REVIEW / SYNTHÈSE

Beneficial role of plant growth promoting bacteria and arbuscular mycorrhizal fungi on plant responses to heavy metal stress

Elisa Gamalero, Guido Lingua, Graziella Berta, and Bernard R. Glick

Abstract: Heavy metal pollution is a major worldwide environmental concern that has recently motivated researchers to develop a variety of novel approaches towards its cleanup. As an alternative to traditional physical and chemical methods of environmental cleanup, scientists have developed phytoremediation approaches that include the use of plants to remove or render harmless a range of compounds. Both plant growth promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF) can be used to facilitate the process of phytoremediation and the growth of plants in metal-contaminated soils. This review focuses on the recent literature dealing with the effects of plant growth-promoting bacteria and AM fungi on the response of plants to heavy metal stress and points the way to strategies that may facilitate the practical realization of this technology.

Key words: phytoremediation, metal contamination, plant stress, ACC deaminase, reactive oxygen.

Résumé : La pollution par les métaux lourds est une préoccupation environnementale mondiale qui a motivé les chercheurs à développer une variété de nouvelles approches de décontamination. Comme alternative aux méthodes physiques et chimiques de décontamination environnementale, les scientifiques ont développé des approches de phytoremédiation qui incluent l'utilisation de plantes pour enlever une variété de composés ou pour les rendre moins dommageables. Les bactéries stimulant la croissance des végétaux et les champignons mycorhizes arbusculaires peuvent être utilisés pour favoriser le processus de phytoremédiation et la croissance des végétaux dans des sols contaminés. Cette revue se concentre sur les données récentes de la littérature portant sur les effets des bactéries stimulant la croissance des végétaux et des champignons mycorhizes arbusculaires sur la réponse des végétaux au stress causé par les métaux lourds, et montre la voie vers des stratégies qui peuvent faciliter la réalisation de cette technologie.

Mots-clés : phytoremédiation, contamination par les métaux, stress des végétaux, ACC désaminase, oxygène réactif.

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Introduction

Heavy metal pollution is one of the major problems negatively affecting both human and environmental health. Sources of heavy metal pollution may be atmospheric or terrestrial and include mining, metal working industries, combustion of fossil fuels, disposal of ash residues from coal combustion, vehicular traffic, and use of pesticides and fertilizers in agriculture (Clemens 2006). Seventeen of the 53 heavy metals are believed to be involved in the functioning of organisms and (or) ecosystems. Thus, (*i*) some heavy metals are important as micronutrients (Fe, Mo, and Mn); (*ii*) some toxic heavy metals have roles as trace elements (Zn, Ni, Cu, V, Co, W, and Cr); and (*iii*) there are some heavy metals without any known nutritional functions but are nonetheless toxic for plants and microorganisms (Hg, Ag, Cd, Pb, and U) (Schützendübel and Polle 2002). In addition to these, there are also a number of metalloids that are toxic for plants, including arsenic. Although some of these metals are essential for life, when present in excess they show toxicity, mainly related to oxidative and (or) genotoxic mechanisms (Briat and Lebrun 1999). To avoid the toxicity associated with these metals, several technologies and methods have been developed to remove them from polluted

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soils. Traditionally, these methods have included soil removal or extraction through chemical or physical means. Unfortunately, these techniques are costly from both an economic and an environmental point of view, and could potentially have a deleterious impact on soil physical, chemical, and biological properties.

As an alternative to these traditional approaches, the use of plants to remediate heavy metal polluted soils is a clean and cost-effective technology that is likely to be readily accepted by a concerned public. The use of the plants for environmental cleanup is called "phytoremediation" (Salt et al. 1995*a*); however, this approach also has its drawbacks, as few plant species can naturally tolerate and accumulate heavy metals. Unfortunately, many of the plants that are most effective at removing metals from the soil, i.e., hyperaccumulators such as *Thlaspi caerulescens* (Alpine pennycress) and *Alyssum bertoloni*, are small and slow growing, thus reducing the potential for metal phytoextraction and restricting their use in this technology (Khan et al. 2000). To be considered effective for the remediation of heavy metal polluted soils, plants must be tolerant to one or more heavy metals, highly competitive, fast growing, and produce a high aboveground biomass. Because of their high biomass and extensive root system, trees are attractive for phytoremediation. Metal accumulation by trees is generally low, but the development of genomic tools, and recent results obtained on the interactions between trees, especially poplar, and beneficial soil microorganisms, may open new perspectives for their use in phytoremediation (Lingua et al. 2008).

Although some organic compounds can be directly degraded and completely mineralized by plant enzymes through phytodegradation (Alkorta and Garbisu 2001; Wild et al. 2005), inorganic pollutants cannot be degraded. Inorganic pollutants must therefore be stabilized in the soil to make them less bioavailable (phytostabilization); extracted, transported, and accumulated in plant tissues (phytoextraction); or transformed into volatile forms (phytovolatilization) (Pilon-Smits 2005). Phytoremediation efficiency for metals is limited by the bioavailability of the metal in soil, plant root development, and the level of tolerance of the plant to each particular metal (Pilon-Smits 2005). Nevertheless, metal phytoextraction can be influenced by soil microorganisms living in intimate association with plant roots (Shilev et al. 2001). Following root exudation, the proliferation of specific groups of microorganisms, able to aggressively colonize the root surface and affect plant growth, occurs (Kloepper et al. 1989). These rhizospheric microorganisms can act on pollutants, mainly organic ones, using their own degradative capabilities (phytostimulation or rhizodegradation) (Kuiper et al. 2004), but also positively affect plants by improving growth and health (Glick 1995), enhancing root development (Gamalero et al. 2002, 2004; Berta et al. 2005), or increasing plant tolerance to various environmental stresses (Glick 2004; Mayak et al. 2004*a*, 2004*b*; Reed and Glick 2005). This review provides a synthetic overview regarding the plant responses to heavy metal stress. Subsequently, the involvement of the microflora in so-called "assisted phytoremediation" or "rhizosphere remediation" is described. In particular, the role of bacteria synthesizing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase and that of arbuscular mycorrhizal fungi (AMF) are detailed. Finally, the possible applications of bacterial-AMF mixed inocula in rhizosphere remediation, as well as future perspectives, are discussed. Given the very large body of literature in this area, for the most part our attention is focused on the more recent literature.

Heavy metal stress and plant responses

A detailed description of all the factors involved in the responses of plants to heavy metal stress is well beyond the scope of this review. Nevertheless, the most relevant aspects are briefly summarized here.

In the first instance, some heavy metals (or similarly behaving metalloids) are essential nutrients (including Ca, Cu, Mg, Mn, and Zn) for human metabolism and some are not (including As, Cd, Co, Cr, Hg, Pb, Mo, and Ni). The nonessential metals are usually toxic at lower concentrations than the essential ones (Clemens 2006). In addition, micronutrients may be taken up in different ways. For example, Cu uptake and translocation by plants is strictly regulated, resulting in very low leaf concentrations, in the range of 2–20 ppm (Wallnofer and Engelhardt 1984), whereas Zn is primarily accumulated in leaves, with concentrations in the order of hundreds of ppm (Rosselli et al. 2003). Moreover, the chemical form of the metal (as free ion, hydroxide, or salt, etc.), oxidation state (e.g., Cr^{III}/Cr^{VI}), and the soil features (pH, redox potential of sorbing soil surfaces, organic matter, and clay content) can all dramatically affect the metal bioavailability (Burken 2003).

Secondly, the ability to accumulate and tolerate high concentrations of heavy metals shows large variations among various plants, and sometimes even within a single species (Castiglione et al. 2009). Plant diversity and evolution driven adaptation have resulted in a range of different strategies for tolerating high concentrations of heavy metals in the growth substrate. Some plants do not take up metals in excess (excluders), whereas some have developed mechanisms to take up, translocate, store, and sometimes even detoxify metals (accumulators). Of course, any interaction with soil microorganisms can affect plant heavy metal uptake and tolerance, as well as the growth, reproduction, and survival of the microbes (Pilon-Smits 2005).

Some of the macroscopic consequences of exposure to toxic levels of heavy metals include reduced plant growth (of both roots and aboveground parts), leaf chlorosis and necrosis, turgor loss, a decrease in the rate of seed germination, and plant death (Bingham et al. 1986; Foy et al. 1978). These effects are related to ultrastructural, biochemical, and molecular changes in plant tissues and cells brought about by the presence of the metal(s). At the molecular level, heavy metals often induce modifications in the protein profile. For example, a study by Bona et al (2007) demonstrated that Cu stress caused the suppression of the expression of 2 proteins, the downregulation of 7 other proteins, and the upregulation of 5 different proteins in *Cannabis sativa* L. (common hemp). These differences in protein expression were indicative of the plant's adaptation to chronic stress, and were directed to a re-establishment of the unstressed plant's cellular and redox homeostasis (Bona et al. 2007).

Independent of the different extents of heavy metal mobilization and accumulation, the exposure of plants to stress-

ful conditions raises the ethylene level, leading to inhibition of root elongation and a stress senescence response (Deikman 1997). In higher plants, ethylene is produced from L-methionine via the intermediates, *S*-adenosyl-L-methionine (SAM) and ACC (Yang and Hoffman 1984) through the enzymes SAM synthetase (Giovanelli et al. 1980) and ACC synthase, respectively. Finally, the enzyme ACC oxidase metabolizes ACC to ethylene, carbon dioxide, and cyanide (John 1991). Ethylene is a gaseous hormone, involved in various events including seed germination, fruit ripening, abscission, and, more interestingly for the purposes of this review, senescence and response to stress. The first suggestion of a possible role in heavy metal stress was provided by Sandmann and Böger (1980), who showed Cu-induced ethylene synthesis, and by Maksymiec (2007) who recently reviewed the topic. Ethylene can increase senescence in plants exposed to excess Cu for long periods (Maksymiec et al. 1995; Maksymiec and Baszynski 1996) inhibit cell growth and increase cell wall rigidity by means of lignification (Enyedi et al. 1992). It has been shown that C can stimulate ethylene production by increasing of ACC synthase activity and over-expressing its genes (Pell et al. 1997), and it has been suggested that heavy metals such as Cu and Zn can induce an increase of ethylene concentrations linked to increased lypoxygenase activity (Gora and Clijsters 1989), which can mediate reactive oxygen species (ROS) formation.

A biochemical pathway related to ethylene biosynthesis catalyzes the production of decarboxylated *S*-adenosyl-L-methionine SAM, which is then used to synthesize polyamines. Polyamines, such as spermidine, spermine, and putrescine and their precursors, are essential for growth and development; they stabilize nucleic acids and favour transcription and translation (Bagni et al. 1993). Polyamines can be conjugated to hydroxycinnamic acid derivatives to produce hydroxycinnamoylamides (also known as soluble conjugated polyamines) or to high-molecular-mass compounds such as cell wall components (insoluble conjugated polyamines), which can be regarded as defence- or stress-related compounds (Flores and Martin-Tanguy 1991). Therefore, an upregulation of polyamine metabolism is expected to occur in response to environmental stress conditions, including the presence of heavy metals (Pirintsos et al. 2004; Scoccianti et al. 2006; Pang et al. 2007; Groppa and Benavides 2008). Upregulation of polyamine metabolism has been reported for poplars exposed to high Zn or Cu concentrations under in vitro (Franchin et al. 2007) or greenhouse (Lingua et al. 2008) conditions, and has been shown to correlate with the extent of metal tolerance. Furthermore, in a study comparing several poplar clones under field conditions, the best performing clone (i.e., the one with the highest accumulation of Cu and Zn, and also the one associated with the best plant survival) was related to the highest concentration of putrescine in the leaves (Castiglione et al. 2009).

Inside the cell, heavy metal toxicity results in the accumulation of ROS (Mithöfer et al. 2004; Rodrìguez-Serrano et al. 2006), alteration of the plant nutrient levels (Sandalio et al. 2001) and water status (Perfus-Barbeoch et al. 2002), reduction of plasma membrane H^+-ATPase activity, and a decrease in photosynthesis, including damage to both the light-harvesting complexes and photosystem II (Krupa 1988; Hsu and Kao 2004; Bačkor et al. 2007). Heavy metals can enter into the nucleus, bind to nucleic acids, and modify both transcription and DNA replication; they can also affect microtubule assembly–disassembly, thereby arresting cell division (Fusconi et al. 2006).

The production of ROS usually occurs through the Fenton or Haber–Weiss reactions; however, in the case of Cd, other mechanisms are most likely involved (Prasad 1995). Beyond their harmful effects on cells, ROS have been proposed to act as signals in stress response (Mittler et al. 2004), possibly through a mechanism involving some Ca-binding proteins (Ouelhadj et al. 2006).

The plants that are able to tolerate high concentrations of heavy metals have evolved a variety of adaptive mechanisms to cope with this stress (Hall 2002). These adaptive mechanisms include metal phytochelation and sequestration, induction of mechanisms contrasting the effects of ROS such as the biosynthesis of antioxidant molecules and stress proteins, and the upregulation of peroxidase synthesis (Sanità di Toppi and Gabbrielli 1999), and the biosynthesis of salicylic acid (Metwally et al. 2003; Choudhury and Panda 2004).

The cell wall and the vacuole are the main sites of heavy metal sequestration. In these compartments, chelated by organic compounds or bound to negatively charged cell wall components, heavy metals are the least toxic (Salt et al. 1995*b*; Bricker et al. 2001). Plant-synthesized metal chelating agents include organic acids (such as malic or oxalic acid) and polypeptides (such as phytochelatins and metallothioneins).

Metallothioneins are low molecular mass cysteine-rich proteins (Roosens et al. 2004) that are able to bind heavy metals and are classified according to the arrangement of their cysteine residues. Plant and fungal metallothioneins are included in class II and further subdivided into 4 types based on their amino acid sequence (Cobbett and Goldsbrough 2002). It has been reported that the different members of the metallothionein gene family show organ-specific expression profiles (Giritch et al. 1998; Chang et al. 2004). In addition, different plant species and (or) different experimental systems can respond differently in terms of metallothionein expression (Kohler et al. 2004; Castiglione et al. 2007).

When ROS are generated (an event also depending on the metal element, e.g., 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 — Stohs and Bagchi 1995; Cho and Seo 2005; Skçrzynska-Polit et al. 2006), the activation of the antioxidant machinery can help plants to overcome heavy metal stress. It involves several enzymes, such as ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase, all working in the ascorbate–glutathione cycle. Glutathione, beyond being the substrate for phytochelatin synthesis, also plays a relevant role as an antioxidant (Grill et al. 1985; Cobbett 2000; Hall 2002). This mechanism, which is active during leaf senescence (del Rio et al. 1998), is also triggered by heavy metals (Seth et al. 2008).

Salicylic acid has been reported to be involved in the response to abiotic stresses, however, data concerning heavy metals are still controversial, as in some cases it has been

shown to potentiate oxidative damages induced by metals, as in the case of *Oryza sativa* L. (rice) and Cd (Panda and Patra 2007), whereas it has been proposed as an inducer of tolerance in other cases (Horvath et al.2007; Maksymiec 2007).

Microbial-assisted phytoremediation of metals

Heavy metals adversely affect bacterial viability (Pennanen et al. 1996), activity (Díaz-Raviña and Bååth 1996*b*), and density (Brookes and McGrath 1984; Fliessbach et al. 1994; Koomen et al. 1990). However, as a consequence of (typically plasmid-encoded) heavy metal resistance, some bacterial populations can adapt to the presence of heavy metals in bulk soil and in the rhizosphere (Díaz-Raviña and Bååth 1996*a*; Malik and Jaiswal 2000; Kozdroj and van Elsas 2000) leading to shifts in microbial community structure (Frostegård et al. 1993, 1996; Gray and Smith 2005; Díaz-Raviña and Bååth 1996*a*). Rhizobacteria have been reported to affect heavy metal availability and accumulation in plants. Recently, Abou-Shanab et al. (2008) demonstrated that *Zea mays* L. (corn) plants inoculated with a mixture of 4 bacterial strains increased Zn, Cr, Pb, and Cu accumulation by 3.9, 2.7, 1.9, and 16 times, respectively, compared with uninoculated plants. On the other hand, goat willow (*Salix caprea* L.) treated with rhizobacteria has shown a unique behaviour as observed by Kuffner et al. (2008). Whereas inoculation with *Streptomyces* AR17 increased the willow plant's uptake of both Zn and Cd, *Pseudomonas* PR04 and *Streptomyces* AR36 reduced Zn uptake. Kuffner et al. (2008) suggested that different gene expression is induced in the plant as a consequence of the binding of the various bacterial strains, thereby conferring differences in plant metal uptake. However, these presumed changes remain to be identified (Kuffner et al. 2008). Soil bacteria may affect gene expression in plants by providing additional nutrients such as fixed N or P, Fe, or other nutrients from the soil, as well as by altering plant hormone levels including those of auxin, cytokinin, and ethylene. In fact, a particular bacterium may affect plants using any combination of these traits.

Despite the fact that heavy metals usually show adverse impacts on soil microflora (Bååth 1989; Giller et al. 1998; Van Beelen and Doelman 1997; Regvar et al. 2001; Rajapaksha et al. 2004), beneficial microorganisms living on plant roots are able to relieve some of the toxicity of metals to plants. This may occur by the microorganisms acting on their bioavailability (via volatilization for bacteria or the sequestering–accumulation of the metal by AMF), through the release of chelators, acidification, or redox changes (Smith and Read 1997; Abou-Shanab et al. 2003), by increasing the tolerance of the plant to heavy metals, or by behaving as plant growth promoting bacteria (PGPB). PGPB can either directly or indirectly support plant growth (Glick 1995). Direct stimulation of plant growth by PGPB is mediated by facilitating the uptake of nutrients from soil (e.g., solubilization of Fe or phosphate), fixing atmospheric N, producing phytohormones such as auxins and cytokinins that can affect various stages of plant development, and synthesizing the enzyme ACC deaminase, which can lower plant ethylene levels (Brown 1974; Kloepper et al. 1989; Lambert and Joos 1989; Glick 1995; Patten and Glick 1996; Glick et al. 1998). In particular, the growth of plants facing biotic or abiotic stress, including metals, organic contaminans, phytopathogens, drought, flooding, or a high level of salt is greatly facilitated by treating the plants with PGPB that express ACC deaminase (Glick 1995, 2004; Glick et al. 1998, 2007*a*, *b*). The indirect promotion of plant growth occurs when these bacteria suppress the development of soilborne diseases because of phytopathogenic organisms (Weller 1988).

The increase of plant tolerance to heavy metal stress by ACC deaminase expressing bacteria as well as the mechanisms of plant protection by AMF are discussed in the next sections.

Action of bacteria synthesizing ACC deaminase on plants subjected to heavy metal stress

The enzyme ACC deaminase, which degrades the plant ethylene precursor ACC to ammonia and α-ketobutyrate, was first isolated from *Pseudomonas* sp. strain ACP (Honma and Shimomura 1978). Further studies demonstrated the presence of ACC deaminase activity in several soil microorganisms such as the fungus *Penicillium citrinum* (Honma 1993), the yeast *Hansenula saturnus* (Minami et al. 1998), and a number of bacterial strains (Jacobson et al. 1994; Glick et al. 1995; Blaha et al. 2006; Campbell and Thomson 1996; Burd et al. 1998; Belimov et al. 2001; Ghosh et al. 2003; Ma et al. 2003). Microorganisms expressing ACC deaminase behave as a sink for ACC, lower ethylene levels in plants, and, as a consequence, promote plant growth, especially during periods of stress (Glick et al. 1998).

In the model described by Glick et al. (1998), PGPB colonize the seed or root of a developing plant and, in response to tryptophan and other small molecules in the seed or root exudates (Whipp 1990; Bayliss et al. 1997; Penrose and Glick 2001), they synthesize and secrete indole acetic acid (IAA) (Patten and Glick 1996, 2002). This IAA, and the endogenous plant IAA, can either stimulate plant growth or induce the synthesis of ACC synthase, which converts SAM to ACC. A portion of the ACC produced by this latter reaction is exuded from seeds or plant roots (Bayliss et al. 1997; Penrose and Glick 2001), taken up by the bacteria, and converted by ACC deaminase to ammonia and α-ketobutyrate. As a result of this bacterial activity, the amount of ethylene produced by the plant is reduced.

Plants growing on metal-contaminated soils have to cope with the toxic effects of high heavy metal concentrations and with a significant reduction of the Fe content, both of which activate the synthesis of stress ethylene. In metal-contaminated soils, plants are typically unable to accumulate sufficient Fe, despite the fact that plants produce phytosiderophores or may affect Fe availability by lowering the pH of the soil. This is because plant siderophores generally have a very much lower (i.e., by 10–30 orders of magnitude) affinity for Fe than bacterial siderophores do. Consequently, plants growing on metal-contaminated soils show inhibition of both chloroplast development, chlorophyll biosynthesis, and chlorosis (Imsande 1998). Nevertheless, it has been observed that bacterial ferrisiderophore complexes (bacterial siderophores typically bind Fe with association constants ranging from 1×10^{30} to 1×10^{50})

could be taken up and used by the plants (Jurkevitch et al. 1988; Marschner and Romheld 1994; Crowley et al. 1992; Bar-Ness et al. 1992). Therefore, inoculation of the plants with bacteria that are able to produce siderophores could help to prevent plants from becoming chlorotic when they are grown in heavy metal polluted soils.

To improve the growth of *Brassica juncea* (L.) Czern. (India mustard), as well as *Brassica rapa* var. *campestris* (L.) (common mustard), in Ni-polluted soil, several indigenous bacterial strains have been isolated (Burd et al. 1998). Among these bacterial strains, *Kluyvera ascorbata* SUD165, was characterized as Ni-resistant siderophore-producing, able to grow at cold temperatures (i.e., 5–10 °C), and capable of synthesizing ACC deaminase (Burd et al. 1998). In addition, strain SUD165 promoted tomato and canola plant growth in the presence of high levels of Ni under a variety of experimental conditions (Burd et al. 1998, 2000; Ma et al. 2001). Although strain SUD165 did not affect the amount of Ni accumulated per gram of plant tissue, it decreased the toxicity of the metal through reduction of the ethylene level by 3- to 4-fold in plants grown on Ni-contaminated soil.

Kluyvera ascorbata SUD165/26, a spontaneous siderophore-overproducing mutant, was selected to improve the performance of the wild-type strain (Burd et al. 2000). Both the SUD165 and the SUD165/26 strains promoted the growth of tomato, canola, and India mustard plants in the presence of inhibitory levels of Ni, Pb, or Zn. However, the SUD165/26 mutant decreased the inhibitory effect of the metals on plant growth to a greater extent than the wild type. In particular, the mutant caused a significant elevation of the chlorophyll content in plant leaves, a trait that is particularly sensitive to the Fe concentration in the plant, in the presence of Ni, Pb, or Zn. This effect was attributed to the increased level of siderophore produced by the mutant strain being able to provide Fe to the plant in the presence of very high levels of other metals.

Several bacterial strains belonging to *Pseudomonas*, *Alcaligenes*, *Variovorax*, *Bacillus*, and *Rhodococcus* genera and expressing the enzyme ACC deaminase have been isolated from the rhizoplane of pea and India mustard cultivated in heavy metal polluted soil or sewage sludge (Belimov et al. 2001). These strains were Cd tolerant and stimulated the root elongation of canola and India mustard in the presence of 300 μmol/L $CdCl_2$. Moreover, a positive correlation was observed between the in vitro ACC deaminase activity and the bacterial effect on plant root elongation (Belimov et al. 2005). These authors attributed the success of their selected inoculants to the presence of ACC deaminase in all of their bacterial strains.

Improvement of phytoremediation by PGPB can occur because of the stimulation of plant growth by the bacterium. When this occurs, the plant may accumulate higher amounts of heavy metals inside its tissues and (or) there may be enhanced immobilization of metals in the soil owing to increased exudation of different organic compounds by plant roots. Recently, Dell'Amico et al. (2008) isolated a strain of *Pseudomonas tolaasii* (ACC23) able to increase the biomass of canola plants under Cd stress (+83% for root and +94% for shoot) and the Cd uptake per plant (+107%) compared with uninoculated plants. Since the specific Cd uptake into shoots and roots did not change after bacterial inoculation, strain ACC23 was not able to affect metal availability and mobility in canola. On the other hand, because this strain increased the plant biomass, the total amount of Cd accumulation was increased (Dell'Amico et al. 2008). The physiological characterization of this bacterium indicated its ability to produce IAA, siderophores, and ACC deaminase. Although this work did not show the clear involvement of any of these plant-beneficial traits in plant growth promotion under Cd stress, it is likely that the success of this bacterial inoculant depends on one or more of these traits. Besides the higher phytoremediation efficiency mediated by rhizobacteria, plant growth promotion on heavy metal polluted soils are believed to be largely based on 2 main mechanisms: (*i*) the lowering of stress ethylene in the plant, leading the plant to develop longer roots (Burd et al. 1998; Glick et al. 1998) and more efficient soil exploration (Berta et al. 1993; 2002); and (*ii*) the synthesis of siderophores, allowing the plant to acquire sufficient Fe for optimal plant growth (Burd et al. 2000). In addition, while its role in promoting plant growth in the presence of heavy metals has not been demonstrated directly, bacterial IAA has been shown to promote root elongation, and it is likely that it plays a key growth-promoting role in metal-contaminated soils (Patten and Glick 2002). In fact, based on microarray data, a model has been suggested in which IAA and ACC deaminase work synergistically to promote plant growth (Glick et al. 2007*a*).

Action of arbuscular mycorrhizal fungi on plants subjected to heavy metal stress

Under natural conditions, 80%–90% of plants are colonized by AMF leading to mutualistic associations that have been found in most vegetative systems and climates, including some aquatic ecosystems (Read 1991; Nielsen et al. 2004; Rosendahl 2008). The extensive extraradical hyphal network produced by these fungi allows the plants to access a greater volume of the soil, leading to the enhancement of plant nutrient absorption and translocation (Giovannetti et al. 2002). Moreover, depending upon the particular host plant and fungus, AMF may modify the architecture and topology of the root system, generally resulting in longer or more branched roots, and therefore resulting in more efficient nutrient absorption (Berta et al. 2002). Besides promoting plant growth, AMF can enhance plant tolerance to environmental stresses, including heavy metals (Leyval et al. 2002). 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. In fact, although a number of studies have been carried out on the relationship between host plants and AMF in heavy metal polluted soils, the impact of mycorrhizal fungi in phytoremediation is still controversial. Thus, metal uptake by plants treated with AMF can vary dramatically as a function of the particular strain of AMF utilized, the type of plant, and the metal in question.

Heavy metals have been reported to reduce, delay (Leyval et al. 1997; Citterio et al. 2005; Repetto et al. 2003; Lingua et al. 2008), or even eliminate AM colonization when they are found at high concentrations in the soil, thus hindering any possible beneficial effects of the mycorrhiza–plant associations (Lingua et al. 2008). However, even in highly contaminated soils, AM fungal propagules never disappear

completely (Vallino et al. 2006). Whenever colonization occurs, even to a small extent, it induces beneficial effects on the host plants (Trotta et al. 2006). Several AM fungal isolates have been found in metal-polluted soils, indicating a potential adaptation of these autochtonous AM fungal populations (Gildon and Tinker 1981, 1983; Weissenhorn et al. 1993; del Val et al. 1999; Hildebrandt et al. 1999; Vallino et al. 2006). However, to maintain heavy metal tolerance of these indigenous isolates from heavy metal polluted soils, it is necessary to continue to cultivate them in the presence of heavy metals (Sudova et al. 2007). Heavy metal resistance in AM fungi, providing protection against heavy metals to both partners of the symbiosis, is modulated depending on the metal and the stage of development. A cDNA encoding a metallothionein-like polypeptide (*GmarMT1*) able to confer increased tolerance against Cd and Cu was identified in germinated spores of the AM fungus *Gigaspora margarita* BEG34. In the absence of metal, the expression of *GmarMt1* occurred both in the presymbiotic and symbiotic stages. On the other hand, Cu exposure upregulated gene expression exclusively in the symbiotic mycelium (Lanfranco et al. 2002). Mycorrhizal colonization efficiency of roots in heavy metal polluted sites depends on the particular metal element. Thus, for example, Cd did not affect the extent of colonization in 3 different pea genotypes (Rivera-Becerril et al. 2002), Cu did not affect colonization in poplar (Todeschini et al. 2007), and As did not affect colonization in *Pteris vittata* (brake fern) colonized by 2 different AM fungi, i.e., *Glomus mosseae* and *Gigaspora margarita* (Trotta et al. 2006).

Mycorrhizal symbiosis can affect plant growth in heavy metal polluted sites by influencing the fate of the metal in the plant and also by increasing the plant's tolerance to this type of stress. Reductions (Heggo et al. 1990; Weissenhorn et al. 1995; Zhu et al. 2001; Lin et al. 2007), increases (Weissenhorn and Leyval 1995; Joner and Leyval 1997, 2001; Tonin et al. 2001; Jamal et al. 2002; Lingua et al. 2008), or no changes (Dueck et al. 1986; Galli et al. 1995) of heavy metal concentrations in plants following mycorrhizal inoculation have all been observed depending on the fungal–plant association (Liao et al. 2003; Wang et al. 2005). In particular, if the AM fungus induces increases of the heavy metal accumulation in plants without enhancing their biomass or tolerance, deleterious effects on plant growth may occur. The effect of a metal on a particular plant may vary depending on the metal, the plant organ, and the species or even the strain of mycorrhizal fungi employed.

Mixed mycorrhizal inoculants seem to be more effective than single ones. For example, Wang et al. (2007) compared the growth and the heavy metal accumulation of *Z. mays* inoculated with *Glomus caledonium* 90036 or with a mix of AM fungi (*Gigaspora margarita* ZJ37, *Gigaspora decipens* ZJ38, *Scutellospora gilmori* ZJ39, *Acaulospora* spp., and *Glomus* spp.). Whereas maize inoculated only with *G. caledonium* showed a higher extent of colonization than plants treated with the mixture, the shoot concentrations of Cu, Zn, Pb, and Cd were lower with *Glomus caledonium* than with the mixture, suggesting that the mixed inoculant was more effective than the single one in promoting heavy metal phytoextraction efficiency.

Although it is very difficult to discriminate heavy metal distribution between fungal and plant cell structures, several mechanisms for metal accumulation have been suggested (Galli et al. 1994; Leyval et al. 1997; Schützendübel and Polle 2002). Heavy metals may (*i*) attach to the fungal cell wall and subsequently be accumulated in the vacuoles, (*ii*) be sequestered by siderophores, deposited into the root apoplasm or into the soil, and possibly taken up by plant ferrisiderophore receptors, (*iii*) complex to metallothioneins or phytochelatins synthesized by the fungus or the plant or, (*iv*) be extruded from the cytosol by specific heavy metal transporters located on plant membranes (Ouziad et al. 2005).

Some years ago a new important factor that impacts these processes was discovered, i.e., glomalin, a glycoprotein produced by AM fungi (Wright and Upadhyaya 1998). More recently, Gonzalez-Chavez et al. (2004) showed that glomalin, extracted from polluted soil or from hyphae, strongly and irreversibly sequesters metals such as Cu, Cd, and Zn. Therefore, AM fungi may stabilize metals in the soil, reduce their availability, and decrease the risk of toxicity to other soil microorganisms and plants growing in the immediate vicinity.

Alteration of the heavy metal content in mycorrhizal plants and, consequently, an improvement in plant tolerance, may be related to extensive changes in gene expression as well as protein synthesis induced by the symbiosis itself. As an example, the Zn transporter *MtZIP2* from *Medicago truncatula* is upregulated by the presence of Zn and downregulated by mycorrhizal colonization, leading to a lower content of Zn within the host plant tissues (Burleigh et al. 2003). Recently, Ouziad et al. (2005) assessed the expression of *LePCS1* (coding for phytochelatin synthase), *Lemt1*, *Lemt3*, and *Lemt4* (encoding metallothioneins), or *LeNramp2* (encoding a broad-range heavy metal transporter) by tomato plants grown under heavy metal stress and colonized or not with the AMF *Glomus intraradices*. Whereas the transcription of the *LePCS1*, *Lemt1*, *Lemt3*, and *Lemt4*, or *LeNramp2* genes was unaffected by the mycorrhizal colonization and by the heavy metals, the *Lemt2* gene (encoding metallothionein) was strongly expressed in non-mycorrhizal plants grown in heavy metal polluted soil. These results suggest that heavy metal stress increases the transcript levels of some, but not all, genes of the Nramp or metallothioneins family. On the other hand, AM fungal colonization results in the downregulation of *Lemt2* and *LeNramp1* genes, presumably because the content of heavy metals is lower in mycorrhizal plants than in non-mycorrhizal ones. The downregulation of plant mRNA (Ouziad et al. 2005; Burleigh et al. 2003) may be related to the ''dilutive effect'' of heavy metals (Burleigh et al. 2003) that occurs when plant growth improves as a result of AM colonization, increasing the demand for other limiting mineral nutrients (Timmer and Leyden 1980; Clark and Zeto 2000), including heavy metals that behave as micronutrients.

Mycorrhizal colonization in plants exposed to heavy metal stress can modulate the pattern of protein expression. A protein band of around 30 kDa, a short-chain alcohol dehydrogenase (ADH), an UTP-1-phosphate uridylytransferase (UDP-glucose pyrophosphorylase, UDPGP or UGPase), and a protein with a high homology to subunit B from a vacuolar H^+-ATP synthase (V-ATPase), were all induced in pea

plants by Cd treatment but downregulated by inoculation with *Glomus mosseae* BEG12. On the other hand, a protein corresponding to an annexin fragment from *Medicago sativa* L. (alfalfa) and a disease resistance response protein Pi49 (PR10) were upregulated by the mycorrhizal treatment (Repetto et al. 2003). In a study on As hyperaccumulation in the absence and presence of *Glomus mosseae* and *Gigaspora margarita* on *P. vittata* plants, the expression of enzymes involved in photosynthesis and carbon fixation (i.e., RuBisCO, RuBisCO activase, and ATP synthase) and sugar metabolism and bioenergetics (e.g., glyceraldehyde-phosphate dehydrogenase and triosephosphate isomerase) were especially affected. AM symbiosis also modulated enzymes involved in the biosynthesis of S compounds (considering the role of some sulphurous compounds as nontoxic osmolytes or protective antioxidant agents) and some antioxidant enzymes such as thioredoxin peroxidase and glutathione peroxidize (Berta et al. 2008). These cases provide tangible examples of how mycorrhizae can overcome some of the effects of heavy metal(loid) stress in plants.

Besides affecting heavy metal accumulation and plant gene expression, the establishment of the mycorrhizal symbiosis can alter the allocation of heavy metals inside the plant. In fact, a limitation of using high biomass producing plants in phytoremediation is that these plants typically retain heavy metals in their roots, making it difficult to harvest metal-containing plant tissues. However, in *C. sativa*, the AM fungus *Glomus mosseae* BEG12 enhanced the translocation of Cd, Ni, and Cr^{VI} from root to shoot compared with non-mycorrhizal *C. sativa* plants (Citterio et al. 2005). Differences in heavy metal translocation within various plant organs as a consequence of mycorrhizal symbiosis are related both to the AM fungus and to the particular plant involved. Lingua et al. (2008) assessed the effects of high Zn concentrations on 2 different clones of poplar (*Populus alba* L. 'Villafranca' and *Populus nigra* 'Jean Pourtet'), inoculated or not with 2 arbuscular mycorrhizal fungi (*Glomus mosseae* or *Glomus intraradices*). In *P. alba* 'Villafranca', *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. On the other hand, in *P. nigra* 'Jean Pourtet', the 2 fungi decreased the Zn content in whole plants and, particularly, in stems. These results suggest that the performance of the poplar clone 'Villafranca' is improved by the presence of *G. mosseae*, whereas 'Jean Pourtet' accumulated more Zn per plant in the absence of mycorrhiza without a significant reduction of leaf biomass, suggesting that this clone has a different protective mechanism, possibly linked to lower Zn translocation to the leaves, more intense colonization, and higher leaf P content.

Additionally, AM fungi may affect tolerance to heavy metals by modulating plant stress reactions. As an example, poplar grown in the presence of Zn showed a 3-fold increase in the content of polyamines compared with untreated plants, inoculation with *G. mosseae* drastically reduced both free and conjugated polyamines, suggesting that in the presence of this AM fungus, given that the amount of Zn accumulated was very high, the toxicity of the metal was reduced (Lingua et al. 2008).

Notwithstanding the amount of work that has been reported, the mechanisms involved in conferring increased tolerance and decreased toxicity to metal-stressed plants by AM fungi have not been definitively elaborated and may be quite diverse. Thus, it is likely that heavy metal tolerance of plants conferred by AM fungal colonization is not simply understood because of the multiplicity of factors involved.

Action of bacteria and AM fungi on plants subjected to heavy metal stress

Although there are a huge number of scientific reports regarding the application of bacteria or AM fungi to improve phytoremediation efficiency and plant growth, the literature regarding the use of a combination of the 2 microorganisms in heavy metal polluted sites is scant. However, interesting results have been reported by the Azçon group in Spain. When *Trifolium repens* L. (white clover) was inoculated both with an indigenous Cd-adapted AM strain of *Glomus mosseae* and a Cd-adapted bacterium (*Brevibacillus* sp.), the doubly inoculated plants produced the highest extent of root biomass and symbiotic structures (nodules and AM colonization). Plant nutrient acquisition (N and P) were consistently enhanced by this mixed inoculum. However, the Cd uptake by *Trifolium* plants decreased in the dual AM fungus–bacterium treatments (Vivas et al. 2003). Similar results have been obtained by treating *T. repens* plants with AM and *Brevibacillus* strains with Zn (Vivas et al. 2006*a*) and Ni (Vivas et al. 2006*b*). These studies point out that indigenous bacteria or AM fungi may be highly efficient in supporting plant growth during phytoremediation of heavy metal polluted soils. Unfortunately, a detailed knowledge of the mechanisms involved in these bacteria–AM fungi–plant interactions under either natural or stressful conditions is lacking. Nevertheless, several possibilities exist: (*i*) both AM fungi and bacteria improve plant nutrition by providing essential minerals such as N and P, therefore stimulating plant growth; (*ii*) the 2 microoorganisms could affect plant growth by the synthesis of phytohormones; (*iii*) the bacterial strain could alleviate stress through ACC deaminase synthesis; and (*iv*) the bacterial strain may behave as a mycorrhiza helper bacterium and stimulate fungal development. In the latter case, if the mycorrhizal mycelium shows a high sorption capacity, an indirect consequence of the mycorrhiza helper activity may be lowering the metal availability to the plant. The ways in which a bacterial strain could stimulate the development of mycorrhizal fungi are still under debate (Frey-Klett et al. 2007). Although ACC deaminase has been shown to be involved in the stimulation of the growth of *Gigaspora rosea* BEG34 by the strain *Pseudomonas putida* UW4 on cucumber plants grown under nonstressful conditions (Gamalero et al. 2008), the synergism between the 2 microorganisms disappears when plants are exposed to salinity.[3] A similar situation was reported in alder plants. Alders can be colonized by both ectomycorrhizal and AM fungi by establishing a tetrapartite symbiosis, *Alnus*–ectomycorrhizae–AM–*Frankia* (Roy et al. 2007). Although positive effects were observed in alder plants inoculated with mycorrhizae (*Paxillus involutus*) and *Frankia* sp., this effect was only observed in the absence of contami-

[3] E. Gamalero, G. Berta, N. Massa, B.R. Glick, and G. Lingua. Submitted for publication.

nants (Markham 2005). By contrast, Lumini et al. (1994) reported that the plant biomass of *Alnus cordata* (Loisel.) Duby (Italian alder) inoculated with the AMF *Glomus* spp. and *Frankia* sp., after 1 growing season on lignite mine tailings (pH 5.7) was higher than when plants were inoculated only with *Frankia* sp. At this point, the potential benefits of *Frankia* sp. and mycorrhizal inoculation of alders in polluted soils have to be evaluated on a case by case basis.

Perspectives and conclusions

The application of transgenic plants in phytoremediation of contaminated environments provides many advantages over the use of hyperaccumulating plants (Zhang et al. 2008). Because of the recent advances in this technology, it is now feasible to increase a plant's capacity to tolerate, accumulate, and (or) metabolize pollutants leading to the production of large biomass (Krämer and Chardonnens 2001; Pilon-Smits and Pilon 2002). Several plants overproducing metal-chelating molecules, e.g., phytochelatins (Pomponi et al. 2006) and metal transporters (Song et al. 2003; Yazaki et al. 2006), showed enhanced heavy metal tolerance and accumulation. Moreover, transgenic tobacco, canola, and tomato expressing bacterial ACC deaminase genes have been constructed (Grichko et al. 2000; Nie et al. 2002; Stearns et al. 2005; Farwell et al. 2006). This approach leads to several advantages in comparison with bacteria: (*i*) during the initial stages of seed germination, bacterial ACC deaminase activity is expected to be lower than the activity measured in transgenic plants (Nie et al. 2002); (*ii*) it can be constitutively expressed (although limited to specific tissues) and thereby stimulate plant growth, which leads to a higher metal accumulation; (*iii*) in some cases plants showed an increase in the aboveground biomass as well as in roots (Grichko et al. 2000); and (*iv*) promotion of the metal uptake of certain fast-growing plants that may be used instead of slow-growing hyperaccumulators (Stearns et al. 2005). However, these transgenic plants perform similarly to plants treated with ACC deaminase expressing rhizobacteria. For this reason, and also taking into account the concerns raised by public opinion about the use of transgenic plants, PGPB may be an effective alternative to improve phytoremediation efficiency.

One fascinating proposal involves the use of endophytes living inside the plants and positively affecting plant growth and health. In fact, it has recently been reported that plant endophytes might be partially responsible for the degradation of environmental pollutants. The endophytic bacterial strain *Methylobacterium populum* sp. nov., BJ001 isolated from poplar is involved in the degradation of organic compounds such as 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and hexahydro-1,3,5-trinitro-1,3,5-triazine. Moreover, most of the endophytes of the Ni hyperaccumulator *Thlaspi goesingense* tolerated higher concentrations of Ni than rhizosphere bacteria (Idris et al. 2004). One endophytic PGPB, *Burkholderia phytofirmans* strain PsJN (Sessitsch et al. 2005), originally isolated from surface-sterilized onion roots colonized by *Glomus vesiculiferum*, has been shown to colonize the inside of different plant species. This strain showed beneficial activities on potatoes (Frommel et al. 1991), vegetables (Nowak et al. 1995), and grapevines (Barka et al. 2000) via reduction of the level of ethylene by a high level of synthesized ACC deaminase. In this regard, the expectation of an improvement of phytoremediation efficiency through the use of a bacterial strain able to synthesize ACC deaminase directly inside the root is intriguing. However, the associations of endophytic organisms with their hosts are varied and complex, and we are only starting to understand these interactions.

The phytoremediation of many organic compounds, both in the presence and absence of added microorganisms, has advanced to the state where this technology is already being employed with success in the field. On the other hand, because metals must be taken up into plants and transported to the shoots and leaves despite their possible toxicity, phytoremediation of inorganics is still at an early stage of development. Nevertheless, the work of a number of researchers indicates that adding both soil bacteria and mycorrhizae can facilitate this process. Thus, eventual successful commercialization of metal phytoremediation is likely to include a more complete understanding of the role of bacteria and mycorrhizae in this process harnessed to exploit their synergistic mechanisms for promoting plant growth and metal uptake in metal-contaminated soils.

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