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

Contribution of Metallophyte/Arbuscular Mycorrhizal Fungi Symbiosis to the Phytoremediation Processes of Copper Contaminated Soils

Doctoral Thesis
In Fulfillment of the
Requirements for the Degree
Doctor of Sciences in Natural Resources
by

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TEMUCO – CHILE 2011

CONTRIBUTION OF METALLOPHYTE/ARBUSCULAR MYCORRHIZAL FUNGI SYMBIOSIS TO THE PHYTOREMEDIATION PROCESSES OF COPPER CONTAMINATED SOILS

Esta tesis fue realizada bajo la supervisión del director de Tesis Dr. PABLO ENRIQUE CORNEJO RIVAS, perteneciente al Departamento de Ciencias Química 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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Por la Gracia de Dios soy lo que soy....
(1 Corintios 15:10)

Agradecimientos/Acknowledgements

Primeramente quisiera agradecer profundamente a mis tutores, Dr. Fernando Borie y al Dr. Pablo Cornejo por haberme aceptado como estudiante para desarrollar mi tesis en su grupo de trabajo, les agradezco por sus constantes enseñanzas, su apoyo incondicional y su infinita PACIENCIA para conmigo.

Por otra parte quisiera agradecer a la Directora del Programa, Dra. María de la Luz Mora, porque siempre me apoyó en todas mis decisiones y al Programa que siempre respondió a mis solicitudes. Gracias por confiar en mi y aceptar mi propuesta de Tesis.

Quisiera de la misma forma agradecer a la Dra. Rosario Azcón y su grupo de trabajo de la Estación Experimental del Zaidín por permitirme realizar una pasantía en sus laboratorios. Gracias a todos los granadinos porque hicieron de mi estadía un momento maravilloso, gracias Anita, Pijo, Poligonero y Bea (Japón y España). Mi mayor y eterno agradecimiento para: Gordo, Pinche , Michel y mi querida Gloria gracias a todos Uds. por darme luz.

I wish to express my sincere gratitude to Prof. Nanthi Bolan for allowing me to do a research internship in his group in The Centre of Environmental Risk Assessment and Remediation, University of South Australia, Adelaide, Australia. I felt his disposition and kindness every time I needed. Moreover, thanks to: Luchun, Jack, Girish and Julia for all the good moments that gave me in Aussie.

Especially I would like saying THANKS to Ruth and Ross Rofe, for opening the doors of their house and love me as a real family. All the Rofe family (Lydia, Simon, Alex, Dorothy, fashion sister and Alex's Parents) will remain always in my heart.

A mis amigos y al grupo de micorrizas de la UFRO mi mas sincero agradecimiento. Especialmente al remanente de la comunidad científica: Anita, Rubén, Gustavo y Gabriela. A Edgardo por todas las risas. A Carmen y Marta. A Loreto por ser siempre mi amiga incondicional

Los agradecimientos mas importantes van dirigidos a mi familia (aunque una familia atípica) A mi padre primeramente, por siempre creer en mi y apoyarme en cada momento. A mis hermanos, especialmente a Anabelle y a mi cuñado Carlos por ser toda una familia para mi. A Fernanda por su amor incondicional e infinita paciencia y a su hermosa familia (Pamela, David, Toti y Cristóbal), gracias por aceptarme como uno de los suyos.

Finalmente el desarrollo de mi tesis fue posible gracias a las variadas becas que la Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) me otorgó, principalmente a la Beca para realización de estudios de doctorado en Chile, Beca de apoyo de Tesis 24091053, Beca para la realización de pasantía Internacional, y Beca de apoyo a la finalización de tesis. Quisiera agradecer también a la Beca de la Agencia de Cooperación Española para el Desarrollo (MAE-AECID) que posibilitó una estadía doctoral en España. Por otra parte esta tesis fue financiada parcialmente por los proyectos Fondecyt 3070052 y 11080131 del Dr. Pablo Cornejo

Thesis outline.

Human activities generate wastes, some of which contain high amounts of metals/metalloids that could enter to the natural ecosystems and alter the activities and functioning of soil micro- and macro-organisms. Some microorganisms can adapt/resist the metal stress, and some of them are able to promote the plants establishment and therefore promote phytoremediation processes. In this context, the use of arbuscular mycorrhizal fungi (AMF), and their role in phytoremediation, has emerged as an interesting choice. In addition to AMF well-known contribution to plant nutrient acquisition and growth, these fungi develop diverse mechanisms that encourage the plant growth in soils with high metal concentrations. Nevertheless, the role of AMF in phytoremediation remains unclear; this is partly due to the existence of two opposing views about the processes used by AMF in soils contaminated by metals. Some reports state that AMF would promote phytoextraction processes whereas others conclude that AMF promote mainly phytostabilization processes. The information generated in the last years has enhanced our understanding of mycorrhizal biology and of the metal tolerance of plants and fungi; however, complementary studies in this area for improving and implementing the use of AMF in phytoremediation programs are necessary.

This thesis aimed to analyze the contribution of AMF symbiosis to the phytoremediation processes of copper contaminated soils. For achieving this, a series of assays were carried out, which are presented in the following chapters:

Firstly in chapter number 2 reviews phytoremediation of metal polluted soils by arbuscular mycorrhizal fungi, which provide the thesis theoretical background.

In chapter number 3, a general screening to evaluate the plant behavior to increasing Cu levels without the AMF use is realized. The plant Cu tolerance and the exudation of molecules by roots in response to increasing Cu doses were evaluated using two Cu-metallophytes (*Oenothera picensis* and *Imperata condensata*) collected from Cu polluted environments and two agricultural plants (*Lupinus albus* and *Helianthus annuus*) proposed as candidates to be implemented in

phytoremediation programs due to the faster growth and the high biomass production rate showed by this kind of plants.

Differences in root exudation patterns were observed among the plants, highlighting the high amounts of succinic acid exuded by *O. picensis* and citric acid by *I. condensata*. In addition, the exudation of phenolic compounds was also specie-dependent, with catechin mainly exuded by *I. condensata*, cinnamic acid by *O. picensis* and coumaric acid exclusively exuded by *H. annuus* at high Cu doses.

The results indicated differences in root exudation patterns among metallophytes and agricultural plants and those could affect the Cu tolerance by plants. Particularly, the higher exudation rate showed by *I. condensata* can be an effective exclusion mechanism to tolerate high Cu doses, and could support the use of this plant in Cu phytostabilization programs, especially in highly Cu polluted soils.

Chapter 4 and 5 evaluate the alleviation of Cu toxicity in *Oenothera picensis* by copper-adapted arbuscular mycorrhizal fungi and biotreated agrowaste residue. This study was realized during the doctoral internship in the Estación experimental el Zaidín (E.E.Z, CSIC, Granada, Spain) we studied the use of AMF either isolated from Cu polluted environments and compared the plant response with AMF isolated from agricultural soils, which are presumably sensitive to high metal levels. In addition, a ubiquitous Spanish agricultural waste, obtained from Sugar Beet (SB) production and its potential use as an organic amendment promoting plant growth were studied. Sugar beet agrowaste amendment application and AMF was evaluated using the metallophyte *O. picensis.* Plants were grown in a Cu-treated soil, either with or without SB application, and inoculated with: Cu-adapted AMF (GA) or *Glomus claroideum* (GC). The application of SB had a positive effect, both increasing shoot biomass, and allowing the plant to survive at the highest Cu levels. In SB absence, only GA-colonized plants survived at the highest Cu doses. No changes in root colonization by GC occurred at increasing Cu supply levels, but a greater root colonization was stimulated by GA in SB treated than in untreated plants. In addition, changes in antioxidant enzyme activities were found, and they were more related to the AM and Cu supply than SB addition. These results denote enhanced plant Cu tolerance as a consequence of adaptive physiological mechanisms provided by GA, which allowed plant survival in Cu polluted soil.

Finally, the effects of arbuscular mycorrhizal inoculation on metallophytes and agricultural plants growing at increasing copper levels were evaluated. A pot culture experiment was carried out to assay the effectiveness of different AMF inocula promoting plant growth and copper uptake using *Oenothera picensis, Imperata condensata* and *Helianthus annuus.* Plants were grown under increasing Cu supply levels and were inoculated or not with: *i)* Cu-adapted AMF (GA) or *ii) Glomus claroideum* (GC). The results showed differences between AMF inoculated and uninoculated plants, which were strictly dependent of the AMF inocula used and the Cu level applied. In addition, the Cu transfer from the soil to the plant was low, remaining mostly at root level; however, AMF produced changes in Cu distribution increasing the translocation to the shoots. Moreover, differences in AMF parameters (root colonization, spore number and glomalin production) were strictly dependent of the Cu level and the AMF inoculum, suggesting a specific compatibility between AMF and plants. Our results indicate that under phytotoxic Cu levels in the soil metal adapted AMF (GA) is a good option to be used in order to promote phytoremediation processes.

In summary, we conclude that in metal contaminated soil the AMF promotes mainly phytostabilization processes rather than phytoextraction ones.

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Chapter 1. General Introduction.

1. General introduction

Chile is the most important copper (Cu) producer in the world, with an annual production of about 5 million tons, which represent a 40% of the total national exportations, a third of total Cu production worldwide (data from Comisión Chilena del Cobre, COCHILCO 2010).

However, the activities associated to the Cu production have produced a negative impact over natural ecosystems, due mainly to the discharge of metals-rich wastes in areas near to production sites, this have caused a progressive Cu enrichment soils (Ginocchio, 2000). Copper is an essential trace element for normal plant growth, but an excessive accumulation of this element in soils might be highly toxic to plants and microorganisms, resulting in vegetation degradation, soil quality decrease and as a consequence affecting the normal functioning of the ecosystem (Adriano, 2001; Wong, 2003).

In Chile only few studies have been carried out on the effects of Cu accumulation to natural ecosystems, which does not relates to the magnitude of the mining activities. For instance, representative reports developed in central Chile (Ginocchio 2000; De Gregori et al. 2003; Ginocchio et al. 2004) described the strong loss of diversity in natural plant communities in a Mediterranean ecosystem affected by the deposition of particles enriched with metals from Ventanas Cu smelter (CODELCO). This degradation process was also associated with strong soil acidification due to the emission of SO_2 to the atmosphere, which resulted in acid rain. Therefore, emission from the Ventanas Cu smelter caused the loss of cover and diversity of plant communities, that at present consist only in some stress tolerant species, which are able to grow in those polluted environments (Ginocchio 2000; Ginocchio et al. 2004; González et al. 2008).

Nowadays, there are several remediation systems for the reclamation of metal contaminated soils, which involve physical, chemical or biological treatments (Mulligan et al., 2001). However, these treatments are expensive, and alter the physicochemical and biological soil properties being environmentally unfriendly (Pilon-Smith, 2005)

Recently, the potential role of higher plants in remediation of metal-polluted soils has acquired relevance (Pilon-Smiths, 2005). The use of vegetation for landscaping, stabilization and pollution control is probably the most realistic approach to the reclamation of the land impacted by metal concentrations (Robinson et al., 2007; Bolan et al., 2011). Nevertheless, an important factor that determines the successful use of vegetation in metal polluted sites is the initial plant establishment, which is often limited by metal toxicity, low nutrient contents and poor soil physical structure (Ye et al., 2002), difficulting long-term success of phytoremediation programs in metal contaminated soils.

Oenothera picensis and *Imperata condensata* are Cu metallophyte plants naturally growing in Cu polluted soils. These plants have been known to tolerate Cu toxicity, offering a potential to be used in phytoremediation programs (Ginocchio, 2000; Cornejo et al., 2008; González et al., 2008). In fact, metallophytes have evolved biological mechanisms to tolerate toxic conditions, and among those highlights the exudation of different compounds by roots, which can modify soil metal bioavailability (Nigam et al., 2001). Exudation of organic molecules by roots is considered one of the most important strategies developed by plants to tolerate high metal concentrations, because these compounds can exclude metals through their chelation in the rhizosphere or in the apoplastic space, thus avoiding their entrance into the symplast (Nigam et al., 2001).

On the other hand, there are other plant species capable to growth and develop in Cu polluted environments such as some agricultural plants. Here we propose the use of *Lupinus albus* and *Helianthus annuus*, which were selected due to their presumably tolerance to high Cu concentrations (Jung et al., 2003), together with be able to grow quickly and develop a higher biomass than metallophytes plants, being alternatives to be implemented in phytoremediation programs. Some studies have investigated the metal tolerance mechanism of those plants (Meier et al 2011a); however, few of them had compared the plant response between metallophytes and agricultural plants and its physiological behavior either alone or with the use of soil microorganisms associations, which could enhance plant establishment in metal polluted soils.

Soil microorganisms are involved in diverse biochemical processes, such as soil formation, energy transfer and nutrient cycling, which enhance and accelerate vegetation processes and thereby increase the stability of polluted ecosystems (Moynahan et al., 2002). However, managing soil microorganisms in phytoremediation should include the use of those forming symbiotic associations with plant roots such as the arbuscular mycorrhizal fungi (AMF) as prerequisite for any soil restoration program to be successful (Haselwandter and Bowen, 1996; Meier et al., 2011b).
It is well known that AMF improves plant establishment in metal polluted soils, and even, some studies conclude that the symbiosis is partly responsible for plant survival in those extreme environments (Carvalho et al., 2006; Hildebrandt et al., 2007, Meier et al., 2011b). In this sense, AM fungal colonization contributes to enhance the plant establishment, through the improvement plant nutrition, particularly phosphate and some trace elements (Reinhardt, 2007; Meier et al., 2011b). In addition, AMF improves the soil structure through the combined actions of external mycelium and glomalin production, which also has the capacity to sequester metals from soils (González-Chavez et al., 2004; Cornejo et al., 2008).

However, the specific role of AMF in phytoremediation remains until unclear. This is partly due to the existence of two opposing views about the processes used by AMF in soils polluted by metals. Some reports conclude that AMF would promote phytoextraction process (Davies et al., 2001; Khan et al., 2000; Trotta et al., 2006) while others conclude that AMF mainly promote phytostabilization ones (Audet and Charest, 2006; Citterio et al., 2005; Giasson et al., 2005; Janousková et al., 2006). The information generated in the last years has enhanced our understanding of mycorrhizal biology and the metal tolerance of plants and fungi; however, complementary studies for improving and implementing the use of AMF in phytoremediation programs are still required.

1.1 Hypothesis and Research Objectives.

1) Under increasing Cu doses, the root exudates patterns of two native metallophytes naturally grown in Cu polluted soils will differ from those of two agricultural plants.

2) In Cu polluted soils, the arbuscular mycorrhizal fungi would mainly promote phytostabilization processes rather than phytoextraction ones.

1.1.1 General objective

The general objective of this research is to evaluate the contribution of the symbiosis between plants and arbuscular mycorrhizal fungi to the phytoremediation processes of copper polluted soils.

1.1.2 Specific objectives

1. To determine the Cu tolerance of two metallophytes (*Oenothera picensis* and *Imperata condensata*) and two agricultural plants (*Lupinus albus* and *Helianthus annuus*).
2. To analyze the exudation of organics acids by metallophytes and agricultural plants, experimentally exposed at increasing Cu supply levels.
3. To evaluate the behavior and development of metallophytes and agricultural plants associated with different AMF ecotypes in Cu contaminated soils.

Chapter 2. Phytoremediation of metal polluted soils by arbuscular mycorrhizal fungi

Paper in press in Critical Review in Environmental Science and Technology, DOI: 10.1080/10643389.2010.528518

Phytoremediation of metal polluted soils by arbuscular mycorrhizal fungi

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Abstract

Human activities generate wastes, some of which contain large amounts of heavy metals/metalloids that could enter natural ecosystems and alter the activities and functioning of soil micro- and macro-organisms. Microorganisms can adapt/resist to metal stress, and some of them are able to promote the plants establishment and therefore phytoremediation process. In this context, the use of arbuscular mycorrhizal fungi (AMF), and their role in phytoremediation, has emerged as a new and interesting choice. In addition to AMF's well-known contribution to plant nutrient acquisition and growth, these fungi develop diverse mechanisms that encourage plants to grow in soils with high toxic metals concentrations. This review is concerned about the AMF metal tolerance mechanisms and its role in the promotion of in phytoremediation processes.

Keywords: Heavy metals; Arbuscular mycorrhizal fungi (AMF); metal tolerance mechanisms; phytostabilization; phytoextraction.

2.1 Introduction

Soils become polluted when potentially toxic substances are discharged in amounts that exceed the background levels, reaching prohibitively high concentrations, which affect normal ecosystem functions. Metals, generated by human activities, are among the most important pollutants of the environment. In general, heavy metals are elements whose specific density is equal to or greater than 5 g mL^{-1}, or whose atomic number exceeds 20

but do not include elements in groups I and II of the Periodic Table (Adriano, 2001). However, classification based on density, atomic weight, atomic number, or other properties does not define these elements' toxicity (Duffus, 2002). Rather, toxicity is defined by the concept of "Potentially Toxic Elements" (PTE) (Gadd, 1993) although this term is the subject of debate (Hodson, 2004). In this review, we will use the term 'metals' because it is extensively used and accepted in environmental studies.

2.2 Soils polluted with heavy metals

In the last two decades of the twentieth century, the worldwide discharge of metals reached approximately 22 Gg for Cd, 939 Gg for Cu, 783 Gg for Pb, and 1350 Gg for Zn (Singh et al., 2003). Metals in soils may derive from lithogenic (geogenic) and anthropogenic sources. The lithogenic source derives from the geological erosion of primary minerals, but this represents only a small fraction of the total input (Kabata-Pendias, 1992). The main source of metals in the environment is derived from human activities, such as mining, agriculture, and domestic wastes (Adriano, 2001), with mining being the most important (Hinde, 2000). This is because solid mine wastes have a large amount of particulate matter, and a high volume of wastewater is generated during the mining and metal extraction processes. Furthermore, in industrialized regions worldwide, the urban-based industrial processes contribute to metal addition through atmospheric deposition (Galloway et al., 1982; Gray et al., 2003). The discharge of metal-enriched solid and effluent wastes into the air, water, and soils, generate a deleterious effect in the ecosystems. This principally occurs in soil where high metal concentrations cause irreversible soil degradation, affecting its physical, chemical and biological properties, which limit the vegetation establishment and their use in agriculture (Bolan et al., 2003; Huising, 1974; Ginocchio, 2000; Ginocchio et al., 2004; Navarro et al., 2008; Reimann et al., 2001).

Metals undergo both chemical and biological transformations including retention, redox and methylation reactions while they are retained in the soil by sorption, precipitation and complexation, and removed by plant uptake and leaching (Adriano, 2001). Although most metals are not subject to volatilization losses, As, Se and Hg tend to form gaseous compounds through redox and methylation reactions (Frankenberger and Karlson, 1995).

When metal solution concentration is low and sorption surfaces large, sorption/desorption processes will govern the soil solution concentration (Tiller, 1989; Bolan et al., 1999) while the fate of metals in the soil depends on both soil properties and environmental factors.

2.3 Phytoremediation of polluted soils

According to most legislative schemes, a soil may require remediation if the concentration of one or more metals exceeds the specified threshold level in the soil profile. A number of environmental remediation systems using physical, chemical, or biological treatments have been developed in the last decades. The physicochemical treatments include soil washing, vitrification, thermal treatment, and excavation and confinement of the soil in special dumps or landfill sites (Macnair et al., 2000; Mulligan et al., 2001a). Besides being expensive (Table 2.1), these treatments may alter the soil's physicochemical and biological properties. For these reasons, physicochemical treatments are not applicable on the scale at which mining activities usually pollute soils (Khan, 2005; Zhu et al., 2004).

Table 2.1 Costs of different technologies for remediating metal-polluted soils and sediments (adapted from Glass 1999).

	Glass (1999)	**Mulligan et al. (2001b)**	
Process	**Cost (US $)**	**Cost (US $)**	**Other factors to be considered**
Containment	----	20-66/m^3	Transport/monitoring
Vitrification	75-425/ton	90-870/ ton	Long-term monitoring
Land filling	100-500/ton	----	Transport/excavation/monitoring
Chemical treatment	100-500/ton	60-290/ ton	Recycling of contaminants
Electrokinetics	20-200/ton	70-170/ton	Monitoring
Bioremediation	----	15-200/ton	Long-term monitoring
Phytoremediation	5-40/ton	----	Long-term monitoring

Recently, the potential role of higher plants in remediation of metal-polluted soils has gained momentum (Pilon-Smiths, 2005), because in contrast to physical and chemical remediation techniques the use of plants do no require the removal of polluted soils and therefore the costs involved and the impact on the ecosystem are correspondingly low.

Phytoremediation refers to a suite of technologies that use plants (and their associated microorganisms) to remove, transfer, stabilize, decrease, and/or decompose pollutants in the environment (Denton, 2007; Chaney et al., 1997; Lasat, 2001; McGrath et al., 2001). Inorganic elements, such as metals, cannot be degraded by biological or physical processes (Reimann et al., 2001); however, they can be immobilized or sequestered by biota including soil microorganisms and higher plants involving different metabolic mechanisms (Horne, 2000). Phytoremediation has been widely used by governmental agencies and industries because of its low implementation cost and acceptance by those who prefer "green technologies" over using chemicals or heavy machinery for remediating metal-polluted soils (Dietz and Schnoor, 2001; Lewandowski et al., 2006). Depending on the nature of the pollutant, soil conditions and required cleaning level, plants and their rhizosphere organisms can be used for phytoremediation in different ways (see Table 2.2):

a. Phytostabilization: Phytostabilization is based on the reduction of pollutant mobility and bioavailability through its immobilization and consequent prevention of its migration. In this way, phytostabilization reduces the possible risks of offsite contamination through leaching to ground water or dissemination by rain and wind (Salt et al., 1995).

b. Phytoextraction: is based on the extraction of metals by plants. After harvesting, the plant material is normally incinerated, and the ashes are treated as hazardous residues (Kumar et al., 1994). The recovery of plant-extracted metals from the final ashes is referred to as 'phytomining' (Meagher, 2000; Nedelkoska and Doran, 2000; Sheoran et al., 2009).

c. Phytovolatilization: Volatilization is an inherent process in the phytoremediation of organically polluted soils (Schnoor et al., 1995) where after uptake in plant tissue certain pollutants can leave the plant in volatile form (Terry et al., 1992). However, phytovolatilization as applied to inorganic contaminants is still in its infancy.

d. Phytofiltration is the use of plant roots (rhizofiltration) or seedlings (blastofiltration) to absorb/adsorb pollutants, mainly metals, from water and aqueous waste streams (Prasad and Freitas, 2003). Plant roots or seedlings grown in aerated water absorb, precipitate and concentrate toxic metals from polluted effluents (Elless et al., 2005).

e. Rhizodegradation: this is the process where plants facilitate biodegradation of organic pollutants by microbes in their rhizosphere via their own enzymatic activities (Pilon-Smits, 2005).

Plants used for phytoremediation must be genetically capable of growing in soils with a high metal concentration and have the ability to accumulate these elements in shoots or roots (Marchiol et al., 2004). Only a limited number of plants, known as 'metallophytes', fulfill the above requirements (Baker, 1987). Metallophytes that can accumulate high concentrations of metals in their shoots are called 'hyperaccumulators' (Baker, 2000; McGrath and Zhao, 2003). The aerial biomass (leaves) of hyperaccumulators may contain more than 0.01% (w/w) of Cd and Se, 0.1% of Ni, Co, Cu, and Cr, or 1% of Zn and Mn irrespective of the metal concentration in soil (Baker, 2000; Whiting et al., 2004).

The principal disadvantage of phytoremediation is that it is a slow process, which requires several years or decades to reduce metal concentrations in soil to levels that are harmless to humans and other organisms (McGrath and Zhao, 2003). This slowness is due to the limited growth and biomass production of hyperaccumulator plants (Peuke and Rennenberg, 2005) and the lack of knowledge about the interactions among plants soils and microbial communities that could limit the metal availability (Bolan et al., 2008).

Table 2.2 Different technologies of phytoremediation

Process	**Mechanism**	**Contaminant**	**Application in soils polluted with metals**	**Reference**
Phytoextraction	Hyper- accumulation	Inorganic and organic	Yes	Kumar et al., 1994
Phytostabilization	Complexation	Inorganic and organic	Yes	Salt et al., 1995
Phytofiltration	Rhizosphere accumulation	Organic and inorganic	No	Dushenkov et al., 1995
Phytovolatilization	Volatilization by leaves	Organic and inorganic	Yes	Pilon-Smits, 2005
Phytodegradation	Degradation by roots and microorganisms	Organic	No	Ji et al., 2004

2.4. Contribution of microorganisms to the phytoremediation of metal-polluted soils

Soil microorganisms are involved in diverse biochemical processes, such as soil formation, energy transfer and nutrient cycling. These processes enhance or accelerate revegetation, thereby increasing the stability of polluted ecosystems (Moynahan et al., 2002; Reynolds et al., 1999). At the same time, metals affect soil microorganisms. Continuous exposure to high concentrations of metals can induce tolerance, and promote the development of some specialized microbial populations (De la Iglesia et al., 2006; Ellis et al., 2003; Gildon and Tinker, 1983) (Table 2.3).

Table 2.3 Mechanisms of metal resistance in microorganisms (adapted and complemented from Bruins et al., 2000).

Mechanisms	**Reference**
Extracellular sequestration (biosorption)	Akthar et al., 1996; Caesar-Tonthat et al., 1995; Joho et al., 1995; Tabak et al., 2005.
Metal exclusion by permeability barrier	Comte et al., 2008; Liu and Fang, 2002; Guibaud et al., 2008.
Intracellular sequestration of metals through metallothioneins	González-Guerrero et al., 2007.
Active transport of the metal away from the cell/organism	Bruins et al., 2000; Ledin, 2000.
Enzymatic detoxication of the metal to a less toxic form	Gadd, 1993.
Reduction in metal sensitivity of cellular targets	Rouch et al., 1995.

Nevertheless, high metal concentrations, as present in mine wastes, are toxic to soil microorganisms, causing a reduction in biomass, population number, and diversity (Edvantoro et al., 2003; Giller et al., 1998; Shukurov et al., 2005). These changes in the structure of microbial communities may eventually affect the whole soil-plant-microbe ecosystem (De la Iglesia et al., 2006; Ramsey et al., 2005).

As previously mentioned, soil microorganisms have developed several mechanisms to resist and/or tolerate the toxic effects of metals, which are described in the Table 2.3 Particularly, in the case of soil fungi, the tolerance/resistance mechanisms developed include: *i)* adsorption of metals to the cell wall surface (biosorption); *ii)* transportation and cellular incorporation (bioaccumulation); and *iii)* transformation of metals through reduction, oxidation, and methylation reactions (Gadd, 1986; 1993). Of these different detoxification mechanisms, biosorption has received increased attention. The wall of fungal cells is mainly composed of polysaccharides and chitin which can act as a barrier to metal ions and other solutes, and control their uptake into the cell (Ahalya et al., 2003; Gadd, 1996). The presence in the cell wall of free amino acids together with hydroxylic, carboxylic, and other functional groups confers a negative charge to the structure, allowing it to bind ionic elements including most metals present in soil. For example, some filamentous fungi like *Rhizopus arrhizus*, *Aspergillus niger*, *Mucor rouxxi*, and *Phanerochaete chryosporium* have a large capacity for adsorbing metal ions such as Cu^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}, Mg^{2+}, $Ni^{2+,}$ and Pb^{2+}. Hence they can serve as sorbents in bioremediation processes (Bhainsa and D`Souza, 2008; Gadd, 1996; Huang and Huang, 1996; Say et al., 2001).

However, managing soil microorganisms in phytoremediation must include the use of those forming symbiotic associations like the mycorrhizal fungi as prerequisites for any soil restoration programs to succeed (Haselwandter and Bowen, 1996). In this respect several studies have shown that ectomycorrhizal and ericoid mycorrhizal fungi play an important role in the phytoremediation of metal-polluted sites (Agerer, 2001; Gadd, 1993; Martino et al., 2003). However, the most prominent symbiotic fungus for potential use in phytoremediation is the arbuscular mycorrhizal fungi (AMF) due to its ubiquity in soil environments and because this fungus have developed several strategies that allow the plant to tolerate high metal concentration in the soil (Díaz and Honrubia, 1993; Hildebrandt et al., 1999; Janousková et al., 2005; Turnau et al., 1996). Although there have been a number of reviews that describe the contributions of the AMF in metal contaminated soils (Göhre and Paszkowski, 2006; Hildebrandt et al., 2007) there has been no comprehensive review linking in detail the AMF metal tolerance mechanisms to its environmental significance.

This review aims to describe and analyze the occurrence of AMF in metal-polluted soils and their role in the promotion of phytoremediation processes.

2.5. Arbuscular mycorrhizal fungi in metal-polluted soils

Arbuscular mycorrhizal fungi belong to the phylum *Glomeromycota* (Schüßler et al., 2001), and are commonly associated with the roots of most terrestrial plants forming the so-called arbuscular mycorrhizal symbiosis (Barea, 1987). The fungus colonizes the root cortex and develops a dense external mycelium around the root. This mycelium acts as an intermediary between the soil and the plant, absorbing nutrients from soil and transporting them to the host root (Bago et al., 2000; Borie et al., 1999: Baligar and Fageria, 1997; Kaldorf et al., 1999; Kernaghan, 2005; Reinhardt, 2007; Smith and Read, 1997). In turn, fungi obtain photosynthates from the plant for their metabolic functions. Since AMF are strict symbionts, they need to interact with the plant to complete their life cycle (Azcón-Aguilar et al., 1999; Bago and Bécard, 2002).

The arbuscular mycorrhizal association is a widespread terrestrial symbiosis, involving 80-85% of vascular plants in almost all ecosystems (Smith and Read, 1997). Their presence even extends to ecosystems that are highly disturbed by human activities, including soils polluted with high metal levels (Brundrett et al., 1996; Cornejo et al., 2008: da Silva et al., 2006; del Val et al., 1999). AMF association contributes to the establishment and growth of plants, especially under adverse conditions, such as in arid and low-fertility soils (Allen, 1996) as well as in sites that are highly polluted by metals (Figure 2.1).

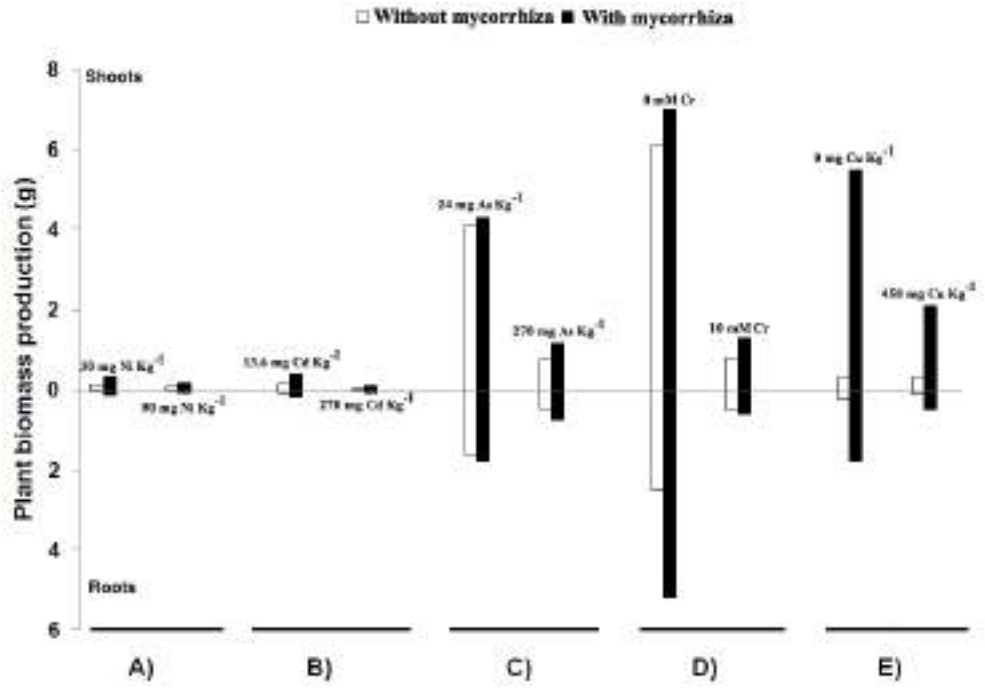

Fig. 2.1 Effectiveness of arbuscular mycorrhizal fungi in promoting plant biomass production (Shoots and Roots) in metal contaminated soils. A) *Trifolium repens* in Ni contaminated soil (Vivas et al., 2006); B) *Trifolium repens* in Cd contaminated soil (Vivas et al., 2003); C) Plants of *Zea mays* in As contaminated soil (Bai et al., 2008); D) *Helianthus annus* in Cr contaminated soil (Davies et al., 2001) and E) Plants of *Coffea Arabica* in Cu contaminated soil (Andrade et al., 2010).

This is because AMF help plants with their nutrient acquisition (Fitter, 1985; Jeffries et al., 2003) specially phosphorus (Figure 2.2) (Bolan, 1991; Jakobsen et al., 1991; Javot et al., 2007), as well other nutrients such as nitrogen (Frey and Schüepp, 1993; López-Pedrosa et al., 2006), calcium, sulfur (Vögel-Mikus., et al., 2006), potassium (Andrade et al., 2010) and zinc (Audet and Charest, 2006; Burkert and Robson, 1994). In addition, AMF improve soil structure by increasing particle aggregation and stabilizing aggregates against wind and water erosion, through the release of a glycoprotein called glomalin (which is described later) (Borie et al., 2000; Rillig and Mummey, 2006; Wright and Upadhyaya, 1998). Each one of these functions contributes to the protection of the plant against environmental stress

thus promoting soil vegetation in ecosystems that have been degraded by discharge of high metal concentrations (Gaur and Adholeya 2004; Leung et al., 2006; Smith and Read, 1997; Turnau et al., 2001).

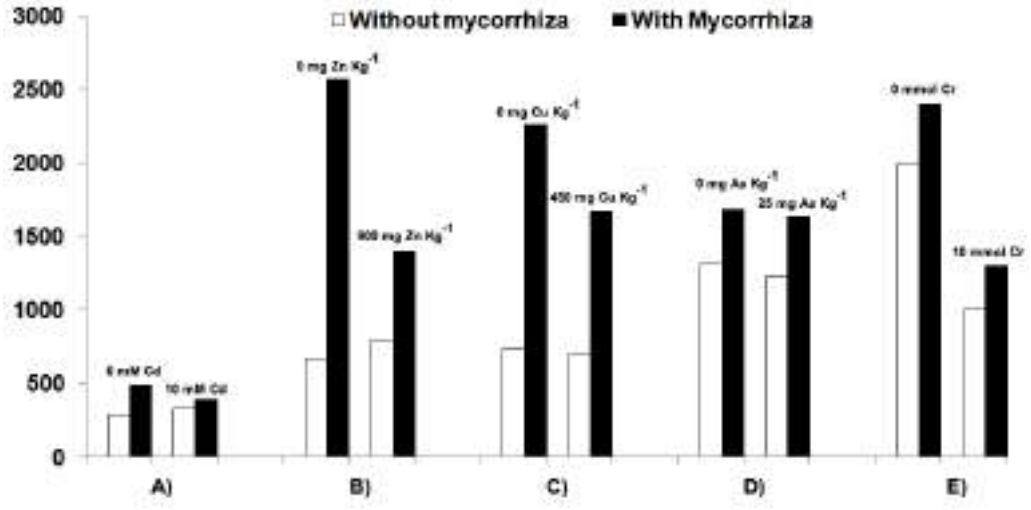

Fig. 2.2 Effectiveness of arbuscular mycorrhizal fungi promoting phosphorus shoot nutrition in metal contaminated soils. A) *Aster tripolium* growing under in Cd contaminated soil (Carvalho et al., 2006); B) and C) *Coffea Arabica* in Zn and Cu contaminated soils (Andrade et al., 2010); D) Plants of *Pteris Vittata* growing in As contaminated soil (Trotta et al 2006); E) *Helianthus annus* growing in Cr contaminated soil (Davies et al., 2001).

The ability of AMF to confer resistance to plants against metals has been reported in several studies (Barea et al., 2002; Hildebrant et al., 2007; Janousková et al., 2005). These observations and the positive effect of mycorrhizal symbiosis on the phytoremediation of metal-polluted soils are of great biotechnological interest, since mycorrhizal plants are as effective in extracting metals (e.g., Cu, Cd, Pb, and Zn) as non-mycorrhizal hyperaccumulator plants (Ebbs and Kochian, 1998; Huang and Cunningham, 1996). Mycorrhizal plants also improve phytostabilization since metals (Zn, Cd, Cu) are confined to hyphae and roots without translocating these elements to aerial parts (Joner and Leyval, 1997; 2001). As such, the metals remain in the soil but because they are less bioavailability, thus toxicity to other organisms is reduced (Leyval et al., 2002).

Therefore, the manipulation and use of the AMF as a tool for polluted soils must be considered when phytoremediation programs are designed. However, the presence and diversity of the AMF in metal-polluted soils must be examined in order to identify the suitable species that are effective in achieving this objective (Ellis et al., 2003; Gildon and Tinker 1983).

2.5.1 Presence and diversity of arbuscular mycorrhizal fungi in metal polluted soil

The presence of AMF in metal-polluted soils must be considered in terms of its ecological diversity (qualitative aspect), and functional compatibility with the endemic metallophytes and hyperaccumulator plants in the ecosystem (evaluated quantitatively through the density of fungal infection). The presences of AMF in metal-polluted soils and their ability to form an effective mycorrhiza symbiosis have been extensively investigated (Chen et al., 2005; da Silva et al., 2003; del Val et al., 1999; Leyval and Weissenhorn, 1996; Shetty et al., 1994). In addition, arbuscular mycorrhizal associations with metallophytes in highly polluted soils have been reported by several authors (Cornejo et al., 2008: del Val et al 1999: Tonin et al., 2001; Whitfield et al., 2004). Furthermore, Leung et al. (2006) have found effective mycorrhizal associations with hyperaccumulator plants, such as *Pteris vitata*, in soils polluted with As and Zn. (Wilkinson and Dickinson, 1995), and also AMF metal genetic adaptation have been observed in native AMF populations from polluted soils which shown a higher metals tolerance than those isolated from non-polluted soils (Gildon and Tinker, 1983; Hildebrant et al., 2007). However the above, the diversity of AMF ecotypes in metal polluted soils is relatively low (Pawlowska et al., 1996), because high metal concentrations tend to reduce both the density and diversity of fungal populations (del Val et al., 1999). In terms of taxonomic diversity, the AMF genera reported in literature belong mainly *Glomus* and *Gigaspora* species (da Silva et al., 2003; 2006; del Val et al., 1999), however AMF taxonomic diversity in metal polluted soils contemplates also other genera (Table 2.4).
Some studies have shown that high metal concentrations can inhibit AMF spore germination (del Val et al., 1999; Hepper and Smith, 1976), growth of the extraradical mycelium (ERM) (del Val et al., 1999), and root colonization (Gildon and Tinker, 1983). Moreover, metal tolerance can vary among the different ecotypes within the same AMF

species (del Val et al., 1999). Likewise, spore density and root colonization rates are highly variable. For instance, AMF spore numbers in metal-polluted soils can vary from 30-460 kg^{-1} soil (Leyval et al., 1995) to 3900-20700 kg^{-1} soil (Zack et al., 1982). This variability has also been observed with respect to the richness and diversity of AMF communities which strongly depend on factors that exert a high selectivity, including soil pollution level, type of metal, and host species involved (del Val et al., 1999).

Table 2.4. Arbuscular mycorrhizal fungal species and associated host plants reported growing in metal polluted soils

Arbuscular mycorrhizal fungi	**Soil contaminant**	**Host plant**	**References**
Acaulospora delicata	As	*Holcus lanatus*	González-Chávez et al., 2002(b)
Acaulospora laevis	Cd, Cu	*Zea mays*	Liao et al., 2003
Acaulospora scrobiculata	Cu, Zn	"Tropical grassland"	da Silva et al., 2003
Acaulospora spinosa	Zn, Cu, Cd, Pb	*Brachiaria sp.*	da Silva et al., 2006
Acaulospora undulata	As	*Holcus lanatus*	Gonzáles-Chávez et al., 2002b
Entrophospora infrequens	As, Cu, Zn	*Holcus lanatus*, "Tropical grassland"	da Silva et al., 2003; González-Chávez et al., 2002
Gigaspora gigantea	Zn, Cu, Cd, Pb	*Brachiaria sp.*	da Silva et al., 2006
Gigaspora margarita	As	*Peteris vitata*	Trotta et al., 2006
Gigaspora rosea	Pb, Zn, Cd, Cu, As	*Fragaria vesca*, *Holcus lanatus*	González-Chávez et al., 2002b; Turnau et al., 2001
Glomus microaggregatum	Cu, Zn	"Tropical grassland"	Da Silva et al., 2003
Glomus aggregatum	Calamine (Cd, Pb, Zn)	*Festuca ovina*, *Leontodon hispidus*	Pawlowska et al., 1996

Glomus albidum	Cu, Zn	"Tropical grassland"	da Silva et al., 2003
Glomus caledonium	Cd, Cu	*Zea mays, Sorghum vulgare*	González-Chávez et al., 2002; Liao et al., 2003
Glomus claroideum	Zn, Cd, Pb, As, Cu	*Plantago lanceolata, Sorghum vulgare, Sorghum bicolor*	del Val et al., 1999; González-Chávez et al., 2002; Orlowska et al., 2005
Glomus constrictum	Cd. Cr, Cu, Pb, Zn, As	*Zea mays, Holcus lanatus*	González- Chávez et al., 2002b; Pawlowska et al., 2000;
Glomus diaphanum	Cu, Zn	"Tropical grassland"	da Silva et al., 2003
Glomus etunicatum	Zn, Cd, Pb, Cu	*Plantago lanceolata,* "Tropical grassland"	da Silva et al., 2003; Orlowska et al., 2005.
Glomus fasciculatum	As	*Holcus lanatus*	González-Chávez et al., 2002
Glomus geosporum	Cd, Cu	*Aster tripolium*	Carvalho et al., 2006
Glomus gerdemannii	Pb, Zn, Cd, Cu, As	*Fragaria vesca*	Turnau, et al., 2001
Glomus intraradices	Cr, Pb, Cd, Cu	*Helianthus annuus, Zea mays, Agrostis capillaris*	Fred et al., 2001 Sudová and Vosátka, 2007 Sudová et al., 2008
Glomus macrocarpum	Zn, Pb	*Anthyllis cytisoides, Lygeum spartan*	Díaz et al., 1996
Glomus manihotis	Cd, Cu	*Zea mays*	Liao et al., 2003
Glomus mosseae	Cd, Cu, Zn, Pb, Cd, Cr, Ni, Hg	*Trifolium subterraneum, Sorghum vulgare, Viola calaminaria, Sorghum*	del Val et al., 1999; González-Chávez et al., 2002; Joner and

		bicolor	Leyval, 1997; Tonin et al., 2001.
Glomus occultum	Pb, Zn, Cd, Cu, As	*Fragaria vesca*	Turnau, 2001
Glomus sinuosum	Cu, Zn	"Tropical grassland"	da Silva et al., 2003
Glomus tortuosum	Cu, Zn	"Tropical grassland"	da Silva et al., 2003
Glomus versifome	Cu, Zn	*Zea mays*	Bi et al., 2003
Paraglomus occultum	Cu, Zn	"Tropical grassland"	da Silva et al., 2003
Scutellospora gilmorei	Cu, Zn	"Tropical grassland"	da Silva et al., 2003
Scutellospora gregaria	Zn, Cu, Cd, Pb	*Brachiaria sp.*	da Silva et al., 2006
Scutellospora heterogama	Zn, Cu, Cd, Pb	"Tropical grassland"	da Silva et al., 2003

On the other hand, in concordance with the AMF spore density, root colonization in metal-polluted soils is low and presents a high variability, differing to that observed for unpolluted soils (Díaz et al., 1996). For example, Gildon and Tinker (1983) found 35% of mycorrhization in clover growing in soils containing up to 863 mg kg^{-1} of Cd, while Orlowska et al. (2005) found 50% of mycorrhization in *Festuca rubra* and *Plantago lanceolata* in soils polluted with Cd, Pb, and Zn (108, 2372, and 12067 mg kg^{-1}, respectively). Working with *Kummerowia striata*, *Ixeris denticulate*, *Lolium perenne*, *Trifolium repens*, and *Echinochloa crusgalli*, Chen et al. (2005) observed a decrease in mycorrhization from 72% to 3.8% when the Pb concentration increased from 300 to 600 mg kg^{-1}.

Therefore, the diversity and functionality of AMF are strongly affected by the metals present in the soil, their concentrations, and the physicochemical characteristics of the soil. Thus, the factors influencing the diversity and functional compatibility of the plant-fungus interaction must be evaluated when analyzing the role of AMF in the phytoremediation of soils affected by high metal levels.

2.5.2 Mechanisms developed by arbuscular mycorrhizal fungi to alleviate metal stress

Most of the mechanisms that plants and AMF adopt to alleviate metal stress are quite similar, because of the strict biotrophy of AMF. The mechanisms used by AMF are summarized in the Figure 2.3: immobilization by chelating substances secreted to soil (mechanism 1, Ernst et al., 1992); binding of metals to biopolymers in the cell wall, such as chitin and glomalin (mechanism 2, González-Chávez et al., 2004); superficial immobilization in the plasmatic membrane once it crosses the cell wall (mechanism 3, Ernst et al., 1992); membrane transporter that mobilizes metals from the soil to the cytosol (mechanism 4); intracellular chelation through metallothioneins (MT, González-Guerrero et al., 2006), organic acids, and amino acids (Clemens, 2001) (mechanism 5); export of metals from the cytosol by membrane transporters (mechanism 6); and confinement of metals into the vacuoles (mechanism 7, González-Guerrero et al., 2008; Hall, 2002).

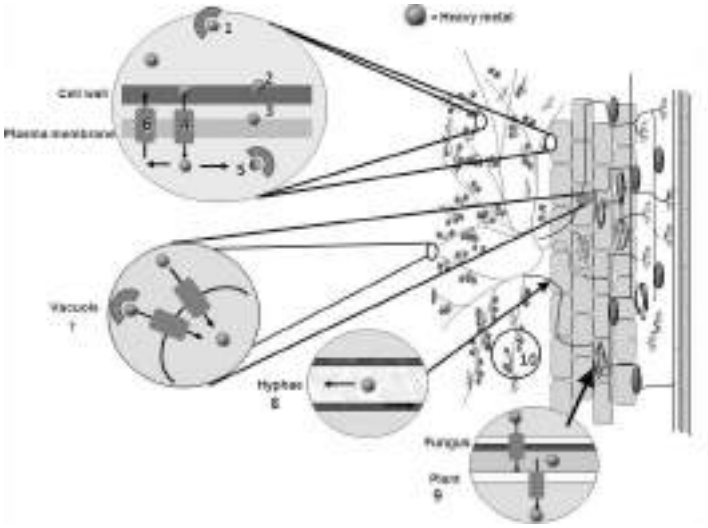

Fig. 2.3 Possible mechanisms by which mycorrhizal fungi enhance resist/tolerate metal toxicity of plants. Modified from Göhre and Paszkowski, 2006.

A mechanism against metal stress, present exclusively in AMF, involves transporting metals by means of the fungal hyphae (mechanism 8); in this case, the cenocitic hyphae (González-Chávez et al., 2002a). Additionally, membrane transporters in AMF arbuscules

may carry metals to the interfacial matrix (the contact zone between the plasma membrane of the fungus and the plant cell), and their subsequent incorporation inside the plant (mechanism 9). This may explain how some mycorrhizal plants can accumulate metals in their shoots (Ebbs and Kochian, 1998; Huang and Cunningham, 1996). It is also possible that the fungi assign some of their structures (spores) to store metals (mechanism 10). The storage of metals in spores has been described only in monoxenic culture (Ferrol et al., 2009), however recently Meier et al (unpublished data) demonstrated the accumulation of Cu in mycorrhizal spores in soils contaminated with high Cu concentration (Figure 2.4).

Thus, AMF symbiosis holds the key to protecting both partners against metal toxicity in metal-polluted ecosystems. This biotechnological tool should be kept in mind when designing phytoremediation programs.

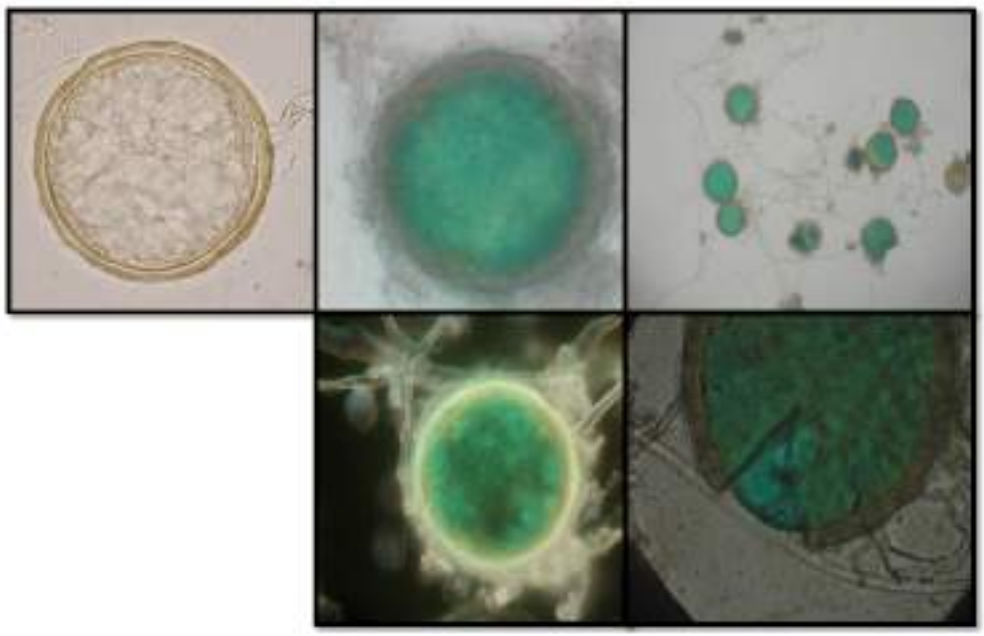

Fig. 2.4. Copper accumulation in AMF spores. A) Spore of *Glomus claroideum* in Cu unpolluted soil. B) Spores of *G. claroideum* in field condition in high Cu polluted soils (450 μg Cu kg^{-1}, Meier et al., unpublished data)

2.6. Role of arbuscular mycorrhizal fungi in the phytoremediation of metal-polluted soils

The benefits of AMF symbiosis under stress conditions produced by metals have already been mentioned and its contribution to the phytoremediation procces are illustrated and summarized in the Fig 5. Nevertheless the above, the role of AMF in phytoremediation are still not completely understood. Several studies have shown that AMF develop mechanisms that allow the metal accumulation in plant roots (Giasson et al., 2005) and prevent its translocation to the shoot, thus AMF promote phytostabilization process (Audet and Charest, 2006; Citterio et al., 2005; Giasson et al., 2005; Janousková et al., 2006). In contrast, other studies have shown that AMF promote phytoextraction, causing an increase in metal translocation to the shoots (Davies et al., 2001; Khan et al., 2000; Trotta et al., 2006). Recently, Audet and Charest (2006) have proposed a model to explain the contribution of AMF to phytoremediation. According to this model, AMF would promote phytoextraction when the metal concentration in the soil is low, while phytostabilization is enhanced at high metal concentrations.

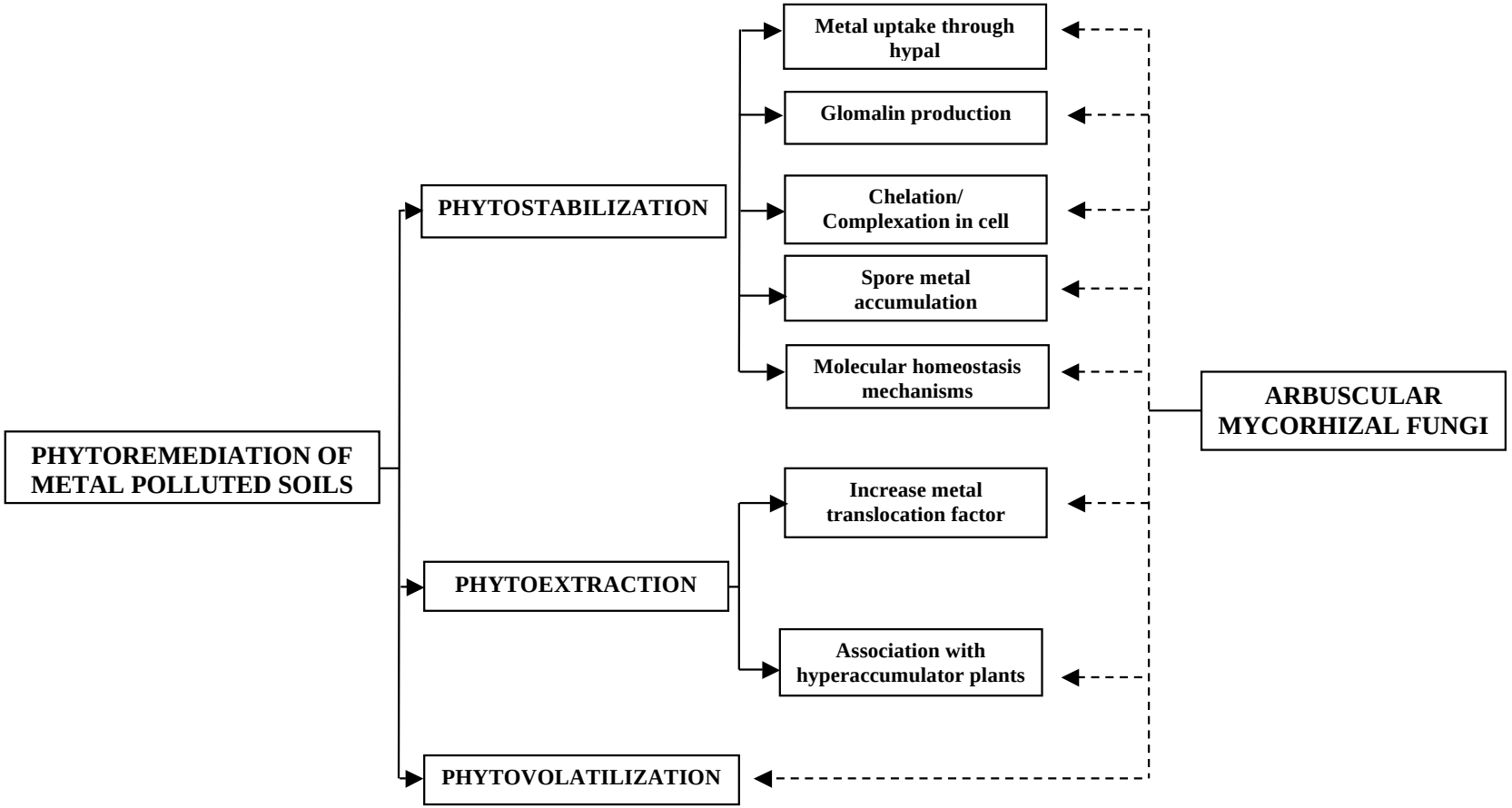

Fig. 2.5. Contribution of arbuscular mycorrhizal fungi to the phytoremediation of metal polluted soils.

2.6.1 Arbuscular mycorrhizal fungi and phytostabilization

Phytostabilization can reduce the spread of metals in soil by decreasing their leaching and consequent contamination of aquifers and also by preventing dust dispersal through wind and water erosion. Arbuscular mycorrhizal fungi participate in the immobilization of metals beyond the immediate vicinity of plant roots, creating an extensive influence zone called the 'mycorrhizosphere' (Barea et al., 2002; Giasson et al., 2005). In this zone, AMF can immobilize metals through mechanisms similar to those used by plants (see Figure 2.3, mechanisms 1 to 7). The most outstanding of these are the extracellular immobilization of metals involving adsorption to the fungal wall (Zhou, 1999) and chelation by different functional groups (González Chávez et al., 2004). There are also intracellular immobilization mechanisms, including chelation of components inside the fungal cell (Gaur and Adholeya, 2004).

2.6.1.1 Metal sequestration through arbuscular mycorrhizal fungal hyphae.

It is known that the extra-radical mycellium (ERM) of AMF can contribute to the remediation of metal-polluted soils (Table 2.5). Joner and Leyval (1997), and Joner et al. (2000) have reported that the outer surface of ERM has a larger capacity for sorbing metals than root cells. In comparing the active sorption of Cd and Zn by several *Glomus* species, these researchers found that the ERM in *Glomus mosseae* could sorb 0.5 mg Cd g^{-1} which was ten times higher than that observed with other fungal biosorbents such as *Rhizopus arrhizus* (Zhou, 1999).

Similarly, González-Chávez et al. (2002a; 2009) concluded that the ERM could sorb and accumulate high levels of Cu (3–14 mg Cu g^{-1} dry hyphae). They also described the existence of several tolerance mechanisms in different AMF isolated from the same Cu-polluted soil. The high metal sorption capacity might be related to the presence of free amino acids in the fungal cell wall, and the presence of negatively charged hydroxyl, carboxyl, and other functional groups capable of binding metallic ions, such as Cu^{2+} and Zn^{+2} (Zhou, 1999).

Other mechanisms by which the ERM of AMF can immobilize metals include the production of extracellular glycoproteins (Cornejo et al., 2008; González-Chávez et al., 2004; Wright and Upadhyaya, 1998), and the inclusion and subsequent intracellular chelation (Lanfranco et al., 2002). By reducing metal bioavailability and promoting phytostabilization, the ERM of AMF minimizes the exposure of plants to metals.

Table 2.5. Extent of beneficial effects of arbuscular mycorrhizal fungi (AMF) in phytostabilization processes of metal polluted soils

AMF	Metal	Host plant	Beneficial effect	Reference
Glomus mosseae	Cd, Zn	*Trifolium subterraneum*, *Lolium perenne*.	AMF adsorbed up to 0.5 mg Cd per g mycelia equivalent to 3 fold binding capacity of non-tolerant fungi or 10 fold higher than reported for *Rhizopus arrhizus* (commonly used as biosorption organism)	Joner et al., 2000
Glomus intraradices	Cr	*Helianthus annus*	AMF increased 5 fold root Cr concentration	Davies et al., 2001
Glomus mosseae, *Glomus caledonium* and *Glomus claroideum*	Cu	*Sorghum vulgare*	ERM increased Cu-sorption from 2.3 to 13.8 mg Cu g^{-1} dry mycelium.	González-Chávez et al., 2002
Glomus mosseae	Zn	*Trifolium pratense* L.	22% of total Zn plant uptake linked to ERM	Chen et al., 2003
Gigaspora rosea and *Glomus mosseae*	Cu	*Zea mayz* and *Sorghum vulgare*	GRSP produced by *Gi. rosea* hyphae bound up to 28 mg Cu g^{-1} and *G. mosseae* ranged 1.0-1.6 mg Cu g^{-1}	González-Chávez et al., 2004
Mixed spores of mycorrhizal fungal species isolated from orchard soil	Pb	*Kummerowia striata*, *Ixeris denticulate*, *Lolium perenne*, *Trifolium repens and Echinochloa crusgalli*	AMF inoculation increased the Pb root concentration from 7.6 to 57.2%.	Chen et al., 2005.
Indigenous mycorrhizal populations from polluted soils	Cu, Zn	*Argemone subfusiformis*, *Baccharis linearis*, *Oenothera affinis*, *Polypogon viridis*	GRSP bound from 1.4 to 28% of total Cu in soil and from 1.4 to 5.8% of total Zn.	Cornejo et al., 2008
Indigenous mycorrhizal populations from polluted soils	Pb, Zn	Degraded ecosystem with presence of *Sesleria caerulea*	GRSP bound Pb attained until 23.4 mg g^{-1} which represents about 16% of total soil Pb.	Vodnik et al. 2008

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2.6.1.2 Metal sequestration by glomalin

Glomalin (*Glomalin-related soil protein*, GRSP) is a glycoprotein produced by the hyphae of AMF (Gadkar and Rillig, 2006). The structure of glomalin has not been completely established; some studies suggest that glomalin consists of monomeric structures linked through hydrophobic interactions (Nichols, 2003). However, for soil studies the glomalin is operationally defined by extraction and detection methods and known as 'glomalin-related soil protein' (GRSP) (Nichols, 2003; Purin and Rillig, 2007; Wright and Upadhyaya, 1996; 1998).

GRSP has been detected in almost all soils in concentrations ranging from 2-15 to more than 60 mg g^{-1} (Nichols, 2003; Wright and Upadhyaya, 1998). In metal-polluted soils (Cu 62-831 mg kg^{-1}), the concentration of GRSP varied widely (6.6–37 mg g^{-1}), suggesting that its quantity depends on the level of soil contamination (Cornejo et al., 2008).

GRSP has a diversity of functions in soil. Besides promoting particle aggregation (Rillig and Mummey, 2006; Rillig, 2004; Wright and Upadhyaya, 1998), GRSP may have a physiological role by decreasing the palatability of AMF to soil microorganisms (Purin and Rillig, 2007). Recent studies suggest that GRSP can also sequester toxic elements through functional groups in its structure (Cornejo et al., 2008; González-Chávez et al., 2004; González-Chávez et al., 2009; Vodnik et al., 2008). The metal-binding capacity of GRSP varies with soil type and some physicochemical parameters, such as pH and redox potential (Chern et al., 2007; Nichols, 2003; Wright and Upadhyaya, 1998). Using soils polluted with Cu, Cd, and Pb, González-Chávez et al. (2004) showed that GRSP could sequester up to 4300 mg of Cu, 1120 mg of Pb and 80 mg of Cd per kg GRSP. Vodnik et al. (2008), working with Pb-polluted soils, found that the quantity of contaminant linked to the GRSP ranged from 690 to 23400 mg kg^{-1}, corresponding to 0.8–15.5% of the total Pb present in the soil. Moreover, the quantity of GRSP was strongly and positively related to the concentration of Pb in the soil ($r = 0.90$, $P < 0.01$). Likewise, Cornejo et al. (2008) observed a high correlation between the GRSP concentration and the content of Cu and Zn in some Cu-polluted soils ($r = 0.89$ and 0.76 for Cu and Zn, respectively, $P < 0.001$), and the metal linked to GRSP corresponding to 1.44–27.5% of the total Cu content and 5.8% of the total Zn content in the soils. This evidence suggests an active role of GRPS in sequestering some

potentially toxic metals, and this protein's efficiency in mitigating metal stress of plants growing in metal-polluted soils.

2.6.1.3 Molecular metal homeostasis mechanisms

Metal tolerance in AMF can be achieved through the action of several homeostatic mechanisms. Besides being able to immobilize metals on their surface (through the GRSP), AMF have developed diverse mechanisms of metal chelation at intracellular level (González-Chávez et al., 2002b; González-Guerrero et al., 2005; 2009). The cell wall constitutes the first protective barrier against metal uptake. However, in highly polluted soils a fraction of the metal ions may cross this barrier, and reach the fungal cytoplasm. Chelating agents in the cytoplasm can deactivate metals, and hence minimize cell damage by excessive intrusion of metals. Among the best-characterized metal chelators in AMF are the metallothioneins (MTs) (Vasak and Hasler, 2000).

Metallothioneins are polypeptides consisting of 70-75 amino-acids with a high content of cysteine, an amino-acid capable of forming stable complexes with cations through its sulfhydryl groups (Kojima, 1991). Metallothioneins are involved in maintaining cellular homeostasis against high metal concentrations (Cobbett and Goldsbrough, 2002). However, the production of MTs may be induced by metals as well as by hormones (Haq et al., 2003). In the case of AMF there are different studies about the interaction between the MTs and metals and their contribution in the phytostabilization processes. This has been confirmed through experiments of MTs over expression in several organisms. For example, the insertion of the gene *CUP1* (from yeast) in cauliflower resulted in an increase of the Cd tolerance and up to 16-fold accumulation (Hasegawa et al., 1997). Transgenic tobacco plants with the same gene and inoculated with AMF, were exposed to 4 different Cd doses. These plants presented a lower Cd concentration in their shoots and leaves when compared to non-transgenic and uninoculated tobacco of the same variety (Janousková et al., 2005).

So far, four AM fungal genes involved in maintaining cellular homeostasis against metals have been characterized: i) *GrosMT1* in *Gigaspora rosea* (Stommel et al., 2001), ii) the Zn transporter *GinZnT1* in *Glomus intraradices* involved in vacuolar Zn compartmentalization (González-Guerrero et al., 2005); iii) *GmarMT1* in *Gigaspora*

margarita (BEG 34), which codes for MTs that regulate the fungal redox potential and protect it against the oxidative stress produced by some metals, such as Zn or Cd (González-Guerrero et al., 2006; 2007); and iv) *GintABC1,* which codes for a polypeptide of 434 amino acids that participates actively in Cu and Zn detoxification (González-Guerrero et al., 2006).

2.6.2 Arbuscular mycorrhizal fungi and phytoextraction

Phytoextraction is a recent, cost-effective and attractive green technology for the remediation of soils affected by mining processes (McGrath and Zhao, 2003; Pilon-Smith, 2005; Quartacci et al., 2006; Shah and Nongkynrih, 2007). The process uses hyperaccumulator plant species able to take up, tolerate, and develop phytomass even in soils with high metal concentrations. Hyperaccumulator plants can accumulate metals in their shoots, allowing the metals to be extracted by harvesting the plant shoots. This represents a considerable advantage over phytostabilization (where the metals remain in the soil in a harmless or not bioavailable form). However, phytoextraction is a slow process in that several years are required to decrease metal concentrations to a harmless level. The slowness of the process is mainly due to the interaction of the two components: the soil and the plant: (Barceló and Poschenrieder, 2003).

i) Soil represents the first component and is the main factor limiting metal bioavailability. Complex chemical interactions between plant, metal and soil make the absorption of metals by plants difficult, and reduce the effectiveness of the process (Adriano, 2004).

In some cases, phytoextraction can be improved by applying synthetic chelating substances, such as diethylene triamine pentaacetic acid (DTPA), ethylenediamine tetraacetate (EDTA), and ethylenediaminedissuccinate (EDDS), thereby increasing metal availability in the soil (Liu et al., 2008; Sudová et al., 2007). These substances can accelerate the absorption of metals in shoots and roots while the AMF promote plant growth (Adel and Hashem, 2009). However, these chelating agents are usually non-biodegradable and non-selective in their action. Their use would also increase the risk of metal leaching and the contamination of subterranean waters (Barona et al., 2001).

ii) The second component is the plant. Hyperaccumulators have to produce a large phytomass because the amount of metals extracted is proportional to the rate of plant growth (Shah and Nongkynrih, 2007). It is also necessary that these species accumulate high metals quantities in their tissues. Unfortunately, there is no wild or identified plant species having both these properties (Li et al., 2003).

Another aspect to consider is that most hyperaccumulator plants, including families like *Brassicaceae, Plumbaginaceae, Juncaceae, Caryophyllaceae, Juncaginaceae, Amaranthaceae*, and some members of the family Fabaceae do not develop arbuscular mycorrhizas. Exceptions have been reported for Ni hyperaccumulators belonging to the *Asteraceae* family (Leyval et al., 1997; Turnau and Mesjasz-Przybylowics, 2003) and some Pteridophytes (Gaur and Adholeya, 2004). Among the above-mentioned plants, *Pteris vittata* L. is a well known As hyperaccumulator, because it can produce a large phytomass, an extensive root system, and is fast-growing and perennial. These characteristics make this species potentially useful for phytoextraction programs (Ma et al., 2001). The association of *P. vittata* with AM increases its capacity to uptake As up to 88.1 mg As kg^{-1} dry weight compared to 60.4 mg As kg^{-1} in the non-mycorrhizal plants (Leung et al., 2006). This agrees with other results demonstrating an increase in As translocation when this plant is associated with AM (Trotta et al., 2006; Table 2.6). However, the information on AMF-As interactions in *P. vittata* is still very limited and more studies are required, particularly on the role of AMF in increasing the efficiency of phytoremediation of As-contaminated soils (Liu et al., 2009).

Despite the many studies carried out during past two decades, the role of mycorrhiza in phytoextraction processes remains uncertain. Tonin et al. (2001) have shown that AM can enhance metal transfer from soil to plant (translocation factor), thus promoting phytoextraction. Whereas, Weissenhorn and Leyval (1995) have indicated that high metal accumulation resulting from AM colonization can in fact inhibit plant growth and development.

Table 2.6 Extent of beneficial effects of arbuscular mycorrhizal fungi (AMF) in phytoextraction processes of metal polluted soils.

AMF	Metal	Host plant	Beneficial effect	References
Glomus geosporum	Multicontaminated soil	*Aster tripolium* L.	AMF increased 2.2 and 4.1 the Cd and Cu translocation factor,	Carvalho et al., 2006
Glomus etunicatum and G. intraradices	As	Grass Mix (*Festuca rubra, F. eliator, Agropyron repens* and *Trifolium repens*)	Inoculated plants extracted 3.8 and 14 fold than uninoculated plants, respectively.	Giasson et al., 2006
Glomus intraradices, G. mosseae	Zn	Grass Mix (*Festuca rubra, F. eliator, Agropyron repens* and *Trifolium repens*	Inoculated plants extracted 5.0 and 7.0 fold than un inoculated plants, respectively.	Giasson et al., 2006
Glomus intraradices and G. mosseae	As	*Pteris vittata* L.	AMF increased As translocation factor by 5,8 and 14,6 fold, respectively	Trotta et al., 2006
Indigenous mycorrhizal populations from polluted soils	Multicontaminated soils	*Pteris vittata* L.	As concentration in leaves increased 24 fold in mycorrhizal plants compared with other species evaluated	Leung et al., 2006

The mycotrophic status of hyperaccumulator plants belonging to the *Brassicaceae* family, with particular reference to *Thlaspi caerulescens*, has received worldwide attention in relation to phytoremediation (Fischerová et al., 2006). Hirrel et al. (1978) have reported that *Thlaspi caerulescens* does not form AM associations. However, carrying out an extensive bibliographic analysis, DeMars and Boerner (1996) found that 18.9% of the 946 species investigated had AM associations. Similarly, Vögel-Mikus et al. (2006) and Pongrac et al. (2009) found that *T. praecox* presented AM association although the colonization rate was low and variable. They have concluded that the AMF association in *T. praecox* plays an important role in enhancing phytoextraction by increasing the translocation of Zn, Cd, and Pb.

These contradictory results suggest that further studies are needed to evaluate the role of AMF in metal phytoextraction. The presence and diversity of metallophytes and hyperaccumulator plants, especially in geographical areas where the information is limited, deserve wider investigation (Ginocchio and Baker, 2004).

2.7. Conclusions and future perspectives

There is plenty of literature, which proves the important role of AMF in promoting both plant establishment and plant survival in metal polluted soils. Nevertheless the above, seems premature still to speak of field application of AMF in the phytoremediation of metal polluted soils This is partly due to the existence of two opposing views about the processes used by AMF in soils polluted by heavy metals (AMF would promote phytoextraction process or the AMF promote phytostabilization process), and of a third mechanistic hypothesis embracing the former two processes. In this last hypothesis, AMF promote phytoextraction when the metal concentration in the soil is low, while phytostabilization is enhanced at high metal concentrations.

The information generated in the last years has enhanced our understanding of mycorrhizal biology and of the metal tolerance of plants and fungi; however, are necessary more studies in this area for improving and implementing the use of AMF in phytoremediation programs

The new researches into this topic should focus on the process optimization, including determining of physiological mechanisms involved in metal absorption, translocation, and

metabolism by the plants, and describing the genetic control mechanisms. Concerning the role of AMF in phytoremediation, it is very important to ascertain their presence and diversity in polluted soils as well as their functional compatibility with metallophytes and hyperaccumulator plants. Also is necessary to study about the physiological mechanisms involved in metal tolerance by AMF, including intracellular metal chelation and the role played by MTs.

In summary, we believe that the future designs of phytoremedation programs must include the presence of AMF for improve it efficiency and effectiveness.

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Chapter 3. Influence of copper on root exudate patterns in some metallophytes and agricultural plants

Paper accepted in Ecotoxicology and Environmental Safety, DOI 10.1016/j.ecoenv.2011.08.029

Influence of copper on root exudates patterns in some metallophytes and agricultural plants

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Abstract

A hydroponic experiment was carried out to determine the root exudation patterns in two Cu-metallophytes (*Oenothera picensis* and *Imperata condensata*) and two agricultural plants (*Lupinus albus* and *Helianthus annuus*). Plants were grown in nutrient solution at increasing Cu doses (0, 0.125, 0.25, 0.5, 1 and 2 mg Cu L^{-1}), and plant growth, root elongation, Cu accumulation and root exudates were measured. All plants showed a decrease of over 60% in root elongation at the highest Cu supply level, being *O. picensis* the most sensitive specie and showing the highest shoot and root Cu concentrations (116 and 2657 μg Cu g^{-1}, respectively), which were six fold higher than the other species. Differences in root exudation patterns of low molecular weight organic acids were found, with extremely high amounts of succinic acid exuded by *O. picensis* (1049 μmol g^{-1} h^{-1}), and citric acid by *I. condensata* (164 μmol g^{-1} h^{-1}). In metallophytes, the organic acid exudation was increased even with no root elongation, meanwhile agricultural plants exuded citric acid at constant levels. Exudation of phenolic compounds was highly species-dependant, with catechin mainly exuded by *I. condensata*, (2.62 μmol g^{-1} h^{-1}) cinnamic acid by *O. picensis* (5.08 μmol g^{-1} h^{-1}) and coumaric acid exclusively exuded by *H. annuus* (13.6 μmol g^{-1} h^{-1}) at high Cu levels. These results indicated that differences in root exudation patterns among metallophytes and agricultural plants could affect their Cu tolerance. Particularly, the higher

exudation rate showed by *I. condensata* can be an effective exclusion mechanism to tolerate high Cu concentrations, supporting its use in Cu phytostabilization programs.

Keywords: Copper toxicity, Metallophytes, Organic acids, Phenolic compounds, Root exudates.

3.1. Introduction

Copper (Cu) mining activities have produced detrimental effects on natural ecosystems due to the high amount of Cu-enriched particulate matter deposited on soils, limiting plant root growth and cover with a concomitant reduction of water and nutrient uptake capacity (Li et al., 2006). Plant communities present in Cu polluted soils are dominated by few metallophyte species, which are able to grow under these restrictive conditions (Baker, 1987; Ginocchio and Baker, 2004). In fact, metallophytes have evolved biological mechanisms to resist/tolerate toxic conditions of metalliferous soils and are typically endemic from the native plant communities present in the area (Whiting et al., 2004). Among the tolerance mechanisms developed by these plants, highlight the exudation of different compounds, which can modify soil metal bioavailability (Nigam et al., 2001). Particularly, the low molecular weight organic acids (LMWOA) exuded by roots have been studied due to their potential to stimulate microbial growth, mobilize low soluble nutrients (e.g., P, Fe, Zn) and produce detoxification of some harmful metals (Jones and Darrah, 1994; Neumann and Römheld, 1999; Dakora and Phillips, 2002). Exudation of organic molecules by roots is considered one of the most important strategies developed by plants to tolerate high metal concentrations, because these compounds can exclude metals through their chelation in the rhizosphere or in the apoplastic space, thus avoiding their entrance into the symplast (Nigam et al., 2001).

The most common and effective root exudates binding metals and metaloids such as As, Cr, Cd and Pb, are citrate, oxalate and malate anions, among others, being the most studied (Jones and Darrah, 1994; Magdziak et al., 2011). In addition, some studies using agricultural plants have shown that stressed roots can exude diverse LMWOA for overcoming such limitations, including citric, oxalic and succinic acid, which could play a

very important role in alleviating Cu phytotoxicity (Evangelou et al., 2006; Yuan et al., 2007).

Nevertheless, few studies have been conducted to assess the exudation of other organic compounds by plant roots that also have the capacity to exclude Cu, such as phenolic compounds (PhC) (Jung et al., 2003; Borges et al., 2005). In recent years, PhC has been reported to act as antioxidants in stressed plants, effectively preventing the oxidative stress produced by high metal concentrations (Górecka et al., 2007). This finding may be due to their high tendency to chelate metals. In particular, the hydroxyl and carboxyl groups of polyphenolic compounds can potentially bind Cu and Fe more strongly than many LMWOA, excluding citric acid (Jung et al., 2003; Martell and Smith, 1989). Root exudation of PhC associated with Cu presence in the rhizosphere can vary among plant species and in different tissues as well as at increasing Cu concentrations (Ali et al., 2006). In this sense, plant species react differently to Cu excess; however, differences in plant responses to this metal ion seem to depend not only on its concentration but also on the capability of plants to increase protection against oxidative stress produced by the metal stress (Azcón et al., 2009; Meier et al., 2011).

In this context, it is possible to hypothesize that Cu-adapted/tolerant metallophyte species may produce higher amounts of root exudates, which can restrict metal acquisition by plants, thus improving their metal tolerance. Nevertheless, root exudation (either LMWOA or PhC) by Cu-metallophyte plants has been scarcely studied. In addition, there are no studies contrasting quantity/quality of root exudates among metallophytes and Cu-sensitive plants and the role of this mechanism in improving metal tolerance. Differences in root exudation levels and/or the identity of LMWOA and PhC either between or within plant species may account for differences on Cu tolerance in plants growing in Cu polluted environments. Therefore, the aim of this work was to determine in hydroponic conditions the effect of increasing Cu doses on the root exudates patterns of two native metallophytes present in highly Cu polluted soils and to compare their behavior with two agricultural plants.

3.2. Material and methods

3.2.1 Plant species

Two metallophytes, *Oenothera picensis* (Onagraceae) (formely named *O. affinis*), and *Imperata condensata* (Poaceae) (formerly named *Polypogon viridis*) were selected. Both species have been previously described as Cu tolerant plants (Cornejo et al., 2008; González et al., 2008). Seeds of *O. picensis* and stolons of *I. condensata* were collected from several plants that were growing in Cu-polluted soils (up to 831 mg Cu kg^{-1} soil) to produce plantlets. The collection area was a Mediterranean ecosystem strongly affected by atmospheric deposition of Cu-enriched particles, located approximately at 1.5 km southeast from the Ventanas copper smelter (CODELCO) in the Puchuncaví Valley, central Chile (32°46′ 30″ S; 71° 28′ 17″ W). On the other hand, commercial seeds of the agricultural plants *Helianthus annuus* (sunflower) and *Lupinus albus* cv. Rumbo-B were selected due to their presumably tolerance to high Cu concentrations **(**Jung et al., 2003**)** together with the potential of both species to be cropped in central Chile. The seeds and stolons of the plants evaluated in this study were surface sterilized with 2% Cloramin-T solution for five minutes and rinsed thoroughly.

3.2.2 Plant growth conditions

Dose-response metal assays in nutrient solutions (Schat and Ten Bookum, 1992) were used to evaluate the root exudate patterns, Cu uptake and plant growth. Plantlets of all species were grown in sterile perlite/sand/vermiculite substrate (1:1:1, v:v:v) supplemented with dH_2O and maintained in a growth chamber (21 ± 2 °C day; 15 ± 2 °C night; photoperiod regime day/night 14/10 h; 60% of relative humidity). After two weeks, the plantlets were transferred to 1-L polyethylene containers, filled with a modified Hoagland nutrient solution and maintained under continuous aeration by air pumping. The composition of the nutrient solution was as follows (in mM): 1.3 $MgSO_4$, 2.0 $Ca(NO_3)_2$, 2.0 KNO_3, 2.0, K_2HPO_4, and (in µM) 0.2 $CuSO_4$, 1.0 $ZnSO_4$, 2.0 $MnCl_2$, 20 H_3BO_3, 0.1 $(NH_4)_6Mo_7O_{24}$ and 200 FeEDTA. The pH of the solution was adjusted to 5.5 using HCl or NaOH solution as required. The

containers were placed into a plant chamber room under controlled conditions (as previously mentioned). After 4 weeks of growing in this culture condition, the longest root of each plant was measured, and then the plants were exposed to increasing Cu concentrations by adding to the basal nutrient solution the equivalent to: 0 (control), 0.125, 0.25, 0.5, 1.0 and 2.0 mg Cu L^{-1}, supplied as $CuSO_4 * 5H_2O$. The final Cu supply dose in the medium was tested using AAS (Perkin-Elmer 3110), and the results were compared through analytical curves obtained by serial dilution of a standard Copper solution 1000 ppm in 1 M nitric acid (Fisher Scientific, UK Limited). Thus, the measured Cu exposure was as follows: 0.01, 0.14, 0.28, 0.57, 1.11 and 2.25 mg Cu L^{-1}. The differences between nominal and real Cu supply doses were not significant by means of a one sample *t*-test; therefore, we used the nominal exposure concentrations in the analysis. This test solution was replaced every two days to prevent depletion of nutrients and changes in pH and Cu concentration. The plants grew in these conditions for 10 days, and then the root elongation was determined after to the collection of root exudates, as indicated below.

3.2.3 Collection and analysis of root exudates

Exudates were collected using the methodology described by Rosas et al. (2007) with minor modifications. Plants with intact roots were rinsed thoroughly with 50 mL of deionized water (<1 μS cm^{-1}) and then immersed in deionized water under constant aeration for 1 h. The solution was filtered (0.45 μm) and freeze-dried. This short period of exudation was chosen to minimize microbial degradation of organic acids, which increases at longer elution periods (Jones and Darrah, 1994). Chromatographic analysis was conducted in an HPLC system (Merck-Hitachi model L-4200) equipped with a UV-visible detector and a Sphere Column Heater (Phenomenex Terma model TS-130). To quantify the LMWOA concentration, the residue was resuspended in 300-500 μL of deionized-sterile water for HPLC injection and filtered (0.22 μm). Chromatographic analysis was conducted on a 250 × 4 mm reverse phase column (LiChrospher 100 RP-18, 5 mm particle size, Merck, Darmstadt, Germany). The mobile phase was H_2O-CH_3OH buffered with 200 mM orthophosphoric acid at pH 2.1. The gradient started with 80/20 H_2O/CH_3OH (v/v) reaching 77/23 H_2O/CH_3OH (v/v) and remained constant for 3 min and returning to 80/20 H_2O/CH_3OH (v/v) for 5 min.

The flow rate used was 1 mL min^{-1}, the injection volume was 20 μL and the detection wavelength was λ=210 nm. Identification of LMWOA was performed by comparison of retention times and by addition of standards for each organic acid. Preliminary observations made with standard of organic acids showed that recovery levels of these compounds were close to 98%.

The identification and quantification of PhC were performed using the same extract utilized for LMWOA detection. However, a general screening was first conducted to determine the plant species and Cu-levels that presented exudation of these kinds of compounds. In this sense, PhC exudation was only detected at lower Cu doses (0, 0.125 and 0.250 mg Cu L^{-1}) and at the highest Cu concentration (2.0 mg Cu L^{-1}). Separation was made in an RP-18 column (12.5 x 0.4 cm, particle size 5 µm, Merck, Darmstadt, Germany) at 25ºC using a mixture of formic acid 5% in water (A) and methanol (B) as the mobile phase. The analysis of the PhC was performed using an isocratic run from 0 to 10 min, with a mixture of 70% A and 30% B, followed by a gradient up to 100% B at 70 min. The compounds were detected at λ=290 nm with 0.001 sensitivity. The injection volume used was 10 µL. The PhC identification was made using the following standards: catechin, ferulic acid, p-coumaric acid, cinnamic acid, myricetin, kaempferol, quercetin, caffeic acid, galangin, pinocembrin, apigenin, caffeic acid phenyl ester and resveratrol (Sigma, USA). For PhC quantification, the signal areas obtained in the chromatogram were integrated and interpolated in a calibration curve of catechin, cinnamic and p-coumaric acids.

3.2.4 Determination of Cu in plant samples

After the end of the assay, the Cu content in roots and shoots were analyzed. The plant roots were thoroughly rinsed in abundant deionized water. After that, plant were separated into roots and shoots and then dried at 60 ºC in a forced-air oven for 48 h and weighed. The tissue samples obtained were crushed, ground, ashed at 550ºC and digested using a H_2O/HCl/HNO_3 mixture (8/1/1, v/v/v). The Cu content was determined in a flame atomic absorption spectroscopy (Perkin-Elmer 3110).

3.2.5 Experimental design and statistical analysis

A nested full-randomized design was used to determine the main effect of the following factors: plant species (four plant species), Cu supply dose (six increasing Cu doses) and the nested factor Cu-into-plant, and its interaction on the variables studied. Each combination had six repetitions (N=6). The main effects were tested by means of a hierarchical ANOVA. If the *P* value indicated significant differences between Cu treated plants and control plants ($P<0.05$), post hoc pair-wise comparisons were performed using an orthogonal contrast test (Petersen, 1977). The exudation of citric and succinic acids were analyzed through lineal regression analysis to establish their dependence respect to the root elongation and root Cu concentration, using the Pearson correlation coefficient (R). The data sets obtained for all plants were subjected to principal component analysis (PCA), and the correlation among the different variables and the principal components (PC) obtained were also analyzed using R. A non-hierarchical cluster analysis using Ward's method was performed for grouping the different experimental treatments. Statistical analyses were performed using the SPSS software v. 10.0 (SPSS Inc., Chicago, Il).

3.3. Results

Most of the analyzed variables were significantly influenced by plant species (root elongation, root and shoot production, root and shoot Cu content and citric, oxalic and succinic acid exudation; Table 3.1), whereas the Cu doses applied to the different plant species only influenced shoot and root Cu concentration (Cu-into-plant). In general terms, plant growth parameters were not affected by the Cu concentrations, with the exception of root elongation (Table 3.1). Citric acid exudation showed a high dependence on the plant species and Cu dose (Table 3.1).

Table 3.1 *F*-values and probabilities of significance for the main effects of Plant species, Cu dose and the nested factor Cu-into-plant for the variables measured and analyzed by means of a hierarchical ANOVA.

	***F*-values**		
Variables	**Plant**	**Cu dose**	**Cu-into-plant**
Root elongation	13.5**	7.1**	0.5ns
Shoot dry weight	71.1**	1.3ns	1.2ns
Root dry weight	59.9**	1.7ns	0.9ns
Shoot Cu Concentration	134.8**	176.7**	107.4**
Root Cu Concentration	323.1**	118.4**	21.8**
Citric acid	305.7**	22.3**	33.1**
Fumaric acid	0.7ns	0.8ns	0.9ns
Oxalic acid	19.4**	11.8**	13.4**
Succinic acid	19.9**	3.7*	3.1**

Significance conventions: *$P<0.05$; **$P<0.01$; ns=no significance. In each combination six repetitions were considered.

Table 3.2 Root elongation (cm) of the main root of the plant species after exposition to increasing Cu doses.

	Cu dose (mg Cu L^{-1})					
Plant species	**0***	**0.125**	**0.250**	**0.5**	**1.0**	**2.0**
Oenothera picensis	2.8 ± 0.4^{a}	2.0 ± 0.62^{ab}	1.6 ± 0.2^{ab}	1.1 ± 0.3^{ab}	0.7 ± 0.2^{b}	0.6 ± 0.3^{b}
Imperata condensata	3.5 ± 0.8^{a}	2.3 ± 0.6^{ab}	1.7 ± 0.6^{abc}	1.7 ± 0.3^{abc}	1.5 ± 0.3^{bc}	1.3 ± 0.3^{c}
Lupinus albus	5.0 ± 0.3^{a}	4.4 ± 1.0^{a}	4.9 ± 0.7^{a}	4.9 ± 0.7^{a}	1.9 ± 0.4^{b}	1.8 ± 0.6^{b}
Heliantus annuus	6.5 ± 0.6^{a}	3.6 ± 0.5^{bc}	4.7 ± 0.8^{ab}	1.9 ± 0.4^{cd}	1.3 ± 0.3^{cd}	1.1 ± 0.1^{d}

Values are mean ± standard error. Different letters on each row indicate significant differences (P<0.05) using the orthogonal contrast test (N=6).*The Cu levels are expressed as mg Cu L^{-1} added to a basal Hoagland nutrient solution.

Table 3.3 Shoot and root dry weight, (mg $plant^{-1}$) of the plant species after exposition to increasing Cu doses.

	Shoot dry weight (mg $plant^{-1}$)						**Root dry weight (mg $plant^{-1}$)**					
Plant species	**0***	**0.125**	**0.250**	**0.5**	**1.0**	**2.0**	**0**	**0.125**	**0.250**	**0.5**	**1.0**	**2.0**
Oenothera picensis	$82{,}5 \pm 4.7^{a}$	85 ± 2.8^{a}	$62{,}5 \pm 8.5^{ab}$	65 ± 2.8^{ab}	60 ± 9.12^{ab}	45 ± 2.8^{b}	14.1 ± 2.4^{a}	18.5 ± 1.0^{a}	16.2 ± 4.2^{a}	15.2 ± 1.1^{a}	12.7 ± 1.9^{a}	10.8 ± 1.6^{a}
Imperata condensata	345 ± 28^{a}	430 ± 75^{a}	467 ± 43^{a}	332 ± 11^{a}	492 ± 55^{a}	322 ± 55^{a}	$98{,}6 \pm 23^{ab}$	147 ± 14^{a}	157 ± 10^{a}	110 ± 17^{a}	161 ± 42^{a}	38.2 ± 6.6^{b}
Lupinus albus	310 ± 66^{a}	385 ± 61^{a}	368 ± 6.5^{a}	331 ± 12^{a}	311 ± 18^{a}	293 ± 17^{a}	66 ± 10^{a}	77 ± 6.4^{a}	68 ± 5.5^{a}	52 ± 3.2^{a}	62 ± 3.9^{a}	53 ± 4.3^{a}
Heliantus annuus	434 ± 16^{ab}	344 ± 28^{bc}	433 ± 19^{ab}	401 ± 28^{ab}	272 ± 63^{c}	482 ± 72^{a}	92.2 ± 6.3^{ab}	68.1 ± 8.2^{b}	101 ± 9.2^{a}	89.4 ± 8.7^{ab}	50.4 ± 5.3^{b}	70.4 ± 16.9^{b}

Values are mean ± standard error. Different letters on each row indicate significant differences (P<0.05) using the orthogonal contrast test (N=6).

*The Cu doses are expressed as mg Cu L^{-1} added to a basal Hoagland nutrient solution

In addition, Cu added produced a significant decrease in root elongation in all plant species (Table 3.2). The metallophyte *O. picensis*, presented 78% inhibition in root elongation, while *Imperata condensata* and *L. albus* presented around 60%. Nevertheless, the most affected plant was *H. annuus*, which showed 83% inhibition in root elongation at the highest Cu dose (Table 3.2). Finally, shoot growth was not significantly reduced due to increasing Cu doses (Table 3.3), with the exception of *O. picensis*, which showed 45% of growth inhibition.

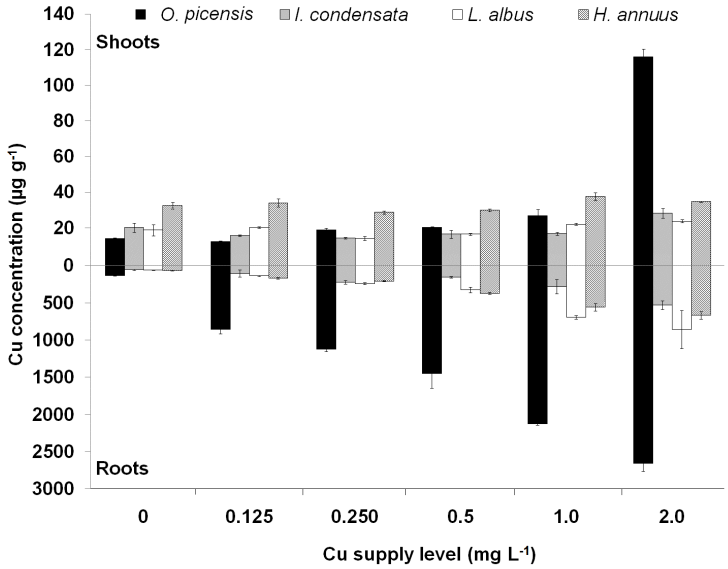

Fig. 3.1 Copper (Cu) concentration in shoots and roots of four plant species after exposition to increasing Cu doses (0; 0.125; 0.250, 0.5; 1.0 and 2.0 mg Cu L^{-1}). Values are means ± S.E (N=6).

The shoot Cu concentrations for almost all species evaluated were about 20 µg Cu g^{-1}, with the exception of *H. annuus* and *O. picensis*, which reached 35 and 116 µg Cu g^{-1}, respectively, at the highest Cu dose (Fig. 3.1). However, the highest differences in Cu concentration among the plant species evaluated were observed in root tissues (Fig. 3.1). Copper root concentrations ranged from 330 to 660 µg Cu g^{-1} at the highest Cu doses; however, *O. picensis* reached up to 2660 µg Cu g^{-1}, which was four-fold higher than in the other plant species (Fig. 3.1).

Only four LMWOAs were detected in the plant species evaluated: succinic, oxalic, citric and fumaric acids, which were strictly dependent on the plant species analyzed and the Cu dose (except fumaric acid) (Table 3.1; Fig. 3.2).

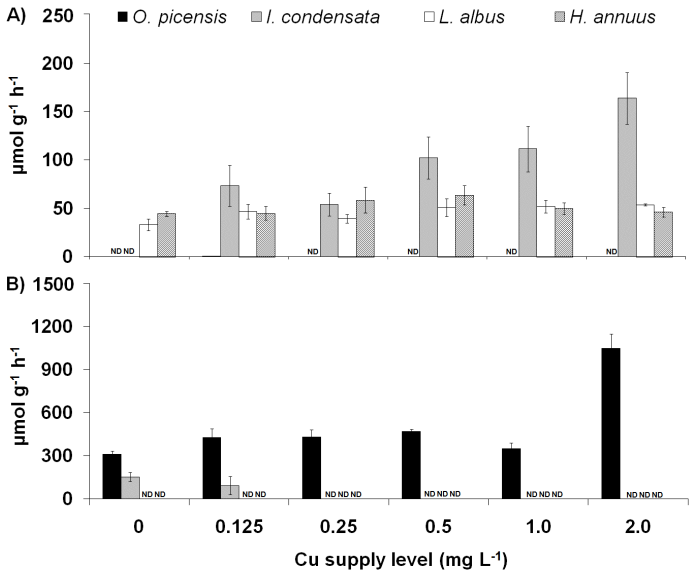

Fig. 3.2 Exudation of low molecular weight organic acids (LMWOA) of four plants species after exposition to increasing Cu doses (0; 0.125; 0.250, 0.5; 1.0 and 2.0 mg Cu L^{-1}). A) citric acid, B) succinic acid. Values are means ± S.E (N=6). The LMWOA exudation is expressed per gram of dry weight. ND= Not detected.

The LMWOA exuded by roots of agricultural plants mainly consisted of citric acid, which was produced in high amounts (Fig. 3.2A), reaching an average of 46 and 51.4 mmol g^{-1} h^{-1} in *L. albus* and *H. annuus*, respectively. However, in both plant species, the exudation of the citric acid remained relatively constant at higher Cu doses. In contrast, metallophytes exhibited a more variable LMWOA exudation at different Cu doses (Fig. 3.2). *Imperata condensata* showed a scarce exudation of succinic acid at low Cu doses (up to 0.125 mg Cu L^{-1}, Fig. 3.2B); nevertheless, at increasing Cu doses, and in particular at the highest Cu dose, this metallophyte exuded high amounts of citric acid, reaching values 3-4 fold higher than

the agricultural species evaluated (Fig. 3.2A). Oxalic acid was only detected in exudates of *I. condensata* but at very low amount, reaching 1.6 μmol g^{-1} h^{-1} at the highest Cu dose (data not shown). Succinic acid was almost exclusively exuded by *O. picensis*, reaching concentrations of 1048.6 μmol g^{-1} h^{-1} at 2 mg Cu L^{-1} (Fig. 3.2B), while fumaric acid was only detected at 0.125 mg Cu L^{-1} and at very low concentrations (0.4 μmol g^{-1} h^{-1}, data not shown). Lineal regression analysis showed constant exudation of citric acid for both agricultural plants, while in *I. condensata* was negatively related to the root elongation (R=-048, *P*<0.01; Fig. 3A). *Oenothera picensis* showed a similar trend, but in this case respect to the succinic acid exudation (R=-0.65, *P*<0.01; Fig. 3.3A).

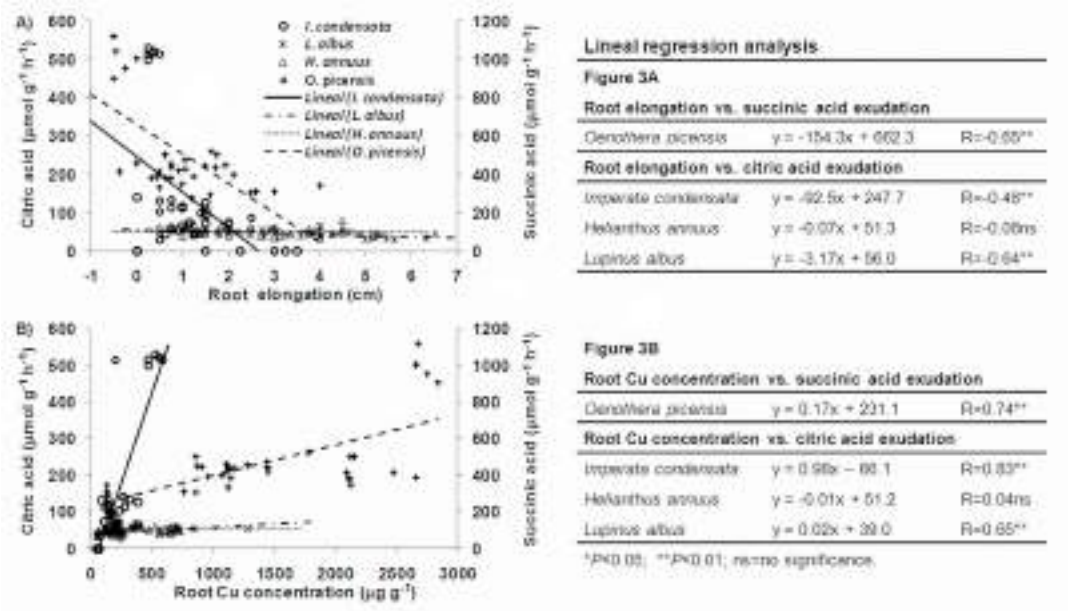

Fig. 3.3. Relationship between citric and succinic acid exudation with the root elongation and root Cu concentration in two metallophytes (*Oenothera picensis* and *Imperata condensata*) and two agricultural plants (*Helianthus annuus* and *Lupinus albus*). A) Relationship between root elongation and citric (for *I. condensata* and agricultural plants) and succinic (for *O. picensis*) acid exudation. B) Relationship between root Cu concentration in the plant and citric (for *I. condensate* and agricultural plants) and succinic (for *O. picensis*) acid exudation. The lineal regression analysis, showing the equations, R Pearson coefficient and significance is showed at right.

Respect to the root Cu concentration, both agricultural plants showed a constant citric acid exudation, while *I. condensata* showed a high increase in its citric acid exudation levels (R=0.83, *P*<0.01; Fig. 3.3B), and *O. picensis* showed a moderate-high increase of succinic acid exudation (R=0.74, *P*<0.01; Fig. 3.3B) at increasing root Cu concentrations.

The amount and type of PhC detected in root exudates were different among the species analyzed and were only exuded at lower (up to 0.25 mg Cu L^{-1}) and higher Cu doses (Fig. 3.4). In *I. condensata*, catechin and cinnamic acid were the main PhC exuded (Fig. 3.4A, and 3.4B) and were strictly dependent on the Cu level applied.

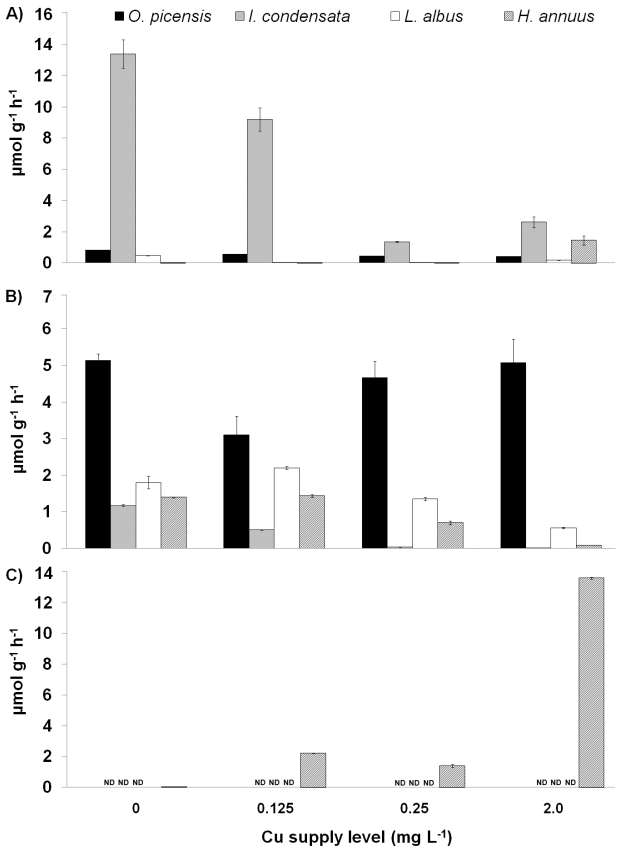

Fig. 3.4 Exudation of phenolic compounds of four plant species after exposition to increasing Cu doses (0; 0.125; 0.250 and 2.0 mg Cu L^{-1}). A) Catechin, B) Cinnamic acid, C) Coumaric acid. Values are means ± S.E (N=6). The exudation of phenolic compounds is expressed on gram per dry weight. ND= Not detected.

Without Cu addition, *I. condensata* exuded high amounts of catechin (13.4 μmol g^{-1} h^{-1}), but at increasing Cu doses, the exudation progressively decreased and was 2.62 μmol g^{-1} h^{-1} at the highest Cu dose. A similar pattern was observed in the exudation of cinnamic acid by this metallophyte, which decreased by about 95% when increasing Cu doses were applied. The main PhC exuded by *O. picensis* was cinnamic acid, with concentrations relatively constant at increasing Cu doses (Fig. 3.4B). In addition, the agricultural plants exuded different types of PhC at different Cu doses. In particular, the root exudates of *H. annuus*, which contained almost exclusively coumaric acid (Fig. 3.4C), were exuded in high amounts at the highest Cu dose (13.6 μmol h^{-1} g^{-1}, 6-10 fold higher than that exuded at lower Cu doses).

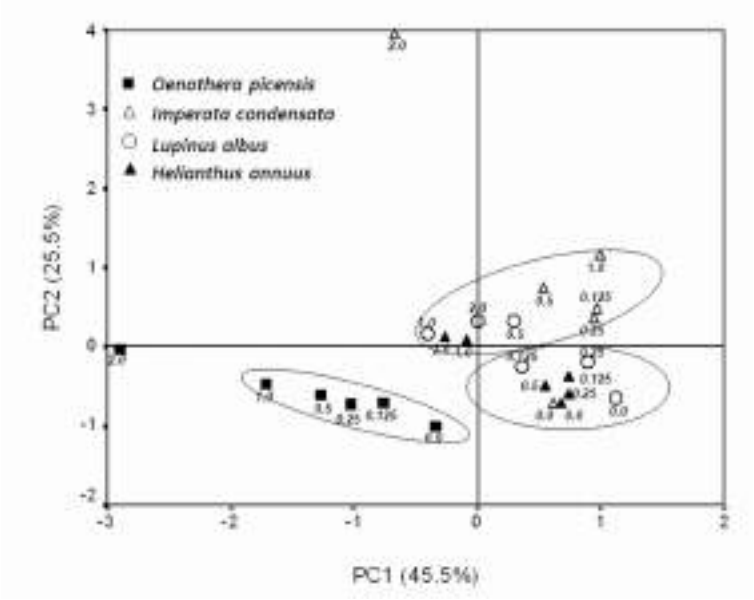

Fig. 3.5. PCA scores for the respective combinations among plant species and Cu doses added in a nutrient solution bioassay. The mean value was used in each situation. Percentage values in parenthesis indicate the variation explained by each PC. The circle comprises individuals of similar characteristics according to the cluster analysis, and should be understood as a visual aid for the discrimination of groups.

Several strong relationships were found among the different variables examined in this study (Table 3.4), highlighting the direct correlation observed between shoot biomass production and citric acid exudation as well as the inverse correlation between biomass

(shoot and root) production and succinic acid exudation. On the other hand, PC1 and PC2 accounted for 71% of total variance, with PC1 being highly correlated with growth parameters and inversely associated with Cu root concentration (Fig. 3.5; Table 3.4). In addition, PC2 was significantly correlated with the citric (and oxalic) acid exudation. Five groups were obtained by non-hierarchical cluster analysis (Fig. 3.5). In general, *O. picensis* formed a homogeneous group, which was very different from the total group of agricultural plants, and *I. condensata* established two similar groups, but *I. condensata* growing at 2.0 mg Cu L^{-1} formed an exclusive group highly correlated with PC2.

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

Variables	**Shoot**	**Root**	**Cu-shoot**	**Cu-root**	**Citric**	**Oxalic**	**Succinic**	**Fumaric**	**PC1**	**PC2**
Root elongation[1]	0.39ns	0.21ns	-0.26ns	-0.60**	-0.04ns	-0.18ns	-0.42*	0.08ns	0.64**	-0.38ns
Shoot[2]	-	0.85**	-0.27ns	-0.71**	0.63**	0.04ns	-0.80**	-0.31ns	0.85**	0.32ns
Root[3]		-	-0.30ns	-0.62**	0.52*	-0.12ns	-0.59**	-0.24ns	0.79**	0.23ns
Cu-shoot[4]			-	0.65**	-0.17ns	0.00ns	0.64**	-0.145ns	0.61**	0.07ns
Cu-root[5]				-	-0.42*	-0.03ns	0.82**	0.08ns	-0.93**	-0.06ns
Citric[6]					-	0.63**	-0.60**	-0.24ns	0.42*	0.87**
Oxalic[7]						-	-0.11ns	-0.01ns	-0.13ns	0.85**
Succinic[8]							-	0.25ns	-0.84**	-0.25ns
Fumaric[9]								-	-0.16ns	-0.15ns
PC1									-	0.00ns

Significance conventions: ns=not significant; *P<0.05; **P<0.01; ns=non significant.

[1]Elongation of the main root (cm); [2]Shoot dry weight (mg plant^{-1}); [3]Root dry weight (mg plant^{-1}); [4]Cu concentration in shoots (μg g^{-1}); [5]Cu concentration in roots (μg g^{-1}); [6]Exudation of citric acid (μmol g^{-1} h^{-1}); [7]Exudation of oxalic acid (μmol g^{-1} h^{-1}); [8]Exudation of succinic acid (μmol g^{-1} h^{-1}); [9]Exudation of fumaric acid (μmol g^{-1} h^{-1}).

3.4. Discussion

3.4.1 Root elongation and plant growth

It is well known that high Cu doses produce harmful effects on plant growth, development (especially at root level), which commonly lead to plant death (Ye et al., 2003). This inhibitory effect on root growth/elongation is due to a reduction and retardation of normal root cell division (Clemens, 2001). In this study, we have found strong inhibition of root elongation, when increasing Cu doses were applied (Table 3.2) being *O. picensis* the most affected metallophyte species. In contrast, lesser reduction in root elongation and dry weight were observed in *I. condensata*, suggesting that this metallophyte has a more effective mechanism to tolerate phytotoxic Cu levels, allowing it root growth at concentrations that normally inhibit growth in non-adapted species, or even in other Cu metallophytes species (Ginocchio et al., 2002). In fact, this plant specie was able to exude high amounts of citric acid even when the plant not showed root growth.

Studies have shown that root growth from cuttings of non-tolerant strains of *Mimulus guttatus* were completely inhibited at 0.5 mg Cu L^{-1}, while tolerant ones were not affected (Macnair, 1987; Macnair et al., 1993). A similar trend was reported in the metallophyte *Mimulus luteus* var. variegatus (Ginocchio et al., 2002), where plants obtained from severely Cu-polluted soils were tolerant to high soil Cu concentrations and root growth was only inhibited by the addition of Cu doses higher than 0.5 mg Cu L^{-1} to the nutrient solution. Our results indicate that the strong reduction in the growth parameters of *O. picensis* suggest that this species does not intrinsically has the ability to tolerate Cu in condition such as those used in this study and probably other factors, such as the formation of arbuscular mycorrhizal symbiosis with adapted fungal populations, could allow their growth in highly Cu polluted soils in natural conditions (Meier et al., 2011). In fact, the high succinic acid exudation rates here reported could be an inefficient way to cope the high Cu levels in the rhizosphere, since represent a squandering of high amount of C not used to form another plant structures, limiting its growth (Tables 3.2, 3.3 and 3.4; Figs. 3.3A, 3.5).

On the other hand, agricultural plants had contrasting behaviors relative to root elongation. Whereas, in *H. annuus*, the root elongation was strongly affected by Cu supplied doses, in *Lupinus albus* root elongation was not inhibited even at high Cu doses (more than 0.5 mg Cu L^{-1}). The above suggests the presence of Cu tolerance mechanisms (in this case particularly the citric acid exudation) that could allow the root growth at phytotoxic Cu concentrations (Jung et al., 2003) and could support it use in phytoremediation programs (Martínez-Alcalá et al., 2010).

3.4.2 Copper concentration in plant tissue

Most of the plants showed normal shoot Cu concentrations (about 20 mg Cu kg^{-1}; Adriano 2001). Nevertheless, at the highest Cu dose *O. picensis* showed Cu concentrations 3-4 fold higher than all the other species in both roots and shoots (Fig. 3.1). This plant specie has been previously described as the major Cu accumulator present naturally in Cu polluted ecosystems in central Chile, reaching shoot Cu concentrations of over 600 μg g^{-1} (González et al., 2008). This high capacity of Cu uptake by roots has also been described in other Cu metallophytes, such as *Elshotzia haichowensis*, *Hirschfeldia incana* and *Mimulus guttatus* (Robinson and Thurman, 1986; Poschenrieder et al., 2001; Lou et al., 2004), with Cu concentrations in plants ranging from 140 to 2000 μg Cu g^{-1}.

The other plant species evaluated exhibited normal Cu concentrations (Fig. 3.1). Nevertheless, the agricultural species were able to accumulate more Cu in their roots than the metallophyte *I. condensata*. The high capacity of Cu accumulation in the agricultural plants studied have been previously reported (Pineda, 2004; Martínez-Alcalá et al., 2010) and could justify their use in phytoremediation programs, if environmental and technological conditions allow their cropping in Cu polluted soils.

3.4.3 Exudation of organic acid and phenolic compounds

The LMWOA exuded by roots were strictly dependent on the plant species analyzed and the Cu doses added to the nutrient solution (Table 3.1; Fig 3.2). Several LMWOAs, mainly citric and oxalic have been reported as root exudates at considerable high metal

levels such as Ni, Zn, Al, Cd, Cr and Cu (Shen et al., 2002; Zeng et al., 2008; Magdziak et al., 2011). In our study the metallophyte *O. picensis* exuded high amounts of succinic acid. There are no previous studies that reported high levels of succinic acid as root response to elevated Cu concentrations, and only low values have been found related to other metals, such as Cr, Zn, Cd and Pb (Schwab et al., 2008; Zeng et al., 2008; Magdziak et al., 2011). Nevertheless, stability constant (K_f) of the succinate-Cu chelate (about 2 x 10^4) is significantly lower than K_f for other organic acids also detected, such as citric and oxalic, with values of 1.7 x 10^7 and 7.9 x 10^6, respectively (Borges et al., 2005). Therefore, the extremely high levels of succinic acid exudation could explain a non-specific or non-efficient response to the physiological stress generated by high Cu levels in the environment, which in this case was highly related with a minor plant growth and a very high Cu concentration in shoots and roots, inferring low Cu exclusion by the comparatively low concentration of succinate-Cu chelate (Table 3.4; Figs. 3.3B, 3.5). However, further studies *in vitro* and *in vivo* conditions analyzing the Cu-chelate formation and Cu-speciation by roots exudates are needed to support the use of this plant species in bioremediation processes.

Additionally, other LMWOAs were detected in this species (fumaric and oxalic acids), but these compounds were detected only in some treatments, and their concentrations were consistently low. Although citric acid exudation has been extensively studied, particularly in plants growing in phosphorus deficient environments (Neumann and Römheld., 1999) and plants exposed to high metal concentrations (Schwab et al., 2008), this organic acid was not detected in this plant at any Cu dose.

Imperata condensata showed a very different LMWOA exudation pattern in response to increasing Cu doses. At low Cu, its roots exuded low amounts of succinic acid, which even decreased at increasing Cu doses and almost completely disappear at 0.25 mg Cu L^{-1}. However, the most important LMWOA exuded by this plant was citric acid (Fig. 3.2A), which was exuded in concentrations 3-4 fold higher than the agricultural plants at the higher Cu doses (Fig. 3.2A). This different behavior among *I. condensata* and the agricultural plants suggests that this metallophyte could have a species-specific LMWOA exudation mechanism to tolerate extremely high Cu concentrations in the environment due to the strong affinity of citric acid to form stable extracellular metal complexes (the K_f of

citrate-Cu chelate is about 1100 times higher than K_f of succinate-Cu chelate) (Nigam et al., 2001) resulting in a effective Cu-exclusion mechanism. In fact, the multivariate analysis showed that *I. condensata* presents a behavior similar to the agricultural plants, with similar growth rates, Cu concentration and LMWOA exudation, but with a highly different citric acid exudation at 2.0 mg Cu L^{-1} (Table 3.4, Figs. 3.2, 3.3 and 3.5).

Even though organic acids are the main components exuded by metallophytes and agricultural plants, the PhC may play a determinant role in decreasing Cu toxicity in the environment, in particular due to the high stability constants that show their ligands with Cu^{+2} ions, which can be several times higher than those of LMWOAs. However, few studies have been conducted evaluating PhC exudation in response to high metal levels (Jung et al., 2003; Borges et al., 2005).

Cinnamic acid, catechin and coumaric acid were detected, both under control, low and high Cu doses (PhC were not detected at intermediate-high Cu doses), showing a variable pattern in terms of composition and amount (Fig. 3.4). Based on our results, we not observed a pattern that explain the levels of PhC exudation at low and high Cu levels; therefore, further studies focused to elucidate the contribution of these compounds in Cu-polluted environments are needed. The presence of PhC has also been reported for *Picea abies* by Martell and Smith (1989), suggesting a relevant role in the formation of organo-metal complexes. Similar results have been reported by Jung et al. (2003) in *L. albus*, who proposed that the complexation of PhC and Cu^{+2} ions in the rhizosphere and in the apoplasm could alleviate Cu toxicity. The same finding was detected in our study, especially for *H. annuus*, which exuded high amounts of coumaric acid, especially at the highest Cu level (Fig. 3.4C). *Imperata condensata* exuded high amounts of catechin at low Cu supply, which could contribute to the Cu tolerance when root exudates did not contain citric acid at low environmental Cu amounts (Fig. 3.2). In this case, *I. condensata* would be the plant that exhibits the more efficient mechanism against Cu phytotoxic levels in the environment, because their root exudates show high Cu^{+2} complexation capacity thus preventing the entry of this metal into the symplast both at low and high Cu levels. These characteristics could explain its presence in extremely Cu polluted soils (more than 300 mg kg^{-1} of available Cu, Cornejo et al., 2008) and may also determine its preferential use in phytostabilization programs in Cu-polluted soil.

3.5. Conclusions

The high exudation rate (either LMWOA and PhC) showed by *I. condensata* and *L. albus*, could had prevented the Cu uptake by plant roots as an efficient exclusion mechanism, allowing their growth at high Cu levels. In opposition, the high Cu concentration found in *O. picensis* is probably due to the low affinity of succinic acid to form stable complexes with Cu ions, allowing its acquisition and reducing significantly its growth. In this sense, root exudation characteristics together with plant growth could be useful parameters for evaluating Cu tolerance in plants, especially at higher Cu levels. Nevertheless, further studies analyzing the metal binding properties of the different exuded compounds by roots are required to assess their role in Cu tolerance in metallophytes and agricultural plants.

Acknowledgments

Financial support for this study was provided by the National Commission for Scientific and Technological Research (CONICYT), through FONDECYT 11080131 grant (P. Cornejo). Sebastian Meier also acknowledges the support of the Scientific and Technological National Commission Research (CONICYT) through project 24091053.

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Chapter 4. Alleviation of Cu toxicity in Oenothera picensis by copper-adapted arbuscular mycorrhizal fungi and treated agrowaste residue.

Paper published in Applied Soil Ecology (2011) 48, 117-124.

Alleviation of Cu toxicity in *Oenothera picensis* by copper-adapted arbuscular mycorrhizal fungi and treated agrowaste residue

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Abstract

The alleviation of Copper (Cu) toxicity in the pseudometallophyte *Oenothera picensis* via arbuscular mycorrhizal fungi (AMF) inoculation and/or sugar beet agrowaste (SB) application was evaluated at increasing soil Cu levels. Plants were grown in Cu-treated soils (0, 100 or 500 mg Cu kg^{-1}), either with or without SB application, and inoculated with: *i*) Cu-adapted Glomeromycotan fungi (GA); *ii*) *Glomus claroideum* (GC); or *iii*) no fungus (uninoculated). Application of SB amendment increased shoot biomass 2-8 fold with respect to the unamended soils, and allowed the survival of non-mycorrhizal- and GC-inoculated plants, even at the highest Cu level. Additionally, SB application increased shoot Cu content at higher Cu levels and shoot P content especially at lower Cu levels. In general, compared to GC-inoculated plants, GA inoculation caused a decrease in both superoxide dismutase and ascorbate peroxidase antioxidant enzyme activities in shoots (up to levels of 100 mg Cu kg^{-1}), as well as glutathione reductase and catalase activities (up to 500 mg Cu kg^{-1}). Finally, in SB treated plants, GA colonization was higher as compared to GC-inoculated plants, especially at the highest Cu level. These results suggest a relevant role of Glomeromycotan fungal populations isolated from Cu-polluted environments in the alleviation of Cu toxicity that could allow their use in remediation programs for Cu-polluted soils.

Keywords: Antioxidant enzymes; arbuscular mycorrhiza; copper pollution; *Oenothera picensis;* sugar beet agrowaste.

4.1. Introduction

Copper-mining activities produce significant detrimental effects on natural ecosystems due to the high amount of Cu-enriched particulate matter deposited in the soil, which reduces plant cover and growth and limits plant establishment (Ginocchio, 2000). For this reason, the long-term success of phytoremediation programs in Cu polluted soils has been limited (Azcón et al., 2009). Among the factors involved, lack of knowledge about the role of microbial communities in soils polluted with metals could explain some failures in the implementation of phytoremediation processes (Arriagada et al., 2009). Metal tolerance has been reported in diverse microorganisms that colonize metal polluted soils (del Val et al., 1999; Ferrol et al., 2009). Furthermore, metal-tolerant plant species (metallophytes, pseudometallophytes) can also grow on metal polluted soils (Ginocchio, 2000), and microorganisms such as metal tolerant arbuscular mycorrhizal fungi (AMF) could be functioning as the dominant population associated with their rhizosphere (Ferrol et al., 2009).

The AMF interact with plants in metal contaminated soils, and some reports conclude that the symbiosis is partly responsible for plant survival in those extreme environments (Carvalho et al., 2006; Hildebrandt et al., 2007). In this sense, the arbuscular mycorrhizal (AM) fungal colonization could enhance metal tolerance by improving plant nutrition and providing a barrier against metals that prevents their uptake by plants (Leyval et al., 1997). Therefore, plants growing under metal stress conditions may require the use and selection of the most effective AMF for surviving. This selection should be supported by the knowledge of metal-tolerant fungal species able to grow and function on polluted soils, and additionally adapted to nutrient-impoverished soils (del Val et al., 1999). To understand the interactions between metals, AMF and plants, it is necessary to: *i*) study and compare the AM fungal diversity in both metal-polluted and unpolluted soils, *ii*) pay special attention to AMF associated with metal-tolerant plants, and *iii*) pinpoint those that are suitable for bioremediation purposes (Vivas et al., 2006).

It is well-known that, when present in excessive amounts, metals cause uncontrolled redox reactions in cells that result in the formation of reactive oxygen species (ROS) (Schutzendubel and Polle, 2002). Results reported by Ouziad et al. (2005) suggest that a primary function of the fungal cells in symbiosis is to cope with this heavy metal-induced oxidative stress. Plant cells contain an array of protective and repair enzyme systems that minimize oxidative damage.

Smirnoff (1993) has divided these systems into two categories. One category comprises enzymes that interact with active forms of oxygen and keep them at low levels, such as ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) (Smirnoff, 1993). The second category comprises enzymes that generate oxidized antioxidant, such as glutathione reductase (GR). The first group of enzymes are involved in the detoxification of $O_2^{\cdot -}$ radicals and H_2O_2, thereby preventing the formation of $^{\cdot}OH^-$ radicals. Glutathione reductase is an important component of the ascorbate glutathione pathway, which is responsible for the removal of H_2O_2 in different cell compartments (Aebi, 1984).

Because the soil biological quality has been deteriorated in metal-contaminated soils due to the gradual decline in organic matter content, the use of an organic amendment is recommended. Sugar beet waste (SB), an inexpensive lignocellulosic residue, has been used as an effective amendment for improving physical, chemical and biological soil properties (Medina et al., 2006). These SB residues have been transformed by *Aspergillus niger* into simple sugar compounds that can be used by rhizosphere microorganisms to promote metabolic activities and growth (Vassilev et al., 1998). Recently, Azcón et al. (2009) reported a positive interactive effect of such treated SB and AM inocula on the development of plants that were sensitive to toxic metals. These results suggest that the use of SB and AM inocula could attenuate metal stress in metallophytes; thus, these inocula have a potential use in phytoremediation programs. Moreover, if SB and/or AMF (indigenous or non-adapted strains) contribute to the amelioration of metal stress in plant tissues, changes in the plant antioxidant defense system will also be expected.

The aim of this research was to evaluate the role of AM fungal inoculation and/or SB addition on the alleviation of Cu toxicity using a native Chilean pseudometallophyte as a model plant. The effectiveness of AM inoculation, either by populations of Cu-adapted autochthonous Glomeromycotan (GA) fungi or the non-adapted *Glomus claroideum* (GC) fungus (with or without agrowaste residue), was tested by analyzing the plant growth, nutrient acquisition and Cu content. Changes in the activities of SOD, CAT, GR and APX enzymes were also determined to analyze the effects of the different treatments on oxidative stress in *O. picensis*.

4.2 Materials and Methods

4.2.1 Plant species

The pseudo-metallophyte *Oenothera picensis* (fragrant evening primrose) was used as a model plant for this bioassay. This species (formerly named *O. affinis*) has been previously described as Cu tolerant plant (González et al., 2008). Seeds of *O. picensis* were collected from a Mediterranean ecosystem area strongly affected by the deposit of Cu-enriched particles (up to 830 mg total Cu kg^{-1} soil and 330 mg DTPA extractable Cu kg^{-1} soil; Cornejo et al., 2008) and located approximately 1.5 km southeast from a copper smelter in the Puchuncaví Valley, Central Chile (32°46′30″S; 71°28′17″W).

4.2.2 Agrowaste

The treated agrowaste used in these experiments was an amendment that had been successfully tested by Vassilev et al. (1998) and Medina et al. (2006). The amendment was prepared with sugar beet waste (SB), which was supplemented with rock phosphate (RP) and mineralized by *Aspergillus niger*. For amendment production, an *A. niger* strain (NB2) was used due to its increased production of organic acids (mainly citric acid) when growing on complex substrates and its ability to mineralize lignocellulosic materials (Vassilev et al., 1998).

4.2.3 Soil and arbuscular mycorrhizal fungi

The soil used in this assay was collected from a zone in Granada, Spain. The soil had a pH_W of 7.2, and was comprised of 1.6% organic matter, 57.8% sand, 19.0% silt, 23.2% clay, and had the following nutrient concentrations (in mg kg^{-1}): N, 2.1; $NaHCO_3$-extractable-P, 1.7; and extractable K, 0.8.

Two inocula of AM fungi were used for this study. A mix of autochthonous Glomeromycotan (GA) fungi was isolated from the rhizosphere soil of *O. picensis* plants growing in Cu polluted areas of the Puchuncaví Valley in central Chile. The fungal reproduction was carried out in an open pot culture using a sepiolite:quartz sand:vermiculite (1:1:1 v:v:v) mix as a

substrate, and *O. picensis* and *Plantago lanceolata* were used as host plants. After 6 months of plant growth, the shoots were removed and the soil and root substrate were used as GA inocula. A preliminary morphological analysis revealed that the majority of the spores present in the inocula belonged to the *Glomus* genus, with *Glomus* aff. *intraradices* being the dominant ecotype. In addition, a strain of *Glomus claroideum* (GC) was isolated from agricultural soils of the Araucanía Region in southern Chile and used as reference of presumably non-Cu-adapted AM fungus. The GC inoculum was obtained similarly to the GA inoculum; however, *Sorghum bicolor* and *Trifolium repens* were used as host plants.

4.2.4 Experimental design and plant growth conditions

There were three AM treatments, two levels of SB amendment, and three Cu levels in a full randomized design with five replicates per combination for a total of 90 experimental units. The AM treatments were: *i*) Non-AM inoculated plants (NM), *ii)* plants inoculated with *Glomus claroideum* (GC), and *iii*) plants inoculated with a mixture of Cu-adapted autochthonous Glomeromycotan (GA) fungi. Each one of these treatments was assayed with or without SB amendment, and plants were grown at Cu concentrations of 0, 100 or 500 mg Cu kg^{-1} soil.

Before starting the experiment, the soil was sieved through a 2 mm mesh and diluted with quartz-sand (< 1 mm particle size; 2:1 soil:sand, v/v), sterilized by tyndallization for three consecutive days, and air-dried for 24 h. The soil mixture was placed into 200-mL pots. After sterilization, the soil/sand mixture was supplemented with 0, 100 or 500 mg Cu kg^{-1} soil, an adequate amount of $CuCl_2 \cdot 2H_2O$ solution was added, and mixtures were allowed to equilibrate for two weeks. The respective treated SB amendment was mixed (5%, w/w) with the soil:sand mixture and left to equilibrate for another two weeks at room temperature.

Seeds of *O. picensis* were surface sterilized with 2% Cloramin-T solution for five minutes and rinsed thoroughly. Seeds were germinated and plantlets were grown before transplanting to the greenhouse, where plants were grown under a 16/8 h light/dark photoperiod with 80-90% relative humidity at 25±3/15±3ºC day/night temperatures. At transplanting, the plantlets were either inoculated with AMF or maintained uninoculated. In both cases, a mixture of rhizosphere substrate containing spores (about 250-300 spores per 100 g), hyphae (about 3-4 m per g), and mycorrhizal root fragments was used as an inoculum. Ten grams of each inoculum were added to

the respective pots just below the seedlings. Uninoculated plants (NM) received an equivalent amount of autoclaved inoculum. Plants were grown for 3 months under greenhouse conditions before being harvested.

4.2.5 Measurements

At harvest, the shoots and roots were separated, and shoot subsamples (1 g) of fresh material were stored at -80ºC for antioxidant enzyme activity assays. Plant samples (shoots and roots) were dried at 70ºC for 2 days and weighed. Then, the samples were ground, ashed at 550ºC and digested using an $H_2O/HCl/HNO_3$ mixture (8/1/1, v/v/v). The plant extracts were used for the determination of S, Cu, Mn, P and Zn in an ICP plasma analyzer (IRIS Intrepid II XDL, Thermo Electron Corporation). Mineral analyses were carried out by the analytical service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

Arbuscular mycorrhizal fungal root colonization was quantified using a dissection microscope (20-40X) after clearing a portion of the roots in 10% KOH (w/v) and staining with 0.05% trypan blue in lactic acid (w/v). The gridline intersection method (Giovannetti and Mosse, 1980) was used to determine the proportion of AM root colonization.

In the shoots, total SOD activity (EC 1.15.1.1; Beyer and Fridovich, 1987) was measured on the basis of the SOD-dependent reduction of nitroblue tetrazolium (NBT) by photochemically-generated superoxide radicals. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25ºC (Beyer and Fridovich, 1987). The CAT activity (EC 1.11.1.6) was measured by H_2O_2 consumption (extinction coefficient of 39.6 mM^{-1} cm^{-1}) at 240 nm for 1 min (Aebi, 1984). The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and 100 mL of enzyme extract in a 2 mL volume. The APX activity (EC 1.11.1.11) was measured in a 1-mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2 and 0.5 mM ascorbate. The H_2O_2 was added to start the reaction, and the absorbance decrease at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Amako et al., 1994). The GR activity (EC 1.20.4.2.) was estimated by measuring the decrease in absorbance at 340 nm at 25ºC due to the oxidation of NADPH (Carlberg and Mannervik, 1985). The reaction mixture (1 mL) contained 0.1 M HEPES-NaOH (pH 7.8), 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM oxidized

glutathione, 150 mL enzyme extract, and 0.2 mM NADPH was added with thorough mixing to begin the reaction. The results were expressed in mmol NADPH per oxidized g of fresh plant material per minute, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH (e_{340}=6.22 mM^{-1} cm^{-1}).

4.2.6 Statistical analyses

Data regarding Cu levels, AM inoculation, agrowaste residue application and its interactions were tested by means of a multifactorial ANOVA. Means were compared using the orthogonal contrast test (Petersen, 1977). Data sets not meeting assumptions for ANOVA were transformed as required, but the results are presented in the original scale of measurement. Statistical significance was determined at $p \leq 0.05$.

4.3 Results

Most of the measured variables responded significantly to the different treatments applied, and the interaction of the different factors was analyzed (Table 4.1). Whereas the application of increasing Cu and the addition of SB agrowaste produced differences in almost all variables, non-significant changes were observed in shoot Cu content, root Mn content, SOD and APX activities as a consequence of inoculation with different AM strains. The triple interaction Cu x SB x AM generated significant changes in the amounts of Cu, P, S, Zn and Mn taken up by plants, and also led to changes in the antioxidant enzyme activities, with the exception of CAT (Table 4.1).

4.3.1 Dry matter yield and nutrient content

The applications of SB increased shoot dry weights by 2- to 8-fold compared to plants not exposed to SB, even when toxic amounts of Cu were applied (500 mg Cu kg^{-1}; Fig. 4.1). However, the above positive effect was not observed in root biomass production, which was lower in plants exposed to SB. Neither NM- nor GC-colonized plants were able to survive at the highest Cu level in the absence of SB. In addition, 100 or 500 mg Cu kg^{-1} in SB treatments significantly increased root dry weight in GA colonized plants by about 60% with respect to NM

treatment. Differences in dry matter yield among the treatments generated changes in the nutrient content of plant tissues. Nevertheless, no apparent dilution effect of the nutrient concentration was observed due to the increase in dry matter production, and both accumulation and concentration of macro- and micronutrients followed a similar trend (data not shown).

Table 4.1
F-values and significance for the main effects and factor interactions for the variable analyzed in an *Oenothera picensis* crop study by means of a multifactorial ANOVA (n=90).

Experimental variable	Cu[1]	SB	AM	Cu x SB	Cu x AM	SB x AM	Cu x SB x AM
Shoot dry weight (g)	26.0***	329.7***	0.2ns	12.1***	1.8ns	2.2ns	0.3ns
Root dry weight (g)	10.5***	0.0ns	5.8**	9.8***	3.8**	8.1**	1.6ns
Shoot Cu content (µg/plant)	9.7***	203.2***	1.1ns	6.9**	1.7ns	0.2ns	2.5ns
Root Cu content (µg/plant)	172.0***	59.6***	52.7***	81.1***	38.1***	21.9***	13.8***
Shoot P content (µg/plant)	92.4***	1209.2***	3.7*	51.3***	5.9***	3.7*	3.7**
Root P content (µg/plant)	24.7***	12.2**	11.5***	17.6***	2.7ns	2.0ns	5.9***
Shoot S content (µg/plant)	73.4***	12.2**	33.0***	40.5***	3.4*	25.3***	1.4ns
Root S content (µg/plant)	37.6***	672.3**	14.7***	55.0***	4.0**	10.1***	1.3ns
Shoot Zn content (µg/plant)	132.9***	849.4***	15.2***	42.5***	3.3*	7.2**	4.4**
Root Zn content (µg/plant)	32.1***	12.4**	36.8***	9.9***	21.5***	19.0***	7.8***
Shoot Mn content (µg/plant)	11.9***	670.2***	7.8**	9.7***	7.7***	16.1***	5.6**
Root Mn content (µg/plant)	12.3***	141.8***	1.8ns	3.9*	9.0***	11.8***	9.8***
GR activity[2] (nmol min^{-1} mg $protein^{-1}$)	31.8***	38.9***	24.4***	1.5ns	14.3***	63.9***	11.8***
SOD activity (units min^{-1} mg $protein^{-1}$)	20.1***	51.4***	0.7ns	24.3***	20.5***	23.8***	4.6**
APX activity (nmol min^{-1} mg $protein^{-1}$)	15.7***	6.9*	3.0ns	15.5***	18.8***	16.2***	6.3**
CAT activity (µmol min^{-1} mg $protein^{-1}$)	10.7***	69.2***	20.2***	6.1**	4.7**	11.7***	0.0ns
Mycorrhization (%)[3]	13.1***	10.8**	96.6***	6.0**	60.5***	5.2*	22.4***

Significance conventions: *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, ns = no significant differences.
[1]Cu = different Cu levels; SB = sugar beet treated agrowaste residue application; AM = arbuscular mycorrhizal inoculation.
[2]GR = glutathione reductase; SOD = superoxide dismutase; APX = ascorbate peroxidase; CAT = catalase.
[3]For mycorrhization determination, only were considered the treatments including AM fungi inoculation

1

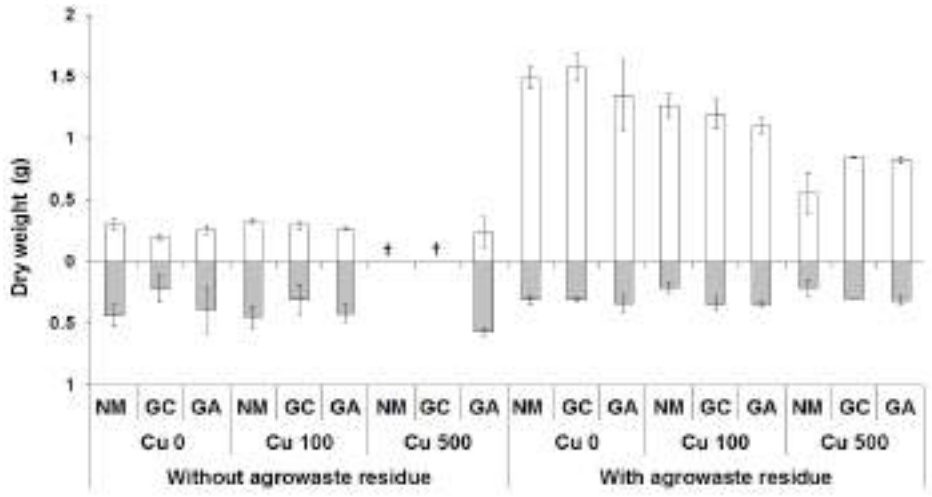

Fig. 4.1 Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on shoot and root dry weight in *Oenothera picensis.* NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; n = 5.

Plant Cu contents were increased by addition of Cu to the soil (Fig. 4.2). At 100 mg Cu kg^{-1}, in the absence of SB, Cu content in the shoots of NM plants was 3.9 fold higher than GA-colonized plants. In contrast, non-significant differences were observed in root Cu contents between NM and GA-colonized plants (Fig. 4.2). Thus, the amount of Cu translocated from root to shoot was greatly reduced (from 9.8% to 2.7%) by mycorrhizal GA inoculation. For SB treatments, non-mycorrhizal plants contained 80% more Cu in roots than GC-colonized plants, and had similar Cu levels as compared to GA plants at the highest Cu level (Fig. 4.2).

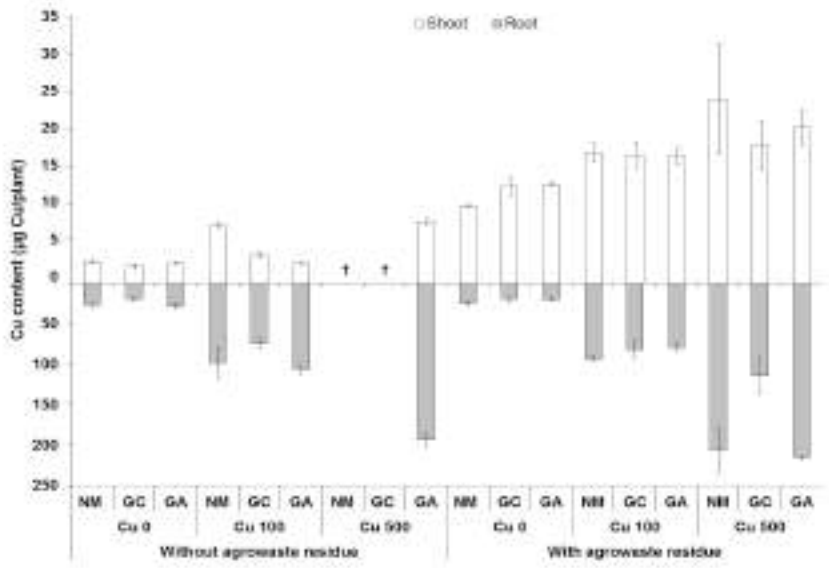

Fig. 4.2. Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on shoot and root Cu content (µg/plant) in *Oenothera picensis.* NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; n = 5.

Application of SB significantly increased shoot P content; particularly at the lowest soil Cu level (Fig. 4.3). The effect of GC and GA on plant P uptake was similar irrespective of Cu treatments. Sugar beet residue greatly increased the S uptake of GA- and GC-colonized plants compared with non-mycorrhizal plants, with larger differences observed in the shoots compared to the roots (data not shown). SB amendment greatly increased Mn uptake by plants (data not shown); however, at the highest Cu level, the lowest Mn acquisition was observed, particularly in the roots of mycorrhizal plants. Similarly, SB residue enhanced the Zn uptake by shoots, and reduced uptake in the roots (data not shown).

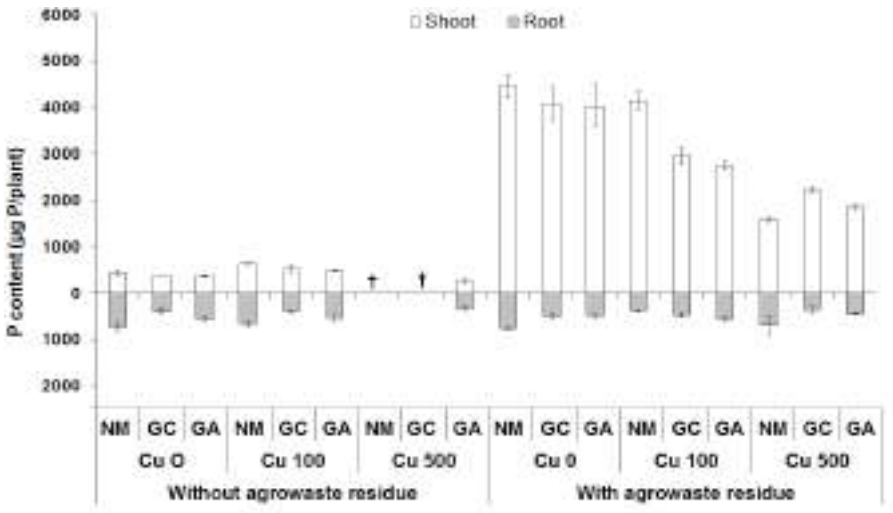

Fig. 4.3 Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on shoot and root P content (µg/plant) in *Oenothera picensis*. NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; n = 5.

4.3.2 Antioxidant enzyme activities

Figures 4.4 and 4.5 illustrate the activities of antioxidant enzymes in shoots under different combinations of SB addition, Cu supply and AM inoculation treatments. Changes in antioxidant enzyme activities were more closely related to the AM and Cu treatments rather than the addition of SB. In general, GR activity increased as Cu concentration increased (Fig. 4.4).

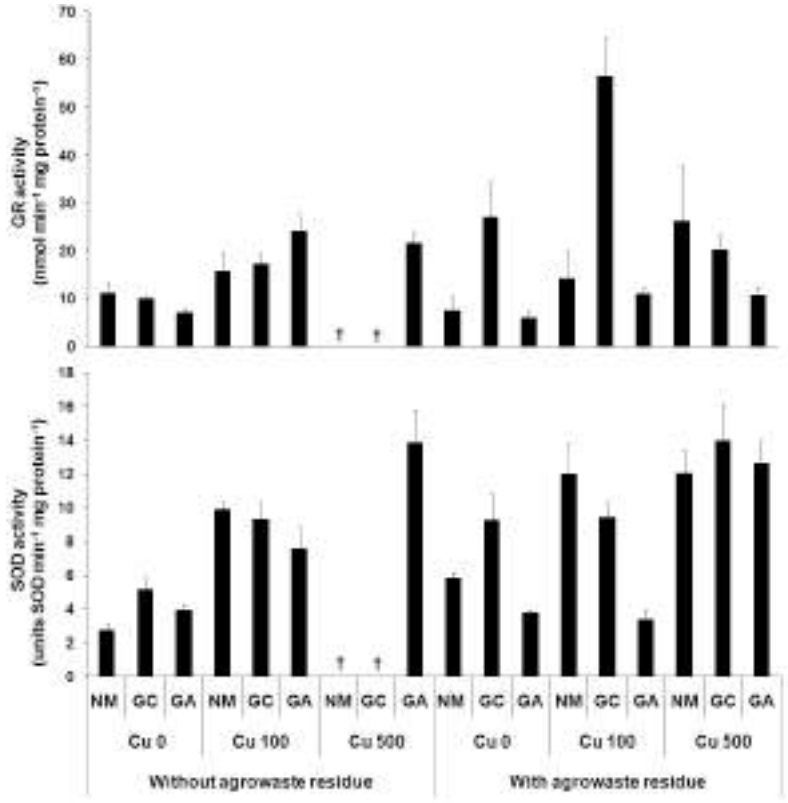

Fig. 4.4. Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on antioxidant activities in *Oenothera picensis.* Glutathione reductase (GR) activity, Superoxide dismutase (SOD) activity. NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; n= 5. N.D = None detected.

For SB treated plants, at 0 or 100 mg Cu kg^{-1}, GC inoculation enhanced GR activity by about 3.6 and 5.1-fold, respectively, and non-significant differences were found between NM- and GA-colonized plants. Changes in GR activity at the highest Cu level were also observed; the enzyme activity increased by 2.5-fold in NM plants subjected to SB amendment, whereas in GA-colonized plants, GR activity increased by only 80%.

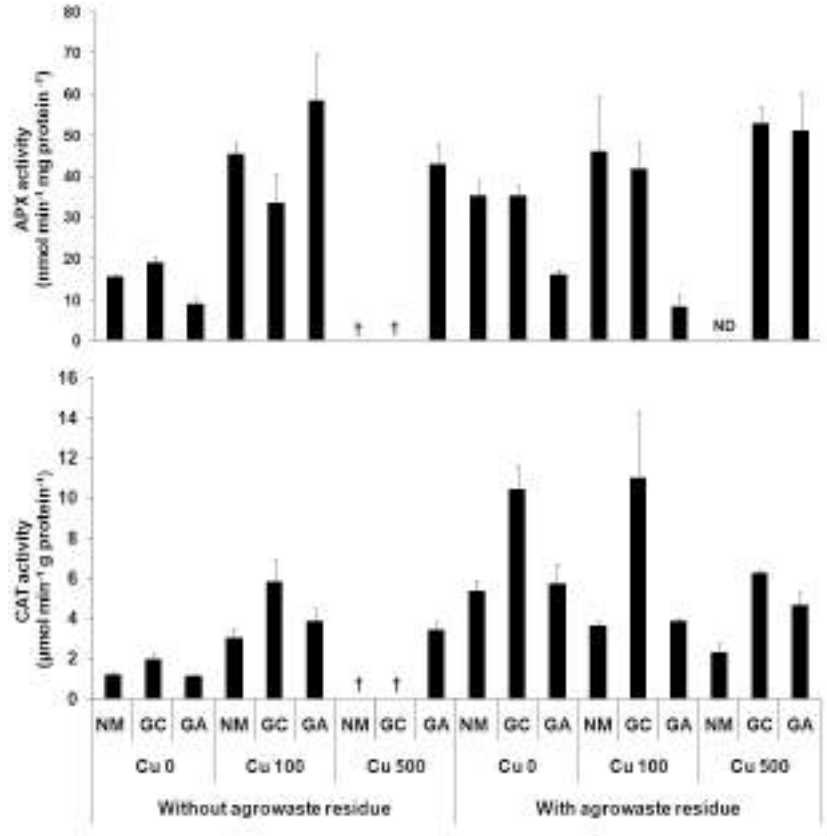

Fig. 4.5 Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on antioxidant activities in *Oenothera picensis.* Ascorbate peroxidase (APX) activity and Catalase (CAT) activity. NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; n= 5. N.D = None detected.

SOD activity increased as Cu levels increased in SB-treated and non-treated plants (Fig. 4.4). Under SB treatment, similarities in SOD activity were observed between NM- and AM- (GC or GA) colonized plants at the highest Cu level. However, GA-colonized plants growing at 0 or 100 mg Cu kg^{-1} exhibited SOD activity that was at least 3.6-fold lower than the activity observed in NM- or GC-colonized plants.

Ascorbate peroxidase activity also increased due to the addition of Cu to the soil (Fig. 4.5). When SB and 100 mg Cu kg^{-1} were supplied, APX activity was reduced by about 5.5 fold in GA-

colonized plants, with respect to NM or GC treatments. Nevertheless, for GA-colonized plants, APX was activated by the addition of 500 mg Cu kg^{-1}, and no differences were detected in enzyme activity among GC- and GA-treated plants.

A differential CAT activity response to Cu addition occurred among SB-treated and non-treated plants, which was dependent on the AM inoculation treatments (Fig. 4.5). Without SB addition, CAT activity was enhanced by the addition of 100 mg Cu kg^{-1} in AM-colonized and non-colonized plants. Under SB treatment, CAT activity decreased as Cu levels increased in NM plants. When SB and 100 mg Cu kg^{-1} were added, CAT activity was not altered in GC-colonized plants, but was significantly reduced at the highest Cu level. Comparatively, the application of SB slightly inhibited CAT in GA-colonized plants at 100 mg Cu kg^{-1}, and non-significant differences in the enzyme activity were observed at either 0 or 500 mg Cu kg^{-1}.

4.3.3 Mycorrhizal root colonization

Mycorrhization remained relatively constant as Cu levels increased in GC-colonized plants (Fig. 4.6), and the addition of SB slightly decreased the percentage of GC root colonization at all Cu levels. In contrast, the above-mentioned negative effect of Cu on symbiotic development was not observed in GA-colonized plants, which exhibited a Cu-tolerant increase in AM colonization by GA inoculum. This effect was observed at 100 and 500 mg Cu kg^{-1} and was irrespective of the presence of the SB amendment. The most evident differences in mycorrhizal colonization were observed at the highest Cu level (500 mg Cu kg^{-1}) with SB agrowaste residue application, under such conditions, the colonizing ability of autochthonous fungi (GA) was much higher than GC

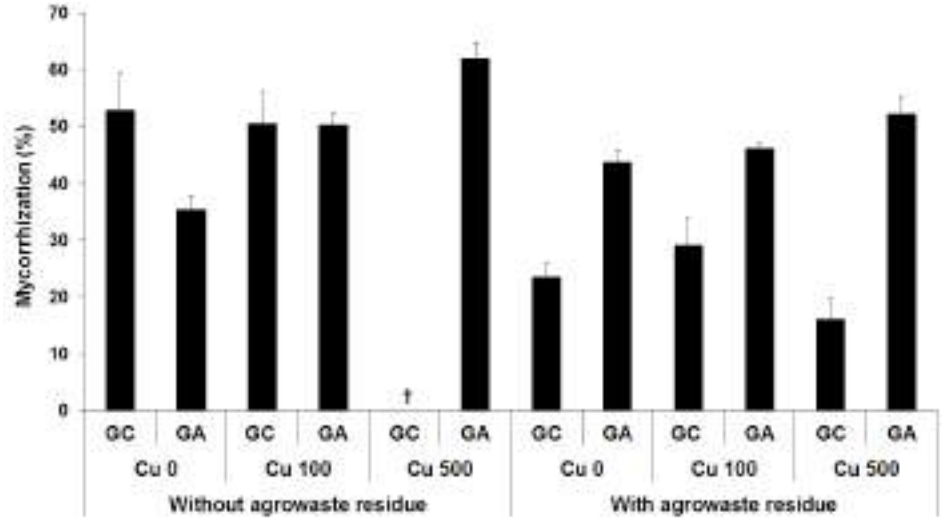

Fig. 4.6. Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on mycorrhizal root colonization in *Oenothera picensis.* NM = non-mycorrhizal plants, † = plants death. Bars denote means ± S.E.; n = 5.

4.4 Discussion

The presence and toxicity of copper in plant tissues depends on complex interactions between soil and plants, as well as microbial rhizospheric activities. AM fungi appear to play a central modulating role in protecting plants from metal toxicity (Schutzendubel and Polle, 2002). According to our results, the most important effect of the Cu-adapted AM inoculum was observed at the highest Cu levels assayed (500 mg Cu kg^{-1}). Under these conditions, only plants colonized by Cu-adapted mycorrhizal strains (GA) were able to survive and grow when no SB residue was added (Fig. 4.1). This mycorrhizal effect at 500 mg Cu kg^{-1} was not observed in SB-treated plants colonized by GC (a strain assumed to be Cu sensitive). The effect of tolerance of indigenous AM fungi versus non-adapted fungus in promoting plant establishment and survival on contaminated soil has been previously reported for soils polluted with different metals like Zn (Hildebrant et al., 2007), Pb (Sudová and Vosátka., 2007), and Cu (Leung et al., 2006).

Different mechanisms seem to be functioning in the mycorrhizal stimulating effect of GA-colonized plants at the highest Cu concentration. Additionally, the SB amendment was required for non-mycorrhizal (NM) or GC-colonized plant survival and growth, especially at the highest Cu concentration. At 500 mg Cu kg^{-1} plus SB, root Cu accumulation of GA-colonized plants was greater than that of GC-colonized plants, but their Cu content in the shoots was similar (Fig. 4.2). In accordance with these results, in the SB treated soil, NM plants had 8-fold increased shoot Cu acquisition, whereas those colonized by GA had only 3-fold increased Cu at 500 mg Cu kg^{-1} soil. The role of the agrowaste amendment in improving plant growth at toxic Cu levels could, in part, be ascribed to the formation of metal-citrate complexes (Bolan and Duraisamy, 2003). In addition, the rich organic matter composition of the amendment, particularly dissolved organic carbon (DOC), plays a vital role in the immobilization of metals (including Cu) by forming metal-DOC complexes, thereby decreasing metal phytotoxicity (Bolan et al., 2003; Bolan et al 2010). Nevertheless, our results did not support a Cu-chelating activity for SB. In this case, the agrowaste residue could be playing mainly a nutritional role and increasing the Cu tolerance by means of plant growth stimulation (Caravaca et al., 2004), as was observed for P (Fig. 4.3) and S.

In this study we were also interested in the different antioxidant responses of plants colonized by Cu adapted/tolerant or Cu sensitive AM fungal strains. This comparative study under non-toxic and increasing Cu levels will allow a better understanding of the Cu stress-tolerance mechanisms of mycorrhizal colonized plants. The role of the AM fungi (GC or GA) and/or Cu level was very important for the activity of the antioxidant enzymes evaluated here. It is well-known that the ability of the plant antioxidative system to counteract toxic levels of ROS determines the extent of oxidative stress. Superoxide dismutase is the first enzyme of defense against ROS; subsequently, CAT and APX act to detoxify the H_2O_2 produced by SOD (Bowler et al., 1992), and GR regenerates glutathione disulfide (GSSG) to GSH in the Asada–Halliwell pathway. In general, the Cu applied to the soil at increasing concentrations steadily activated GR, SOD and APX in the shoots, irrespective of the AM treatment (Figs. 4.4 and 4.5). However, at 100 mg Cu kg^{-1}, GA-colonized plants exhibited lower enzyme activities than NM- or GC-colonized plants. In addition, the root growth was stimulated approximately 60% by GA inoculation as compared to NM plants at this Cu level (Fig. 4.1). These results give reason to infer that, at a Cu concentration of 100 mg Cu kg^{-1} soil, symbiosis with native AM fungi decreased the need of such enzymes to detoxify ROS in plant tissues.

At the highest Cu level, a noticeable reduction of shoot growth occurred (Fig. 4.1), which was accompanied by a significant increase in shoot Cu concentration (data not shown). Furthermore, the addition of 500 mg Cu kg^{-1} significantly activated the antioxidant responses in GA-colonized plants, and in most of cases non-significant differences in the enzyme activities were observed when plants were inoculated with AM. These facts indicate that Cu toxicity occurred at the highest Cu dose, and this toxicity was likely due to: *i*) increased ROS production by the Fenton reaction (Schutzendubel and Polle, 2002) and/or *ii*) the impairment of photosynthetic function (Küpper et al., 2002). Furthermore, these results suggest that the availability of Cu to the plant, Cu toxicity, and plant antioxidant responses depend on exchange processes between soil, plants and root-colonizing microorganisms (Azcón et al., 2009).

On the other hand, Cu levels did not affect GC root colonization, regardless of whether SB was applied or not (Fig. 4.6). The opposite trend was observed in the roots of plants colonized by GA. Thus, GA colonization increased by about 15% at the highest soil Cu concentration. These results provide evidence that native AM fungus adapted to Cu polluted soils may have developed a differential mechanism that improves their tolerance to a wide range of metal concentrations in soil (Hildebrant et al., 2007). In addition, reports of González-Chávez et al. (2002) suggest that extraradical AM mycelia are able to sorb and/or accumulate Cu, and different tolerant isolates from the same polluted soil have different metal tolerance abilities. This provides further evidence for functional diversity within AM fungal populations in Cu-polluted soils (del Val et al., 1999).

4.5 Conclusion.

Marked differences between mycorrhizal and non-mycorrhizal *Oenothera picensis* plants were observed at increasing soil Cu concentrations, with the use (or omission) of a treated SB amendment together with the inoculation of both a Cu-adapted and a non-adapted AMF strain. Data obtained suggest that Cu-adapted mycorrhizal fungi (GA) provide physiological traits that allow for plant survival at phytotoxic Cu levels, possibly through exclusion mechanisms favoring a decrease of the oxidative stress produced by this element. The mechanism of resistance triggered by Cu-adapted AMF strains requires further study before *O. picensis* can be used in remediation of soils polluted with Cu.

Acknowledgements

This research was supported by the Plan National CICYT AGL2003-05619-CO2-02 (Spain) and by the National Commission for Scientific and Technological Research through Project FONDECYT 11080131 (Chile). Sebastian Meier acknowledges the support of MAE-AECID (Spain).

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Chapter 5. Interaction between mycorrhizal fungi and biotreated agrowaste residue improves nutritional status in a metallophyte growing in Cu-poluted soils.

Paper submitted to Chemosphere

Interaction between arbuscular mycorrhizal fungi and biotreated agrowaste residue improves nutritional status in a metallophyte growing in Cu-polluted soils

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Abstract

The interactive effect of sugar beet agrowaste (SB) and arbuscular mycorrhizal (AM) fungi inoculation in response to increasing soil Cu levels was evaluated in the metallophyte *Oenothera picensis*. Plants were grown in a Cu-treated soil (0, 100 or 500 mg Cu kg^{-1}), in presence or absence of SB, and inoculated with: *i*) Cu-indigenous mycorrhiza (IM) isolated from Cu-polluted soils; *ii*) *Glomus claroideum* (GC); or *iii*) maintained uninoculated. Sugar beet application produced an increase in shoot biomass production of 2-7 times, improving plant nutritional status and allowing their survival at the highest Cu level. On the other hand, AM fungi utilization had a positive effect promoting plant establishment; however, the Cu plant concentration, AM colonization, AM spore density and glomalin production were strictly dependent of the AM fungi strains used. In addition, remarkable differences between AM fungi strains were observed at the highest soil Cu concentration where only plants colonized by IM were able to survive and grow when no SB residue was added. In summary, there were positive effects of the AM fungi inoculation and SB application. A combination of physiological and nutritional changes caused by the interaction of both SB and AMF might be of interest to improve phytoremediation strategies in Cu-polluted soils.

Keywords: Arbuscular mycorrhiza fungi; copper pollution; glomalin-related soil protein; *Oenothera picensis;* sugar beet agrowaste.

5.1. Introduction

Copper (Cu) is an important essential trace element for normal plant growth and development. However, an excessive amount of this element in soil is highly toxic to plants, and often results in vegetation degradation and soil quality decrease with the consequent negative effect on the normal ecosystem functions [34]. In this sense, for the reclamation of contaminated sites, a number of environmental remediation systems involving physical, chemical or biological treatments have been developed in the last decades [18]. However, these treatments are expensive and alter the soil physicochemical and biological properties, and therefore are considered environmentally invasive.

Phytoremediation can be defined as the combined use of plants, soil amendments and agronomic practices to remove pollutants from the environment or attenuate their toxicity [25]. Due to the economic costs of growing a crop are lower than those associated to soil removal and/or replacement, the use of vegetation for landscaping, stabilization and pollution control is probably the most feasible approach to the soil reclamation in sites polluted by metals. Nevertheless, one key factor that determines the success of phytoremediation is the initial plant growth, which is often limited by metal toxicity, low nutrient availability and poor physical structure of soil [36].

On the other hand, it is well known that soil biological quality declines in metal contaminated soils as a consequence of the progressive decrease of soil organic matter content. Thus, the use of organic amendments such as sugar beet (SB), an inexpensive lignocellulosic residue, appear to be a feasible alternative to improve physical, chemical and biological soil properties [15].

Additionally, some microorganisms can facilitate plant establishment in Cu polluted soils. For example, it has been widely reported that arbuscular mycorrhizal (AM) fungi improve plant establishment in metal polluted soils, and even, some studies have concluded that the symbiosis is partly responsible for plant survival in those extreme environments [3, 11, 17]. In this sense, AM fungi have been proven to enhance the plant nutrition [23], and improve the soil structure through the actions of external mycelium as well as the production of glomalin, a glycoprotein that can sequester Cu in the soil [4, 10]. Therefore, AM fungi colonization constitutes a functional component of the soil-plant system, and

could be considered as a key factor for attenuation of metal stress in polluted environments [1, 2].

Different AM fungi isolates can differ in their metal tolerance as well as in their ability to protect plants against metal toxicity [5]. Even thought AM fungi from polluted areas are generally more resistant to environmental stresses [6, 11, 17], we consider particularly relevant to determine if AM fungi from non-polluted environments posses intrinsic capacities to tolerate elevated Cu levels, and also to predict whether they are able to survive and growth in metal polluted sites. In this context, we hypothesized that the use of AM fungi (either an indigenous Cu-adapted mycorrhiza fungus (IM) or non-adapted *Glomus claroideum* (GC) fungus) alone or in combination with SB application could attenuate plant Cu stress, due to an improved nutritional status and growth, thus contributing to facilitate the plant-soil system management in Cu-polluted environments.

Therefore, the aim of this research was to evaluate the interactive effect between the AM fungi inoculation and/or the SB application on the amelioration of nutritional status and growth in *Oenothera picensis*, a plant that naturally grows in Cu polluted soils. The effectiveness of either AM fungi inoculation or agrowaste residue application was tested by analyzing plant growth, nutrients concentration in plant tissues and mycorrhizal parameters such as root colonization, spores density and glomalin accumulation in soil.

5.2. Materials and Methods

5.2.1 Biological material

The metallophyte *Oenothera picensis* (formerly named *O. affinis*), which has been described as Cu-tolerant plant [9], was used for this bioassay. Seeds of *O. picensis* and rhizosphere soil were collected from a Cu-polluted area in a Mediterranean ecosystem strongly affected by atmospheric deposition of Cu-enriched particles (up to 830 mg total Cu kg^{-1} soil and 330 mg DTPA extractable Cu kg^{-1} soil) [4]. It was located approximately at 1.5 km Southeast from Ventanas copper smelter (CODELCO), in the Puchuncaví Valley, Central Chile (32°46′ 30″ S; 71° 28′ 17″ W).

Indigenous mycorrhizal inocula (IM) were isolated from the rizosphere of Cu-polluted soil following the methodology proposed by Vivas et al. [32]. The fungal reproduction was made in an open pot culture using sepiolite:quartz sand:vermiculite (1:1:1 v:v:v) mix as substrate, and *O. picensis* and *Plantago lanceolata* were used as host plants.

Glomus claroideum (GC) was used as reference of a non Cu adapted AM fungus. GC strain was isolated from agricultural soils in the Araucanía Region (Southern Chile) and it was obtained in a similarl way to that for IM, but using *Sorghum bicolor* and *Trifolium repens* as host plants. In both cases, after 6 months of plant growth, shoots were eliminated and a mixture of rhizosphere substrate was used as inoculum, containing spores (about 250-300 spores per 100 g), hyphae (about 3-4 m per g) and mycorrhizal root fragments.

5.2.2 Agrowaste Residue

The residue used as amendment was sugar beet (SB) agrowaste. The SB characteristics were: cellulose (29%), hemicellulose (23%) and lignin (5%). The solid residue was dried at 60°C and then ground to 2-mm-pore screen. Portions of 15 g of the solid substrate were placed in 250-mL Erlenmeyer flasks. Czapek-DOX mineral salt solution (0.01g L^{-1}, $FeSO_4$ x103 $7H_2O$; 0.5g L^{-1}, Mg SO_4 x 7 H_2O; 0.5 g L^{-1}, KCl; 3 g L^{-1}, $NaNO_3$; 1.0 g L^{-1}, K_2 HPO_4 and 30.0 g L^{-1}; sucrose) was added (40 mL) to each flasks. Rock phosphate (Morocco fluorapatite, 12.8 % soluble P, 1 mm mesh) was added at rate of 0.75 g per flask. These culture media were sterilized by autoclaving at 120°C for 30 min. The *Aspergillus niger* NB2 strain was used throughout this study to mineralize P in the agrowaste residue. For inoculum preparation, *A. niger* was grown at 30°C for 7 days in plates containing and spores were scraped in sterile distilled water. Three mL of *A. niger* spore suspension (about of 1.2 x 10^6 spores mL^{-1}) were spread carefully over the surface of the respective flask containing SB agrowaste. The fermentation process was carried out at 30°C for 20 days.

5.2.3 Plant growth conditions and experimental design

The experiment was performed in a fully randomized design using the following mycorrhizal treatments: non-AM inoculated plants, inoculated either with *G. Claroideum*

(GC) or a mixture of indigenous mycorrhizal fungi (IM) isolated from Cu-polluted areas. Each one of these three mycorrhizal treatments was assessed with or without SB amendment, and Cu was added at concentrations of 0, 100 or 500 mg Cu kg^{-1} soil. Five replicates were used for each combination totaling 90 experimental units.

Seeds of *O. picensis* collected from Cu-polluted areas were surface sterilized using 2% Cloramin-T solution for five minutes and rinsed thoroughly. After that, they were germinated and grown under greenhouse conditions (25±3/15±3 ºC day/night temperatures; 16/8 h light/dark photoperiod; 80-90% relative humidity) during 3 weeks before transplanting.

The soil used in this assay was collected in a semi-arid ecosystem from South-eastern of Spain (Granada). Soil physicochemical characteristics were reported by Marulanda-Aguirre *et al*. [13] and these were pH_W 7.2, 1.6% organic matter, 57.8% sand, 19.0% silt, 23.2% clay, and the following nutrient concentrations (in mg kg^{-1}): N, 2.1; $NaHCO_3$-extractable-P, 1.7; and K, 0.8. The soil was sieved through a 2 mm mesh and diluted with quartz-sand (2:1 soil:sand, v/v), sterilized by tyndallization for three times, and after 24 h air dried. Soil mixture was placed in 200 mL pots. After sterilization, the soil/sand mixture was supplemented with 0, 100 or 500 mg Cu kg^{-1} by adding the adequate amounts of $CuCl_2$ x $2H_2O$, then left to stabilize for two weeks.

The respective treated SB amendment was mixed (5%, w/w) with the soil:sand mixture and left to stabilize for another two weeks at room temperature. Lately, ten g of each inoculum (either IM or GC) were applied to the respective pots and uninoculated plants (NM) received an equivalent amount of autoclaved inoculum.

After transplanting, *O. picensis* plants were grown for 3 months in a glasshouse with temperature ranging from 25±3/15±3ºC day/night; 16/8 h light/dark photoperiod; and 80-90% relative humidity. Plants were watered daily with distilled water, and shoots and roots were harvested for chemical analyses.

5.2.4 Measurements

At harvest, shoots and roots were separated and dried at 70ºC for 2 days and after that weighed. Then, the samples were ground, ashed at 550ºC and digested by using a H_2O/HCl/HNO_3 mixture (8/1/1; v/v/v). The plant extracts were used for the determination

of K, Mg, Cu, B, Mn, P and Fe in an ICP plasma analyzer (IRIS Intrepid II XDL, Thermo Electron Corporation). Mineral analyses were carried out by the Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain. The Cu translocation factor was defined as the ratio between Cu foliar concentration and Cu root concentration [14].

Arbuscular mycorrhizal fungal colonization was quantified using a dissection microscope (20–40X) after cleaning the roots in 10% KOH (w/v) and staining them in trypan blue [21]. A variation of the gridline intersection method, developed by Giovannetti and Mosse [8] was used to determine the proportion of root colonized by AMF. Arbuscular mycorrhizal spores were isolated from 50 g of soil through the wet sieving and decanting method, followed by sucrose centrifugation. After centrifugation, the supernatant was poured through 50-μm-pore-size mesh and quickly rinsed with tap water. Spores were counted in a Doncaster dish under the dissecting microscope and grouped according to morphological characteristics [26].

Glomalin related soil protein (GRSP), operationally measured as Bradford-reactive soil protein [24] was determined according to the method described by Wright and Upadhyaya [35] with minor modifications. For the easily extractable fraction of GRSP (EE-GRSP) samples of 1 g soil were subjected to extraction with 8 mL of 20 mM citrate, pH 7.0, and autoclaving for 30 min at 121 °C. The total GRSP (T-GRSP) was extracted from 1 g of soil with 8 ml of 50 mM citrate, pH 8.0, and autoclaving for 1 h at 121 °C. For T-GRSP, the procedure described was repeated several times on the same sample until the reddish-brown color typical of GRSP disappeared from the supernatant, combining all extracts from a soil sample. In both cases, the supernatant was separated by centrifugation at 8000 g for 20 min and filtered using paper Whatman No 1. The protein content in the crude extract was determined by Bradford assay (Bio Rad Protein Assay; Bio Rad Laboratories) with bovine serum albumin as the standard.

5.2.5 Statistical analyses

The data of main effects of Cu levels, AM inoculation, agrowaste residues application and its interactions were tested by means of a multifactorial ANOVA. Means were compared by the orthogonal contrast test [20]. Data sets not meeting assumptions for

ANOVA were transformed as required, but the results are presented in the original scale of measurement. Statistical significance was determined at $p \leq 0.05$. In all cases statistical analyses were performed using the SPSS software v. 10.0 (SPSS Inc., Chicago, Il).

5.3 Results

In general terms, all the analyzed variables were significant and highly affected by the applied treatments and the interactions between them (Table 5.1). In particular, the multiple interactions produce significant changes in all the biomass production and nutritional variables analyzed. Under the experimental conditions here applied, the different AM fungi treatments used (uninoculated, or inoculated with GC or IM) produced a minor effect in some of the variables measured, for example the Cu translocation factor, the P, and Mn shoot concentrations and the EE-GRSP in soil (Table 1).

5.3.1 Growth and elements content.

A positive effect on shoot biomass production was found as a consequence of SB application (Table 5.2). In fact, SB application increased shoot biomass production by 2-7 times allowing also plant survival at the highest Cu concentrations. However, this positive effect was not observed at root level, where no differences were observed (Tables 5.1, 5.2). Non-differences on shoot and root biomass production were found in AMF inoculated plants (either inoculated with GC or IM) in comparison with the control treatments (NM) at all Cu supply levels (Table 5.2). However, at the highest Cu concentration and without SB application, only the plants colonized by IM were able to survive (Table 5.2).

Plant Cu concentrations were increased due Cu addition, and it was accumulated principally at root level (about 95% of the total Cu in plants). Thus, a low translocation factor was observed independent of the Cu supply level applied (Table 5.3). When no SB was added, AM fungi inoculation reduced the translocation by at least 50% in plants treated with 100 mg Cu kg^{-1}. A significant decrease of translocation was also observed in SB treated plants at the highest Cu supply level. The above effect was more evident at root level and with SB addition, was at the highest Cu concentration the NM plants concentrated 2.3 and 1.3 times higher Cu than GC and IM inoculated plants, respectively (Table 5.3).

Table 5.1 *F*-values and significance for the main effects and factor interactions for the variable analyzed in an *Oenothera picensis* crop study by means of a multifactorial ANOVA (n=90).

Experimental variable	**Cu[1]**	**SB**	**AM**	**Cu x SB**	**Cu x AM**	**SB x AM**	**Cu x SB x AM**
Shoot dry weight (g)	507.4***	3288.8***	196.7***	1050.0***	30.0***	731.9***	464.0***
Root dry weight (g)	65.6***	2.2ns	38.6***	19.4***	19.1***	37.0***	36.3***
Shoot Cu concentration (μg g^{-1})	15.7***	26.6***	4.4*	20.5***	3.2*	1.9ns	9.4***
Root Cu concentration (μg g^{-1})	161.3***	143.1***	31.1***	91.4***	23.5***	33.2***	16.1***
Cu translocation factor	64.5***	5.1*	0.5ns	6.2**	3.4*	9.0***	5.9***
Shoot P concentration (μg g^{-1})	55.5***	442.5***	4.6*	36.3***	2.5*	10.6***	5.5**
Root P concentration (μg g^{-1})	13.8***	31.5***	6.3**	10.8***	3.3*	6.0**	5.4**
Shoot K concentration (μg g^{-1})	205.6***	728.2***	26.5***	3.2*	20.8***	3.6*	15.8***
Root K concentration (μg g^{-1})	96.1***	43.7***	10.7***	43.8***	6.2***	16.8***	7.7***
Shoot Mg concentration (μg g^{-1})	6.3**	119.6***	59.2***	257.9***	85.6***	58.9***	101.0***
Root Mg concentration (μg g^{-1})	28.5***	25.0***	11.7***	47.0***	7.1***	13.0***	2.2ns
Shoot Fe concentration (μg g^{-1})	1.6ns	3.8ns	2.0ns	13.6***	6.3***	1.5ns	6.0***
Root Fe concentration (μg g^{-1})	2.6*	0.8ns	18.7***	0.0ns	54.7***	8.0**	6.7***
Shoot Mn concentration (μg g^{-1})	5.5**	234.4***	2.0ns	34.7***	15.7***	28.9***	19.5***
Root Mn concentration (μg g^{-1})	4.8*	148.6***	4.5*	0.5ns	9.4***	12.8***	8.5***
Shoot B concentration (μg g^{-1})	69.8***	27.9***	5.8**	189.8***	11.0***	9.1***	8.4***
Root B concentration (μg g^{-1})	21.6***	246.5***	3.9*	10.7***	12.1***	6.7**	6.9***
Mycorrhizal colonization (%)	126.0***	149.9***	4767.6***	85.4***	532.4***	80.3***	180.3***
AM spore density (spores in 50 g of soil)	13.3***	12.0**	1673.4***	32.0***	186.5***	5.3**	26.7***
EE-GRSP (mg g^{-1})	10.6***	159.5***	0.0ns	3.8*	10.2***	8.0**	2.6*
T-GRSP (mg g^{-1})	63.0***	334.7***	25.3***	41.3***	16.9***	13.8***	15.4***

Significance conventions: **P*<0.05, ***P*<0.01, ****P*<0.001, ns = no significant differences.

[1]Cu = different Cu levels; SB = sugar beet biotransformed residue application; AM = arbuscular mycorrhizal inoculation.

1

Table 5.2 Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted fungal populations (IM) on shoot and root dry weight in *Oenothera picensis.* Values are mean ± standard error. Different letters on each row indicate significant differences (P<0.05) using orthogonal contrasts test (N=5).

Cu levels	**AM Inoculation**	***Shoot***		***Root***	
		-SB	+SB	-SB	+SB
Control	NM	$0.33 \pm 0.02^{(f)}$	$1.43 \pm 0.06^{(b)}$	$0.54 \pm 0.06^{(a)}$	$0.38 \pm 0.02^{(de)}$
	GC	$0.20 \pm 0.01^{(g)}$	$1.52 \pm 0.08^{(ab)}$	$0.18 \pm 0.01^{(k)}$	$0.33 \pm 0.02^{(efg)}$
	IM	$0.26 \pm 0.02^{(f)}$	$1.63 \pm 0.01^{(a)}$	$0.43 \pm 0.02^{(bc)}$	$0.32 \pm 0.03^{(fgh)}$
Cu 100	NM	$0.30 \pm 0.01^{(fg)}$	$1.10 \pm 0.08^{(c)}$	$0.49 \pm 0.09^{(ab)}$	$0.22 \pm 0.03^{(jk)}$
	GC	$0.29 \pm 0.02^{(fg)}$	$1.10 \pm 0.06^{(c)}$	$0.29 \pm 0.02^{(fghi)}$	$0.34 \pm 0.02^{(ef)}$
	IM	$0.23 \pm 0.02^{(fg)}$	$1.01 \pm 0.07^{(c)}$	$0.43 \pm 0.04^{(cd)}$	$0.27 \pm 0.01^{(hij)}$
Cu 500	NM	---	$0.67 \pm 0.11^{(de)}$	---	$0.28 \pm 0.06^{(ghi)}$
	GC	---	$0.77 \pm 0.01^{(de)}$	---	$0.29 \pm 0.03^{(fghi)}$
	IM	$0.66 \pm 0.05^{(e)}$	$0.79 \pm 0.02^{(d)}$	$0.44 \pm 0.02^{(bcd)}$	$0.23 \pm 0.01^{(ijk)}$

Table 5.3 Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal populations (IM) on shoot and root Cu concentration and metal translocation factor in *Oenothera picensis.* Values are mean ± standard error. † = death plants. Different letters on each row indicate significant differences (P<0.05) using orthogonal contrasts test (N=5).

Cu levels	**AM Inoculation**	***Shoot***		***Roots***		***Translocation factor***	
		-SB	+SB	-SB	+SB	-SB	+SB
Control	NM	$7.75 \pm 0.2^{(f)}$	$6.41 \pm 0.2^{(f)}$	$60.7 \pm 2.1^{(g)}$	$72.7 \pm 2.8^{(g)}$	$0.12^{(abc)}$	$0.09^{(bcdef)}$
	GC	$7.12 \pm 0.09^{(f)}$	$7.74 \pm 0.6^{(f)}$	$89.1 \pm 3.2^{(g)}$	$59.5 \pm 3.1^{(g)}$	$0.08^{(bcdef)}$	$0.14^{(ab)}$
	IM	$7.11 \pm 0.2^{(f)}$	$9.28 \pm 0.3^{(f)}$	$75.1 \pm 3.1^{(g)}$	$62.7 \pm 2.2^{(g)}$	$0.10^{(abcde)}$	$0.17^{(a)}$
Cu 100	NM	$21.4 \pm 0.2^{(bc)}$	$13.5 \pm 0.7^{(de)}$	$219 \pm 13^{(f)}$	$416 \pm 13^{(d)}$	$0.12^{(abcd)}$	$0.03^{(g)}$
	GC	$9.95 \pm 0.5^{(ef)}$	$14.0 \pm 0.9^{(d)}$	$244 \pm 7.9^{(f)}$	$240 \pm 8.8^{(f)}$	$0.04^{(efg)}$	$0.06^{(cdef)}$
	IM	$6.78 \pm 0.2^{(b)}$	$14.7 \pm 1.2^{(d)}$	$252 \pm 6.0^{(f)}$	$325 \pm 8.9^{(e)}$	$0.03^{(efg)}$	$0.05^{(efg)}$
Cu 500	NM	†	$45.5 \pm 1.8^{(a)}$	†	$866 \pm 20^{(a)}$	†	$0.05^{(defg)}$
	GC	†	$20.9 \pm 0.1^{(c)}$	†	$380 \pm 12^{(d)}$	†	$0.05^{(defg)}$
	IM	$21.2 \pm 0.09^{(c)}$	$25.0 \pm 1.92^{(b)}$	$501 \pm 8.6^{(c)}$	$671 \pm 5.3^{(b)}$	$0.04^{(efg)}$	$0.04^{(efg)}$

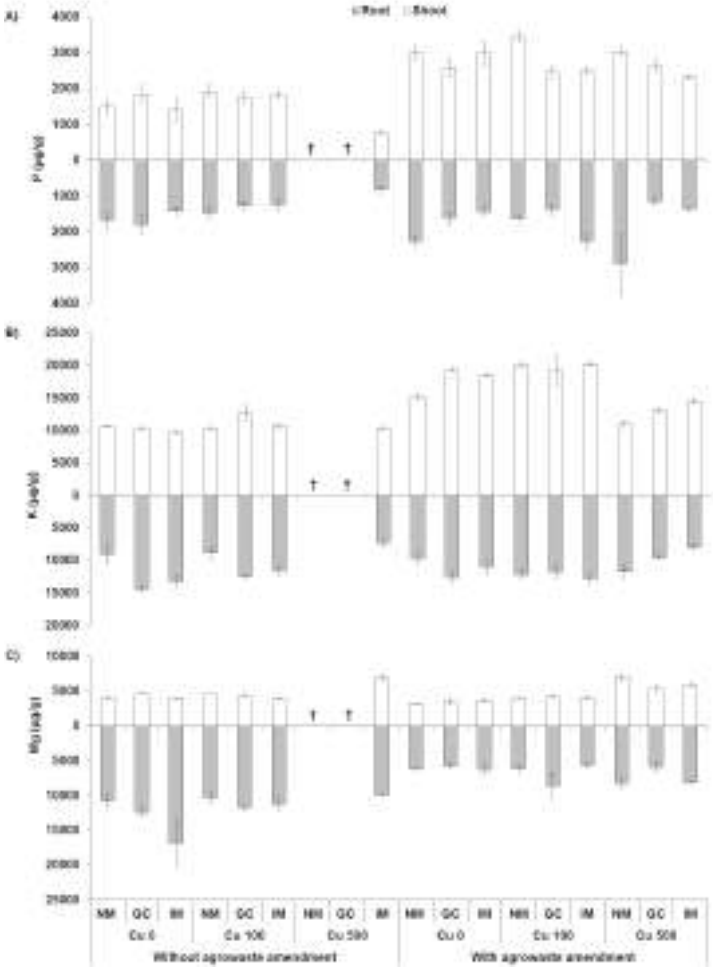

Fig 5.1 Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal populations (IM) on shoot and root macronutrients extraction in *Oenothera picensis.* (A) P concentration, (B) K concentration and (C) Mg concentration NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E. n=5.

On the other hand, shoot macronutrient concentration was more affected by the amendment application than AM colonization, except for Mg (Fig. 5.1), producing between 1.7 to 2.3 fold increase in P and K concentration. Nevertheless, such effect was not detected at root level. A similar trend was observed in micronutrient concentrations, especially at root levels, which were highly affected by the SB use. In addition, Mn and B root concentrations were 2-4 fold higher in SB treatments compared to unamended soils (Fig. 5.2).

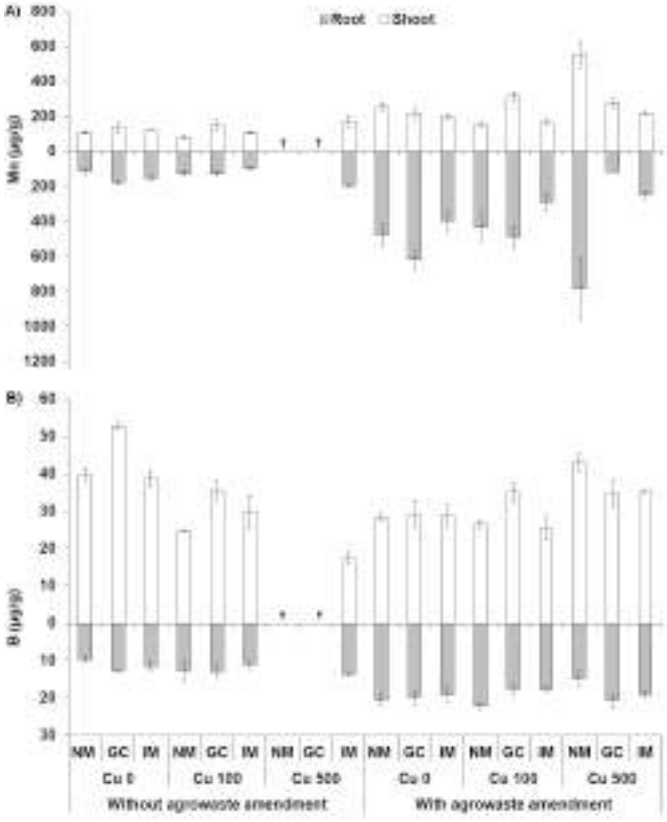

Fig 5.2 Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal populations (IM) on shoot and root micronutrients concentration in *Oenothera picensis.* (A) Mn concentration and (B) B concentration NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; n = 5.

5.3.2 Arbuscular mycorrhizal parameters

As expected, no root colonization was found in all control treatments (i.e. without AMF inoculation). The other two independent variables (Cu supply levels and SB application) affected significantly fungal colonization, spore number and glomalin production (Tables 5.1, 5.4, and Fig. 5.2).

In particular, plants inoculated with IM increased their root colonization and spore number at high Cu supply levels, irrespectively of the SB addition, whereas an opposite effect was observed in GC inoculated plants. For IM inoculated plants, root colonization and spore production were about 1.7 and 2 fold higher at highest Cu dose (Table 4).

In contrast, in SB treated soil, GC spores number decreased significantly at increasing Cu supply levels, from 56 spores per 50 g of dry soil in control soil to only 7 spores at the highest Cu dose (Table 5.4).

Table 5.4 Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal populations (IM) on root colonization and spore number in *Oenothera picensis.* Values are mean ± standard error. Different letters on each row indicate significant differences (P<0.05) using Tukey test (N=5).

Cu levels	AM Inoculation	Colonization (%)		Spore number	
		-SB	+SB	-NSB	+SB
Control	GC	54.6 ± 2.7 $^{(ab)}$	26.0 ± 0.6$^{(fg)}$	14.7 ± 0.3$^{(d)}$	56.0 ± 2.5$^{(b)}$
	IM	35.3 ± 1.5 $^{(de)}$	42.7 ± 1.5$^{(cd)}$	46.3 ± 2.4$^{(bc)}$	50 ± 0.5$^{(b)}$
Cu 100	GC	53.6 ± 4.3 $^{(ab)}$	29.3 ± 0.9$^{(ef)}$	35.3 ± 4.4$^{(c)}$	16.7 ± 2.5$^{(d)}$
	IM	50.3 ± 0.9 $^{(bc)}$	45.6 ± 0.9$^{(bc)}$	57.3 ± 2.4$^{(b)}$	58.0 ± 5.9$^{(b)}$
Cu 500	GC	---	16.3 ± 0.3$^{(g)}$	---	7.2 ± 0.6$^{(d)}$
	IM	62.0 ± 0.6 $^{(a)}$	52.7 ± 2.1$^{(b)}$	92.3 ± 1.2$^{(ab)}$	94.7 ± 4.3$^{(a)}$

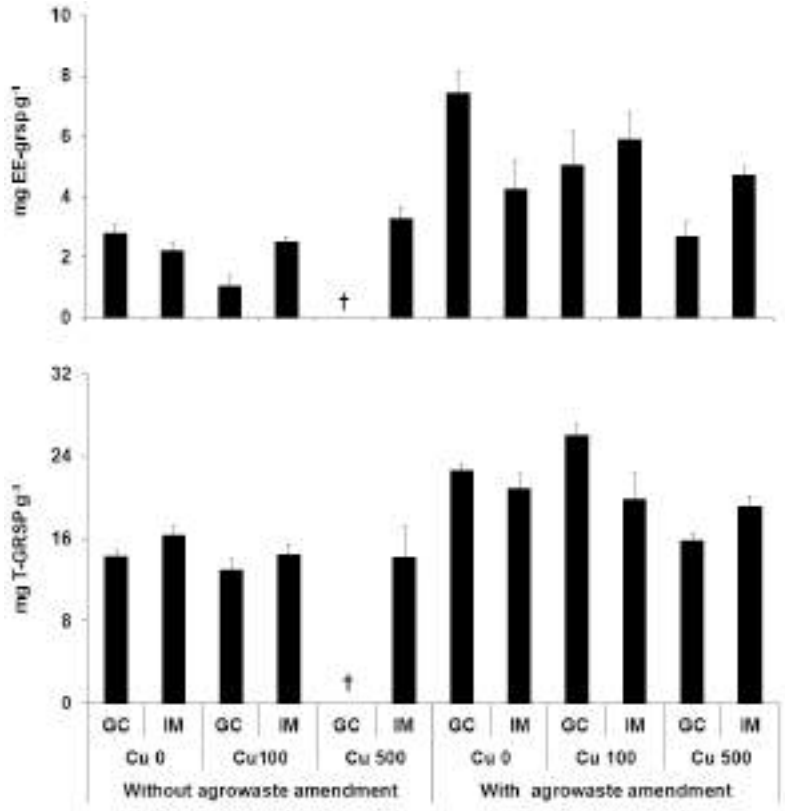

Fig 5.3 Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal populations (IM) on easly extractable glomalin and (EE-GRSP) total glomalin related soil protein (T-GRSP). NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; n = 5.

Glomalin related soil protein production (either EE-GRSP and T-GRSP) seems to be more dependent of the SB application than AMF inoculation (Fig. 4). However, we found differences between the AMF strains studied. In this sense, the amount of EE-GRSP and T-GRSP increased by 1.2- and 1.8- fold, respectively, in IM inoculated plants as compared with the GC strain, under SB application and at the greatest Cu addition level (Fig. 3).

5.4 Discussion

Our results showed an important interactive role between SB and AMF inoculation in promoting plant establishment and nutrition in Cu polluted soils. The SB application produced an increase in the dry matter production and allowed the plant survivor at the highest Cu supply level (Table 2). This finding is supported by previous reports, which indicate the crucial role played by SB improving plant metal tolerance in soils contaminated with Cd [16], Zn [15], Pb [1] and Cu [17]. Furthermore, the benefits of SB amendment on plant growth could be related to its polysaccharide composition, which is principally rich in P due to the phosphate rock applied during the fermentation process [28].

Other reports conclude that the polysaccharide composition of the amendment can also bind metals into its structure [1]. Thus, the metal binding capacity has been correlated with the density of polysaccharides acids capable of complexing cations [22]. However, our results did no support a Cu chelating activity by the amendment. Moreover, SB addition increased both the plant Cu concentration and the Cu translocation to the shoots (Table 3). Therefore, this agro-waste residue appears to be playing principally a nutritional role, increasing Cu tolerance through a stimulating effect on plant growth (Fig. 1 and 2).

On the other hand, AM fungi inoculation promoted the plant survival in Cu polluted soil (Table 2). Nevertheless, this response was strictly dependent of AMF strain, which denotes the presence of metal adaptation mechanisms [19]. In fact, according to our results, the most important effect of the Cu adapted IM was observed at the highest soil Cu concentration (500 mg Cu kg^{-1}). Under these conditions, only plants colonized by IM were able to survive and grow when no SB residue was added (Table 2). Such protective effect has been previously observed in plants colonized by other strains of indigenous AM fungi in soils polluted with Zn [11], Pb [27], or As [12].

In addition, different mechanisms seem to be functioning in mycorrhizal stimulating effect on the Cu accumulation. In fact, we found a substantial reduction of Cu concentration in shoots and roots in mycorrhizal plants, and such reduction became more noticeable under the more toxic Cu level. Under this condition, IM was able to decrease in about 29% and 82% Cu in roots and shoots than control plants (NM), respectively (Table 3). In a related report Meier et al., [17] found a decrease in the Cu uptake by *O. picensis* plants inoculated

with IM. The above was related with the decrease on its antoxidative enzyme activity, suggesting that IM enhanced plant Cu tolerance as a consequence of adaptive physiological mechanisms provided by the fungi. In addition, the impact of AM colonization on metal uptake by host plants has been earlier proved for several metals like Pb [32, 37], Cd [30], Ni [29] and Zn [31], suggesting the presence of metal exclusion mechanisms for IM against high Cu concentration in soils.

On the other hand, an increase of both the AMF root colonization and the AM spores production occurred as a consequence of IM inoculation, especially at the highest Cu supply, indicating that the autochthonous fungus was more tolerant to the Cu applied, compared to the GC strain (Table 4). Moreover, the increased AM colonization in IM plants together with a decrease in root Cu concentration could denote that plants may regulate the AM fungal colonization by its own benefits [7, 11]. These facts confirm the protective role of AM fungi in metal polluted soils, and also allow establish tolerance differences between metal-adapted and non-adapted AM fungi strains against toxic concentrations of Cu in the soil [6]. In fact, AM spore production in Cu non-adapted fungus (GC) suffered a decrease when high Cu levels were applied. Although the spores production did not completely disappear in GC at the highest soil Cu level, the low level of propagules presumably resulted insufficient to reach a good colonization able to support plant growth without the application of SB amendment.

Finally, differences on GRSP production were observed. Such differences seem to be more related to soil Cu supply and SB application than the AM fungi inoculums used (Fig. 3). For IM colonized plants, the high GRSP production and the low Cu concentration in their roots suggest that this compound could act as a relevant exclusion mechanism developed by the autoctonus fungus to cope toxic metal concentrations in the soil [4, 33].

5.5 Conclusion

The combination between sugar beet (SB) amendment and indigenous mycorrhyza (IM) constitute a successful biotechnological tool for improving the *Oenothera piscensis* establishment in Cu-polluted soils. The IM strain was able to control the Cu uptake by plants, allowing its survival at the highest Cu concentration, whereas SB had a direct effect on plant growth by improving plant nutrition. In addition, the use of IM produces a highest density of AM propagule, which can produce a faster plant establishment under metal stress conditions. Therefore, we conclude that the combination of SB and arbuscular mycorrhizal fungi might be of interest to improve phytoremediation strategies in Cu-polluted soils.

Acknowledgements

This research was supported by the Plan National CICYT AGL2003-05619-CO2-02 (Spain) and by the National Commission for Scientific and Technological Research through Project FONDECYT 11080131 (Chile). Sebastian Meier acknowledges the support of MAE-AECID (Spain).

5.6. References

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Chapter 6. Effects of arbuscular mycorrhizal inoculation on metallophytes and agricultural plants growing at increasing copper levels.

Paper submitted to Applied Soil Ecology, APSOIL-D-11-00268R1

Effects of arbuscular mycorrhizal inoculation on metallophyte and agricultural plants growing at increasing copper levels.

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Abstract

A pot culture experiment was carried out to assay the behavior of different arbuscular mycorrhizal (AM) fungal inocula on plant growth and copper (Cu) uptake using two metallophytes (*Oenothera picensis* and *Imperata condensata*) and one agricultural plant (*Helianthus annuus*) grown at increasing Cu supply levels. Plants were established in a Cu polluted soil spiked with 0, 150, 300 or 450 mg Cu kg^{-1}, and inoculated or not with: *i)* Cu-adapted AM fungi (GA) or *ii)* the Cu non-adapted strain *Glomus claroideum* (GC). Differences in plant biomass between inoculated and uninoculated plants were found, which were dependent on the AM fungal inocula used and the Cu level applied. Although the beneficial effect of AM fungi in promoting plant biomass production was not observed in metallophytes plants, a positive interaction between GA and *H. annuus* increased the shoot growth, especially at higher Cu levels. In addition, the Cu transfer from the roots to the shoots was low, remaining mostly at root level, especially in non-mycorrhizal plants; however AM fungi produced changes in Cu distribution increasing the translocation to the shoots. Differences in AM fungal parameters (root colonization, spore number and glomalin production) were strictly dependent on the Cu level and the AM fungal inoculum, suggesting the existence of certain compatibility, which was dependent on the particular combination AM-plant used. Specifically, the glomalin accumulation and Cu-bound to glomalin were significantly higher in AM colonized *H. annuus* plants, which could suggest a highly efficient way to reduce the Cu toxicity levels in soil. Therefore the use of *H. annuus* with AM fungal could promote phytostabilization processes.

Keywords: copper contaminated soils, Cu-adapted AM fungi, glomalin-related soil protein, phytoremediation.

6.1 Introduction

Copper (Cu) is an essential trace element for normal plant growth and development. However, an excessive amount of this element in soil is highly toxic to both higher plants and microorganisms, often resulting in vegetation degradation, soil quality decrease and, as a consequence, the normal functioning of the ecosystem is affected (Adriano, 2001, Bolan et al., 2003, Wong, 2003). Several environmental remediation systems involving physical, chemical, or biological treatments have been developed for reclamation of metal contaminated soils in the last decades (Mulligan et al., 2001). However, these treatments are expensive, and alter the soil's physicochemical and biological properties and therefore are considered environmental unfriendly.

Recently, the potential role of higher plants in remediation of metal-polluted soils has acquired relevance (Pilon-Smiths, 2005). The use of vegetation for landscaping, stabilization and pollution control is probably the most realistic approach to the reclamation of the land impacted by high metal concentrations (Robinson et al., 2007; Bolan et al., 2011). Nevertheless, an important factor that determines the successful vegetation in metal polluted sites is the initial plant establishment, which is often limited by metal toxicity, low nutrient contents and poor soil physical structure (Ye et al., 2002). For the above reasons, the long-term success of phytoremediation programs in metal contaminated soils has been limited. Among factors involved, the lack of knowledge about the role of microbial communities could explain some failures.

Soil microorganisms are involved in diverse biochemical processes, such as soil formation, energy transfer and nutrient cycling, which enhance and accelerate vegetation processes and thereby increase the stability of polluted ecosystems (Moynahan et al., 2002). However, managing soil microorganisms in phytoremediation must include the use of those forming symbiotic associations with plant roots such as the arbuscular mycorrhizal (AM) fungi as prerequisite for any soil restoration program to be successful (Haselwandter and Bowen, 1996; Meier et al., 2011b).

It is well known that AM fungi improve plant establishment in metal polluted soils, and even, some studies concluded that the symbiosis is partly responsible for plant survival in those

extreme environments (Carvalho et al., 2006; Hildebrandt et al., 2007; Meier et al., 2011a). In this sense, AM fungal colonization contributes to enhancement of plant establishment, through improving plant nutrition, particularly phosphate and some trace elements (Reinhardt, 2007; Meier et al., 2011b). In addition, AM fungi can improve soil structure through the combined actions of network of external mycelium and the production of a glycoprotein known as glomalin, which also has the capacity to sequester heavy metals from soil (González Chavez et al., 2004; Cornejo et al., 2008). Therefore, plants proposed for phytoremediation might require the use and selection of the most effective AM fungi in order to survive in such constraint conditions. This selection should be supported by the knowledge of metal-tolerant fungal species able to grow and function on polluted as well as adapted to nutrient impoverished soils (del Val et al., 1999). In order to understand the interactions among metals, AM fungi and higher plants, it is necessary to study and compare the diversity of AM fungi associated with metal-tolerant and non-tolerant plants in metal polluted and unpolluted soils for making a selection of those which are suitable for phytoremediation purposes; however, at present only few studies have been reported on this aspect (Vivas et al., 2006).

Oenothera picensis and *Imperata condensata* are Cu metallophyte plants naturally growing in metal polluted soils at Central Chile. These plants have been known to tolerate Cu toxicity, thereby offering a potential to be used in phytoremediation programs (Ginocchio, 2000; Cornejo et al., 2008; González et al., 2008). Some studies have investigated the Cu tolerance of these plants (González et al., 2008; Gaete et al., 2010); however, few of them have been focused on the role of their rhizosphere associations, especially with AM fungi. Recent studies have demonstrated the positive role of the indigenous AM fungi in promoting metallophyte establishment in Cu polluted soils (Meier et al., 2011b). Nevertheless, there are no reports comparing the effectiveness of different AM fungal strains, either isolated from Cu contaminated soils or from non-polluted areas enhancing plant establishment of both Cu tolerant and non-metal adapted plants.

Therefore, the aim of this research was to compare the behavior of an indigenous Cu tolerant mycorrhizal fungal (GA) mix versus a non-metal adapted AM fungus strain (*Glomus claroideum* -GC) using two Cu metallophytes and one agricultural plant, all growing at increasing Cu supply levels. The effectiveness of AM fungal inoculation was tested through the analysis of plant

biomass production, Cu concentration in plant tissues and AM development parameters, including glomalin accumulation and Cu sequestration by this glycoprotein.

6.2 Materials and Methods

6.2.1 Experimental design

The soil used in this study corresponded to a Cu contaminated soil (830 mg total Cu kg^{-1} soil and 330 mg DTPA extractable Cu kg^{-1} soil; Cornejo et al., 2008) obtained near to Ventanas Cu smelter (CODELCO), in the Puchuncaví Valley, Central Chile (32°46′ 30″ S 71° 28′ 17″ W). The Cu contaminated soil was spiked with four additional Cu levels: 0, 150, 300 or 450 mg Cu kg^{-1} (applied as $CuCl_2$ x $2H_2O$, Sigma reagent). In addition, there were three plant specie and three AM fungi treatments, which included: *i)* a mixture of autochthonous Glomeromycota (GA) fungi, *ii)* a strain of *Glomus claroideum* (GC), and *iii)* uninoculated plants. A completely randomized design with three replicates per each combination was used, with a total of 108 experimental units.

6.2.2 Plant species

Two metallophytes, *Oenothera picensis* (formerly named *O. affinis*) and *Imperata condensata* (Poaceae) were used. Both plants have been described as Cu tolerant species (González et al., 2008; Cornejo et al., 2008). Seeds of *O. picensis* and stolons of *I. condensata* were collected *in situ* from Cu-polluted areas to produce plantlets. The plant collection area was a Mediterranean ecosystem strongly affected by the deposit of metal-rich particles, located approximately at 1.5 km southeast from the Ventanas Cu smelter (CODELCO), at the Puchuncaví Valley, Central Chile (32°46′ 30″ S 71° 28′ 17″ W). In addition, commercial seeds of the agricultural plant, sunflower (*Helianthus annuus*) was included due to its potential ability to accumulate Cu without being overly sensitive to Cu toxicity (Lin et al., 2003)

6.2.3 Soil characteristics and plant growth conditions

The soil used in this study belonged to Chilicauquén series, fine sandy loam in texture, moderately deep, formed on a substrate of sandstone cemented with clay from the upper horizons. This soil was sieved through a 2 mm mesh and diluted with quartz-sand (< 1mm) (2:1 soil:sand, v/v), sterilized by tyndallization for three consecutive days, and air dried. Soil mixture was placed in 500 mL pots. After sterilization, the soil/sand mixture was treated with 0, 150, 300 or 450 mg Cu kg^{-1} soil by adding known amounts of $CuCl_2 \cdot 2H_2O$ solution, then left to equilibrate for two weeks.

Seeds of *O. picensis* and *H. annuus*, and stolons of *I. condensata* were surface sterilized with 2% Cloramin-T solution for five minutes and rinsed thoroughly. The seeds and stolons were grown under greenhouse conditions (25±3/15±3 ºC day/ night temperatures; 16/8 h light/dark photoperiod; 80-90% relative humidity) for 4 weeks using a sepiolite:quartz sand:vermiculite (1:1:1 v:v:v) mix as substrate before transplanting. At transplanting, the plants were or not AM fungal inoculated. The Cu-adapted indigenous AM fungi (GA) were isolated by wet sieving and decanting (Gerdemann and Nicholson, 1963) from rhizosphere soil of *O. picensis* and *I. condensata* plants growing in Cu polluted soils of Puchuncaví Valley (Central Chile), mixed and transferred to an open pot culture using sepiolite:quartz:vermiculite (1:1:1 v:v:v) mix as substrate. *Oenothera picensis* and *Plantago lanceolata* were used as host plants. After 6 months of plant growth, shoots were eliminated and the substrate containing about 200-250 spores per 100 g, 2.5-3.0 m of AM hyphae per g, and fragments of colonized roots was used as GA inoculum. A preliminary morphological analysis revealed that almost all the spores present in the inocula belonged to *Glomus* genus, being *Glomus* aff. *intraradices* the dominant ecotype. Other present ecotypes were *Acaulospora* aff. *lacunosa, Entrophospora infrequens* and others, wich belong to *Gigaspora* and *Scutellospora* genus, but such ecotypes were found in a very low density and only in some inoculum samples. *Glomus claroideum* (GC) strain was isolated from agricultural soils of the La Araucanía Region (Southern Chile) and used as a reference of non- Cu adapted AM fungi. The GC inoculum was obtained in similar way that for GA, but using *Sorghum bicolor* and *Trifolium repens* as host plants. In both cases, a mixture of rhizosphere substrate containing spores (about 250-300 spores per 100 g), hyphae (about 3-4 m per g) and mycorrhizal root fragments was used as inoculum. Ten g of each inoculum were added to the respective pots just

below the seedlings. Uninoculated plants (NM) received an equivalent amount of autoclaved inoculum. Plants were grown for 3 months under greenhouse conditions and then harvested.

6.2.4 Plant and AMF analysis

At harvest, shoots and roots were separated, dried at 70ºC for 2 days and weighed. Then, the samples were ground, ashed at 550ºC and digested using a H_2O/ HCl/ HNO_3 mixture (8/1/1 v/v/v). The Cu concentrations were determined in a flame atomic absorption spectroscopy (Perkin-Elmer 3110).

Arbuscular mycorrhizal root colonization was quantified using a dissection microscope (20-40X) after clearing a portion of the roots in 10% KOH (w/v) and staining in 0.05% trypan blue in lactic acid (w/v). The gridline intersection method (Giovannetti and Mosse, 1980) was used to determine the proportion of AM root colonization. The AM fungi spores were separated from the soil by wet sieving and decanting in a 70% w/v sucrose solution (Gerdemann and Nicholson, 1963), and quantified under a magnifying glass with a 30-50 magnification.

Glomalin related soil protein (GRSP), operationally defined as Bradford-reactive soil protein (Rillig, 2004) was determined according to the method described by Wright and Upadhyaya (1998), with minor modifications. The GRSP was extracted from 1 g of soil with 8 ml of 50 mM citrate, pH 8.0, and autoclaving for 1 h at 121 °C. After that, the supernatant was separated by centrifugation at 8000 g for 20 min and filtrated using Whatman paper No 1. The above procedure was repeated several times on the same sample until the reddish-brown color typical of GRSP disappeared from the supernatant, combining all extracts from a soil sample. The protein content in the crude extract was determined by Bradford assay (Bio Rad Protein Assay; Bio Rad Laboratories) with bovine serum albumin as the standard.

To determine GRSP-bound Cu (GRSP-Cu) we used the following methodology. GRSP was precipitated by slow addition of 2M HCl up to pH 2.5, centrifuged at 8000g for 20 min, re-dissolved in 0.5M NaOH, extensively 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 GRSP-Cu was determined by atomic Absorption Spectroscopy (-AAS-, Perkin-Elmer 3110).

6.2.5. Statistical analyses

The data for the main effects of plant species, Cu levels, AM inoculation (either GA or GC), and its interactions were tested by means of a multifactorial ANOVA. Means were compared by the orthogonal contrast test (Petersen, 1977). Data sets not meeting assumptions for ANOVA were transformed as required, but the results are presented in the original scale of measurement. Statistical significance was determined at $P \leq 0.05$.

6.3 Results

6.3.1 Dry matter production

Toxicity symptoms induced by high Cu levels were observed during plant growth in *O. picensis*, which at the highest Cu supply level were not able to survive when GA was used (Fig 6.1). Shoot biomass dry yield was significantly affected in the metallophytes species colonized by AM fungi; whereas, in general, non-differences were detected at root level ($P > 0.05$). In particular, the shoot biomass production in *I. condensata* remained relatively constant independent of the AM inoculum used or the Cu supply level applied to the soil.

Helianthus annuus plants inoculated with indigenous mycorrhizal fungi (GA) produced in general an increase in shoot biomass production ($P < 0.05$) and the effect was more striking at the highest Cu level. The GA inoculated plants produced five-fold higher shoot growth than the uninoculated ones at the highest Cu supply level (Fig 6.1). In addition, differences in shoot growth between GA and GC strains were observed (Fig. 6.1).

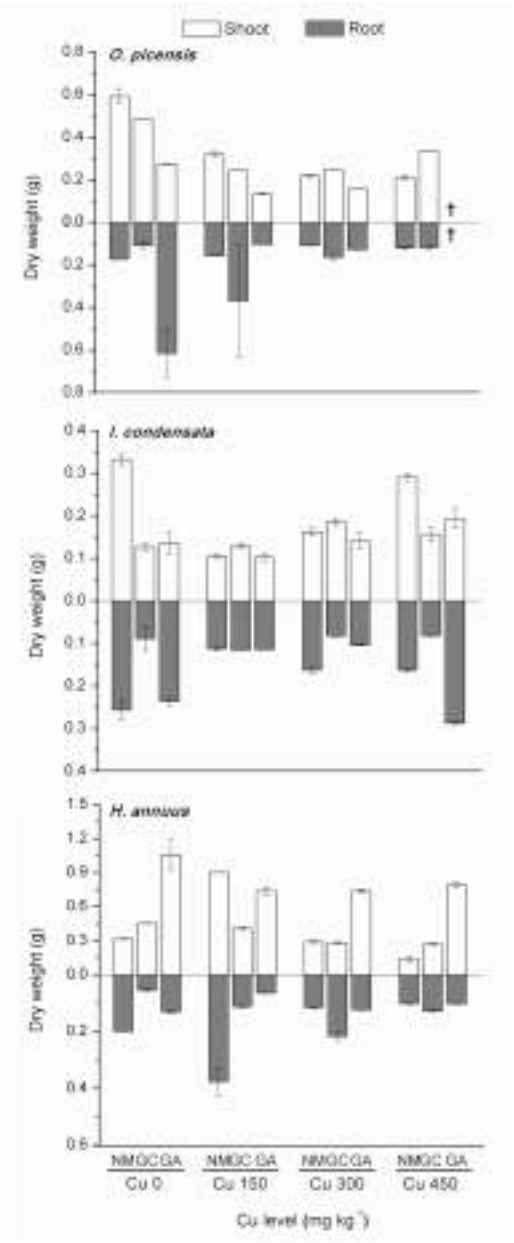

Fig 6.1 Effect of Cu supply levels (0, 150, 300 or 450 mg Cu Kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal population (GA) on shoot and root dry weight (DW). NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E. n = 3.

6.3.2 Plant metal concentration

Plant Cu concentration was increased due to Cu supply, accumulating it principally at root level (more than 95% -Fig. 2-). High tissue Cu concentrations were observed in *O. picensis* with values over 70 and 690 μg Cu g^{-1} in shoots and roots, respectively (Fig. 6.2). Differences in Cu concentration and distribution were also found in AM fungal colonized plants. Therefore, GA inoculated plants increased its shoot Cu concentration by about 1.5 to 2.6 fold, whereas no significant difference was found between GC-inoculated and uninoculated plants. However, an opposite behavior was observed in Cu concentrations in root at high Cu supply levels (>300 μg Cu g^{-1}), where lower root Cu concentrations were found in AM fungal colonized plants (25% and 51% lower in GA and GC) in comparison with uninoculated *O. picensis* plants.

The AM colonized *I. condensata* plants markedly increased their shoot Cu concentration by about 10-15 fold compared with control plants, especially using GA strain, which accumulated Cu from 25 to 50% more than GC colonized plants in the majority of Cu levels applied (Fig. 6.2). In addition, a similar behavior was observed in roots, where AM (either GC and GA) promoted a higher Cu concentration compared with uninoculated plants, with some exceptions.

The *H. annuus* plants inoculated with GA significantly reduced shoot Cu concentration ($P < 0.01$), whereas non-significant differences were found in uninoculated and GC inoculated plants with the exception of GC plants at 450 μg Cu g^{-1}, which exhibited Cu concentrations several times higher than the other treatments (Fig. 6.2). In contrast, root Cu concentrations were higher in AM fungi inoculated plants only at low Cu supply levels; at increasing Cu levels the uninoculated plants showed higher Cu accumulation (about of 33% more) than the AM inoculated plants.

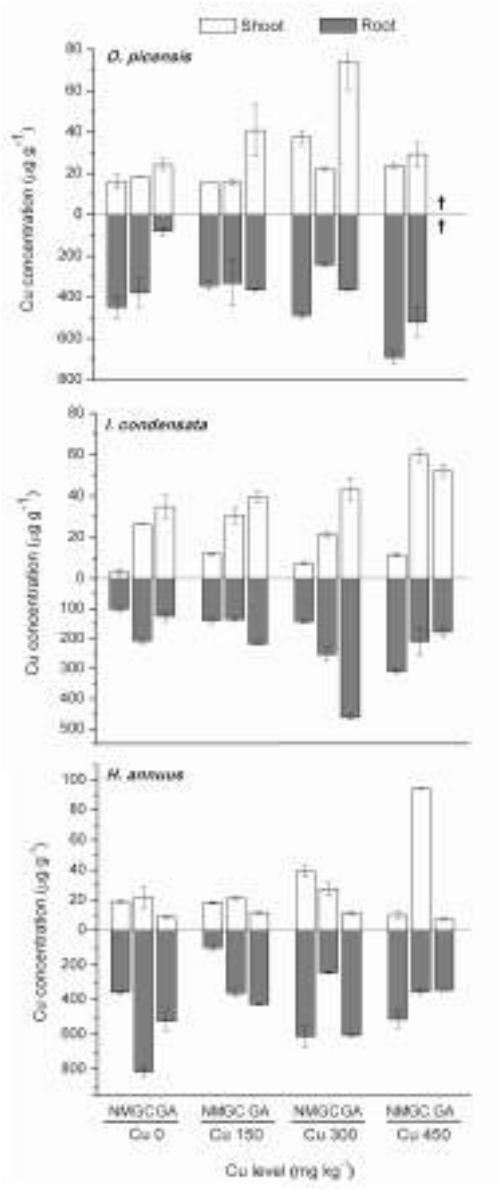

Fig 6.2 Effect of Cu supply levels (0, 150, 300 or 450 mg Cu Kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal population (GA) on shoot and root Cu concentrations (μg g^{-1}). NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E. n = 3.

6.3.3 Arbuscular mycorrhizal parameters

No AM root colonization was found in any uninoculated treatment. In AM inoculated treatments, the AM root colonization was highly influenced by the plant species, following the order *H. annuus*>*I. condensata*>*O. picensis*. Moreover, the Cu application influenced root colonization depending on the plant species and the type of AM inoculum used. In metallophytes the colonization was in general higher at increasing Cu supply levels in GA treatments, but a decrease or non-effect was observed in GC treatments at increasing Cu levels. In turn, there was no significant effect of Cu levels on *H. annuus* root colonization. Particularly, in *O. picensis* the maximal colonization was observed using GC which, compared with the control (Cu0) soil was increased by about 3-fold at the highest Cu level. A similar trend was observed in *I. condensata* plants colonized by GA (Table 6.1) which, at the highest Cu concentration increased the colonization by about 5-fold with respect to the control soil (Cu0-GA) and 2-fold with respect to GC colonized plants (Table 6.1).

The spore production was increased at high soil Cu levels (except in *O. picensis*, Table 1). Remarkable differences between indigenous AM fungal and GC strains were found in *H. annuus*. In fact, GA produced 2-fold more spores than GC at the highest Cu supply level; however, differences in spore production were not observed in the metallophyte plants (Table 6.1).

Finally, glomalin related soil protein production (GRSP) and its ability to sequester Cu were highly dependent on both plant species and the AM fungal strain used (Figs. 6.3 and 6.4). In this sense, GRSP production in metallophyte plants remains relatively constant (about 13 mg glomalin g^{-1} soil) independent of the Cu application levels, and no differences between the use of indigenous and non-adapted AM fungi were detected (Figure 6.5). However, it was noteworthy that independent of the Cu level used, in general the GRSP produced by GA was able to sequester twice more Cu into it structure than GC, with some exceptions (Fig. 6.4). The opposite was observed in *H. annuus* plants colonized by GC, which showed the highest glomalin production (39 mg glomalin g^{-1}) together with the highest Cu concentration into its proteic structure (38 μg Cu g $GRSP^{-1}$ -Figs. 6.3 and 6.4-).

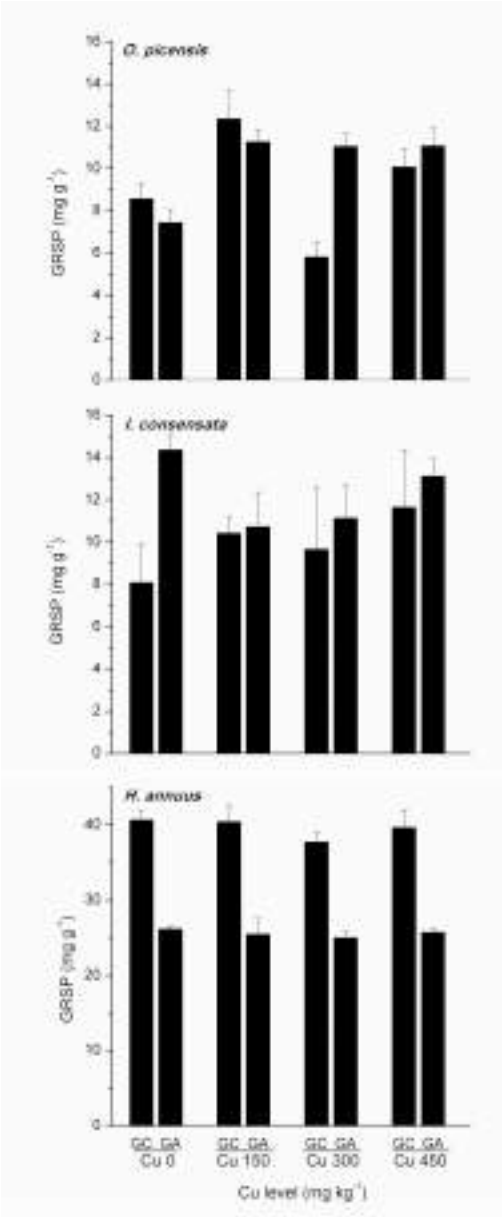

Fig 6.3 Effect of Cu supply levels (0, 150, 300 or 450 mg Cu Kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal population (GA) on glomalin production (GRSP). † = Death plants. Bars denote means ± S.E. n = 3.

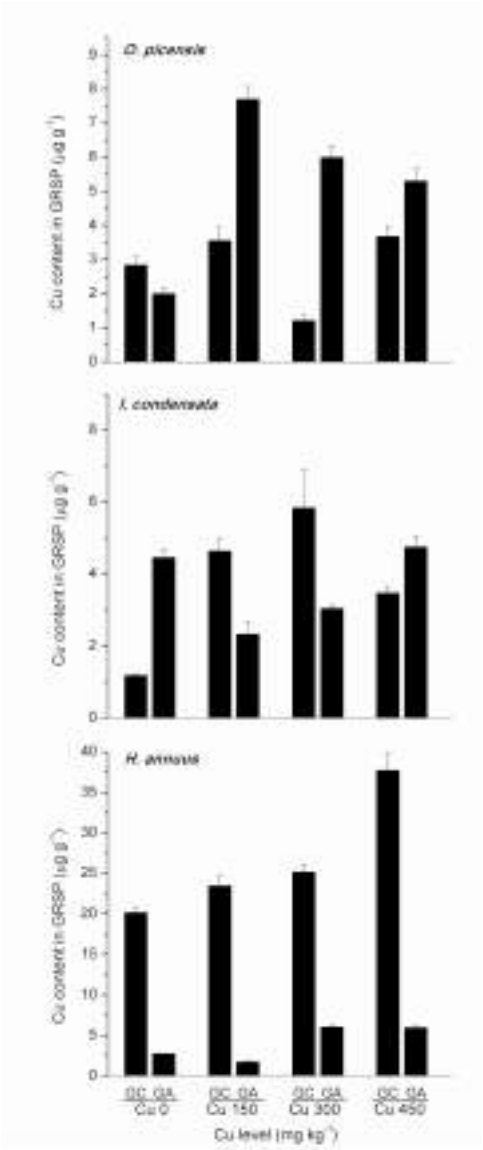

Fig 6.4 Effect of Cu supply levels (0, 100 or 450 mg Cu Kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal population (GA) on glomalin related soil protein bound Cu (GRSP-Cu). † = Death plants. Bars denote means ± S.E. n = 3

Table 6.1. Effect of Cu supply levels (0, 150, 300 or 450 mg Cu Kg^{-1}) and arbuscular mycorrhizal (AM) fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous fungal population (GA) on root colonization and spore number (20 g ds^{-1}) in *Oenothera picensis, Imperata condensata* and *Helianthus annuus*. Values are mean ± standard error. Different letters on each row indicate significant differences (P<0.05) using Tukey test (N=5).

Treatment	**AM Inoculation**	***Oenothera picensis***		***Imperata condensata***		***Helianthus annuus***	
		Root colonization (%)	Spore number	Root colonization (%)	Spore number	Root colonization (%)	Spore number
Cu 0	GA	13.9 ± 2.6^{bc}	53.3 ± 7.1^{a}	31.2 ± 5.4^{bc}	27.6 ± 1.5^{ab}	25.9 ± 3.4^{bc}	28.5 ± 2.6^{d}
	GC	20.7 ± 3.9^{abc}	36.7 ± 3.8^{a}	19.7 ± 2.7^{cde}	37.3 ± 3.0^{ab}	27.3 ± 2.9^{bc}	44.3 ± 2.9^{bcd}
Cu 150	GA	25.0 ± 1.0^{abc}	33.4 ± 3.7^{a}	37.3 ± 1.2^{ab}	31.3 ± 3.2^{ab}	30.9 ± 4.3^{bc}	70.2 ± 3.9^{ab}
	GC	25.1 ± 4.4^{abc}	24.7 ± 3.4^{a}	25.6 ± 1.9^{bcde}	44.3 ± 3.8^{ab}	38.1 ± 3.2^{ab}	29.3 ± 6.3^{cd}
Cu 300	GA	28.9 ± 0.3^{ab}	27.1 ± 3.1^{a}	31.2 ± 1.4^{bc}	42.5 ± 1.6^{ab}	36.5 ± 4.3^{abc}	88.5 ± 11^{a}
	GC	13.7 ± 1.4^{bc}	29.0 ± 1.5^{a}	26.1 ± 1.8^{bcd}	32.1 ± 3.8^{ab}	38.9 ± 3.6^{ab}	36.3 ± 10^{bcd}
Cu 450	GA	-----	31.3 ± 2.9^{a}	50.8 ± 5.1^{a}	43.7 ± 7.3^{ab}	24.9 ± 1.5^{bc}	83.7 ± 7.8^{a}
	GC	33.7 ± 6.9^{a}	32.6 ± 3.4^{a}	24.8 ± 3.3^{bcde}	51.7 ± 12^{a}	35.6 ± 2.3^{abc}	41.4 ± 2.4^{bcd}

6.4 Discussion

In this study the AM fungal effect on plant growth was not uniform and depended principally on plant specie and the AM strain utilized, which suggests a certain compatibility between some AM fungi and host plant (Dodd et al., 2000), which could determine the use of specific AM fungi-plant species combinations at high soil Cu levels, when both simbionts are included in phytoremediation programs. The known beneficial effect of plant mycorrhization on promoting root biomass production (Smith, 2000) was in the most of cases not observed in this study. In contrast, the decrease of root biomass of some inoculated plants indicated that the bulk of the carbon saves was used for fungal biomass production instead of supplying nutrients to the plants (Smith, 2000), due to hyphal production being much more economical in terms of organic carbon than the production of an equivalent length of root system (Jacobsen et al., 2002). However, some exceptions to the above were found in *H. annuus*, in which the use of Cu-tolerant AM fungi promoted the shoot growth, even at phytotoxic Cu levels (Fig. 6.1). This positive response of metal tolerant AM compared with non-indigenous strains have been reported by several studies, concluding that the use of metal tolerant AM fungal could be partly responsible for plant survival in metal contaminated soils (Hildebrandt et al., 2007, Azcon et al., 2009). In addition, this result provides evidences about the functioning of tolerance mechanisms, which could allow the inclusion of *H. annuus* in phytoremediation programs if other management aspects, such as irrigation, fertilization, among others, are also considered.

In contrast, the death of *O. picensis* plants colonized by GA at the highest Cu concentrations suggest that this plant is non-tolerant to high Cu concentrations, which is in disagreement with other reports (González et al., 2008). Probably this plant in natural condition would be tolerant to other limiting factors of the Cu polluted environments such as soil acidity, poor nutrient availability and/or drought. Indeed, in natural conditions Cornejo et al. (2008) found this plant species (named as *O. affinis*) growing in a not heavily Cu polluted soil (158 mg total Cu kg^{-1} soil and 18.8 mg DTPA extractable Cu kg^{-1} soil), which is in accordance with a high micro-spatial variability of soil Cu levels in the ecosystems in the Puchuncaví valley (Ginocchio et al., 2004). Moreover, recent studies demonstrated that *O. picensis* present a high capacity for exuding succinic acid at high Cu levels; in contrast, *I. condensate* exuded high amount of citric acid, which is able to form stable Cu complexes more efficiently than succinic acid, thus allowing to

tolerate the metal by means of exclusion mechanisms at higher Cu levels in the environment (Meier et al., 2011a).

A number of mechanisms have been proposed for the mycorrhizal stimulating effect on metal accumulation in plants (Meier et al., 2011b). Nevertheless, some of these mechanisms are not completely understood and are still matter of investigation. Several studies have shown that AM fungi have developed mechanisms, which avoid metal transference to the shoots, thus promoting the phytostabilization process (Audet and Charest, 2006; Janousková et al., 2006). In contrast, other reports have shown that AM fungi promote phytoextraction, producing an increase in metal translocation to shoots (Davies et al., 2001; Trotta et al., 2006). In our study, plant Cu concentration was related not only with the soil Cu contamination but also with mycorrhizal interactions in the rhizosphere. Thus, the Cu traslocation was low, remaining mostly at root level (more than 95%). Nevertheless, AM fungi inoculation produced changes in Cu distribution within plants, which was strictly dependent on the AM fungal inocula used and the host plant colonized. For instance, metallophytes plants colonized by indigenous AM fungal increased Cu concentration in shoots instead roots (in comparison with GC and uninoculated plants), supporting the hypothesis that the AM increases the metal traslocation from roots to shoots (Trotta et al., 2006). However, this effect was not evident in *H. annuus,* where at the highest Cu concentration the AM inoculation prevented both Cu uptake and its subsequent transfer to shoot (Fig. 6.2). These differences in AM fungal effects on soil-plant transfer of Cu at higher Cu supply levels could suggest the existence of specific tolerance mechanisms of each AM fungal strain not only related to the Cu level in the soil, but also to the specific host plant evaluated. In this sense, considering that AM used as inoculum (GA) corresponded to a mix of different strains, further studies aimed to characterize and evaluate their effectiveness are necessary.

On the other hand, AM root colonization and spores production were observed at high soil Cu levels indicating that AM fungal (either GA or GC) were tolerant to the supplied Cu levels. Nevertheless, AM responses were not uniform and presented high variability, which had been observed in other studies. For instance, AM fungal spore numbers ranged from 30 to 460 kg^{-1} soil in a Zn contaminated soil (Leyval et al., 1995) and from 3900 to 20700 kg^{-1} soil in a multicontaminated soil (Zack et al., 1982). This variability has also been observed with respect to the AM root colonization, which is strongly dependent on a number of factors that exert a high selectivity, including soil pollution level, type of metal, and host species involved (del Val et al.,

1999). High AM root colonization values, especially in GA-colonized plants, were observed in the plants evaluated at high soil Cu levels, with the exception of *O. picensis* at the highest metal concentration (Fig. 6.2). In addition, the positive relation between Cu levels and AM root colonization (Table 6.1) could indicate that the plants may regulate AM colonization by its own benefits, possibly due to the protective role of AM fungi against metal toxicity (Hildebrant, 2007; Ferrol et al., 2009). Specifically, the higher biomass produced by *H. annuus* could be responsible for the higher spore density when Cu was applied, since the AM fungi potentially received higher amounts of organic compounds originated by the plant. This aspect could be highly important if efficient AM-plant associations (such as GA-*H. annuus* in the present study) promote not only an increased plant biomass, but also a higher density of AM propagules or resistance spores able to colonize other plants, thus accelerating the phytoremediation process.

The differences in glomalin production observed in this study seem to be dependent on the soil Cu level supplied, the plant evaluated and the AM inocula used (Fig. 6.3). This specific effect on glomalin production has been observed under *in vitro* conditions, by González-Chavez et al., (2004) who reported that this glycoprotein were able to sequester up to 28 mg Cu/g glomalin. In addition, Cornejo et al., (2008) observed a high positive correlation (r = 0.89) between Cu concentration in soil and GRSP contents. These results, together with the high glomalin production by *H. annuus* GC colonized plants and the low Cu concentration here found in their roots suggest that this protein could be a special exclusion mechanism developed by non-adapted AM fungal to cope high Cu in the soil. In this sense, the higher GRSP accumulation in *H. annuus* rhizosphere soil compared to metallophytes soil indicated a *de novo* synthesis of glomalin by both GC and GA fungi. This high capacity to produce and accumulate GRSP by AM colonized *H. annuus* plants could be explained by an improved development of AM fungal structures and the subsequent increased mycelium and spores turnover, since glomalin appears to be a component of the hyphae and spore wall (Driver et al., 2005). Moreover, explaining the high increase of GRSP in GC colonized *H. annus* plants, the low density of spores could indicate an enhanced turnover of fungal structures and especially in this case where a non-adapted fungus was used. However, molecular mechanisms could be also involved in this process, due to the high homology between glomalin and heat shock proteins (Gadkar and Rillig, 2006), which are a kind of proteins related to diverse environmental stresses. Therefore, unspecific tolerance mechanisms could be acting in non Cu-adapted AM strains, indirectly enhancing the GRSP

content in soil and the Cu-binding capacity. Certainly, this Cu-binding capacity by GRSP and AM fungal structures could be more strong if compared with other metal pollutant, as phytotoxic Al (Aguilera et al., 2011). These aspect deserve further studies, since the use of AM fungal strains with a high capacity for producing glomalin and sequestering potentially toxic elements could be a way for efficiently improving the development of phytostabilization programs (Cornejo et al., 2008; Vodnik et al., 2008; Meier et al., 2011c).

6.5 Conclusion.

Marked differences between mycorrhizal inoculated and uninoculated plants were found in response to increasing Cu levels in the soil and the effect was dependent on the AM fungal strain used. This could indicate the existence of certain compability between the host plant and some AM fungal, which deserves to be evaluated in further studies. The lower root metal concentration at higher Cu level in mycorrhizal plants suggest the presence of specific root Cu exclusion mechanism developed by the AM fungi, probably due to metal sequestration by glomalin, which could be related with the plant biomass production and other molecular mechanisms present in certain AM fungal strains. Finally, the high biomass produced by *H. annuus* inoculated with GA, at high Cu levels, indicates that further studies are required to include this plant in phytostabilization programs for this or other metal pollutants.

Acknowledgments

Financial support for this study was provided by the National Commission for Scientific and Technological Research (CONICYT), through a FONDECYT 11080131 grant (P. Cornejo). Sebastian Meier also acknowledges the support of the Scientific and Technological National Commission Research (CONICYT) through project 24091053.

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General discussion

7. General discussion

In the present study we aimed to evaluate the contribution of the arbuscular mycorrhizal fungi to the phytoremediation of Cu polluted soils. In order to achieve the above we divided the subject into three main areas.

Firstly in Chapter 3, the physiological responses of metallophytes and agricultural plants to increasing Cu doses were studied. In this aspect, significant differences in all the parameters evaluated were found. Highlighted the high LMWOA exudation patterns of the metallophyte plants, which suggest the existence of specific mechanisms in response to high Cu doses. For example, *I. consensata* presented an effective exclusion mechanism principally through the citric acid exudation. On the contrary, the high Cu concentration on plant tissues in *O. picensis* seems to indicate that the high succinic acid exudation is an unspecific Cu response due to the low affinity of this acid to form stable complexes with Cu ions, which allowed its acquisition, and therefore reducing significantly the plant growth. The high exudation rates by *L. albus*, could had prevented the Cu uptake by plant roots as an efficient exclusion mechanism, allowing their growth at high Cu levels. In addition, a similar behavior was observed in *H. annuus*, showing also a good response in terms of dry mass production and Cu tolerance, which indicate that both agricultural plants could be a good option for the implementation of phytoremediation programs ifagronomic aspects are also considered.

Secondly, the effect of the use of organic amendment together with different strains of AMF was evaluated using *O. picensis* as host plant (Chapter 4 and 5). In this sense, the presence and toxicity of copper on plant tissues were dependent on complex interactions between soil and plants, as well as microbial rhizosphere activities. In fact, significant differences between mycorrhizal and uninoculated *O. picensis* plants were observed at increasing soil Cu doses, with the use (or not) of a biotransformed sugar beet amendment together with the inoculation of both Cu-adapted and a non-adapted AMF strain. The most important results were achieved using Cu-adapted AM inoculum, which provided physiological traits that allowed the plant survival at phytotoxic Cu levels, possibly through

exclusion mechanisms, which favoring a decrease of the oxidative stress produced by this element.

A synergic effect between sugar beet (SB) amendment and Cu-adapted AM inoculum is proposed. Whereas the AMF controlled the Cu uptake by plants, the SB had a positive effect on plant growth through improving the plant nutrition. Therefore both constitute a biotechnological tool for improving the *Oenothera piscensis* establishment in Cu-polluted soils.

The effectiveness of different arbuscular mycorrhizal fungal inocula on plant growth and Cu uptake in some metallophytes and one agricultural plant was evaluated in the Chapter 6. Although the AMF effect on plant growth was not uniform, marked differences between mycorrhizal inoculated and uninoculated plants were found in response to increasing Cu doses in the soil being the AMF effect strictly dependent of AMF strain used, which could suggest a specific compatibility between AMF and host plant. In addition, interesting and new mechanisms developed by AMF to avoid the Cu entrance to the plant were found. Among them highlights the glomalin production and the presence of green spores (see Fig 2.4) whose role would be storing Cu in their structures, which here is proposed as a new mechanism present in AMF to cope high metal levels in soils.

Summarizing, differential behaviors in response to increasing Cu doses were observed between metallophytes and agricultural plants. In this sense, the plant response was dependent on complex interactions among the plant specie, the soil, the Cu dose and the arbuscular mycorrhiza activities on the rhizosphere. In this study, the AMF association contributed to enhancement the plant establishment, through improving plant nutrition, and provided physiological traits, which allow the plant survival at phyto-toxic Cu levels. In addition, several metal exclusion mechanisms (e.g. glomalin production, or green “reservoir spores) were developed by AMF, which resulted in lesser Cu uptake by plants. The above could suggest that in Cu polluted environments the AMF promotes predominantly phytostabilization instead phytoextraction processes. Nevertheless, and important aspect to have into consideration is that AMF response over the plant grow was not uniform,

depending on the Cu doses and the AMF strain used, therefore further studies are necessary to corroborate this subject.

General conclusions

8. General Conclusions

Taking into account the main results, it can be concluded that:

- The exudation of compounds by roots is a mechanism that influences the plant Cu tolerance. The high exudation rate of citric acid showed by *I. condensata* and *L. albus*, could prevented the Cu uptake due to the high stability constant (K_f) of this acids (1.7×10^7 and 7.9×10^6, respectively) by plant roots as an efficient exclusion mechanism, allowing their growth at high Cu levels. On the contrary, the high Cu concentration found in shoot and root of *O. picensis* is probably due to the lower affinity of succinic acid to form stable complexes with Cu ions (K_f 2×10^4) allowing its acquisition and significantly reducing its growth.

- The combined use of sugar beet (SB) and Cu-adapted mycorrhizal strain may constitute an interesting model of biotechnological tool not only for improving the plant establishment but also allowing their survival in Cu-polluted soils, which have an ecological relevance. The Cu-adapted mycorrhizal fungi presented some metal exclusion mechanisms, which controlled the Cu uptake by *Oenothera picensis*, whereas SB had a direct effect over plant growth, improving plant nutrition and water retention capacity. Thus, an interactive and synergic effect between both AMF and the amendment is proposed.

- Cu-adapted mycorrhizal fungi provide physiological traits that allow for plant survival at phytotoxic Cu levels, possibly through exclusion mechanisms favoring a decrease of the oxidative stress produced by this metal. The mechanism of resistance triggered by Cu-adapted AMF strains requires further study before *O. picensis* or other plant can be used in remediation of Cu polluted soils.

- A specific compatibility between the host plant and AMF is proposed, which depended of the plant specie, Cu supply levels and AMF strain. Nevertheless mores studies on this subject are needed.

- The high shoot biomass production and low metal content in roots by *H. annuus* inoculated with Cu-adapted mycorrhizal (GA), indicates that mostly of Cu remained at root level. Several exclusion mechanisms are here proposed such as metal chelation by citric and

coumaric acid exudation as well by the glomalin production. In addition this specie has a high growth rate, which plus to the above suggest that could be a choice in the implementation of phytostabilization programs.

- In Cu polluted soils, the arbuscular mycorrhizal fungi symbiosis promotes mainly pytostabilization processes rather than phytoextraction ones.

Outlook

The information generated in this thesis has enhanced our understanding of mycorrhizal biology and plant metal tolerance; however, deeply studies are necessary in this area for improving and implementing the use of AMF in phytoremediation programs. New research into this topic should focus on the process optimization, including the physiological mechanisms involved in metal absorption, translocation, and metabolism by the plants, and describing the genetic control mechanisms. Concerning the role of AMF in phytoremediation, it is very important to evaluate their presence and diversity in polluted soils as well as their functional compatibility with metallophytes and autochthonous flora in order to chose the best combination AMF/plant to be used as a biotechnological tool to the remediation of metal polluted soils. Also it is necessary to study about the physiological mechanisms involved in metal tolerance by AMF, including intracellular, molecular studies and structural localization of metals. Finally, the role of the use of amendments (either organic as well inorganic ones) together with Cu-adapted mycorrhizal fungi using either metallophytes or agricultural plants to accelerate phytoremediation process could be an interesting subject of study in the near future.
In summary, we suggest that in the future designs of phytoremediation programs must be included the technological use of AMF to improve it efficiency and effectiveness.