UNIVERSIDAD DE LA FRONTERA
Facultad de Ingeniería, Ciencias y Administración
Programa Doctorado y Magíster en Ciencias de Recursos Naturales

MECHANISMS OF TOLERANCE TO PHYTOTOXIC LEVELS OF ALUMINIUM IN ARBUSCULAR MYCORRHIZAL SYMBIOSIS IN ACID SOILS

DOCTORAL THESIS IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **DOCTOR OF SCIENCES IN NATURAL RESOURCES**

ALEX MAURICIO SEGUEL FUENTEALBA

TEMUCO – CHILE
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Esta tesis fue realizada bajo la supervisión del Director de Tesis Dr. FERNANDO BORIE BORIE, perteneciente al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

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... Dedico esta Tesis de Doctorado a mis padres
Gladys y Luis

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Abstract

Soil acidity is a limiting factor to agricultural production on a significant portion of arable land worldwide. Low productivity of these soils is mainly due to nutrient limitation and the presence of high levels of aluminum (Al), which causes deleterious effects on plant physiology and growth. In response to acidic soil stress, plants have evolved various mechanisms to tolerate high concentrations of Al in the soil solution. In this sense, arbuscular mycorrhizal (AM) fungi can play an important role protecting the roots against phytotoxic Al levels and mycorrhization increases plant resistance to acidity and phytotoxic levels of Al in the soil environment. However, despite the well known positive response of AM fungi to Al tolerance by different plants it is necessary to know some basis of the Al tolerance mechanisms present in the AM symbiosis. For this reason, the general objective of this Doctoral Thesis was to study the role and/or contribution of arbuscular mycorrhizal (AM) fungi in the mechanisms involved in Al tolerance of plants growing in acid soils. Firstly, a theoretical background is presented in Chapter 2 including a general presentation of the investigated problem and a critical review on the state of the art of the research worldwide carried out. To know the AM fungi-Al interaction on the Al tolerance of some-selected cereal cultivars, *in vivo* experiments were conducted using several wheat and barley Al tolerant cultivars. The results showed greater increase of AM structures at high Al-saturation. It would produce an improved nutritional status of wheat and barley and would represent an indirect Al tolerance mechanism provided by AM fungi to the host plant. Glomalin accumulated great Al amount in its molecule assuming an important role of AM fungi due to the possible prolonged Al immobilization in soil. In addition, the results here obtained suggest that an early AM colonization can be an important factor in Al tolerance for agricultural plants cropped in acid soils. In the Third and Fourth Chapters are detailed these experiments and results.

On the other hand, some AM fungal strains can confer a higher Al tolerance to plants through an induced higher organic acid exudation which decrease the concentration of free Al on their root zones. This can be consequence of a substantial genetic variation among and within AM

fungal species. However, in the Al tolerance, in addition to the known the response of each ecotype it is necessary to know what happens with fungal dynamics in the field between community species in the field. For that reason, it was hypothesized that the variation in soil chemical characteristic along a vegetation gradient would influence the Al tolerance of AM fungal communities colonizing the vegetation and these AM fungal would mediate Al tolerance of host plants (Chapter 5). The results showed that Glomalin and citrate production was increased when the plants (*A. virginicus*) inoculated with AM native populations were exposed to 100 µM Al. All AM fungal treatments showed higher malate production than non-mycorrhizal plants. A high presence of *A. morrowiae* and *G. clarum* presence (Al-tol. AM fungal ecotypes) in the natural AM community could explain the higher Al tolerance of *A. virginicus* in this experiment. The data here presented provide evidence that there is a functional variation among AM fungi and that the level of Al tolerance conferred to host plants may vary amongst AM species.

In summary, it was observed a bioprotector and bioremediator role of AM fungi on Al tolerance of plants selected in this research through direct and indirect mechanisms expressed by the AM symbiosis as an improved nutritional status, early AM root colonization and glomalin and organic acid production.

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Chapter 1. Introduction and objectives

1. Introduction and objectives

1.1. Introduction

Soil acidity is one of the most important constraints to agricultural productivity worldwide, with acidic soils representing about 40% of the total arable land (Summer and Noble, 2003). Acid soils increase aluminum activity (Al) which is one of the main limiting factors for plant growth (Kochian *et al*. 2004). In addition, the excess of protons (H^+) and deficiencies of some essential nutrients such as phosphorus (P), calcium (Ca) magnesium (Mg) and molybdenum (Mo) are also important stress factors in plants growing in those soils (Marshner, 1995; Mora *et al*. 2002).

Aluminum is earth's third most abundant element after oxygen and silicon, accounting for almost seven percent of the earth's crust (Ma *et al*. 2001). The solubilization of Al is related to the degree of soil acidification caused by natural and anthropogenic sources of soil acidity. Acid soils favor the solubilization of Al and speciation to the phytotoxic Al^{3+} ion, producing the main limiting factor for plant growth (Kochian *et al*. 2004). The sites of these toxicity effects within the plant have been broadly reported to occur in the cell wall matrix of the root tip, at the plasma membrane interface, within the cytoplasm, and within subcellular compartments including the cytoskeleton (Jones *et al*. 2006; Staß and Horst, 2009; Bose *et al*. 2010). Together, these primary and secondary effects ultimately disrupt cell homeostasis and limit cell division, root elongation, and the capacity of Al-sensitive plant genotypes to exploit water and nutrient reserves in the soil, reducing the health and productivity of crop and forest plants growing on acidic soils (Barceló and Poschenrieder 2002; Kochian *et al.* 2005; Ma, 2007; St. Clair *et al*. 2008).

Aluminum-tolerant plant species and/or cultivars within species have evolved mechanisms that detoxify Al and reduce its impact on cell physiology, allowing these species/cultivars to grow when exposed to Al in the environment. The exudation of organic (carboxylic) acids by roots and the external detoxification of Al by chelation with these compounds is one of the most widely reported mechanisms used by plants to overcome Al stress (Delhaize *et al*. 1993; Kollmeier *et al*. 2001; Piñeros *et al*. 2002; Zhao *et al*. 2003). Exudation or organic

acids leads to the chelation of Al^{3+} in the rhizosphere and consequently reduces Al uptake by roots, preventing the subsequent deleterious impacts on metabolism and growth.

On the other hand, arbuscular mycorrhizal (AM) symbiosis plays an important role protecting the roots against the Al toxicity through interaction Al - phosphorus (P) (Marschner, 1995). This role may be especially important in acid soils with high contents of Al and low P (Lux and Cumming, 2001), as many of the volcanic soils from southern Chile (Sadzawka, 2006). In addition to all the known benefits of AM fungi in nutrient acquisition (some of them with amelioration capacity for overcoming Al damage), they may play important roles in conferring Al resistance to their plant hosts. Klugh and Cumming (2007) and Klugh-Stewart and Cumming (2009) reported that some AM fungi strains give higher Al tolerance to plants through a higher organic acid exudation which decrease the concentration of free Al on their root zones. In the case of acid soils and / or with high Al contents, there is variation between tolerant AM fungi ecotypes with not related to other adaptations to this type of stress, showing increased adaptation to these conditions by a difference in spore germination, hyphal growth and colonization percentage (Klugh and Cumming, 2007). This can be consequence of a substantial genetic variation among and within AM fungal species (Bever *et al*. 2001) which may provide different benefits depending on the edaphic environments (Vosátka *et al*. 1999; Kelly *et al*. 2005).

Additionally, some studies have shown that Glomalin-Related Soil Protein (GRSP), a glycoprotein produced by AM fungi and released to the soil in high amounts (Wright and Upadhyaya, 1996, 1998; Gadkar and Rillig, 2006), would be able to immobilize large quantities of potentially toxic elements (Gonzalez-Chavez *et al*. 2004; Vodnik *et al.* 2008, Cornejo *et al*. 2008). Therefore, this molecule may have the ability to bind Al due to its complexing capacity, as it has been recently reported by Aguilera *et al*. (2011), representing a very important external mechanism related to AM fungi to take into account in reducing the toxicity of this element. Across the studies noted above, a limitation on the absorption and translocation of Al to host plant shoots is often the variable associated with AM-mediated Al tolerance. This reduction is associated with elevated P acquisition, suggesting AM species and ecotypes that confer Al tolerance alter the chemistry of the mycorrhizosphere.

1.2. Hypotheses

It is recognized that plants colonized by arbuscular mycorrhiza (AM) have advantages in their growth and development limitations in soils with high acidity and Al, compared with plants that do not form this association. For this reason, this study tends to test, through *in vivo* experiments, the following hypotheses:

Arbuscular mycorrhizal fungi, isolated from ecosystems with phytotoxic levels of Al in Andisols of Chile, confer increased Al tolerance to plants against such stress through:

1. Nutritional changes in AM colonized plants such as enhanced P, Mg and Ca acquisition which neutralize Al phytotoxicity.

2. Production of glomalin which incorporate free Al from soil solution.

3. The increase on the release of exudates with chelating properties exacerbating the mechanism involved in Al exclusion.

1.3. General objective

To study the role and/or contribution of arbuscular mycorrhizal fungi in some mechanisms involved in Al tolerance of mycorrhizal plants growing in acid soils.

1.4. Specific objectives

- To identify the main direct and indirect mechanisms involved in Al tolerance of selected cereal cultivars.

- To study the early effect of soil Al on AM fungal propagule density and root colonization of Al-tolerant wheat and barley cultivars.

- To elucidate potential mechanisms of Al tolerance operating in AM fungi ecotypes.

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Chapter 2. Theoretical background: The role of arbuscular mycorrhizas in decreasing aluminum phytotoxicity in acidic soils

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The role of arbuscular mycorrhizas in decreasing aluminum phytotoxicity in acidic soils: a review

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Abstract

Soil acidity is an impediment to agricultural production on a significant portion of arable land worldwide. Low productivity of these soils is mainly due to nutrient limitation and the presence of high levels of aluminium (Al), which causes deleterious effects on plant physiology and growth. In response to acidic soil stress, plants have evolved various mechanisms to tolerate high concentrations of Al in the soil solution. These strategies for Al detoxification include mechanisms that reduce the activity of Al^{3+} and its toxicity, either externally through exudation of Al-chelating compounds such as organic acids into the rhizosphere or internally through the accumulation of Al-organic acid complexes sequestered within plant cells. Additionally, root colonization by symbiotic arbuscular mycorrhizal (AM) fungi increases plant resistance to acidity and phytotoxic levels of Al in the soil environment. In this review, the role of the AM symbiosis in increasing the Al resistance of plants in natural and agricultural ecosystems under phytotoxic conditions of Al is discussed. Mechanisms of Al resistance induced by AM fungi in host plants and variation in resistance among AM fungi that contribute to detoxifying Al in the rhizosphere environment are considered with respect to altering Al bioavailability, maintaining the acquisition of nutrients from acidic soils, and generally increasing host stress metabolism.

Keywords: AM fungal diversity, exudation, glomalin related soil protein, GRSP, organic acids, aluminium tolerance mechanisms

Contents

2.1. Introduction

2.1.1. The importance and origin of acidic soils

Soil acidity is one of the most important constraints to agricultural productivity worldwide, with acidic soils representing about 40% of the total arable lands (Summer and Noble, 2003). Plant growth on acidic soils is limited by a set of conditions, including the excess of protons (H^+), aluminium (Al) and manganese (Mn) phytotoxicities, and deficiencies of essential nutrients, such as phosphorus (P), calcium (Ca), magnesium (Mg), and molybdenum (Mo) (Driscoll *et al*. 2001; Bolan *et al*. 2003; Fageria and Baligar 2008). Moreover, the limited agricultural productivity of acidic soils is due to diminished microbial activity as a consequence of the presence of high concentrations of deleterious chemical species of Al (Robert, 1995; Fageria and Baligar, 2003; Dahlgren *et al*. 2004).

Natural sources of soil acidity include the decomposition of organic matter, microbial respiration, and plant absorption of cations, especially ammonium (NH_4^+), processes that have a direct impact on soil pH (Martens, 2001; Tang and Rengel, 2003). Erosion and leaching of basic cations, such as potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}), also contribute to the acidification of soils, which is increased in areas with excessive rainfall. Furthermore, excessive addition of acidifying fertilizers, especially ammonium salts, and other agricultural practices are anthropogenic contributors to the acidification of soils (Bolan *et al*. 2003). Other human activities, including industrial emissions of sulfur dioxide (SO_2) and nitrogen oxides (NO_x) that generate acid precipitation and mining that generates acidity in soil/surface substrates, also acidify soils (Evangelou, 1995; Driscoll *et al*. 2001; Frazer, 2001; Norton and Veselý, 2004; Clair and Hindar, 2005). Thus, natural soil acidification is widespread naturally and is exacerbated by human activity, which limits plant productivity in many regions around the world.

2.1.2. Aluminium forms in soil and phytotoxicity

Aluminium is a metal that comprises approximately 8% of the earth's crust, being the third most abundant element after oxygen and silicon (Ščančar and Milačič, 2006). Most Al is present as oxides and alumino silicates, solid amorphous or crystalline minerals that are not harmful to plant roots. However, many of these Al-containing minerals exhibit pH-dependent solubility and the diverse ionic species of Al exhibit pH-dependent speciation that contribute to Al phytotoxicity in varying degrees. In acidic solutions (pH <5.0), Al exists as octahedron hexahydrate, $Al(H_2O)_6^{3+}$, which by convention is named Al^{3+}. When pH increases, Al^{3+} undergoes successive hydroxylations to form $Al(OH)^{2+}$, $Al(OH)_2^{+}$, $Al(OH)_3$ and $Al(OH)_4^{-}$ at pH 7-8 (Stumm and Morgan, 1996). Acidic soils favor the solubilization of Al-containing minerals and generate the phytotoxic Al^{3+} ion, producing the main limiting factor for plant growth on such soils (Wagatsuma and Ezoe, 1985; Pintro *et al*. 1998; Watanabe and Okada, 2005). Aluminium toxicity to plants has been convincingly demonstrated only for Al^{3+} and the complex $AlO_4Al_{12}(OH)_{24}(H_2O)_{12}^{7+}$ (Al_{13}) (see Kochian 1995). However, some experimental results also indicate the toxicity of hydroxylated Al compounds, mainly $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ (Kinraide, 1997). The Al^{3+} ion has a high affinity for oxyanions and various elements and compounds in the soil solution, such as organic acids, which modify Al availability and phytotoxicity.

Due to its importance in limiting agricultural and forest productivity, there have been numerous studies undertaken that describe the effects of Al on plant root growth and physiology. The sites of these toxicity effects within the plant have been broadly reported to occur in the cell wall matrix of the root tip (Horst *et al*. 1999; Jones *et al*. 2006; Staß and Horst, 2009), at the plasma membrane interface (Rengel and Zhang, 2003; Ahn and Matsumoto, 2006; Bose *et al*. 2010a), within the cytoplasm (Rengel *et al*. 1995; Jones *et al*. 1998; Rengel and Zhang, 2003; Guo *et al*. 2007), and within subcellular compartments including the cytoskeleton (Vazquez *et al*. 1999; Blancaflor *et al*. 1998; Yamamoto *et al*. 2001). Many of these phytotoxic effects of Al induce broad-ranging secondary effects, such as disruption of signaling pathways and the production of reactive oxygen species (ROS). Together, these primary and secondary effects ultimately disrupt

cell homeostasis and limit cell division, root elongation, and the capacity of Al-sensitive plant genotypes to exploit water and nutrient reserves in the soil, reducing the health and productivity of crops and forests growing on acidic soils (Driscoll *et al*. 2001; Barceló and Poschenrieder, 2002; Kochian *et al* 2005; Ma 2007; St. Clair *et al*. 2008).

2.2. Aluminium tolerance mechanisms in higher plants

Plants markedly differ in their capacity to tolerate Al and mechanisms involved have received extensive research focus in the past 20 years (Delhaize and Ryan, 1995; Ma *et al*. 2001; Ryan *et al*. 2001; Kochian *et al*. 2004, 2005). Aluminium-resistant plant species and/or genotypes within species have evolved mechanisms that detoxify Al and reduce its impact on cell physiology, allowing these species/genotypes to grow when exposed to Al in the environment. These mechanisms fall broadly into two categories that function within the rhizosphere to alter the chemical form and toxicity of Al in the environment and/or function within plant cells to reduce the negative effects of Al on plant metabolism (Delhaize and Ryan, 1995; Jones *et al*. 1998; Ma *et al*. 2001; Barceló and Poschenrieder, 2002; Kochian *et al*. 2004, 2005; Panda and Matsumoto, 2007). The exudation of organic (carboxylic) acids from roots and the external detoxification of Al by chelation with these compounds is one of the most widely reported mechanisms used by plants to overcome Al stress (Delhaize *et al*. 1993; Li *et al*. 2000; Kollmeier *et al*. 2001; Piñeros *et al*. 2002; Shen *et al*. 2002; Zhao *et al*. 2003). Exudation or organic acids leads to the chelation of Al^{3+} in the rhizosphere and consequently reduces Al uptake by roots and its subsequent impacts on metabolism and growth. There is a close relationship between the alleviation of Al toxicity and the effectiveness of the different carboxylic anions produced by plant roots in forming stable Al complexes based on their stability constants (log K_s), ranging between 7.4-12.3 for citrate > 6.1-7.3 for oxalate > 5.1-5.4 for malate > 3.2-4.6 for succinate, among other organic acid anions, with variation dependent on method of measurement (Martel and Smith, 1977; Charlet *et al*. 1984; Hue *et al*. 1986; Pawlowski, 1998).

The Al-activated efflux of organic acids, which is mediated by different systems in different plant species, is often specific for Al and may exhibit rapid or delayed kinetics (Ryan *et al*. 2001; Barceló and Poschenrieder, 2002; Panda and Matsumoto, 2007). Organic acid exudation in response to Al exposure has received considerable attention and the underlying physiology and molecular biology are being elucidated (Wang *et al*. 2007; Liu *et al*. 2009, Maron *et al*. 2010). For example, the release of malate by Al-resistant *Triticum aestivum* genotypes reduced the accumulation of Al in Al-sensitive root tips and allowed root growth under Al exposure (Delhaize *et al*. 1993, Ryan *et al*. 1995). This response has been ascribed to the *Alt1* gene in *T. aestivum* that functions to rapidly release malate into the rhizosphere, chelating Al^{3+} and reducing its interactions with the cell wall, plasma membrane, and subsequent uptake into the cell (Hoekenga *et al*. 2006). Similar systems have been identified for a variety of species, including *Zea mays* (Piñeros *et al*. 2002; Maron, *et al*. 2010), *Hordeum vulgare* (Zhao *et al*. 2003; Wang *et al*. 2007), and *Arabidopsis thaliana* (Goodwin and Sutter, 2009; Liu *et al*. 2009).

In addition to carboxylic acids, the exudation of diverse phenolic compounds may confer Al tolerance due to the ability of phenolic compounds to form stable complexes with metals, such as Al, in the rhizosphere (Barceló and Poschenrieder, 2002). Kidd *et al*. (2001) reported that, while Al exposure induced oxalate exudation in *Zea mays* varieties, patterns of production were not correlated with Al resistance and were modified by the composition of the rooting media. However, constitutive or induced Al resistance in these genotypes was associated with the exudation of catechol, catechin, quercitin, and/or curcumin that quantitatively far exceeded the exudation of organic acids. The function of phenolic compounds as an Al tolerance mechanism is not well characterized, and their lesser affinity for Al^{3+} compared with organic acid anions, especially at acidic pH where H^+ and Al^{3+} ions would compete for binding sites within phenolic compounds, may reduce their efficacy to chelate Al^{3+} (Ofei-Manu *et al*. 2001).

As an alternative to these extracellular Al-detoxification systems, an increase in the production of compounds that chelate Al intracellularly and reduce its interactions with plant metabolic processes has been proposed as an internal Al tolerance mechanism. Internal detoxification of Al

is limited to Al-accumulating species, such as *Fagopyrum esculentum* (Ma *et al*. 2001) and *Hydrangea macrophylla* (Ma *et al*. 1997). In these species, the accumulation of Al to levels as high as 15,000 μg g^{-1} was related to high intracellular concentrations of oxalate and citrate, respectively. Moreover, Klug and Horst (2010) noted that Al exposure of *F. esculentum* also led to the exudation of oxalate into root intracellular spaces and that Al resistance in this species may rely on both protection of the cell wall from Al binding and uptake and detoxification of Al internally. In addition, the up-regulation of ATP-binding cassette type transporters in many species exposed to Al suggests that there may be a broad-based expression of metabolic systems that compartmentalize metal-complexes, in this case Al-complexes, in the vacuole (Sasaki *et al*. 2002; Larsen *et al*. 2005; Zhen *et al*. 2007; Goodwin and Sutter, 2009).

In addition to these reported mechanisms of Al resistance in plants, the vast majority of higher plants form associations with soil microorganisms that may synergistically promote or stimulate these mechanisms in the plant host or confer Al resistance to plant hosts through the operation of microbially-based systems. Among these microorganisms, arbuscular mycorrhizal (AM) fungi play a key role in fostering growth of most agricultural species and increase the productivity and environmental stress resistance of many ecologically and economically important tree species as well (Smith and Read, 2008).

2.3. Arbuscular mycorrhizal fungi and plant response to soil Al

The AM symbiosis is the oldest and most extensive plant-fungus association present in the world (Wang and Qiu, 2006; Bonfante and Genre, 2008), occurring in about 85% of all the vascular plants in almost all terrestrial ecosystems (Öpik *et al*. 2006). It is a mutualistic association formed between specific soil fungi and plant roots in which the fungal symbiont facilitates the acquisition of nutrients, especially P, from soil to host plants in exchange for fixed carbon (C) (Marmeisse *et al*. 2004; Cavagnaro, 2008; Javaid, 2009; Podila *et al*. 2009; Plassard and Dell, 2010; Smith *et al*. 2011).

The AM fungal association plays a crucial role in the alleviation of diverse abiotic stresses present in the soil environment (Jeffries *et al*. 2003; Evelin *et al*. 2009; Gamalero *et al*. 2009; Gianinazzi *et al*. 2010), including the presence of phytotoxic levels of Al (Rufyikiri *et al*. 2000, Yano and Takaki, 2005, Klugh and Cumming, 2007). AM fungi may increase the capacity of their host plants to withstand abiotic soil stresses through modulation of the edaphic environment and detoxification of harmful compounds in the mycorrhizosphere. The production of low molecular weight exudates or glomalin by mycorrhizas and the biosorption of metals to fungal hyphae will modulate interactions between plants and soil Al (Barceló and Poschenrieder, 2002; Janouskova *et al*. 2005; Borie *et al*. 2006; Gohre and Paszkowski, 2006; Bedini *et al*. 2009; Podila *et al*. 2009; Zhang *et al*. 2009). In addition, increased host plant stress resistance may result from elevated uptake of P and other essential nutrients, the changes in tissue metabolite concentrations, and/or elevated activity of stress resistance pathways that are induced by the symbiosis (Tanaka and Yano, 2005; Javot *et al*. 2007; Andrade *et al*. 2009; Abdel Latef and Chaxing, 2011; Karimi *et al*. 2011; Meier *et al*. 2011). These metabolic changes resulting from AM colonization may serve to prime physiological systems against stress-induced perturbations to homeostasis and may contribute to conferred Al resistance in higher plants.

2.3.1. Contribution of the AM symbiosis to plant Al resistance

The majority of studies on Al resistance species have utilized non-mycorrhizal plants or species that do not form the symbiosis, e.g., *Arabidopsis thaliana*. This work on non- mycorrhizal plants clearly informs the limits of acclimation to Al exposure in plants. However, there is a robust literature on the differences in physiology and environmental stress response between non-mycorrhizal and mycorrhizal plants, and the ecological, physiological, and molecular processes underlying these differences have the capacity to extend the limits of Al resistance in higher plants.

The benefits of the AM association for host plants are ideal for acidic soils because of the increased access to limiting nutrients and induced general stress resistance metabolism of host plants. The AM fungal association is prevalent in well-weathered tropical soils (Cardoso and Kuyper, 2006), deciduous forests (Berliner and Torrey, 1989; Yamato and Iwasaka, 2002; Postma *et al*.2007; Diehl *et al*. 2008), and in extremely acidic environments (Cumming and Ning, 2003; Maki *et al*. 2008; Taheri and Bever, 2010), the soils of which are dominated by Al, indicating that AM fungi may play important functions in the protection of roots against Al toxicity.

2.3.1.1. Al resistance of AM plants – Al binding to hyphae, exudates, and glomalin

Limiting the interactions of the Al^{3+} ion with sensitive plant physiological and metabolic processes is a unifying mechanism of Al resistance (Delhaize and Ryan, 1995; Ma *et al*. 2001; Ryan *et al*. 2001; Kochian *et al*. 2004, 2005). The association of AM fungi with the roots of plants may extend the thresholds of Al resistance by extending or augmenting the resistance mechanisms of their host plants or by providing new Al-resistance mechanisms that serve to detoxify Al in the root environment. The extensive hyphal networks produced by AM fungi have the capacity of directly binding Al (Joner *et al*. 2000; Gohre and Paszkowski, 2006) or creating an expanded mycorrhizosphere in which Al is detoxified (Li *et al*. 1991; Tarafdar and Marschner, 1994). Several studies have reported that increased Al resistance was associated with elevated Al binding in root systems colonized by AM fungi. For example, when compared to non- mycorrhizal plants, concentrations of Al in roots of AM-colonized plants were 51% greater for *L. tulipifera* colonized by *G. clarum* and *Glomus diaphanum* in sand culture (Lux and Cumming, 2001), 210% greater for *Ipomoea batatas* grown with *Glomus margarita* in an acidic soil (Yano and Takaki, 2005), and 210% greater in *Clusia multiflora* inoculated with several *Acaulospora* species grown in soil (Cuenca *et al*. 2001). In these cases, Al may be bound extracellularly to AM fungal cell walls or be sequestered intracellularly in fungal vacuoles and polyphosphate granules (Toler *et al*. 2005; González-Guerrero *et al* 2008; Zhang *et al*. 2009).

Such Al immobilization and exclusion mechanisms active in the roots of AM-colonized plants may contribute to acquired stress resistance in the host plant.

The exudation of metal-binding compounds by mycorrhizal roots also plays a role in Al resistance facilitated by AM fungi. While there is not yet any direct indication that novel Al-binding exudates are induced in host plants by AM fungi, several studies indicate that the association of some AM fungi with plant roots maintains exudation by roots under Al exposure. There were strong relationships between Al phytotoxicity and free Al^{3+} concentrations in *L. tulipifera* that occurred as a result of differential organic acid exudation, notably citrate, among four AM symbionts and the non-mycorrhizal control, with exudation by roots associated with *G. clarum* being the greatest and these plants exhibiting the greatest Al resistance (Figure 2.1) (Klugh and Cumming, 2007). Exudation altered the activity of Al^{3+} in the root zone and, across the four AM symbionts and a non-mycorrhizal control, biomass and leaf P concentration were negatively correlated with free Al^{3+} in the root zone, whereas leaf Al was positively correlated with free Al^{3+} (Figure 2.1). In *A. virginicus*, a similar relationship was noted among six AM fungi and non-mycorrhizal treatments, with citrate again being the dominant organic acid that was produced under Al exposure (Klugh-Stewart and Cumming, 2009).

The accumulation of Al in root tissues of mycorrhizal plants is not always associated with induced Al resistance or reduced Al burden in tissues of host plants, however. Several studies on *L. tulipifera* indicated that AM either increased (Lux and Cumming, 2001) or did not affect (Klugh-Stewart and Cumming, 2009) Al accumulation in leaves and roots. In addition, Cumming and Ning (2003) noted that colonization by an acid-selected AM fungal consortium reduced Al concentrations in roots, but not in leaves, of *A. virginicus*. In these cases, patterns of Al accumulation may reflect changes in the activity of Al^{3+} caused by plant/fungal exudates and functional characteristics of root systems that differ among host species increased translocation of Al to shoots may occur passively and at an elevated level when AM fungi stimulate exudation and the formation of Al complexes in the mycorrhizosphere that are subsequently more mobile within the plant root and more readily enter the xylem (Lux and Cumming, 2001).

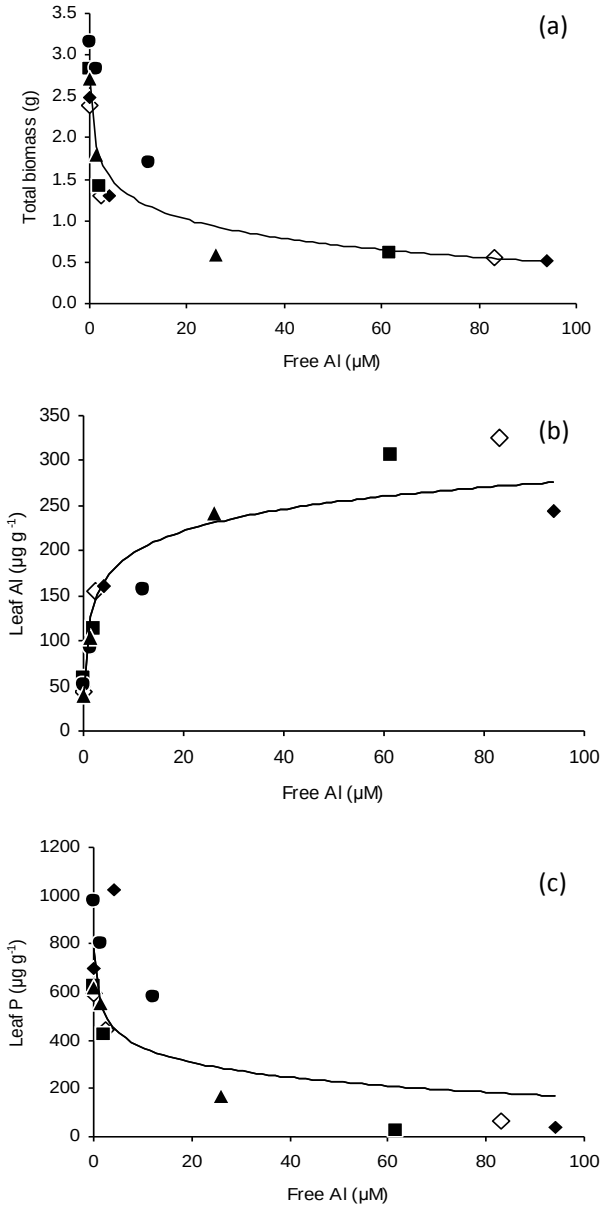

Figure 2.1. Relationships between free Al (Al^{3+}) concentrations estimated in root zones and (a) biomass, (b) leaf aluminum (Al) concentration, and (c) leaf phosphorus (P) concentration of non-mycorrhizal and mycorrhizal *Liriodendron tulipifera*. Symbols: ◇, non-mycorrhizal;◆, *Acaulospora morrowiae*; **n,** *Glomus claroideum*;**l,***Glomus clarum*; and▲,*Paraglomus brasilianum* (Reprinted from Klugh and Cumming, 2007 with permission).

In addition to maintaining exudation by host roots under Al exposure, AM fungi also have the capacity to provide novel biochemical mechanisms that may confer Al resistance to their plant hosts. Glomalin is a component of hyphae and AM fungal spore walls (Driver *et al*. 2005) and quantitatively represents a significant fraction of the pool of soil protein due to its persistence and recalcitrance in native soils (Wright and Upadhyaya, 1996; Rillig and Mummey, 2006, Bedini *et al*. 2007). It is of significance in considering metal resistance and AM fungi because it has high cation exchange capacity and high affinity for polyvalent cations.

Some studies show that glomalin has the potential to immobilize high amounts of metals (González-Chávez *et al*. 2004; Vodnik *et al* 2008; Cornejo *et al*. 2008a). Furthermore, studies on the nature of this protein indicate that glomalin (measured as glomalin-related soil protein, GRSP) production by AM fungi increases when AM fungi are subjected to adverse soil conditions (Vodnik *et al*. 2008; Cornejo *et al*. 2008a), including acidic soils with elevated Al (Lovelock *et al*. 2004). It is probable that, in soils with high Al content, a large quantity of this protein has accumulated as an AM fungal response to Al exposure. Results obtained by Etcheverría (2009) in acidic soils in temperate forest from southern Chile showed that GRSP has the capacity to sequester substantial quantities of Al (4.2 to 7.5% by weight), and thus may represent an Al-binding mechanism in AM fungi that can be very important in the reduction of Al toxicity to plant roots as well. Aguilera *et al*. (2011) have shown that GRSP has the ability to sequester Al within the glomalin molecule, which may sequester Al in a highly recalcitrant form, since some studies have indicated a high residence time of glomalin in soils (Rillig *et al*. 2001).

Thus, the capacity of some AM fungal species and ecotypes to maintain organic acid or glomalin exudation to the mycorrhizosphere in acidic soils may be effective Al resistance mechanisms that reduce the concentration of free Al^{3+} in acidic soil solutions, reducing direct Al phytotoxicity, uncouple the interactions between this toxic metal and $H_2PO_4^-$, Ca^{2+}, and Mg^{2+}, and facilitate root growth and exploration of the soil to support plant productivity.

2.3.1.2. Al resistance of AM plants – improved nutrient relations

The uptake of plant nutrients is critical to the maintenance of homeostasis and growth of plants under edaphic stress and resistance to Al is often, but not always, reflected in both limited perturbations to P, K, Ca, and Mg acquisition and maintained concentrations of these elements in root and shoot tissues (Andrade *et al*. 2009).

The interaction between Al^{3+} and $H_2PO_4^-$ in the root zone may lead to the precipitation of $AlPO_4$, limiting the capacity of the plant root to obtain P. AM fungi, potentially due to higher affinity uptake systems (Smith *et al*. 2011) or maintenance of C flux to the rhizosphere (Klugh and Cumming, 2007; Klugh-Stewart and Cumming, 2009), may obviate this stress. Numerous studies with a variety of plant hosts and AM symbionts have noted mycorrhizal protection of P acquisition (Medeiros *et al*. 1994; Borie and Rubio, 1999; Rufyikiri *et al*. 2000; Kelly *et al*. 2005; Klugh and Cumming, 2007, Cornejo *et al*. 2008b). Rufyikiri *et al*. (2000), using *Musa acuminata* colonized by *Glomus intraradices*, noted a positive effect of AM fungi under Al exposure (78 and 180 μM). In this study, the shoot dry weight of mycorrhizal plants was greater than non-mycorrhizal plants and the contribution of AM fungi to water and nutrient uptake, including P, was particularly pronounced. These benefits were related to a marked decrease in Al content in roots and shoots and delay in the appearance of Al-induced leaf symptoms. *Liriodendron tulipifera*, a significant forest tree species in the eastern United States, is especially sensitive to soil acidification and Al-induced P limitation (Lux and Cumming, 2001; Klugh and Cumming, 2007). For this species, the maintenance of Pi acquisition under Al exposure (50, 100, and 200 μM) by *G. clarum* was critical in maintaining growth and this strong linkage between AM-mediated Pi acquisition and Al toxicity may relate to the highly mycotrophic nature of this tree species. In contrast, Al had marginal effects on root and shoot P concentrations in *A. virginicus* (Cumming and Ning, 2003; Kelly *et al*. 2005), with shoot P often increasing under Al exposure. These effects were ascribed to growth dilution/concentration effects, where significant reductions in the growth of non-mycorrhizal plants without concomitant reductions in Pi uptake led to elevated tissue P concentrations.

The Al^{3+} ion, bound within the root apoplast, may also affect cation uptake by limiting the diffusion of Ca^{2+}, Mg^{2+}, and other multivalent cations to the plasma membrane surface (Huang *et al*. 1992ab; Kinraide *et al*. 2004; de Wit *et al*. 2010). Indeed, Ca and Mg limitation are classic Al toxicity symptoms in non-mycorrhizal plants (Foy *et al*. 1978). The AM symbiosis may alter these charge-based interactions within the plant root by absorbing cations through hyphae and transferring them to host plants (Ryan *et al*. 2003; Lee and George, 2005; Ryan *et al*. 2007). In addition, AM fungi may alter reactions of Al^{3+} with the plant root cell wall through the production of metal-chelating compounds of fungal or host origin (Klugh and Cumming, 2007; Cornejo *et al*. 2008a). Borie and Rubio (1999), Rufyikiri *et al*. (2000), and Lux and Cumming (2001) all noted that AM fungi moderated Al-induced reductions in Ca and/or Mg concentrations in roots and shoots and these changes were often associated with reductions in Al accumulation.

From the above, it is evident that differences in the accumulation of nutrients in tissues may or may not be a good indicator of the mycorrhizal benefit under Al exposure. The nutrient benefit may be the result of increased C flux and Al chelation in the mycorrhizosphere, discussed above, or may reflect greater nutrient uptake effectiveness by AM fungi or changes in plant nutrient use efficiency resulting from colonization by AM fungi (Smith and Read, 2008). Differences in plant host, AM species, and Al exposure conditions will all influence the uptake and translocation of nutrients within host plants and plant growth may be the best integrated response of the efficacy of the AM association in providing Al resistance.

2.3.1.3. Al resistance of AM plants – elevated host stress metabolism

Interactions between AM fungi and their host plants bring about broad ranging changes in metabolism, which may prime plant cells to cope with abiotic stresses in the root zone (Hohnjec *et al*. 2007; Goodwin and Sutter, 2009). Changes in the regulation of antioxidant enzyme activities or the induction of specific stress-related systems resulting from AM colonization would allow the host plant to overcome stresses induced by unfavourable levels of soil Al by

inducing metabolic stress resistance pathways that relieve the effects of Al on plant cell homeostasis (see Ouziad *et al*. 2005; Zhu *et al*. 2010, Abdel Latef and Chaxing, 2011). The induction by AM fungi of metal transporters (Repetto *et al*. 2002; Ouziad *et al*. 2005), antioxidant enzymes (Garg and Manchanda, 2009), and the accumulation of secondary compounds and other metabolites (Peipp *et al*. 1997; Garg and Manchanda, 2009) may all function to enhance plant resistance to Al. For example, Garg and Manchanda (2009) noted elevated activities of superoxide dismutase, catalase, and peroxidase in roots and leaves of *C. cajan* colonized by *G. mosseae* and these were associated with reduced lipid peroxidation in roots.

Little information directly linking AM fungi and metabolic priming of host plants against Al stress is available. However, the impacts of Al on plants include increased oxidative stress (Naik *et al*. 2009; Hossain *et al*. 2011; Ma *et al*. 2012) and the induction by AM colonization of ROS enzymes or other compounds that would reduce the toxic effects of Al on metabolism could contribute to acquired Al resistance in colonized host plants. This area represents a vital area for continued investigation.

2.3.2 Variation in the Al tolerance of AM fungal species and ecotypes

The Al tolerance benefit that AM fungi provide to plants is variable among AM fungal species and host plant species in terms of Al exclusion, nutrient acquisition, or plant growth (Borie and Rubio, 1999; Kelly *et al*. 2005, Klugh-Stewart and Cumming, 2009). This is a consequence of a substantial genetic variation among and within AM fungal species (Bever *et al*. 2001; Avio *et al*. 2009). Natural ecosystems contain native populations and communities of AM fungi that provide variation in benefits to plant growth and variation in response to the environment (van der Heijden *et al*. 1998; Clark *et al*. 1999; Bever *et al*. 2001). Changes in the soil environment may also modify the abundance and distribution of the AM fungi species, as those capable of acclimating to the new environment may become more prevalent; such changes in AM communities may have implications for host plant performance in ecosystems (Bever *et al*. 2001;

Taheri and Bever, 2010). In general, AM fungi have been found in soils from pH 2.7 to 9.2, but different isolates of the same species have varied tolerance to acidity and most AM fungal isolates appear to be adapted to soil pH conditions close to those from which they were collected (Siqueira *et al*. 1984; Sylvia and Williams, 1992; Bartolome-Esteban and Schenck, 1994; Clark, 1997). This has resulted from natural selection favouring the presence of better adapted AM fungal ecotypes in acidic soils and displacing from such environments those with lesser competitive ability (Ashen and Goff, 2000).

2.3.2.1.Variation in AM fungal Al resistance – spore germination, germ tube growth, and colonization

In the case of acidic soils and/or soils with elevated Al levels, there may be variation among ecotypes of potentially Al-tolerant AM fungi related to microsite variation, persistence within roots, and differences in sensitivity of life stage events, such as the germination of spores, germ tube growth, hyphal growth, and root colonization capability For example, Lambais and Cardoso (1989) reported that germ tube growth in *Glomus macrocarpum, Gigaspora margarita,* and *Scutellospora gilmorei* decreased in response to Al concentrations ranging from 0 to 130 μM Al in sand at pH 4.5. However, while spore germination of *Gi. margarita* was not significantly influenced by Al, spore germination in *G. macrocarpum* and *S. gilmorei* was deleteriously affected by Al. *G. macrocarpum* was the most sensitive AM fungus assayed, with no spore germination or germ tube growth at 90 μM Al or higher levels (Lambais and Cardoso, 1989). In another study, Bartolome-Esteban and Schenck (1994) assessed spore germination and germ tube growth at different Al saturation levels and found that *Gigaspora spp.* exhibited high Al tolerance, *Scutellospora spp.* were variably affected by high Al saturation, and isolates of *Acaulospora scrobiculata* were relatively sensitive to high Al saturation, consistent with the findings of Lambais and Cardoso (1989). Experiments where fungal response to soil acidity and Al are summarized in Table 2.1 and Figure 2.2. Across many studies, there is no clear pattern of sensitivity of fungal colonization of host plant roots exposed to Al, although some fungal species/isolates exhibit reductions in colonization in response to Al in the environment (Figure 2.2).

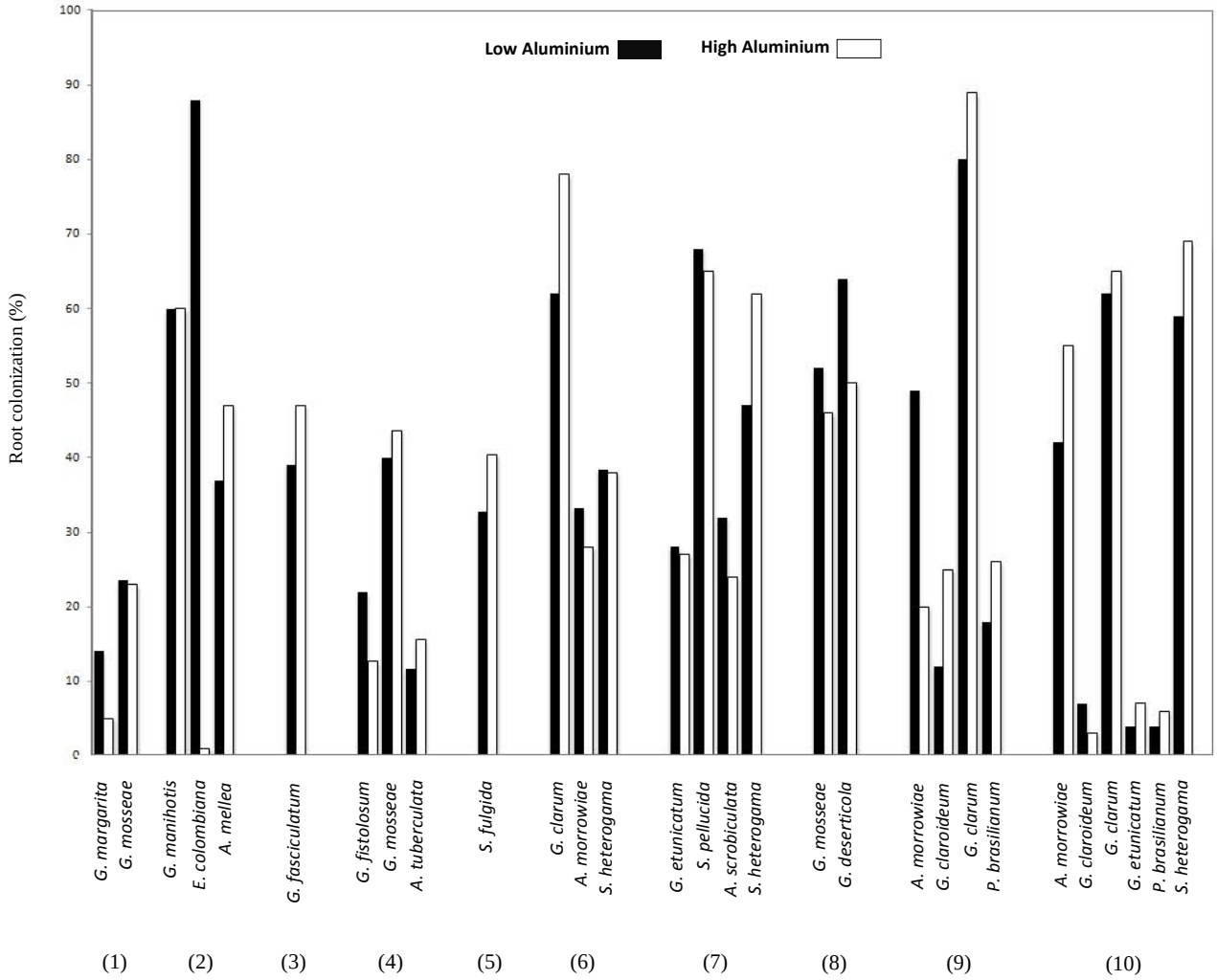

Figure 2.2. Root colonization (%) by AM fungal ecotypes for plants grown under low and high Al conditions. (1) *Zea mays* was grown in an ultisol amended with 12 (Low Al) or 0 meq $CaMgCO_3$ (100 g soil)$^{-1}$ (High Al) (Siqueira *et al*. 1984); (2) *Manihot esculenta* was grownin an acid tropical soil and watered with solutions of pH 6.3 (Low Al) or 3.9 (High Al) (Howeler *et al*. 1987); (3) *Hieracium pilosella* was grown in a strongly weathered sandy soil and watered with nutrient solution with pH 5.5 (Low Al) or 2.5 (High Al) (Heijne *et al*. 1996); (4) *Zea mays* was cultivated in sand-vermiculite and supplied with acid rain solution (Low Al) or acid rain solution with 3 mM Al (High Al) (Vosatka *et al*. 1999); (5) *Clusia multiflora* was grown in an ultisol and watered with distilled water (Low Al) or acidified water at pH 3 (High Al) (Cuenca *et al*. 2001); (6) *Andropogon virginicus* was exposed to 0 (Low Al) or 400 µM Al (High Al) in sand culture (Kelly *et al*. 2005); (7) *Malus prunifolia* plants were grown in limed soil (pH 6, Low Al) or unlimed soil (pH 4, High Al) (Cavallazzi *et al*. 2007); (8) *Eucalyptus globulus* was grown in sand:vermiculite:sepiolite substrate amended with 0 (Low Al) or 600 mg Al kg^{-1} (High Al) (Arriagada *et al*. 2007);

(9) *Liriodendron tulipifera* was exposed to 0 (Low Al) or 200 μM Al (High Al) in sand culture (Klugh and Cumming, 2007); (10) *Andropogon virginicus* was exposed to 0 (Low Al) or 100 μM Al (High Al) in sand culture (Klugh-Stewart and Cumming, 2009).

Table 2.1. Arbuscular mycorrhizal fungal response to Al exposure. A) Data from Lambais and Cardoso (1989 as reported in Clark 1997); B) Bartolome-Esteban and Schenck (1994); C) Klugh-Stewart and Cumming (2009).

		Aluminium concentration (μM)					
A)		0	40	130	0	40	130
		Spore germination (%)			Germ tube growth rating*		
Gigaspora margarita		81	76	78	3	2.3	2
Scutellospora gilmorei		70	38	31	2.5	1.8	1.3
Glomus macrocarpum		11	3	0	1.5	1	0
		Aluminium saturation (%)					
B)	INVAM	6	27	100	6	27	100
	designate	Spore germination (%)			Hyphal growth (mm)		
Gigaspora albida	GABD 185	13	25	65	79	51	46
Gigaspora margarita	GMRG 444	70	55	66	210	185	197
Gigaspora gigantea	GGGT 109	92	93	67	138	225	304
Gigaspora gigantea	GGGT 663	40	19	18	215	240	166
Scutellospora heterogama	CHTG 139	35	41	40	97	104	80
Scutellospora pellusida	CPLC 288	75	80	75	31	20	16
Scutellospora calospora	CCLC 269	30	32	19	39	40	31
Scutellospora calospora	CCLC 348	56	46	54	45	40	28
Glomus manihot	LMNH 980	86	89	91	40	45	27
Glomus etunicatum	LETC 236	83	4	0	27	1	0
Glomus etunicatum	LETC 329	60	5	0	8	2	0
Glomus etunicatum	LETC 455	80	17	0	22	5	0
Glomus clarum	LCRL 551	36	26	3	18	4	2
Acaulospora scrobiculata	ASCB 456	14	4	14	1	1	1
		Aluminium concentration (μM)					
C)	INVAM	0		100	0		100
	designate	Spore germination (%)			Hyphal growth (mm)		
Acaulospora morrowiae	WV107	45.4		50.9	22.7		12.7
Glomus claroideum	WV109E	25.3		6.8	10.1		6.4
Glomus clarum	WV234	71.7		44.4	80.2		47.4
Glomus etunicatum	VZ103A	33.5		29.6	18.7		9.6
Paraglomus brasilianum	BR105	39.4		16.9	24.5		8.2
Scutellospora heterogama	WV108	67.5		75.2	177.7		165.6

*Germ tube growth rating: 0 = no growth; 1 = 0-5 mm; 2 = 5-10 mm; 3 > 10 mm.

Recently, Klugh-Stewart and Cumming (2009) reported that spore germination rates of *Acaulospora morrowiae*, *G. etunicatum*, and *Scutellospora heterogama* were unaffected by exposure to 100 μM Al, whereas germination was reduced in *G. clarum*, *Paraglomus brasilianum*, and greatly inhibited in *Glomus claroideum*. However, hyphal length per spore suggested that germ tube growth and spore germination were differentially affected by Al exposure (Table 2.1) (Klugh-Stewart and Cumming, 2009). Such differences may reflect the variation in genotypes among spores within a single-species trap culture (Bever and Morton, 1999) and subsequent selection and survival under imposed Al stress (Klugh-Stewart and Cumming, 2009). Additionally, in this study, Al did not affect mycorrhizal colonization with *A. virginicus*, which suggests that Al does not inhibit the formation of the symbiosis by Al-resistant or -sensitive AM fungi (Figure 2.2). However, growth and protection of *A. virginicus* from Al among AM species was not associated with any of the AM fungal resistance traits, again suggesting that selection of Al resistance may occur at the AM germination and growth stages, but that the Al resistance mechanisms in AM fungi may not be translatable to host plants (Cuenca *et al*. 2001; Klugh-Stewart and Cumming, 2009).

2.3.2.2.Variation in AM fungal Al resistance – colonization and plant performance

The protection of plant growth under exposure to Al may be the best indicator of fungal resistance to Al in soils. In Figure 2.3, we present data from 13 studies where multiple AM fungi were used in studies and Al was a controlled variable. An analysis of these data together indicates that there are significantly different growth benefits (fold increases) from AM depending on both Al level (F: 179; $p<0.001$) and AM ecotype (F: 1,384; $p<0.001$). Moreover, the positive effect of plant growth under Al exposure depends on the Al-by-AM species interaction (F: 9,529; $p<0.001$), reflecting the AM species-specific dependence of induced Al resistance.

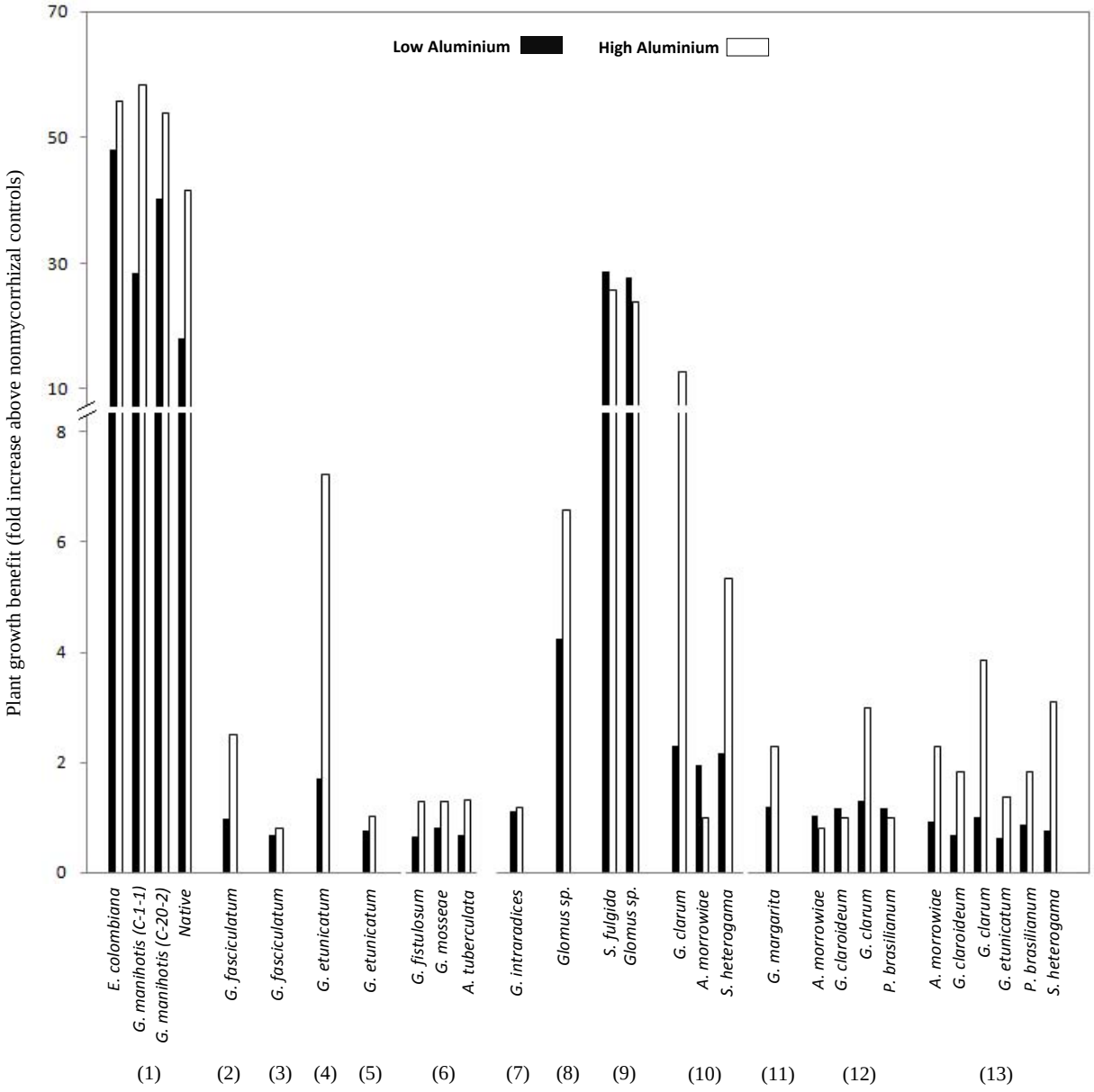

Figure 2.3. Plant growth benefit (fold increase above nonmycorrhizal controls) from AM fungal ecotypes for plants grown under low and high Al conditions. (1) *Manihot esculenta* grownin an acid tropical soil limed to pH 5.3 (Low Al) or 3.9 (High Al) (Sieverding, 1991 as reported in Clark, 1997); (2) *Hieracium pilosella* and(3) *Deschampsia flexuosa*grown in a strongly weathered sandy soil and watered with nutrient solution with pH 5.5 (Low Al) or 2.5 (High Al) (Heijne *et al*. 1996); (4) Al-tolerant *Hordeum vulgare* and (5) Al-sensitive *H. vulgare* grown in an acidic andisol that was limed (pH 5.3, Low Al) or unlimed (pH 4.6, High Al) (Borie and Rubio, 1999); (6) *Zea mays* was

cultivated in sand-vermiculite and supplied with acid rain solution (Low Al) or acid rain solution with 3 mM Al (High Al) (Vosatka *et al*. 1999); (7) *Musa acuminate* plants were grown in sand culture with 0 (Low Al) or 180 μM Al (High Al) (Rufyikiri *et al*. 2000); (8) *Liriodendron tulipifera* was exposed to 0 (Low Al) or 200 μM Al (High Al) in sand culture (Lux and Cumming, 2001); (9) *Clusia multiflora* was grown in an ultisol and watered with distilled water (Low Al) or acidified water at pH 3 (High Al) (Cuenca *et al*. 2001); (10) *Andropogon virginicus* was exposed to 0 (Low Al) or 400 μM Al (High Al) in sand culture (Kelly *et al*. 2005); (11) *Ipomoea batatas* plants were cultivated in an acidic silty loam soil that was limed (pH 5.2, Low Al) and unlimed (pH 4.2, High Al) (Yano and Takaki, 2005); (12) *Liriodendron tulipifera*was exposed to 0 (Low Al) or 200 μM Al (High Al) in sand culture (Klugh and Cumming, 2007); (13) *Andropogon virginicus* was exposed to 0 (Low Al) or 100 μM Al (High Al) in sand culture (Klugh-Stewart and Cumming, 2009).

Several studies have used a host plant with several AM fungal ecotypes and assessed different responses reflecting Al resistance. Cavallazzi *et al*. (2007) showed that the mycorrhizal colonization of apple was significantly influenced by acidic soil selected fungal isolates of *G. etunicatum*, *Scutellospora pellucida*, *S. heterogama*, and *A. scrobiculata* in soils varying in pH (4.0, 5.0, 6.0) and Al availability (2.7, 0.3, and 0 cmolc kg^{-1}). Under the highest Al condition, plants colonized by S. *heterogama* exhibited the greatest leaf P concentration and the lowest leaf Al concentration, whereas plants inoculated with *A. scrobicalata* exhibited reductions in colonization and had the lowest biomass and tissue P and highest tissue Al (Cavallazzi *et al*. 2007). In studies with *L. tulipifera* and *A. virginicus*, Klugh and Cumming (2007) and Klugh-Stewart and Cumming (2009) showed different benefits of AM fungal ecotypes to Al in diverse host plants. In general, their results suggest that Al tolerance in host plants depends on the adaptability of the AM fungi to edaphic conditions, including high Al levels, and the specificity of the host plant with a particular AM fungal ecotype, which may explain why, in some cases, the same fungal ecotype gives different responses in association with different plant species.

In a study utilizing several ecotypic isolates of three AM fungi and *A. virginicus* at different Al levels, Kelly *et al*. (2005) found that *G. Clarum* isolates provided the greatest resistance to toxic levels of Al (400 μM), *S. heterogama* isolates showed intermediate benefits for plant growth, and

plants colonized by *A. morrowiae* isolates were the least Al resistant (Kelly *et al*. 2005) (Figure 2.3). Across these species and ecotypes, Al resistance as measured by plant biomass was positively correlated with root colonization and negatively correlated with the accumulation of Al in leaf tissue. However, there was no association between Al Tolerance Index (biomass with Al/biomass without Al) and pH at the site of fungal isolation, suggesting that broad patterns of AM species behaviour for Al resistance for the host *A. virginicus* may override ecotypic variation in Al resistance within AM species or that Al resistance as a trait is not stable (see section 2.3.2.4).

2.3.2.3. Variation in AM fungal Al resistance – AM mechanisms of Al resistance

Differences in Al absorption and translocation by host plants associated with different AM fungal ecotypes under high Al levels may reflect the function of underlying mechanisms of Al resistance that vary according to the fungal symbiont. The biosorption and sequestration of Al in the mycelium (Joner *et al*. 2000) and changes in the chemical speciation of Al, which implies the production of root exudates (Lux and Cumming, 2001; Cumming and Ning, 2003), are mechanisms that may vary among different AM fungal species and ecotypes and may confer Al tolerance to plant plants.

Across the studies noted above, a limitation of the absorption and translocation of Al to host plant shoots is often the variable associated with AM-mediated Al resistance. This reduction is often associated with elevated P acquisition, suggesting AM species and ecotypes that confer Al resistance alter the chemistry of the mycorrhizosphere, as discussed in section 2.3.1.1. As noted in Figure 2.1, the growth of *L. tulipifera* with several AM symbionts could be related to the concentration of Al^{3+} in the root zone, which also influenced the accumulation of Al in tissues (Table 2.2). A similar pattern has been noted for the host species *A. virginicus* (Klugh-Stewart and Cumming, 2009), with patterns of resistance consistent across multiple ecotypes within AM species (Kelly *et al*. 2005).These broad patterns suggest that the stimulated flux of C, primarily

as citrate, to the mycorrhizosphere appears to be a major mechanism of Al resistance in AM plants just as it functions in numerous non-mycorrhizal plant species.

Table 2.2. Accumulation of Al in plants exposed to low and high Al levels with and without AM fungi. Some values extrapolated from figures in each reference.

Plant	AM treatment	Shoot Al (mg/kg)		Root Al (mg/kg)		Reference
		Low Al	High Al	Low Al	High Al	
Hordeum vulgare (Al tol.)	*G. etunicatum*	145	296			Borie and Rubio (1999)
	Nonmycorrhizal	246	405			
Hordeum vulgare (Al sens.)	*G. etunicatum*	147	312			
	Nonmycorrhizal	307	252			
Musa acuminata	*G. intraradices*	200	700	5,500	5,750	Rufyikiri *et al*. (2000)
	Nonmycorrhizal	300	1500	7,000	8,500	
Liriodendron tulipifera	*Glomus spp.*	180	423	800	930	Lux and Cumming (2001)
	Nonmycorrhizal	140	180	500	610	
Clusia multiflora	*S. fulgida*	125	160	12,000	12,500	Cuenca *et al*. (2001)
	Glomus spp.	100	95	9,000	20,000	
	Nonmycorrhizal	220	200	20,000	17,500	
Andropogon virginicus	*G. clarum*	10.9	43.2	342	1,868	Kelly *et al.* (2005)
	A. morrowiae	9.9	165.2	416	3,089	
	S. heterogama	12.5	93.3	334	2,494	
	Nonmycorrhizal	15.0	225.4	583	2,721	
Ipomoea batatas	*G. margarita*	240	360	4,780	4,690	Yano and Takaki (2005)
	Nonmycorrhizal	370	480	5,340	2,230	
Malus prunifolia	*G. etunicatum*	1.6	5.2			Cavallazzi *et al*. 2007
	S. pellusida	3.1	5.4			
	A. scrobiculata	2.4	7.6			
	S. heterogama	3.3	3.2			
	Nonmycorrhizal	7.1	4.0			
Liriodendron tulipifera	*A. morrowiae*	45	240			Klugh and Cumming (2007)
	G. claroideum	50	300			
	G. clarum	43	155			
	P. brasilianum	30	235			
	Nonmycorrhizal	40	330			
Andropogon virginicus	*A. morrowiae*	25	75			Klugh-Stewart and Cumming (2009)
	G. claroideum	15	63			
	G. clarum	18	70			
	G. etunicatum	14	72			
	P. brasilianum	15	91			
	S. heterogama	10	73			
	Nonmycorrhizal	14	70			

2.4.2.4. Stability of Al resistance in AM fungi

One additional factor should be considered when assessing metal resistance of AM fungi. Many isolates used in experiments on the role of AM fungi on host metal resistance, whether focusing on growth, physiology, or molecular responses, utilize inocula generated from common soil trap cultures (Morton *et al*. 1993). Many factors influence the community composition of a trap culture and the genetic makeup of its AM fungi, including plant host species, seasonality of collection, and the abiotic factors in the trap environment, including substrate chemistry. When assessing metal resistance and extrapolating from AM fungi maintained in cultures, consideration should be made of potential changes in the genetic make-up of the AM fungal isolates in culture. Bever and Morton (1999) noted that considerable heritable variation for spore shape was maintained in cultures of *S. pellucida* in trap cultures. In an analogous fashion, such trap culture may enrich variation over time in field-collected metal-resistant AM ectotypes because the selection pressure for metal resistance is removed and nuclei that do not carry metal-resistant genes may proliferate. Such a process was suggested by Kelly *et al*. (2005) for three AM fungal species that did not exhibit clear patterns of Al resistance in relation to the pH of the sites of their original collection. Similarly, Malcová *et al* (2003) and Sudová *et al*. (2007) noted that metal-free culture of metal-resistant *Glomus* ecotypes reduced their resistance to metals compared to the same lines maintained under metal exposure. Clearly, care must be taken when culturing metal-selected isolates for long-term studies of metal resistance in AM fungi.

2.4 Conclusions

Soil acidity is a major limitation to agricultural production throughout the world. The AM fungal symbiosis has great potential to increase plant growth by mediating the soil solution chemistry of the root-soil interface, improving nutrient acquisition, and altering plant stress responses, some or all of which positively contribute to plant performance on acidic soils. The mechanisms that alter Al^{3+} bioavailabilty in the mycorrhizosphere, which will secondarily ameliorate Al impacts on nutrient uptake, may underlie Al tolerance of plants associated with Al-resistant AM fungi.

Currently, data suggest the biosorption of Al to hyphae and perhaps glomalin and sustained organic acid exudation from roots of plants colonized by Al-resistant AM fungi are Al resistance mechanisms conferred to host plants that are not yet fully understood. Continued research is needed to understand the roles played by AM fungi in increasing the Al resistance in crops and trees growing in acidic soils where Al is the principal limiting factor.

In agronomic systems, it is a common practice to apply amendments, such as lime, gypsum and phosphate fertilizer, to enhance the quality and quantity of agricultural production on acidic soils. However, limited reserves of raw material (phosphate rock) are increasing input prices of phosphate fertilizers and sustained inputs of these materials are not feasible, especially in developing economies. For agricultural systems on acidic soils, however, it is also common to use genotypes of Al-tolerant crop species and/or genotypes with high P use efficiency. Thus, it is possible to reduce fertilizer inputs, especially on marginal soils or where the process of P fixation is very intense, as in acid or allophanic soils. Within this same context, the use of AM fungal ecotypes adapted to high levels of Al in soil, and their management, or enhancement by inoculation with native fungi, may provide significant increase to agricultural production on acidic soils. The use of diverse AM fungal species adapted to Al in soils as biofertilizers should be considered as part of integrated nutrient management, which is projected to be an important avenue to improve crop yields through better nutrient supply and may be especially important for agriculture on acidic soils with phytotoxic Al levels.

2.5. References

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Chapter 3. Effect of Arbuscular Mycorrhizal Symbiosis on Aluminum tolerant cultivars of wheat (Triticum aestivum L) growing in acid soils

Article under revision by the co-authors

Effect of Arbuscular Mycorrhizal Symbiosis on Aluminum tolerant cultivars of wheat (*Triticum aestivum* L) growing in acid soils

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Abstract

Arbuscular mycorrhizal (AM) fungi can play an important role protecting the roots against phytotoxic Al levels. To know the AM fungi-Al interaction, six Al- tolerant cultivars of *Triticum aestivum* L ('Crac', 'BT-200', 'Invento', 'Otto', 'Bakan', 'Porfiado') were cultivated in an acid soil at two Al saturations levels (natural [80% Al-sat.] -NS- and limed soil [7% % Al-sat.] -LS-). The harvests were carried at three phenological stages, tillering (60 days after sowing, DAS); anthesis (90 DAS) and physiological maturity (150 DAS). The high Al levels affected shoot and root growth, whereas AM fungi colonization was not inhibited and was greater at high Al saturation. Arbuscular mycorrhizal fungi spores increased from 10 to 131% at high Al levels. There was a trend for increased glomalin related soil protein (GRSP) in 'Porfiado' under NS. In addition, the GRSP bound Al (GRSP-Al) was significantly higher in NS than LS in 'Crac' and 'Porfiado'. These cultivars accumulated lesser Al in tissues than the others and macronutrients concentration increased in LS. Colonized root length correlated negatively with shoot accumulated Al in plants growing under NS and LS (r=-0.42 and -0.49 respectively, $p<0.001$). Moreover, a relationship was found between Al bound to GRSP and root accumulated Al (r=-0.57, $p<0.001$) and translocated Al to shoot (r=-0.57, $p<0.001$) in all wheat cultivars grown under high Al saturation. Relative growth rate and mycorrhizal parameters showed that 'Crac', 'Invento' and 'Porfiado' were the more Al tolerant cultivars and a principal components analysis showed a similar behavior between those wheat cultivars. Glomalin accumulated a significant concentration of Al in its molecule assuming an important role of AM fungi in the possible tolerance of these cereals to high Al levels.

3.1. Introduction

Acid soils increase the solubilization of aluminum (Al) which produces one of the main limiting factors for plant growth (Kochian *et al*. 2002). In addition, the excess of protons (H^+) and deficiencies of some essential nutrients such as phosphorus (P), calcium (Ca), magnesium (Mg) and molybdenum (Mo) are also important stress factors in plants growing in those soils (Marshner, 1995; Driscoll *et al*. 2001; Tang *et al*. 2003).

The arbuscular mycorrhizal (AM) symbiosis plays an important role protecting the roots from Al toxicity through Al - phosphorus interactions (P) (Marschner, 1995). This role may be especially important in acid soils having high levels of exchangeable Al (Lux and Cumming, 2001), as many of the volcanic soils from southern Chile (Sadzawka, 2006). In addition to all the known benefits of AM fungi in nutrient acquisition (some of them with amelioration capacity for overcoming Al damage), Am fungi may play important roles in conferring Al resistance to their plant hosts as has been demonstrated in *Panicum virgatum* (Koslowsky and Boerner, 1989), Al-tolerant cultivars of *Hordeum vulgare* (Borie and Rubio, 1999), *Musa acuminata* (Rufyikiri *et al*. 2000), *Clusia multiflora*, a tropical woody species, (Cuenca *et al*. 2001); *Liriodendrum tulipifera* (Lux and Cumming, 1999; Klugh and Cumming, 2007), *Andropogon virginicus* (Cumming and Ning, 2003; Klugh-Stewart and Cumming, 2009), *Vigna unguiculata* (Rohyadi *et al*. 2004), *Ipomoea batatas* (Yano and Takaki, 2005) and *Gmelina arborea* (Dudhane *et al*. 2012). In all of these species, mycorrhizal plants were more Al-tolerant than non-mycorrhizal plants and absorbed more water and nutrients; also, reactive Al concentration in roots differed significantly in plants growing in symbiosis (Lux and Cumming, 2001; Cumming and Ning, 2003). In relation to mechanism involved, Klugh and Cumming (2007) and Klugh-Stewart and Cumming (2009) concluded that some AM fungi strains give higher Al tolerance to plants through a higher organic acid exudation which decrease the concentration of free Al on their root environments. This can be consequence of a substantial genetic variation among and within AM fungi species (Bever *et al*. 2001) which may provide different benefits depending on the edaphic environments (Vosátka *et al*. 1999; Kelly *et al*. 2005). In the case of acid soils and / or soils with high Al levels, there is variation amongst AM fungi

ecotypes to acid conditions exhibited by differences in spore germination, hyphal growth rate and root colonization percentage (Klugh and Cumming, 2007). In addition, an early colonization can be an important factor in Al tolerance and, consequently, to be beneficial against Al toxicity effects (Seguel *et al*. 2012).

Additionally, recent studies show that Glomalin-Related Soil Protein (GRSP), a glycoprotein produced by AM fungi and released to the soil in high amounts (Wright and Upadhyaya 1996, 1998; Gadkar and Rillig, 2006), would be able to immobilize large quantities of heavy metals (Gonzalez-Chavez *et al*. 2004; Vodnik *et al.* 2008, Cornejo *et al.* 2008a). Glomalin represents a significant fraction of the soil pool of proteins (such as GRSP) due to its persistence, contributing significantly to the binding of particles and the stability of soil aggregates (Rillig and Mummey, 2006) and to carbon sequestration (Bedini *et al*. 2007). Therefore, this molecule may have the ability to bind Al due to its complexing capacity, as it has been recently evidenced by Aguilera *et al*. (2011). This would represent a very important external mechanism related to AM fungi to take into account in reducing the toxicity of this element. The aim of this study was to assess the role of AM fungi in the Al tolerance of six Al-tolerant cultivars of *Triticum aestivum* L.

3.2. Materials and Methods

3.2.1. Soil, plants and growing conditions

The test soil used was a Gorbea Andisol soil series (medial, mesic, Typic Hapludands) collected at 0 to 20 cm depth. The soil was air dried and sieved through a 5 mm mesh. Then, it was amended or not with lime ($CaCO_3$) at the equivalent to 4 ton lime ha $^{-1}$ and incubated for two weeks. Some characteristics of natural and limed soils are described in Table 3.1. All the analytical techniques were according to the Normalization and Accreditation Commission of the Chilean Soil Science Society (Zagal and Sadzawka, 2007). Each 1 L pot was filled with 800 g of limed or unlimed soil where six Al-tolerant cultivars of *Triticum aestivum* L. (wheat) 'Crac', 'BT-200', 'Invento', 'Otto', 'Bakan' and 'Porfiado' (von Baer, 2007) were sown. Seeds were surface-sterilized with 2% Cloramin-T solution for 3 min and rinsed thoroughly. Fifty seeds per cultivar were

germinated between wet tissue paper and thirty seedlings per cultivar were transplanted 7 days after seed germination. Plants were grown under greenhouse conditions with temperatures ranging from 25 ± 3ºC day to 15 ± 3ºC night, a 16/8 h light/dark photoperiod and a relative humidity of 80–90%. A photosynthetic photon flux density of 400–500 mmol m^{-2} s^{-1} as supplementary light when necessary was applied. The plants were irrigated manually with distilled water as needed during the experiment. Nitrogen (N) was supplied in two portions, at establishment (30% total N) and at 6 wk of cultivation (70% total N) to an equivalent amount of 0.113 g N kg^{-1} soil. The P was supplied with 0.016 g P kg^{-1} soil as NaH_2PO_4 and 0.063 g K kg^{-1} soil as KCl, respectively, both applied as solution. Three harvest stages were considered. The first stage was at tillering (60 days after sowing (DAS)), the second stage was at anthesis (90 DAS) and the last stage was at physiological maturity (150 DAS).

Table 3.1. Selected chemical properties of the soil used.

	Natural soil*	Limed soil 4 ton lime ha^{-1}*
Available P, mg kg^{-1}	17.33±3.31	24.04±1.98
pH	4.73±0.92	5.32±1.16
Organic matter, %	9.22±1.25	8.14±1.88
K, $cmol_{(+)}$ kg^{-1}	0.21±0.02	0.19±0.01
Na, $cmol_{(+)}$ kg^{-1}	0.04±0.01	0.04±0.02
Ca, $cmol_{(+)}$ kg^{-1}	0.29±0.03	4.91±0.03
Mg, $cmol_{(+)}$ kg^{-1}	0.07±0.01	0.21±0.01
Al, $cmol_{(+)}$ kg^{-1}	2.39±0.51	0.40±0.02
ECEC, $cmol_{(+)}$ kg^{-1}	3.00±0.72	5.75± 0.98
Al sat, %	79.66±9.88	6.96±1.25
Bases sat, $cmol_{(+)}$ kg^{-1}	0.61±0.01	5.35±1.44

*Means followed by standard error (n=5)

[a]Extractable by Olsen method
[b]Measured in H_2O
[c]Walkley and Black method
[d]Extracted by 1M ammonium acetate
[e]Extracted by 1M potassium chloride
[f]Effective cation exchange capacity

3.2.2. Measurements

At all three harvest stages, root samples were collected from pots, gently washed under tap water and stained with trypan blue after boiling in 10% KOH following the method of Phillips and Hayman (1970). The mycorrhizal colonization was determined by the grid-line intersect method (Giovannetti and Mosse, 1980). Total and colonized root length was calculated by Tennant's gridline intersect method (1975). Relative growth rate was determined by Gardner *et al*. (1985) from total root length. Other fungal parameters such as spores number, total hyphal length and glomalin were determined before sowing and at final harvest (150 DAS). Arbuscular mycorrhizal spores were determined by Sieverding (1991), total hyphal length was measured according to Rubio *et al*. (2003) and quantified by the grid-line intersection method (Giovannetti and Mosse 1980) and total GRSP was determined according to the method described by Wright and Upadhyaya (1998) with minor modifications. To determine GRSP-bound Al (GRSP-Al) total GRSP was precipitated by slow addition of 2 M HCl up to pH 2.0, centrifuged at 8000 g for 20 min, redissolved in 0.5 M NaOH, dialyzed against deionized H_2O and freeze-dried. Dried GRSP was mineralized by acid-digested in H_2O/HCl/HNO_3 (8/1/1 v/v/v) and Al was determined by atomic absorption spectrophotometer (AAS, Perkin-Elmer 3110). The tissue samples obtained were crushed, ground, ashed in a furnace at 550ºC and digested using a H_2O/HCl/HNO_3 mixture (8/1/1 v/v/v). After digestion treatment, P in plant tissues was determined colorimetrically using the vanado-molybdate method and Al, Ca and Mg concentration were determined by AAS as above.

3.2.3. Data analysis

The design was fully factorial, with six Al tolerant wheat cultivars, two Al saturation and five replicates in each combination. Data were analyzed using analyses of variance (ANOVA) followed by Tukey-Kramer's LSD to identify significant differences among treatment means. All the data sets obtained were subjected to principal component analysis (PCA) and the correlation among the different variables and the principal components (PC) obtained were analyzed using the Pearson correlation coefficient. All statistical analyses were carried out using SPSS software v. 10.0 (SPSS, Inc., Chicago, Il.).

3.3. Results

3.3.1. Response of the wheat cultivars

The results showed an inhibitory effect of Al on root growth and a great response to lime was observed in all wheat cultivars. At 60 DAS, the total root length of plants grown under natural soil (NS), with high Al saturation (80%), was higher in 'Porfiado' and 'BT-200' (0.67 and 0.58 m plant $^{-1}$ respectively) showing significant difference ($p<0.001$) between wheat cultivars. However, some wheat cultivars as 'BT-200', 'Invento', 'Otto' and 'Bakan' did not show a total root length increment over time. The relative grown rate (RGR) of roots was greater in 'Crac' and 'Porfiado' (5.32 and 3.78 mm plant^{-1} day^{-1} respectively) under natural soil (NS). In limed soil (LS) the higher RGR was in 'Invento' and 'Otto' (7.79 and 6.41 mm plant^{-1} day^{-1} respectively) presenting significant differences with others wheat cultivars ($p<0.05$) (Table 3.2).

In general, arbuscular mycorrhizal colonized root length was increased across time in all wheat cultivars from 60 to 150 DAS. In plants grown under 80% Al-saturation the higher AM colonized root length was in 'Porfiado' reaching 0.38 m plant $^{-1}$ and the AM colonization ranged between 30 (60 DAS) and 60% (150 DAS). On the other hand, 'Invento', 'Bakan' and 'Porfiado' showed the highest root colonization in limed soil at 150 DAS reaching 52, 53 and 57%, respectively (calculated from Table 3.2).

3.3.2. Arbuscular mycorrhizal parameters

The soil originally had 106 spores gss^{-1} and such level increased at the two Al saturations levels at 150 DAS in all wheat cultivars. Moreover, soils with high Al levels produced greater AM spores number than the limed soil. In addition, 'Crac' showed the highest number of spores in natural soil, which was significantly different in relation to limed soil. However, 'Porfiado' showed a greater spore number in natural soil resulting on an average increase of nearly 1300% over the limed soil (Figure 3.1). Density of extraradical mycorrhizal hyphae in original soil was 2.1 m g $^{-1}$ and it was increased by lime in almost all wheat cultivars at 150 DAS. In addition, 'Bakan' showed the greater density of extraradical mycorrhizal hyphae in limed soil. At the beginning of the experiment, glomalin was 7.42 mg g^{-1}. Moreover, 'Invento' and 'Porfiado'

Table 3.2. Total root length (m plant^{-1}) and Arbuscular mycorrhizal colonized root length on six Al-tolerant wheat cultivars at three phenological stages and Relative root growth rate from 60 to 150 DAS of plant growing in natural and limed soil.

	Wheat	**Total root length (m plant $^{-1}$)**			**AM colonized root length (m plant^{-1})**			**Relative root growth rate (mm plant $^{-1}$ day^{-1})**
Soil	Cultivars	60 DAS	90 DAS	150 DAS	60 DAS	90 DAS	150 DAS	60 to 150 DAS
Natural soil	'Crac	0.49±0.06^{cd}	0.41±0.03^{c}	0.77±0.11^{bc}	0.17±0.02^{bcd}	0.15±0.01^{bc}	0.31±0.01^{cd}	5.32±1.16^{abc}
	BT-200	0.58±0.23^{cd}	0.59±0.03^{bc}	0.58±0.07^{bc}	0.21±0.09^{bcd}	0.23±0.02^{abc}	0.23±0.03^{cd}	1.39±0.55^{bcd}
	Invento	0.46±0.03^{d}	0.39±0.06^{c}	0.48±0.07^{c}	0.14±0.006^{cd}	0.12±0.03^{c}	0.20±0.02^{cd}	0.29±0.11^{d}
	Otto	0.39±0.02^{d}	0.54±0.06^{bc}	0.42±0.08^{c}	0.14±0.01^{d}	0.20±0.13^{bc}	0.16±0.08^{d}	0.11±0.06^{d}
	Bakan	0.61±0.10^{d}	0.76±0.07^{bc}	0.61±0.07^{bc}	0.15±0.005^{d}	0.24±0.06^{abc}	0.24±0.09^{cd}	1.01±0.61^{cd}
	Porfiado	0.67±0.09^{bcd}	0.76±0.06^{bc}	0.86±0.08^{bc}	0.20±0.03^{bcd}	0.24±0.03^{abc}	0.38±0.005^{cd}	3.78±1.13^{abcd}
Limed soil	Crac	1.17±0.12^{ab}	1.47±0.11^{a}	1.96±0.19^{a}	0.36±0.07^{b}	0.43±0.05^{a}	1.01±0.08^{ab}	5.74±0.85^{ab}
	BT-200	0.94±0.16^{bcd}	0.87±0.06^{bc}	1.32±0.16^{abc}	0.30±0.09^{bcd}	0.32±0.03^{ab}	0.52±0.05^{bcd}	4.12±1.18^{abcd}
	Invento	0.72±0.07^{bcd}	1.19±0.05^{ab}	1.47±0.13^{abc}	0.27±0.13^{bcd}	0.46±0.01^{ab}	0.77±0.07^{bc}	7.79±1.20^{a}
	Otto	0.79±0.06^{bcd}	0.78±0.12^{bc}	1.40±0.09^{ab}	0.37±0.05^{bc}	0.35±0.01^{ab}	0.68±0.03^{bc}	6.41±1.20^{a}
	Bakan	1.21±0.12^{bc}	1.04±0.13^{ab}	1.80±0.21^{ab}	0.45±0.02^{ab}	0.40±0.01^{a}	0.94±0.16^{ab}	4.11±1.14^{abcd}
	Porfiado	1.80±0.18^{a}	1.47±0.15^{a}	2.17±0.24^{a}	0.61±0.04^{a}	0.56±0.09^{a}	1.20±0.10^{a}	4.45±0.70^{abcd}
ANOVA								
F lime		66.08***	101.78***	156.39***	62.66***	86.21***	101.19***	42.34***
F cultivars		7.63***	6.36***	6.95***	4.56**	5.17***	23.01***	2.86*
F lime x cultivars		3.28*	6.41***	1.62ns	3.67**	2.80*	21.31***	4.98***

Means (±S.E) followed by different letter in a column are significantly different from each other by to orthogonal contrasts test ($p < 0.05$; n = 5). Significance conventions: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

had a higher glycoprotein production (10.75 and 11.98 mg g^{-1} respectively) in natural soil and no differences were observed between treatments with and without lime in all wheat cultivars (Figure 3.1).

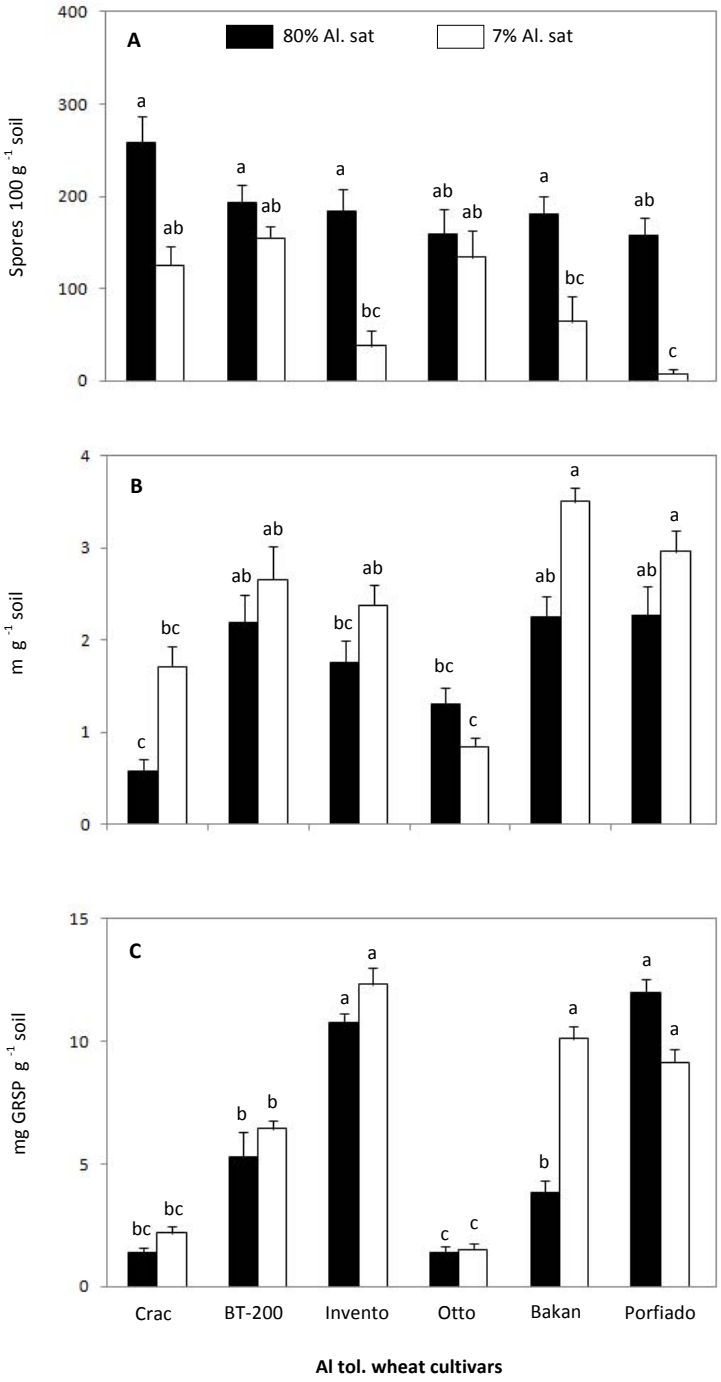

Figure 3.1. Spores number in 100 g of dry soil (A), density of extraradical mycorrhizal hyphae (B) and total glomalin-related soil protein (T-GRSP) on six Al-tolerant wheat cultivars at 150 DAS growing in natural and limed soil. Bars denote mean ±S.E. (n = 5).

3.3.3. Effect of glomalin as an external mechanism of Al tolerance

Aluminum bound to glomalin (Al-GRSP) ranged from 3.5 to 7% and was higher in natural soil with great Al saturation in all wheat cultivars. It was decreased significantly in limed soil where were grown 'Crac', 'Otto' and 'Porfiado' (27, 44 and 33% respectively) over natural soil (Figure 3.2). Three of the six wheat cultivars tested ('BT-200', 'Invento', 'Bakan') did not show significantly differences between natural and limed soil. In addition, Al-GRSP quantified from limed soil was also important. The iron bound to GRSP (Fe-GRSP) was significant lesser than Al-GRSP and followed a similar trend at the natural and limed soil, but a significant increase at the soil with high Al saturation was observed in 'Porfiado' where Al and Fe represented the same percentage (about 7% each one).

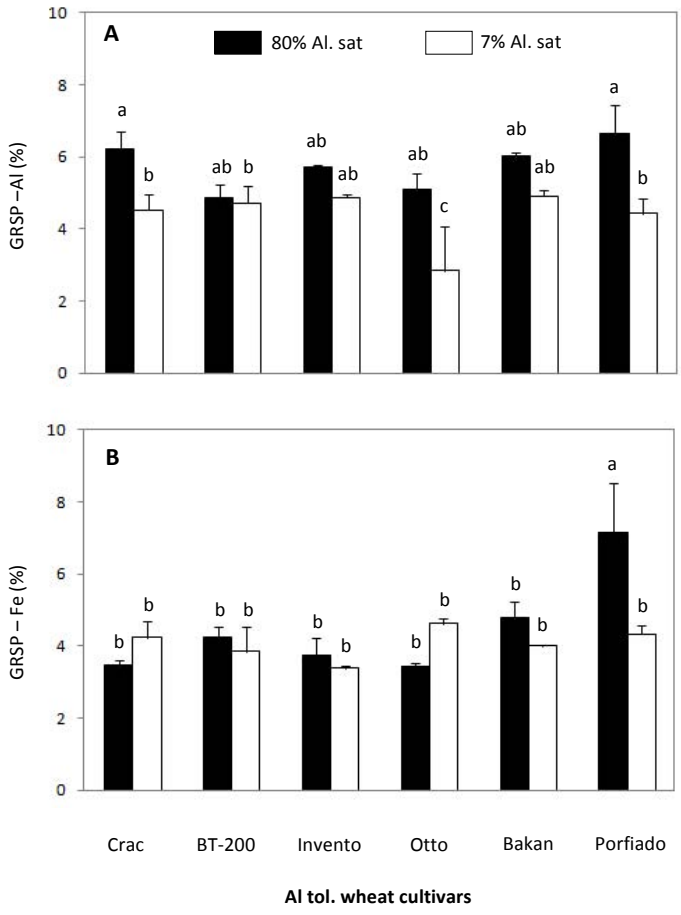

Figure 3.2. Glomalin-related soil protein (GRSP) bound Al (GRSP-Al) (A) and GRSP-Fe (%) (B) on six Al-tolerant wheat cultivars at 150 DAS growing in natural and limed soil. Bars denote mean ±S.E. (n = 5).

3.3.4. Nutritional status of wheat cultivars as an indirect mechanism of Al tolerance

Aluminum concentration was higher in roots than in shoots. Moreover, shoots of all wheat cultivars had a significantly higher (p<0.001) Al concentration in plants grown at higher Al saturation compared with those from limed soil. In addition, 'Porfiado' and 'Crac' exhibited lower Al accumulation in shoot (0.08 and 0.13 mg g^{-1} respectively) and 'Crac and 'Invento' showed lesser Al accumulation in root (9.95 and 13.06 mg g^{-1} respectively) when the plants were grown in soil under 80% Al-saturation (Table 3.3). Calcium and Mg concentrations were increased by lime in all cultivars. In addition, 'Invento' and 'Porfiado' showed greater Ca concentration in root (5.08 and 4.45 mg g^{-1} respectively) and significance differences with the others cultivars ($p<0.001$) in NS. On other hand, 'Otto', 'Bakan' and 'Porfiado' exhibited higher Ca concentration in shoots than 'Crac', 'BT-200' and 'Invento', but their differences were not significant. Additionally, 'Porfiado' had greater root Mg concentration (1.88 mg g^{-1}) in NS showing significance differences with the other wheat cultivars. On other hand, liming produced an increase in P concentration in shoots and roots, but in some cultivars this enhancement was not significant. Additionally, 'Crac' presented the higher phosphorus (P) concentration in shoot and showed significant differences with the other wheat cultivars ($p<0.01$). The P concentration was increased by lime application and that increase was more significant in shoot than in root and 'Porfiado' presented the highest P root concentration (3.48 mg g^{-1}) (Table 3.3)

In this study, a negative relationship was obtained between total root length and AM fungi spores (r = -0.29; p<0.01) in limed soil. In addition, Al accumulated in shoot had a good and negative relationships with the colonized root length in limed soil (r=-0.49; p<0.001) and natural soil (r=-0.42; p<0.001). Moreover, the Al bound to GRSP was negatively correlated with Al accumulation in root in the soil with high Al saturation (r=-0.57; p<0.001) and limed soil (r=-0.33; p<0.001). Also, the relationship between Al-GRSP and Al translocated to shoot presented a negative relationship in NS (r=-0.37; p<0.001) and that relation was incremented in LS (r=-0.56; p<0.001) (Figure 3.3)

Table 3.3. Macronutrient and Aluminum concentration (mg g^{-1}) of shoot and root tissue of six Al-tolerant wheat cultivars at 150 DAS.

Soil	Wheat Cultivars	P		Ca		Mg		Al	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Natural soil	Crac	2.38 ± 0.26^{ab}	1.18 ± 0.21^{c}	3.65 ± 0.60^{bc}	3.85 ± 0.83^{abc}	1.82 ± 0.15^{a}	0.96 ± 0.10^{cd}	0.13 ± 0.03^{bcd}	9.95 ± 1.21^{bc}
	BT-200	1.49 ± 0.45^{b}	0.66 ± 0.05^{c}	2.08 ± 0.21^{c}	2.35 ± 1.07^{c}	1.32 ± 0.41^{a}	0.47 ± 0.02^{d}	0.30 ± 0.06^{a}	18.17 ± 2.02^{a}
	Invento	2.57 ± 0.49^{ab}	1.53 ± 0.15^{bc}	4.85 ± 0.48^{abc}	5.08 ± 0.09^{ab}	1.65 ± 0.19^{a}	1.32 ± 0.19^{bc}	0.26 ± 0.05^{ab}	13.06 ± 1.52^{b}
	Otto	1.21 ± 0.32^{b}	2.04 ± 0.08^{ab}	5.74 ± 0.95^{abc}	3.51 ± 0.07^{bc}	2.56 ± 0.36^{a}	1.10 ± 0.02^{bcd}	0.28 ± 0.04^{a}	19.45 ± 0.77^{a}
	Bakan	1.78 ± 0.18^{b}	1.58 ± 0.48^{bc}	5.71 ± 0.65^{abc}	2.82 ± 0.74^{bc}	1.96 ± 0.32^{a}	0.67 ± 0.09^{c}	0.19 ± 0.06^{abc}	14.76 ± 1.23^{ab}
	Porfiado	1.83 ± 0.19^{b}	3.48 ± 0.56^{a}	5.13 ± 0.39^{abc}	4.45 ± 0.29^{abc}	2.71 ± 0.36^{a}	1.88 ± 0.27^{ab}	0.08 ± 0.02^{cd}	13.22 ± 2.67^{b}
Limed soil	Crac	3.39 ± 0.53^{a}	1.39 ± 0.37^{bc}	5.90 ± 0.82^{abc}	6.46 ± 0.72^{a}	2.81 ± 0.63^{a}	1.43 ± 0.16^{abc}	0.02 ± 0.005^{d}	3.70 ± 0.21^{c}
	BT-200	1.79 ± 0.67^{b}	1.24 ± 0.09^{bc}	3.63 ± 1.50^{bc}	4.23 ± 0.21^{abc}	1.28 ± 0.56^{a}	1.11 ± 0.18^{bc}	0.03 ± 0.008^{d}	5.94 ± 0.85^{c}
	Invento	2.55 ± 0.46^{ab}	1.17 ± 0.24^{c}	6.21 ± 1.09^{abc}	6.01 ± 0.59^{a}	3.36 ± 0.85^{a}	1.78 ± 0.03^{ab}	0.01 ± 0.003^{d}	10.04 ± 2.78^{bc}
	Otto	2.02 ± 0.29^{ab}	2.29 ± 0.14^{ab}	7.56 ± 1.66^{ab}	6.22 ± 1.91^{a}	2.92 ± 0.56^{a}	1.93 ± 0.08^{ab}	0.04 ± 0.006^{d}	13.36 ± 0.89^{b}
	Bakan	2.69 ± 0.33^{ab}	1.63 ± 0.36^{bc}	7.85 ± 0.64^{a}	3.41 ± 0.20^{bc}	2.60 ± 0.38^{a}	0.96 ± 0.18^{cd}	0.01 ± 0.004^{d}	10.42 ± 2.62^{ab}
	Porfiado	2.43 ± 0.20^{ab}	2.74 ± 0.34^{a}	8.54 ± 0.79^{a}	5.47 ± 0.46^{ab}	3.29 ± 0.59^{a}	2.07 ± 0.07^{a}	0.01 ± 0.004^{d}	8.09 ± 1.09^{c}
ANOVA									
F lime		9.28**	3.03*	15.70***	42.06***	5.08*	113.46***	116.01***	128.52***
F cultivars		4.19**	43.97***	6.38***	10.92***	2.69*	71.19***	5.37***	22.01***
F lime x cultivars		1.00ns	4.02**	0.34ns	2.11ns	1.09ns	4.50**	3.65**	5.81***

Means (±S.E) followed by different letter in a column are significantly different from each other by to orthogonal contrasts test ($p < 0.05$; n = 5). Significance conventions: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

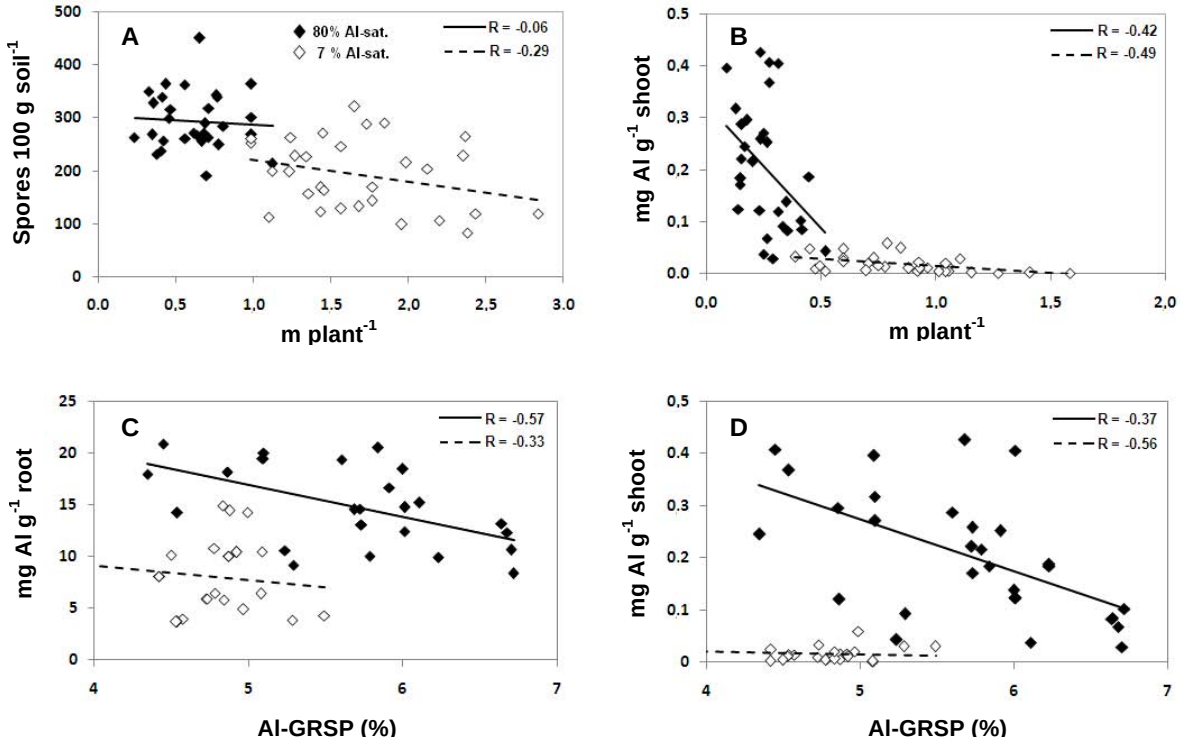

Figure 3.3. Relationships between responses of six Al tolerant wheat cultivars exposed to two Al saturation levels with respect to: A) AM spores and total root length; B) Al shoot and colonized root length ; C) Al root accumulation and Al bound to GRSP and D) Al shoot accumulation and Al bound to GRSP. The average response of each wheat cultivar is shown (n=5).

A general correlation analyzes showed that AM spores number was negatively correlated with root colonized length (r = -0.66; p<0.001) and GRSP was positively correlated with total hyphal length (r = 0.52; p<0.001). Other correlations are shown in a general matrix in Table 3.4. On the other hand, the principal components 1, 2 and 3 accounted for 59% of the total experimental variance (34.9, 13.2 and 11.2% for PC1, PC2 and PC3 respectively) (Figure 3.4). PC1 and PC2 showed a high correlation with all the variables studied (Table 3.4). Moreover, wheat cultivars in unlimed and limed soil formed 2 homogeneous groups and within the group, some cultivars were clearly differentiated by their distance. In limed soil, 'Porfiado', 'Invento' and 'Otto' formed a group, while, BT-200, formed another one. Moreover, in natural soil 'Porfiado', 'Bakan' and 'Invento' formed a homogeneous group with great distance from the group formed by 'BT-200' under similar soil conditions (Figure 3.4).

Table 3.4. Correlation matrix of some selected variables studied and the principal components (PC) obtained

	Root length	Root col.lenght	Spores	Hyphae	GRSP	Al-GRSP	Fe-GRSP	Al shoot	Al root	P shoot	P root
Root length [a]											
Root col.lenght [b]	0.95***										
Spores [c]	-0.65***	-0.66***									
Hyphae [d]	0.01ns	-0.02ns	-0.09ns								
GRSP [e]	-0.08ns	-0.01ns	0.03ns	0.52**							
Al-GRSP [f]	-0.26*	-0.25*	0.42***	0.10ns	0.30**						
Fe-GRSP [g]	-0.01ns	-0.01ns	0.23*	0.09ns	0.26*	0.50***					
Al shoot [h]	-0.67***	-0.64***	0.52***	-0.12ns	-0.06ns	0.21ns	-0.04ns				
Al root [i]	-0.62***	-0.60***	0.26*	0.04ns	-0.03ns	-0.09ns	-0.18ns	0.62***			
P shoot [j]	0.46***	0.47***	-0.32**	-0.04ns	-0.10ns	-0.16ns	-0.18ns	-0.42***	-0,39***		
P root [k]	0.26*	0.25*	-0.36**	-0.08ns	0.07ns	-0.05ns	0.28*	-0.22ns	0.04ns	0.04ns	
Al/Ca shoot [l]	-0.56***	-0.54***	0.44***	-0.02ns	-0.02ns	0.16ns	-0.04ns	0.78***	0.46***	-0.43***	-0.32**
PC1 [m]	-0.82***	-0.81***	0.69***	0.06ns	0.01ns	0.31**	0.03ns	0.83***	0.69***	-0.56***	-0.39***
PC2 [n]	-0.10ns	-0.04ns	0.10ns	0.26*	0.60***	0.47***	0.63***	0.03ns	0.13ns	-0.21ns	0.49***
PC3 [o]	0.11ns	0.16ns	-0.47***	0.03ns	-0.13ns	-0.57***	-0.41***	0.17ns	0.52***	-0.07ns	0.19ns

Pearson correlation coefficients (r) were calculated from five replicates of each sampling situation (n=5). Significance conventions: ns = not significant; *p<0.05; **p<0.01; ***p<0.001

[a] Total root length (m plant^{-1})
[b] Colonized root length (m plant^{-1})
[c] Spores number (spores 100 g dry soil^{-1})
[d] Total hyphal length (m g^{-1})
[e] Glomalin- related soil protein (mg g^{-1})
[f] Glomalin-related soil protein bound Al (%)
[g] Glomalin-related soil protein bound Fe (%)
[h] Shoot aluminium concentration (mg g shoot^{-1})
[i] Root aluminium concentration (mg g shoot^{-1})
[j] Shoot phosphorus concentration (mg g shoot^{-1})
[k] Root phosphorus concentration (mg g shoot^{-1})
[l] Al Ca^{-1} shoot rate
[m] Principal component 1
[n] Principal component 2
[o] Principal component 3

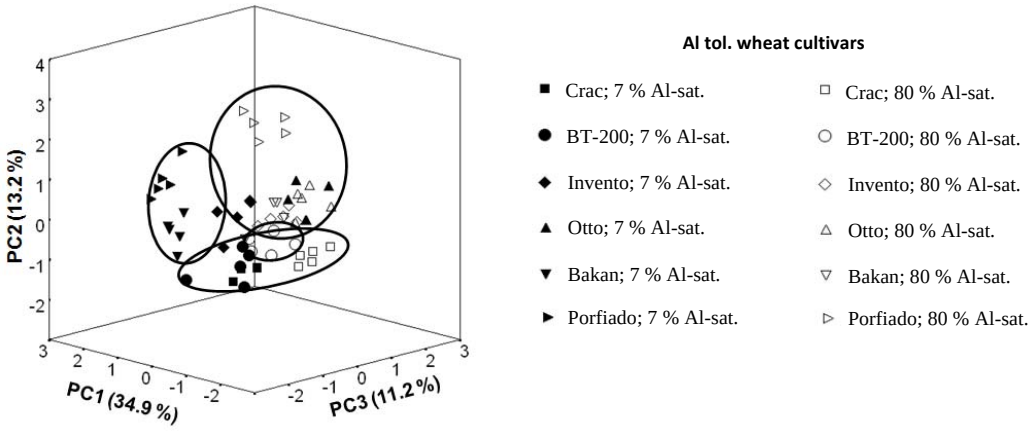

Figure 3.4. PCA scores on six Al tolerant wheat cultivars at 150 DAS growing in natural and limed soil. Five replicates of each sampling situation. Percentage values in parenthesis indicate the variation explained by each PC. The color comprise individuals of similar characteristics according to the cluster analysis, and should be understood as a visual aid for the discrimination of groups.

3.4. Discussion

Despite the application of lime, the soil showed a rather high Al saturation in both treatments. As these wheat cultivars have been produced by masive breeding programs in acid soils (von Baer, 2007), they tolerated such toxic soil conditions. In relation to biomass production, a clear effect on the plant growth and development was observed under lime application in all wheat cultivars. This is mainly due to the growth and health status of plants growing in acid soils that are also strongly responsive to base cation amendments (Long *et al*. 1997; Moore *et al*. 2000).

The primary effect of Al toxicity is a retarded root growth (Rengel, 1996), root biomass of wheat cultivars growing at high Al saturation level decreases, directly affected by the high presence of phytotoxic Al especially in root apical regions producing changes in the cellular

pattern that induces the inhibition of root elongation (Doncheva *et al*. 2005; Jones *et al*. 2006). However, that decreased biomass was not observed in all wheat cultivars. In this sense, Ciamporavá (2002) reported that Al presence produces alterations of root morphology including root thickening. For that reason, in this study, the plant development under Al stress and the relative growth rate (RGR) were determined by total root length in presence of AM symbiosis. In addition, the relative root growth rate showed that 'Crac' and 'Porfiado' were less affected by lime over time suggesting they are the cultivars with the highest Al tolerance. On other hand, 'BT-200' and 'Invento' cultivars had a lesser response to lime application, and their RGR were affected in a higher degree by high Al saturation (Table 3.2). In this sense, Borie and Rubio (1999) showed that non-mycorrhizal plants of Al tolerant barley cultivars were lees affected by lime treatments than mycorrhizal plants, but in this study that response was not observed because lime application was over natural soil, with the presence of native fungal propagules.

In this experiment, greater AM root colonization and root length were observed at 60 DAS in all cultivars and treatments. In addition, at 150 DAS some cultivars showed greater colonized root length under high Al saturation and limed soil as in 'Crac' and 'Porfiado'. This can be due to an increased Al tolerance across time by some cultivars better adaptated to such conditions, or by the effect of different AM fungal species colonizing the plants. In this sense, Klugh and Cumming (2007) reported different AM colonization in the same host (*Liriodendron tulipifera*) when in was colonized by different AM fungal species. Other AM fungal species presented lesser capacity to colonize *L. tulipifera* roots, and plant responses to Al exposure did not significantly differ from non-mycorrhizal ones. In general, mycorrhizal colonization in all cultivars was almost the same than those reported similar to the one found in other studies using the same wheat cultivars at low Al saturation (Cornejo *et al*. 2008b; Rubio *et al*. 2003; Valarini *et al*. 2009). A study conducted by Borie and Rubio (1999), with and without lime in the same soil (Gorbea series) with Al tolerant and Al sensitive barley cultivars showed similar settlement in the host Al tolerant and higher colonization rate in limed soil in sensitive cultivar.

As it was tested for other elements, in the case of Al, the strong negative relationship between the biomass production or total root length and the high Al levels do not affect the colonization and their consequent contribution to the association to tolerate Al phytotoxicity. This was reported by Kelly *et al*. (2005), who showed that AM fungi maintain high colonization levels when roots are exposed to high Al concentration. For this reason, it is not easy to establish degrees of Al tolerance based on the AM colonization of different wheat cultivars, because at 60 DAS almost all cultivars presented a significant rate of AM colonized root. However, the early colonization must be an important factor to establish Al tolerance. In this sense, Seguel *et al*. (2012) reported an early AM colonization in all cultivars assayed when growing at high Al saturation being higher in cultivars apparently more Al-tolerant. Regarding to AM spores, the highest sporulation occurred in the natural soil with highest Al saturation. Whereas, some studies has shown that spores abundance decreases by stress factor (Del Val *et al*. 1999; Ortega-Larrocea, 2001). Borie and Rubio (1999) reported highest spore number at high Al saturation in Al-tolerant barley cultivars compared with Al-sensitive one. This trend could suggest to be a plant response to such environmental stress. In addition, Seguel *et al*. (2012) have observed that high Al saturation increases the presence of AM spores in soil in wheat and barley cultivars. In general, in treatment with higher Al saturation, lesser AM colonized root length and more AM spores were observed.

In the last years, several studies related to role of GRSP in the plant tolerance to metals in the soil have been carried out (González-Chávez *et al*. 2004). Accordingly, it can be stated that AM are able to keep metals out of plants or reduce concentrations into plant tissues (Hildebrandt *et al*. 2007). In this sence, Dudhane *et al*. (2012) informed that GRSP production increased with increasing Al concentration after 45, 75, and 100 days of AM inoculation in Gmelina plants. In general, in this study GRSP production did not show significant differences between Al saturation levels. However, in some cultivars, GRSP bound to Al (Al-GRSP) was significantly higher in natural soil. This may be suggesting mechanisms of Al tolerance related with the GRSP binding capacity of Al, as has been reported for other metals such as Cu, Cd, Pb and Zn (Vodnik *et al*. 2008; Miransari, 2010). On other hand, Fe-GRSP

was lower than Al-GRSP in a similar way that Etcheverría (2009) who reported in acid Andisols from four native forests of southern Chile (6% vs. 4.7%). Moreover, carbon (C) and nitrogen (N) content into GRSP were about 15 ± 1.9 % and 1.8 ± 0.8 %, respectability, in all wheat cultivars and both Al saturations levels. In addition, C associated to GRSP (GRSP-C) represented between a 3 and 6 % of total C in the soil. These results are concomitant with some studies that have shown that GRSP-C ranged 3 and 8% of total C (Rillig *et al*. 2001; Lovelock *et al*. 2004). However, other studies showed greater C content into GRSP (30-35%) and GRSP-C (12-15%) in a Mollisol and acid Andisols, respectability (Etcheverría, 2009; Curaqueo *et al*. 2010). In a recent study about Al-GRSP Aguilera *et al*. (2011), by using confocal laser scanning microscopy (CLSM), showed direct evidence of GRSP ability to sequester Al in the molecule. They suggest 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, especially where Al phytotoxic is high. These benefits obtained by AM activity could be transient or for longer term according to the residence time of fungal structures in the soil or if the bulk of Al immobilized is through GRSP-Al complex formation. However, evidences indicate that these effects could be prolonged, since GRSP turnover time has been estimated on several years (6-42 years; Rillig *et al*. 2001), and AM spores can survive and germinate for longer periods (Tommerup, 1992; McGee *et al*. 1997).

On other hand, it has been suggested that Al excess competes or inhibits Ca and/or Mg absorption capacity affecting normal plant development (Watanabe and Osaki, 2002; Silva *et al*. 2005). In addition, Ca addition can also increase foliar Mg, K and P concentrations, presumably by the displacement of this element from soil exchange sites or by increasing fine root growth (Long *et al*. 1997; Kobe *et al*. 2002), as ocurred in the soil with lime added (Table 3.1). In this study, an improvement in Ca, Mg and P acquisition was obtained in 'Crac' and 'Porfiado' cultivars and a decrease in Al in roots and Al shoots (Table 3.3). Since some wheat cultivars produced simultaneously decreases in shoot and root Al concentration and increases in Ca, Mg and P levels, the best performance between the different cultivars may be better represented by using the Ca/Al, Mg/Al or P/Al molar ratios. In this sense, Ca/Al relation is

strongly associated with growth and development in a wide variety of plants (Schaberg *et al*. 2006). Thus, Ca/Al molar ratios in soil solution and in plant tissues have been proposed as superior indicators than Al concentration itself for evaluating Al toxicity stress (Cronan and Grigal, 1995). The lower Ca/Al molar ratio in shoot was calculated in 'BT-200' reaching 6.11 and it was significantly different (p<0.01) with other cultivars in NS. Moreover, 'Crac', 'Bakan' and 'Porfiado' showed greater Ca/Al molar ratio in shoot (23.8, 20.0 and 47.4 respectability) than 'BT-200'. On other hand, 'BT-200' showed the same trend in Ca/Al molar ratio in root. However, 'Crac', 'Invento' and 'Porfiado' cultivars reached higher Ca/Al molar ratio (0.28, 0.27 and 0.25 respectability) in NS than other wheat cultivars suggesting a better response of those cultivars to Al toxicity. In addition, the positive effect in Al tolerance by AM fungi can be observed in the negative relationships between plant aspect and AM fungi responses (Figure 3.3). In fact, 'Crac' and 'Invento' showed a strong negative correlation between Al concentration in root and Al-GRSP (r=-0.54; p<0.001 and r= -0.61; p<0.001, respectively). Additionally, 'Crac' and 'Porfiado' exhibited a higher negative correlation between Al concentration in shoot and Al-GRSP (r=-0.38; p<0.01 and r=-0.51; p<0.001, respectively).

Aluminum though many studies that analyze the presence of different genes in the plant that provide tolerance to the wheat, the AM fungi play a very important role in the protection of roots against the toxicity by Al (Marschner, 1995, Lux and Cumming, 2001). It has been proposed that mycorrhizal plants increase their tolerance to high Al levels either by the improvement of nutrient absorption or by the reduction of Al exposure, which may result from the enhanced production of organic acids by AM colonized roots (Klugh-Stewart and Cumming, 2009) or the production of glomalin (reported here) that function to reduce the concentration of phytotoxic Al in the rhizosphere.

3.5. Conclusions

This work showed that the better performance of in some wheat cultivars related with high Al tolerance is concomitant with an enhancement in the plant nutritional status, higher presence of AM propagules and greater Al bound to GRSP. Moreover, among the six wheat cultivars used in this study, 'Crac', 'Invento' and 'Porfiado' showed the lesser responsiveness to lime application in term of vigorous development at three phenological stages when growing in an Andisol. In addition, those wheat cultivars showed higher AM propagules and Al-GRSP. Arbuscular micorrhizal fungi colonization was not inhibited with high levels of Al saturation and the propagules correlated well with shoot and root biomass. Mycorrhizal arbuscular symbiosis may be giving tolerance mechanisms to wheat cultivars through increased sporulation and production of glomalin that could be demonstrating that the presence of AM fungi populations adapted to these conditions are raising the adaptation of plants present in natural ecosystems.

3.6. References

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Chapter 4. Effects of soil aluminum on early arbuscular mycorrhizal colonization of aluminum tolerant wheat and barley cultivars

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Effects of soil aluminum on early arbuscular mycorrhizal colonization of aluminum tolerant wheat and barley cultivars

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Abstract

Aluminum (Al) phytotoxicity in acid soils is an important environmental stress that negatively affects crop production, but arbuscular mycorrhizal (AM) fungi performance would allow plants to better withstand this environmental condition. This study aimed to analyze the effect of soil Al on early AM colonization of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) cultivars. Near-isogenic 'Crac', 'Invento', and 'Porfiado' wheat cultivars and 'Sebastián' and 'Aurora' barley cultivars were sown in pots in an acid soil at three Al saturation levels (60, 34, and 11%). At 20 days after sowing (DAS) 'Crac' presented higher AM colonization (27%) than other cultivars. However, 'Invento' had the fastest colonization at 41 DAS, which was inhibited in short term at lower Al-saturation. Moreover, roots of 'Aurora' were colonized 28 and 51% at 20 and 66 DAS, respectively, and also decreased at lower Al-saturation. In soil with 60% Al-saturation a great spore production was observed at 41 DAS, 'Aurora' had the highest spore density at 66 DAS. At 20 DAS a negative relationship ($r=-0.37$; $p<0.001$) was observed between the early root colonization and root weight. In addition, such relation was stronger ($r=-0.49$; $p<0.001$) when plants were grown at high Al saturation. An early AM colonization was observed in all cultivars essayed when growing at high Al saturation being higher in cultivars apparently more Al tolerant, suggesting that an early AM colonization can be an important factor in Al tolerance for agricultural plants cropped in acid soils.

Key words: Acid soils, arbuscular mycorrhizal propagules, cereal crop, soil aluminum saturation.

4.1. Introduction

Aluminum (Al) phytotoxicity in acid soils represents a major limitation to crop production. For overcoming such constraints, farmers usually apply liming for decreasing Al activity or the use of Al-tolerant cultivars. In this sense, plants differ greatly on their capacity to tolerate diverse chemical species, such as Al; and arbuscular mycorrizal (AM) fungi would play a very important role in the protection of colonized plants against Al toxicity. Arbuscular mycorrhizal symbiosis is an association established between specific soil fungi and host plant roots. The main function of AM is related to the acquisition of nutrients for the plant, such as P, Ca, Mg, NH_4^+, Cu, and Zn (Clark and Zeto, 2000; Jeffries *et al.* 2003; Cardoso *et al.* 2006; Cornejo *et al.* 2008b). In addition, AM is also known for its role such as protective agent of pathogens and enhancing some mechanisms of tolerance to several environmental stresses (Smith and Read, 1997; Finlay, 2008; Smith and Read, 2008). AM association plays an important role in alleviation of abiotic stresses in acid soils, specially with high levels of Al through the interaction Al-P in colonized roots (Marschner, 1995), an improvement of nutrient absorption, especially P, Ca^{2+}, and Mg^{2+}, (Borie and Rubio, 1999; Clark and Zeto, 2000; Lux and Cumming, 2001) all of them antagonistic to Al damage, or even through the Al-sequestration by an enhancement of root organic acid excretion (Klugh and Cumming, 2009) and glomalin production by AM fungal structures (Aguilera *et al*. 2011).

In these conditions, there would a variation among Al tolerant AM fungal ecotypes in relation to others that probably have a lack of adaptations to this type of stress, providing a major ability to cope these conditions through an enhanced germination of spores, hyphal growth and/or root colonization intensity (Klugh and Cumming, 2007). The AM fungal ecotypes differ significantly in their external mycelium and these differences likely contribute to differences in their host root colonization strategies (Hart and Reader, 2005); however, the environmental factors could also affect the AM colonization pattern. In this sense, Klugh and Cumming (2009) reported that *Acaulospora morrowiae* and *Scutellospora heterogama* associated to *Andropogon virginicus* at high Al levels did not show effect on the AM colonization in the short term, which suggests that this element was not able to inhibit

the formation of the AM association and its beneficial effects. Moreover, Goransson *et al.* (2008) showed that AM colonization was more common in soils with high pH and relatively low Al concentrations, and that most of this association is explained by ecosystem biodiversity. On other hand, Nurlaeny (1995) concluded that root colonization of both maize (*Zea mays* L.) and soybean (*Glycine max* [L.] Merr.) by *Glomus intraradices* increased when pH increased from 4.7 to 6.4; Silva *et al.* (1994) showed that AM colonization of wheat was lower at more acid conditions, and Cavallazzi *et al*. (2007) concluded that AM colonization on apple plants was differentially influenced by fungal isolates, being an ecotype of *S. heterogama* the principal root colonizer (62%) at lowest soil pH (4.0), and consequently at the highest Al level. Based on the above, we hypothesized that the different AM colonization pattern observed under Al stress suggest that Al tolerance of AM host plants depends of the adaptability of the fungi to high Al levels and the specificity of the host plant to be colonized by a specific AM fungi ecotype; however, the AM colonization at different plant developing stages has yet not been considered in this analysis, being here only presented results from the early growing stages. For this reason, the aim of this work was to study the early effect of soil Al on AM fungal propagule density and root colonization of wheat and barley cultivars to correlate with plant growth and the overcoming of soil acidity constraints.

4.2. Materials and Methods

We used an acid Andisol Gorbea series (medial, mesic, Typic Hapludands) collected at 0 to 20 cm deep (soil bulk density: 0.8 g cm^{-3}) The soil was air dried, sieved through a 5 mm mesh, amended or not with commercial lime (91% of $CaCO_3$, 5% of $Ca(OH)_2$ and 2% of S and Mg) at the equivalent to 1.25 and 2.50 g kg^{-1} soil, and incubated for 2 wk to obtain three Al-saturation levels corresponding to 60, 34, and 11%, respectively. Some other characteristics of natural and limed soil are described in Table 4.1. Each 1 L pot was filled with 800 g of the natural and limed soils, and seeds of three wheat (*Triticum aestivum* L.) cultivars and two barley (*Hordeum vulgare* L.) were sown. Near-isogenic 'Crac', 'Porfiado', and 'Invento' wheat cultivars, and 'Aurora' and 'Sebastian' barley cultivars were provided by a local breeder (Semillas Baer™). Seeds were surface-sterilized with 2% Cloramin-T

solution for 3 min and rinsed thoroughly. Fifty seeds per cultivar were germinated between wet tissue paper and then 30 seedlings were transplanted 7 d after seed germination. The pots were thinned to one plant after establishment.

Table 4.1. Selected chemical properties of the soil used*

	Natural soil	Limed soil 1.25 g kg^{-1}	Limed soil 2.5 g kg^{-1}
[a]Available P, mg kg^{-1}	17.00	31.00	26.00
[b]pH	4.91	5.06	5.45
[c]Organic matter, %	12.00	9.00	10.00
[d]K $cmol_{(+)}$ kg^{-1}	0.31	0.23	0.24
[d]Na $cmol_{(+)}$ kg^{-1}	0.04	0.03	0.03
[d]Ca, $cmol_{(+)}$ kg^{-1}	0.49	1.42	3.29
[d]Mg, ($cmol_{(+)}$ kg^{-1}	0.03	0.15	0.23
[e]Al $cmol_{(+)}$ kg^{-1}	1.32	0.96	0.45
[f]ECEC $cmol_{(+)}$ kg^{-1}	2.19	2.79	4.24
Al sat, %	60.27	34.41	10.61
Bases sat, $cmol_{(+)}$ kg^{-1}	0.87	1.83	3.79

[a]Extractable by Olsen method
[b]Measured in H_2O
[c]Walkley and Black method
[d]Extracted by 1M ammonium acetate
[e]Extracted by 1M potassium chloride
[f]Effective cation exchange capacity

*All the analytical techniques were according to the Normalization and Accreditation Commission of the Chilean Soil Science Society (Zagal and Sadzawka, 2007).

On other hand, plants were grown under greenhouse conditions at temperature ranging from 25 ± 3 ºC day to 15 ± 3 ºC night, 16:8 h photoperiod, and a relative humidity of 80-90%. A photosynthetic photon flux density of 400-500 mmol m^{-2} s^{-1} as supplementary light was applied when necessary. The plants were irrigated manually with distilled water as needed during the experiment. Nitrogen (N) was supplied in two portions, at establishment (30% total N) and at 6 wk of cultivation (70% total N) to an equivalent amount of 0.113 g N kg^{-1} soil. The P was supplied with 0.016 g P kg^{-1} soil as NaH_2PO_4 and 0.063 g K kg^{-1} soil as KCl, respectively, both applied as solution. In general, nutrient doses were low to avoid inhibit the AM colonization by native propagules (Rubio *et al.* 2003). Three harvest stages were considered. The first stage was three leaves (20 d after sowing –DAS), the second stage was tillering (41 DAS) and the last stage was ear emergence (66 DAS).

The plants were separated into root and shoot and dried at 65 ºC in a forced-air oven for 48 h and then weighed. Before drying, a portion of roots was separated and AM colonization was measured, root samples were gently washed under tap water and stained in trypan blue after boiling in 10% KOH following the Phillips and Hayman's method (1970). The mycorrhizal colonization was determined by the gridline intersect method (Giovannetti and Mosse, 1980). Total and colonized root length was calculated by Tennant's gridline intersect method (Tennant, 1975). Arbuscular mycorrhizal spores were collected from soils by wet sieving and decanting according to the methodology described by Sieverding (1991). The spores were transferred to Petri dishes and counted under stereoscopic microscope at 50X.

The experiment was established as a two way factorial design (three Al saturation levels × five cultivars at three harvest time, with four replicates per treatment (N = 180). Data were analyzed using ANOVA followed by orthogonal contrasts to identify significant differences among treatment means, and the correlation among the different variables obtained were analyzed using the Pearson correlation coefficient (r). All statistical analyses were carried out using SPSS software v. 10.0 (SPSS, Chicago, Illinois, USA).

4.3. Results and Discussion

At 66 DAS all wheat and barley cultivars showed less biomass production at highest soil Al saturation, and a positive effect on plant growth was observed when the soil was limed, decreasing the Al levels. In these conditions, 'Porfiado' wheat and 'Sebastian' barley showed the higher increase by lime application (Figure 4.1). In short term, it was not observed significant lime effect on biomass production, probably due to Al presence produces alterations of root morphology including root thickening (Čiamporová, 2002), usually related to a higher root weight. For that reason, total root length was here used to analyze the Al effect in plant development. In this sense, 'Invento' wheat and 'Aurora' barley, in natural soil (60% Al-saturation), showed the greater total root length in the short term (Figure 4.2), which suggest that these cultivars are the most Al tolerant cultivars.

In a previous experiment, we have observed a greater AM colonization at 60 DAS in several wheat and barley Al tolerant cultivars at high Al saturation levels (unpublished data). For this reason, in this work we studied the early AM colonization, focusing in the wheat and barley cultivars that previously showed marked responses in parameters as plant and fungal growth. In general, AM fungal colonization was not inhibited by Al saturation and an early AM colonization was observed in all wheat and barley cultivars, growing in natural soil, at high

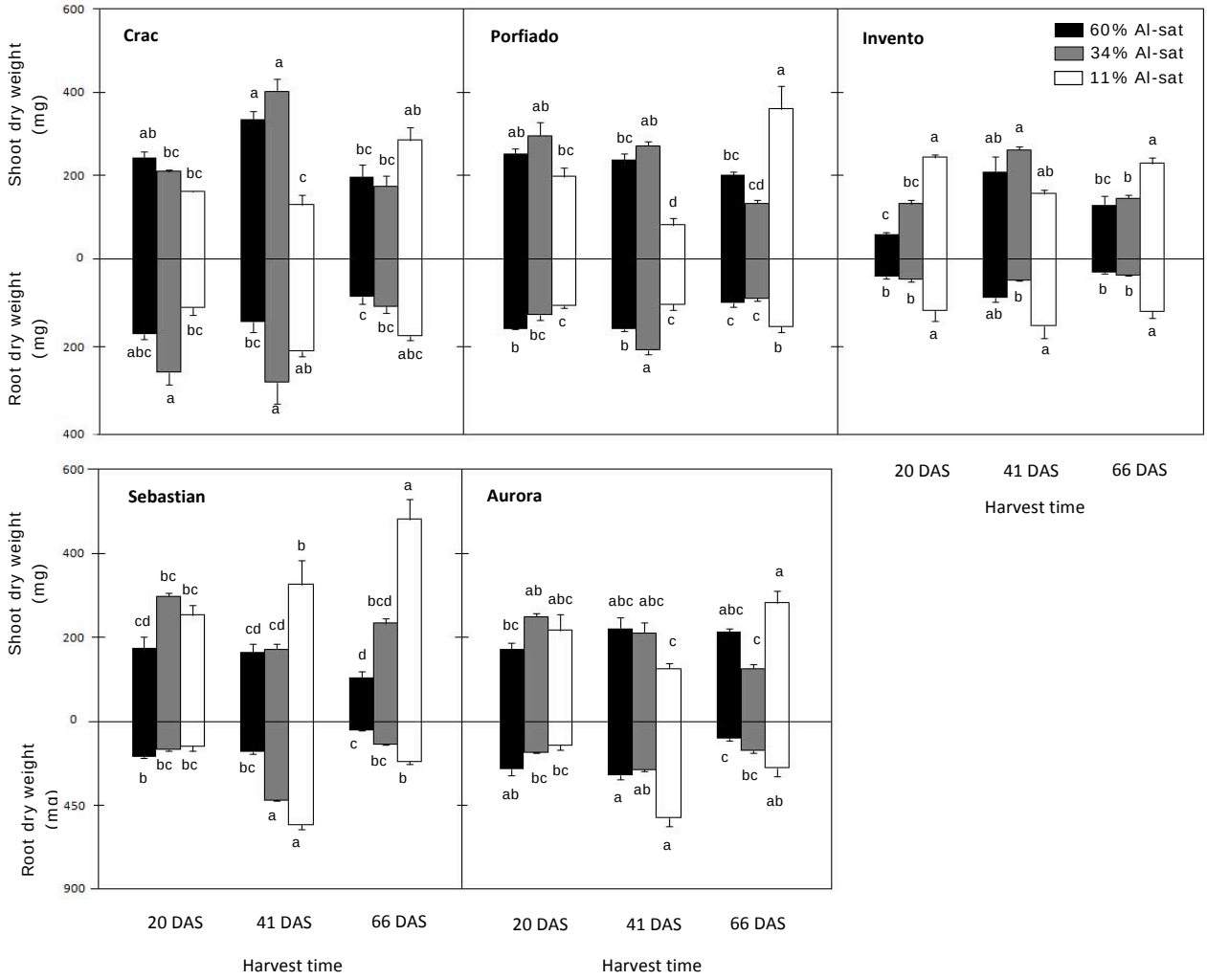

Figure 4.1. Shoot and root biomass production in three wheat (Crac, Porfiado, and Invent) and two barley (Sebastian and Aurora) cultivars at three plant growth stages, under three Al saturation levels. Bars denote means ± SE (n = 4) and different letter for each cultivar represent a mean difference between treatments by orthogonal contrasts test (p<0.05).

Al saturation levels. 'Crac' wheat presented higher AM colonization (27%) at 20 DAS than other wheat cultivars. However, 'Invento' wheat had the fastest colonization at 41 DAS, reaching about 60% and a typical sigmoid colonization function according to Allen (2001). The fast AM colonization in some cultivars is probably due to the presence of more infective fungal structures and the difference in the architecture of the external mycelium (Hart and Reader, 2005). In addition, some studies have shown the close relationship between P uptake at early stages and its final yield (Elliott *et al.* 1997; Snyder *et al.* 2003). Other studies have observed the AM effect of increasing the P uptake when plants grow at high Al levels (Clark, 1997; Siqueira and Moreira, 1997; Borie and Rubio, 1999), suggesting that an early colonization could be an important AM factor in Al tolerance. However, the effect of AM fungi in Al tolerance cannot be regarded as a single consequence of an improved P uptake. Moreover, roots of 'Aurora' barley were colonized in a 28 and 51% at 20 and 66 DAS, respectively; and it was inhibited in lime treatments (Figure 4.3).

Different AM colonization levels in different cultivars can be due to an increased Al tolerance across time by some cultivars that have a better adaptation to these conditions, or by the effect of different AM fungal species colonizing the plants. In this sense, Klugh and Cumming (2007) reported different AM colonization in the same host (*Liriodendron tulipifera*) but colonized by different AM fungal species. In general, AM colonization levels in all cultivars, at 66 DAS, were similar to those reported for same wheat cultivars of this study, but at low Al saturation (Rubio *et al.* 2003; Cornejo *et al.* 2007; 2008a; Valarini *et al.* 2009). However, principal differences in AM colonization by Al presence were observed in the short term in the present study.

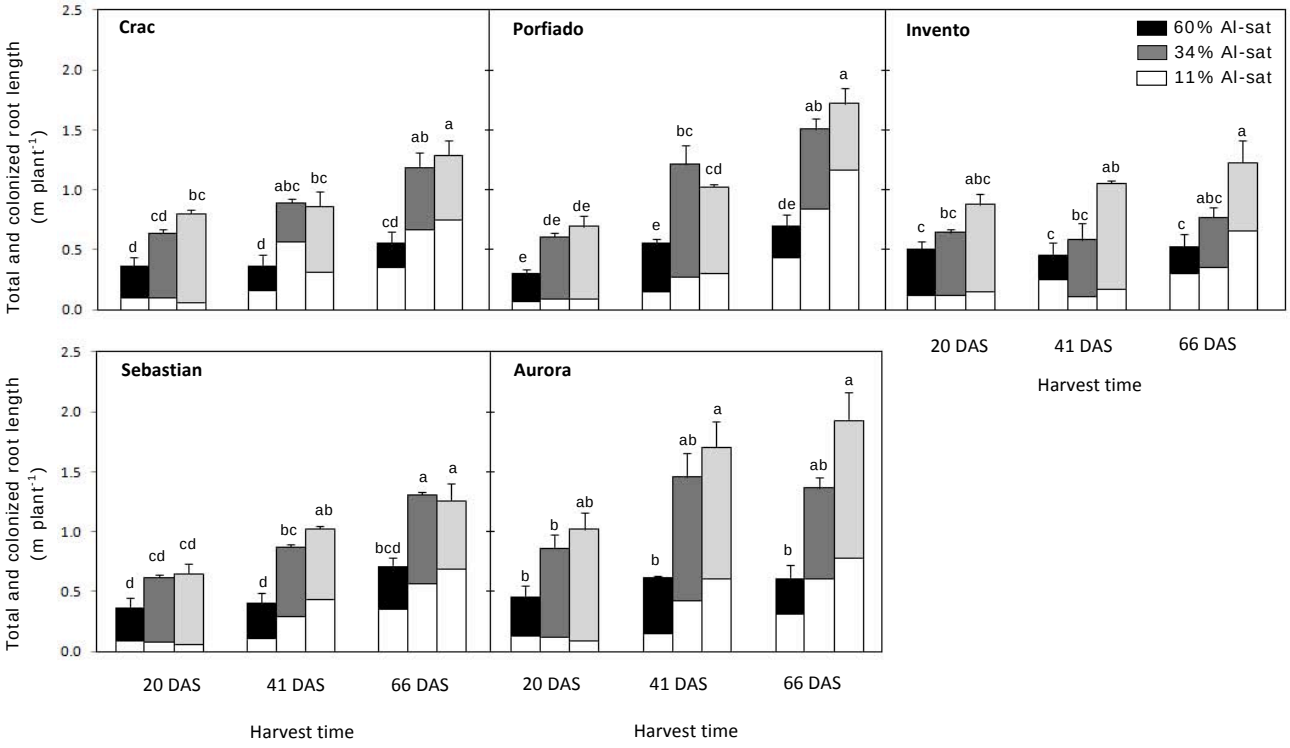

Figure 4.2. Total and colonized root length in three wheat (Crac, Porfiado, and Invent) and two barley (Sebastian and Aurora) cultivars at three plant growth stages, under three Al saturation levels. White bars means colonized root length. Bars for total root length denote means ± SE (n = 4) and different letter for each cultivar represent a mean difference between treatments by orthogonal contrasts test (p<0.05).

In this study, a significant and negative relationship was obtained between AM colonization and root dry biomass at 20 DAS (r=-0.37; p<0.01). This correlation was stronger (r=-0.49; p<0.001) in natural soil at high Al saturation; and lower (r=-0.15; p<0.01) when plants growed at 10% Al saturation. In short term, the relationship between plant growth, expressed as the total root length, and AM colonization was greater (r=-0.64; p<0.001), reinforcing the idea that in the short term the Al stress is highly related with the root thickening.

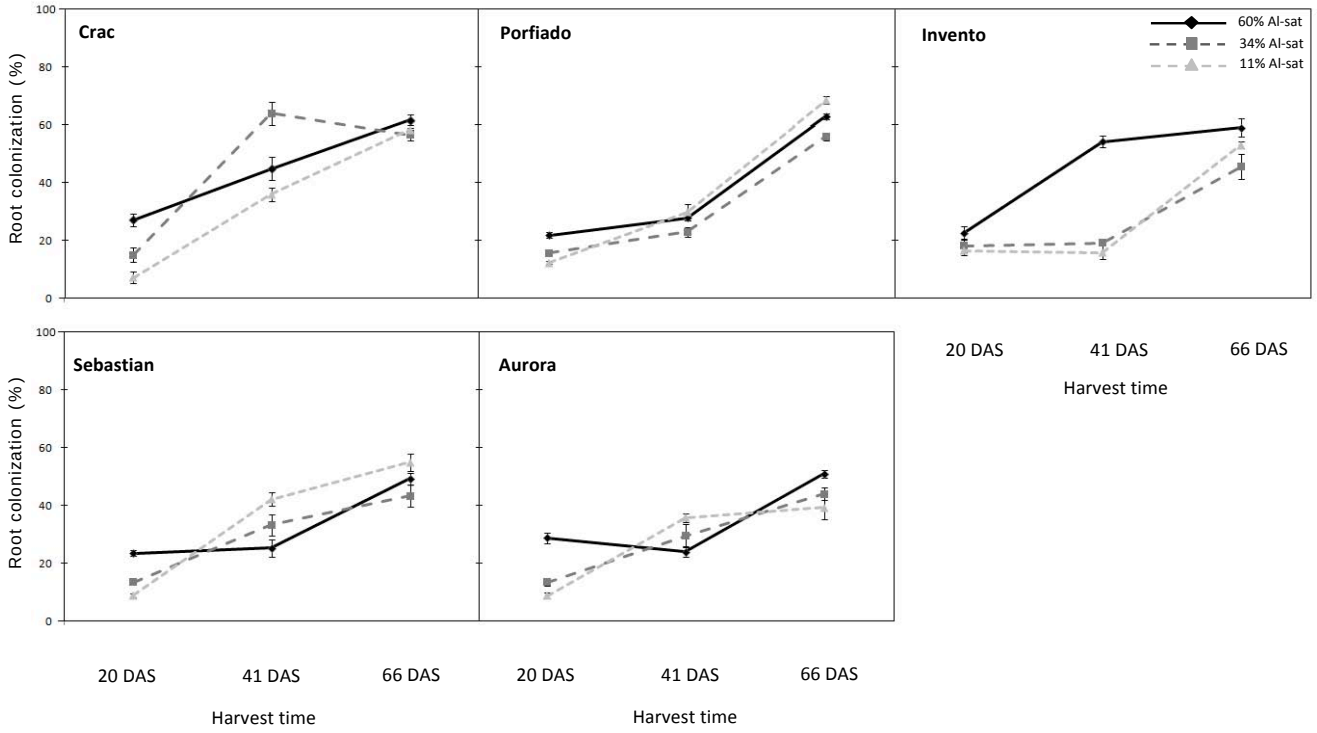

Figure 4.3. Arbuscular mycorrhiza root colonization in three wheat ('Crac', 'Porfiado', and 'Invento') and two barley ('Sebastian' and 'Aurora') cultivars, at three plant growth stages, under three Al saturation levels. Bars denote ± SE (n = 4).

As it was proved for other elements, in the case of Al, the strong negative relationship between the biomass production and/or total root length at high Al levels do not affect the colonization and their consequent contribution to tolerate Al phytotoxicity. Similar results were reported by Kelly *et al*. (2005), who concluded that AM fungi maintain high colonization levels when they are exposed to high Al concentration. Also, it was observed that all cultivars essayed presented the highest root colonization degree at the first growth stage (three leaves) and also at ear emergence stage, suggesting a positive relationship between root mycorrhizal colonization and Al activity in the soil. All cultivars showed an increased spore density when host plants were grown at high Al saturation. In natural soil great spore production was observed at 41 DAS and 'Aurora' barley presented the highest increase of spores with limed soil at 66 DAS (Figure 4.4).

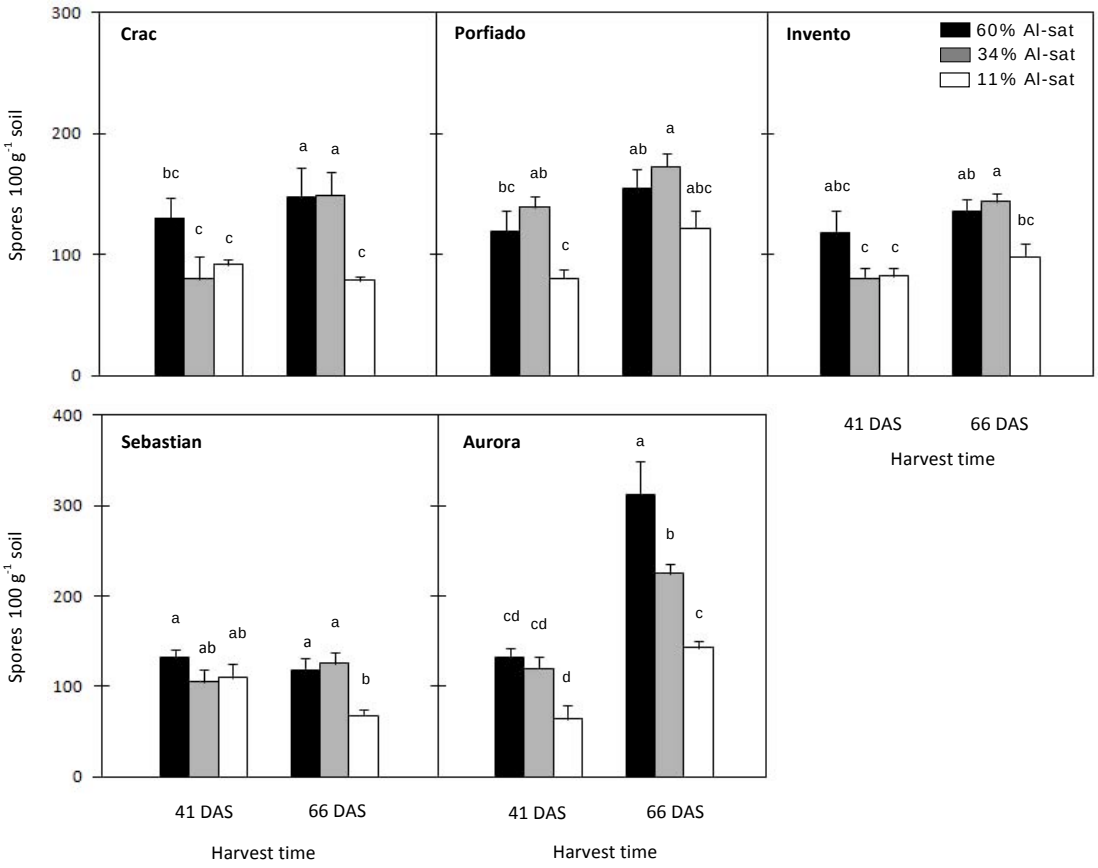

Figure 4.4. Spores number in 100 g of dry soil in three wheat ('Crac', 'Porfiado', and 'Invento') and two barley ('Sebastian' and 'Aurora') cultivars at three plant growth stages, under three Al saturation levels. Bars denote means ± SE (n = 4) and different letter for each cultivar represent a mean difference between treatments by orthogonal contrasts test (p<0.05).

Regarding to AM fungal spores, the highest sporulation occurred in the natural soil with high Al saturation. Whereas some studies have shown that spores abundance is decreased by some stress factors as heavy metals (Del Val *et al.* 1999) or wastewater pollution (Ortega-Larrocea, 2001), other authors (Borie and Rubio, 1999) have observed that AM spores in the rhizosphere of Al-tolerant barley cultivar showed a higher number than the sensitive one and, in turn, the tolerant cultivar showed the greatest spore number in the soil with high Al saturation. This trend has also been observed in soils polluted with other metals as Cu, where the use of Cu-adapted AM fungal inoculum produced a significant increase in the spore

density at the highest pollution levels (Meier *et al.* 2012). Therefore, other aspect to take into account is that one of the results of AM fungal adaptation to environmental stress conditions (as high Al or other metals levels) is the enhanced propagule production, which could ensure root colonization in other plants or in further annual crops. Moreover, spores number also were significant and negatively related (r=-0.25; $p<0.01$) with total root length. This trend could suggest a response to extreme environmental stress level at which it is subjected. In barley, a major spore increase by high Al saturation was observed in both treatments, with and without lime, showing the degree of Al tolerance, principally, in Aurora cultivar. Supported in the recent findings reported by Aguilera *et al*. (2011), it is possible that an early eclosion of AM fungal structures may produce a decrease in the activity of toxic Al.

4.4. Conclusions

This work showed that arbuscular mycorrhizae colonization was not inhibited at high Al saturation levels, suggesting that an early colonization can be an important factor in Al tolerance and, consequently, to be beneficial against Al toxicity effects. In addition, high Al saturation increased the presence of AM propagules in soil. This spores increase together with an early AM root colonization could produce an improved nutritional status of wheat and barley cultivars in soils with high Al levels, representing a feasible indirect mechanism of Al tolerance showed by mycorrhizal plants, which could in part explain the plant Al tolerance; aspect to be considered by farmers in crop cereals under soils with high Al levels.

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Chapter 5. Ecotypic variation in aluminum tolerance of arbuscular mycorrhizal fungi from a re-vegetation gradient on an abandoned coalmine

Article in preparation

Ecotypic variation in aluminum tolerance of arbuscular mycorrhizal fungi from a re-vegetation gradient on an abandoned coalmine

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Abstract

Arbuscular mycorrhizal (AM) fungi can play an important role protecting roots against phytotoxic Al levels. However, it is necessary to know what happens with fungal dynamics in the field between members of the community species. The aim of this report was to elucidate potential mechanisms of Al tolerance operating in some AM fungi ecotypes. A soil-less experiment was carried out with *Andropogon virginicus* inoculated with AM fungi native communities isolated from a vegetation successional gradient in an abandoned acidic mine in West Virginia, USA. The vegetation gradient was dominated by trees, grass swards, the edge of the grass sward and the bare or open soil. Plants were growth for 5 weeks under controlled conditions and shoot and root biomass, root colonization and glomalin, organic acid and total phenol production were determined. In this experiment, twelve AM ecotypes were isolated and identified in the different microsities. According to index of Al tolerance, the plants inoculated with AM fungi from Bare and Tree treatments under Al presence decreased their growth. Mycorrhizal colonization percentages varied among microsite and Al treatments ranging from 43% in edge without Al to 67% in tree treatment with Al. Glomalin and citrate production increased when the plants inoculated were exposed to 100 µM Al. In addition, GRSP production in *A. virginicus* inoculated with AM fungi isolated from sward and tree microsites was higher than other treatments (135 and 153 µg g substrate, respectively). All AM fungal treatments shown higher malate production than non-mycorrhizal plants. An important percentage of *A. morrowiae* and *G. clarum* presence (Al-tol. AM fungi ecotypes), in sward, could explain the higher Al tolerance of *A. virginicus* in this treatment. The data presented here provide evidence that there is a functional variation among AM fungi and that the level of Al tolerance conferred to host plants may vary among AM species.

Key words: Arbuscular Mycorrhiza - Al tolerance – Glomalin – Organic acid production

5.1. Introduction

Soil acidity is produced by many factors that are both natural and human-induced in origin. In fact, human activities produce an important change on the pH of the soil through industrial emissions of sulfur dioxide (SO_2) and nitrogen oxides (NO_x) that generate acid rain (Driscoll *et al*. 2001). In addition, mining generates conditions that produce acidity in the soil/surface substrate. Therefore, coal surface mining has had a significant impact on the landscape of the Appalachian region of the United States (Holl and Cairns, 1994). In West Virginia alone, hundreds of thousands of acres are affected each year (Klugh, 2006). While the Surface Mining Control and Reclamation Act of 1977 minimizes the impact of mining on the environment, mines not reclaimed before 1977 are exempt and continue to impact on the landscape. Unreclaimed sites differ considerably from neighboring areas. The soils and vegetation often have different composition from that which existed before mining activity. Soils on unreclaimed sites in the Appalachian region are often highly acidic and have high aluminum (Al) and low nutrient concentrations (Klugh, 2006). Plant growth on such sites, therefore, often is severely constrained. Most Al containing minerals exhibit pH-dependent solubility and the Al ion exhibits pH-dependent speciation that contributes to the acid soil problem. The solubilization of Al is related to the degree of soil acidification caused by the reasons above mentioned. Acid soils favor the solubilization of Al and speciation to the phytotoxic Al^{3+} ion, producing the main limiting factor for plant growth (Kochian *et al*. 2002; Darko *et al*. 2004). Plants adapted to acid soils have mechanisms to resist Al toxicity that enable their survival. The increment in the production of the components, which are able to chelate Al has been reported as the widely used mechanism by plants, in which the exudation of organic acids from the root apexes highlights (Matsumoto, 2000; Ma, 2007). It reduces the availability of Al^{3+} in the rhizosphere, with a consequent decrease of Al concentration in tissues (Delhaize *et al*. 1993). However, some plants associated with a certain kind of micro-organisms of the soil may promote such tolerance mechanism.

Arbuscular Mycorrhizal (AM) symbiosis is related to the acquisition of nutrients from the plant; however, it is also known for its role such as a protective agent against pathogens and providing some mechanisms of tolerance to several environmental stresses (Smith and Read, 1997; 2008; Finlay, 2008). In relation to the alleviation of abiotic stresses where the mycorrhizal association plays an important role in acid soils with high Al

levels, several studies have shown that nonmycorrhizal plants are more sensitive to Al than mycorrhizal plants, being larger and absorbing more nutrients and water (Lux and Cumming, 1999; Cuenca *et al*. 2001; Cumming and Ning, 2003; Yano and Takaki, 2005; Klugh and Cumming, 2007; Klugh-Stewart and Cumming, 2009). The natural ecosystems contain several native populations of AM fungi, which present different degrees of variation in their effects on plant growth and also in nutrient acquisition (Clark *et al*. 1999; Bever *et al*. 2001). Because of this, edaphic changes in the environment have had an effect in changes on the abundance and distribution of the species of AM fungi (Bever *et al*. 2001). In the case of acid soils and/or elevated Al levels, there is a variation among these Al tolerant AM fungi ecotypes in relation to others that probably have a lack of adaptations to this type of stress. They provide a major adaptation to these conditions through a difference in the germination of spores, hyphal growth and root colonization (Clark, 1997; Klugh and Cumming, 2007; Cavallazzi *et al*. 2007; Klugh-Stewart and Cumming 2009) an adaptability between host plant and different AM fungi ecotypes (Sieverding, 1991; Kelly *et al*. 2005) and through the exudation of organic acids. Klugh and Cumming, 2007; Klugh-Stewart and Cumming, 2009) reported the relationship between different AM fungi ecotypes and organic acid exudation in studies with *Liriodendron tulipifera* and *Andropogon virginicus*. They showed that plants colonized by *G. clarum* and *S. heterogama* exhibited the least reduction in growth when those were exposed to Al, produced the highest concentrations of Al-chelating organic acids, while malate and citrate had the lowest concentrations of free Al in their root zones. However, apart from knowing the response of each ecotype it is necessary to know what happens with fungal dynamics in the field between members of the community species in the field. We hypothesized that the variation in soil chemistry along this vegetation gradient would influence the Al tolerance of AM fungal communities colonizing the vegetation and these AM fungal would mediate Al tolerance of host plants. The aim of this report was to elucidate potential mechanisms of Al tolerance operating in some AM fungal ecotypes.

5.2. Materials and Methods

5.2.1 Field Sampling and Spores Reproduction

AM fungal species were extracted and characterized at three spatially separated vegetative gradients at the Stewartstown mine site, Morgantown WV. The vegetation gradient at each of these three sites was dominated by trees, grass swards, the edge of the grass sward and the bare or open soil. These areas are referred to as microsites within each gradient. The length of these vegetation gradients (designated A, B and C) are approximately 15 m in length from tree to bare soil (Figure 5.1). Each of the three vegetation gradients was separated by a distance of approximately 20 m. Soil was taken from a microsite of each gradient to characterize and reproduced the AM fungal community present in each soil microsite. Some characteristics of soil are described in Table 5.1.

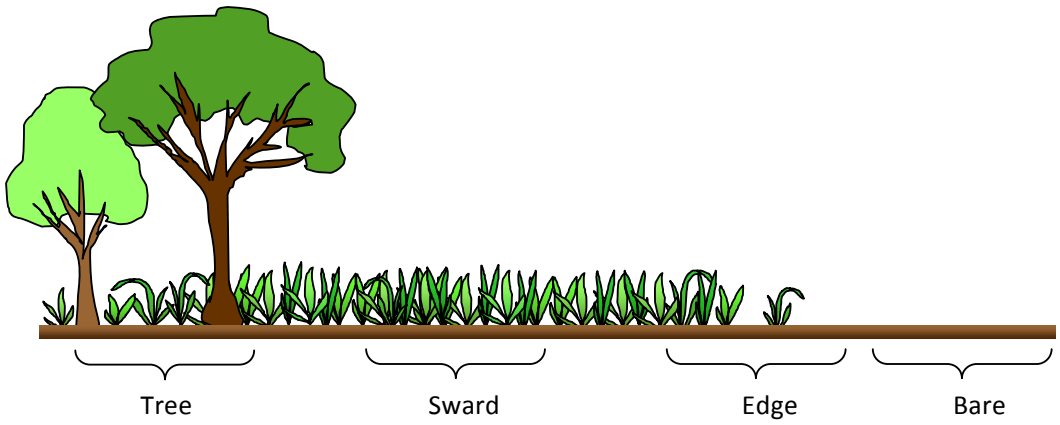

Figure 5.1. Schematic of the microsites on each of the three vegetative gradients on the Stewartstown coal stripmine site.

Collected soil was air dried for two days and then mixed 1:1 (v/v) with coarse sand. Each mixture was placed in 15x10 cm pots pre-sterilized by washing in 10% bleach for at least 30 min. For each of the 5 treatments (four microsite locations plus control) there were five replicate pots. Pots were seeded with *Sorghum sudanense* and were placed in a greenhouse and watered as needed. Pots received extra light from mixed metal halide lamps for two hours in the morning and two hours in late afternoon. After 4 months, plants were left at ambient temperature without watered for 2 weeks. Once dried, plants were harvested and pots were stored in a cold room for 30 days to insure spores have

gone through their dormancy phase. AM spores were collected from soils by wet sieving and decanting according to the methodology described by Sieverding (1991). The spores were transferred to Petri dishes and viewed under a stereoscope. Spores were visually identified to species utilizing morphological characters such as size, color and spore wall features. Identification procedures involved referencing a species database published online (http://invam.caf.wvu.edu) with verification using reference accessions in the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) at WVU.

Table 5.1. Soil chemical properties in the four sites comprising a vegetation gradient at the Stewartstown mine site. Values are means across gradients (n = 3). Values sharing the same letters are not significantly different between sites utilizing Tukey HSD tests with a significance level of 0.05.

	Bare	Edge	Sward	Tree
pH	2.97±0.04^{a}	3.11±0.03^{a}	3.34±0.06^{b}	3.30±0.05^{b}
%OM	3.01±0.19^{a}	3.67±0.67^{a}	4.31±0.56^{a}	11.13±2.61^{b}
Al (mg/kg)	190.11±24.15^{a}	284.23±25.54^{a}	564.78±80.85^{b}	656.33±73.57^{b}
%N	0.06±0.01^{a}	0.09±0.01^{a}	0.22±0.04^{ab}	0.36±0.07^{b}
K (mg/kg)	34.41±1.45^{a}	36.96±2.63^{a}	63.25±6.25^{b}	80.41±6.74^{c}

5.2.2. Measure of infection potential Assay

A mean infection percentage (MIP) assay (Moorman and Reeves, 1979) was performed before the experiment to compare the extent of colonization of the AM fungal isolates and standardize the volume of inoculum of each species for the experiment. Five soil samples from each microsite were mixed 1:5 (v/v) inoculum: acid washed sand and placed into 4x20 cm (width x height) containers (Cone-tainers; Stuewe and Sons, Corvallis, OR). Containers were seeded with *Sorghum sudanense* a highly mycotrophic species and common host plant. Plants were harvested after 21 days of growth. Root samples collected from pots were gently washed under tap water and stained in trypan blue after boiling in 10% KOH and mycorrhizal colonization was calculated by the gridline intersect method (Giovannetti and Mosse, 1980). In this experiment, inoculum was mixed with sand at a ratio of 1.5:5 for bare, 1.3:5 for edge, 1:5 for sward and 0.9:5 for tree based on

MIP results (Figure 5.2). For the control group of nonmycorrhizal seedlings, the pots contained sieved inoculum from roots of non-mycorrhizal sudangrass.

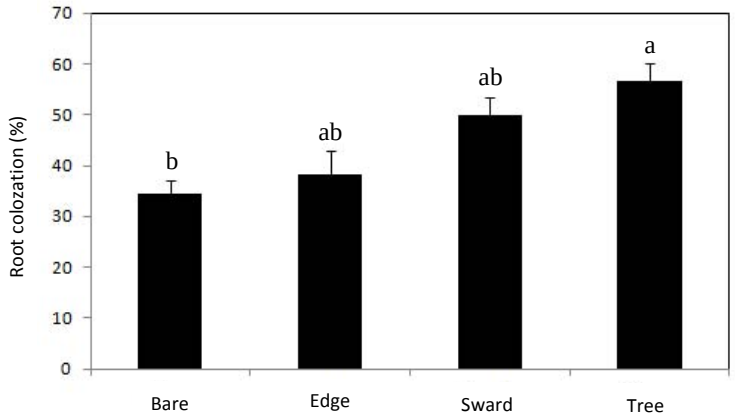

Figure 5.2. Mean infection percentage in *Sorghum sudanense* inoculated with native populations of arbuscular mycorrhizal (AM) fungi. Bars denote means ± SE (n = 5) and different letter for each cultivar represent a mean difference between treatments by orthogonal contrasts test ($p < 0.05$).

5.2.3. Plant and fungal materials

Seeds of *A. virginicus* were germinated in growth chambers after stratification in moist perlite at 4 °C for 90 days. Fungal inoculum, which was generated on roots of sudangrass, was wet-sieved (500 µm and 38 µm mesh sizes) to remove most sudangrass roots, sand and soil before adding the concentrated inoculum (collected on the 38 µm sieve and according to previous MIP assay), containing mostly spores, to acid-washed and autoclaved sand. Plants were growth in chambers for 5 weeks. They received light (400 mmol m^{-2} s^{-1} from fluorescent and incandescent sources) each day and day/night temperatures of 28 C/20 C and 60% relative humidity. Nutrient solutions containing 1.2 mM NO_3, 0.4 mM NH_4, 0.5 mM K, 0.2 mM Ca, 0.05 mM H_2PO_4, 0.1 mM Mg and SO_4, 50.5 mM B, 2 mM Mn and Zn, and 0.5 mM Cu, Na, Co, and Mo and modified to deliver Al (as $Al_2(SO_4)_3$) concentrations of 0 or 100 mM Al (pH 4.0) were supplied three times, approximately 15 mL, daily to pots with *A. virginicus* plants. Selection of this Al level was based on previous finding where 100 mM Al induced moderate impacts on *A. virginicus* (Cumming and Ning, 2003) and *L. tulipifera* (Klugh and Cumming, 2007).

5.2.4 Analysis

Al concentrations were measured every week by collecting leachate flowing from the deepots following delivery of nutrient solutions. Solution Al was determined using the eriochrome cyanine method. Plants were harvested following 6 weeks exposure to Al. At harvest, the contents of each pot were placed in a beaker with 20 mL of water and allowed to stand for 5 min. This water extract of the root zone was decanted and filtered (0.45 μm) for analysis of root zone organic acid profiles (see below). The roots and shoots biomass were dried at 65 ºC and weighed. Before drying, a portion of roots was separated and AM colonization was measured (see above). Bradford-reactive soil protein (BRSP) was determined according to the method described by Wright and Upadhyaya (1998) with minor modifications. To determine GRSP-bound Al (GRSP-Al) total GRSP was precipitated by slow addition of 2 M HCl up to pH 2.0, centrifuged at 8000 g for 20 min, redissolved in 0.5 M NaOH, dialyzed against deionized H_2O and freeze-dried. Dried GRSP was acid-digested (H_2O/HCl/HNO_3; 8/1/1 v/v/v) and Al was determined by atomic absorption spectrophotometer (Perkin-Elmer 3110).

To prepare root zone extracts for organic acid analysis, 50 μL of 50 μM Na_2-EDTA and a drop of 0.1 N NaOH were added to 10 mL of each extract. This procedure promoted chelation of Al in the solution and prevented suppression of organic acid detection by Al (Cumming *et al*. 2001). Samples were freeze dried and stored frozen until analyzed. Residual salts were dissolved in 3 mL of nanopure water and organic acids were separated and quantified by ion chromatography on a Dionex ICE-AS6 column (Dionex Corp., Sunnydale, CA) as described by Cumming *et al*. (2001). The concentration of total phenolics was determined spectrophotometrically according to Heim *et al*. (2001). Solutions were analyzed at 725 nm and Phenol (Fisher) was used as a standard. Exuded total phenol concentrations were expressed as μMol g^{-1} DW. Dried shoots and roots were ground to pass through a 20-mesh screen and then digested in nitric acid and hydrogen peroxide following procedures of Jones and Case (1990). Tissue digests were analyzed for Al concentrations using a graphite furnace atomic absorption spectrophotometer (Varian, Inc., Mulgrave, Victoria, Australia).

5.2.5. Statistical analysis

The experiment was established as a two way factorial design (2 Al concentrations x 5 fungal treatments, 5 replicates per treatment). Data were log-transformed wherever necessary in order to achieve homogeneity of variance. Data were analyzed using analyses of variance (ANOVA) followed by Tukey-Kramer's LSD to identify significant differences among treatment means. All statistical analyses were carried out using SAS JMP v.7 software (SAS Institute, Cary, NC, USA).

5.3. Results and discussion

The benefit that AM fungi give to the plants is variable among species in terms of nutrients acquisition or effect on the plant (Borie *et al.* 1999). This is a consequence of a substantial genetical variation among AM fungi species (Bever *et al.* 2001). In this experiment, eight AM ecotypes were isolated and identified in the different microsities from the acidic mine. The relative abundance of *P. occultum* was significantly higher in the bare and edge microsite than others and tree microsite showed a higher spores number of *A. trappei*. Moreover, spores of *A. morrowiae* were significantly more abundant in the grass sward than in other microsites and *G. clarum* just was found in this micrositie (Table 5.2). In general, AM fungi have been found in soils from pH 2.7 to 9.2, but different isolates have varied pH tolerances and the most AM fungi species appear to be adapted to soil pH conditions close to those from they were isolated (Sylvia and Williams, 1992; Bartolome-Esteban and Schenck, 1994; Clark, 1997).

Aluminum reduced the shoot and root biomass production in control (non mycorrhizal plants), Bare and Tree treatments. According to index of Al tolerance, the plants inoculated with AM fungi from Bare and Tree treatments under Al presence decreased their growth in shoot by 34 and 27% respectively and by 16 and 32 respectively % in root. On other hand, plants growing with AM native populations isolated from edge and sward shown higher Al tolerance degree (Figure 5.3).

Table 5.2. Abundance of AM fungal at different microsites in all soil gradients (%).

Species	Microsite				ANOVA	Log Likelihood
	Bare	Edge	Sward	Tree	*p*-microsite	*p*-microsite
A. koskei	0.0a	1.0a	1.0a	1.2a	0.170	0.001
A. lacunosa	0.0b	0.0b	1.0b	8.5a	<0.001	<0.001
A. mellea	0.3a	0.0b	0.0b	0.0b	0.017	<0.001
A. morrowiae	0.8c	8.8b	7.1b	30.9a	0.001	<0.001
Acaulospora SM2	0.0b	10.0b	38.0a	5.3b	<0.001	<0.001
Ar. Trappei	2.1b	8.2b	13.0b	26.7b	<0.001	0.007
E colombiana	0.0a	0.0a	0.2a	0.0a	0.060	0.029
E. contigua	23.5a	20.2a	1.1b	0.0b	<0.001	<0.001
G. clarum	6.6b	27.1a	27.7a	10.2b	0.000	0.006
G micoaggregatum	0.9a	0.0b	0.0b	0.0b	0.015	0.003
Glomus SM1	34.9a	9.3b	2.1c	6.5bc	<0.001	0.039
P. occultum	30.9a	15.4ab	5.4b	3.3b	0.001	<0.001
Total	100	100	100	100		

Means followed by different letter in a line are significantly different from each other by to orthogonal contrasts test (p<0.05; n=5).

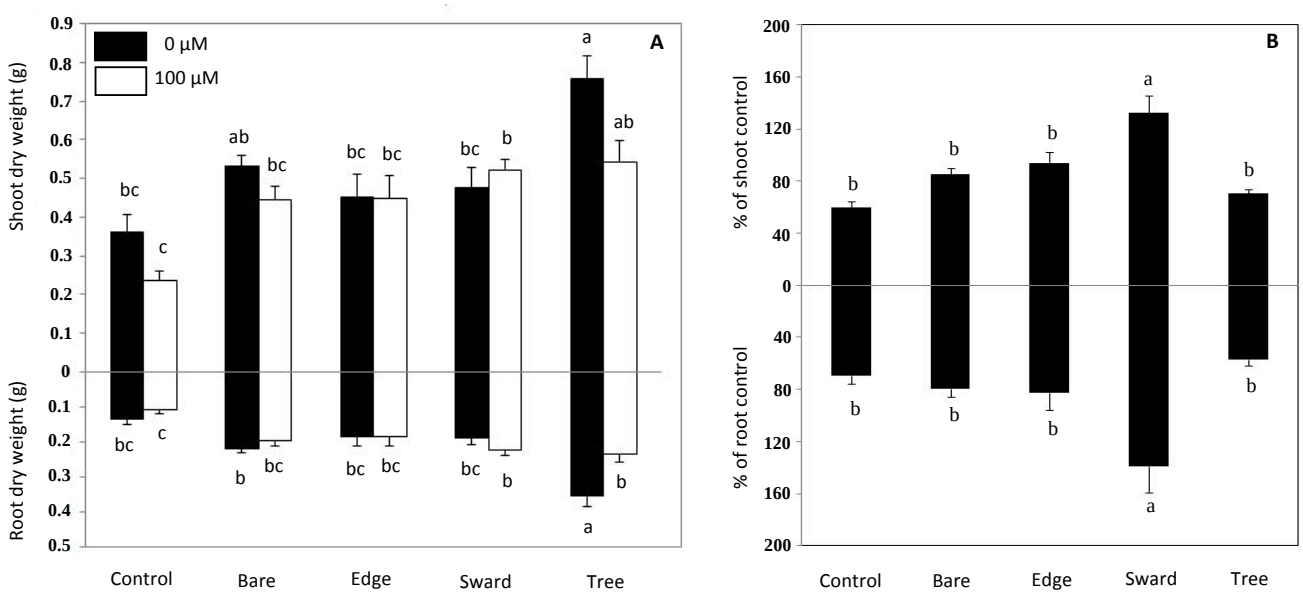

Figure 5.3. Shoot and Root biomass production (A) and tolerance index (B) in plants inoculated with native populations of arbuscular mycorrhizal (AM) fungi and non-mycorrhizal (NM) plants exposed to two Al levels. Bars denote means ± SE (n = 5) and different letter for each cultivar represent a mean difference between treatments by orthogonal contrasts test (p< 0.05).

In general, high Al levels did not affect the mycorrhizal colonization in the short term, which suggests that this element does not inhibit the formation of the symbiosis and its beneficial early effects, providing the plants with a major tolerance (Cuenca *et al*. 2001; Klugh-Stewart and Cumming, 2009). Mycorrhizal colonization percentages varied among microsite and Al treatments and ranged from 43% in edge without Al to 67% in tree treatment with Al. Plants inoculated with AM native populations isolated from bare and tree had the highest colonization with and without Al, plants colonized by ecotypes isolated from sward were intermediate, while edge colonized *A. virginicus* the least (Figure 5.4). Colonization was not affected by Al treatments. However, some other studies have shown that root colonization is lower in soil with higher Al saturation or low pH level. For example, Silva *et al*. (1994) showed that root colonization in *Triticum aestivum* was 5% at soil pH 4.9–5.0, and 77% at pH 5.2–5.5. Additionally, Nurlaeny (1995) studied the response of both *Zea mays* and *Glycine max* colonizated by *G. intraradices* using an Oxisol and an Ultisol at diferent pH levels. They concluded that the root colonization increased in relation to a pH increase from 4.7 to 6.4.

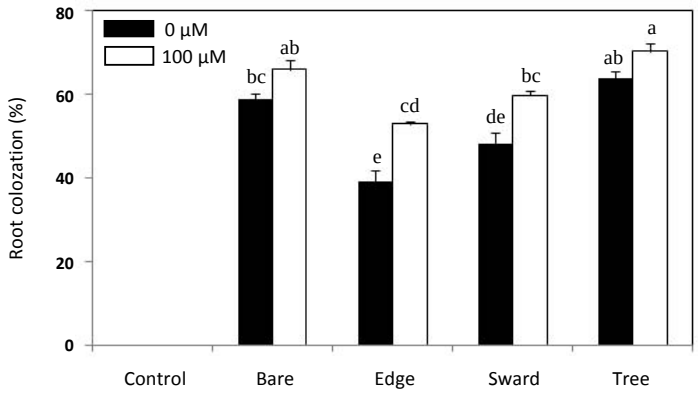

Figure 5.4. Root colonization (%) in *A. virginicus* inoculated with native populations of arbuscular mycorrhizal (AM) fungi and non-mycorrhizal (NM) plants exposed to two Al levels. Bars denote means ± SE (n=5) and different letter for each cultivar represent a mean difference between treatments by orthogonal contrasts test (p<0.05).

Cavallazzi *et al*. (2007) have showed that the mycorrhizal colonization on apple plants was significantly influenced by different fungal isolates. Moreover, Klugh and Cumming (2007) and Klugh-Stewart and Cumming (2009) in studies with *Liriodendron tulifera* and *Andropogon virginicus*, respectively, had showed different responses of AM fungi ecotypes to Al tolerance in diverse host plant. There is not a consensus about the relationship between AM root colonization with Al tolerance. However, Seguel *et al*. (2012) suggested that an early colonization can be an important factor in Al tolerance and, consequently to be beneficial against Al toxicity effects. Klugh and Cumming (2007) and Klugh-Stewart and Cumming (2009) reported changes in the availability of Al in AM colonized plants of *Liriodendron tulipifera* and *Andropogon virginicus*, concluding that some ecotypes of AM fungi provide tolerance to plants as a result of higher production of organic acids and the consequent decrease on the activity of Al^{3+} in the rhyzosphere.

Several recent studies related to the role that GRSP play in the plant tolerance to the presence of metals in the soil have been carried out (González-Chávez *et al*. 2004). Accordingly, it can be stated that AM are able to keep metals out of plants or reduce concentrations into plant tissues (Hildebrandt *et al*. 2007). Gillespie *et al*. (2011) showed that the current extraction procedure that defines GRSP yields a mixture of compounds and thereby overestimates glomalin stocks when quantified using the Bradford assay. However, this artifact is decreased to quantify glomalin produced in a soil-less system as this study.

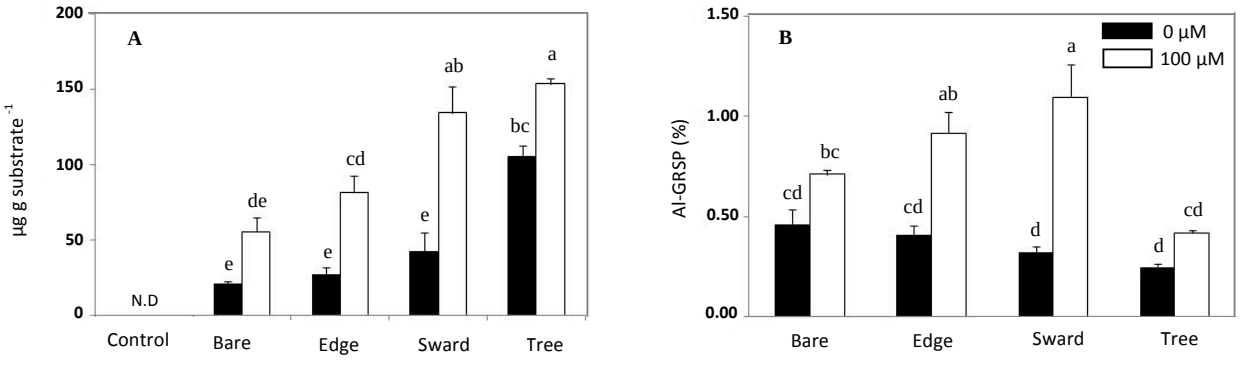

Figure 5.5. Total glomalin-related soil protein (T-GRSP) production (A) and aluminium bound to glomalin-related soil protein Al-GRSP (B) in *Andropogon virginicus* inoculed with AMF native populations isolated from different microsities. Bars denote means ± SE (n = 5) and different letter for each cultivar represent a mean difference between treatments by orthogonal contrasts test ($p< 0.05$).

Glomalin production was increased when the plants inoculated were exposed to 100 uM Al. In addition, GRSP production in *A. virginicus* inoculated with AM fungi native populations isolated from sward and tree microsites was higher than other treatments (135 and 153 ug g susbtrate, respectively). However, sward treatment showed the highest increasing (216%) with respect to treatment without Al and tree treatment showed the less increasing by Al exposure (45%). Aluminum bound to glomalin related soil protein (Al-GRSP) ranged 0.4 to 1.1% in glomalin extracted from substrate with Al exposure and was higher than the glycoprotein extracted from substrate without Al. Sward treatment showed the highest Al-GRSP percentage (1.1%) with Al presence and the higher increasing (244%) with respect to treatment without Al (Figure 5.5).

In our recent study about GRSP bound Al using confocal laser scanning microscopy (CLSM), Aguilera *et al*. (2011) showed direct evidence of GRSP ability to sequester Al in the molecule. Here they suggest 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 addition, Seguel *et al*. (2012) found that GRSP production was greater in the soil sample that presented higher Al saturation and Al-GRSP was higher in the most Al tolerant wheat and barley cultivars. In a previous study, Cornejo *et al*. (2008a) reported that the high GRSP content observed in some Cu-polluted soils could operate as an important factor in soil remediation, 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 term according to the residence time of fungal structures in the soil, or, if the bulk of Al immobilized is 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 for longer periods (Tommerup, 1992; McGee *et al*. 1997).

In addition to the nutritional aspects, changes in the plant architecture, root colonization and glomalin production, the root exudation to the rhizosfere and the activation of plant defence (internal mechanism) may be all relevant in the Al tolerance. Exudation of organic acid (OA) has been proposed as an effective Al tolerance mechanism that chelates

Al externally in the rhizosphere, rendering it non-phytotoxic (Delihaze and Ryan, 1995; Barcelo and Poschenrieder, 2002; Kochian *et al*. 2005; Naik *et al*. 2009).

A variety of five organic acids were measured in the rhizospheres of *A. virginicus* plants, citric acid, malic acid, lactic acid, formic acid and acetic acid. In this sense, citrate and malate are two organic acids commonly cited as Al chelators and associated with Al tolerance. Oxalate, another Al chelator, was not detectable. Citric acid is a strong Al chelator (pKf: 9.6) and can effectively protect wheat seedlings against Al toxicity (Ownby and Popham, 1989; Ma, 2000; 2007; Barcelo and Poschenrieder, 2002). Citrate concentrations in root zones of *A. virginicus* colonized by AM native populations from sward remained constant across both Al treatments. In non-mycorrhizal plants and plants colonized by AM species isolated from bare and edge, citrate concentrations increased slightly in response to 100 µM Al. However, citrate concentration declined considerably when plants inoculated with AM fungi isolated from tree microsite were exposed to Al. Although malic acid is not the greatest chelator of Al, with a pKf: 5.7 (Kochian *et al*. 2005), Delhaize *et al*. (1993) showed in Al tolerant wheat cv. higher malate than citrate exudation. In this study, malate concentrations were highest in treatment tree and it was affected by Al treatment. Across mycorrhizal seedlings in the 100 µM Al treatment, malate concentrations were not different. Production of organic acids (µmol g^{-1} root mass) was calculated to investigate potential changes in exudation induced by Al in root systems of *A. virginicus* colonized by different AM fungi native populations. Citrate production was stimulated at 100 µM Al, although the extent of this stimulation varied with the different AM fungal treatments. Although production of malate by plants colonized by AM fungal isolated was not stimulated with Al exposure, all AM fungal treatments shown higher malate production than non-mycorrhizal plants (Figure 5.6). After six weeks under Al exposure, the organic acid exudation declined compared with plants growing without Al. This suggests that, after prolonged exposure to Al, organic acid exudation may not be a primary mechanism of Al tolerance. Many reported studies about organic acid exudation have used short Al exposure time (48 hours or less); (Jones, 1998; Heim *et al*. 2001; Nguyen *et al*. 2003; Qin *et al*. 2007) and in consequence is difficult to conclude that organic acid exudation is a permanent characteristic of Al tolerance over time.

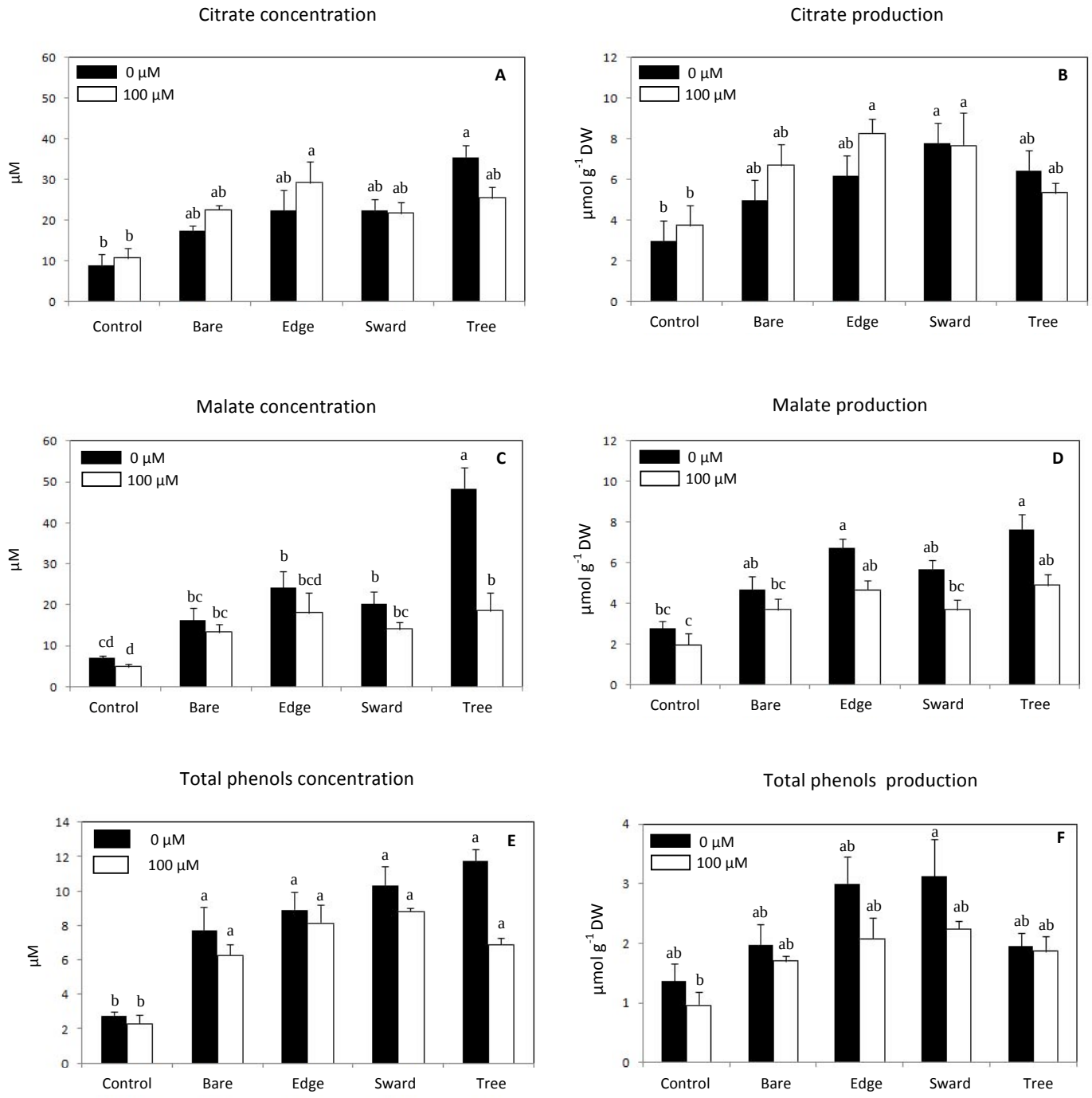

Figure 5.6. Citrate concentration (A) and production (B); Malate concentration (C) and production (D) and total phenols concentration (E) and production (F) measured in *Andropogon virginicus i*noculed with AM fungi native populations isolated from different microsities. Bars denote means ± SE (n = 5) and different letter for each cultivar represent a mean difference between treatments by orthogonal contrasts test (p< 0.05).

The total phenols may chelate Al in the rhizosphere, playing a role in protecting plants agaist the perturbations associated with metal exposure (Heim *et al*. 2000; Kidd *et al*. 2001: Nguyen *et al*. 2003). However, the function of the total phenols in the Al tolerance as an exclusion mechanism in the rhyzosphere is not so clear, especially at acid pH, due to Al^{3+} and H^{+} compete for the union sites with phenolic compounds, reducing the chelating capacity of the metal with the compound, which is different to the most of the organic acids under acid conditions (Kochian *et al*. 2004). This study showed that total phenols production was decreased in response to Al treatments at all AM fungal treatments. This suggests that exudation of phenols do not play a significant role in external Al detoxifications to long term. However, the total phenols production was higher in plants colonized by all AM fungal than nonmycorrhizal plants, under Al exposure.

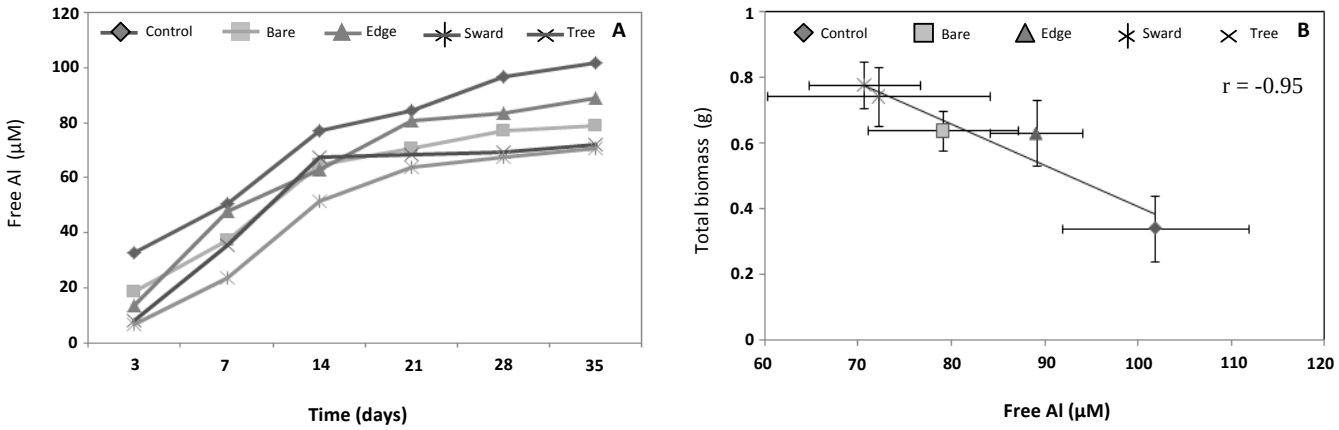

Figure 5.7. Concentration free Aluminum across time (A) and relationship between total biomass of *A. virginicus* plants exposed to 100 µM Al and concentrations free Al in the rhizosphere (B). Points represent means (n =5).

Moreover, the glomalin and organic acids production by *A. virginicus* roots was very important, but the variation of this glycoprotein and root exudates led to differences in free Al concentrations among AM fungal treatments that affected differentially the plant response to Al in the rhizosphere. Based on the concentration of aluminum leaching, eriochrome cyanine method was indirectly used to estimate the concentration of free Al in the rhizosphere.

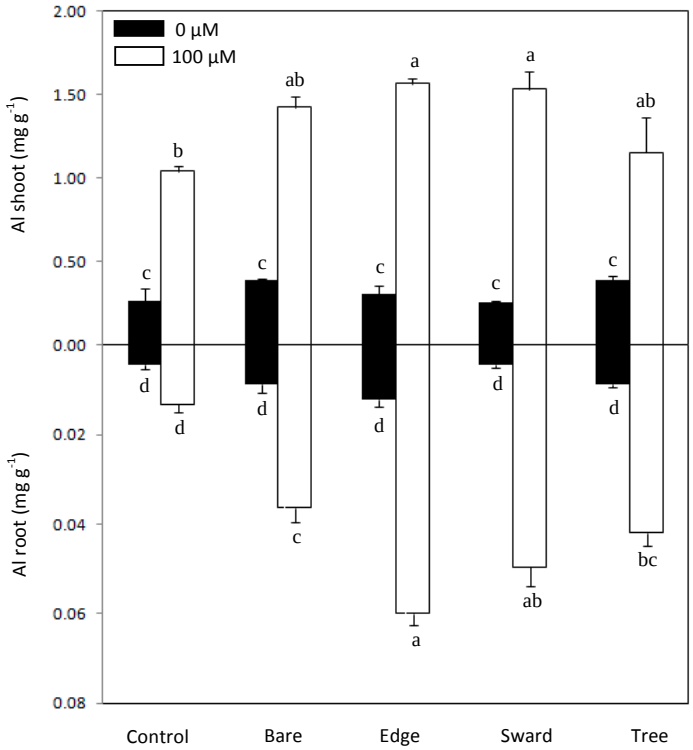

Figure 5.8. Shoot and root aluminum concentration (mg g^{-1}) in *Andropogon virginicus i*noculed with AM fungi native populations isolated from different microsities. Bars denote means ± SE (n = 5) and different letter for each cultivar represent a mean difference between treatments by orthogonal contrasts test (p< 0.05).

Aluminium accumulation in root was higher than shoot Al translocated and total shoot and root Al concentrations showed marked patters across microsities. Moreover, Al accumulated in root showed different comportment between microsities, at 100 uM Al treatment. In this sense, edge showed the highest Al concentration in root and control plants did not show important Al accumulation. On other hand, Al accumulated in shoot was significantly not different between the Al and microsititie treatments (Figure 5.8).

According to the above, plants inoculated with AM fungi isolated from sward and tree microsites presented lower concentration free Al in the rhizosphere, 72 and 70 µM Al respectively. Non mycorrhizas plants showed the highest concentration free Al in its rhizosphere, about 100 µM (Figure 5.7).

In relation to results, citrate should be the primary organic acid responsible for chelation of Al in the rhizosphere, chelating an important percentage of the available Al. Malate and other organic acids should play substantially lesser roles and chelated the available Al in solution. Moreover, glomalin should also be an importante Al quelator. Plant growth across all AM treatments responded consistently to the concentration of free Al in the rhizosphere, with the biomass of *A. virginicus* being reduced by 38% at about 100 uM free Al in the root zone (Figure 5.8). Plants colonized by AM isolated from sward exhibiting the greatest Al tolerance and had the least free Al in the root zone.

Mechanisms related to exudation, nutrient uptake, or nutrient use efficiency may play roles in the observed Al tolerance in mycorrhizal plants. In support of the current study's findings, previous studies that have measured and modeled free Al and organic acid exudation in plants colonized by various AM species all suggest that organic acids played roles in Al resistance of host plants (Lux and Cumming, 2001; Cumming and Ning, 2003; Klugh and Cumming, 2007; Klugh-Stewart and Cumming, 2009).

The data presented here provide evidence that there is functional variation among AM fungi and that the level of Al tolerance conferred to host plants may vary among AM species. Klugh-Stewart and Cumming (2009) reported that some AM ecotypes as *G. clarum*, *S. heterogama* and *A. morrowiae* confer higher degree of Al tolerance to their host plants under Al exposure. Those results are consistentes with the results here shown, because the edges and sward microsites presented an important percentage (27.1 and 27.7%, respectively) of *G. clarum*, the AM ecotype most important in Al tolerance.

5.4. Conclusions

There are few studies related to the exudation of organic acids by AM fungi tolerant for ecotypes adapted to Al and acidic conditions. This study has provided the quantitative data on organic acid exudation and glomalin production by mycorrhizal plants inoculated with AM fungi native populations exposed to Al.

Plants inoculated with AM fungi isolated from sward showed higher Al tolerance index related with shoot and root biomass production, glomalin production and organic acid exudation.

According to abundance of AM fungal at different microsites in all soil gradients, we found that *A. morrowiae* and *G. clarum* had an importante percentage in AM fungi native populations of sward microsite. Previous studies showed that both ecotypes are the most important in related with Al-chelation. This is in concordance with the higher glomalin and organic acid production and GRSP bound to Al observed in this micrositie.

While these results provides supporting evidence of functional variation in conferred Al tolerance to *A. virginicus* among AM fungi native populations, this study suggests that the diversity of AM species in ecosystems is likely the most important factor in promoting benefits related to tolerance environmental stresses.

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Chapter 6. General discussion - conclusions and future perspectives

General discussion, conclusions and future prospects

Soil acidity is a major limitation to agricultural production throughout the world. In agricultural systems developed in acidic soils, it is a common practice to apply some amendment materials, such as lime, gypsum and phosphate fertilizer, to enhance the quality and quantity of plant production. However, as limited reserves of raw material (rock phosphate) have increased prices of phosphate fertilizers the application of sustained inputs of these materials are not feasible, especially in developing economies. For overcoming such limitations, it is common to use genotypes of Al-tolerant crop species and/or genotypes with high P use efficiency. Thus, it is possible to reduce fertilizer inputs, especially on marginal soils or where processes of P fixation are remarkable, as in Andisols. Within the same context, the management of AM fungal ecotypes adapted to high levels of Al in soil or their enhancement by inoculation emerges as a feasible alternative to provide a significant increase to plant production on acidic soils.

This research has shown that the AM symbiosis in acid soils has great potential increasing plant growth by mediating changes on the soil solution chemistry of the root-soil interface, improving nutrient acquisition, and altering plant Al stress responses, some or all of which positively contribute to plant performance on such environments. These results would suggest the Al bioabsorption to AM structures and glomalin, and a sustained organic acid exudation from roots of plants colonized by AM fungi, some of them Al-tolerant. These Al tolerance mechanisms conferred to host plants had not been reported before. The better behaviour in some wheat cultivars related to high Al tolerance is concomitant with an enhancement in the plant nutritional status, a higher presence of AM colonization and AM propagule in the soil and a greater amount of Al bound to GRSP. Thus, from a practical point of view among the six wheat cultivars used in this study, ‘Crac’, ‘Invento’ and ‘Porfiado’ showed the lesser responsiveness to lime application in terms of vigorous development at three phenological stages when growing at high Al saturation and at the same time those wheat cultivars showed higher AM propagules and Al-GRSP, being consequently the better cultivars to be used in southern Chile agriculture.

Arbuscular micorrhizal fungal colonization was not inhibited at high Al saturation levels, suggesting that an early colonization can be an important factor in Al tolerance and, consequently, to be beneficial against the initial Al toxicity effects. The increased of AM propagule under high Al exposure together with an early AM root colonization could produce an improved nutritional status of cereal cultivars in soils with high Al levels, representing a feasible indirect mechanism of Al tolerance showed by mycorrhizal plants, which could in part explain the plant Al tolerance. These aspects should be considered by farmers in crop cereal production under soils with high Al levels.

Although there are studies showing an increase of organic acids exudation in roots colonized by Al adapted AM fungi ecotypes in acidic conditions, this study provides some quantitative data on organic acid exudation and glomalin production by mycorrhizal plants inoculated with native populations of AM fungi exposed to Al. This trend could be of biotechnological importance, since the ability for modifying the plant organic acid exudation and the glomalin production could be used as a tool for the selection of the more efficient AM fungal ecotypes to cope Al stress. In this sense, in our study the plants inoculated with AM fungi isolated from sward microsite showed higher Al tolerance index related with a higher shoot and root biomass production, glomalin content and organic acid exudation. According to abundance of AM fungi at different microsites under increasing free Al contents, here we found that *G. clarum* had a higher importante presence in the AM fungi native communities of edge and sward microsite. Previous studies have shown that *G. clarum* is one of the most important AM fungi related with a decreased of free Al in soil solution. This is in concordance with the higher glomalin and organic acid production and Al bound to GRSP observed in those microsities. While the previous results support evidences of a functional variation in conferring Al tolerance to *A. virginicus* through Al adapted AM fungi native populations, this study suggests that the diversity of AM fungal species in Al stressed ecosystems is likely the most important factor in promoting benefits related to the tolerance against environmental stresses.

Based on the above, the use of diverse AM fungal species adapted to Al in soils as biofertilizers should be considered as part of integrated nutrient management, which is projected to be an important avenue to improve crop yields through a better nutrient supply which would be of great interest for agriculture on acidic soils with phytotoxic Al

levels. The feasibility of using AM fungal inoculants, in general, could be preferential in certain types of production systems where crops are confined to a reduced surface area, such as nurseries, horticultural, or ornamental systems established on acidic soils with high Al^{3+} levels. In such cases, the cost related to the application of inoculants would represent a marginal fraction of all production costs and the development of AM fungal inoculants could be a viable alternative for improving the quality, yields, and sanitary status of production. The use of inoculants might also be beneficial under conditions where native soils/ecosystems have been severely disrupted, such as reclamation programs following strip mining or in the installation of ornamental plants and trees in urban settings where soils have been stockpiled or soil substrates created as part of these activities. Several studies have also demonstrated the high impact of different agricultural practices on the density, diversity and functionality of AM propagule. In these cases, the alignment of management inputs and activities with the goal of maintaining a diverse and functionally beneficial AM fungal community may foster sustainable agronomic production. Thus, the correct choice of agronomic management to be implemented in acidic soils, particularly when extensive crops are established, represents a way to increase the positive effects of AM fungi without requiring elevated and expensive inoculations.

Ongoing and future research on AM symbioses and acidic soils with high Al levels should be extended to include a characterization of Al tolerance of natural AM communities and selection of the most feasible ecotypes to be adopted as biofertilizers. Among parameters to be used in these selections, in addition to the previous mentioned (an increased in organic acid and glomalin production) should be: *i)* high native resistance to Al^{3+} and *ii)* high ability to produce significant amounts of hyphae and spores in the soil and roots. The use of technological tools such as monoxenic culture could give an opportunity to understand the interactions between root and AM fungi, and what are the specific mechanisms by which the fungus can tolerate Al phytotoxicity. In this sense, the study of the role of Al tolerant AM fungi strains through specific AM exudates in detoxifying Al^{3+} in the mycorrhizosphere, would be an interesting way to know the metabolism induced by specific AM fungi and their effect in the plant Al homeostasis at high Al levels. Moreover, the development of adequate and easily performable molecular tools to

monitor the persistence and seasonal cycles of AM strains used as inoculants in colonizing roots of host plants need to be studied.

Considering the relationship here studied, between the early AM colonization and/or increased AM propagules presence under high Al exposure, with an improvement in Al tolerance in host plants, future studies related to the stimulus and/or signals of mycorrhization operating under these conditions are necessary. Strigolactones (SLs) are specific hormones that stimulate the mycorrhization and they are produced at significant levels in the roots of many plant species under P deficient conditions. It is known that there is a close relationship between Al tolerance and P efficiency in Al-tolerant plants. Therefore, Al-tolerant wheat cultivars should be more efficient in P acquisition than sensitive ones when grown in these soils. Moreover, AM symbiosis should be beneficial to Al-P interactions in wheat growing in volcanic acid soils mainly through SLs exuded by wheat roots, which increase hyphal branching and producing an early AM colonization. Thus, SLs levels would affect the rate of AM fungal colonization producing early benefits to plant hosts growing under Al stress conditions. These future studies would allow deeply understand the Al-P interactions and how AM symbiosis, which habitually increase P uptake by plants, would affect the growth and development of Al tolerant wheat cultivars when cropped in acidic volcanic soils.

In the field P and Al along the root axis are quite different. Thus, whereas at top level available P is high, free Al is low at deeper layers. On the contrary, available P is very low and Al levels very high. Then, along the root profile different mechanism could be functioning at the same time or at different rate, depending on plant growth rate. Whereas the studies here presented proposes possible Al mechanisms operating separately and under specific Al exposure levels, future studies would be required to establish the magnitude and significance over time of these Al mechanisms when the host plants grown in the field under several and different Al phytotoxic levels in the soil. In addition, an analysis at the local scale of the effects of different agronomic practices on the functionally of the native AM populations is needed, particularly when annual extensive crops are used in the rotation systems as an agronomic practice to improve the diversity and functionality of AM communities, where the use of AM inoculants cannot be implemented due to technical and economic limitations.

In summary, AM fungi play a bioprotector, bioremediator and bionutritional role in mycorrhizal plants through Al tolerance mechanisms of plants associated with AM fungi that would alter Al^{3+} bioavailabilty in the mycorrhizosphere and may underlie to ameliorate Al impacts on nutrient uptake. However, deeper research is needed to understand the specific roles played by AM fungi in increasing such Al resistance in crops and trees growing in acidic soils where Al is the principal limiting factor.