungi has not been successfully developed mainly due to difficulties in establishing culture. Specifically, it is necessary to promote the use of monoxenic cultures, to study the role of glomalin in these soils and select efficient ecotypes. Thus, it would be possible to optimize plant performance in agricultural crops growing in acid soils.

In Chapter III we expose the main results referring to the first specific objective "To analyze Al-binding capacity of AM fungal structures and GRSP from soils, *in vitro* culture of *Glomus intraradices* and in a soilless system". These results have appeared in a recent article "Fluorescence detection of aluminum in arbuscular mycorrhizal fungal structures and glomalin by using confocal laser scanning microscopy". In this article, AM fungal spores and glomalin-related soil protein (GRSP) isolated from acid soils were analyzed using confocal laser scanning microscopy (CLSM) for Al detection. Mycorrhizal structures of *Glomus intraradices* produced under *in vitro* conditions as well as spores and GRSP from neutral and Cu-polluted soils were used as contrasting criteria. Spores and GRSP from soils with 7 and 70% Al saturation showed autofluorescence which increased especially at the highest soil Al level and when Al solution was added. *G. intraradices* spores showed fluorescence only when exogenous Al^{3+} was added. On the contrary, spores and GRSP from neutral and Cu-polluted soils showed little or no significant fluorescence. This fluorescence shown by fungal structures and GRSP when subjected to high Al (of endogenous or exogenous origin) suggests a high capacity for Al immobilization, which could be an effective way to reduce Al activity and phytotoxicity in acidic soils.

Chapter IV refers to the second specific objective "To analyze the AM fungal diversity present in *Triticum aestivum* L. plants growing in acid soils with phytotoxic Al levels". Results obtained from this study were exposed in the article "Diversity of

arbuscular mycorrhizal fungi associated with *Triticum aestivum* L. plants growing in an Andosol with high aluminum level". The AM fungal species identification was performed based on spore morphological analyses. In this study, we additionally observed AM fungal propagules (spore density and colonization levels) at two crop stages (grain filling and postharvest). At grain filling stage, "Bakan", "Porfiado" and "Maxi" cultivars showed higher colonization levels than "Otto", "Crac" and "Invento" ones. Spore density increased at postharvest stage and "Maxi", "Otto" and "Invento" cultivars presented higher spore densities than the other three cultivars. Twenty-four AM fungal species were identified and subsequently classified into genera *Acaulospora*, *Pacispora*, *Claroideoglomus*, *Glomus*, *Funneliformis*, *Septoglomus*, *Simiglomus*, *Scutellospora*, *Cetraspora*, *Ambispora*, *Archaeospora* and *Paraglomus*. The Shannon–Wiener index showed no significant difference in AM fungal diversity associated with wheat cultivars, but species richness was significantly different among cultivars. Simpson's index was significantly different among AM fungal communities being *Acaulospora* and *Scutellospora* the most dominant genera. In this study, AM fungal community structure was different for each specific wheat cultivar; hence the use of target AM fungal species could be determining factor for the appropriate AM community establishment in potential inoculation assays, especially in Andosols with high aluminum levels.

In Chapter V, we present results obtained from study of the third objective "To evaluate the role of AM fungi on Al phytotoxicity in *Triticum aestivum* L. plants". The aim of this work was to evaluate the effect of Al, AM fungal inoculum and its origin, and wheat varieties on Al phytotoxicity and AM fungal indicators. Therefore, an experiment based on a soilless system of rhizoboxes with three compartments with mycorrhizosphere, rhizosphere and hyphosphere was implemented. Two Al tolerant

“Crac” and “Invento” and one Al sensitive “Tukan” wheat varieties were used as host plants, which were inoculated with AM fungal spores extracted from acidic soils with high (Gorbea) and low (Remehue) Al contents. Besides, 0, 40 and 400 µM of $AlCl_3$ were added to each zone. All factors included in the assay affected Al phytotoxicity and AM fungal indicators being AM fungal inocula application the main factor of influence. Origin of AM fungal spores and wheat variety had specificity of responses in analyzed zones when wheat plants were exposed to two Al concentrations. Thus, our results suggest the usefulness of evaluating specific indicators when a Program guided to the development of inoculants based on AM fungi is performed in Andosols with high Al levels.

In Chapter VI we described a general discussion, the concluding remarks and the future directions.

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CHAPTER I

General Introduction

1.1 General Introduction

Wheat (*Triticum aestivum* L.) crop is the most representative cereal species produced in Chile, mainly due to the economic value of its production, to the surface area designated to the crop and consumption that reach 150 kg per pearson per year. Wheat production is concentrated in the central southern Chile, especially in the Araucanía Region (37 to 40°S latitude), where it covers about 248.000 ha (ODEPA, 2014). Wheat is cropped predominantly in acidic soils characterized by a pH ranging from 4.5 to 5.5 and high exchangeable Al. In general, acidic soils have problematic properties, such as high P adsorption capacity and high Al^{3+}, Mn^{2+} and H^{+} ion levels.

Furthermore, the phytotoxicity caused by Al under these edaphic conditions generates the first symptoms of reduced plant growth, primarily due to the damage to cell membrane, resulting in slower growth and root cell elongation (Magalhaes et al., 2007; Seguel et al., 2013). For counteracting negative effects of these soils on plant growth, several management practices have been implemented, among which: a) liming materials application, b) P fertilization, and c) the use of Al-tolerant wheat plants are the most important. These varieties have been generated through breeding programs that are based on the introduction of cultivars that have been checked against landraces improving germplasm characteristics with respect to their ability to tolerate the conditions of toxicity present in these soils. Thus, the enhancement of arbuscular mycorrhizal (AM) fungal activity through management practices emerges as an interesting alternative.

Arbuscular mycorrhizal (AM) symbiosis is an association established between soil fungi and most terrestrial plants. The association is mutually beneficial for both partners based on a bidirectional interchange of nutrients (Barea et al., 2013; Smith and

Read, 2008), particularly phosphorus (P) (Borie et al., 2010; Borie and Rubio, 1999; Larsen et al., 2009; Smith and Smith, 2013). Essentially, the fungus improves nutrient and water absorption capacity of the host plant while the fungus receives carbon compounds from plant photosynthesis (Barea et al., 2013). Arbuscular mycorrhizal fungal diversity has been widely studied as the abundance and variety of spore species in a specific habitat. It is determined by their morphological characteristics, biochemical composition and nucleotide sequences. Characterization of AM fungal species gives us basic information to assess the impacts generated by various stresses on AM fungal communities, which is very important since such impacts affect directly the biological behaviour in host plant. In general, AM fungi are adapted to the environment from which they were isolated and they colonize plants growing in soils with pH ranging 2.7-9.2 (Clark and Zeto, 1996; Siqueira et al., 1984). However, it has been reported that among AM fungal species, apart from natural phylogenetic diversity, there is also functional diversity mainly due to different behavior under diverse environmental stresses (Klugh and Cumming, 2007; Klugh-Stewart and Cumming, 2009; Medeiros et al., 1994). When an AM fungal application program is undertaken, it is fundamental to assess first the AM fungal diversity by estimating the abundance and variety of AM fungal species in the target agroecosystem. In addition, the study of AM fungal diversity provides basic information to assess the impacts generated by some agricultural practices on AM fungal community structure; these impacts affect directly the sporulation dynamics, AM fungi presence and plant interactions (Oehl et al., 2003, 2004, 2009; van der Heijden et al., 1998). For example, it has been reported that low pH and high aluminum (Al) contents in soils have negative impacts on AM fungal colonization (Göransson et al., 2008; Seguel et al., 2013). Several studies have concluded that AM fungi improve plants nutritional status under different stress

conditions, having a potential use in agriculture (Hijri et al., 2006). Therefore, more studies regarding the ecology of AM fungi and their physiological behavior are required, since their status as obligate symbionts create difficulties in establishing pure cultures or managing the indigenous AM communities through agronomic practices (Barea et al., 2013; Oehl et al., 2005, 2009, 2010), at the present limiting the massive use of AM inocula. The benefits provided by AM fungi not only vary with the different strains that effectively colonize a host root, which influences their abundance and diversity, but also with their physiological effectiveness which depends on the ability to survive and grow, as affected by the prevalent environmental factors (Barea et al., 2013;Morton et al., 1995; Morton and Bentivenga, 1994). Several studies have reported that some AM fungal species associated with specific host promote plant fitness in acidic soils with high Al levels (Borie et al., 2010; Borie and Rubio, 1999; Cumming and Ning, 2003; Klugh-Stewart and Cumming, 2009; Lux and Cumming, 2001). Whereas, AM fungi promote tolerance of plants growing under unfavorable conditions, it is necessary to consider that high concentrations of Al can decrease the spore's germination ability and the subsequent roots colonization; although some spores do produce hyphae and subsequently roots colonization even in extreme Al concentrations (Rohyadi, 2005).

As mentioned above, the attenuation of Al toxicity is related to the Al availability and may be favored not only by Al binding to AM fungal structures such as spores, hyphae and vesicles, but also Al can be immobilized on a glomalin-related soil protein (GRSP) a glycoprotein produced by AM fungi reported in many soils in large amounts (Wright and Upadhyaya, 1996, Wright, 2007), and also bound to hyphae and spores (Driver et al, 2005; Aguilera et al, 2011). Various reports have focused on determining

glomalin ability to metals accumulation, presumably due to complex formation (González-Chávez et al, 2004; Cornejo et al, 2008; Vodnik et al, 2008). More specific analyses have been implemented in the study of glomalin in order to produce without the interference of the compounds present in soil, for which inert substrates which have separate zones of rhizosphere and hyphosphere have been used (Nichols, 2010).

1.2 Hypotheses

Arbuscular mycorrhizal (AM) fungi involved in plants adaptation to acidic conditions, improve plants ability to acquire nutrients, reduce phytotoxic element translocation, promote organic acid exudation with chelant capacity and increase plant biomass. Several studies have reported that certain AM fungi species give protection against stress produced by heavy metals and aluminum (Al) through possible immobilization in fungal structures or compounds generated by AM fungi such as glomalin related soil protein (GRSP). However, the beneficial aspects provided by AM fungi are related to functional diversity. Therefore, the beneficial aspects of AM fungi contributing to Al phytotoxicity alleviation in acidic soils are recognized. Therefore, this Thesis proposes the following hypotheses:

- There are AM fungal species and ecotypes giving great tolerance to *Triticum aestivum* L. plants growing in acidic soils with phytotoxic Al levels.

- The AM fungal structures and GRSP have a high capacity for Al-binding.

1.3 General objective:

- To determine AM fungal diversity and their contribution to Al tolerance of *Triticum aestivum* L. plants growing in acidic soils with phytotoxic Al levels.

1.4 Specific objectives:

1. To analyze Al-binding capacity of AM fungal structures and GRSP from soils, *in vitro* culture of *Glomus intraradices* and in a soilless system.

2. To analyze the AM fungal diversity present in *Triticum aestivum* L. plants growing in acidic soils with phytotoxic Al levels.

3. To evaluate the role of AM fungi on Al phytotoxicity in *Triticum aestivum* L. plants.

CHAPTER II

Diversity of arbuscular mycorrhizal fungi in acidic soils and their contribution to aluminum phytotoxicity alleviation. A REVIEW

Diversity of arbuscular mycorrhizal fungi in acidic soils and their contribution to aluminum phytotoxicity alleviation

Paula Aguilera, Pablo Cornejo and Fernando Borie*

Center of Amelioration and Sustainability of Volcanic Soils. BIOREN-UFRO.

Universidad de La Frontera, P.O. Box 54-D, Temuco, Chile.

Abstract

Acidic conditions limit crop production in 40% of the world's soils. These soils are characterized by a pH between 4.5-5.5, low phosphorus (P) availability, high contents of Aluminum (Al) and Manganese (Mn), and low soil basic cations. Edaphic conditions of acidic soils results in a decrease in plant growth, mainly due to Al^{3+} phytotoxicity. Aluminum is one of the main metals causing phytotoxicity due to negative effects on root growth; it directly reduces water and nutrient acquisition from soils as well as plant functions. To withstand negative effects of acidic soils on plant growth, several management practices have been implemented, among which are lime application, P fertilization and the use of Al-tolerant plants. In this regard, the inoculation of use of arbuscular mycorrhizal (AM) fungi appears as another management alternative, because AM contribution has been extensively documented for plants growing in acidic soils at phytotoxic Al levels. Several reports have demonstrated that AM fungal structures and glomalin-related soil protein (GRSP) a glycoprotein produced by AM fungi, protect plants against stress produced by high levels of heavy metals and Al. However, among AM fungal genera or species there is a functional diversity. Therefore, the aim of this review is to summarize AM fungal diversity present in acidic soils as well as relate their presence with Al phytotoxicity alleviation.

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References

2.1 Introduction

About 30-40 % of the world soils have been classified as acidic and show pH<5.5 (Kochian, 1995). Generally, soil acidity is associated with high levels of Aluminum (Al) (Kochian, 1995; Ma et al., 2001; Piñeros and Kochian, 2001; Kochian et al., 2005; Piñeros et al., 2008). These soils have some edaphic disadvantages to crop production such as high P adsorption capacity, high Al^{3+} or H^{+} levels, low basic soil cations and high Al saturation (Mora et al., 1999; 2002). The most important diagnostic index of Al toxicity is Al saturation (%) of effective cation exchange capacity, which corresponds to the amount of Ca, Mg, K, Na and exchangeable Al (USDA, 1996). The acidification process is generated by natural processes like high rainfall, which produces a soil base leaching or by anthropogenic origin by the use of ammonium fertilizers (Bolan, 1991; Kochian et al., 2004). Moreover, acidification implies an increase in Mn and Al concentration, both generating a detrimental effect on plants, reducing growth and crop production (Cuenca et al., 2001; Mora et al., 2004; Mora et al., 2006). Aluminum is one of the prevalent metals generating phytotoxicity due to strong negative effects on plant root growth, directly reducing the capacity for water and nutrient acquisition (Borie and Rubio, 1999; Cumming and Ning, 2003). In non-tolerant plants, Al inhibits root growth and functions (Piñeros and Kochian, 2001). Severe changes in specific membrane lipids have been reported as the main mechanism of Al phytotoxicity. To withstand negative effects of acidic soils on plant growth, several management practices have been implemented, among which are liming application, P fertilization and using Al-tolerant plants. In this regard, the inoculation of arbuscular mycorrhizal (AM) fungi appears as another management alternative (Borie et al., 2010).

Several studies have demonstrated that AM symbiosis protects plants against stress produced by high levels of diverse toxic elements such as heavy metals (González-Guerrero et al., 2008; Bissonnette et al., 2010; Janoušková and Pavlíková, 2010; Miransari, 2010) and Al (Borie and Rubio, 1999; Cumming and Ning, 2003). However, there is a functional diversity among AM fungal genera or species (Clark and Zeto, 1996; Clark et al., 1999b; Clark and Zeto, 2000), which can determine the role of AM fungi on Al phytotoxicity alleviation in acidic soils.

2.2 Classical and novel methods to study AM fungal diversity

2.2.1 Phylogeny and taxonomic identification of AM fungi

Glomeromycota Phylum is composed by four orders: *Glomerales*, *Diversiporales, Archaeosporales* and *Paraglomerales*. Within these, eleven families have been defined: *Glomeraceae, Claroideoglomeraceae, Gigasporaceae, Acaulosporaceae, Entrophosporaceae, Pacisporaceae, Diversisporaceae, Paraglomeraceae, Geosiphonaceae, Ambisporaceae and Archaeosporaceae* and, seventeen genera have seen proposed: *Glomus, Funneliformis, Sclerocystis, Rhizophagus, Claroideoglomus, Gigaspora, Scutellospora, Racocetra, Acaulospora, Entrophospora, Pacispora, Diversispora, Otospora, Paraglomus, Geosiphon, Ambispora and Archaeospora* (Schüßler and Walker, 2010).

Arbuscular mycorrhizal fungi have been evaluated by several methods based on their three forms present in soil, either as spores, hyphae or colonized roots (Douds Jr. and Millner, 1999). Taxonomic classification of AM fungi has been based largely on spore morphological characteristics (Morton and Bentivenga, 1994; Morton et al., 1995). Taxonomic classification requires isolated spores from soil by means of

techniques such as wet sieving and decanting method (Gerdemann and Nicolson, 1963; Sieverding, 1991), flotation-adhesion (Sutton and Barron, 1972), differential water-sucrose centrifugation (Allen et al., 1979), sucrose gradient centrifugation (Ianson and Allen, 1986), and adhesion and flotation-centrifugation (Horn et al., 1992). However, after spore isolation, it is necessary to complement these methods with traditionally used morphological identification and quantification methods such as root staining (Phillips and Hayman, 1970; Koske and Gemma, 1995). Among root staining methods trypan blue, fuchsin and congo red have been used for fungal structural observation by optical microscope. After stain applications root colonisation percentage is determined by means of gridline intercept method (Giovannetti and Mosse, 1980). Taxonomic approach has been used for different characteristics of AM fungal structures. Finally, appropriate results interpretation and AM fungi species identification are performed by accessing available databases such as INVAM, invam.caf.wvu.edu/cultures/cultsearch.htm (Morton et al., 1997).

Additionally, biochemical methods that are based on the sterols and chitin quantification present in AM fungi cell walls have also been used. However, none of them is exclusive for AM fungi (Alkan et al., 2004). In some genera, the morphological characters, which may be included in AM fungi identification are scarce; therefore, the identification by means of molecular techniques has made a contribution to AM fungal ecology due to its simplicity because only a simple colonized root is required (Redecker, 2000; Redecker., 2002).

2.2.2 Molecular methods

In AM fungal studies, the development of the monoxenic culture has been common by using a transformed root with one mycorrhizal fungus growing under *in vitro* conditions (Bécard & Piché, 1992; St-Arnaud et al., 1996). This kind of technology has been implemented for obtaining adequate amount of AM fungi produced under sterile conditions (Bago et al., 1996; 2004). This kind of culture has allowed the study of fungi under specific environmental factors. In addition, several molecular aspects of AM simbiosis have been reported using monoxenic culture such as responses to oxidative stress produced by Cu (González-Guerrero et al., 2007), Zn (González-Guerrero et al., 2005), herbicides (Benabdellah et al., 2009) and AM fungal physiology (López-Pedroza et al., 2006; Berguero, 2007).

In general, molecular methods have contributed to AM fungal diversity characterisation without needing to implement trap cultures (Clapp et al., 1995; Redecker, 2000; Krüger et al., 2011; Oehl et al., 2005b; Oehl et al., 2006). Techniques such as temporal temperature gel electrophoresis (TTGE) analysis have been used in *Glomus* identification colonizing plant roots from NS31-Glo1 region obtained by nested PCR (Cornejo et al., 2004) and denaturing gradient gel electrophoresis (DGGE) to assess AM fungi community structure of *Acaulospora*, *Gigaspora, Glomus* and *Scutellospora* (Oliveira et al., 2009). These applications have been included in the identification of AM fungal diversity colonising several crops (Daniell et al., 2001) by the use of small ribosomal units (SSU) differences which are then amplified, cloned and subsequently performing a screening which allows to observe restriction pattern length polymorphism (RFLP) (Helgason et al., 1998).

Nuclear ribosomal genes (n-rDNA) are markers used in the AM fungal identification, which have highly conserved regions as well as variable regions, being used for various purposes, from the phylogenetic level of Phylum to species distinction. (Raab et al., 2005). The SSU portion has been amplified by using PCR, where NS31, AM1, AML1 and AML2 primers have been commonly used for Glomeraceae, Diversisporaceae, Gigasporaceae and Acaulosporaceae (Helgason et al., 1998; Lee et al., 2008). Furthermore, this technique has been used for identifying AM fungi colonising roots in heavy metals polluted sites (Bedini et al., 2010), and in semi-natural forests (Helgason et al., 1999). Several studies, have shown that AM fungi cannot be identified as reliably below the genera level, except when using the molecular methods (Helgason et al., 1998; Helgason et al., 1999).

In addition, colonisation in plant has been quantified by qRT-PCR, using 28S rDNA sequence. The first application was made by Alkan et al., (2004), which reported the benefits of this application in *Glomus intraradices* detection and quantification.

2.2.3 Phospholipid fatty acid (PLFA) analysis

Phospholipid fatty acid (PLFA) analysis in microbiological studies of soil aspects have been implemented (Zelles, 1999). This method provides information on changes that exists in microbial community structure, distribution and interaction among AM fungi in soil (Olsson, 1999**;** Marshall, 2011). The PLFA analysis is performed by fatty acids characterisation of cell membranes phospholipids.

This method is based on the specificity and relative abundance of fatty acids for certain groups of microorganisms, but it provides no information on species

individually; therefore, it is recommended to identify superfamilies and domains. Whereas, molecular techniques are recommended for identifying phylogenetic characterisation (Zelles, 1999) the PLFA study allows comparisons between community composition and its associated biomass.

The PLFA 16:1ω5c have been used as biomarker to estimate mycorrhizal biomass and its distribution as well as its relationship with the percentage of colonisation (Aliasgharzad et al., 2010; Carrasco et al., 2010). In addition, comparisons of PLFA with the metals contents present in the rhizosphere have been performed, being also possible to establish ratios between bacterial and fungal communities (Carrasco et al., 2010).

2.2.4 Visualisation with fluorochromes

New technologies have been incorporated in the AM fungal observation, including confocal microscopy and flow cytometry (Gutiérrez et al., 2003; Vierheilig et al., 2005; Dreyer et al., 2006; 2008; 2010). These observations have been possible because some AM fungal structures exhibit autofluorescence, as hyphae (Ames et al., 1982). Some structures that produce no fluorescence are observed by using fluorochromes, such as arbuscles, spores (Dickson, 1999; Vierheilig et al., 2001; Schweiger et al., 2002) and vesicles (Cuenca et al., 2001).

2.3 Diversity of AM fungi in acidic soils

Arbuscular mycorrhizal fungi are obligated symbionts of about 80% terrestrial plants, some of them growing in soils with serious limitations (Smith and Read, 2008).

Arbuscular mycorrhizal symbiosis is an association, which is established between soil fungi and the most vascular plants, allowing a bidirectional interchange of nutrients and energy (Barea, et al., 2005; Smith and Read, 2008). Essentially, host plants improve their water and nutrient absorption capacity and fungi receive carbon compounds (Barea et al., 1980; Smith and Read, 2008).

Arbuscular mycorrhizas are generally adapted to the place, from which they were isolated and they are colonizing plants growing in soils with a pH between 2.7 and 9.2 (Siqueira et al., 1984; Clark and Zeto, 1996a; 1996b). However, among AM fungi genera it has been reported that there is a functional diversity mainly due to different AM behaviors under diverse environmental stresses (Medeiros et al., 1994a; 1994b; Klugh and Cumming, 2007; Klugh-Stewart and Cumming, 2009). On the other hand, it has been reported that low pH and high Al contents in soil can impact negatively on AM colonisation (Göransson et al., 2008).

Arbuscular mycorrhizal fungal diversity has been defined as the abundance and variety of spore species in a specific habitat. Characterisation of AM fungal species gives us basic information to assess the impacts generated by various stresses on AM fungal communities; these impacts affect directly the biological aspects of host plant.

Several species have been reported in acidic soils with high Al contents (Table 2). Morton (1986) described three new species from abandoned coal minesoils. Species were identified by using pot culture; subsequently, their morphological characterisation was made by taxonomic classification. These species were: *Acaulospora dilatata*, *Acaulospora lacunosa* and *Acaulospora rugosa*. Whereas, other species have been found in acidic soils from upland Scotland. These species were established in pot culture, then they were analized by morphological and molecular methods, and finally,

they were identified as *Acaulospora alpina* and *Acaulospora brasiliensis* (Krüger et al., 2011).

On the other hand, Acaulosporaceae, Glomaceae and Gigasporaceae families have been found by using DGGE in maize rhizosphere from acidic soils of Brazil. The PCR amplification of 18S was made by means of nested PCR by using NS1, NS4 VANS1 and NS21 primers. Other primers were used for each specific AM fungus (Oliveira, 2009).

Arbuscular mycorrhizal fungi can determine ecosystem sustainability as they promote establishment of plants growing under unfavourable environmental conditions. In this regard, AM fungal diversity was studied in native and reforested areas of *Araucaria angustifolia* present in soils with pH 3.7. In this location *Acaulospora* (8 species), *Entrophospora* (1 species), *Gigaspora* (2 species), *Glomus* (9 species), *Scutellospora* (4 species) genera were found (Moreira et al., 2003).

The soil type, soil tillage and land use influences AM fungal diversity (Jansa et al., 2002; 2003; Oehl et al.**,** 2003; 2009). In this regard, Oehl et al., (2010) reported AM fungal diversity present in temperate climate location of Europe. Community composition was evaluated by morphological identification obtained from AM fungi reproduced in trap culture. In this investigation the biodiversity was characterised as "specialist" or "generalist" according to their presence in different locations evaluated. Glomeraceae and Acaulosporaceae family were the most found in acidic soils. Community structure depend on soil depth, in this respect, Oehl et al., (2005a) found high spore number of *Glomus etunicatum* at 50-70 cm of soil depth and pH 4.0.

On the other hand, species diversity depends of farming system. Oehl et al., (2004) described the most AM fungal abundance and species diversity in organic farming compared to conventional system. However, Hijri et al., (2006) studied AM fungal diversity in agricultural field by molecular tools and reported that diversity is non dependent of management practices.

Additionally, AM fungal diversity characterisation has been carried out in forest and agricultural ecosystems of Southern Chile. In these soils about 39 AMF species were found, some of them reported for the first time. The genera found in the order of the highest to lowest abundance were: *Acaulospora, Glomus Scutellospora* and *Archaeospora*. Whereas, in agricultural ecosystems, 22 AMF species were found belonging to the genera *Glomus* spp. *Acaulospora* spp. and *Scutellospora* spp. (Castillo, 2005; Castillo et al., 2005).

2.4 Contribution of AM fungi to aluminum phytotoxicity alleviation

2.4.1 Aluminum phytotoxicity

Phytotoxicity caused by Al in acidic soils has been investigated in several studies, due principally to its negative effect on growth and cellular elongation (Caldwell, 1989; Delhaize et al., 1993; Delhaize and Ryan, 1995; Jones and Kochian, 1995). Root tip tissues correspond to the first specific sites receiving negative effects of cellular elongation (Kinraide et al., 1992; Ryan et al., 1992).

Current reports have been focused on Al^{3+} phytotoxicity study in wheat plants, suggesting that Al^{3+} can be associated strongly with phospholipids present in plasma membrane. Aluminum can interact with proteins either by competition with other

cations for binding sites or may induce changes in membrane proteins conformation (Jones and Kochian, 1997).

Cadwell (1989) analyzed the interaction of Al^{3+} with membrane proteins of wheat plants, suggesting that Al^{3+} can displace Ca^{2+} that is bound to membrane proteins. This process corresponds to the beginning of phytotoxicity. On the other hand, it has been shown that exposure of wheat roots at phytotoxic Al^{3+} levels produces alterations in signal transduction pathway. It inhibits specifically inositol 1,4,5-triphospate formation that incides on cellular elongation and growth (Jones and Kochian, 1995).

Phytotoxicity tolerance in wheat has been associated with some properties of radical apices, because they have shown a differential behavior in respect to Al accumulation among Al-tolerant and sensitive genotypes. Knowledge about Al accumulation in roots is still limited. In addition, radioactive tracer for Al is not available. For this reason, Al detection in radicals tissues have been based on several staining methods. Among staining techniques there is hematoxylin forming a colored complex when it reacts with Al (Delhaize et al., 1993; Cançado et al., 1999; Ownby, 1993; Vázquez et al., 1999).

Additionally, the use of new technologies such as visualization by confocal laser scanning microscope (CLSM) has been also implemented. It also requires stain use forming a fluorescence complex to detect Al in the observed structure. Lumagallion staining corresponds to another stain used for detecting the location of Al by CLSM in vegetal tissues. On the other hand, it has been reported that the fluorescence emission lumagallion-Al complex correlates positively with the Al content present in the observed tissue (Kataoka et al., 1997).

Furthermore, studies made with genotypes of *Triticum aestivum* L. tolerant and sensitive to Al, have shown that Al-tolerant plant meristems have the ability to

accumulate less Al in their tissues, suggesting that these plants have a mechanism of exclusion allowing protect tissues against Al phytotoxicity (Rincón and Gonzáles, 1992; Silva et al., 2000).

2.4.2 Beneficial contribution of AM fungi to plant growing in acidic conditions

2.4.2.1 Mycorrhizal benefits to plants growing under acidic conditions

The AM symbiosis, involved in plants adaptation to stressful soil conditions (Smith and Read, 2008), allows to plants improve their ability to acquire water and nutrients such as P, Zn, Cu, Ca, S, Na, N, K and Fe (Borie and Rubio, 1999; Borie et al., 2010). Studies carried out with mycorrhizal plants have shown a decrease of Al or Mn translocation (Clark and Zeto, 1996a; 2000; Clark et al., 1999b; Khan et al., 2000; Smith and Read, 2008).

Several studies have reported that certain AM fungal species when are to be associated with specific hosts promote growth, providing protection against heavy metals stress (Cornejo et al., 2008a; González-Chávez et al., 2002; González-Chávez et al., 2004; González-Guerrero et al., 2008; 2009) and Al (Mendoza and Borie, 1998; Borie and Rubio, 1999; Lux and Cumming, 2001; Cumming and Ning, 2003; Arriagada, 2007; Klugh-Stewart and Cumming, 2009; Borie et al., 2010).

Besides, mycorrhizal benefits to plants growing under acidic conditions have been shown in *Ipomoea batatas* (Yano and Takaki, 2005), *Vigna unguiculata* (Rohyadi et al., 2004; Rohyadi., 2005; Rohyadi, 2009), *Andropogon virginicus* L. (Cumming and Ning, 2003; Klugh-Stewart and Cumming, 2009), *Liriodendrum tulipifera* (Lux and Cumming, 2001; Klugh and Cumming, 2007;), *Musa acuminata* (Rufyikiri et al., 2000), *Panicum virgatum* (Clark et al., 1999a; 1999b).

Mechanisms such as chelation or sequestration have been reported to influence stress attenuation of heavy metals, because it reduces the direct availability of the metal ion, reducing phytotocicity in the rizosphere (Rufyikiri et al., 2000; Cumming and Ning, 2003; Rufyikiri et al., 2003). Studies reported by Cuenca et al., (2001) have shown that Al is present in the mycelium of the AM fungi, principally in vesicles.

2.4.2.2 Species of AM fungi included in technological application based on acidic condition tolerance

Some species have been recognised for their tolerance to acidic conditions (Table 2). Yano and Takaki (2005) studied beneficial aspects of Gigaspora on plant growth parameters at pH 4.2 and 5.2 (with lime application) and found that this fungus favors Al tolerance of *Ipomea batatas* growing in acidic soils with high Al levels. Whereas, Cavallazy et al., (2007) selected AM fungi for improving the establishment of plants in acidic soils with high levels of Al and Mn and demonstrated that *Glomus etunicatum* and *Scutellospora pellucida* were the most efficient inocula. In these conditions *G. etunicatum* and *S. pellucida* promoted plant growth and nutrient absorption.

Inoculation of *Glomus clarum, Scutellospora heterogama and Glomus etunicatum* genera at high Al levels has demonstrated an increase in citrate and malate exudation from colonised plant roots. Additionally, inoculant-plants presented the highest P amount and lowest Al concentration in their shoots. Both fungi and plant parameters were favoured (Klugh-Stewart and Cumming, 2009).

These technological applications are beneficial especially in tropical areas where Al toxicity reduces crop production. Rufyikiri et al., (2000) demonstrated that

inoculated plants with *Glomus intraradices* showed great resistance under high Al concentrations.

Cuenca et al., (2001) described AM fungi tolerance to acidic conditions with high Al contents by using inoculants plant with AMF from acidic and neutral soils growing at pH 3.0-5.0 and found that inocula from acidic condition promoted a higher tolerance of *Clusia multiflora* to acidic soil. In this assay *Gigaspora* and *Scutellospora* genera were found as dominant. On the other hand, Bartolome-Esteban and Schenck (1994) have studied AM fungal tolerance to Al by assessment of spore germination and hyphae growth at 6, 27 and 100% Al saturation, and observed that *Gigaspora* was the most efficient genus. However, Rohyadi (2005) evaluated the effect of Al increasing concentrations on germination of *Gigaspora margarita* spores and found that Al inhibited spores development; therefore, AM fungal colonisation was reduced. However, some spores produced hyphae and subsequently colonized plant roots under excessive Al conditions.

Kelly et al., (2005) evaluated diferential behaviour of AM fungi testing five isolates of each *Glomus clarum*, *Acaulospora morrowiae and Scutellospora heterogama* species using *Sorghum sudanense* as host plant. Isolates whitin species were extracted from acidic and neutral soils and they were distributed in several locations. Plants were exposed at high Al levels and they shown different responses when they were exposed to fungal isolates from same species. Functional diversity observed in Al resistance can influence in plant stability growing under phytotoxic Al levels. Species as *Glomus clarum, Glomus etunicatum and Gigaspora margarita* have been reported like efficient isolates with high ability to withstand acidic environment and Al phytotoxicity (Clark et al., 1999a; 1999b). Species as *G. margarita* and *G. etunicatum* are found commonly

under acidic conditions, being *G. etunicatum* the most efficient (Rohyadi et al., 2004). Moreover, others species found in acidic soils are also *Acaulospora* sp., *Gigaspora* sp. and *Glomus manihotis*, which have been identified as particularly tolerant (Clark, 1997).

Picone (2000) evaluated the species composition, dominance diversity curves and Simpson diversity index in Ultisols with pH ranging from 3.9 to 5.6, reporting the presence of Acaulosporaceae (*Acaulospora foveata*, *Entrophospora* aff *colombiana*), Glomaceae (*Glomus* "small brown", *Glomus occultum*) and Gigasporaceae (*Scutellospora pellucida*, *Gigaspora* sp.) as the most frequent species.

2.5. Possible role of GRSP on Al alleviation in acidic soils

Glomalin is a glycoprotein released from AM fungi (Wright et al., 1996). It has been reported in many soils (Wright and Upadhyaya, 1996; Wright, 2007) in abundant amount (Wright and Upadhyaya, 1996). Glomalin is known to be tightly bound in hyphae and spores (Driver et al., 2005). This glycoprotein has been recognized as heat-shock protein (Gadkar and Rillig, 2006). Subsequently, glomalin was found in spore wall layers of *in vitro* culture of *Glomus intraradices* by using transmission electron microscopy (Purin and Rillig, 2008). A monoclonal antibody has been used for identifying location of this protein in AM fungi (Purin and Rillig, 2008; Rosier et al., 2008). Chemical analyses have shown that this protein consists of C, N, 42-49% of aromatic, 24-30% carboxyl and 4-11% aliphatic compounds (Schindler, 2007).

Several reports have been focused on determining the ability of GRSP in sequestering diverse heavy metals (González-Chávez et al., 2004; Cornejo et al., 2008a;

Vodnik et al., 2008). It has been shown in these studies, that purified glomalin can sequester several heavy metals, especially Cu, Pb and Cd (González-Chávez et al., 2004) presumably, due to complex formation. In addition, AM fungi and GRSP are able to live under adverse environmental conditions with high levels of Cu, Pb and Zn (Bedini, 2010). For improving glomalin analyses, it has been produced without the interference of other spurious compounds and soil constituents under a soilless system (Nichols, 2010). It allows develop a culture system where rhizosphere is separated from hyphosphere by means of nylon mesh.

Recent evidence has shown a strong relationship between soil exchangeable Al levels and glomalin-related to soil protein (GRSP), a glycoprotein produced by AM fungi (Etcheverría, 2009). Additionally, it has been demonstrated that agricultural practices can influence glomalin concentration in soil (Borie et al., 2006; Valarini et al., 2009).

Cornejo et al., (2008a) reported high glomalin content in a high Cu polluted soil. This would suggest a possible role of GRSP in soil remediation and the same role could be played in high Al content soils. Current assays made in our laboratory have shown that glomalin is able to sequester Al in the molecule. This assay was carried out for detecting Al^{3+} presence in glomalin extracted and purified from acidic soil by using CLSM (Fig. 1). This suggests that this glycoprotein could form a very stable complex with Al explaining the benefits of some AM strains, which give higher tolerance to crops that grow in areas where Al phytotoxic is high. The same behavior was shown in other structures of AM fungi isolates from soils and *in vitro* culture of *Glomus intraradices*. Fluorescence emission represents Al content inside the observed structures.

2.6 Agricultural practices favoring AM contribution

The interaction among AM fungi and different agronomic management practices has been studied in acidic soils, showing an increase in wheat production, P acquisition and colonisation rates when plants were inoculated with *Glomus etunicatum* and a no-tillage system (Rubio et al., 2003).

Arbuscular mycorrhizas increase nutrient absorption capacity of plants mainly P, N and some microelements. However, with respect to N, the kind of fertilizer could influence the colonisation development. Recent studies have shown that $N\text{-}NO_3^-$ fertilization in wheat plants inoculated with *Glomus etunicatum* favors mycorrhizal development and its function when they were compared with other plants fertlized with $N\text{-}NH_4^+$ (Cornejo et al., 2007). However, this response will be conditioned by the plant genotype. Other studies carried out in acidic soils have shown that $N\text{-}NO_3^-$ fertilization favors AM propagule development associated with wheat plants. In this regard, more than 4.000 AMF spores per 100 g of soil were found at postharvest stage. It is of special relevance to the amount of fungal propagules that remains in the soil and encourages the next establishing crop (Cornejo et al., 2008b).

In addition, another important factor that influences mycorrhizal behavior in acidic soils is the kind of tillage. In this sense, no-tillage promotes soil chemical properties by increasing P, C, N and S concentrations after wheat harvest as well as mycorrhizal colonisation indicators and GRSP amounts produced by AM fungi (Borie et al., 2006). Besides, it has been reported that native ecotype *Glomus etunicatum* favors Ca and Mg acquisition in Al-tolerant wheat plants. This effect is enhanced when liming applications are conducted (Borie and Rubio, 1999).

According to previous reviews, agronomic management practices determine the role of mycorrhizal fungi associated with wheat plants growing in acidic soils. In this respect, AM can contribute to plant in alleviation of Al stress, through nutrient acquisition improving plant nutrition, by organic acidic exudation with chelating ability (Cumming and Ning, 2003; Klugh and Cumming, 2007; Klugh-Stewart and Cumming, 2009) or by producing GRSP, which has a potential capacity of Al sequestration/immobilization. However, further research is needed for defining AM fungi contribution on stress alleviation produced by Al phytotoxicity.

Several agronomic practices can incide on AM fungi diversity and functionality, by this way it is possible to favours the role of AM fungi associated to plants growing in acidic soil under phytotoxic Al levels especially in an extensive agricultural system.

2.7 Conclusions and future research trends

This review performs a compilation of AM fungi species that have been studied in acidic soils with high levels of Al and analyzes the main contributions of AM fungi to plants growing in Andisols with high levels of Al phytotoxicity. Among the species found in acidic soils *Glomus, Acaulospora, Gigaspora* and *Scutellospora* highlight, which have shown differences in the alleviation of Al phytotoxicity evaluated in terms of biomass production, organic acid exudation and nutrient acquisition. Information provided in this review emphasizes the contribution of AM fungi to plants growing in acidic soils mainly based on increased nutrient acquisition, contributing to plant resistance, decrease in Al and Mn translocation into the shoot and possible Al immobilization/sequestration by AM fungal structures and GRSP.

The above mentioned it has been target of studies focused mainly on technological applications; however, inoculant formulation for use in biofertilization requires a better understanding of physiological, molecular and ecological bases regulating AM fungi diversity and their contribution to plants growing in soils with phytotoxic Al levels.

Biofertilization with AM fungi has not been succesfully developed mainly due to difficulties in culture establishing. Specifically, it is necessary to promote the use of monoxenic cultures, to study the role of glomalin in these soils and select efficient ecotypes. In this way, it would be possible to optimize plant performance in agricultural crops growing in acidic soils.

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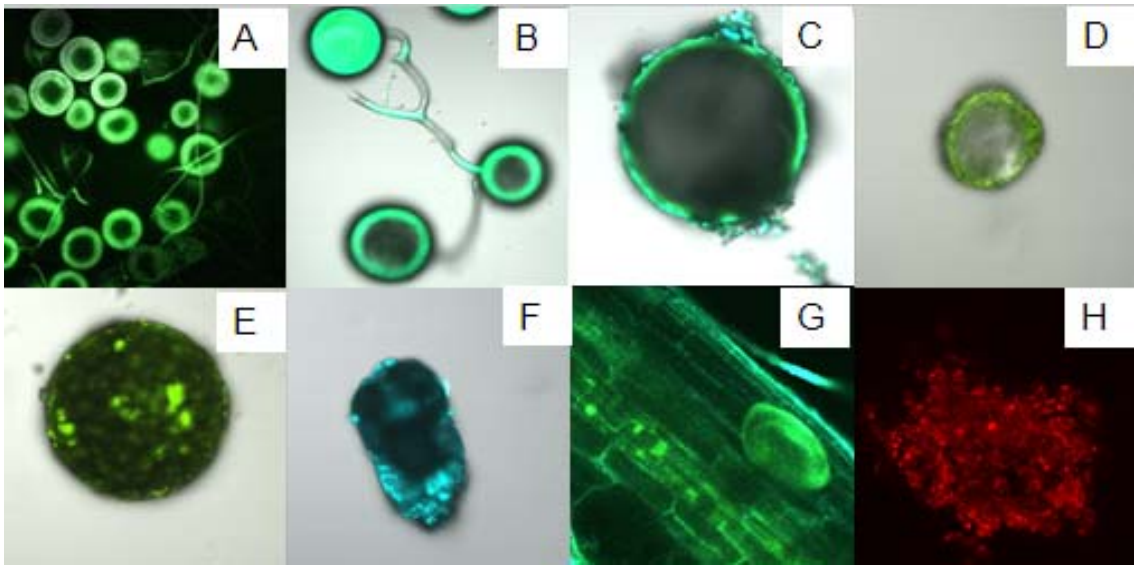

Figure 1. Visualization of AM fungi structures by confocal laser scanning microscopy. A-B: spores and hyphae from *in vitro* cultures of *Glomus intraradices* with Al^{3+} addition. C-D-E-F: Spores from rhizosphere of *Triticum aestivum* plants growing in acidic soils with high Al saturation. G: Fluorescence of spores and cell walls from colonized roots of *in vitro* culture. H: Glomalin-related soil protein from acidic soil with Al^{3+} addition. (Aguilera et al., unpublished data).

Table 1. Arbuscular mycorrhizal fungal diversity associated to host plant growing in acidic soils.

Arbuscular Mycorrhizal Fungi	Host Plants	Condition	Identification Method	References
Acaulospora dilatata, Acaulospora lacunosa and Acaulospora rugosa	*Andropogon virginicus* L.	pH 3.7; Al-Sat* 76 % pH 3.64; Al-Sat 91 % pH 4.2; Al-Sat 33 %	Taxonomic clasification	Morton, 1986
Glomus deserticola and Glomus mosseae	*Medicago sativa*	Increasing aluminum concentrations	Previously identified	Arriagada et al., 2007
Glomus etunicatum	*Hordeum vulgare L.*	Acidic soil	Previously identified	Mendoza and Borie, 1998; Borie and Rubio, 1999
Glomus etunicatum	*Triticum aestivum L.*	Acidic soil	Previously identified	Rubio et al., 2002; Rubio et al., 2003; Cornejo et al., 2008b;
Glomus etunicatum	*Sorghum bicolor* L.	Acidic soil	Previously identified	Cornejo et al., 2007
Acaulospora alpine and *Acaulospora brasiliensis*	*Plantago lanceolata*	pH 4.0-5.0	Morphological analyses and Molecular characterisation	Krüger et al., 2011
Acaulospora alpine	Grasslands of the Swiss Alps	Acidic soil	Morphological and molecular identification	Oehl et al., 2006a

Acaulospora bireticulata *A. gerdemannii A. laevis A. scrobiculata A. spinosa A. rehmii* *Acaulospora* sp. *Acaulospora* sp. *Entrophospora colombiana* *Gigaspora margarita* *G. decipiens Glomus agreggatum* *G. clarum* *G. diaphanum G. etunicatum G. fasciculatum G. geosporum G. macrocarpum G. microcarpum* *G. pansihalos Scutellospora gilmorei* *S. nigra* (Redhead) *S. pellucida* *Scutellospora* sp.	Sorghum bicolor Moench. L	pH 3.7	Morphological identification	Moreira-Souza et al., 2003
Acaulospora longula Acaulospora rugosa *Acaulospora scrobiculata* *Acaulospora morrowiae Glomus caledonium*	Acid savannas of Brazil	pH 5.2	Molecular characterisation	Oliveira et al., 2009

*Aluminum saturation.

Table 2. Arbuscular mycorrhizal fungal species and their efficiency in technological applications studies based on plant exposed to acidic conditions.

Acidic Conditions	AM Fungi	Host Plant	Research Highlights	References
0-50-200 uM Al	*Acaulospora morrowiae* *Glomus claroideum* *Glomus clarum* (1) *Paraglomus brasilianum*	*Liriodendron tulipifera* L.	(1) Showed 88 % colonisation rates, promoted Al plant resistance, colonized plants acumulated less Al in leaves, 2- to 10-fold higher P in leaves than other mycorrhizal plants and promoted citrate, malate and oxalate exudation.	Klugh and Cumming, 2007
0-100 uM Al	*Acaulospora morrowiae,* *Glomus claroideum,* *Glomus clarum* (1) *Glomus etunicatum* (2), *Paraglomus brasilianum,* *Scutellospora heterogama (*3 *)*	*Andropogon virginicus*	(1) and (3) showed high hyphae lengths as well as citrate and malate exudation from colonized plant roots (1) It was the most favored colonized plant growth. While, Al exposition affect all others colonized plants. And it presented the greatest colonisation. (2) acumulated greatest P amount in shoot under Al exposition (1) gived highest Al resistance to plants and acumulated lowest Al concentrations in plant shoots.	Klugh-Stewart and Cumming, 2009
pH 4.2 (originally) pH 3.0, 4.0 and 5.0 (experiment)	*Acaulospora scrobilculata* *Glomus* spp *Gigaspora* spp *Scutellospora fulgida*	*Clusia multiflora*	Mycorrhizal plants produced higher dry matter of roots. Roots length was no afected by pH 3.0 and they showed low Al concentration. Inoculant plants showed higher AM colonisation.	Cuenca et al., 2001

pH 4 and 5	*Glomus clarum* (1), *Glomus diaphanum* (2), *Glomus etunicatum*, *Glomus intraradices*, *Gigaspora Gigaspora albida*, *Gigaspora. margarita*, *Gigaspora rosea* and *Acaulospora morrowiae*	*Panicum virgatum* L.	Mycorrhizal plants increased 52- and 26- fold in dry matter at pH 4 an 5, respectively. (1) and (2) showed the highest dry matter increase	Clark et al., 1999a
4.48 pH_W 88% Al saturation	*Glomus clarum* (1), *Glomus diaphanum* (2), *Glomus etunicatum*, *Glomus intraradices*, *Gigaspora albida*, *Gigaspora margarita* (3), *Gigaspora rosea*, and *Acaulospora morrowiae*	*Panicum virgatum* L.	(1) and (2) increased 42- and 36- fold in dry matter (shoot and root), respectively at pH 4 in relation to nonmycorrhizal plants. (1) increased 64- and 19- fold in root dry matter at pH 4 and 5, respectively. (3) showed the greatest root colonisation at pH 4 and 5.	Clark et al., 1999b
0-400 uM Al	*Glomus clarum* (1) *Acaulospora morrowiae* *Scutellospora heterógama*	*Andropogon virginicus*	(1) The most efficient species that showed high colonisation rates (78%) and low Al traslocation to shoots.	Kelly et al., 2005
pH 4.2-5.2	*Gigaspora Margarita*	*Ipomoea batatas*	Mycorrhizal plants showed 2- fold in dry weight at pH 4.2 and these plant reduced their	Yano and Takaki, 2005

			toxic symptoms.	
pH 4.0; 5.0	*Glomus etunicatum* (1) *SCT110* *Scutellospora pellucida* (2) *SCT111* *Acaulospora scrobiculata SCT112* *Scutellospora heterogama SCT113*	*Malus prunifolia*	(2) showed high colonisation levels; 68 and 66% at pH 4.0 and 5.0, respectively. (1) and (2) increased 132 and 146% of plant heigh respect of non-mycorrhizal plants.	Pereira et al., 2007
180 μM Al	*Glomus intraradices*	*Musa acuminata*	Increase in biomass, water and nutrient absorption. Al in roots and shoots was decreased and Al symptoms in plants were disminished.	Rufyikiri et al., 2000
pH 4.7	*Gigaspora margarita*	*Vigna unguiculata* L	Under 11.9 mg kg^{-1} of available Al spores germination decreased about 40%	Rohyadi, 2005

CHAPTER III

"Fluorescence detection of aluminum in arbuscular mycorrhizal fungal structures and glomalin by using confocal laser scanning microscopy"

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Fluorescence detection of aluminum in arbuscular mycorrhizal fungal structures and glomalin by using confocal laser scanning microscopy

Paula Aguilera, Fernando Borie, Alex Seguel and Pablo Cornejo*

Center of Amelioration and Sustainability of Volcanic Soils. BIOREN-UFRO. Universidad de La Frontera, P.O. Box 54-D, Temuco, Chile.

*Corresponding Author: Pablo Cornejo

Tel.: +56 45 325433

Fax: +56 45 325440

E-mail address: pcornejo@ufro.cl

Abstract

Arbuscular mycorrhizal spores and glomalin-related soil protein (GRSP) isolated from acidic soils were analyzed using confocal laser scanning microscopy (CLSM) for Al detection. Mycorrhizal structures of *Glomus intraradices* produced under *in vitro* conditions as well as spores and GRSP from neutral and Cu polluted soils were used as contrasting criteria. Spores and GRSP from soils with 7 and 70% Al saturation showed autofluorescence which increased especially at the highest soil Al level and when Al^{3+} solution was added. *Glomus intraradices* spores showed fluorescence only when exogenous Al^{3+} was added. On the contrary, spores and GRSP from neutral and Cu-polluted soils showed low or no fluorescence. This fluorescence shown by fungal structures and GRSP when subjected to high Al (be endogenous or exogenous) suggest a high capacity for Al immobilization, which could be an effective way to reduce Al activity and phytotoxicity in acidic soils.

Keywords: Acidic soils; arbuscular mycorrhizal propagules; Cu-polluted soil; glomalin-related soil protein; *Glomus intraradices*.

Aluminum is one of the prevalent metals generating phytotoxicity due to the strongly negative effects on plant root growth, reducing directly water and nutrient acquisition (Ma et al., 2001; Kochian et al., 2004; Kochian et al., 2005). Primary and the earliest symptoms of Al phytotoxicity are a fast constraint of root growth, especially in root apexes (Kochian et al., 2005). Severe changes in specific membrane lipids have been reported as the main Al phytotoxicity mechanisms (Jones and Kochian, 1997). Additionally, arbuscular mycorrhizal (AM) fungi are obligated symbionts of about 80%

terrestrial plants (Smith and Read, 2008), some of them growing in soils with serious limitations. Various studies have demonstrated that AM symbiosis protect plants against stress produced by high levels of diverse toxic elements such as heavy metals (González-Guerrero et al., 2008; Bissonnette et al., 2010; Janoušková and Pavlíková, 2010; Miransari, 2010) and Al (Borie and Rubio, 1999; Cumming and Ning, 2003). This protection could be performed indirectly by a better nutrient acquisition as P, Ca and Mg, all elements having antagonistic effects against Al phytotoxicity, or directly, through its immobilization in fungal structures or compounds produced by AM fungi (Etcheverría, 2009; Borie et al., 2010).

Several techniques to identify AM fungal structures have been used, including confocal laser scanning microscopy (CLSM), through the autofluorescence exhibited by structures as hyphae (Ames et al., 1982) or using fluorochromes to observe arbuscles and spores (Dickson and Kolesik, 1999; Vierheilig et al., 2001; Schweiger et al., 2002; Gutiérrez et al., 2003; Vierheilig et al., 2005; Dreyer et al., 2006; 2008; 2010).

Recent evidence in our research group shows a strong relationship between soil exchangeable Al levels and glomalin-related soil protein (GRSP) (Etcheverría, 2009), a glycoprotein produced by AM fungi (Wright and Upadhyaya, 1996). Hence, the aim of this study was using CLSM to visualize the behavior of spores and GRSP isolated from acidic soils with 7 and 70% Al saturation (Al-sat) and compare them to those isolated from neutral and Cu-polluted soils, and also with *Glomus intraradices* spores produced under *in vitro* conditions. In all cases, the samples were directly analyzed by CLSM, observing fluorescence emission before and after Al^{3+} addition.

Spores from neutral, acidic and Cu-polluted soils were isolated by wet sieving and decanting (Sieverding, 1991) and finally observed by CLSM with or without Al^{3+} addition (0.2 M $AlCl_3$). Rhizosphere soil samples were obtained from: *i)* two acidic Andisols (pH 4.5, soil organic matter -SOM- 12.2%, Al-sat. 70%; and pH 5.3, SOM 11.8%, Al-sat. 7%, respectively); *ii)* one neutral Mollisol (pH 7.4; SOM 2.9%; Al-sat. < 0.2%); and finally, *iii)* one Cu-polluted Entysol (pH 4.6; SOM 2.1% Al-sat < 0.5%; total Cu 830 mg kg^{-1} soil and DTPA extractable Cu 330 mg kg^{-1} soil).

Total GRSP was extracted from soils using Wright and Upadhyaya procedure (1996). Briefly, 1 g soil in 8 mL of 50 mM citrate buffer pH 8.0 was autoclaved for 1 h at 121°C, and this procedure was repeated until no dark coloured was obtained. Then, the supernatant was filtered through Whatman n° 1 paper, precipitated at pH 2.5 using 2M HCl, pelletized by centrifugation, re-suspended in 0.5 M NaOH, extensively dialyzed against ddH_2O and freeze dried. Total GRSP was directly observed by CLSM and also after Al^{3+} addition. We performed a screening of spores and GRSP by the use of λ-scan that generated fluorescence emission at 405, 488 and 633 nm in a Fluoview FV1000 Confocal Laser Scanning Biological Microscope (Olympus, Japan). The FV10-ASW v. 2.0c software was used for obtaining emission intensities.

Arbuscular mycorrhizal spores obtained from acidic soils with 7 and 70% Al-sat showed autofluorescence, mainly localized in the cell wall in the first case (Fig. 1A). In addition, a higher fluorescence covering the whole spore structure was also observed in the second case (Fig. 1B). The Al^{3+} addition allowed the observation of high fluorescence in the whole spore contents isolated from soil with 7% (Fig. 1C), being equivalent to the

emission of spores growing at 70% Al-sat (Table 1; Fig. 1D-E). Additionally, spores growing at 7% and 70% Al-sat with addition of Al^{3+} only slightly ($P>0.05$) increased their emission levels with respect to spores without Al^{3+} addition (Table 1). These findings suggest the existence of a maximum capacity of Al sequestration/immobilization by different AM spore components, which could be achieved under *in vivo* conditions in soils with Al-sat levels near 70%. Spores, hyphae and AM colonized carrot (*Daucus carota*) transformed roots from monoxenic cultures of *G. intraradices* (St-Arnaud et al., 1996) were extracted, sectioned and placed on slides; some of them maintained in ddH_2O and the others in 0.2 M $AlCl_3$. Under light microscopy, *G. intraradices* exhibited a normal morphology and colonization (Fig. 2A). In addition, the *G. intraradices* hyphae showed autofluorescence without Al^{+3} or fluorochromes addition under laser excitation (Fig. 2B). On the other hand, spores only showed fluorescence after Al^{3+} application (Table 1; Fig. 2C-D). Autofluorescence of some AM fungal structures have been previously described, especially in arbuscles (Ames, 1982; Gange et al., 1999), but the reason of this trend is still unclear, and the proposed fluorescent component is widely diverse, including the matrix or host plasmalemma (Ames et al., 1982), phenolic compounds (Vierheilig et al. 2001) and chitin (Dreyer et al., 2006). However, these hypotheses do not explain the absence of autofluorescence in the spores, suggesting that fluorescence in the AM spores observed here is produced by the interaction of Al^{3+} with compounds present on the spore cell wall surface (as glomalin; Driver et al., 2005) and also inside the spore. Within carrot colonized transformed roots, a slight autofluorescence was observed (Fig. 2E), which increased when Al^{3+} was added, allowing detection of intraradical spores and vesicles. An increase of fluorescence in root cell walls was also observed (Fig. 2F).

Furthermore, spores from Cu-polluted soil showed low autofluorescence (Fig. 1F), maintaining a similar fluorescence after Al^{3+} addition (Table 1; Fig. 1G). Similar behavior was observed in spores isolated from neutral soil, where the Al^{3+} addition did not produced an increase in fluorescence (Table 1; Fig. 1H 1I). These results suggest the formation of a stable complex between metals and fungal components through immobilization sites, especially in cell walls, limiting the substitution of Cu or other metallic ions usually present in neutral soils by Al^{3+} ions. This aspect may be relevant in multicontaminated soils, where AM fungus could play an important role mitigating some of them, and reaching a maximum level of metal immobilization in their structures. In addition, heavy metal immobilization and accumulation capacities by AM fungal structures have been previously observed for Cu in extraradical mycelium (González-Chávez et al., 2002) as well as Zn, Cu and Cd in the cell wall, vacuoles and cytoplasm (González-Guerrero et al., 2008).

Aluminum salts (as $AlCl_3$) have been previously used in microscope observations as an enhancer of contrast (Silva, 2000). In this context, Tannous et al., (2003) successfully used Al^{3+} for increasing brightness of nuclei in tumor cells, thus enhancing CLSM visualization of cutaneous neoplasms. However, Al location in AM fungal studies has not been previously reported; our study is the first that uses Al fluorescent properties when coordinated with other chelating agents to visualize Al^{3+} location on fungal structures. It also suggests an important role of AM fungi in the attenuation of Al phytotoxicity in soils with high exchangeable Al levels through stable Al complex formation with GRSP, which is known to be tightly bound in mycorrhizal hyphae and spores (Driver et al., 2005).

In this sense, we also included Al detection in GRSP here, which is known for its high metal sequestration capacity (González-Chávez et al., 2004; Cornejo et al., 2008; Vodnik et al., 2008). In this regard, there was a strong increase in fluorescence emission in GRSP extracted from soil with 7% Al-sat (Fig. 1J), when Al^{3+} was added (Table 1; Fig. 1K). In contrast, GRSP extracted from soil with 70% Al-sat showed a higher autofluorescence (Fig. 1L). However, when exogenous Al^{3+} was added, the autofluorescence did not increase (Table 1; Fig. 1M). This could indicate that Al sequestration in GRSP was near its maximum level of immobilization in soil with 70% Al-sat.

On the other hand, CLSM observation of GRSP extracted from Cu-polluted soil showed no fluorescence either before or after Al^{3+} addition (Table 1), suggesting a strong Cu-binding capacity to GRSP, which probably saturated all binding sites explaining the high Cu proportion represented by its elemental composition (2.7%; Cornejo et al., 2008). In addition, GRSP from neutral soil showed no fluorescence (Fig. 2N), but it was generated when Al^{3+} solution was added (Table 1; Fig. 2O). This behavior could be due to Al absence in its original structure. Etcheverría (2009) reported that Al linked to GRSP was higher than Fe-GRSP in Andisols from four native forests of southern Chile. As GRSP have been obtained from *in vitro*/soilless growth (Gadkar et al., 2006; Nichols, 2010) as well as from soils, this study is the first direct evidence of GRSP ability to sequester Al in the molecule, suggesting that this glycoprotein could form stable complexes with Al, explaining the benefits of some AM fungal strains in terms of increasing Al-tolerance of crops growing in soils, where phytotoxic Al is high. In a previous study, we reported that the high GRSP content observed in some Cu-polluted soils could operate as an important factor in soil

remediation (Cornejo et al., 2008), and the same role could be played in soils with high Al content. These benefits obtained by AM activity could be transient or for longer time according to the residence time of fungal structures in the soil or if the bulk of Al immobilized is performed through GRSP-Al complex formation. However, some evidence indicates that these effects could be prolonged, since the turnover time of GRSP has been estimated on several years (6-42 years; Rillig et al., 2001), and AM spores can survive and germinate in soil after a lot of years (Tommerup, 1992; McGee et al., 1997).

In conclusion, direct observation by CLSM without normally used fluorochromes allowed us to evidence the ability to sequester and possibly accumulate Al in AM fungal structures and GRSP, hence suggesting an important role of AM fungi in alleviating the phytotoxicity of this element in acidic soils with high Al levels. Notwithstanding, the observations here made should be complemented with other techniques, such as ultrastructural localization, to know the specific location and the amount in which Al is immobilized. The physiological and molecular bases by which these fungi are capable of sequester, transport and compartmentalize this element inside their structures also need further studies.

Acknowledgements

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Figure legends.

Figure 1. Fluorescence of AM fungal spores and glomalin-related soil protein (GRSP) isolated from acidic, neutral and Cu-polluted soils. (A) Spores from acidic soils at 7% aluminum saturation (Al-sat); (B) spores from acidic soils at 70% Al-sat; (C) 7% Al-sat with Al^{3+} addition; (D) 70% Al-sat with Al^{3+} addition; (E) spores from soil with 70% Al-sat with Al^{3+} addition measured with 8 slices. (F) Fluorescence of AM spores from Cu-polluted soil; (G) fluorescence of spores from Cu-polluted soil with Al^{3+} addition. (H) Fluorescence of AM spores from neutral soil; (I) fluorescence of spores from neutral soil with Al^{3+} addition. Fluorescence (Merge) of GRSP from soils at different Al saturation: (J) acidic soils at 7% Al-sat; (K) acidic soils at 7% Al-sat with Al^{3+} addition; (L) acidic soils at 70 % Al-sat; (M) acidic soils at 70% Al-sat with Al^{3+} addition; (N) GRSP from neutral soil; (O) GRSP from neutral soil with Al^{3+} addition.

Figure 2. Observation of monoxenic cultures of *Glomus intraradices* growing in association with *Daucus carota* transformed roots. (A) Light microscopy observation. (B) Autofluorescence detection of hyphae by confocal laser scanning microscopy (CLSM). (C) Visualization of hyphae and spores with Al^{3+} addition by CLSM (bright field). (D) Visualization of hyphae and spores with Al^{3+} addition by CLSM (Merge). (E) Fluorescence of colonized roots. (F) Fluorescence of AM spores, vesicles and cell walls from colonized roots with Al^{3+} addition.

Table 1. Fluorescence intensity from diverse structures of arbuscular mycorrhizal fungi (spores and hyphae) and glomalin-related soil protein (GRSP) under different aluminum conditions.

Soil or Medium	Fungal structure	Al^{3+} added	Mean relative fluorescence intensity[1]	
			Relative fluorescence μm^{-2}	*T*-Student value
Monoxenic culture	Spores	-	0	-5.90**
		+	3.21×10^{-2} (5.4×10^{-3})	
	Hyphae	-	3.02×10^{-2} (2.3×10^{-3})	-0.60ns
		+	3.67×10^{-2} (1.1×10^{-2})	
Acidic soil 7 % Al saturation	Spores	-	3.62×10^{-2} (5.2×10^{-3})	-1.76ns
		+	0.18 (8.3×10^{-2})	
	GRSP	-	2.23×10^{-3} (9.2×10^{-5})	-11.64***
		+	1.09×10^{-2} (7.4×10^{-4})	
Acidic soil 70 % Al saturation	Spores	-	6.79×10^{-2} (2.9×10^{-2})	-0.58ns
		+	9.07×10^{-2} (2.5×10^{-2})	
	GRSP	-	6.53×10^{-3} (5.7×10^{-4})	-1.14ns
		+	1.07×10^{-2} (3.6×10^{-3})	
Cu-polluted soil	Spores	-	7.32×10^{-3} (1.2×10^{-4})	1.43ns
		+	5.00×10^{-3} (1.1×10^{-4})	
	GRSP	-	0	nd
		+	0	
Neutral Soil	Spores	-	5.91×10^{-3} (9.3×10^{-4})	0.39ns
		+	5.44×10^{-3} (7.0×10^{-4})	
	GRSP	-	0	-11.73***
		+	1.99×10^{-2} (1.7×10^{-3})	

[1] Significance conventions: *P<0.05; **P<0.01; ***P<0.001; ns=no significance; nd= not detected. In each case four samples were considered. The means are followed by the standard errors in brackets.

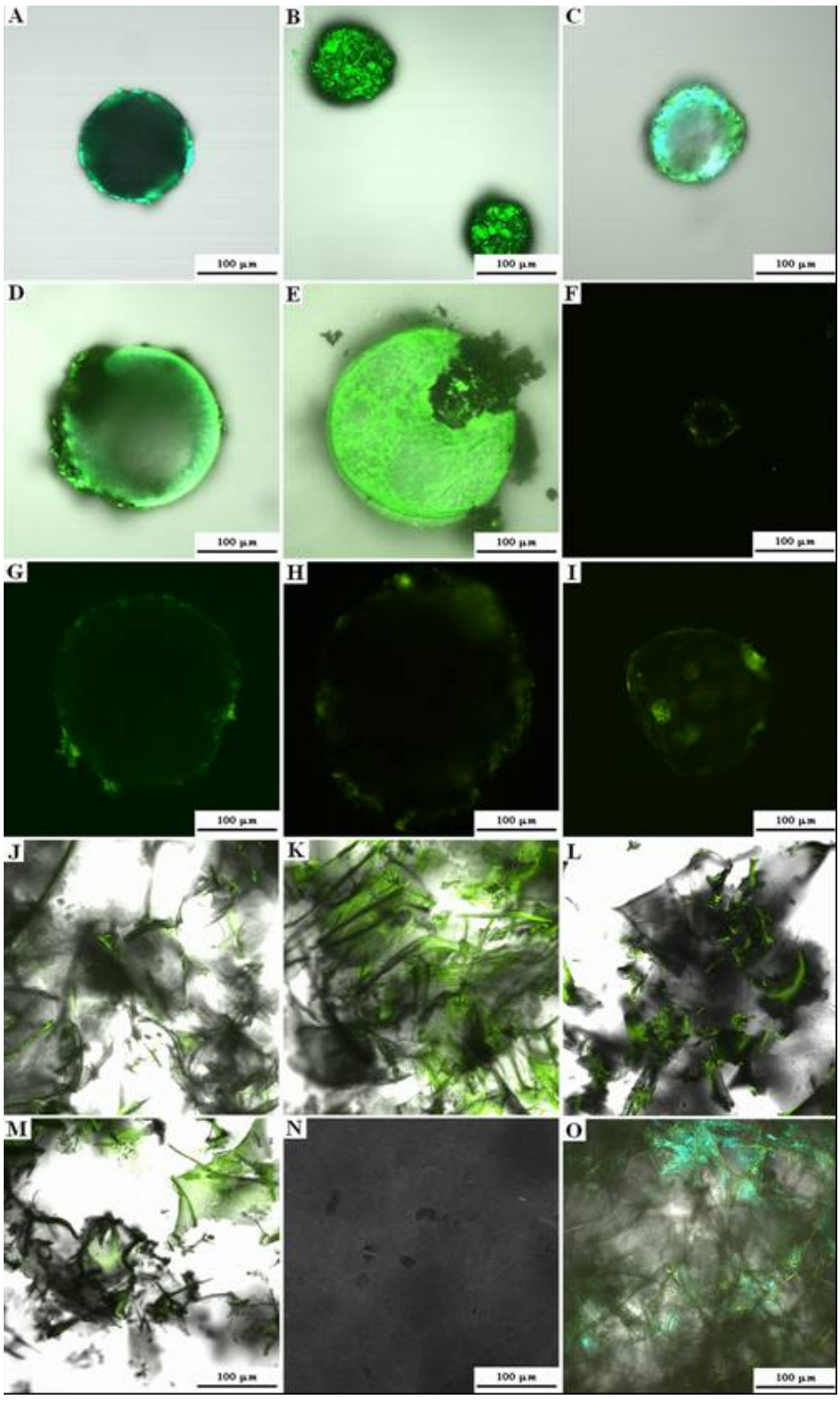

Figure 1.

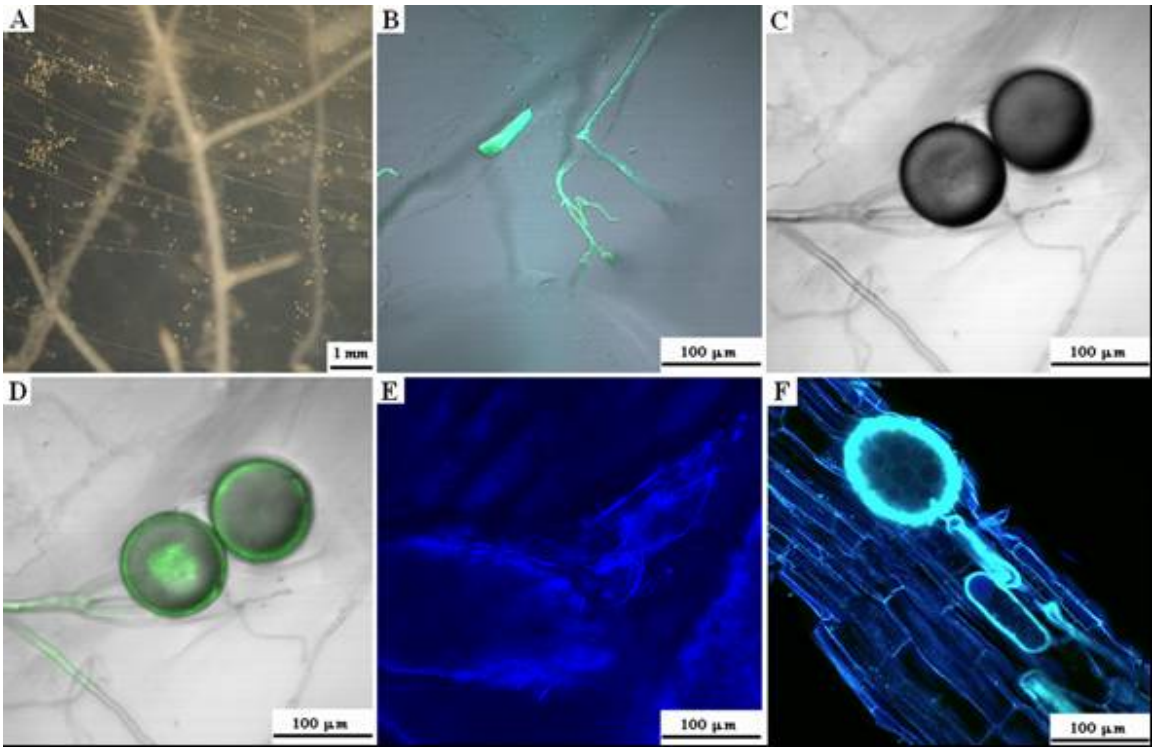

Figure 2.

Research highlights

-We detected interactions between aluminum and AM fungal structures using CLSM.

-Fungal and GRSP fluorescence emission could be related with Al levels in the medium.

-We suggest a key role of AM structures

CHAPTER IV

"Diversity of arbuscular mycorrhizal fungi associated with Triticum aestivum L. plants growing in an Andosol with high aluminum level"

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Type of contribution: regular paper

Diversity of arbuscular mycorrhizal fungi associated with *Triticum aestivum* L. plants growing in an Andosol with high aluminum level

Paula Aguilera[a], Pablo Cornejo[a*], Fernando Borie[a], José Miguel Barea[b], Erik von Baer[c] and Fritz Oehl[d]

[a]Center of Amelioration and Sustainability of Volcanic Soils, BIOREN-UFRO, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[b]Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain.

[c]Campex Semillas Baer. Gorbea, Casilla 87-Temuco-Chile.

[d]Agroscope, Institute of Sustainability Sciences, Plant-Soil-Interactions, Ecological Farming Systems, Reckenholzstrasse 191, CH-8046 Zürich, Switzerland.

*Corresponding Author: Pablo Cornejo

Tel.: +56 45 2325433

Fax: +56 45 2325440

E-mail address: pablo.cornejo@ufrontera.cl

Abstract

Arbuscular mycorrhizal (AM) fungi contribute to agricultural productivity by improving plant nutrient and water acquisition, and modifying some physiological traits under various environmental stresses. Notwithstanding, there is a lack of knowledge about the ecology and behavior of AM fungi in acidic soils with high levels of exchangeable aluminum (Al). Therefore, we have carried out a preliminary research based on the taxonomic identification of AM fungal species associated to winter wheat habitually cropped in an Andosol from Southern Chile. Rhizosphere soils from six winter wheat cultivars ("Bakan", "Porfiado", "Maxi", "Crac", "Invento" and "Otto") were collected from field plots. AM fungal species identification was performed based on spore morphological analyses. In this study, we additionally observed AM fungal propagules (spore density and colonization levels) at two crop stages (grain filling and post harvest). At grain filling stage, "Bakan", "Porfiado" and "Maxi" cultivars showed higher colonization levels than "Otto", "Crac" and "Invento" ones. Spore density increased at post harvest stage and "Maxi", "Otto" and "Invento" cultivars presented higher spore densities than other three cultivars. Twenty-four AM fungal species were identified and subsequently classified into genera *Acaulospora, Pacispora, Claroideoglomus, Glomus, Funneliformis, Septoglomus, Simiglomus, Scutellospora, Cetraspora, Ambispora, Archaeospora* and *Paraglomus.* The Shannon-Wiener values showed no significantly difference in AM fungal diversity associated to wheat cultivars, but species richness was significant different among cultivars. Simpson´s index was significantly different among AM fungal communities being *Acaulospora* and *Scutellospora* the most dominant genera. In this study, AM fungal community structure was different for specific wheat cultivar; hence the use of target AM

fungal species could be determinant factor for the appropriate AM community establishment in potential inoculation assays, especially in Andosols with high aluminum levels.

Keywords: Acidic soil, Al tolerant wheat cultivars, AM fungal diversity.

4.1 Introduction

Arbuscular mycorrhizal (AM) symbiosis is an association established between soil fungi and most terrestrial plants. The association is mutually beneficial for both partners based on a bidirectional interchange of nutrients (Barea, et al. 2013; Smith and Read, 2008), particularly phosphorus (P) (Borie et al. 2010; Borie and Rubio, 1999; Larsen et al. 2009; Smith and Smith, 2013). Essentially, the fungus improves nutrient and water absorption capacity of the host plant while the fungus receives carbon compounds from plant photosynthesis (Barea et al. 2013).

In general, AM fungi are adapted to the environment from which they were isolated and they colonize plants growing in soils with pH ranging 2.7 to 9.2 (Clark and Zeto, 1996a; 1996b; Siqueira et al. 1984). However, it has been reported that among AM fungal species, apart from natural phylogenetic diversity, there is also functional diversity mainly due to different behavior under diverse environmental stresses (Klugh and Cumming, 2007; Klugh-Stewart and Cumming, 2009; Medeiros et al. 1994a; 1994b).

When a AMF application program is undertaken, it is fundamental to assess first the AM fungal diversity by estimating the abundance and variety of AM fungal species in the

target agroecosystem. In addition, the study of AM fungal diversity provides basic information to assess the impacts generated by some agricultural practices on AM fungal community structure; these impacts affect directly the sporulation dynamics, AM fungi presence and plant interactions (Oehl et al. 2003; 2004; 2009; van der Heijden et al. 1998). For example, and relevant to this study, it has been reported that low pH and high aluminum (Al) contents in soils have negative impacts on AM fungal colonization (Göransson et al. 2008; Seguel et al. 2013).

Various studies have concluded that AM fungi improve plants nutritional status under different stress conditions, having a potential use in agriculture (Hijri et al. 2006). Therefore, more studies regarding the ecology of AM fungi and their physiological behavior are required, since their status as obligate symbionts create difficulties in establishing pure cultures or managing the indigenous AM communities through agronomic practices (Barea et al. 2013; Oehl et al. 2005; 2009; 2010), at the present limiting the massive use of AM inoculants.

The benefits provided by AM fungi not only vary with the different strains that effectively colonize a host root, which influences their abundance and diversity, but also with their physiological effectiveness which depends on the ability to survive and grow, as affected by the prevalent environmental factors (Barea et al. 2013; Morton et al. 1995; Morton and Bentivenga, 1994).

Several studies have reported that some AM fungal species associated with specific hosts promote plant fitness in acidic soils with high Al levels (Borie et al. 2010; Borie and Rubio, 1999; Cumming and Ning, 2003; Klugh-Stewart and Cumming, 2009; Lux and

Cumming, 2001). This is the case with wheat (*Triticum aestivum* L.), the most widely grown cereal crop species in Chile, mainly due to the economic value of this crop. Wheat production is concentrated in Central Southern Chile, especially in the Araucanía Region (37 to 40°S latitude), where it covers about 248,000 ha chiefly in Andosols which represent 60% of the arable soil in the country (Besoaín, 1985). These soils are characterized by a pH ranging from 4.5 to 5.5 and high exchangeable Al. In general, volcanic soils have problematic properties, such as high P adsorption capacity and high Al^{3+}, Mn^{2+} and H^{+} ion levels.

The objective of the present study was to analyze in different wheat crop varieties the changes in AMF propagule densities and of overall AM fungal community composition in such acidic soils with high Al^{3+} levels, these varieties had especially been bred for Chilean crop production systems. Colonization rates, spore densities, species richness and several diversity indices were determined in the rhizospheric topsoils of six Al tolerant wheat cultivars used.

In previous studies we have found a high attenuation ability of high Al levels in AM fungi associated with Al tolerant wheat cultivars (Seguel et al. 2012). Additionally, this ability of AM symbiosis could be related with the specific analyzed AM species. Therefore, the aim in this study was to determine the AM fungal community structure and composition and to understand their interaction with six Al tolerant wheat cultivars under field conditions. This work is part of a research line focused on elucidating the main mechanisms by which AM fungi help to reduce negative Al effects and to find efficient AM fungal strains able to be used as inoculants in crop production in soils with high Al levels.

4.2 Materials and Methods

4.2.1. Soil sampling

The soil used was a Dystric Andosol (pH 4.5, -SOM- 12.2%, Al-Sat. 25%) sampled in an Experimental Station (39°06'14''S and 72°41'16''W). In this soil a field assay has been established including three replicate plots of six Al tolerant *Triticum aestivum* L. cultivars ("Bakan", "Porfiado", "Maxi", "Crac", "Invento" and "Otto"). These wheat cultivars have been developed previously by means of mass selection using this soil.

Rhizosphere soil was considered the soil adherent to roots of wheat plants obtained at 0-20 cm depth. Six rhizosphere soil subsamples were obtained from each plot and then combined, air dried and sieved through a 2 mm mesh and analyzed as one individual sample per plot. Soil samples were taken at two crop stages: grain filling (November, 2011) and one month postharvest (March 2012). Previously, in November, 2010, trap cultures were established using field samples (0-20 cm) according to the methodology proposed by Oehl et al. (2003) in order to improve detection of the whole AM fungal diversity. Trap cultures of AM were maintained for one year prior to analysis. According to Cornejo et al. (2007) postharvest is the best time for AM diversity analyses in wheat rhizosphere, because there it possible to found higher AM fungal spore densities and diversity at this stage in annual cereal crops.

4.2.2. Spore isolation and identification

Spores were isolated from soils using wet sieving and sucrose density gradient centrifugation. Briefly, 25 g of soil were passed through sieves of 500, 125 and 32 μm and

thoroughly washed with distilled water. The last soil portion collected in 32 µm mesh and the soil fraction between 500 and 125 µm were distributed into plastic tubes. 25 mL of the spore suspensions were transferred to 50 mL centrifugation tubes. 25 mL of a 70% sugar solution were inserted at the bottom of the tubes and centrifuged at 2000 rpm for 2 min. Samples were decanted after centrifugation, washed and transferred to Petri dishes for sorting and quantification under the dissection microscope at up to 400 fold magnification. The number of AM fungal spores was expressed as spores in 100 g of dry soil. Finally, spores were mounted on microscope slides in polyvinyl alcohol-lactic acid glycerol (PVLG) medium (Oehl et al. 2003; Sieverding, 1991) for identification.

The spores were identified based on morphological characteristics such as spore wall structures, subtending hyphae and germination structures. Identification reports (Błaszkowski, 2012; Oehl et al. 2011a, 2011b) and institutional collection of original species descriptions were used for all AM fungi. These analyses were carried out in Agroscope Reckenholz-Tänikon Research Station, Zürich, Switzerland.

4.2.3. Root colonization

Mycorrhizal colonization levels were determined by the gridline intersect (Giovanetti and Mosse, 1980) after clearing the roots in 2.5% KOH solution (wt/vol) and by staining then with 0.05% trypan blue (Phillips and Hayman, 1970). The gridline intersect method of Tennant (1975) was defined for estimating the total root length.

4.2.4. Diversity indices and statistical analysis

Arbuscular mycorrhizal fungal species richness (S), Shannon-Wiener index (*H'*), Evenness (*E*), Simpson index (*D*), AM colonization and spores densities in soil were subjected to one-way analyses of variance and means were tested by multiple range Tukey test. Significant differences were established at $P<0.05$. A hierarchical cluster using the farthest neighbor method based on structure and propagule densities was performed to determine similarities among the AM fungal communities associated with the wheat cultivars.

4.3 Results

4.3.1 Species and genera richness

In total twenty-four species of AM fungi were detected in the AM fungal community in this study. They belong to all classes and orders, and to nine families and twelve genera of the Glomeromycota (Table 1) according to Oehl et al. (2011a). Eighteen species could be unequivocally identified, whereas six others might correspond to undescribed species.

From the obtained data we analyzed total number of species in each AM fungal community by means of species richness (Whittaker, 1975). The AM fungal diversity was based on Shannon-Wiener diversity index (Hutchenson, 1970) and we determined AM fungal community structure by Simpson's dominance index (Simpson, 1949) and it was calculated from the mean of spore densities in each rhizospheric soil of wheat cultivars. Species evenness was also calculated. Highly significant differences were found in AM

fungal species richness among the six wheat cultivars (Table 1). Wheat cultivars were associated with range of 15-19 AM fungal species. Rhizosphere from “Porfiado” and “Invento” cultivars presented the highest species richness values (19), whereas from “Otto” cultivar the lowest value was observed (15).

4.3.2 Diversity indices

Shannon-Wiener index values showed no significant difference in relative abundance of each species of different AM fungal communities reflecting a proportional distribution of AM fungal species that was maintained at constant level across six wheat cultivars (Table 1). Calculation of the AM fungal community evenness did not shows significant differences indicating equitable distribution among AM fungal communities found in all wheat cultivar rhizospheres.

Simpson´s index was significantly different among AM fungal communities reflecting differences in AM fungal species dominance being “Porfiado” and “Invento” cultivars which showed the highest Simpson´s index values. Specific density analyses of 24 AM fungal species showed that the most representative genera were *Scutellospora* and *Acaulospora* (Fig. 1) although represented by different species numbers per genus. These AM fungal species were the most commonly identified and they represented more than 50% of the total found species.

4.3.3 AM spore densities and colonization

In this study, we also analyzed spore densities and colonization rates associated with the wheat cultivars at two crop stages (grain-filling and post harvest). At the grain filling-stage, “Bakan”, “Porfiado” and “Maxi” cultivars showed higher colonization rates than “Otto”, “Crac” and “Invento” (Fig.2). Spore densities increased in the post harvest stage, and “Maxi”, “Otto” and “Invento” cultivars had the highest spore densities (Fig. 3).

4.3.4. Similarities of Al tolerant wheat cultivars

A cluster analysis (Fig. 4) was used for grouping wheat cultivars in agreeing with similarities of AM fungal communities by using species richness, Shannon-Wiener diversity index, evenness, Simpson´s dominance index, specific density of each AM fungal species, spores number and colonization levels. Three groups were observed: group I including “Maxi”, “Porfiado” and “Invento” cultivars, although group II showed “Bakan” cultivar is associated with “Crac” cultivar and group III which is formed exclusively by “Otto” cultivar.

4.4 Discussion

4.4.1 Al tolerant wheat cultivars in an Andosol

The high Al saturation levels near 30%, low P availability and soil acidity are the main constraint characteristics which reduce potential cereal yields in Southern Chile

(Borie and Rubio, 1999). To overcome problematic conditions of acidic soils, farmers use alternatives as the application of high quantities of P fertilizers, lime application or use of Al tolerant wheat cultivars as the genotypes here studied. Negative effects caused by Al in acidic soils have been studied in several studies, but principally relating its negative effect on growth and root cellular elongation (Caldwell, 1989; Delhaize et al. 1993; Jones and Kochian, 1995; Seguel et al. 2013), which determines a low ability of nutrients, water absorption and losses in grain yield and quality. Furthermore, studies made with genotypes of *Triticum aestivum* tolerant and sensitive to Al have shown that Al tolerant root meristems have the ability to accumulate less Al in their tissues (Rincón and Gonzáles, 1992; Silva et al. 2000). Additionally, Gallardo et al. (1999) demonstrated a high increase of root growth in Al tolerant wheat cultivars growing under acidic conditions as compared to sensitive cultivars. Under this context, use of Al tolerant wheat cultivars is a reasonable alternative to cope with high Al levels (von Baer, 2007), and the relation of these genotypes with the indigenous microbial populations deserves to be analyzed, since some recent results have demonstrated that an important part of the Al-tolerance can be alleviated by the presence of Al-adapted AM fungi (Borie and Rubio 1999; Aguilera et al. 2011; Seguel et al. 2012).

Since 1956 some wheat cultivars were introduced in acidic soils of Southern Chile and contrasted with local cultivars, improving the germplasm in respect to tillering, yield and baking quality. Moreover, all selected materials here used were evaluated under breeding programs development at Experimental Station “Campex Semillas Baer”. Additionally, the most important cultivars obtained had higher association ability with AM fungi (von Baer, 2007), but this is the first study oriented to relate the role of indigenous

(presumably Al-adapted) AM fungi communities and their role in promoting certain degree of Al-tolerance in cultivars of wheat host plants developed under Al-restrictive conditions.

It is well-known that AM fungi improve the establishment of plants, especially when growing under unfavorable environmental conditions (Barea et al. 2013). In this context, several studies have demonstrated that some AM fungal species are able to survive and overcome acidic conditions and negative effects of Al presence (Borie and Rubio, 1999; Clark et al. 1999a, 1999b). In fact, interactions between Al tolerant wheat cultivars in combination with autochthonous AM fungal isolates have been largely documented in acidic soils with high levels of Al saturation, including cultivar "Otto" (Borie et al. 2002; Cornejo et al. 2007; Rubio et al. 2003) and more recently "Crac", "Invento" and "Porfiado" cultivars (Seguel et al. 2012). In our study cultivar "Otto" corresponds to the first cultivar to be released to the local market in 1989, offering yield stability to farmers in Southern Chile. In detail, "Invento" and "Porfiado" cultivars have been developed using cultivar "Otto" to obtain F1 progeny within the program to obtain Al tolerant wheat cultivars. Noticeably, in the cluster analysis (Fig. 4) "Otto" cultivar was included as a dendrogram root. Additionally, "Maxi", "Invento" and "Porfiado" cultivars were clustered in a same group, which could suggest a degree of co-adaptation between wheat cultivars and the most compatible or specialized AM fungal community.

4.4.2 AM fungal communities characterization associated to Al tolerant wheat cultivars

Some previous studies have characterized many AM fungal species into the fungal community as ecological "specialist" or "generalist" fungi according to its wide or

restricted distribution in agro-ecosystems (Castillo et al. 2006; 2010; Oehl et al. 2004; 2010). According to our results this characterization could be applied at genotypic level, since the changes in community structure are evident between the distinct cultivars of Al tolerant wheat here tested. In this context, some AM fungal species here found could be characterized as generalist fungi, because they are present in a homogeneous relative density across all the analyzed wheat cultivars (Table 1, Fig.1). Specifically, ten AM fungal species (*Acaulospora laevis*, *Acaulospora* sp CL1, *Acaulospora sieverdingii*, *Acaulospora longula*, *Claroideoglomus etunicatum*, *Claroideoglomus claroideum*, *Glomus aureum*, *Glomus* sp CL3, *Archaeospora trappei*, *Scutellospora calospora*, *Ambispora* sp CL5) were found in association with all wheat cultivars. By the contrary, other species were associated in high proportion with a specific genotype, such as *Glomus aureum* with "Otto", *Scutellospora calospora* with "Bakan", "Porfiado", "Maxi", "Crac" and "Invento". At genus levels *Acaulospora*, *Ambispora*, *Archaeospora*, *Claroideoglomus*, *Glomus* and *Scutellospora* are generalists, while *Cetraspora*, *Funneliformis*, *Pacispora*, *Paraglomus*, *Septoglomus* and *Simiglomus* are specialists (Table 1; Fig. 1). Additionally, the cluster analysis (Fig. 4) evidenced that wheat genotype modifies the structure of the AM fungal community. In detail, "Maxi", "Porfiado" and "Invento" cultivars selected homogeneous AM fungal communities; whereas "Crac" and "Bakan" cultivars were associated with a different type of AM fungal communities, and "Otto" selected another distinct AM fungal community. In general the differences were based on the distinct AM fungal species richness and the dominance of some AM fungal genera in specific wheat cultivars, specifically *Acaulospora* and *Scutellospora* in the rhizosphere of "Porfiado" and "Invento",

independently of their high differences in root colonization and spores production (Figs. 2, 3).

Based on the previous, our results have shown that the selection of wheat genotype could be used to manage the AM symbiosis development (Figs. 2, 3) and the composition of AM fungal communities, which is of agronomic and environmental importance since some AM fungal species could provide specific characteristics, as improved Al-tolerance (Aguilera et al. 2011; Seguel et al. 2013) or an increased glomalin production (Bedini et al. 2010). Reinforcing, studies by Aguilera et al. (2011) have demonstrated that AM fungi play an important role in alleviation of Al toxicity by means of Al immobilization in glomalin and Al compartmentalization in AM fungal structures. Nevertheless, further studies are needed to elucidate the effects of predominant AM fungal species here found on production, such as wheat crop yield and glomalin production capacity.

It should be pointed out that AM fungal diversity characterization has also been carried out in grassland ecosystems of Southern Chile and some comparisons can be performed. Castillo et al. (2006) identified about 29 AM fungal species and 8 genera being *Acaulospora* and *Glomus* the most representative genera. In the present report only *Acaulospora laevis* and *Acaulospora longula* coincide with this previous characterization. In total, only ten AM fungal species here found were also found in the previous studies and 14 AM fungal species were found exclusively in our study. On the other hand, Castillo et al. (2010) reported only eight AM fungal species associated with wheat crops under conventional tillage in an acidic Andosol of Southern Chile, being four species coincident with our study (*Glomus diaphanum*, *Claroideoglomus etunicatum*, *Glomus intraradices* and

Scutellospora calospora). These previous results obtained in edaphoclimatic conditions similar to our study, support the fact that AM fungal community composition not only is modified by highly disrupting agricultural practices such as crop species or tillage system, but also by subtle changes such as the cultivar selection. In this sense, AM fungal species dominance has been associated to plant species tolerance to unfavorable conditions (Mathimaran et al. 2005). Particularly in our study, *Scutellospora* and *Acaulospora* genera have a significant high dominance in "Porfiado" and "Invento" cultivars, which could be associated to a higher Al-tolerance and the potentiality to be included in programs to develop inocula based on AM fungi and oriented to the management of acidic soils with high Al levels.

4.5 Conclusions

Accordingly, this study shows that there is an effect of wheat cultivar on AM fungal diversity and an important level of AM fungal specificity in Al tolerant wheat cultivars grown in Andosols with high Al^{3+} levels. On the other hand, our results suggest the utility of some ecological indexes, as species richness and dominance of AM fungal communities, for the selection of the most compatible Al tolerant wheat cultivars growing in acidic soils with high Al^{3+} levels, especially associated to high densities of *Acaulospora* and *Scutellospora* into fungal community. However, more antecedents will be needed to understand the role of specific AM fungal species on the Al-tolerance in plants able to grow in environments with high Al^{3+} levels as a part of programs oriented to the development of AM fungi based inoculants to be used in soils with high Al^{3+} levels.

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Figure captions

Figure 1. Relative densities of spores of arbuscular mycorrhizal (AM) fungal genera found in rhizosphere soil of Al tolerant *Triticum aestivum* L. cultivars growing in an Andosol with high Al^{3+} levels at postharvest.

Figure 2. Arbuscular mycorrhizal (AM) colonization (%) of roots of six Al tolerant *Triticum aestivum* L. cultivars growing in an Andosol with high Al^{3+} levels at grain filling stage. Different letters indicate significantly different means according to Tukey's multiple range test ($P < 0.05$).

Figure 3. Arbuscular mycorrhizal (AM) spore density in rhizosphere soil of Al tolerant *Triticum aestivum* L. cultivars growing in an Andosol with Al^{3+} levels at grain filling (A) and postharvest (B). For each sampling stage, different letters indicate significantly different means according to Tukey's multiple range test ($P < 0.05$).

Figure 4. Hierarchical cluster analysis using farthest neighbor as agglomerate method based on the principal components obtained from specific densities of AM fungal

propagules, species richness, Shannon-Wiener index, Evenness index, Simpson's index, colonization (%) and spore densities of arbuscular mycorrhizal (AM) fungal communities in rhizosphere soil of six wheat varieties. Note the formation of three different groups that showed "Otto" as root cultivar of cluster. Scale bar, 0.5 U.

Table caption

Table 1. Arbuscular mycorrhizal (AM) fungal species and diversity indices of AM fungal communities associated to six Al tolerant *Triticum aestivum* L. cultivars growing in an Andosol with phytotoxic aluminum level.

AM fungal class and order	AM fungal family	AM fungal species	Wheat cultivars*					
			Maxi	Porfiado	Invento	Bakan	Crac	Otto
Glomeromycetes								
Diversisporales	Acaulosporaceae	*Acaulospora laevis* Gerd. & Trappe	□	□	□	□	□	□
		Acaulospora sp. CL1**	□	□	□	□	□	□
		Acaulospora sieverdingii Oehl, Sýkorová, Błaszk. & G.A. Silva	□	□	□	□	□	□
		Acaulospora longula Spain & N.C. Schenck	□	□	□	□	□	□
	Pacisporaceae	*Pacispora dominikii* Oehl & Sieverd.	■	□	□	□	□	■
Glomerales	Entrophosporaceae	*Claroideoglomus etunicatum* A. Schüssler	□	□	□	□	□	▨
		Claroideoglomus claroideum A. Schüssler	□	□	□	□	□	□
	Glomeraceae	*Glomus aureum* Oehl & Sieverd.	□	□	□	□	▨	□
		Glomus sp CL2	□	■	□	■	■	□
		Glomus sp CL3	□	□	□	□	□	▨
		Glomus intraradices N. C. Schenck & G.S. Sm.	■	■	■	■	□	■
		Glomus diaphanum J.B. Morton & C. Walker	□	□	□	□	▨	■
		Funneliformis mosseae (T.H. Nicolson & Gerd.) C. Walker & A. Schüssler	■	■	■	□	■	▨
		Septoglomus constrictum (Trappe) Sieverd., G.A. Silva & Oehl	■	■	■	□	□	▨

		Simiglomus hoi (S.M. Berch & Trappe) G.A. Silva, Oehl & Sieverd	■	□	□	□	□	▨
Gigasporales	Scutellosporaceae	*Scutellospora calospora* (T.H. Nicolson & Gerd.) C. Walker & F.E. Sanders	□	□	□	□	□	▨
	Racocetraceae	*Cetraspora gilmorei* (Trappe & Gerd.) Oehl, F.A. Souza & Sieverd.	□	□	□	■	■	■
		Cetraspora sp CL4	□	□	■	■	■	■
Archaeosporomycetes								
Archaeosporales	Ambisporaceae	*Ambispora gerdemannii* (S.L. Rose, B.A. Daniels & Trappe) C. Walker, Vestberg & A. Schüssler	■	■	□	■	■	■
		Ambispora sp CL5	□	□	□	□	□	□
	Archaeosporaceae	*Archaeospora trappei* (R.N. Ames & Linderman) J.B. Morton & D. Redecker	□	□	▨	□	□	■
		Archaeospora myriocarpa (Spain, Sieverd. & N.C. Schenck) Oehl, G.A. Silva, B.T. Goto & Sieverd.	□	□	■	□	□	▨
		Archaeospora sp CL6	■	□	□	■	■	■
Paraglomeromycetes								
Paraglomerales	Paraglomeraceae	*Paraglomus occultum* (C. Walker) J.B. Morton & D. Redecker	■	□	□	□	■	■
AM fungi species richness (F=67.8; P < 0.001)			16c	19a	19a	18ab	17bc	15d

Shannon-Wiener index (F=1.96 N.S.)	2.20	2.08	2.16	2.25	2.39	2.28
Evenness index (F=1.28 N.S.)	0.55	0.46	0.53	0.54	0.52	0.53
Simpson's diversity index (F=3.3; P < 0.05)	0.137ab	0.176a	0.188 a	0.14ab	0.103b	0.107b

* ☐ present in all samples, ▨ present in two samples, ■ not detected. **CL1 to CL6 corresponds to undescribed AM fungi species. For diversity indices, different letters indicate significantly different means according to Tukey's multiple range test. ANOVA and P value in parenthesis.

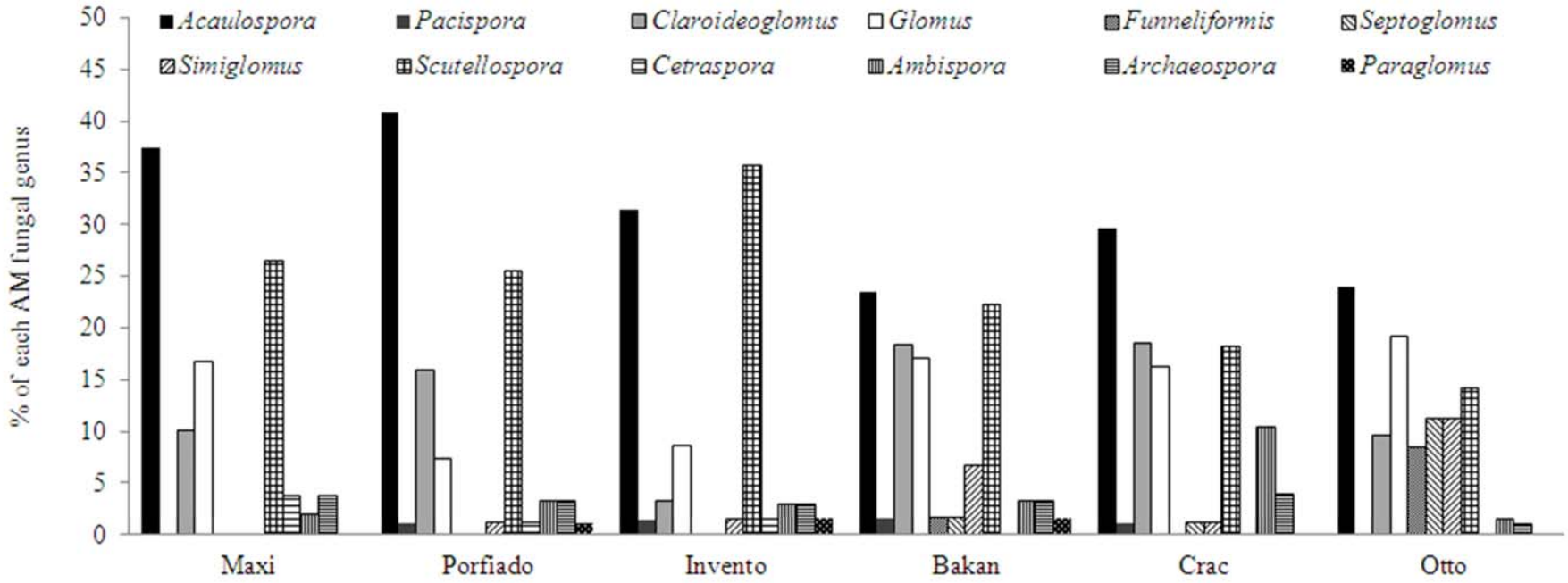
% of each AM fungal genus
50
45
40
35
30
25
20
15
10
5
0
Acaulospora
Pacispora
Claroideoglomus
Glomus
Funneliformis
Septoglomus
Simiglomus
Scutellospora
Cetraspora
Ambispora
Archaeospora
Paraglomus
Maxi
Porfiado
Invento
Bakan
Crac
Otto

Fig. 1

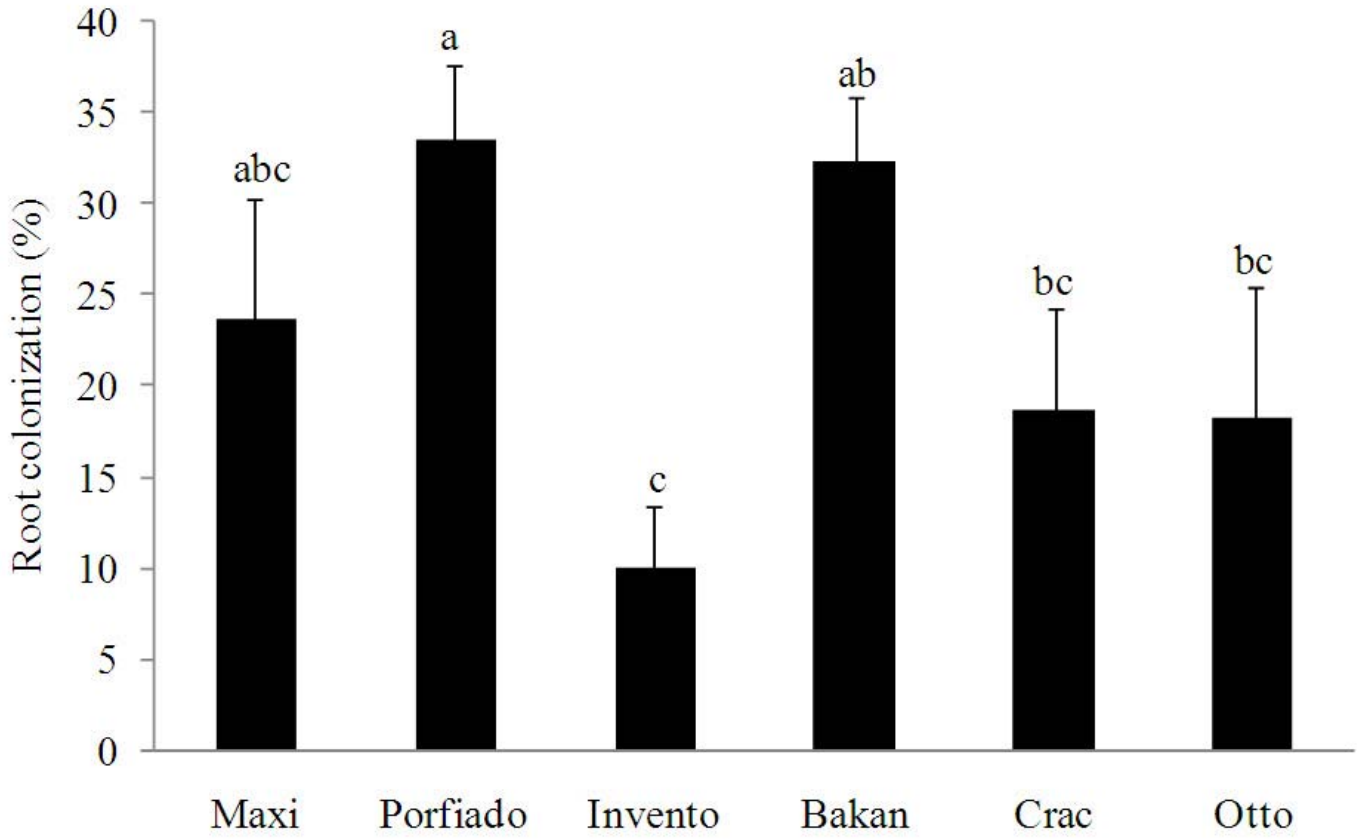
Root colonization (%)
40
35
30
25
20
15
10
5
0
abc
a
c
ab
bc
bc
Maxi
Porfiado
Invento
Bakan
Crac
Otto

Fig. 2

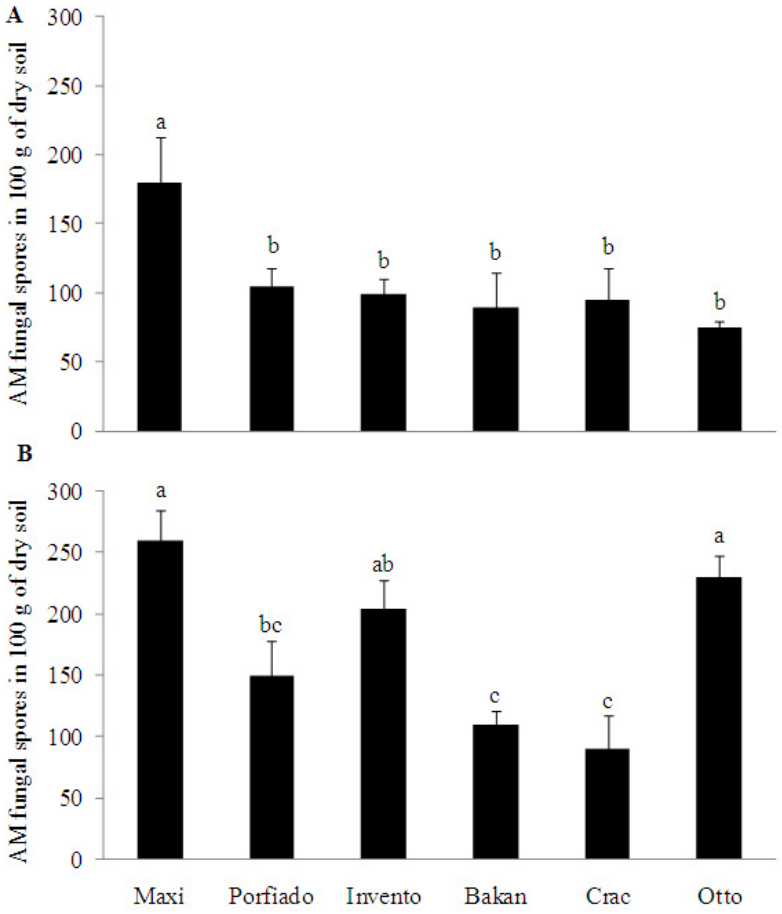
A
AM fungal spores in 100 g of dry soil
300
250
200
150
100
50
0
a
b
b
b
b
b
B
AM fungal spores in 100 g of dry soil
300
250
200
150
100
50
0
a
bc
ab
c
c
a
Maxi
Porfiado
Invento
Bakan
Crac
Otto

Fig. 3

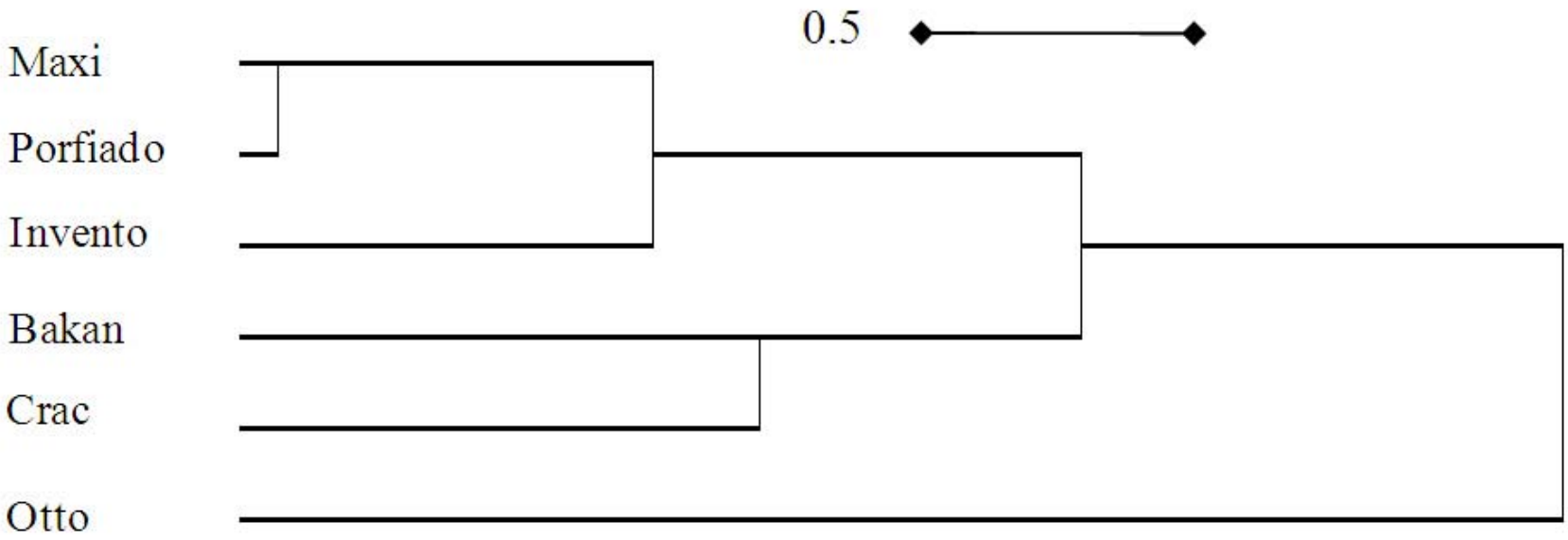

Fig. 4

CHAPTER V

Arbuscular mycorrhizal fungi isolated from two acidic soils and their incidence on Triticum aestivum L. growth under aluminum phytotoxicity conditions

Manuscript in preparation.

Type of contribution: regular paper

Arbuscular mycorrhizal fungi isolated from two acidic soils and their incidence on *Triticum aestivum* L. growth under aluminum phytotoxicity conditions

[a]Center of Amelioration and Sustainability of Volcanic Soils, BIOREN-UFRO, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[c]Campex Semillas Baer. Gorbea, Casilla 87-Temuco-Chile.

*Corresponding Author: Dr. Pablo Cornejo

Tel.: +56 45 2325433

Fax: +56 45 2325440

E-mail address: pablo.cornejo@ufrontera.cl

Abstract

Wheat cropping in Chile is mainly located in Central Southern zone on acidic soils that have some edaphic disadvantages to crop production generating a decrease in plant growth, mainly due to aluminum (Al) phytotoxicity. Factors that allow implementing wheat production, specifically on Andosols, are principally related to the introduction of Al-tolerant wheat cultivars with ability to tolerate these unfavorable conditions. Additionally, several researches have proposed that arbuscular mycorrhizal (AM) symbiosis contributes to improve water and nutrient acquisition and modify some physiological characteristics under metal stress conditions. In this context, the aim of this work was to evaluate the effect of Al, AM fungal inoculum and wheat cultivar on growth of wheat genotypes differing in Al tolerance. Therefore, a soilless system of rhizoboxes was implemented including three compartments for studying mycorrhizosphere, rhizosphere and hyphosphere. Two Al tolerant "Crac" and "Invento" and one Al sensitive "Tukan" wheat cultivars were used as host plants that were inoculated with AM fungal spores extracted from two volcanic soils (Gorbea "25 % Al-saturation" and Remehue "9% Al-saturation"). Besides, 0, 40 and 400 μM of $AlCl_3$ were added to each zone. All factors included in the assay affected Al phytotoxicity and AM fungal indicators being AM fungal inoculum application the main factor of influence. The AM fungi, its origin, wheat cultivar and Al doses applied had specificity of responses in the three compartments evaluated. Thus, our results suggest the utility of evaluating specific indicators when a Program guided to the development of inoculants based on AM fungi is performed in soils with high Al levels like as Andosols.

Keywords: hyphosphere, mycorrhizosphere, rhizosphere

5.1 Introduction

Phytotoxicity caused by aluminum (Al) in acidic soils involves low productivity of plant growing there due to acid (4.5-5.5), low phosphorus (P) availability, high levels of exchangeable Al^{3+} and Mn^{2+}, and low basic cations (Kochian, 2005). Furthermore, the phytotoxicity originated by Al under these edaphic conditions generates the first symptoms of reduced plant growth, primarily due to the damage to cell membrane, resulting in slower growth and root cell elongation (Magalhaes et al., 2007; Seguel et al., 2013).

For counteracting the negative effects produced on plant growth by such soil environments conditions, several management practices have been implemented, among which: a) liming material application, b) P fertilization, and c) the use of Al-tolerant wheat plants are the most important ones. These cultivars have been generated through breeding programs that are based on the introduction of cultivars that have been checked against landraces improving germplasm characteristics with respect to their ability to tolerate the conditions of Al toxicity present in these soils. Thus, the enhancement of arbuscular mycorrhizal (AM) fungal activity through management practices emerges as an interesting alternative.

The AM symbiosis is an association established between soil fungi and most terrestrial plants. The association is mutually beneficial for both partners based on a bidirectional interchange of nutrients (Barea, et al., 2013; Smith and Read, 2008), particularly phosphorus (P) (Borie et al., 2010; Borie and Rubio, 1999; Larsen et al., 2009; Smith and Smith, 2013). Essentially, the fungus improves nutrient and water absorption capacity of the host plant, while the fungus acquires carbon compounds from plant photosynthesis (Barea et al., 2013). In this context, several

studies have shown that AM fungi favor the biological adaptation of plants living under abiotic stress conditions, highlighting the heavy metals (Bissonnette et al, 2010;. Janoušková and Pavlíková, 2010) as well as Al (Cumming and Ning, 2003; Seguel et al., 2013).

Whereas, AM fungi promote tolerance of plants growing under unfavorable conditions, it is necessary to consider that high concentrations of Al can decrease the spore's germination ability and the subsequent root colonization; although some spores do produce hyphae and, subsequently, root colonization even in extreme Al concentrations (Rohyadi, 2005).

As mentioned above, the attenuation of Al toxicity is related to the Al availability and may be favored not only by Al binding to AM fungal structures such as spores, hyphae and vesicles (Driver et al, 2005; Aguilera et al, 2011), but also Al can be immobilized on glomalin-related soil protein (GRSP) a glycoprotein produced by AM fungi reported in large amounts in many soils (Wright and Upadhyaya, 1996, Wright, 2007). Several reports have focused in determining glomalin ability to metal accumulation, presumably due to complex formation (González-Chávez et al, 2004; Cornejo et al, 2008; Vodnik et al, 2008). More specific analyses have been implemented in the study of glomalin in order to produce analytical signals without the interference of humic compounds present in soil, for which inert substrates which have separate zones of rhizosphere and hyphosphere have been used (Nichols, 2010).

The objective of the present study was to evaluate the effect of Al, AM fungal inoculum isolated from two volcanic soils with different Al level and wheat cultivar on: *i)* growth of *Triticum aestivum* L. cultivars differing in Al-tolerance and *ii)* AM

fungal indicators (spores number, hyphal density, colonization intensity and glomalin) in a plexiglas rhizobox system with three study zones (hyphosphere, rhizosphere and micorrhizosphere).

5.2 Materials and Methods

5.2.1 Experimental design

A soilless system was implemented according to the adaptation of methodology described by Tarafdar and Marsher (1994) and Hodge et al., (2001). The system is composed by plexiglas rhizobox (Fig. 1) with three compartments, the central one (mycorrhizosphere) for growth of *Triticum aestivum* L. seedlings that was used as host plant. One wall of the central compartment was separated from the adjacent one by nylon mesh of 45 μm allowing AM fungal hyphae to pass through but blocking the passage of plant roots (hyphosphere). A second group of seedlings were grown in a third compartment that it was completely separated from mycorrhizosphere by plexiglas wall which block the hyphae passage (rhizosphere).

5.2.2 Biological material

Plants of two Al tolerant *Triticum aestivum* L. “Crac” and “Invento” and one sensitive “Tukan” cultivars were grown in the mycorrhizosphere and rhizosphere compartments in sterile substrate composed by vermiculite/sand (1:1). Wheat seeds were surface sterilized with 2% of sodium hypochlorite solution for 10 min, then, they were washed with distilled water. In each compartment (mycorrhizosphere and rhizosphere) 8 plants were grown. For 60 days, this assay was carried out under controlled conditions at 21°C, 8 h dark (15°C) cycle, 50% relative humidity and a

photosynthetic photon flux density of 500–650 μmol m^2s^{-1}. For maintaining humidity, wheat plants received Hewit (1996) nutrient solution for each compartment. A field capacity was maintained about 60% w/w. Three Al treatments were applied weekly of 0, 40 and 400 μM of $AlCl_3$ solution and each treatment had three replicates.

In the mycorrhizosphere, wheat plants were inoculated with a mix of spores from soils with two different exchangeable Al level (Gorbea and Remehue). Gorbea soil serie is a Dystric Andosol (pH 4.5, -SOM- 12.2%, Al-Sat. 25%) sampled in a Gorbea Experimental Station (39◦06'14''S and 72◦41'16''W). Remehue soil serie is a Typic Hapludands (pH 4.6, -SOM- 15%, Al-Sat. 9%) sampled in a Remehue Research Station (40º35'S, 73º12'W). The AM fungal community structure of Gorbea has been recently reported (Aguilera et al., 2014).

Spores were isolated from 100 g soil using wet sieving and sucrose density gradient centrifugation. Briefly, 25 g soil were passed through sieves of 500, 125 and 32 μm and washed thoroughly with distilled water. The last soil portion collected in 32 μm mesh and the soil fraction between 500 and 125 μm were distributed into plastic tubes. The 25 mL of the spore suspension were transferred to 50 mL centrifugation tubes. The 25 mL of a 70% sugar solution were inserted at the bottom of the tubes and centrifuged at 2000 rpm for 2 min. Spore samples were decanted after centrifugation, washed, sterilized with 0.2% of chloramin-T and applied in each mycorrhizosphere compartment. Wheat plants were harvested after 60 days.

5.2.3 Determinations

For phytomass quantification, shoot and roots were dried in oven at 65° C for 48 h and weighted. Aluminum contents in shoots and roots were determined by atomic absorption spectroscopy, for which, the samples were previously calcined for 5 min and subsequently placed in a furnace at 500 °C for 8 h. Ashes were digested in an acid mix of $H_2O/HNO_3/HCl$ (8:1:1). The Al exclusion index was defined as: (mean Al root concentration in non mycorrhizal plants at 400 μM)-(mean Al root concentration in mycorrhizal plants at 400 μM)/(mean Al root concentration in non mycorrhizal plants at 400 μM)*100.

Mycorrhizal root colonization levels were determined by the gridline intersect method (Giovannetti and Mosse, 1980) after clearing the roots with 2.5% KOH solution (wt/vol) and staining with 0.05% trypan blue (Phillips and Hayman, 1970). The gridline intersect method of Tennant (1975) was defined for estimating the total root length. Fungal AM spores were isolated from soils as explained above and, subsequently, they were transferred to Petri dishes for sorting and quantification under the dissection microscope at up to 400-fold magnification (Oehl et al., 2003; Sieverding, 1991).

Hyphal density was determined by the method described in Borie et al. (2000). Briefly, substrate samples (1g) were mixed with 4 mL solution containing glycerol/ 12 M HCl/water (12:1:7) and 10 mL acid fuchsin. Then, the samples were shaken for 30 min, afterwards, 16 mL water were added and maintained at 60°C for 10 min. An aliquot (1 mL) was taken from suspension that was transferred to a membrane filter of 0.45 μm pore size. To quantify the total hyphal density Newman's intersect gridline method (1966) was used.

Total glomalin was extracted according to Wright and Upadhyaya (1996). Briefly, 1 g substrate in 8 mL of 50 mM citrate buffer pH 8.0 was autoclaved for 1 h at 121 °C. This procedure was repeated until no dark color was obtained. Then, the supernatant was filtered through Whatman No.1 paper, and the extracted protein in the supernatant was analyzed using the Bradford assay with bovine serum albumin as standard.

Acid phosphatase activity (P-ase) was determined in roots according to method reported by Tabatabai and Bremner (1969). Roots were incubated with 1mL of 50 mM *p*-nitrophenol phosphate as substrate and 4 mL 0.1 M buffer Tris pH 5.5, for 1 h at 20 °C; after incubation, a 0.5 M $CaCl_2$ solution was added, filtered and centrifuged at 2500 g for 10 min. The released *p*-nitrophenol (*p*-NP) was determined spectrophotometrically at 400 nm and expressed as μmol *p*-NP $g^{-1}min^{-1}$. Dry weight of roots was included in all calculations.

Visualization of Al in roots was carried out according to methodology proposed by Kataoka et al. (1997). Briefly, roots were carefully washed and fixed in 4% paraformaldehyde solution for 2 hours. Then, the roots were stained with 10 mM of lumogallion at pH 5.2, 50°C for 60 min and placed on slices for observation under Fluoview FV1000 Confocal Laser Scanning Biological Microscope (Olympus, Japan). The FV10-ASW v. 2.0c and ImageJ v. 1.43u software programs were used for obtaining emission intensities.

5.2.4 Statistical analyses

Experimental design included experimental variables that were determined in different compartments (Rhizosphere, Mycorrhizosphere or Hyphosfere), for a better understanding of the statistical procedures we listed the details for each group of determinations. For shoot and root dry matter, shoot and root Al concentration and P-ase activity we used a tetra-factorial ANOVA, including as factors the wheat cultivar (Crac, Invento or Tukan), the Al dose (0, 40 or 400 μM), the inoculum origin (Gorbea or Remehue) and the compartment (Rhizosphere or Mycorrhizosphere). For colonization and spore density (only determined in mycorrhizosphere compartment) we used a tri-factorial ANOVA, including as factors the wheat cultivar, the Al dose and the inoculum origin. Finally, for hyphal density and glomalin we used a tetra-factorial ANOVA, including as factors the wheat cultivar, the Al dose, the inoculum origin and the compartment (Hyphosphere or Mycorrhizosphere). Statistical significance was determined at $P < 0.05$. Means were compared by means of Tukey's multiple range test. Data sets not meeting assumptions for ANOVA were transformed as required, but the results are shown in their original measure scale.

5.3 Results and Discussion

5.3.1 Effect of Al, AM fungal inoculum and wheat cultivar on phytotoxicity indicators

At the end of assay all wheat cultivars showed higher shoot dry matter weight in mycorrhizal than non mycorrhizal plants (Table 1), highlighting a positive effect

of Gorbea on Al-tolerant “Invento”, which had the highest shoot biomass at two different Al levels added, and on Al-sensitive “Tukan” at 400 µM Al. Additionally, Gorbea associated with “Crac” showed an increase of shoot biomass in mycorrhizal plants non exposed to Al. However, Al addition diminished the growth of these plants. Similar effects on biomass increase have been observed for “Invento” cultivar in a previous report where this cultivar is described as high Al tolerant probably due to high root length at initial growing period (Seguel et al., 2012).

The AM fungal application had a discordant effect on root biomass obtained from Al-tolerant cultivars (Table 1), which only “Invento” associated to Gorbea inoculum showed an increase of root biomass. However, a clear negative effect of Al addition on root biomass was observed in Al-sensitive cultivar. This effect can be explained due to inhibition of root elongation that has been reported as the main damage indicator in Al-sensitive plants genotypes (Delhaize, 2007; Horst et al., 2010; Ma, 2007). The AM fungal application increased root biomass being Gorbea inoculum the most effective one. The AM fungal association has been promoted as an important Al-tolerance mechanism in host plants (Seguel et al., 2013). The Al-tolerant wheat plants here used have been developed under selective breeding programs implemented in acidic soils in which AM presence gives tolerance to Al phytotoxicity (von Baer, 2007).

Comparing Al root concentrations in non mycorrhizal plants (Table 1), Al-tolerant cultivars showed the highest value of Al root concentration. Several reports have indicated that toxic forms of Al can bind cell wall, vacuoles or others compounds with negative charges as pectin; however, the mechanisms among Al excluders and Al-includers plants remains unclear (Horst et al., 2010). Although,

intracellular exclusion correspond to one of detoxification mechanisms which it has been associated to internal chelating by organic anions (Ma et al., 2001; Seguel et al., 2013). Three analyzed wheat cultivars showed higher Al root concentrations in non mycorrhizal plants than in mycorrhizal ones. “Crac” in presence of the highest Al dose showed 2.2-fold and 1.8-fold greater Al accumulation in roots respect to inoculated plants with Gorbea and Remehue inocula, respectively. Under the same condition, “Invento” showed significant differences of 4.2-fold and 3.4-fold higher Al content in roots of non inoculated plants respect to inoculated plants with Gorbea and Remehue inocula, respectively. On the other hand, Al-sensitive cultivar accumulated 2.2 and 1.8-fold higher Al in roots, in non inoculated plants exposed to 40 μM of Al and 2.5 and 1.8-fold higher Al in roots, in non inoculated plants exposed to 400 μM of Al. Thus, Gorbea inoculum generated a greater decrease in Al roots contents in all wheat cultivars, where this effect was accentuated when AM fungal were associated to “Invento”. Among different responses conferred by mycorrhizal it has been demonstrated that AM fungal symbiosis can decrease Al roots content probably due to Al extracellular exclusion mechanism (Borie et al., 2002; Cumming and Ning, 2003). The fluorescence intensities based on visualization of Al-lumogallion complex under confocal microscopy allow us localize Al in root tips of Al tolerant and Al sensitive cultivars. High values of fluorescence intensity were related with the highest Al addition, which it was related with Al root concentrations here determined (Kataoka et al., 1997).

In non mycorrhizal plants at the highest Al addition, Al-sensitive cultivar had a higher Al concentration than Al-tolerant. In all cultivars, AM fungal application decreased Al translocation at 400 μM Al. “Crac” showed 5.8 and 2.4-fold,

"Invento" showed 3.2 and 2.4-fold and "Tukan" showed 2.7 and 2.4-fold less Al concentration in shoots of Gorbea and Remehue inoculated plants, respectively. Our results are consistent with previous reports (Kelly et al., 2005; Klugh-Stewart and Cumming, 2009), which AM fungal application was related to diminished of Al availability reducing Al toxicity in AM fungal hosts.

We considered Al exclusion index related to Al root concentration in mycorrhizal plants respect to the non mycorrhizal plants under the highest Al addition. Therefore, in all cultivars associated with Gorbea inoculum showed a lower accumulation index than inoculated plants with Remehue inoculum. Especially, in "Invento" cultivar.

Phosphatase activity (P-ase) showed higher values in Al tolerant cultivars inoculated with Gorbea inoculum in presence of the highest Al application, but this behavior was not observed in Al sensitive cultivar (Fig. 2). Likewise, P-ase activity among Al-tolerant plants grown in rhizosphere zone of "Invento" not showed significant differences related to Al application. In general, AM fungal application increased P-ase with both inocula although Gorbea inoculums produced higher activity coinciding with results reported by Tarafdar and Marschner (1994) who quantified P-ase activity in mycorrhizosphere of wheat plants and they obtained an increase of this activity by inoculation of AM fungi. However, Borie et al (2002) reported a decrease in P-ase activity in wheat mycorrhizal plants growing under acidic conditions. Nonetheless, has been described that plants growing under P-limiting conditions favor phosphatase exudation and AM fungi can induce this mechanism (Tarafdar and Marschner, 1994; Finlay, 2008). Additionally, Gorbea inoculum is composed by AM fungal species and ecotypes isolated from acidic

soils under P-limitant conditions which could suggest a high compatibility among these AM fungi and Al-tolerant hosts (Cumming and Ning, 2003).

In general, at the end of the assay Al phytotoxicity indicators (Table 2) in mycorrhizosphere and rhizosphere of wheat plants (shoot dry matter, root dry matter, Al shoot and Al root concentration and phosphatase activity) were affected by analyzed factors (5 by Al dose, 6 by AM fungal application, 5 by wheat cultivar and 3 by AM fungal origin) being AM application the main factor that contributes significantly in all analyses. Additionally, interactions of Al dose*AM application, Al*wheat cultuvar and Al*inoculum origin significantly affected to Al phytotoxicity indicators.

5.3.2 AM fungal indicators

In this assay, we performed comparisons among three wheat cultivars inoculated with Gorbea and Remehue inocula at three different Al additions in each mycorrhizosphere zones. Results of AM fungal colonization (Fig. 3) indicated that, in general terms, Gorbea inoculum favored root colonization at 400 μM Al in all cultivars being higher than Remehue inoculums, especially in "Invento" and "Tukan" cultivars. Whereas, "Tukan" cultivar increased root colonization at 400 μM Al showing the highest and significant colonization percentage value, which could be related to the high biomass associated to Gorbea inoculum obtained under the same condition. This behavior has been observed in previous reports (Clark et al., 1999; Kelly et al., 2005).

On the other hand, results in the mycorrhizosphere zones of all cultivars showed a trend of a higher spore density associated to Remehue than Gorbea

inoculum (Fig 3). Whereas, the highest spore density was found in the control plants rhizosphere of “Invento” associated with Remehue inoculum. Although, in the same cultivar, spore density showed a reduced spore density at 40 and 400 µM Al treatments respect to the control. An inverse effect in spore number and root colonization has been previously reported (Aguilera et al., 2014), where a greater spore number could be favored when low colonization intensity occurs, which could be associated to countervailing effect among both AM fungal indicators when they are associated to “Invento” growing in Andosols with high Al contents. Additionally, this cultivar had the highest hyphal density.

In the mycorrhizosphere zone, a significant effect on spore number was generated by Al doses, wheat cultivars and inocula origin (Table 3). The AM fungi promote plant tolerance under Al phytotoxicity conditions, it is necessary to consider that high Al concentrations can decrease spore germination ability (Rohyadi, 2005). In contrast, Al doses and wheat cultivar did not affect significantly AM fungal colonization. Inocula origin and interactions that include inocula origin affected colonization rates.

Results of hyphal density and glomalin are referred to hyphosphere and mycorrhizosphere zones. With respect to the hyphal density analyses (Fig. 4); in general, there was a trend to develop more mycelium in the mycorrhizosphere respect to the hyphosphere. In “Crac” and “Tukan” hyphosphere associated to Gorbea inoculum, hyphal density showed a significant increase with high Al level applied compared with 0 and 40 µM Al. On the contrary, in “Invento” hyphosphere the hyphal density was higher in Remehue than Gorbea inoculated plants which significant decrease at 400 µM Al. In “Crac” mycorrhizosphere, hyphal density not

showed significant differences between both inocula. In "Invento" mycorrhizosphere, a greater hyphal density was found in Remehue inoculated plants. In addition, in "Tukan" mycorrhizosphere, there was a high mycelium growth in Remehue inoculated plants at 400 μM Al. According to our results, extraradical mycelium could be declining Al content in root, which is possible relating with an Al exclusion observed in "Invento" mycorrhizal plants, this has been suggest by compartmentalization inside of AM fungal mycelium (Driver et al., 2005; Joner et al., 2000; Meharg, 2003).

According to our results, after 60 days, we found glomalin in a soilless system. Broadly, studies related to metal-glomalin interactions in contaminated soils, they are in order of mg/g, whereas in our results, glomalin levels were found in order of μg/g (Bedini et al., 2010; Cornejo et al., 2008; González-Chávez et al., 2004). Regarding glomalin results (Fig. 5), in mycorrhizosphere of Al-tolerant cultivars associated with Gorbea inoculum, Al addition had a negative effect on glomalin concentration. Conversely, glomalin was obtained in more quantities in "Tukan" mycorrhizosphere. In the hyphosphere of three cultivars we found glomalin at slightly lower rates respect to the mycorrhizosphere, which could be related to: *i)* glomalin production by AM fungal mycelium present in this compartment and visualized in Fig. 4, *ii)* nylon mesh of 45 μm allowed the passage of this protein from mycorrhizosphere; or *iii)* visualization of other protein like as P-ase generated from mycorrhizosphere that could react with Bradford analysis.

On the other hand, in a soilless system, glomalin is produced in minimal quantities under *in vitro* conditions (Driver et al., 2005) or using inert substrate (Nichols, 2010) who suggests that this glycoprotein can be washed outside of

compartments. Hence, futures studies oriented to glomalin potential should be consider that glomalin production begin after 3 weeks (Driver et al., 2005) and high glomalin amounts need more volume of containers that support AM fungal symbiosis (data not shown). Notwithstanding, in our results, glomalin could be associated to hyphal density; specifically, in "Tukan" due to glomalin correspond to structural component of AM fungal mycelium.

In the hyphosphere and mycorrizosphere zone glomalin was affected significantly by wheat cultivar and 4 interactions (Table 4), whilst hyphal density was influenced significantly by wheat cultivars, inocula origin, hyphosphere/mycorrizosphere zones and 3 interactions.

The Al-phytotoxicity alleviation by AM fungi has been extensively demonstrated based on AM fungal indicators determinations like as root colonization, spore and hyphal density, and glomalin production. The AM fungal symbiosis allow plants withstand limitants for growth under adverse conditions of acidic soils by synergic effect on own plants mechanisms against metals tolerance or by means of bioaccumulation/sequestration of metals in AM fungal spores, extensive spread of mycelium reaching several meters around of host plant, and glomalin have a high affinity for metals (Aguilera et al., 2011; Clark, 1997; Cornejo et al., 2008; González-Chávez et al., 2004; Driver et al., 2005; Seguel et al., 2013).

5.4 Conclusions

Based on our results of biomass in shoots and roots, Al contents in shoots and roots, and Al exclusion index obtained from all cultivars, we can conclude that

Gorbea inoculum have a more pronounced potential respect to Remehue inoculum. Besides, Al tolerant wheat cultivars have a high Al tolerance that is improved with AM fungal inoculum. In this regard, AM fungal community of Gorbea soil has been previously described and it reported a high AM fungal species richness and AM fungal dominance of *Scutellospora* and *Acaulospora* genera that has been associated to Al-tolerance in acidic conditions. Additionally, this work showed there is an effect of Al, AM fungal inocula and their origin, and wheat cultivar on Al phytotoxicity and AM fungal indicators. The AM fungal inoculum application is the main factor influencing them. The AM fungal origin and wheat cultivar had specificity of responses in analyzed zones, when wheat plants were exposed to two Al concentrations. Thus, our results suggest the utility of evaluation of specific indicators when a Program is focused to the inoculants development based on AM fungi, mainly in Andosols with high Al levels.

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Table and Figure captions

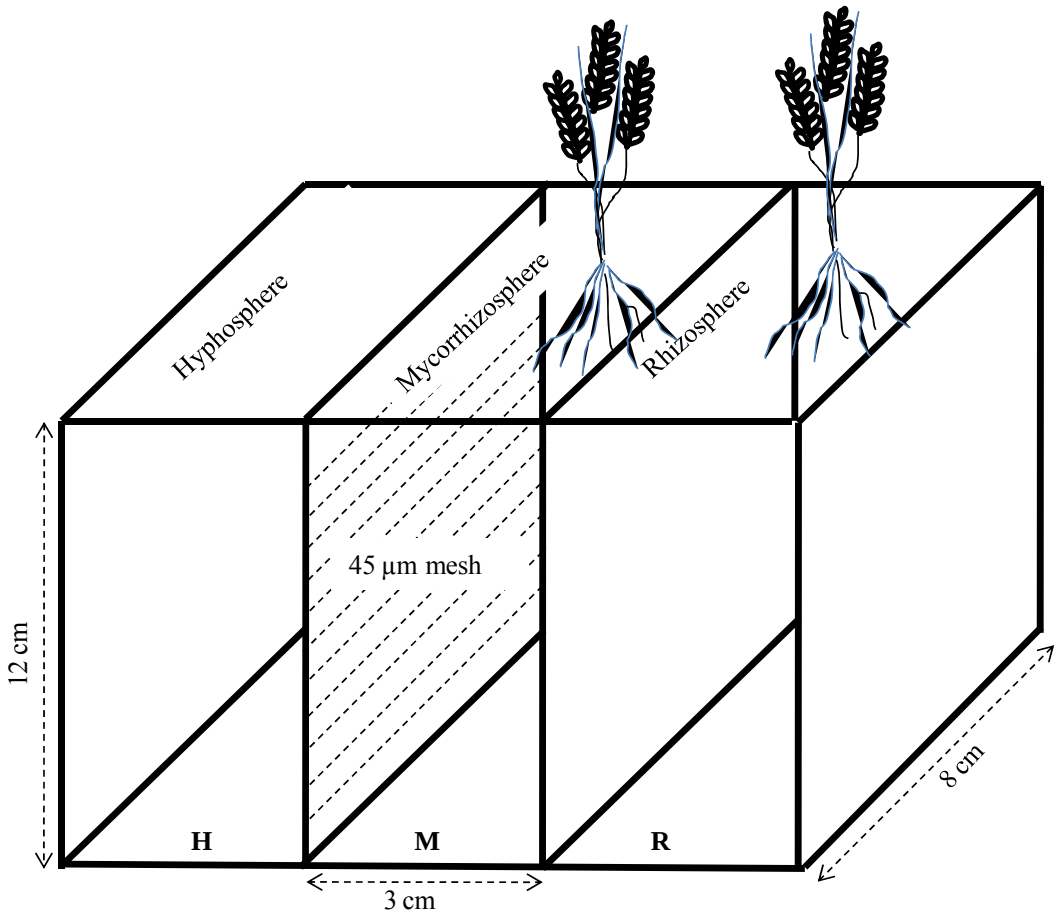

Figure 1. Schematic representation of rhizobox system. H: hyphal compartment, M: plant compartment with inoculum addition, R: plant compartment without inoculum addition. H and M compartments are separated by 45 µm nylon mesh that allows hyphae pass through but not plant roots**.**

Table 1. Shoot and root dry matter, Al contents in root and shoot and Al exclusion index for mycorrhizosphere and rhizosphere zones.

Wheat cultivar[a]	Mycorrhizal inoculation	Al[b]	Shoot dry matter (mg)	Root dry matter (mg)	Al shoot (µg/g)	Al root (µg/g)	Al exclusion index[c] (%)
Crac	Non mycorrhizal (rhizosphere)	0	110 c	10 d	nd	nd	
		40	130 c	20 c	1.0 c	130 d	
		400	110 c	30 b	5.3 b	1,190 b	
	Gorbea Inoculum (mycorrhizosphere)	0	200 b	10 d	nd	nd	
		40	160 c	20 c	0.8 c	400 d	
		400	140 c	30 b	0.9 c	550 c	54 c
	Remehue inoculum (mycorrhizosphere)	0	120 c	20 c	nd	nd	
		40	130 c	20 c	0.9 c	90 d	
		400	110 c	30 b	2.2 b	660 c	45 d
Invento	Non mycorrhizal (rhizosphere)	0	120 c	20 c	nd	nd	
		40	190 c	10 d	1.1 c	310 d	
		400	130 c	20 c	4.2 b	1,900 a	
	Gorbea Inoculum (mycorrhizosphere)	0	190 c	30 b	nd	nd	
		40	210 a	30 b	1.1 c	200 d	
		400	210 a	30 b	1.3 c	450 d	76 a
	Remehue inoculum (mycorrhizosphere)	0	180 c	20 c	nd	nd	
		40	160 c	10 d	0.4 c	200 d	
		400	200 c	20 c	1.7 b	550 c	71 a
Tukan	Non mycorrhizal (rhizosphere)	0	110 c	20 c	nd	nd	
		40	110 c	10 d	0.9 c	840 c	
		400	150 c	10 d	6.0 a	850 c	
	Gorbea Inoculum (mycorrhizosphere)	0	130 c	40 b	nd	nd	
		40	130 c	50 a	0.8 c	380 d	
		400	210 a	60 a	2.2 b	330 d	61b
	Remehue inoculum (mycorrhizosphere)	0	100 c	30 b	nd	nd	
		40	120 c	30 b	0.5 c	460 d	
		400	190 c	40 b	2.5 b	450 d	47 d

Means followed by the same letter within the same determination (column) are not significantly different at $P < 0.05$ by ANOVA with Tukey's Multiple Range Test. a: Al tolerant wheat cultivars "Crac" and "Invento", and Al sensitive "Tukan"; b: 0, 40 and 400 µM de $AlCl_3$ solution; c: (mean Al root concentration in non

mycorrhizal plants at 400 μM)-(mean Al root concentration in mycorrhizal plants at 400 μM)/(mean Al root concentration in non mycorrhizal plants at 400 μM)*100.

. We not detected (nd) Al in roots with 0 Al dose.

Table 2. *F* values and significance for the main effects and factor interactions in mycorrhizosphere and rhizosphere zones.

Experimental variable	Aluminum (Al)[a]	Compartment (C)[b]	Wheat cultivar (WV)[c]	Inoculum origin (I)[d]	Al*C	Al*WV	Al*I[e]
Shoot dry matter (g)	5.1**	30.8***	17.3***	4.8*	4.2*	7.5***	0.2ns
Root dry matter (g)	6.7**	21.5***	15.6***	5.1*	0.6ns	4.0**	0.7ns
Al shoot (mg/g)	32.2***	6.2*	0.3 ns	0.9 ns	2.3 ns	0.4 ns	1.0 ns
Al root (mg/g)	101.5***	43.1***	8.2**	0.3 ns	71.7***	30.0***	0.4 ns
Phosphatase activity (μmol *p*-NP $g^{-1}min^{-1}$)	1.1 ns	13.3***	3.2*	0.1 ns	0.1 ns	3.0*	1.5 ns

∗P<0.05, ∗∗P <0.01, ∗∗∗P <0.001, ns = no significant differences. a: 0, 40 and 400 μM de $AlCl_3$ solution; b: Mycorrhizosphere and Rhizosphere compartments; c: Al tolerant wheat cultivars “Crac” and “Invento” and Al sensitive “Tukan”; d: inoculum from acidic soils (Gorbea and Remehue); e: we did not found any significant triple and quadruple interactions.

Table 3. *F* values and significance for the main effects and factor interactions in mycorrhizosphere.

Experimental variable	Aluminum (Al)[a]	Wheat cultivar (WV)[b]	Inoculum origin (I)[c]	I*WV	Al*I	Al*WV	I*WV*Al
Spore number (N°/100 g)	5.0*	11.2***	17.8***	0.5 ns	0.6 ns	0.5 ns	0.3 ns
Colonization (%)	0.2 ns	1.9 ns	8.7**	5.5**	3.3*	0.4 ns	2.1*

∗P<0.05, ∗∗P <0.01, ∗∗∗P <0.001, ns = no significant differences. a: 0, 40 and 400 μM de $AlCl_3$ solution; b: Al tolerant wheat cultivars "Crac" and "Invento" and Al sensitive "Tukan"; c: inoculum from Gorbea or Remehue.

Table 4. *F* values and significance for the main effects and factor interactions in hyphosphere and mycorrhizosphere.

Experimental Variable	Aluminum (Al)[a]	Weat cultivar (WV)[b]	Inoculum origin (I)[c]	Zone (Z)[d]	I *WV	I *Z	WV *Z	I *WV *Z	I *Al	WV *Al	I *WV *Al	Z *Al	I *Z *Al	WV *Z *Al	I *WV *Z *Al
Glomalin	0.7	5.6	0.1	1.6	6.7	3.4	0.1	1.8	0.1	3.6	3.0	0.1	0.1	0.3	0.6
(mg/g)	ns	**	ns	ns	**	*	ns	ns	ns	**	*	ns	ns	ns	ns
Hyphal density	0.5	41.2	27.7	58.2	20.5	2.3	1.3	0.8	1.7	6.2	2.8	0.4	0.7	1.0	0.6
(m/g)	ns	***	***	***	***	ns	ns	ns	ns	***	*	ns	ns	ns	ns

∗P<0.05, ∗∗P <0.01, ∗∗∗P <0.001, ns = no significant differences. a: 0, 40 and 400 µM de $AlCl_3$ solution; b: Al tolerant wheat cultivars "Crac" and "Invento" and Al sensitive "Tukan"; c: inoculum from Gorbea or Remehue; d: Mycorrhizosphere and Rhizosphere compartments.

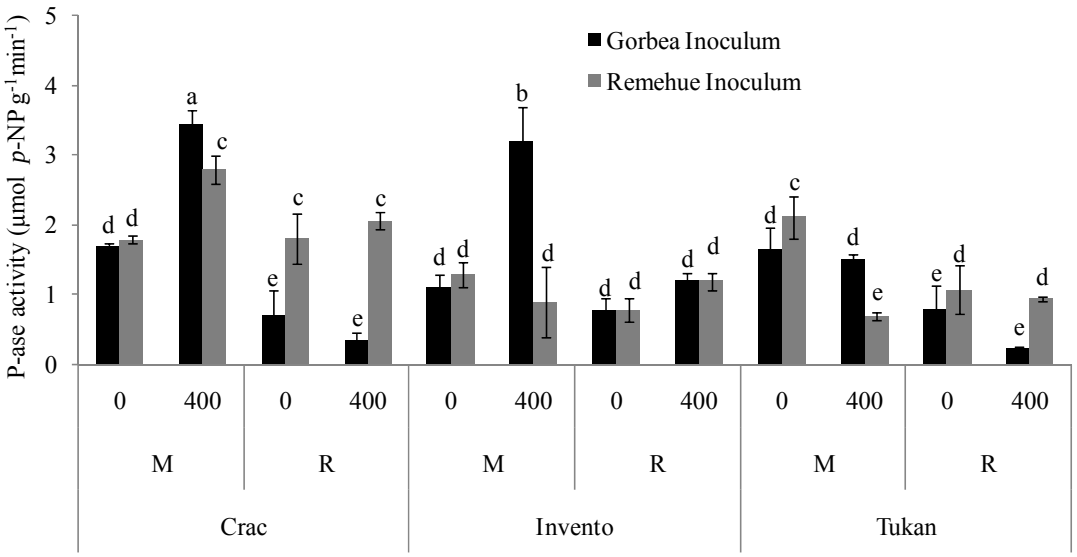

Figure 2. Effect of mycorrhizal inoculation on root phosphatase activity (µmol *p*-NP $g^{-1}min^{-1}$) in mycorrhizosphere (M) and rhizosphere (R) of two Al tolerant wheat cultivars "Crac" and "Invento" and one Al sensitive "Tukan" inoculated with spores from acidic soils (Gorbea and Remehue) at 0 and 400 µM de $AlCl_3$ solution. Different letters indicate significantly different values, ANOVA with Tukey's Multiple Range Test ($P < 0.05$).

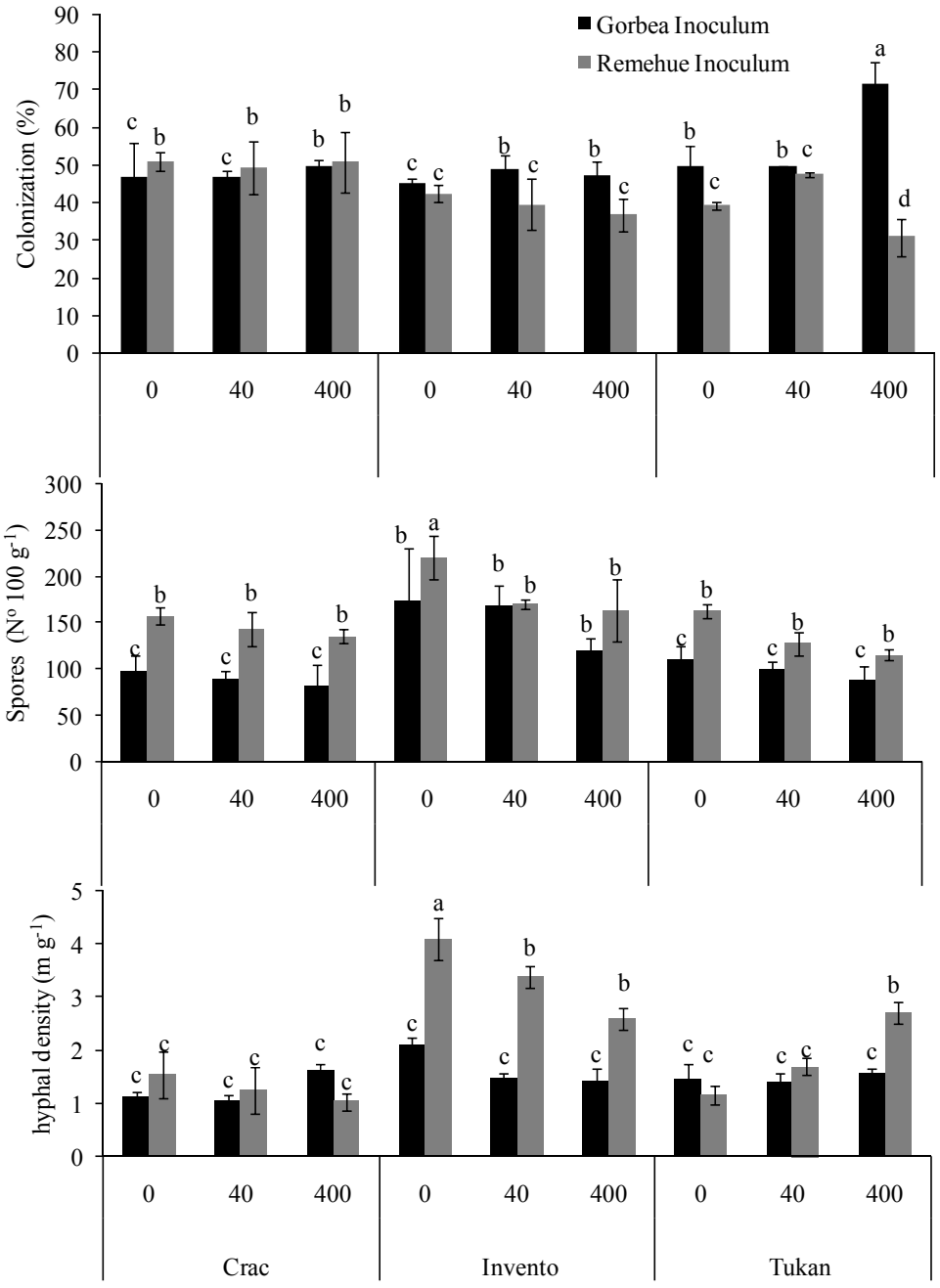

Figure 3. Arbuscular mycorrhizal (AM) colonization (%) of roots, arbuscular mycorrhizal (AM) spore number and hyphal density on mycorrhizospheres of two Al tolerant *Triticum aestivum* L. cultivars "Crac" and "Invento" and one Al sensitive "Tukan" inoculated with Gorbea and Remehue inocula at three Al application levels (0, 40 and 400 µM de $AlCl_3$ solution). Different letters indicate significantly different

values according to ANOVA with Tukey's multiple range test ($P < 0.05$). Hyphal density on mycorrhizosphere was added in the graph for better visualization of AM fungal propagules.

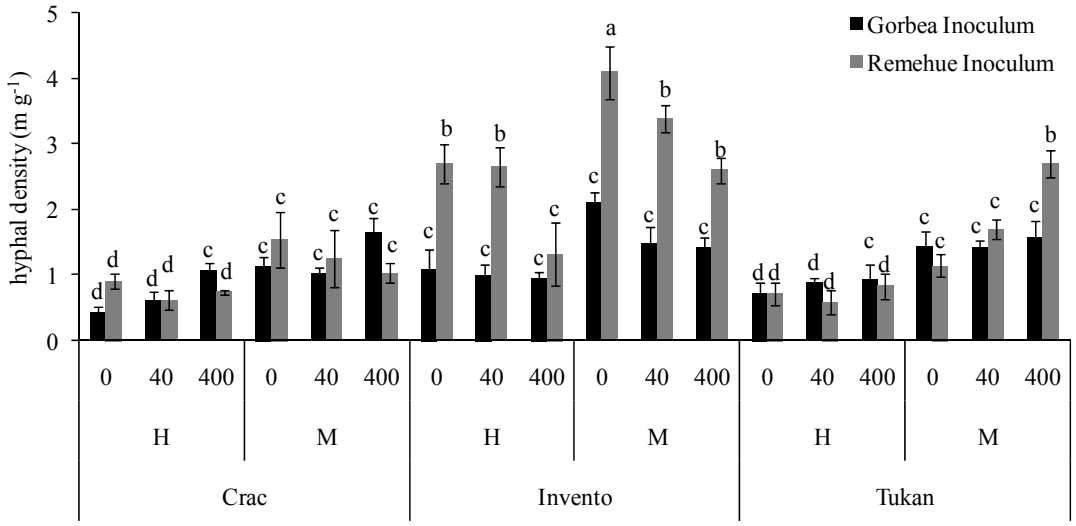

Figure 4. Hyphal density in mycorrhizosphere (M) and hyphosphere (H) compartment of two Al tolerant *Triticum aestivum* L. cultivars "Crac" and "Invento" and one Al sensitive "Tukan" inoculated with Gorbea and Remehue inocula at three Al application levels (0, 40 and 400 μM of $AlCl_3$ solution). Different letters indicate significantly different means according to ANOVA with Tukey's multiple range test ($P < 0.05$).

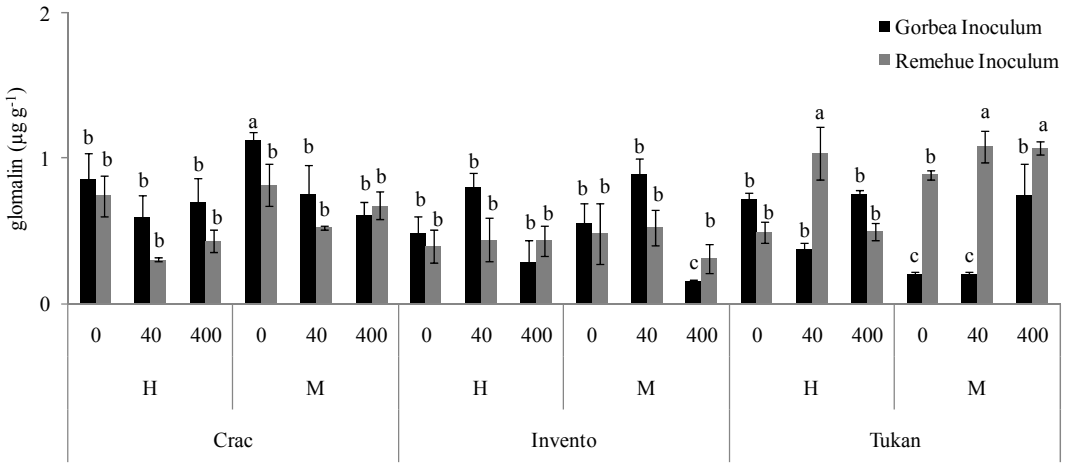

Figure 5. Glomalin extracted from mycorrhizosphere (M) and hyphosphere (H) compartment of two Al tolerant *Triticum aestivum* L. cultivars "Crac" and "Invento" and one Al sensitive "Tukan" inoculated with spores from acidic soils (Gorbea and Remehue) at three Al application levels (0, 40 and 400 µM of $AlCl_3$ solution). Different letters indicate significantly different means according to ANOVA with Tukey's multiple range test ($P < 0.05$).

Chapter VI

General discussion, concluding remarks and future directions

5.1 General discussion

Aluminum (Al) phytoxicity is a constraint that affects the plant production in about 40% of arable soils in the world. The Al effects on crops have been well documented and several mechanisms have been described in relation to response or tolerance that exhibit plants growing under Al stress conditions. However, knowledge of the physiological, genetic and molecular bases related to these deleterious effects has been rather limited. Besides, there are plants that have certain mechanisms related to Al tolerance, such as organic acid exudation into rhizosphere, which participate in Al chelation and make this element less toxic for plants. Related to this, the root association with arbuscular mycorrhizal (AM) fungi also favors plant adaptation to acidic soils. The AM fungi have the ability for reducing the phytotoxicity of some elements such as Al or Mn, or decrease mineral deficiencies due to increased absorption of nutrients. Moreover, it has been reported that AM fungi can sequester metals within the roots, thus decreasing metal translocation to plant shoots.

In this Thesis, we suggested a high ability to sequester and possibly accumulate Al in AM fungal structures and glomalin, a glycoprotein produced by AM fungi, hence suggesting an important role of AM fungi in alleviating the phytotoxicity of this element, which could be an effective way to reduce its activity in acidic soils. On the other hand, several studies have shown that Al is present in the mycelium, vesicles and auxiliary cells. Our work is the first evidence respect to the Al accummulacion inside of AM fungal structures and GRSP, suggesting the existence of a maximum capacity of Al sequestration/immobilization by different AM spore components, which could be achieved under *in vivo* conditions in soils with Al-sat levels near 70%. Autofluorescence of some AM fungal structures have been previously described,

especially in arbuscles, but the reason of this trend is still unclear, and the proposed fluorescent component is widely diverse, including the matrix or host plasmalemma, phenolic compounds and chitin. However, these hypotheses do not explain the absence of autofluorescence in the spores. For this reason, we suggest that fluorescence in the AM spores observed here is produced by the interaction of Al^{3+} with compounds present on the spore cell wall surface as glomalin and also inside the spore.

A low autofluorescence observed in spores from Cu-polluted soil and spores isolated from neutral soil was increased when Al exogenous was added. These results suggest the formation of a stable complex between metals and AM fungal components through immobilization sites, especially in cell walls, limiting the substitution of Cu or other metallic ions. This aspect may be relevant in multicontaminated soils, where AM fungus could play an important role mitigating some of them, and reaching a maximum level of metal immobilization in their structures. It also suggests an important role of AM fungi in the attenuation of Al phytotoxicity in soils with high exchangeable Al levels through stable Al complex formation with GRSP, which is known to be tightly bound in mycorrhizal hyphae and spores.

Aluminum salts ($AlCl_3$) have been successfully used in microscope observations for increasing brightness in medical applications. However, Al location in AM fungal studies has not been previously reported; our study is the first that uses Al fluorescent properties when coordinated with other chelating agents to visualize Al^{3+} location on fungal structures.

We also included Al detection in GRSP here, which is known for its high metal sequestration capacity. The GRSP extracted from Cu-polluted soils showed no fluorescence either before or after Al^{3+} addition suggesting a strong Cu-binding capacity

to GRSP, which probably saturated all binding sites explaining the high Cu proportion represented by its elemental composition. The GRSP from neutral soil showed no fluorescence, but it was generated when Al^{3+} solution was added. This study is the first direct evidence of GRSP ability to sequester Al in the molecule, suggesting that this glycoprotein could form stable complexes with Al, explaining the benefits of some AM fungal strains in terms of increasing Al-tolerance of crops growing in soils, where phytotoxic Al is high. These benefits obtained by AM activity could be transient or for longer time according to the residence time of fungal structures in the soil or if the bulk of Al immobilized is performed through GRSP-Al complex formation.

As it is well known, the AM symbiosis involved in plant adaptation under stressful soil conditions allowing plants to improve their ability to acquire water and nutrients, principally phosphorus. However, the beneficial aspects that provide the AM fungi are related to their genetic variation, which influences the richness and diversity allowing survive and grow according to other environmental factors. Related to the above mentioned, several studies have reported that certain AM fungal species, associated with specific hosts promote plant growth, and provide an adequate protection against the stress produced by high heavy metal or Al levels. Mechanisms such as chelation or sequestration have been reported to be involved in heavy metal stress attenuation, due to reduction on activity of the metal ion, lowering its phytotoxicity in the rizosphere. Besides, mycorrhizal benefits to plants growing under acidic conditions have been shown in diverse plant species.

In this Thesis, we also described the AM fungal diversity in acidic soils with high levels of exchangeable aluminum, reporting twenty-four AM fungal species, which were identified and subsequently classified into genera *Acaulospora, Pacispora,*

Claroideoglomus, Glomus, Funneliformis, Septoglomus, Simiglomus, Scutellospora, Cetraspora, Ambispora, Archaeospora and *Paraglomus.* Eighteen species could be unequivocally identified, whereas six others might correspond to undescribed species. The Shannon-Wiener values showed no significant difference in AM fungal diversity associated to wheat cultivars, but species richness was significant different among cultivars. Simpson´s index was significantly different among AM fungal communities being *Acaulospora* and *Scutellospora* the most dominant genera, which are AM fungal species habitually found under similar conditions in acidic soils in others countries.

According to our results this characterization could be applied at genotypic level, since the changes in community structure are evident between the distinct cultivars of Al tolerant wheat here tested. In this context, some AM fungal species here found could be characterized as generalist fungi, because they are present in a homogeneous relative density across all the analyzed wheat cultivars. Specifically, ten AM fungal species (*Acaulospora laevis*, *Acaulospora* sp CL1, *Acaulospora sieverdingii*, *Acaulospora longula*, *Claroideoglomus etunicatum*, *Claroideoglomus claroideum*, *Glomus aureum*, *Glomus* sp CL3, *Archaeospora trappei*, *Scutellospora calospora*, *Ambispora* sp CL5) were found in association with all wheat cultivars. By the contrary, other species were associated in high proportion with a specific genotype, such as *Glomus aureum* with "Otto", *Scutellospora calospora* with "Bakan", "Porfiado", "Maxi", "Crac" and "Invento". At genus levels *Acaulospora*, *Ambispora*, *Archaeospora*, *Claroideoglomus*, *Glomus* and *Scutellospora* are generalists, while *Cetraspora*, *Funneliformis*, *Pacispora*, *Paraglomus*, *Septoglomus* and *Simiglomus* are specialists. Additionally, the cluster analysis evidenced that wheat genotype modifies the structure of the AM fungal community. In general the differences were based on the distinct AM fungal species

richness and the dominance of some AM fungal genera in specific wheat cultivars, specifically *Acaulospora* and *Scutellospora* in the rhizosphere of "Porfiado" and "Invento". Based on the previous, our results have shown that the selection of wheat genotype could be used to manage the AM symbiosis development and the composition of AM fungal communities, which is of agronomic and environmental importance since some AM fungal species could provide specific characteristics, as improved Al-tolerance or an increased glomalin production. The AM fungal community composition not only is modified by highly disrupting agricultural practices such as crop species or tillage system, but also by subtle changes such as the cultivar selection. *Scutellospora* and *Acaulospora* genera have a significant high dominance in "Porfiado" and "Invento" cultivars, which could be associated to a higher Al-tolerance and the potentiality to be included in programs to develop inocula based on AM fungi and oriented to the management of acidic soils with high Al levels.

In our country, a significant number of Al tolerant cereal genotypes are commercialized, but mechanisms involved in such as Al tolerance, and the role played by the AM fungi in natural conditions are still unknown.

Additionally, in this Thesis, we evaluated the effect of Al, AM fungal inoculum origin, compartment (hyphosphere, mycorrhizosphere and rhizosphere) and wheat cultivar on plant performance and AM fungal symbiosis. Mycorrhizal plants showed higher shoot dry matter weight than non mycorrhizal plants highlighting a positive effect of inoculum from AM fungal community found in soil in where habitually growth Al-tolerant wheat plants under high Al saturation. This positive effect of Gorbea inoculum was observed in Al-tolerant and Al-sensitive wheat cultivars. The AM fungal application favored root biomass of Al-tolerant cultivar "Invento", being Gorbea

inoculum the most effective one. In this sense, the AM fungal association has been promoted as an important Al-tolerance mechanism in host plants. On the other hand, the Al-tolerant wheat plants here used have been developed under selective breeding programs implemented in acidic soils in which AM presence gives tolerance to Al phytotoxicity. Respect to the Al root concentrations, three analyzed wheat cultivars showed higher Al root concentrations in non mycorrhizal plants than in mycorrhizal ones. Several reports have indicated that toxic forms of Al can bind cell wall, vacuoles or others compounds with negative charges as pectin; however, the mechanisms among Al excluders and Al-includers plants remains unclear. Although, intracellular exclusion correspond to one of detoxification mechanisms which it has been associated to internal chelating by organic anions. Gorbea inoculum generated a greater decrease in Al roots contents in all wheat cultivars, where this effect was accentuated when AM fungal were associated to "Invento". Among different responses conferred by mycorrhizal it has been demonstrated that AM fungal symbiosis can decrease Al roots content probably due to Al extracellular exclusion mechanism. Our results are consistent with previous reports, which AM fungal application was related to diminished of Al availability reducing Al toxicity in AM fungal hosts.

We considered Al exclusion index related to Al root concentration in mycorrhizal plants respect to the non mycorrhizal plants under the highest Al addition. Therefore, in all cultivars associated with Gorbea inoculum showed a high exclusion index than inoculated plants with Remehue inoculum. Especially, in "Invento" cultivar.

In general, AM fungal application increased P-ase with both inocula although Gorbea inoculum produced higher activity. Nonetheless, has been described that plants growing under P-limiting conditions favor phosphatase exudation and AM fungi can

induce this mechanism. Additionally, Gorbea inoculum is composed by AM fungal species and ecotypes isolated from acidic soils under P-limitant conditions which could suggest a high compatibility among these AM fungi and Al-tolerant host.

In general, Gorbea inoculum favored root colonization at 400 µM Al in all cultivars being higher than Remehue inoculums, especially in "Invento" and "Tukan" cultivars. Whereas, "Tukan" cultivar increased root colonization at 400 µM Al showing the highest and significant colonization percentage value, which could be related to the high biomass associated to Gorbea inoculum obtained under the same condition.

On the other hand, results in the mycorrhizosphere zones of all cultivars showed a trend of a higher spore density associated to Remehue than Gorbea inoculum. An inverse effect in spore number and root colonization has been previously reported, where a greater spore number could be favored when low colonization intensity occurs, which could be associated to countervailing effect among both AM fungal indicators when they are associated to "Invento" growing in Andosols with high Al contents. Additionally, this cultivar had the highest hyphal density.

Results of hyphal density and glomalin are referred to hyphosphere and mycorrhizosphere zones. With respect to the hyphal density analyses in general, there was a trend to develop more mycelium in the mycorrhizosphere respect to the hyphosphere. In "Crac" and "Tukan" hyphosphere associated to Gorbea inoculum, hyphal density showed a significant increase with high Al level applied compared with 0 and 40 µM Al. According to our results, extraradical mycelium could be declining Al content in root, which is possible relating with an Al exclusion observed in "Invento" mycorrhizal plants, this has been suggest by compartmentalization inside of AM fungal mycelium.

In this assay, after 60 days, we found glomalin in a soilless system. Broadly, studies related to metal-glomalin interactions in contaminated soils, they are in order of mg/g, whereas in our results, glomalin levels were found in order of μg/g. Al addition had a negative effect on glomalin concentration. In the hyphosphere of three cultivars we found glomalin at slightly lower rates respect to the mycorrhizosphere, which could be related to: *i)* glomalin production by AM fungal mycelium present in this compartment and visualized in Fig. 4, *ii)* nylon mesh of 45 μm allowed the passage of this protein from mycorrhizosphere; or *iii)* visualization of other protein like as P-ase generated from mycorrhizosphere that could react with Bradford analysis.

The Al-phytotoxicity alleviation by AM fungi has been extensively demonstrated based on AM fungal indicators determinations like as root colonization, spore and hyphal density, and glomalin production. The AM fungal symbiosis allow plants withstand limitants for growth under adverse conditions of acidic soils by synergic effect on own plants mechanisms against metals tolerance or by means of bioaccumulation/sequestration of metals in AM fungal spores, extensive spread of mycelium reaching several meters around of host plant, and glomalin have a high affinity for metals.

5.2 Concluding remarks

According to our results we can conclude that the direct observation by confocal laser scanning microscope (CLSM) without normally used fluorochromes allowed us to evidence the ability to sequester and possibly accumulate Al in AM fungal structures

and glomalin. Additionally, we used $AlCl_3$ salts as dye, this salts only has been used previously in medical applications.

On the other hand, we suggested an effect of wheat cultivar on AM fungal diversity and an important level of AM fungal specificity/compatibility in Al tolerant wheat cultivars grown in Andosols.

Additionally, AM fungi inocula, its origin, wheat cultivar and Al have a specificity of responses in each cultivar, evidencing that the inoculum from acidic soil with the highest Al level had a better performance in the symbiosis.

5.3 Future directions

Our results indicated that physiological and molecular bases by which these fungi are capable of sequester, transport and compartmentalize Al inside their structures also need further studies. The above mentioned it has been target of studies focused mainly on technological applications; however, inocula formulation to use in biofertilization requires a better understanding of ecological bases regulating AM fungal diversity and its contribution to plants growing in soils with phytotoxic Al levels.

Biofertilization with AM fungi in extensive agriculture has not been successfully developed mainly due to difficulties in culture establishing. Specifically, it is necessary to promote the use of monoxenic cultures, to implement molecular tools oriented to diversity, as well as, to deep in the study of the role played by glomalin in these soils and select efficient ecotypes. Hence, futures studies oriented to glomalin potential should be consider that glomalin production begin after 3 weeks and high glomalin amounts need more volume of containers that support AM fungal symbiosis. In

addition, it is crucial to select AM fungal ecotypes with a high Al tolerance and promote the establishment of germplasm bank based on pure culture of AM fungal species that allow us generates research focused on technological application in our country.

To promote the study of chemical signals present in AM fungal symbiosis and their relationship with phosphorus adquisition efficiency, like as the strigolactones. Therefore, it would be possible to optimize plant performance in agricultural crops growing in acidic soils.