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"Arbuscular Mycorrhizal fungi restore normal growth and alter polyamine content and gene expression in AL35 poplar clone grown on highly heavy metal contaminated soil"

Tutor

Ch.mo Prof.
Castiglione Stefano

Dottoranda

Dott.ssa
Cicatelli Angela

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Introduction

Introduction

1. Heavy metals

Metals are natural constituents of soils, waters and organisms. Seventeen of the 53 metals are involved in the functioning of organisms and ecosystems.

Heavy metals (HMs), such as Fe, Mo, and Mn, are important as micronutrients; others have roles as trace elements, Zn, Ni, Cu, V, Co, W, and Cr. There are some HMs without any known nutritional function, but they are nonetheless toxic for animals, plants and microorganisms (Hg, Ag, Cd, Pb, and U) (Schu¨tzendu¨bel, 2002). In addition to these HMs, some metalloids, including As, are toxic for plants.

The definition "heavy metal" is based on the density of the elemental form of the metal, on the reactivity of metal, on atomic number and on other chemical properties and toxicity (Duffus, 2002). Generally, HMs are classified as those metals with elemental density greater than 5 g/cm^{-3}, with atomic number superior to 20, and with aptitude to form some complexes.

Although HMs are natural constituents of soils and occur naturally in the environment, nowadays contamination of soils by toxic metals and metalloids is a major concern worldwide. A portion of HMs in soils derives from geochemical processes (weathering of rocks, volcanic eruptions, continental dusts) and in many regions there are natural mineral deposits (Maksymiec, 2007; Memon, 2009). Moreover a noteworthy pollution by metals has been accelerated by civil, agricultural and industrial activities since the beginning of the Industrial Revolution (Padmavathiamma, 2007). Sources of pollution include the burning of fossil fuels, mining and smelting, industrial emissions, municipal wastes, mineral fertilizers and pesticides. About 235 million hectares are contaminated by HMs (Giordani, 2005). Extensive pollution is adversely affecting environment and human health (Bodar, 2006). Soil can be considered a source of pollution with the capacity to transfer pollutants to the groundwater, into the

food chain and finally also into human body. Metals are the most studied soil pollutants because of their ubiquity, toxicity, bioavailability, mobility and persistence.

1.1 *Heavy metals stress and plant responses*

HMs have become one of the main abiotic stress agents for living organisms. The exposure to toxic levels of HMs induces some of the macroscopic consequences in plants (the topic of this thesis), such as the inhibition of plant growth (of both roots and aboveground parts), leaf chlorosis and necrosis, turgor loss, a decrease of seed germination and of photosynthetic apparatus, often correlated with progressing senescence processes, or with plant death (Foy, 1978). All these effects are related to ultrastructural, biochemical and molecular changes in plant tissues and cells brought about by the presence of the HM (Gamalero, 2009).

The effects of HMs on plants may be the result of their direct effect on membranes and on the photosynthetic apparatus, or their indirect effect caused by the induction of some signaling pathways through ethylene synthesis and ROS (Reactive Oxygen Species) production (Miller, 2008; Shao, 2008). The photosynthetic apparatus can be damaged by a direct effect of HMs excess, affecting all cell membranes, including thylakoids. Several studies report that HMs can determine the release of proteins, lipids and element components of thylakoid membranes, causing damage to light-harvesting complexes and Photosystem II (Hsu, 2004; Backor, 2007). However, some HMs can replace Mg in the chlorophyll (Chl) . Chl synthesis reduction, which is usually observed after HMs stress, may be a consequence of enzyme inhibition involved in the pathway of its synthesis (Boddi, 1995). In the cell nucleus, HMs can bind to nucleic acids and mutagenize them and even modify both transcription and DNA replication; HMs can also affect microtubule assembly–disassembly, thereby inhibiting cell division (Fusconi, 2006). The exposure of plants to

stressful conditions raises the ethylene level, a gaseous hormone, which affects several plant responses, including senescence and stress (Deikman, 1997). In higher plants, Cu-induced ethylene synthesis can increase senescence (Maksymiec, 2007), inhibit cell growth and increase cell wall rigidity by means of lignification (Enyedi, 1992). It has been shown that HMs (Cu, Zn) can stimulate ethylene production by over-expression of genes involved in its synthesis (1-aminocyclopropane-1-carboxylate synthase - ACC synthase) (Pell, 1997) and by increased lypoxygenase activity (Gora, 1989), which can mediate ROS formation. HMs cellular toxicity can result in the accumulation of ROS, such as superoxide anion radical (O_2^-), H_2O_2 and hydrogen peroxide radical ($OH^{\cdot}$), which usually damage the cellular components, membranes, nucleic acids and chloroplast pigments. Accumulated H_2O_2 can rapidly increase cell wall rigidity and Jasmonic Acid (JA). HMs induce stress responsive genes and secondary metabolites (Schutzendubel, 2002). ROS production is dependent upon the particular metal elements; Cu can directly generate ROS, whereas Cd is a redox inactive metal and can only generate ROS indirectly by inducing the expression of lipoxygenases in plant tissues and therefore causing oxidation of polyunsaturated fatty acids (Cho, 2005; Skcrzynska-Polit, 2006). ROS action may result in cell disturbances and it enhances senescence processes in cooperation with ethylene and JA, although the activation of the antioxidant machinery can help plants to overcome HM stress (Gamalero, 2009).

1.2 HM contaminated sites in Italy and in the Campania region

Soil contamination by toxic and persistent HMs, mainly associated to intensive agriculture, urban-industrial expansion and illegal waste disposal, has adverse effects on human health and ecosystems. The remediation of pollutant sites is a widespread and urgent problem. The attention of many scientists and Institutions, such as the US Environment Protection Agency, or the European Environment Agency, have focused on a common objective, namely, to improve

significantly the understanding on the complexities involved in identifying contaminated sites, determining liability and defining remediation standards. In the European Thematic Strategy for Soil Protection (ETSSP), recently published by the European Commission (EC 2002), improving knowledge on HM contents and on sources to European soils is a priority objective for the European Union (EU) to protect soils, using a common and specific strategy and to control the emissions of pollutants. Many nations in the EU, as in the developed countries, have adopted legislation to assess soil pollution.

Italy has a land use oriented law with the threshold limits for pollutants changing according to the use of the soil (Table 1). The Legislative Decree N° 471/99 (1999) includes the regulations and methods for remediation and recovery of polluted sites.

These norms and their implementation define the 'environmental damage', the thresholds set values of pollutants, introduce soil assessment methods to identify affected areas and contamination sources and define methods to permanently reduce the concentration of contaminants and/or capacity to infiltrate environmental or biological systems. The Italian government has approved some laws implementing institutional controls of criminal offences that affect the environment (Legislative Decree N° 471/9, Legislative Decree N° 468/2001, etc.). Therefore, the presence of pollutants in the environment, or in the soil matrix is insufficient to classify a site as contaminated. In accordance with italian law, it is possible to define a site as polluted site, when the HMs thresholds exceed the set norms. In order to plan a remediation procedure, it is necessary to assess the pollution level in the soil, through official methods National Soil Sampling Guidelines (NSSG - Theocharopoulos, 2001), based on the assessment of total metal concentration in the soil as established by the Italian law (Legislative Decree N° 152/2006), even if, recently, scientific literature offers new and more sensitive investigation procedures (such as Diffusive Gradients in Thin Film) (Clarisse, 2006). Although the contamination

Table 1: Threshold limits for HM pollutants according to the use of soil (Legislative Decree N° 152/06)

Heavy metal	Private and residential soil (mg kg^{-1} D.W.)	Industrial soil (mg kg^{-1} D.W.)
Arsenic	20	50
Cadmium	2	15
Chromium	150	800
Mercury	1	5
Nikel	120	500
Lead	100	1000
Copper	120	600
Zinc	150	1500

evaluation can be very difficult and cost effective, the identification of polluted areas and sites is growing. On the basis of European Environmental Agency (EEA) data, principal sources of soil contamination include industrial or municipal waste disposal sites, industrial and commercial activities, mining sites, oil spills sites, etc. In Italy, industrial and commercial activities, often associated with illegal disposal of pollutants cause significant environmental contamination, releasing a large quantities of pollutants, such as mineral oil, Polycyclic Aromatic Hydrocarbons (PAH), Aromatic and Chlorinated Hydrocarbons (BTEX and CHC). These pollutants contribute to soil contamination to a different extent amongst the European nations (Fig. 1).

A picture of the number of sites requiring cleanup, including HMs, in Campania region has been reported in the environmental report entitled " The State of the Environment Report 2009 – Campania region", produced by Campania Regional Environmental Protection Agency (ARPAC). The report presents an extensive collection of validated data on air, water and soil monitoring in Campania region. According to the existing legislation in Italy, ARPAC has been committed to detect and include in the census polluted and potentially polluted sites in Campania. From 2005 to 2008, the number of these sites has doubled. In 2005, the agency recorded 2.599 polluted, or potentially polluted sites, among which, 766 areas were characterised by massive illegal waste disposal and 1.833 sites were contaminated with several harmful substances. To date, the agency identified 5.281 polluted, or potentially polluted sites in Campania: 1.548 sites are areas featuring massive illegal waste disposal and 3.733 are polluted, or potentially polluted sites. Among those, 462 sites revealed trespassing of law limits for some contaminants. The province of Caserta is the leader amongst the areas featuring massive illegal waste disposal (851 sites). Fig. 2 illustrates the distribution of the major classes of pollutants and their combinations in the soil. Inorganic pollutants, including HMs (ca 6% of pollutants, defined "special") are

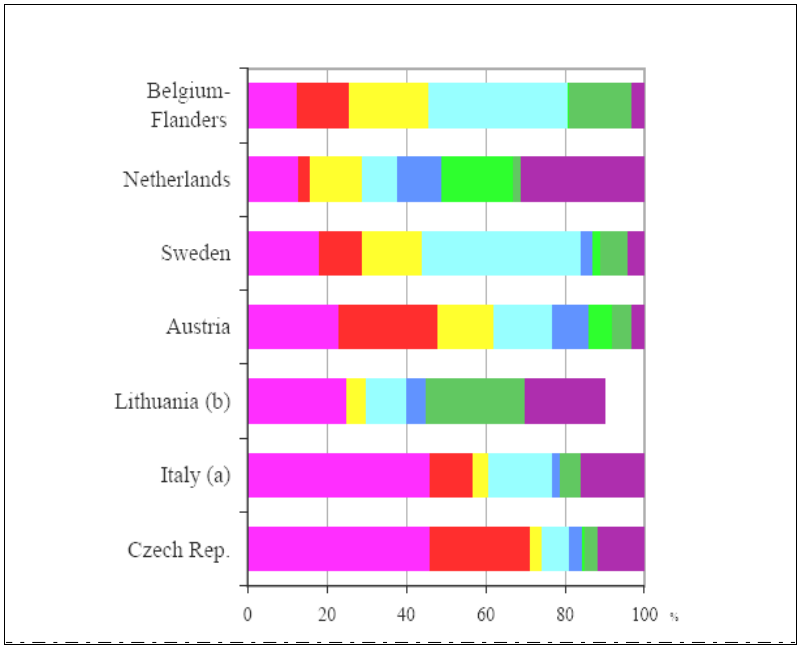

Fig. 1: Environmental contamination by Mineral oil, PAH, Phenols, BTEX, CHC, Heavy metals, Cyanide and others polluted in European nations (European Environmental Agency, 2005)

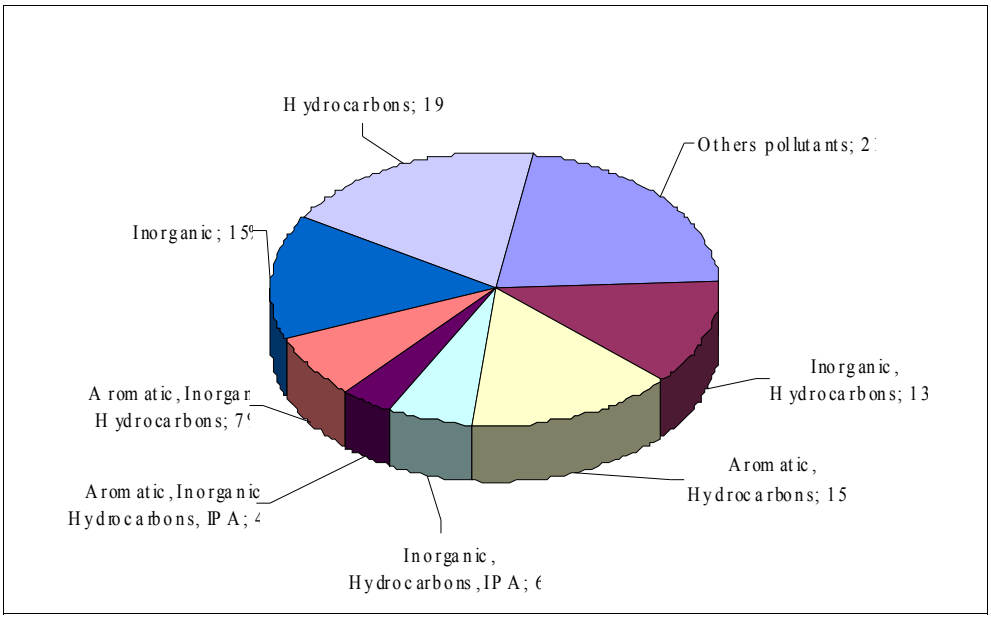

Fig. 2: Distribution of major classes of pollutants in soil and their combinations (Campania region) (Campania Regional Environmental Protection Agency, 2009)

localized in Salerno and Caserta provinces and are essentially produced after treatment of civil wastes.

After evaluation of soil contamination, the choice of a remediation technique that will eliminate or at least reduce the concentration of contaminants is not simple. At present, contaminated sediments are often not remediable due to their high content of pollutants. Several remediation technologies are available for contaminated soils, but they are very expensive, have detection limits and sometimes secondary effects on ecosystems.

2. Plant survival strategies

Plants are generally highly sensitive and show various physiological symptoms in response to toxic HMs. However, some plant species adopt specific and useful strategies in order to grow on soil with high concentrations of a particular metal. Generally, plants can be considered as metal excluders, metal indicators or metal accumulators (Baker, 1990). Metal excluders prevent metal uptake from polluted soil; however, they may still contain large amounts of metals in their roots. Metal indicator species can accumulate metals in their tissues; the translocation of metal is regulated, in fact metal levels in the living tissues generally reflect metal levels in the soil. Metal accumulator group includes plants that concentrate metals in their above-ground tissues to levels far exceeding those present in the soil. Usually, this latter category includes hyperaccumulator plants capable of accumulating potentially phytotoxic metals to concentrations more than 100 times those found in nonaccumulators. However, some plants, considered as tolerant, can grow in HM contaminated soils, taking up high amount of the pollutants.

Plants that are able to tolerate high concentrations of HMs have evolved several mechanisms that control and respond to the uptake and accumulation of both essential and nonessential HMs. Physiological, biochemical and molecular approaches continue to be employed to identify the underlying mechanisms of metal accumulation, tolerance and adaptive mechanisms to cope HMs stress. Some adaptive mechanisms evolved by tolerant plants (Hall, 2002) include: synthesis of specific metal transporters; chelation and sequestration of HMs by particular ligands (Phytochelatins - PCs, Metallothioneins - MTs) (Cobbett, 2002); induction of mechanisms contrasting the effects of ROS, such as the biosynthesis of antioxidant molecules and stress proteins; the up-regulation of peroxidase (Sanita` di Toppi, 1999); the biosynthesis of salicylic acid (Choudhury, 2004).

2.1 Metal uptake

Inorganic contaminants of soil (HMs) are taken up by plants via membrane transporter proteins. Although exact mechanisms of up-take, transport, and accumulation of HMs in plants are only partially understood, several genes that are likely to be involved in these processes have been described. They include: Zn-regulated transporter (ZRT); Fe-regulated transporter (IRT)-like proteins (ZIP); natural resistance associated macrophage proteins (NRAMP); and cation diffusion facilitator (CDF) (Thomine, 2000; Hall, 2003).

A number of ZIP genes that are expressed upon Zn deficiency have been isolated from Arabidopsis (*Arabidopsis thaliana* (L.) Heynh. (Wintz, 2003), in rice (*Oryza sativa* L. - Ishimaru, 2005), in soybean (*Glycine max* L.- Moreau, 2002) and in Alpine Pennycress (*Thlaspi caerulescens* L. - Pence, 2000). Other classes of metal transporting protein identified in plants belong to the large family of cation-transporting P-type ATPases and to the ATP-binding cassette (ABC) family transporters (Hall, 2003). Recently, some Arabidopsis ABC transporters were found to participate in detoxification processes as well as in plant growth and development (Geisler, 2005).

2.2 Metal chelation and detoxification: phytochelatins and metallothioneins

One recurrent general mechanism for HM detoxification in plants is the chelation of metal by a ligand and, in some cases, the subsequent compartmentalization of the ligand-metal complex either in the cell wall or in vacuoles. In these cell compartments, pollutants, chelated by organic compounds or bound to cell wall components, are less toxic for cells. To this purpose plants can produce several kinds of metal chelating agents, such as organic acids (malic or oxalic acid) or ligand peptide as PCs and/or MTs.

2.2.1 Phytochelatins

Phytochelatins are a family of Cys-rich polypeptides with several repetitions of the γ -Glu-Cys dipeptide followed by a terminal Gly. PCs have been identified in a wide variety of plant species and in some microorganisms. They play an essential role in the detoxification of HMs, such as Cd, Cu, Zn, Hg, Pb, and metalloids (As, and Se) (Cobbett, 2002; Vivares, 2005). PCs are structurally related to glutathione (GSH) and are synthesized in response to several HMs (Cobbett 2002), as products of a biosynthetic pathway involving γ-glutamylcysteine synthetase (GCS), GSH synthetase (GS), phytochelatin synthase (PCS), HM tolerance 1 (HMT1), ABC type vacuolar membrane transporter of PC–HM complexes. Expression of PCS genes has been examined in several studies (*A. thaliana,* Indian Mustard- *Brassica juncea,* Lotus - *Lotus japonicus)* (Ramos 2007*)*, indicating that PCs play a central role in homeostasis of HMs in plants and that they regulate cation availability in plant cells (Guo, 2008). Ramos and co-workers (2007) showed a complex and multiple regulatory mechanisms of PC gene expression in plant tissues and of their protein products in Lotus in response to HMs and to other environmental stimuli. In addition, PCS expression studies in garlic (*Allium sativum* L.) plants exposed to HMs (Zhang, 2005) and in *in vitro* analysis in the marine alga *Dunalliela tertiolecta* (Tsuji, 2002) suggested a role of PCs also in the detoxification of HMs and in the mitigation of oxidative stress.

2.2.2 Metallothioneins

Metallothioneins (MTs) are ubiquitous proteins that can bind HMs and may play a role in their intracellular sequestration. They are members of a superfamily characterized by a common low molecular weight (5.000–10.000 Da) and a large content of cysteine residues arranged in typical pattern. Since they were first purified from horse kidney (Kägi, 1960), MT genes and polypeptides have been founded in many prokaryotic micro-organisms (cyanobacteria, γ- and α-

Proteobacteria and some Firmicutes) and also in many eukaryotes (protists, yeasts, fungi, and higher plants) (Cobbett, 2002). In animals and fungi, MTs form complexes with HMs and the transcription of MT genes is regulated by HM (Thiele, 1992). In plant, the first Cys-rich polypetide was isolated more than 25 years ago from wheat (*Triticum* sp.) (Lane, 1987). These polypeptides present distinct differences from members of the other MT families, such as the three Cys-rich regions as well as the longer linker sequences (Freisinger, 2008). The MT plant family is divided into four subfamilies: p1, p2, p3, and pec. Assignment of a new plant MT with two Cys-rich domains to one of the subfamilies p1, p2, or p3 is usually straightforward and is based on the number of Cys residues and their distribution pattern in the N-terminal domain. With few exceptions, a typical plant MT1, from the p1, contains 6 Cys residues in its N-terminal domain, a MT2, from p2, 8 Cys residues and an MT3, from the p3, contains 4 Cys residues. In contrast, the distribution of Cys residues within the C-terminal domain follows the consensus sequence CxCxxxCxCxxCxC, with x denoting any amino acid other than Cys. Consensus sequence is strictly conserved in all three subfamilies and even is shared by the central Cys-rich region of the Ec proteins from the subfamily pec (Freisinger, 2008). Several data demonstrated the role of MTs in HM detoxification and homeostasis, but metal-inducibility of plant MTs has not always been demonstrated.

In plant, MTs are induced in response to oxidative stress, abscisic acid (ABA), heat/cold shock, wounding, viral infection, senescence, salt stress and sucrose starvation (Chyan, 2005). However, the situation in plants is complicated by the in fact that plants are able to synthesize MTs enzymatically from phytochelatins (Cobbett, 2002). Studies on plant MT structure and function have been limited, in part due to the difficulties encountered in purifying these proteins from native sources, with the exception of proteins from seeds. In recent years, it has been argued that MTs may play a role in HM detoxification either because they bind HMs, or because they function as antioxidants (Akashi, 2004). The evidence is

largely based on MT gene expression studies, and on yeast complementation experiments with plant MT genes (Kohler, 2004; Hassinen, 2009). Gene expression studies were performed to quantify mRNA levels in different tissues, at different developmental stages and under stress conditions such as HM exposure. MT genes appear to be differentially regulated in a tissue-specific manner and in relation to developmental stage and also in response to a number of stimuli, including HMs (Castiglione, 2007). Kohler and co-workers (2004) analysed the mRNA levels of multiple isoforms of hybrid P. x generosa MTs after supplying a single metal (Cd, Zn, Cu) at different concentrations to hydroponically growning plants. The analysis of *Pa*MT1, *Pa*MT2 and *Pa*MT3 gene in response to high zinc concentrations, in a micropropagated white poplar clone, showed a differential expression (Castiglione, 2007).
Further information regarding the structures and properties of MTs could clearify their mechanism of action and the functions.

2.3 Oxidative stress and metal tolerance: GSH and polyamines

Abiotic stress, including HMs, disrupts the metabolic balance of cells, resulting in enhanced production of ROS, that may cause wide-ranging damage to proteins, nucleic acids and lipids, eventually leading to cell death (Miller, 2008). However, plants have developed a complex and efficient network of scavenging mechanisms that allows them to overcome ROS toxicity and use some of these toxic molecules as signal transduction mediators. Detoxification of ROS can occur via specific enzymes, such as peroxidase, catalase and others, that detoxify ROS and/or via antioxidant compounds as ascorbate, GSH and tocopherol, which play an important role in the regulation of the cellular ROS homeostasis, influencing gene expression associated with abiotic and biotic stresses (Miller 2008). GSH, the tripeptide γ-glutamylcysteinylglycine (γ-glu-cys-gly) is involved in both direct and indirect control of ROS concentrations. GSH is a component of the ascorbate-glutathione pathway and takes part in the

removal of the H_2O_2 excess (Noctor, 1998), in a reaction in which GSH is oxidized. GSH also induces some defence mechanisms through a redox signalling pathway, where GSH interacts with ROS, redox molecules and hormones to protect plant against stress effects (Shao, 2008).

2.3.1 Polyamines

Polyamines (PAs), which are small organic polycations, are found in all living organisms. The common PAs in plants are spermidine (Spd), spermine (Spm) and their diamine precursor, putrescine (Put). They often occur in free, bound and conjugated forms to low-molecular mass compounds, mainly phenolics, and in some cases, they also serve as precursors for secondary metabolites such as nicotin (Bagni, 2001). The principal enzymes involved in PA biosynthesis in plants are Arginine Decarboxylase (ADC), Ornithine Decarboxylase (ODC), S-AdenosylMethionineDecarboxylase (SAMDC), Spermidine Synthase (SPDS) and Spermine Synthase (SPMS) (Fig. 3- Page, 2007). Put is the obligate precursor of Spd and Spm; it is synthesized via ADC and ODC from Arginine or Ornithine, while Spd and Spm biosynthesis requires the activities of SAMDC and of SPDS. PAs are essential for normal growth and development through transcriptional and translational regulations (Kusano, 2008), although their physiological significance derives from their involvement in various kinds of biotic and abiotic stress responses. PAs have a protective role with respect to membrane damage and lipid peroxidation (Groppa, 2008 a) and function in quenching the accumulation of ROS (Papadakis, 2005). Thus, enhancement of cellular PAs levels is associated with osmotic, salt, and drought stress as well as toxic HM concentrations (Groppa, 2008 a, b, c). Several genes coding for the enzymes involved in PAs metabolism have been characterized and cloned from different plant species. ADC genes have been identified in many plant species and their expression under several stress conditions have been analyzed (Minguet, 2008). Not surprisingly, therefore, PAs over-production in engineered

plants, over-expressing PAs biosynthetic genes, confers increased tolerance to multiple environmental stresses (Prabhavathi, 2007; Wen, 2008), including HMs (Franchin, 2007). PAs biosynthesis and accumulation in response to high concentrations of Zn and Cu were investigated in the white poplar (*Populus alba* L.) commercial clone 'Villafranca' (Franchin, 2007). In leaves and stems, *Pa*ADC and *Pa*ODC transcript levels were enhanced by increasing Zn concentrations, while increasing Cu concentrations cause leaf toxicity. Tobacco BY-2 cells exposed to $CdCl_2$ produced a significant accumulation of total, free and conjugates PAs, (Kuthanova´, 2004). Although there are some evidences for a positive involvement of PAs in ecto-mycorrhizal interactions (as assessed through measurement of free and conjugated Put, Spd and Spm in plant tissues during mycorrhiza formation - Niemi, 2007), there are poor informations regarding their biosynthetic genes, except for the fact that no difference in ADC expression was found between roots of Scots Pine (*Pinus sylvestris* L.) inoculated and not inoculated with *Suillus variegatus* (Niemi, 2007). To date, the PA profile in inoculated plant using Arbuscular Mycorrhizal (AM) fungi and exposed (or not) to HMs has not been examined.

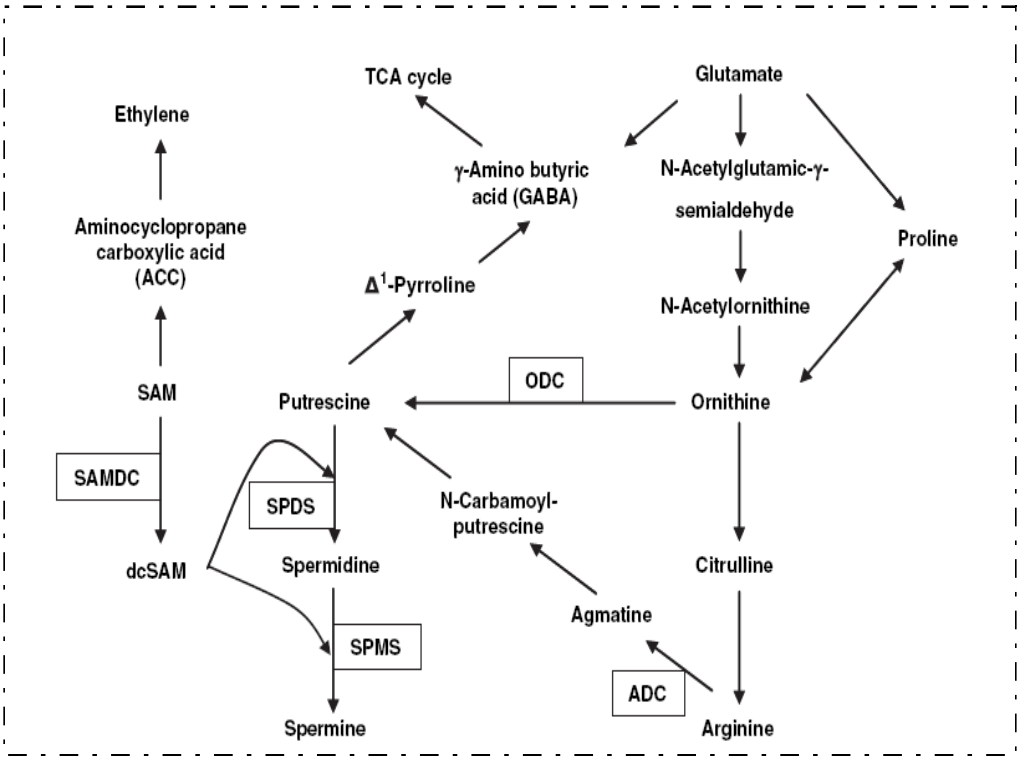

Fig. 3: Abbreviated polyamine biosynthetic pathway and related pathways. The enzymes are ODC, ornithine decarboxylase; ADC, arginine decarboxylase; SAMDC, S-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase (Page, 2007)

3. Phytoremediation technology

Some HMs are essential for life, but when present in excess, they are toxic and cause soil deterioration. To avoid the toxicity associated with these metals, several technologies and methodologies have been applied to remove them from the environment. Conventional *ex situ* and *in situ* methods include soil removal, or extraction through chemical or physical means, such as excavation and incineration, off-site storage, soil washing and *in situ* capping for stabilization (Mulligan, 2001).

Although traditional methods are adequate for treating and removing high concentrations of contaminants, unfortunately, these techniques are generally invasive causing changes to the structure and the physical, chemical and biological properties of soils, and are cost effective. The estimated cost of land filling, or incineration of a ton of soil is between $200–$1500 (Pilon-Smits, 2005). Recently, phytoremediation has become a promising remediation technique that is readily accepted by a concerned public, to clean-up soils contaminated by organic and inorganic pollutants, including HMs. This natural technology, based on the ability of selected plant species and genotypes to interact and/or adsorb specific types of contaminants, has received increasing interest due to its cost effectiveness (70-100 $, for decontamination of HM polluted soils - Glass, 2000) and its environment-friendly characteristics. Different phytoremediation technologies are suitable for different classes of pollutants, organic and inorganic. The first class of pollutants includes polychlorinated biphenyls, polycyclic aromatic compounds, nitroaromatics, or linear halogenated hydrocarbons. They can be mineralized, degraded, sequestered or volatilized by plants. The second group includes contaminants, such as toxic HMs and radionuclides that cannot be degraded, and only a few remediation techniques are available to remove them.

Some phytoremediation technologies (Pilon-Smith, 2005) used for remediating polluted matrix are:

- Phytostabilization: use of plant to stabilize pollutants in soils or waters; in this way, the plant can prevent soil erosion, leaching or runoff of pollutants by stabilization, or by converting them to less bioavailable forms;
- Phytovolatilization: after uptake in plant tissues, pollutants can be released in volatile forms by plant into the atmosphere;
- Phytodegradation: degradation of pollutants through intracellular accumulation, or via enzymatic transformation in plants, or through transformation by specific plant associated microorganisms;
- Rhyzoremediation: degradation of pollutants in the rhyzosphere by microbes. The degradation of compounds in the rhyzosphere can be enhanced by Plant Growth-Promoting Rhizobacteria (PGPR) and fungi;
- Phytoextraction: use of plants for cleaning up, sequestering (in the harvestable tissues), or detoxifying pollutants.

3.1 Phytoextraction of heavy metals

Among the various approaches included in phytoremediation, phytoextraction is probably the most studied and potentially the most feasible in low or moderately HM contaminated soils.

Two prerequisites for successful phytoextraction process are: the ability of the plants used to tolerate the type and level of contamination in the soil, and the capacity of the plants to take up large amounts of pollutants into their biomass.

Few plant species naturally tolerate and accumulate HMs, in fact plants are generally highly sensitive even to small amounts of HMs. However, there are some plant species that are able to adsorb specific pollutants in quantities that exceed several times their natural contents (0,1-1% dry matter), during normal growth and reproduction (McGrah, 2003).

These plants are commonly know as hyperaccumulators and they have adapted to live on metalliferous soils and are thus able to survive in extreme soils with a

high metal content. Around 400 terrestrial and aquatic species are recognized to be natural hyperaccumulators of metals. Thay belong to 45 different plant families including: Asteraceace, Brassicaceae, Caryophyllaceae, Poaceae, Violaceae and Fabaceae (Verbruggen, 2009). New metal hyperaccumulating species, or populations are continuously identified. The Brassicaceae family is the best represented amongst these families, with 87 *Brassica* spp. classified as metal hyperaccumulators. This plant family includes Alpine pennycress and Arabidopsis extensively studied for their ability to hyperaccumulate different HMs, mainly Zn, Cd and Ni (Khan, 2000; Verbruggen, 2009). However, not all metal hyperaccumulating plants have a high biomass, in fact many are small and slow growing plants. These characteristics thus reduce the potential for metal phytoextraction and restrict their use in this technology (Khan, 2000).

For the purpose of HM remediation soil, a promising alternative to the use of hyperaccumulators is the utilization of plants able to tolerate one or more HMs, highly competitive, fast growing, and producing a high aboveground biomass.

Deep-rooted tree of the Salicaceae family, such as willows and poplar have been considered suitable for phytoextraction (Pulford, 2002; Di Baccio, 2003; Castiglione, 2007 and 2009). These species are perfect indicators of pollution (Mertens, 2004) and they are characterized by a low environmental requirement, rapid growth and strong transpiration rate (Pulford, 2002). Particularly, *Populus* spp. present several attributes that make them good candidates for remediation purposes. They can be easily propagated, they are excellent biomass producers and they adapt to *in vitro* cultures and to genetic transformation (Castiglione 2007; Franchin, 2007). At the same time, several studies revealed a remarkable clonal variability in metal accumulation in their organs (Castiglione, 2009; Utmazian, 2007).

The efficiency of the phytoextraction process on HM polluted soils depends on many factors that are closely related (Mleczek, 2009). Some important factors are soil parameters (pH, granulometric composition, type of soil, etc.), climatic

factors (humidity, latitude, ect.), bioavailability of pollutants and biological activity of soils (Pilon-Smith, 2005).

The bioavailability of pollutants, which depend upon chemical properties, on soil characteristics and on environmental conditions, is important for pollutant remediation. It has been possible to improve the phytoextraction capacity of plants by adding to soil, contaminated by HMs, synthetic chelants such as EDTA (Etilendiamminotetracetic acid), EGTA (Etileneglicoletetracetic acid), or biodegradable as EDDS (S,S-etilendiaminodisuccinic acid) and NTA (Nitrilotriacetic acid). Chelant addition is capable to solubilize and complex HMs into the soil solution as well as promoting HMs translocation from roots to the harvestable parts of the plant (Komarek, 2007). Nevertheless, these chelants and the formed EDTA–metal complexes are phytotoxic. In addition, they have low biodegradability and high solubility in soil, which results in a high risk of eco-incompatibility for their persistence and in an alteration of the ground micro-flora (Komarek, 2007).

HM phytoextraction can be enhanced by coupling the technology to soil bioaugmentation. Rhizospheric microorganisms, which live in tight association with plant roots, can act on pollutants, mainly organic ones, using their own degradative capabilities (phytostimulation or rhizodegradation). They also positively affect plant growth and health, enhancing root development, and/or increasing plant tolerance to various environmental stresses (Gamalero, 2009) Phytoextraction can also be improved by increasing plant biomass, using PGPR (Lebeau, 2008). Several strains of PGPR have been isolated to date, each with one or more traits that might enhance plant growth. Some of these bacteria directly influence plant growth, *e.g.*, by synthesizing plant hormones, or facilitating uptake of nutrients from the soil. Others exert their beneficial effects indirectly by enhancing HM uptake, thanks to the production of enzymes, siderophores (Braud, 2009), or even organic acids.

The natural ability of plants to degrade or remove contaminants, as HMs, can be integrated and improved by AM fungi (see section 4).

An alternative and very promising method to improve plant phytoremediation capacity is to generate transgenic plants that have a higher capacity to tolerate, accumulate and metabolize pollutants, through the overproduction of HMs chelating molecules (e.g., phytochelatins - Cobbett, 2002; Vivares, 2005), or metal transporters involved in metabolism, uptake or transport of pollutants. To date, transgenic tobacco (*Nicotiana tabacum*), canola (*Brassica napus*), and tomato (*Solanum lycopersicum*) expressing bacterial ACC deaminase genes, or Indian mustard plants over-expressing enzymes involved in GSH accumulation have been constructed (Pilon-Smith, 2005).

Another fascinating proposal for phytoremediation application is the use of endophytic bacteria, that live inside the plant and promote growth and resistance to pathogens and to stresses. Recently, it has been reported that plant endophytes might be partially responsible for the degradation of environmental pollutants. *Pseudomonas* (Germaine, 2006) and other endophytic strains isolated from poplar (Moore, 2006) have in fact been shown to improve tolerance to HMs and ability to degrade organic pollutants. Engineering endophytes have also been tested for enhancing remediation of metals (Doty, 2008). Although genetic engineering of plants for enhanced phytoremediation capacity has obvious environmental benefits, their use could be associated to some hypothetical risk and is not well accepted by public opinion in Europe, in particular, where a strong Green movement is present.

3.2 Phytoextraction: risks, feasibility, perspectives

Phytoextraction is the use of plants and their associated microbes to remove HMs from contaminated sites: soil, water and sediment. Although this technique seems to be a simple method to remediate contaminated matrices, few studies in the field report a successful decontamination and the applicability might in some

cases be limited. Essentially, a metal phytoextraction protocol might consist of three steps: cultivation of the appropriate plant species on the contaminated site, removal of harvestable metal-enriched biomass from the site, and postharvest treatments to generate a cost–benefit, such as energy recovery from thermal treatment (Vassilev, 2004), or contaminant recovery through a series of chemical and physical methods.

At present, phytoextraction is limited by the long period required for soil cleanup, by the restricted types of HMs that can be extracted and by limited production of biomass in some selected species. Some authors have determined theoretical feasibility of phytoextraction, as the amount of metals being removed from soil in relation to total amount in contaminated matrix (Mertens, 2004). For example, it has been estimated it would take 15 years to obtain Cd soil reduction of 1,0 mg kg^{-1}. However, to increase efficiency and for a shorter duration of phytoextraction, it is possible to choose some plant species, especially selected for metal uptake. The phytoextraction can be ameliorated by using of engineered plants and soil amendments. Chemically assisted phytoextraction is based on the combined use of fast-growing tree (natural, or engineered species), that produce high biomass and metal chelating agents to increase the metal soluble fraction. Moreover long duration of soil cleanup could be accepted in phytoextraction projects, if the cost of application were sufficiently low and if, for instance, the phytoextraction were combined with a profit making operation, such as forestry or energy production.

Phytoextraction of HMs from soil might cause ecological risks due to dispersion of metals in the environment, to accumulation of HMs in food chain, to leaf fall and to accumulation of pollutants in topsoil. However, the risk of cumulative accumulation of HMs in herbivores can be calculated only with theoretical methods and it should be further investigated. To avoid plant material diffusion (leaves, branches, litter) and a redistribution of HMs in the upper soil profile, during growth and phytoextraction, it is possible to control the dispersion into

adjacent environments, to remove the litter, or to apply a coppice regime. Phytoextraction techniques need some ameliorations because they may be economically feasible. However, further investigation is necessary to ensure the protection of the environment and to prove its sustainability on a field scale.

4. Arbuscular mycorrhizal symbiosis

Arbuscular mycorrhizal (AM) symbiosis is the most common type of mycorrhizal association. Fossil evidences suggest that early land plants formed similar associations with ancestral AM fungi, and thus the symbiosis has existed for over 450 million years. Under natural conditions, 80%–90% of plants are colonized by AM leading to mutualistic associations, that are present in almost all terrestrial, including some aquatic ecosystems (Smith, 1997; Iaccarino, 2006). The fungi that form AM symbiosis are all members of the Glomeromycota, a sister group to the Ascomycota and Basidiomycota (Iaccarino, 2006). AM fungi are obligate biotrophs. The development of an AM symbiosis initiates with an exchange of signals between the two symbionts: the plant and the fungus. Germinating fungal spores detect the presence of a plant root through exuded from P- or N-deprived roots. In turn, AM fungal signal molecules elicit changes in gene expression in the plant (Siciliano, 2007). When the fungus interacts with root cells, it forms an appressorium, through which it invades the root cortical cells. AM fungus colonizes the plant root and forms differentiated hyphae, called arbuscules. The plant cell envelops it in a novel membrane, providing a symbiotic interface across which bi-directional nutrient transfer between the plant and AM occurs (Smith, 1997). Outside the root, the AM fungi develop an extensive extraradical mycelium that ramifies and modifies the architecture and topology of the plant root system. These associations generally result in longer, or more branched root system, called Wood Wide Web, and therefore in more efficient absorption and translocation of minerals, such as inorganic phosphate and nitrogen. While large amounts (up to 100%) of plant P can be supplied via the mycorrhizal pathway, AM fungi also have an important role in uptake of other nutrients. It has been demonstrated that up to 60% of plant Cu, 25% N, 25% Zn and 10% K, can be delivered by the external hyphae of AM fungi (Marschner, 1994). AM fungi have a positive impact on plant mineral nutrition, but, also, on plant health, influencing nutrient

availability via their effects on soil physico-chemical properties, nutrient cycling and microbial communities. Besides promoting plant growth, AM fungi can enhance plant tolerance to environmental stresses, including HMs (Leyval, 1997). Many plants growing on metal-contaminated soils possess mycorrhizae, indicating that these fungi have evolved a tolerance to HMs. Colonized plants by AM fungi are usually more tolerant to certain HMs than plants that bare. These positive effects on plant development result from an improved nutrient supply and can partly be ascribed to the complex, and not fully understood, interactions between the plant and the fungus.

It has been suggested that the AM effects on host plant tolerance to HMs may depend on enhanced HM uptake and root-to-shoot transport and immobilization (phytoextraction) (Khan, 2000), and also on reduced metal transfer from roots to shoots (Joner, 2000). In other cases, AM fungi contribute to HM immobilization within the soil and thereby improve phytostabilization. Several mechanisms for metal accumulation have been suggested: immobilization of metals by compounds, such as MTs, or PCs synthesized by the fungus, or by the plant; extrusion from the cytosol by specific HM transporters located on root cell membranes by the fungus; precipitation in polyphosphate granules in the soil; chelation of HMs to fungal cell walls; sequestration by siderophores, deposited into the root apoplasm, or into the soil (Schu¨tzendu¨bel, 2002; Ouziad 2005). Glomalin is an example of an insoluble glycoprotein, produced and released by AM fungi, in fact it is able to bind HMs in the soil (Gonzalez-Chavez, 2004). Gonzalez-Chavez and co-workers (2004) showed that glomalin, extracted from polluted soil, or from fungus hyphae, strongly and irreversibly sequesters HMs, such as Cu, Cd, and Zn. Through HM stabilization in soil, AM fungi reduce their availability, and decrease the risk of toxicity to soil microorganisms and plants growing in the immediate vicinity.

Binding of HMs in mycorrhizal structures and immobilization of HMs in the plant mycorrhizosphere have been reported in several studies (Ouziad, 2005;

Gohre, 2006; Chen, 2007; Hildebrandt, 2007), reflecting the suitability of AM fungi to phytostabilization applications.

The colonization by AM fungi can influence the uptake and subsequent accumulation of HMs in above-ground tissues of plants (Davies, 2002). Mycorrhizal symbiosis can affect plant growth in HM polluted sites by influencing the fate of the metal in the plant and also by increasing the plant tolerance to this type of stress. Reduction (Lin, 2007), increase (Joner, 1997; Lingua, 2008), or no change (Galli, 1995) of HM concentrations in plants, following mycorrhizal inoculation, have been all observed depending on the fungal–plant association (Liao, 2003; Wang, 2005). Thus, HM uptake and accumulation, in AM fungi inoculated plant, can vary dramatically and they are also influenced by soil conditions and HM concentration in soil. Audet and Charest (2007) have designed a meta-analytical approach that relates plant growth and HM uptake to tolerance. This conceptual model, proposed to determine the role of AM symbiosis in HM phytoremediation, fuses two antithetical hypotheses. At low soil HM concentrations, AM fungi enhance the HM sequestration process in the plant/root, increasing root uptake capacity with the extra-radical hyphal network and resulting in an extra HM uptake in plant tissues ("Enhanced uptake" model). At high soil HM levels, the plant mycorrhizosphere furnishes additional metal binding sites, resulting in soil HM immobilization and in their decreased availability for plants ("Metal binding" model). In this condition, the AM symbiosis increases plant biomass because HM immobilization reduces potential toxic effects and enhances plant tolerance through HM stress avoidance (Audet, 2007).

The tolerance towards HMs observed in mycorrhized plants may vary depending on the species, or even on the strain of mycorrhizal fungi employed. Mixed mycorrhizal inoculants seem to be more effective than single ones in promoting phytoextraction efficiency. HM concentrations of several metals in maize (*Zea*

mays) plant inoculated with *Glomus caledonium* have resulted lower than in plants treated with a mixture of AM fungi (Wang, 2007).

Alteration of the metal content in mycorrhizal plants and, consequently, an improvement in plant tolerance, may be related to extensive changes in gene expression and protein synthesis induced by the symbiosis itself. Several experiments have been performed to study the specific expression of selected fungal genes in extraradical mycelium and in mycorrhizal-colonized roots of different plant, grown on polluted soils. Analyses on mycorrhizal-colonized tomato (*Solanum lycopersicum*) roots revealed that HM stress influences differentially the transcript levels of genes of MT family (Lemt1, Lemt3 and Lemt4) and of Nramp, (Ouziad, 2005). These data have demonstrated the HM-dependent expression of different AM fungi genes in the intra- and extra-radical mycelium.

Use of metal tolerant mycorrhizal plants is a very promising approach for phytoremediation of metal-contaminated soils.

5. Poplar

The genus *Populus* L. belongs to the Salicaceae family. It is collectively known as poplar and includes a large variety of species widely distributed in different regions of the boreal hemisphere. Discrepancies in the recognised number of the species, from 22 to 85, could be attributed to difficulties to clearly recognise and classify species. The extensive interspecific hybridization and the consequent morphological variation, in fact, influenced and posed limitations to comparative evolutionary studies. The genus *Populus* (Eckenwalder, 1996; Hamzeh, 2004) includes 29 species classified in six sections (*Abaso, Aigeiros, Tacamahaca, Populus, Turanga, Leucoides*). All poplar species are fast-growing, economically and ecologically important multipurpose forest trees. Generally, poplar species are dioecious, and their reproduction is mainly sexual, but sometimes a single tree can spread around through rootsuckers (Brundu, 2008)

P. alba belongs to the section *Populus*; it also referred to as European white poplar and is widely distributed in river basins over northern Africa, southern Europe and central Asia. This tree can reach considerable height (up to 30 metres), and diameter (up to 2 metres). The crown is broad, rounded and thick. The bark is white, powdery and soft to the touch when young, but it tends to fissure with the age and becomes rough and black, particularly at the base of the trunk. Some specimen of white poplar (Fig. 4) can be 300-400 years old. Over the past decade, several species of *Populus* genus have emerged as model plants for phytoremediation purposes (Laureynses, 2004; Castiglione, 2007 and 2009) because they display a range of different growth characteristics, including an excellent resistance to insects and pathogens, and tolerance towards various stress conditions, such as HMs, drought, wind, salinity and high temperatures (Dickmann, 2001). Generally poplar trees can be easily propagated, they establish and displays rapid growth, high biomass production as well as a high transpiration rate and a widespreading root system.

Fig. 4: *Populus alba* trees

In addition, it is amenable to coppicing and short rotation harvesting, as well as to *in vitro* propagation and genetic transformation (Franchin, 2007; Balestrazzi, 2009). The whole genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) of *P. trichocarpa* T., is sequenced and available on public data bases (Tuskan, 2006). Thus there is an array of tools that are still not available for any other forest tree species.

5.1 Phytoremediation capability of white poplar

For several characteristics some poplars and willows are ideal candidates for phytoremediation purposes, not only for their high biomass production and deep wide-spreading root system, but also for their excellent tolerance and accumulation capacity of pollutants.

Poplar trees have been studied for cleaning up contaminated soils and water with organic pollutants such as atrazine (Burken, 1997), trichloroethylene (Newman, 1997), petroleum hydrocarbons, such as benzene, toluene and xylenes (Jordahl, 1997), herbicides (Gullner, 2001).

In addition, poplars are selected for high biomass production, high growth vigour, and disease resistance and used for short rotation coppice (SRC) cultures (Laureysens, 2004; Baum, 2009). The SRC includes the cutting of a tree at the base of its trunk, resulting in the emergence of new shoots from the stump and/or roots, and permits an intensively managed plantations for rotations shorter than 15 years. *Populus* species are currently grown as a renewable energy source and for economically use, such as for plywood, pulp and paper industry (Dutton, 2005). At the same time, several studies have focused their attention on the potentiality of poplars in phytoextraction of HMs (Pulford, 2003; Laureysens, 2004; Castiglione, 2007 and 2009). Moreover tolerance characteristic, metal uptake and compartmentalization are highly variable among poplar tree species and hybrids (Laureysens, 2004) and sometimes even within a single species (Castiglione, 2009). The ability to accumulate and

tolerate high concentrations of HMs have been studied in controlled environments particularly suitable for preliminary screenings, such as *in vitro*, hydroponic or pot conditions (Di Baccio, 2003; Franchin, 2007; Castiglione, 2007; Lingua, 2008), but also on polluted fields (Castiglione, 2009). Castiglione and co-workers (2009) report a comparative study on two poplar collections, derived from *P. alba* and *P. nigra* natural populations, growing on heavily Cu and Zn contaminated site, to select the best performing clone that shows improved tolerance and uptake capacity for remediation of HM polluted soil. The study evidenced a high genetic dissimilarity within clonal collections growing in contaminated soil. After one growth season, clonal differences in plant survival and growth were observed, and the six best performing clones were selected for their survival capacity and consistent amounts of metals in plant organs. One of these clones belonging to *P. alba*, named by the authors AL35, had a distinctly higher concentration of both metals in the leaves and in the roots, making it particularly suitable for both phytoextraction and phytostabilization. Plant diversity and adaptation can be associated to a range of different strategies for tolerating high concentrations of HMs in the growth substrate, such as the synthesis of PAs and of MTs and/or PCs. In particular, the high metal accumulating clone AL35 exhibited a extremely high concentration of free and conjugated Put. Up-regulation of PA metabolism has been also reported for poplars exposed to high Zn or Cu concentrations under in vitro (Franchin, 2007), or greenhouse (Lingua, 2008) experiments. Different poplar species can respond differently in terms of MT expression (Kohler, 2004; Castiglione, 2007). The analysis of *Pa*MT1, *Pa*MT2 and *Pa*MT3 gene expression in response to high Zn concentrations in a micropropagated white poplar commercial clone (Villafranca) showed that they were also differentially expressed in organ-specific manner (Castiglione, 2007). *Pa*MT1 and especially *Pa*MT3 gene expression is stimulated by high concentrations of Zn preferentially in leaves. The response of these genes is generally not linear with

respect to metal concentration and/or exposure time. The treatment with high concentrations of Zn interferes with important metabolic processes and negatively affects the photosynthetic machinery. In fact, total chlorophyll declined markedly upon Zn treatment and also reduced the chl a:chl b ratio, in Villafranca clone (Castiglione, 2007).

Poplar tolerance and translocation of HMs in different organs can be influenced by mycorrhizal symbiosis with AM fungi. Lingua and co-workers (2008) assessed the effects of high Zn concentrations on different clones of poplar (*P. alba* clone 'Villafranca' and *P. nigra* clone 'Jean Pourtet'), inoculated or not with AM fungi (*G. mosseae* or *G. intraradices*). In the Villafranca clone, *G. mosseae* increased the total amount of Zn in the plant, but reduced its accumulation in leaves, whereas *G. intraradices* did not affect Zn levels in any organ. In *P. nigra*, the AM fungi decreased the Zn content in the whole plant. These results suggested that the performance of the Villafranca clone is improved by *Glomus spp.* inoculation. In contrast, the Jean Pourtet clone accumulated more Zn in the absence of mycorrhiza without a significant reduction of leaf biomass, probably with a different protective mechanism, linked to reduction of translocation to the leaves, more intense colonization. Additionally, AM fungi may affect tolerance to HMs by modulating plant stress reactions.

The aim of work

The main objectives of the present work were those of, i) investigating the impact of AM symbiosis on metal tolerance in a *P. alba* clone, named AL35, selected on a HM polluted site for its high survival ability, on metal accumulation capacity (Castiglione, 2009), and ii) detecting the stress-responsive gene modulation in poplar leaves, after prolonged exposure to high concentrations of Cu and Zn.

In AL35 plants, pre-inoculated with the AM fungi *G. mosseae* or *G. intraradices*, growth, HM contents, expression of MT, *Pa*SPDS and *Pa*ADC genes and PA accumulation were investigated during the first (2006) and second growing season (2007), and then compared with that of plants grown on unpolluted soil, inoculated or not with the same AM fungi. The plants were grown in pots and under controlled conditions (greenhouse) in order to minimise the effects of other environmental stress factors (such as drought, pests, etc.), but under conditions as similar as possible to those of the field. Thus, the soil has been collected from a multi-metal (Cu and Zn) contaminated site, where the large field scale trial was established to evaluate the phytoremediation potential of a large clonal collection of poplars belonging to different species (Castiglione, 2009).

A major objective of this study was also to correlate improved nutrition, sequestration of HMs, enhanced tolerance and functional stress-related genes to AM fungi colonization. Results are discussed in relation to the differences in plant biomass, metal uptake and translocation observed in mycorrhizal vs non mycorrhizal AL 35 plants.

Materials and Methods

1. Plant material

The poplar clone AL35 used in the present study, was selected during a field trial that tested different poplar clones on a heavily Cu and Zn contaminated site, located next to the KME-Italy S.p.A. factory (Castiglione, 2009). Twenty-cm long cuttings were collected in February 2006 from plants growing in the field, and stored at 4°C until use.

2. Fungal inoculation

In March 2006, the poplar cuttings were placed overnight under running tap water. They were then put into 20-cm high plastic pots (750 mL) containing heat-sterilised (180°C, 3 h) quartz sand (3-4 mm diameter). Pots were either separately inoculated with *Glomus mosseae* (Gerd. and Nicol.) Gerdemann and Trappe BEG 12, or *Glomus intraradices* (Schenck and Smith) BB-E (supplied by Biorize, Dijon, France as described by Lingua and co-workers, (2008), or were uninoculated (controls). The inoculum was provided at 50% (v/v) concentration, using a 50-mL bottomless Falcon tube placed around the cutting. Cuttings were fed on alternate days with 80 mL of Long Ashton solution (Table 2), modified according to Trotta and co-workers (1996). After one month, cuttings were transferred into sterilized 7.5-L plastic pots containing either polluted, or unpolluted autoclaved soil.

3. Analysis of growth and mycorrhizal colonisation

At the end of the experiment (July 2007), growth was evaluated on the basis of leaf, stem and root fresh and dry weights. The degree of mycorrhizal colonisation of all plants, pre-inoculated or not, was evaluated microscopically using the method of Giovannetti and Mosse (1980), with some modifications as described below. Roots of 1 cm in length were cleared in 10% KOH for 3 h at

90 °C, washed thoroughly in distilled water, bleached for 1 min in 1% $KMnO_4$ followed by 10 min in 5% oxalic acid. After acidification in HCl for one night, treated roots were stained with Aniline Blue for 1 h at 90 °C and finally differentiated in glycerin:water (1:1) for at least one week before observation under bright field microscopy. Microscopy observations were carried out at 50-630x magnifications. Results are expressed as intensity of colonisation, i.e., percentage of colonised roots (M%). The number of arbuscules and vesicles was also evaluated.

4. Experimental design and growth conditions

The soil originating from the polluted area is a sandy loam (according to USDA specifications: sand 31%, silt 46%, clay 23%), and has the following chemical features: organic matter 2.24% DW; N< 0.01% DW; K 0.0237% DW; P 0.0026% DW; pH 6.2, with a mean soil total Zn concentration of 950 mg kg^{-1} DW and 1300 mg kg^{-1} DW of Cu (Castiglione, 2009). The unpolluted soil, collected from a nearby uncontaminated area, had mean Zn and Cu concentrations of 60 and 14 mg kg^{-1} DW, respectively. The chemical analyses were carried out on the soil (see above paragraph of Materials and Methods). The experimental design therefore consists in growing of plants pre-inoculated with either *G. mosseae* (Gm plants) or *G. intraradices* (Gi plants) for two vegetative seasons (from March 2006 to July 2007), in pots containing either polluted or unpolluted soil. Ten plants per treatment were prepared, placed in pots and grown in a glasshouse and automatically watered (from above) twice a week before dawn for 3 min; in July and August, plants were watered for 8 min on alternate days. A commercial organic slow release fertilizer (Grenagro Medio Plus, Grena, San Bonifacio, Verona, Italy) was supplied (16,5 g per pot) once. The same number of uninoculated plants were grown under the same conditions.

Table 2: Chemical composition and concentration of basal nutrient solution, based on Long Ashton Formula

Compound	**gL^{-1}**	**mM**	**Element**	**mgL^{-1}**
KNO_3	0,505	5,0	K	195
			N	70
$Ca(NO_3)_2$	0,656	4,0	Ca	160
			N	112
NaH_2PO_4. $2H_2O$	0,208	1,33	P	41
			Na	31
$MgSO_4$.7 H_2O	0,369	3,0	Mg	24
Fe.citrate.5 H_2O	0,0245	0,1	Fe	5,6
$MnSO_4$	0,00223	0,01	Mn	0,55
$CuSO_4$.5 H_2O	0,00024	0,001	Cu	0,064
$ZnSO_4$.7 H_2O	0,000296	0,001	Zn	0,065
$(NH_4)_6.Mo_7O_{24}$.4 H_2O	0,000035	0,0002	Mo	0,019
NaCl	0.00585	0,1	Cl	3,55

5. Sampling procedure

Samples were taken in July 2006 (first sampling) and in July 2007 (second sampling, end of experiment). In the first year, only leaf samples were taken. In the second year, the whole plant was harvested; root, stem and leaf samples were collected and stored separately for fresh and dry weight measurements, and for determination of Cu and Zn concentrations. Leaf samples of each treatment were pooled together at each sampling time, frozen in liquid nitrogen, and stored at -80°C for RNA extraction and polyamine determination.

6. Chemical analysis

Approximately 0,5 g DW of material was used for the determination of Zn and Cu concentration in leaves, stems and roots, separately. Samples were weighed and dried at 75 °C up to constant weight, pulverized in an agate mortar (Eatchs, Retsch, Germany) and digested with an acid mixture (HNO3 65%:HF 50% = 2:1 = v:v). Metal concentration was assessed by means of a calibration curve, after measurement determined using an atomic absorption spectrometer (AAnalyst 100, PerkinElmer, Wellesley, MA, USA). Atomic absorption spectroscopy is a technique used to asses the concentration of metal elements in a sample. All atoms can absorb the electromagnetic radiation and the wavelengths at which radiation is absorbed or emitted is exclusive for a particular chemical element. Generally in atomic absorption spectroscopy, the radiation source is a hollow cathode lamp. In this lamp, filled with argon or neon gas, there is a metal cathode containing the metal for excitation, and an anode. When a high voltage is applied across the anode and cathode, gas particles are ionized and excited; these atoms emit light with the frequency characteristic of the metal. The sample to analyze, containing some metals, must be atomized. Generally the sample is atomized with a flame, but other atomizers, such as a graphite furnace, can be used. The atoms, produced by the sample, absorb the light produced by the radiation source. The quantity of energy, put into the atomizer from source, is

known, and the quantity transmitted by the atomized sample can be measured by a detector. According to the Beer-Lambert law, the quantity of transmitted light is proportional to the concentration of the element being measured. All the chemical analyses were carried out on three subsamples. Certified standards (BCR 062, 100, 129 and 145R, by the Institute for Reference Materials and Measurements, Ratieseweg, Belgium), with known element concentration, were analyzed with the samples in order to confirm the correctness of the procedure. The same method was used for the determination of HMs in soil.

7. RNA extraction

Total RNA was extracted from approximately 100 mg of frozen leaf tissues using the RNeasy PlantMini Kit (Qiagen, Milano, Italy) and the buffers provided with the kit, designed specifically for RNA purification from different plant organs. Plant tissue were ground in mortar with liquid-nitrogen. 450 µl of lysis buffer and 4 µl of β-mercaptoethanol were added to tissue powder. A short incubation at 56°C promotes the disruption of the tissues. Tissue lysates were transferred onto spin columns to remove cell debris. After centrifugation the cleared lysate was mixed to 0,5 volume of ethanol and transferred onto the column that retains RNA on the membrane. To eliminate traces of genomic DNA, on-column DNA digestion is performed according to the manufacturer's instructions. Two wash buffers were added to spin column membrane to wash RNAs. RNA-free water was directly added to the column to elute the RNA.

8. RNA electrophoresis

The integrity and size distribution of total purified RNA is checked by formaldehyde agarose (FA) gel electrophoresis and ethidium bromide staining. Agarose powder is mixed to 1X FA gel buffer (Table 3a and b) to prepare FA 1,2 % agarose gel. After agarose melting, 37% formaldehyde and ethidium

Table 3: Composition of 10X and 1X Formaldehyde Agarose (FA) gel buffer

a) 10X FA gel buffer

Component	Concentration (mM)
MOPS	200
Sodium acetate	50
EDTA	10
NaOH (to pH 7,0)	

b) 1X FA gel buffer

Component	Volume (ml)
10X FA gel buffer	100
37% Formaldehyde (12,3M)	20
RNase-free water	880

Table 4: Composition of 5X RNA loading buffer

Component	Volume (ml)
Saturated aqueous bromophenol blue solution	0,016
EDTA (500 mM pH 8,0)	0,080
37% Formaldehyde (12,3M)	0,720
100%Glycerol	2,0
Formamide	3,084
10X FA gel buffer	4
RNase-free water	to 10 ml

bromide are added to the gel. One volume of 5X loading buffer (Table 4) is added to 4 volumes of RNA samples. After incubation for 3 minutes at 65°C (in this way hairpins and double strand RNAs are removed) and chilling on ice, the RNA is loaded on the equilibrated FA gel.

9. *cDNA synthesis*

Total RNA, extracted from leaf tissues and treated with RNAse-free DNAase (Qiagen, Milano, Italy), was used to generate cDNA using SuperScript III Reverse Transcriptase synthesis kit (Invitrogen, Milano, Italy). 1-2 µg of total RNA were added to 500ng of oligo$(dT)_{18}$ and dNTPs Mix (10 mM). The mixture was heated to 65°C for 5 min and incubated on ice for, at least, 1 min to disrupt high secondary structures. To generate cDNA, 5X First-Strand buffer, 0,1 M DTT and 200 U SuperScript III RT enzyme were added to the mix containing RNA and incubated at 50°C for 60 min.

10. *Quantitative Reverse Transcription- Polymerase Chain Reaction (qRT-PCR)*

The polymerase chain reaction (PCR – Mullis, 1994) has revolutionized the detection of DNA and RNA. As little as a single copy of a particular DNA sequence can be specifically amplified. A special kind of amplification technique is Real-time PCR. Reactions are characterized at specific time points during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. Real-time systems were improved by probe-based, or intercalator-based (*e.g.* SYBR green dye) PCR product detection. When the starting copy number of the nucleic acid target is high, a rapid and significant increase in fluorescence is observed. Real-time PCR was performed in a reaction mixture containing 2x iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA), specific primers, cDNA template and RNase-free sterile water in a final volume of 20 µl. Gene-

specific primers used for PCR experiments and relative annealing temperatures (T) are listed in Table 5. All the primers were designed on poplar sequences available at the *P. trichocarpa* database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) using the primer design software Primer3 (version 0.4.0). The cDNA was used as a template for amplification using the BioRad iQ5 cycler (Bio-Rad Laboratories). Each reaction was performed in duplicate to verify the reproducibility of the system and for standard error calculation. The following thermal cycle conditions were used for the amplifications of the target and housekeeping genes: an initial denaturing step at 95°C for 3 min was followed by 45 cycles, each cycle consists of denaturation at 95°C for 10 s, annealing at T °C for 40 s, and extension at 55°C for 10 s. In each experiment, a negative (no-template) control was used to test for false-positive results or contaminations. Primers, specific for *P. trichocarpa* actin B gene, were used for the normalization of reactions. The housekeeping gene actin B was chosen as a control for all RT-PCR experiments after testing also poplar ubiquitin and 18S-rDNA genes. The actin gene was the most reproducible and stable in time and among samples. Data collection and analysis were performed using the Optical System Software (iQ5 version 2.0). Fold-changes in RNA expression were estimated, using threshold cycles, by the comparative C_T method ($2^{-\Delta\Delta Ct}$) (Livak, 2001). The ΔCt value is calculated for each sample as the difference between the Ct values of the gene of interest and the housekeeping gene. The ΔΔCt value is the difference between the ΔCt values of the experimental and the control samples. The fold-change in gene expression is therefore equal to $2^{-\Delta\Delta Ct}$ if the PCR replication efficiency for all analyzed genes is 100%. Threshold cycle (C_T) values were in the range of 25-27 cycles for actin, and 20-22 for the genes of interest. Data are the means (± SD) of two biological replicates. Genes were considered to be up-regulated in mycorrhizal plants relative to uninoculated controls when mRNA levels were > 2-fold, and down-regulated when they were < 0.5-fold.

Table 5: List of primer pairs and of annealing temperatures used for RT-PCR amplifications of *Pa*MT gene family, *Pa*SPDS, *Pa*ADC and Actin genes

Primer name	Primer sequence (5'-3')	Annealing T (°C)
MT1a_for	ATGTCTGGCTGTAGCTGTGG	60
MT1a_rev_UTR	CCATGTCCATGTGTCCTCAT	60
MT1b_for	CCTAAAGAAAATGTCTGGTT	55
MT1b_rev_UTR	TATAGGCCACAATAACTACTT	55
MT2a_for	ATGCT TGCTGTGGTGGAAGC	55
MT2a_rev_UTR	GAATCAACGCAGCCAGC	55
MT2b_for	CAGATGCAGCATGTACCCA	55
MT2b_rev_UTR	GTTTTCTCATTTGCAGGAGC	55
MT3a_for	ATGTCTAGCACCTGCGACAA	55
MT3a_rev_UTR	ACACATGACGGTTTACGTG	55
MT3b_for	AATCATCATGTCTAGCACCT	55
MT3b_rev_UTR	CATGATAGTTGATGTGCTTG	55
PaADC_for	TGGTGATAGCGATCATGGAA	55
PaADC_rev	CGGGGATGTTACTCTCAAGC	55
PaSpds1_for	TCGATTCCATCTCCCAAAAC	55
PaSpds1_rev	CCTCAAATCCAACAGCCAAT	55
PaSpds2_for	TGACGTAGCAATCGGGTATG	55
PaSpds2_rev	TGTGCTCACAACTCCTCCTG	55
Actin_for	GCCCAGAGGTCCTCTTCCAA	55-60
Actin_rev	GGGGCTAGTGCTGAGATTT	55 – 60

11. HPLC analysis of polyamine content

Plant material (0.3-0.5 g FW leaves) was homogenized in 10 volumes of 4,0% perchloric acid, kept for 1 h on ice, and centrifuged at 15'000 *g* for 30 min. The pellets were washed twice by resuspension in perchloric acid, centrifuged and resuspended in the original volume of perchloric acid and free and conjugated PAs (Put, Spd and Spm) were extracted. Aliquots of the supernatants and standard solutions of Put, Spd and Spm were subjected to acid hydrolysis with 6,0 M HCl at 110° C overnight, derivatized with dansyl chloride, essentially to release polyamines from their perchloric acid insoluble and soluble conjugates, as described by Bregoli and co-workers (2002). Dansylated derivatives were extracted with toluene, taken to dryness and resuspended in acetonitrile. PAs were separated and quantified by HPLC (PU-980 Jasco, Tokyo, Japan) using a reverse phase C18 column (Spherisorb ODS2, 5-mm particle diameter, 4.6, 250 mm, Waters, Wexford, Ireland) and a programmed acetonitrile:water step gradient, as that allowed polyamine separation in 15 min.

12. Statistical analyses

Mean values and standard errors were calculated, and the data compared by one-way analysis of variance (ANOVA), followed by a post-hoc F-test with $P<0.05$ as the significance cut-off. ANOVA, ANalysis Of VAriance, is a powerful and common statistical procedure used to evaluate the observed variance partitioned into components due to different explanatory variables. In its simplest form, ANOVA represents a statistical test of whether the means of several groups are all equal. This analysis can be different depending on the number of variables, dependent and independent.

The one-way ANOVA is used to test for differences among two or more independent groups. In this case, there is only one dependent variable and one independent variable.

Two-way ANOVA is used for repeated measures in the experiments.

Factorial ANOVA is used to study the effects of two or more independent variables on one dependent variable.

ANOVA analysis is often followed up by one or more different follow-up tests, such as F-test to assess if differences in groups are statistically significant.

Results

1. Mycorrhizal colonisation

At the end of the experiment, AL35 plants were harvested and the extent of mycorrhizal colonisation of all plants, pre-inoculated or not, was evaluated microscopically after staining. Percentage of colonised roots (M%), was below 1% in uninoculated plants, on both polluted and unpolluted soil. By contrast, plants inoculated with *G. intraradices* or *G. mosseae* showed levels of M% ranging from 5 to 23% without significant differences between the two fungal species, and without any significant difference between polluted and unpolluted soil (Fig. 5). Although many vesicles were observed, no arbuscules were seen in any observed root samples.

2. Biomass production

In July 2007, at the end of the experiment, fresh and dry weight measurements were made for all plants that underwent the various treatments. Fig. 6 shows that root, stems and leaf biomass of plants grown on unpolluted soil was not significantly affected by mycorrhization, with exception for leaves of Gm plants. By contrast, when AL35 plants were grown on polluted soil, in the absence of AM fungi, their biomass was severely affected, with decreases of approximately 85% relative to controls (unpolluted soil). On polluted soil, both fungal species exerted a positive effect on the growth of all plant organs, except for leaves of Gi plants, with about 4- to 6-fold increases in mycorrhizal plants relative to non mycorrhizal ones.

3. Copper and zinc concentrations in plant organs

3.1 Copper concentration

Cu concentration was determined in leaves after first sampling and separately in all organs at the end of the experiments (Fig. 7). On unpolluted soil, the concentrations of Cu in roots, stems and leaves were similar in

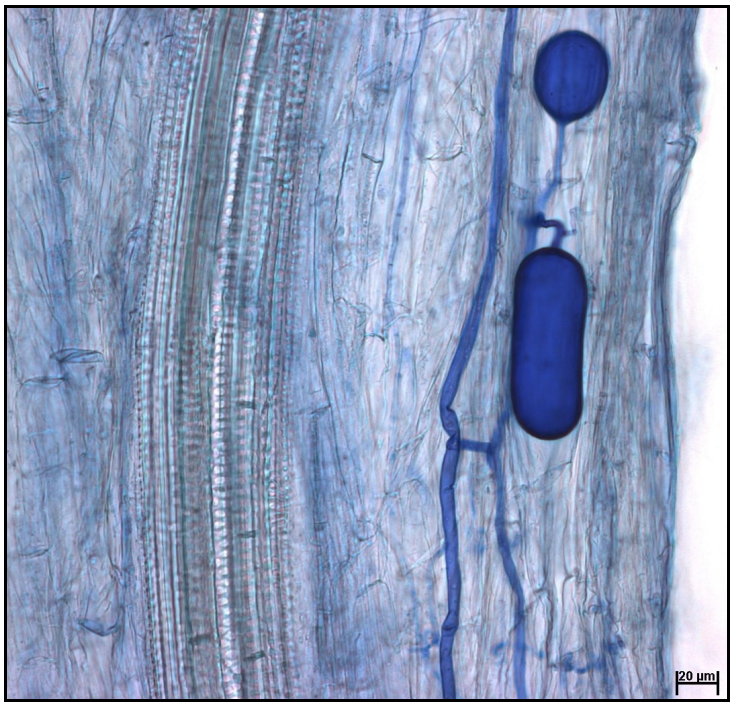

Fig. 5: vesicles of *G. mosseae* in colonized AL35 roots

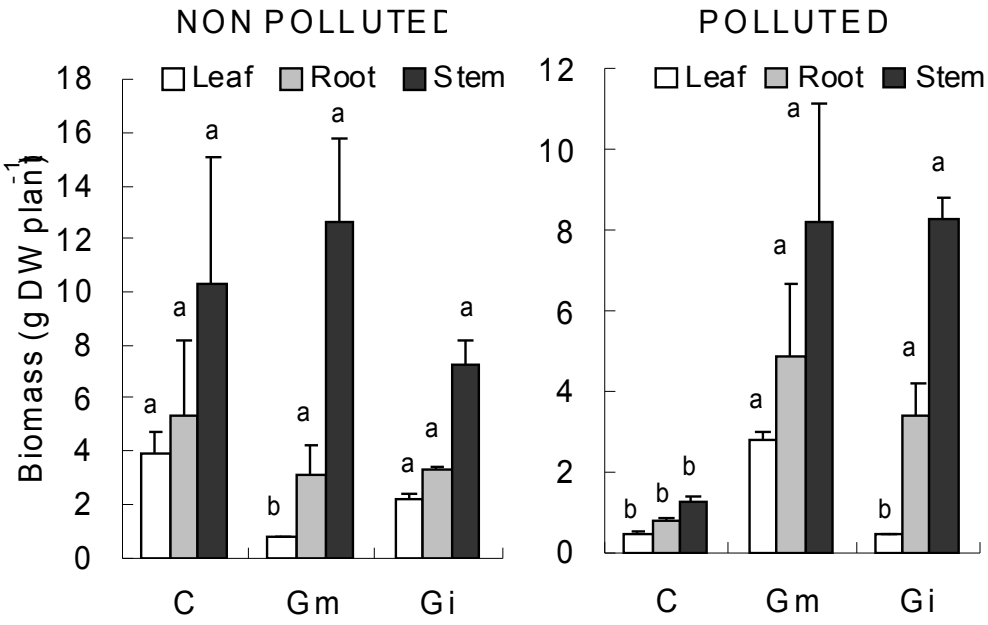

Fig. 6: Root, stem and leaf biomass of AL35 plants after two growth seasons on unpolluted or polluted soil. Plants were either uninoculated (C) or inoculated with *G. mosseae* (Gm) or *G. intraradices* (Gi). Values are means ± standard deviation (n = 3). Different letters indicate significant differences for amoung treatments ($P<0.05$).

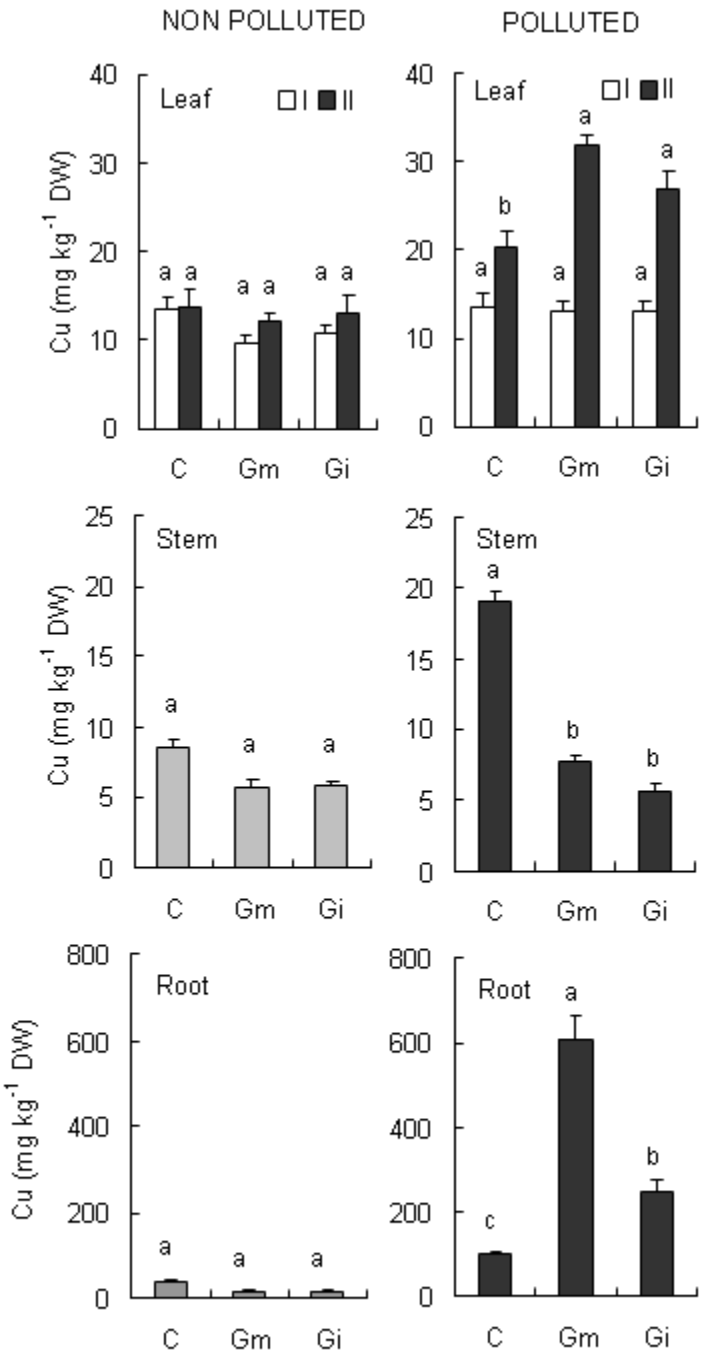

Fig. 7: Cu concentrations in leaves, stems and roots of AL35 plants grown on non-polluted or polluted soil, in the presence or absence (C) of either *G. mosseae* (Gm) or *G. intraradices* (Gi). White bars indicate Cu concentration in leaves, collected after one growth seasons; stems and roots were harvested only at the second growth season (end of experiment). Different letters indicate significant differences ($P<0.05$) for treatments referred to the same organ and for each sampling time separately.

mycorrhizal and non mycorrhizal plants. On polluted soil, Cu reached the highest concentration in roots up to 600 mg kg^{-1} DW. In Gm plants, in fact, it was detected at concentrations over 6 times that of non-mycorrhizal plants. In stems, Cu concentration was comparatively low, ranging from about 5 to 20 mg kg^{-1} DW. On unpolluted soil, differences were not significant among treatments. While on polluted soil, significant differences were observed among mycorrhizal plants and controls. In leaves, Cu was always rather low (ranging from ca 10 to 30 mg kg^{-1} DW) as compared to roots. Although at the second sampling date, on polluted soil, AL35 plants inoculated with Gm and Gi showed a significant Cu concentration increase.

3.2 Zinc concentration

Zn was mainly accumulated in the leaves (Fig. 8). On unpolluted soil, no differences were detected in leaves and stems of mycorrhizal and non mycorrhizal plants. In roots of Gm and Gi plants, Zn content was less than half that of control (Fig. 8).

On polluted soil at the first sampling date (white bars), leaves of Gm plants accumulated a significant and higher concentration (about twice) with respect to control and Gi plants. At the second sampling (black bars), leaf Zn concentrations reached the highest values, about 400-500 mg kg^{-1} DW, although differences between mycorrhizal and non-mycorrhizal plants were no longer significant. In stems, Zn concentrations were lower than in leaves (60-120 mg kg^{-1} DW) and higher in uninoculated and Gi plants than in Gm plants. In roots, Zn concentrations were of the same order of magnitude as in stems. However, metal quantities detected in roots were higher in Gm plants than in control and Gi plants (which were not significantly different among them).

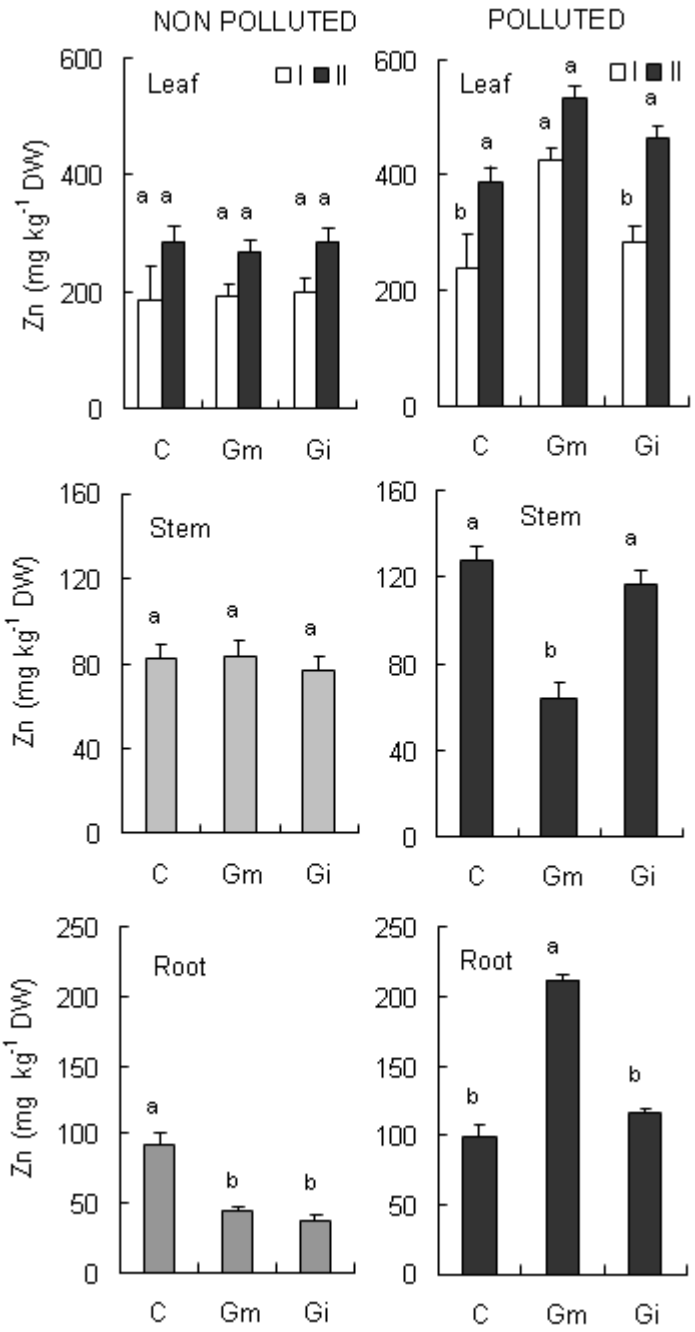

Fig. 8: Zn concentrations in leaves, stems and roots of AL35 plants grown on non-polluted or polluted soil, in the presence or absence (C) of either *G. mosseae* (Gm) or *G. intraradices* (Gi). White bars indicate Zn concentration in leaves, collected after one growth seasons; stems and roots were harvested only at the second growth season (end of experiment). Different letters indicate significant differences ($P<0.05$) for among treatments referred to the same organ and separately for each sampling time.

In Table 6, total amount of HMs, extracted by the plants for each organ, and the Translocation Factor (TF, shoot to root ratio) are reported. Total amount of Zn and Cu extracted by AL35 plants increased in all organs after inoculation with AM fungi.

Cu concentration increased dramatically in roots after mycorrhization. An increase, albeit smaller, was also observed in leaves and stems. Thus, the total amount of Cu extracted by Gm plants as a whole, relative to uninoculated ones, rose 30-fold. The organ distribution of Cu (in percentage) was also different in non-mycorrhizal and mycorrhizal plants. In control plants, (uninoculated plants) the percentage of Cu concentration in roots was 68,7. In Gm and Gi plants, total amount of Cu was 95,0 and 93,5% respectively. Consequently, the TF was also much higher in uninoculated plants compared to mycorrhizal plants.

Zn concentration was always higher in mycorrhizal plants, with the exception of Gi leaves, as compared with non mycorrhizal ones. The TF for Zn was reduced in mycorrhizal plants relatively to the control.

4. PaMT and PaSPSD gene expression patterns in AL35 leaves

MT proteins and PA synthesis can be induced by a wide variety of chemical and physical stimuli in plant. To determine the effect of HM stress on gene expression of *Pa*MT and *Pa*SPDS gene family and *Pa*ADC gene, qRT-PCR experiments were performed in AL35 leaves.

4.1 Transcript levels of PaMT genes

Gene expression analyses were performed to evaluate steady state transcript levels of *Pa*MT genes in leaves of AL35 poplar plants grown on polluted and unpolluted soil, in the presence or absence of AM fungi, during the first (Fig. 9) and second vegetative season (Fig. 10).

Table 6: Total Cu or Zn content (calculated as the product of mean metal concentration by mean DW) in leaves, stem and roots of AL35 plants after two growth seasons on polluted soil in the absence (C) or in the presence of *G. mosseae* (Gm) or *G. intraradices* (Gi). Percentage content (%) in each organ relative to the total (leaves+stems+roots), and the shoot (leaf+stem)–to–root ratios (Translocation Factor, TF) are also given.

	Leaves		Stems		Roots		
	total (mg)	%	total (mg)	%	total (mg)	%	TF
Copper							
C	0.010	8.9	0.025	22.3	0.077	68.7	0.45
Gm	0.089	2.9	0.064	2.1	2.955	95.3	0.05
Gi	0.012	1.3	0.047	5.3	0.832	93.5	0.07
Zinc							
C	0.194	44.1	0.166	37.7	0.078	17.7	4.6
Gm	1.502	50.0	0.525	16.9	1.035	33.7	2.0
Gi	0.203	12.8	0.967	61.9	0.394	15.2	3.0

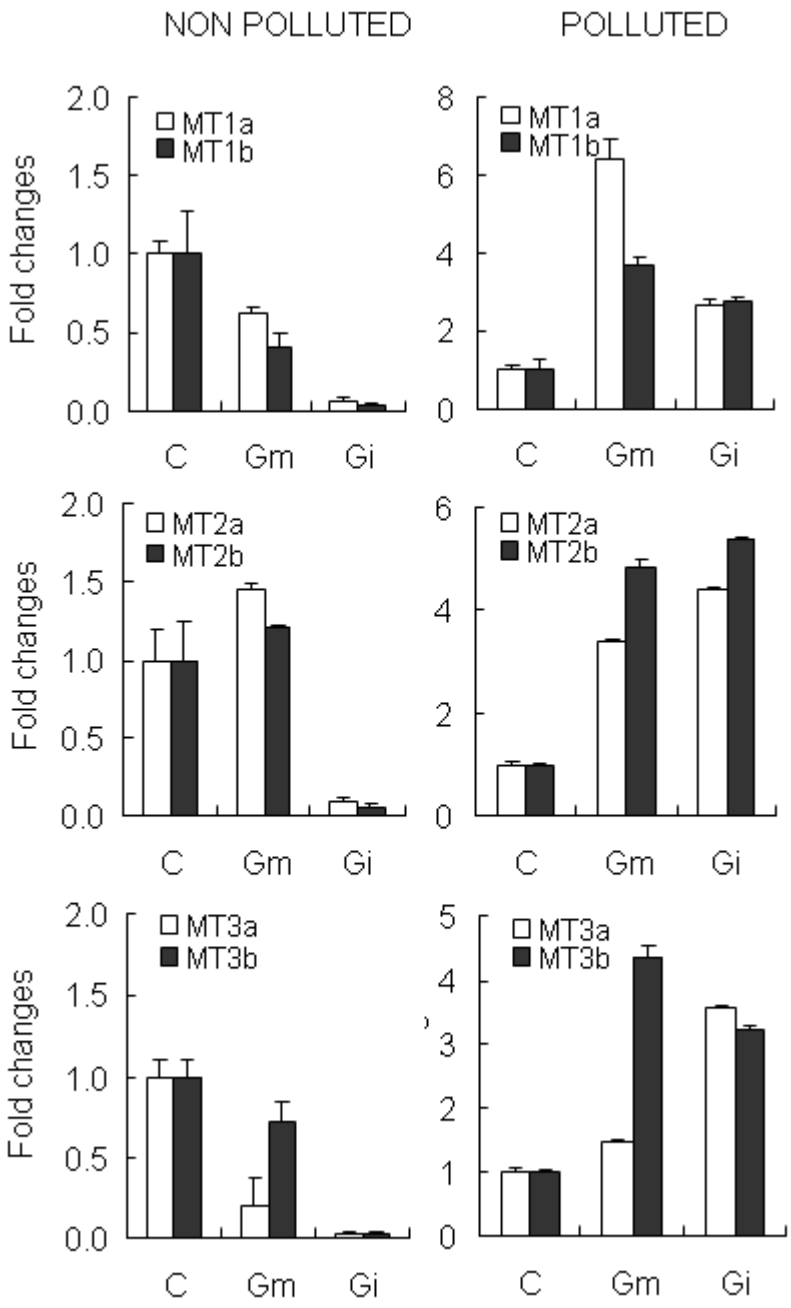

Fig. 9: Transcript levels of *Pa*MT genes in leaves of *P. alba* clone AL35 at the first sampling date, on polluted or non-polluted soil in the presence or absence (C) of either *G. mosseae* (Gm) or *G. intraradices* (Gi). The value 1 was arbitrarily given to control plants (C). Bars represent 95% confidence intervals calculated on two biological replicates.

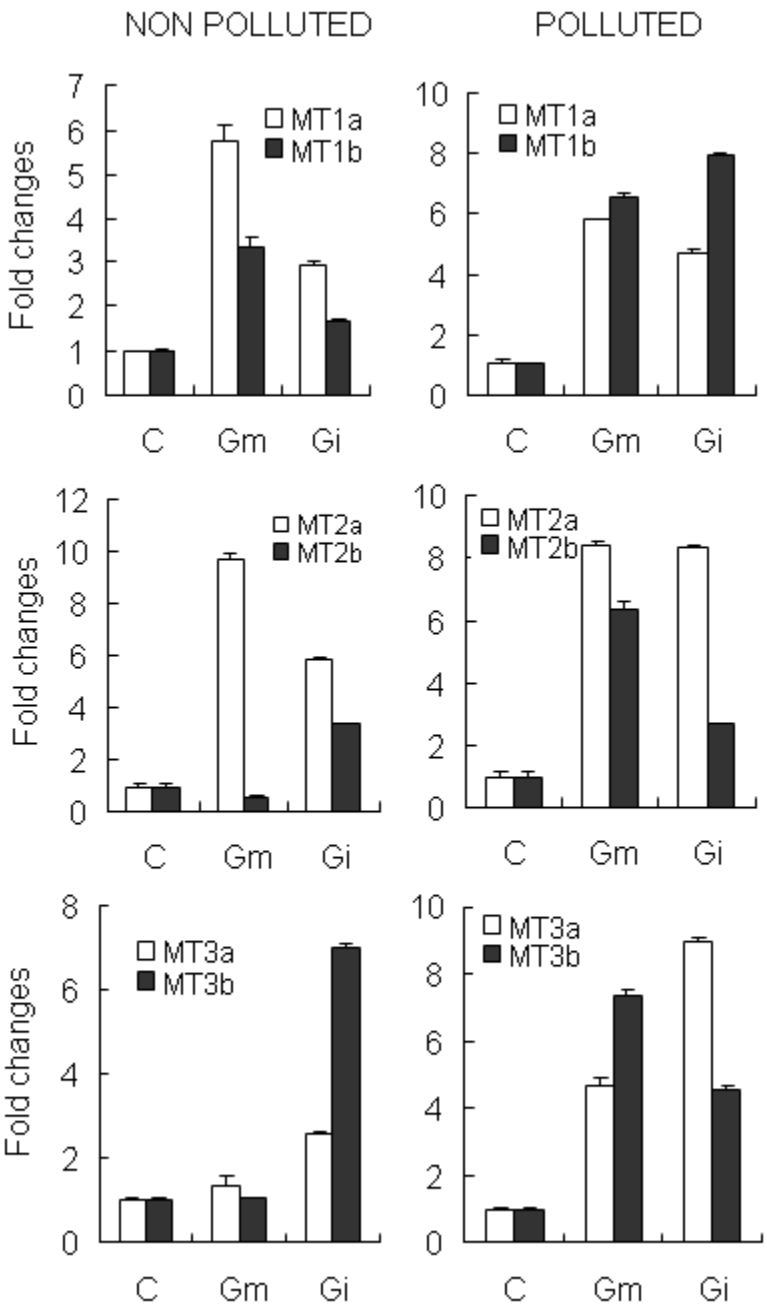

Fig. 10: Transcript levels of *Pa*MT genes in leaves of *P. alba* clone AL35 at the second sampling date, on non-polluted or polluted soil in the presence or absence (C) of either *G. mosseae* (Gm) or *G. intraradices* (Gi). The value 1 was arbitrarily given to control plants (C). Bars represent 95% confidence intervals calculated on two biological replicates.

At the first sampling date (Fig. 9), transcript levels of all *Pa*MT genes in leaves of all plants, grown on unpolluted soil, were either lower in the presence of AM fungi, or unaffected compared with uninoculated controls. In particular, the transcription levels of all *Pa*MT gene isoforms were strongly down-regulated in Gi mycorrhizal plants. While, in Gm plants, all *Pa*MT genes were weakly affected by mycorrhization. On polluted soil, gene expression of all *Pa*MT genes was strongly up-regulated by AM fungi (from ca 3 to ca 6-fold), with the exception of *Pa*MT3a in Gm plants. At the end of the experiment (Fig. 10), on polluted soil, the up-regulation of MT genes, induced by AM fungi, was always very high (up to 8-9 folds). At second sampling, compared to the first one, however, transcription of *Pa*MT1a, *Pa*MT1b and *Pa*MT2a was enhanced in all plants by the presence of AM fungi. In contrast, *Pa*MT2b, *Pa*MT3a and *Pa*MT3b genes were strongly enhanced, also in Gi plants, on unpolluted soil.

4.2 Transcript levels of PaADC and PaSPDS genes

qRT-PCR analyses were performed to evaluate steady state transcript levels of the *Pa*ADC and *Pa*SPDS, key genes of PA biosynthetic pathway (Fig. 11 and 12). The mycorrhization of AL35 trees on unpolluted soil caused a slight down-regulation of *Pa*ADC gene at both sampling dates (Fig. 11 a and c). By contrast, on contaminated soil, an induction of *Pa*ADC transcription (7-fold in Gm and 4-fold in Gi plants - Fig. 11 b) was observed in mycorrhizal plants at the first sampling date. At the second sampling, the expression of *Pa*ADC gene was down-regulated in inoculated plants as compared to uninoculated ones, and to the same extent on polluted and non polluted soil (Fig. 11, c and d).

The transcription of both *Pa*SPDS genes at first sampling date was lower in Gi plants, or unchanged in Gm, relatively to uninoculated control (Fig. 12 a) grown on unpolluted soil.

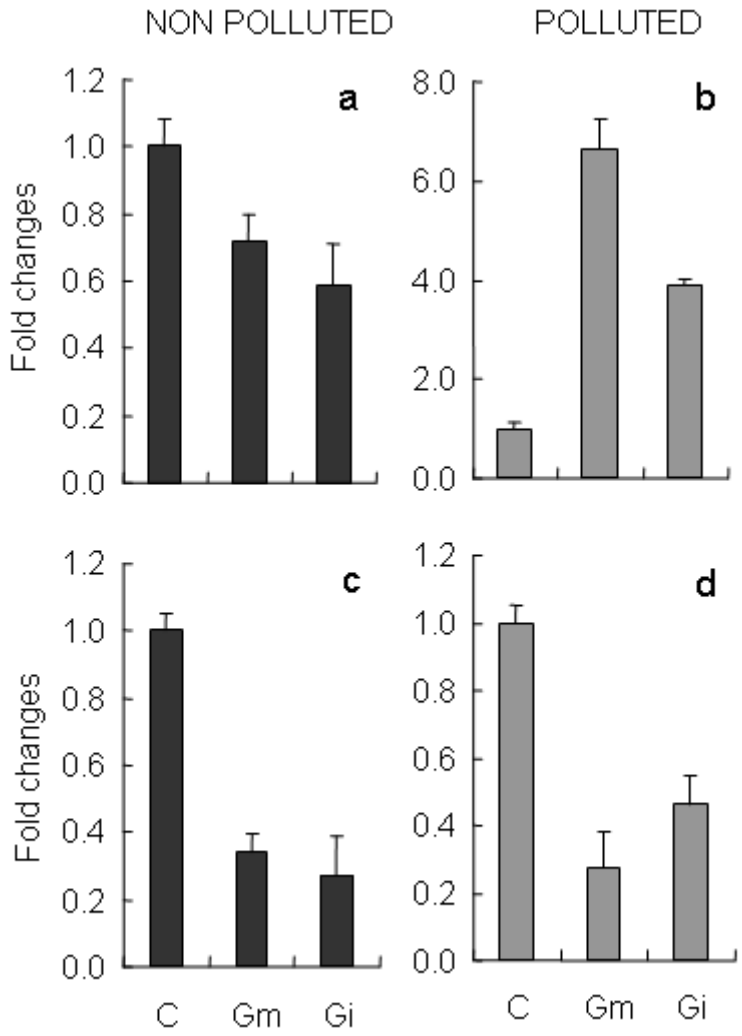

Fig. 11: Transcript levels for *Pa*ADC in leaves of AL35 plants at the first (a, b) and second (c, d) sampling date. Plants were grown on non-polluted or polluted soil in the presence or absence (C) of either *G. mosseae* (Gm) or *G. intraradices* (Gi). The value 1 was arbitrarily given to control plants (C). Bars represent 95% confidence intervals calculated on two biological replicates.

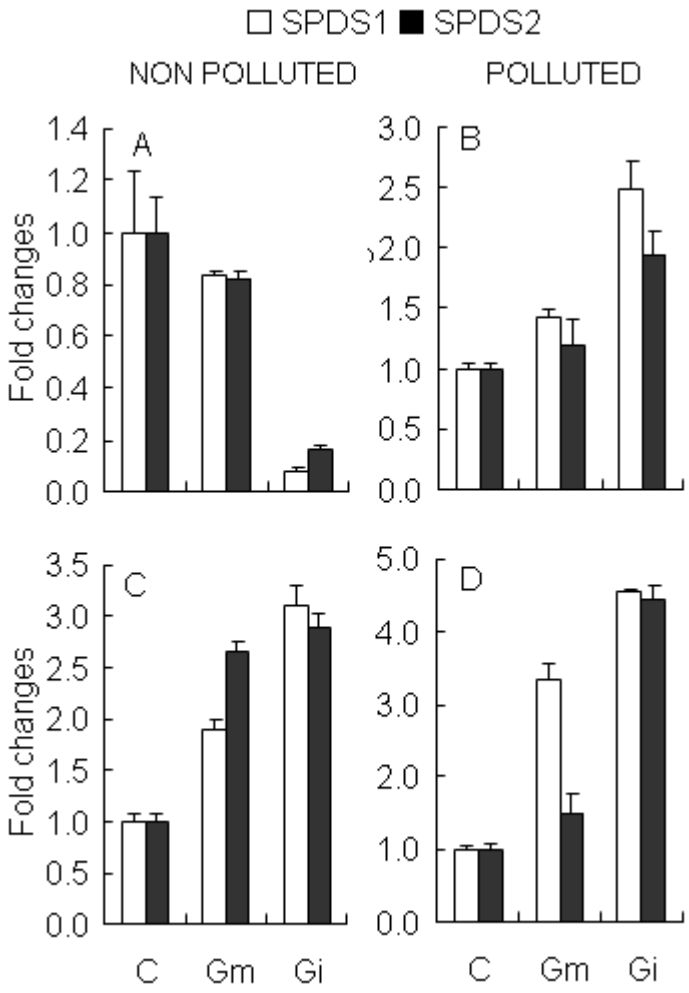

Fig. 12: Transcript levels for *Pa*SPDS1 and *Pa*SPDS2 in leaves of AL35 plants at the first (a, b) and second (c, d) sampling date. Plants were grown on non-polluted or polluted soil in the presence or absence (C) of either *G. mosseae* (Gm) or *G. intraradices* (Gi). The value 1 was arbitrarily given to control plants (C). Bars represent 95% confidence intervals calculated on two biological replicates.

In contrast, on contaminated soil both *Pa*SPDS1 and *Pa*SPDS2 gene were up-regulated by AM fungi, with a strong induction in Gi plants (Fig. 12 b).

At the second sampling date, both genes were up-regulated by the presence of AM fungi, either on polluted (Fig. 12 d) or unpolluted soil (Fig. 12 c), with the exception of *Pa*SPDS2 gene in Gm plants (Fig. 12 d), where only a low induction is observed.

5. Analysis of PA content

The most common PAs in plants are Spd, Spm and their diamine precursor, Put. All of them were detected in their free (Fig. 13 a and b) and soluble conjugated form (Fig. 13 c and d) both at first and second sampling date, in leaves of AL35 plants grown on polluted and unpolluted soil. At first sampling, free Put and Spd levels were significantly higher in plants grown on contaminated soil when compared with the plants grown on unpolluted soil. At first sampling, on unpolluted soil (Fig. 13 a), free PA levels, particularly Put and Spd contents, were strongly reduced in the presence of AM fungi, especially in Gm plants. While leaf contents of conjugated Put, Spd and/or Spm were significantly higher in mycorrhizal plants, if compared with uninoculated control (Fig. 13 c). On polluted soil (Fig. 13 b), free Put titers were similar in AL35 leaves either in the presence or in the absence of AM fungi. Spd and/or Spm levels were significantly higher in Gm plants (Fig. 13 b). On polluted soil, conjugated Spd and Spm titers were also dramatically enhanced, up to 5-folds, only in Gi plants (Fig. 13 d), relative to uninoculated controls.

At the end of the experiment (June 2007), free and conjugated PA levels in mycorrhizal plants vs uninoculated controls were not significantly different on both soils.

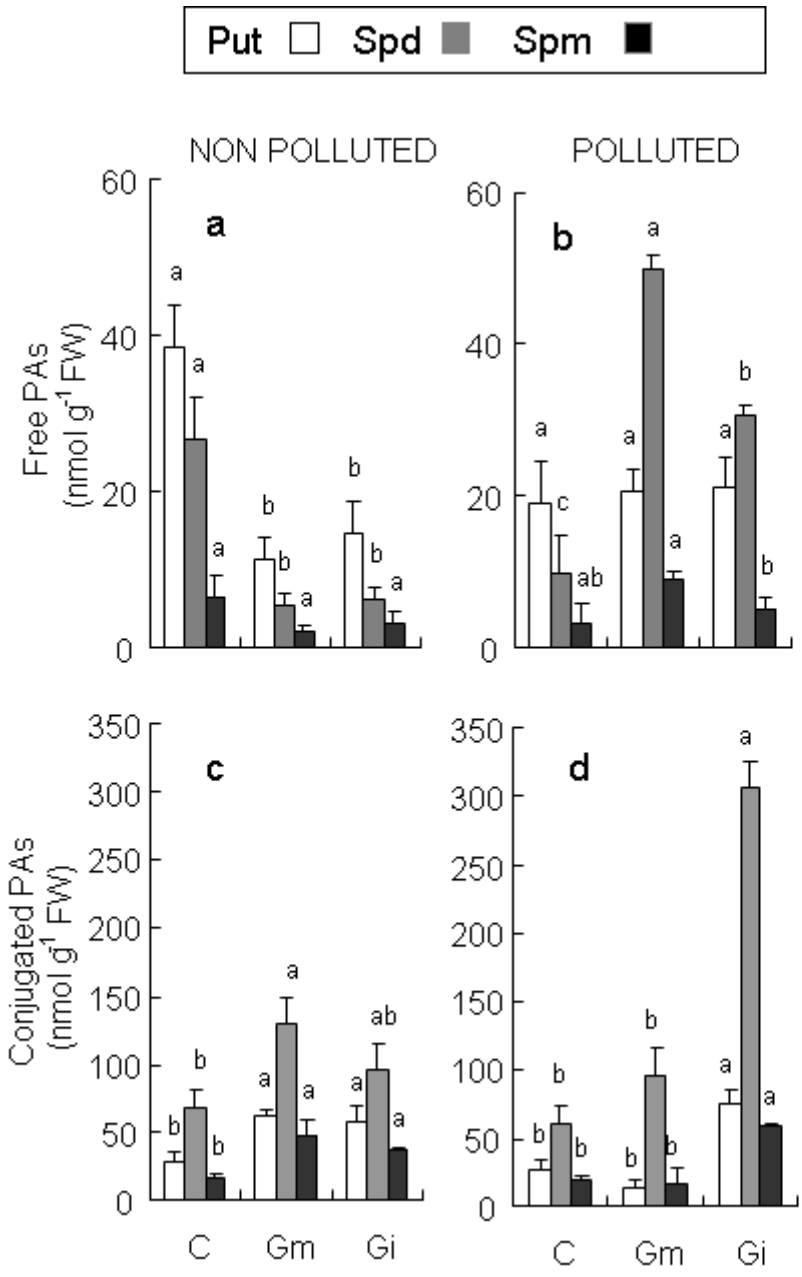

Fig. 13: Free (a, b) and soluble conjugated (c, d) putrescine (Put), spermidine (Spd) and spermine (Spm) levels in leaves of *P. alba* clone AL35 (first sampling date) grown on non-polluted (a, c) or polluted (b, d) soil in the presence or absence (C) of either *G. mosseae* (Gm) or *G. intraradices* (Gi). Different letters indicate significant differences ($P<0.05$) for each polyamine referred to uninoculated control.

Discussion

1. G. mosseae and G. intraradices fungi restore plant biomass despite a higher copper and zinc accumulation in AL35 plant organs

The natural ability of plants to remove contaminants can be integrated and improved by symbiosis with AM fungi (Smith, 1987). They colonize roots of almost all plant species. AM fungi may, depending upon the particular host plant and fungus, promote a better plant growth and nutrient acquisition, P in particular, and modify the architecture of root system, through their extensive extraradical mycelium (Smith, 1987; Lebeau, 2008). Mycorrhizal fungi can also play an important role in the protection of plants against root pathogens (Schelkle, 1996).

Populus genus is known to form both ectomycorrhizal and endomycorrhizal associations occasionally present in the same root system (Cripps, 1993). This flexibility in a double colonization capacity can result from several factors (successional stages, local soil conditions, geographical location - Rooney, 2009) and may contribute to its widespread geographic distribution in temperate regions of Boreal hemisphere (Khasa, 2002).

The potential of AM fungi to buffer HM stress has been demonstrated in wide range of studies (Hildebrandt et al., 1999; Janouskova, 2006; Chen, 2007), also including poplar (Lingua, 2008). Moreover, a considerable variability in plant and fungal interaction has been observed in contaminated soils (Liao, 2003; Wang, 2005). Therefore, it has been proposed that specific fungal tolerance mechanisms may contribute to increased HM tolerance of mycorrhizal plants.

In order to evaluate in AL35 clones the potential benefits of pre-inoculation with AM fungi on growth metal accumulation and HM tolerance, it is important to determine how the establishment of the symbiosis is affected by contaminants. In the present work, percentage of mycorrhization with either *G. mosseae* or *G.*

intraradices of AL35 poplar roots was comparable in plants grown on unpolluted and polluted soil, suggesting that the two AM fungi possess specific and unique mechanisms of HM resistance/tolerance, or that the capacity to rapidly adapt to HM stress, due to pre-AM fungi inoculation could overcome the reduced (or lack of) spontaneous mycorrhization frequently encountered in contaminated soils. HMs, in fact, have been reported to reduce, delay, or even eliminate AM fungi when they are present at high concentrations in the soil (Leyval, 1997; Citterio, 2005; Repetto, 2003; Lingua, 2008), thus hindering any possible beneficial effects of mycorrhization (Lingua et al. 2008). However, even in highly contaminated soils, AM fungi propagules never totally disappear (Vallino, 2003), indicating a genetic possibility to evolve HM tolerance under selection pressure of the environment. Recently, progress has been made towards understanding cellular, constitutive and adaptive mechanisms of AM fungi to contaminated soils, in order to control HMs and to avoid their toxicity (Hildebrand, 2007; Ferrol, 2009). Moreover the *G. intraradices* genome sequence project will contribute to the disclosure of new information on genes potentially involved in HM tolerance and homeostasis, not only in the of *G. intraradices*, but also in other fungus species.

In AL35 the percentage of mychorrizal colonization by *G. mosseae* and *G. intraradices* varies from 5 to 23% and it is in line with previous reports for *Populus* genus (Khasa, 2002; Takàcs, 2005; Todeschini, 2007; Lingua, 2008; Quoreshi, 2009). However in AL35 roots many vesicles, but not arbuscules, were observed, contrary to other clones of *P. alba* (Lingua, 2008) and *P. nigra* (Todeschini, 2007), that have been shown to have low levels of arbuscule formation, both on polluted or unpolluted soil, albeit the soil used for the trial was not an industrial contaminated soil, but an artificial contaminated soil. The lack of arbuscules in our case could be related to specific characteristic of clone-fungal species and soil interaction, or to the high level of P in the soil (Smith, 1987). Investment in symbiosis therefore means that plants can indirectly access

nutrients beyond the nutrient depletion zone of roots via extensive mycelial networks. When soil concentration of available P is high, it may be more efficient for plants to absorb P directly, than to take it up via fungus (Jakobsen, 2001), or the cellular exchange between plant and fungi must be localized in structures different from arbuscules. Consistent with this hypothesis, it has been reported that tomato mycorrhizal roots expressed five phosphate transporter genes (LePT1-LePT5) and one of them (LePT5) was also expressed in cells that did not contain arbuscules (Balestrini, 2007).

In AL35 plants grown on polluted soil, the inhibitory effect of the HMs on its growth ability is evident and that is a frequent symptom of phytotoxicity. It is noteworthy, however, that biomass production was restored to levels comparable with that of plants grown on unpolluted soil by inoculation with either of the two AM fungi. These data clearly indicate that mycorrhization exerted a strong protective effect capable of removing the negative consequences of HM stress at the cellular and tissue levels. Only leaf biomass in Gi plants, grown on polluted soil, was not restored by plant mycorrhization.

Some histological analyses on leaves of a *P. alba* clone (Villafranca), preinoculated with Gm or Gi fungi and grown on Zn supplemented soil, have revealed interesting differences at chloroplast level. Chloroplasts of Gi plants, which accumulated a very large concentration of Zn (ca 2500 ppm/mg kg^{-1}) had almost a total absence of starch, and the leaves were thinner and smaller. In contrast, in Gm plants, where Zn concentrations were comparable (ca 2000 ppm/mg kg^{-1}), leaf size and starch content were equivalent to the plant controls grown on uncontaminated soil in the presence of AM fungi (Todeschini and co-workers, manuscript submitted). These data concordant with previous studies describing differentiatl effects on plants of the white poplar and black poplar exposed (or not) to HMs, associated to a specific AM fungi (Lingua, 2008). Other examples of disparity between the effects of *G. mosseae* as compared with

G. intraradices are related to PA levels and to *Pa*MT gene expression in AL35 clones.

AM fungi improve nutrient uptake, especially P, thus enhancing plant growth (Janouskova, 2005; Wang, 2005). However, at the first sampling date, in AL35 organ tissues, no significant difference was observed in P concentration between inoculated and uninoculated plants. By contrast, on polluted soil, at the second sampling, restoration of growth to control levels in mycorrhizal plants was associated with increased P concentrations relative to non-mycorrhizal plants (at least in roots of both Gm and Gi plants). This observation suggests that growth recovery was at least in part due to a general effect on nutritional status. The beneficial effects of mycorrhizal colonization through improved P nutrition may become evident only in a highly contaminated soil and under long-term stress-inducing conditions.

Mycorrhizal fungi may induce the alleviation of HM stress either by immobilisation of pollutants in the mycorrhizosphere (Lebeau, 2008; Hildebrandt, 2007 and refs therein; Gonzalez-Guerrero, 2008) and by promoting their immobilization in the substrate (Janouskova, 2006), or by decreasing HM concentration in different plant organs (Kapoor, 2007).

Variable effects in plants following mycorrhizal inoculation have been observed relative to metal uptake and sequestration, depending on many factors. AM fungi are able to transport non-essential elements (Joner, 1997; Hutchinson, 2004) and to promote their sequestration in roots, or in plant leaves (Galli, 1994; Janouskova, 2006). However, in some plants, AM fungi can induce metal uptake without enhancing growth of the host plant (Hildebrandt, 2007).

A stress alleviating mechanism resulting from enhanced growth in the presence of microorganisms is the “dilution effect” (Audet, 2007). Thus, if mycorrhizal plants display a greater biomass, then tolerance may derive from the fact that total amount of metal extracted increases in the whole plant but not the organ concentrations.

Thus, dilution effects cannot be assumed in present study. In fact, stress alleviation due to AM fungi, in terms of growth recovery on polluted soil, occurs in spite of the fact that AL35 plants, in the presence of either *G. mosseae* or *G. intraradices*, accumulated more Cu and Zn in the plant organs (roots and leaves), than those of non-mycorrhizal ones.

Although it is difficult to compare pot greenhouse experiments with field trials, it is remarkable that the Zn concentrations accumulated over a similar time period (i.e., first growth season, i.e., 3-6 months) by AL35 leaves in the present study were much lower than in a previous field study on the same contaminated soil (Castiglione, 2009). This could be due to several factors, such as soil sterilization, lower transpiration rate, limited root development, and/or lower metal bioavailability (evaluated at ca 10-15 % at the second sampling date). However, HM concentrations were comparable, both on polluted and unpolluted soil, with those recently reported by Hassinen and co-workers (2009) for hybrid aspen (*P. tremula* x *P. tremuloides*) grown on a multi-metal contaminated site, except for Cu concentrations in roots, which were considerably higher in AL35 plants. However, in the case of hybrid aspen, the presence of AM fungi was not investigated.

In uninoculated AL35 plants grown on polluted soil, Cu was taken up and accumulated mainly in the roots (approx 5-fold stem/leaf levels); this organ-distribution is in accordance with other studies on woody plants, including *Salix* (Vandecasteele, 2005), and *Populus* (Todeschini, 2007). By contrast, Zn was accumulated mostly in leaves, again according with the expected pattern (Di Baccio, 2003; Lingua, 2008).

Inoculation with AM fungi seems to contribute to this redistribution of some HMs inside the plant and affect shoot/root partitioning of metals. Thus, although at the end of the experiment, Gm plants had higher leaf Cu concentrations than non-mycorrhizal ones, probably due to the overall increase in uptake of this metal (root Cu concentration was 6 times higher and the total amount in plants

30 times higher), TF reveals an altered shoot/root partitioning of HMs in mycorrhizal vs non-mycorrhizal plants for both metals. In particular, Gm plants, which grew much better than controls on polluted soil, had substantially higher root concentrations of both HMs. Thus, increased tolerance in Gm/Gi mycorrhizal plants did not depend upon an exclusion mechanism (i.e., reduced metal uptake into roots). Nevertheless, plants that preferentially accumulate HMs in roots generally display higher tolerance, since photosynthetic apparatus is protected by toxicity due to the presence of HMs (Pulford, 2002). Reduced HM toxicity has been associated with diminished root-to-shoot translocation due to the presence of AM fungi in a tropical grass (*Brachiaria decumbens*) (Soares, 2008). In an another greenhouse pot experiment, the effect of *G. mosseae* has been investigated in three leguminous species grown on multi-metal contaminated soil (Lin, 2007). These plants grew better, indicating that mycorrhization enhanced HM plant tolerance. However, in mycorrhizal plants, root/shoot ratios of Cu in all three species, and of Zn in one of them (alfa-alfa), were also greater, indicating reduced metal translocability.

Total amount of extracted HMs, in our study, was always greater in mycorrhizal plants. The white poplar AL35 clone, in fact, seems to be a promising plant for phytoremediation purposes, especially if preinoculated with *G. mosseae*. The present study confirms that AL35 is particularly suitable for phytostabilization of HMs as previously reported (Castiglione, 2009). Moreover, probably because hyphae display a higher affinity for HMs than plant cells (Joner, 2000), HMs become immobilized in mycorrhizal roots more than in non mycorrhizal ones, thereby increasing phytostabilization, rather than the phytoextraction. This can avoid some of the potential risks described with respect to phytoextraction of toxic metals, such as the reintroduction of HMs in the environment due to leaf fall.

2. Metallothionein genes expression in AL35 white poplar and metal tolerance

In the present research, AM fungi improved growth and HM accumulation in organs of mycorrhizal AL35 plants. This effect depends on different molecular and biochemical processes, resolving in an alleviation of HM stress. Sunflower plants inoculated with *G. intraradices* were less sensitive to Cd stress than non inoculated plants, despite the greater up take of the metal; this phenomenon was associated to a higher amount of photosynthetic pigments as well as to shoot P concentrations, and, to a lesser extent, to the increased guaiacol peroxidase activity (de Andrade, 2008). Galli and co-workers (1995) have shown that AM fungi protect maize roots against metal toxicity by enhancing metal chelating compounds, as cysteine and glutathione.

Several studies have indicated that MT gene expression responds to various developmental, or environmental signals (Kohler, 2004; Castiglione, 2007). Some of these studies have attempted to establish a role for these compounds in HM plant tolerance, including poplar. Kohler and co-workers (2004) showed that *Ptd*MTs from the hybrid poplar *P.* x *generosa* were able to restore Cd tolerance in a Cd-hypersensitive yeast mutant. In addition, *Ptd*MT1 and *Ptd*MT2 gene expression was up-regulated by Zn, but just slightly by 50 μM Cu (and totally inhibited by higher concentrations). The diverse expression patterns of MT genes in plants suggest that the multiple MT isoforms (in poplar as in other plants) may have diverse functions in HM sequestration/homeostasis in different plant organs (Cobbett, 2002).

In a previous study (Castiglione, 2007), a detailed expression analysis of the 'Villafranca' MT genes was carried out on micropropagated plantlets grown under *in vitro* conditions, revealing that the MT genes were differentially stimulated by high Zn concentrations, although the observed response was not linear with respect to metal dosage and exposure time. At present, few studies have investigated gene expression patterns in mycorrhizal plants exposed to

HMs. Tomato plants colonized with *G. intraradices* (Ouziad, 2005) grew better than non-mycorrhizal plants on HM polluted soil. An analysis of gene expression patterns revealed that LeMT2 was strongly up-regulated on contaminated soil, and that fungal colonization reduced the amount of Lemt2 transcripts. Other MT genes, as well as LeNramp2 (coding for HM transporter) and LePCS1 (coding for phytochelatin synthase) genes were not differentially expressed. Thus, despite higher tissue (root, and in some cases leaf) metal concentrations, up-regulation of MT gene expression may have contributed to stress alleviation in mycorrhizal plants, allowing them to grow distinctly better than in the absence of AM fungi colonization.

In the present work, *Pa*MT gene expression in AL35 plants was strongly affected by inoculation with AM fungi, at the first sampling date. The growth of plants on different soils (unpolluted, or polluted, with Cu and Zn) causes an opposite effect on *Pa*MT gene transcription. On HM polluted soil gene transcription was enhanced, while, on unpolluted soil, *Pa*MT gene expression was reduced, or unchanged.

Moreover, no direct relation between leaf metal concentrations and MT transcript levels has been observed, at first sampling, hence the former cannot explain the observed induction of the latter in mycorrhizal plants. However, although roots were not harvested at the first sampling date, data, from the second sampling, indicate that roots of Gm/Gi plants had accumulated higher concentrations of one, or both HMs than controls. This suggests that leaf mRNA levels are induced by a signal coming from roots (namely, the high metal concentration and/or the AM fungi). Since results from unpolluted soil showed that the fungus alone does not have this effect, the two stimuli (HMs + AM fungi) appeared to be necessary for this induction to take place.

Based on biomass data, AL35 mycorrhizal plants performed better under HM stress than non-mycorrhizal ones. This improved tolerance was probably associated, in general, to a higher MT gene expression, suggesting that these

polypeptides may afford protection against HM stress. Recently, transgenic *P. alba* 'Villafranca' plantlets, over-expressing a pea MT2 gene (*Ps*MTA1), were shown to exhibit improved tolerance to Cu, lower ROS accumulation, and reduced paraquat-induced photo-oxidative stress (Balestrazzi, 2009). Thus, increased protection against HM stress may be the direct consequence of the, as yet uncertain, metal-binding capacities of plant MTs, or an indirect effect deriving from reduction of oxidative stress through their purported free radical scavenging properties (Akashi, 2004; Wong, 2004).

At the second sampling date, non mycorrhizal and mycorrhizal AL 35 plants showed a similar trend (up-regulation) on polluted and unpolluted soils. Thus, by the middle of the second growth season, probably the plants/fungi symbiosis may have adapted to the high concentration of HMs in the polluted soil (metal sequestration, etc.) and the effect observed on *Pa*MT gene expression was mainly associated to the presence of the AM fungi.

3. Polyamine metabolism in mycorrhizal plants grown on polluted or unpolluted soil

It has been well documented that HM toxicity induces changes in PA metabolism in plants (Geuns, 1997; Lin, 1999; Groppa, 2008 a, b, c).

In plants, Kuthanová and co-workers (2008) found an abnormally high levels of Put in Cd treated tobacco cells, and hypothesized that PAs might be involved in the process of apoptosis. In tobacco plants, Spd and Spm induced hypersensitive cell death after pathogen attack, probably through the production of hydrogen peroxide during their catabolism (Yoda, 2003).

Proline content was found to be significantly increased after treatments at the Cd higher concentrations in roots, stems, and leaves (Wu, 2004) of barley plants (*Hordeum vulgare*). This amino acid was probably involved in detoxification of HMs by its direct function, or by the biosynthesis of chelating peptides (Wu, 2004). PA production was related, at least in part, to the inhibition of root

growth observed in Cd and Cu treated sunflower seedlings (Groppa, 2008b) and in Cd treated wheat plants (Groppa, 2008c). Probably, PA levels are involved in the signaling cascade triggered in response to HM stress. Lei and co-workers (2007) observed in *P. cathayana* exposed to high Mn concentrations, the accumulation of free amino acids, the activation of the anti-oxidant enzymes, superoxide dismutase and ascorbate peroxidase, and the enhanced synthesis of ABA and PAs. A regulation of PA metabolism has been reported for white poplar exposed to high Zn or Cu concentrations in *in vitro* (Franchin, 2007) growth, or in greenhouse (Lingua, 2008) conditions, and has been shown to correlate with the extent of HM tolerance.

Very little information is available about the changes of PA levels caused by AM fungi, and almost nothing as regards the combination of HM stress and AM fungi.

In this study, it was observed that PA contents in AL35 poplar leaves after three months of exposure to high HM concentration were increased in the presence of AM fungi, as revealed by the higher concentration of free and conjugated PAs in Gm and Gi plants, and by an up regulation of *Pa*ADC, *Pa*SPDS1 and *Pa*SPDS2 genes. These data support the capability of different white poplar clones to increase PA metabolism in response to abiotic stress induced by HMs (Franchin, 2007), but also provide a new insight into the combined effect of HMs and AM fungi.

In a previous field study on HM-contaminated soil, it was shown that leaves of AL35 clone contained a large amount of Put associated with the very high Zn and Cu concentration accumulated in all plant organs (leaves, roots, stems) (Castiglione, 2009). Enhanced Put titres seem to be a common physiological response to HM stress (Groppa, 2008 a). However, in the present greenhouse study, much lower amounts of these HMs were taken up by the AL35 plants, probably due to lower transpiration rate, limited root development in pots, lower HM bioavailability, and/or lack of aerial metal deposition. This condition could

account for the limited HM induced PA response. A modest increase in Put concentration (ca 40-50%) was likewise reported for *P. cathayana* plants exposed to high Mn concentrations (Lei, 2007). Although, in the present study, at first sampling date, AL35 plants don't accumulate Put, ADC transcripts were increased by the presence of HM and AM fungi. The lack of accumulation of the Put, diamine precursor of the higher PAs, may depend upon the fact that it was converted into Spd, in accordance with the importance of the ADC pathway (for Put biosynthesis) in response to different types of stress, such as salt (Liu, 2006) and HM (Prabhavathi, 2007).

Gene expression analysis indicated that *Pa*SPDS1 and *Pa*SPDS2 up-regulation, in inoculated plants growing on polluted soil was stronger in Gi plants, possibly indicating the greater need for these plants, compared with Gm ones, to contrast HM induced stress. Moreover, it is not surprising that different fungus species exert different effects. Transcriptional changes associated with colonization by *G. mosseae* and *G. intraradices* in mycorrhizal roots of the model legume barrel clover revealed that approximately 200 genes were significantly co-induced, while several hundred other genes were up-regulated specifically by one of the two symbiotic fungi (Hohnjec, 2005).

As a result of the transcriptional up-regulation of *Pa*SPDS genes, concentrations of free and conjugated Spd and, to a lesser extent, Spm, were significantly higher than in controls in mycorrhizal plants on polluted soil. Similarly, in Narrow-leaf Bird's-foot Trefoil (*Lotus glaber*), the PA balance was modulated by inoculation with *G. intraradices*, since in two month-old mycorrhizal plants grown under salt stress, the free (Spd+Spm)/Put ratio increased in roots and shoots relative to uninoculated plants (Sannazzaro, 2007). Also in alfalfa, free Spd and Spm concentrations were enhanced in leaves and roots under water stress conditions in the presence of *G. fasciculatus* (Goicoechea, 1998). Taken together, these data suggest that the accumulation of PAs, rather than their

diamine precursor Put, was typical for mycorrhizal plants, in response to an abiotic stress factor.

In AL35 plants, also, the conjugated PAs, mainly phenylamides (i.e., products of the covalent bonding between hydroxycinnamic acids and aliphatic di- and polyamines), increased in leaves of mycorrhizal plants grown on polluted soil, especially in those inoculated with *G. intraradices*. Mn treatment, in *P. cathayana*, strongly influenced the composition and concentration of free amino acids, with the greatest increases observed for His, Pro, Phe, Tyr and Ser (Lei, 2007). Phe is (via phenylalanine ammonia-lyase) an important precursor of many phenolics, and hence also of the phenylamides. There is previous evidence suggesting that mycorrhizal roots produce a long-distance signalling, resulting in the activation in leaves of secondary metabolite production (Guerrieri, 2005; Copetta, 2006).

The phenylamide conjugates operate as singlet oxygen quencher (Velikova, 2007) and have scavenging properties against free radicals (Son, 2002). Thus, it is proposed that phenylamides may modulate oxidative stress and ROS-based signalling in the response of plants to environmental cues, including HM stress (Balestrazzi, 2009).

When compared with Gm, Gi plants accumulated more conjugated PAs; they also exhibited a lower leaf biomass, comparable with that of non mycorrhizal plants grown on polluted soil, and, therefore, indicative of a reduced capacity to recover from HM-induced stress. This would suggest a different role for the free and conjugated forms of PAs, with the latter regarded as secondary metabolites (Edreva, 2007), and thus more closely related to defense and, in some cases, to growth inhibition associated to NO (Tun, 2006). NO is considered to be a stress-inducing agent (Leshem, 1997), but it can exercise a protective role, functioning as an antioxidant (ROS scavenger), limiting in this way cellular damages (Laspina, 2005). Likewise, NO seems to be a signalling compound in the molecular cascade leading to changes in gene expression (Delledonne, 2005;

Lamattina, 2003). Thus, NO could be a link between PA-mediated stress responses and other stress mediators (Groppa, 2008 b).

At first sampling date, AL35 plants grown on unpolluted soil, showed a different transcriptional pattern relative to plants grown on polluted soil. This pattern was associated to an opposite PA profile: in mycorrhizal plants, free PA concentrations decreased, while those of conjugates increased slightly. A decrease in free PA levels was also observed in English Plantain (*Plantago lanceolata*) mycorrhizal plants in the presence of limiting, or excess concentrations of P, suggesting that PA decrease could depend upon the plant's nutritional status (Paradi, 2003). The limited increase in conjugated PAs, observed in AL35 inoculated or not, grown on unpolluted soil, can be interpreted as a response to the systemic signal generated by the AM fungi per se, in the absence of abiotic stress. Peipp and co-workers (1997) reported that PA-derived phenylamides, such as coumaroyl- and feruloyl-putrescine, accumulated in barley upon *G. intraradices* colonization, and this was interpreted as a defence response. Moreover, the increase in the amount of conjugates in mycorrhizal plants of AL35 plants occurred at the expense of the respective free forms, suggesting that PA conjugation to phenols (probably due to increased availability of the latter) was stimulated, but not by a biosynthetic activity (as revealed by the down- rather than up-regulation of *Pa*ADC and *Pa*SPDS genes).

As observed for *Pa*MT genes, at the second sampling, differences between mycorrhizal and non-mycorrhizal plants with regard to PA biosynthetic genes, followed the same pattern on both polluted and unpolluted soil. A down-regulation for *Pa*ADC gene and an up-regulation for *Pa*SPDS genes are evident. This reflected the absence of significant differences in PA concentrations on both types of soil, and may again be indicative of the plant's long-term adaptation to HM stress; at this point, the effect observed on PA gene expression was associated only to the presence of the AM fungi.

In conclusion, present results point to an induction of the synthesis/accumulation of PAs, in particular Spd and Spm, in plants grown on contaminated soil and colonized by AM fungi, through the transcriptional up-regulation of at least two of their biosynthetic genes (*Pa*SPDS1 and *Pa*SPDS2 *)*. Moreover AL35 plants, colonized by *G. mosseae* or *G. intraradices,* also showed improved biomass production, suggesting that stress recovery may also be the result of enhanced PA metabolism.

There is ample evidence indicating a role for PAs in alleviating stress, including HM damages (Papadakis, 2005), and this has been extensively discussed previously for the poplar (Franchin, 2007; Lingua, 2008; Todeschini, 2007; Castiglione, 2009). In addition to their putative anti-oxidative role, PAs block one of the major vacuolar channels (the fast vacuolar cation channel), and their accumulation could facilitate metal ion compartmentation (Brüggemann, 1998) by affecting ion conductance at the tonoplast level. This idea is corroborated by studies showing that over-expression of PA biosynthetic genes enhances tolerance to multiple environmental stresses (Prabhavathi, 2007; He, 2008). Wen and co-workers (2008) reported that European pear (*Pyrus communis*) shoots, overexpressing an apple SPDS gene (MdSPDS1), showed enhanced tolerance not only to salinity and hyperosmosis, but also to Cu.

Conclusions

This study has showed how symbiosis of AM fungi *G. mosseae* or *G. intraradices* with a *P. alba* clone (AL35) is able to restore to control levels the growth of the plants on HM contaminated soil and this occurs despite the high tissue accumulation of both Cu and Zn. During the first vegetative season, the enhanced stress related gene expression in leaves and foliar accumulation of PAs, in mycorrhizal plants vs non mycorrhizal ones, are part of a systemically AM fungi-induced response, as the result of an improved protection of the plant from HM stress.

MTs and PAs appear to play a role in the "mycorrhiza-buffering" of HM stress, in AL35 clone. An enhanced transcription of stress-responsive genes, in fact, was observed in leaves of plants grown on polluted soil, but not in mycorrhizal plants grown on unpolluted soil. These data reaffirm the potential of *P.alba* to up-regulate PA metabolism and *Pa*MT gene expression in response to external factors, such as HMs, and suggest a possible use of AL35 clones in combination with AM fungi for phytostabilization purposes. An enhanced phytostabilization would reduce potential risks of phytoextraction avoiding a possible return of HMs into the soil and, as consequence, into the food chain. Thus, the establishment of plant-AM fungi association with improved stress tolerance and/or stabilization capacity, or phytoremoval of HMs from soils is a promising strategy for the advancement of plant-based environmental clean-up.

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