13 Role of Arbuscular Mycorrhiza and Associated Microorganisms in Phytoremediation of Heavy Metal-Polluted Sites

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13.1 INTRODUCTION

Various technologies exist that enable the detoxification/deactivation and removal of toxic compounds from the soil, mostly based on physicochemical extraction methods. They are costly and totally destroy soil microorganisms. The restitution of life on polluted sites or areas that were subjected to conventional technologies usually takes a very long time, is difficult, and often requires human intervention.

Phytoremediation is an alternative to physicochemical methods; it involves the use of plants in the process of decreasing the level of toxic compounds in soil, stabilizing the soil, and inhibiting erosion [1]. However, plants need appropriate below-ground ecosystems to establish diverse communities, especially on difficult sites [2–4]. Mycorrhizal fungi play a key role in increasing the volume of soil explored by the plant in search for nutrients and trace elements. Their activity directly or indirectly influences the microbial populations, qualitatively and quantitatively, including bacteria and fungi from the zone called the mycorrhizosphere.

The present chapter will focus on arbuscular mycorrhizal fungi (AMF). Under natural conditions, they are accompanied by bacteria such as legume symbiotic nodular bacteria, plant growth-promoting rhizobacteria (PGPR), and fungi, including other mycorrhizal symbionts and saprobic fungi. All of these organisms build specific consortia that influence the plant by means of interactions with abiotic [5] and biotic components of the soil [6] or stimulate plant growth through the production of vitamins and hormones [7,8]. Rebuilding/establishing such consortia is of utmost importance for the effectiveness of phytoremediation processes.

13.2 MYCORRHIZA AND ITS ROLE IN THE ENVIRONMENT

Mycorrhizal fungi are widespread and form symbiosis with a large majority of plant species on Earth [2]. A common trait of all mycorrhizal fungi is that their mycelium overgrows the soil surrounding the plant roots; the hyphal net stabilizes the soil and furthermore produces substances that bind or glue soil particles together [9]. The way in which the root and its surface are colonized depends on the type of mycorrhiza.

There are two main types of mycorrhiza: ecto- and endomycorrhiza. In the first case, the mycelium forms a more or less compacted fungal mantle on the surface of the root. Its protective properties depend on the species of the symbiotic fungus, the mantle's water-absorbing capacity, the production of pigments, and organic acids. The mycelium penetrates between cortical cells of the root, forming the so-called Hartig net, which is the site of exchange of compounds between the partners. Ectomycorrhiza is formed by several thousands of fungal species, more or less specific towards host plant species (usually trees from the temperate zone). These trees are obligate symbionts.

Endomycorrhiza is far more diverse. Its characteristic feature is the possibility to penetrate not only spaces between cells, but also the inside of live cortical cells, crossing the cell wall and then developing in touch with the plasma membrane of the plant cell. This type of symbiosis includes orchid, ericoid, and arbuscular mycorrhiza. In the first two types, the mycelium forms coils inside cortical cells, but in arbuscular mycorrhiza, characteristic tree-like structures termed arbuscules develop. Arbuscular mycorrhiza (AM) is the most widespread type of mycorrhiza, occurring in 80% of plant species; it is formed by about 120 species of fungi belonging to the Glomeromycota [10]. This symbiosis is believed to be phylogenetically the most ancient type of mycorrhiza.

Molecular and paleobotanical studies seem to support the hypothesis of a close relationship of the AMF with plants since they appeared on land [11,12]. This mycorrhiza plays a key role in the productivity, stability, and diversity of natural ecosystems. Natural soils with low levels or completely devoid of AMF propagules are rare. Several factors can influence the quantity (i.e., the number of propagules) and the quality (i.e., the composition in species) of AM fungi in the soil. The presence of heavy metals and/or other pollutants, the use of amendments to remediate pollution, and the kind of vegetation heavily affect the composition and abundance of the Glomalean fungi [13–15].

The disappearance of the propagules leads to serious consequences, such as the degradation of plant communities; decreased availability of essential elements; and loss of ecosystem stability. Among examples in which it is necessary to introduce AMF propagules during creation and rebuilding of plant communities are sites resulting from volcanic activity and cutting down of forests; industrial wastes; postmining open areas; excessively fertilized agricultural lands; and soils strongly polluted by toxic compounds such as heavy metals (HM) and xenobiotics [2]. In such situations, the introduction of mycorrhizal inoculum involving selected fungal strains adapted to survive in a given toxic environment and under given climatic conditions becomes a key tool in decreasing the toxicity of these compounds to the plants and in establishing a stable vegetation cover.

Fungi adapted to polluted soils should be a choice of preference for the production of inoculum for soil remediation. The number of spores in polluted areas can be affected by the presence of heavy metals, but different fungi show different sensitivity [15] and species-specific (or even strain-specific) behaviors can be observed. In order to reduce the costs of inoculation, the choice of plants is also a relevant point. Plants should be efficient in removing or stabilizing the pollutants, and they should also promote the establishing of strong mycorrhizal and microbial communities because different plant species can affect the species composition of the Glomalean community [15].

13.3 PHYTOREMEDIATION AND THE BEGINNING OF INTEREST IN MYCORRHIZA

At first, the necessity to include soil microorganisms in phytoremediation was neglected. People used compounds that increase the availability of toxic compounds, therefore stimulating the accumulation of metals in plants [16–18], as well as fertilizers to boost plant biomass production [19]. The most efficient varieties were selected; techniques involving genetic engineering were also used [20–23]. The plants' ability to produce organic compounds influencing the rhizosphere and increasing the availability of metals was also acknowledged [24–26].

Among plants that produce high amounts of organic acids in the rhizosphere, researchers' attention was drawn to the order *Lupinus*. Its cultivation can successfully replace the use of chemicals increasing the availability of soil metals. At last the fact that the activity of microorganisms is a factor strongly influencing the processes of mobilizing and immobilizing metals, by means of precipitating sulphides and hydrated iron oxides or their binding to polysaccharides, was noticed [24,27]. Elements such as Pb, Zn, and Cu can also bind to carbonates and oxalates produced by microorganisms [28]. Metals can as well bind to functional groups localized on the surface of the microorganisms' cell walls [29].

Biological methods of cleaning up contamination mainly use bacteria and saprobic fungi; the role of mycorrhizal fungi is still underestimated. Well-developed mycorrhiza can enhance plant survival in difficult areas because it increases the availability of biogens; reduces stress due to low water availability; increases the resistance to pathogens; stimulates the production of phytohormones; and generally improves the soil structure. These factors can significantly enhance bioremediation.

Among the usually considered bioremediation practices, special attention is due to phytostabilization, phytodegradation, and phytoextraction. Briefly, phytostabilization involves the immobilization of toxic compounds in the soil by means of plants that reduce soil erosion; leaking of contaminants into the ground waters; and their dispersion through wind erosion [30]. Phytodegradation includes various metabolic processes of plants and accompanying microorganisms leading to the breaking up of organic compounds such as polyaromatic hydrocarbons, pesticides, and explosives. Phytoextraction takes advantage of the ability of plants to hyperaccumulate metals. Plants are considered useful if they can take up over 1% of a given metal in the dry mass of their shoots. Such plants are grown on the given area and their above-ground parts are harvested, dried, and burned [31,32].

According to Gleba et al. [33], during phytoextraction the "giant underground networks formed by the roots of living plants function as solar driven pumps that extract and concentrate essential elements and compounds from soil and water." This observation has some important implications and consequences.

First, because heavy metals are taken up and transported in water solution, increased plant transpiration would increase metal translocation to the shoot. There is no doubt that mycorrhizal colonization affects the water relations of plants (Smith and Read [2] and references therein). A number of papers indicate that transpiration rates of mycorrhizal plants are significantly higher than those observed in nonmycorrhizal ones [34–39]. Mycorrhizal root systems are usually more branched [40] and therefore they present a larger absorbing surface even in the absence of changes in root biomass [34]. Also, the increased leaf area can be an important factor leading to increased transcription [41]; however, even comparing plants of the same size and root system length, the transpiration rates of mycorrhizal plants remain superior, due to the reduced stomatal resistance [38].

It has been noted that the high stomatal resistance observed in P-deficient nonmycorrhizal plants is a nutritional effect [38,39]. Nevertheless, stomatal behavior is affected by hormonal changes, which can depend on P nutrition as well as on mycorrhizal colonization [35,42–44]. Abscissic acid (ABA) is known to block transpiration and, consequently, metal accumulation in shoots [17]. According to Allen [35], ABA concentrations in leaves of *Bouteloua gracilis* decreased following mycorrhizal colonization by *Glomus fasciculatum*; on the other hand, Danneberg et al.

[43] report higher concentrations of ABA in leaves and roots of maize colonized by *Glomus* sp. (isolate T6) in comparison to nonmycorrhizal plants. Once more, the different combination of plant and fungus species might be important, as well as the growth conditions and the methods utilized for the measurements.

In the second place, roots and hyphae of mycorrhizal root systems explore an incredibly larger volume of soil in comparison with nonmycorrhizal root systems. Even if the contribution of the external hyphae to water uptake and its translocation to the roots has not been clarified [2], it was proved that the external mycelium may contribute to the uptake and translocation of some heavy metals (including Zn, Cu, and Cd [45–47]) to the host roots.

The preceding considerations show, once more, how important it is to gain deeper knowledge on the basic functioning of the mycorrhizal symbiosis for its exploitation in biotechnology and environmental applications.

13.4 MYCORRHIZA IN PHYTOSTABILIZATION

Mycorrhiza proved to be especially useful in phytostabilization. Although the first plants that colonize areas with increased heavy metal levels usually belong to nonmycorrhizal species [48,49], the development of a dense vegetation cover and improvement of the soil structure require the presence of symbiotic fungi. This is especially important for sites where postflotation material originating from zinc and lead ore processing is deposited. Such material is almost devoid of nitrogen and phosphorus compounds, has poor water-holding capacities, and is vulnerable to wind erosion [50]. Possible mechanisms of improved resistance of AMF-colonized plants to HMs include the enhancement of nutrient uptake, particularly phosphorus, and water supply [51,52], as well as metal sequestration through the production of binding substances or absorption of metals by microbial cells [47,53].

Recently, a gene called *GmarMT1,* encoding for a fungal metallothionein, has been identified in *Gigaspora margarita* (BEG 34) [54]. Metallothioneins (MT) are Cys-rich polypeptides able to chelate metal ions and important in the buffering of their intracellular concentration. Heterologous complementation in yeast revealed that the polypeptide encoded by *GmarMT1* confers increased tolerance to Cu and Cd. The gene expression in the symbiotic mycelia is up-regulated upon Cu exposure [54]. Spontaneous colonization of polluted substrate by arbuscular fungi takes a long time. However, it is possible to introduce propagules of selected strains of mycorrhizal fungi in the form of inoculum. Individual strains show a pronounced diversity in the effectiveness of metal binding and therefore also in reducing the toxicity of the substratum. Because the mycelium of certain strains of species, like *Glomus mosseae*, are tolerant to high heavy metal concentrations, they can bind a few times more metals than the mycelium of a saprobic species commonly used in bioremediation — *Rhizopus arrhizus* [55].

It was demonstrated for cadmium and zinc that, although these elements are detected in the mycelium developing inside plant roots, their accumulation in above-ground parts might be limited [46,56]. However, the analysis of tissue concentration and total shoot uptake of Cd, Zn, and Pb in *Plantago lanceolata*, grown in rhizoboxes on substratum collected from zinc wastes, inoculated with a number of arbuscular mycorrhizal fungal (AMF) strains has shown that metal uptake by the plant differs depending on AMF strain/species [57] (Figure 13.1).

The ability to bind and detoxify heavy metals in underground parts of plants might be of importance also for the stimulation of the growth of crops cultivated on polluted soils. Such plants were inoculated with a *Glomus intraradices* strain isolated from a metallophyte — *Viola calaminaria* [56,58]. AMF also eliminated the toxic effect of Cd on several pea genotypes [59]. It was noted that strains isolated from polluted areas are far more useful than strains originating from nonpolluted sites [60–62]. Differences in the effectiveness of metal detoxification and accumulation exist also among strains and species occurring on polluted sites [63]. This underlines the importance of the identification

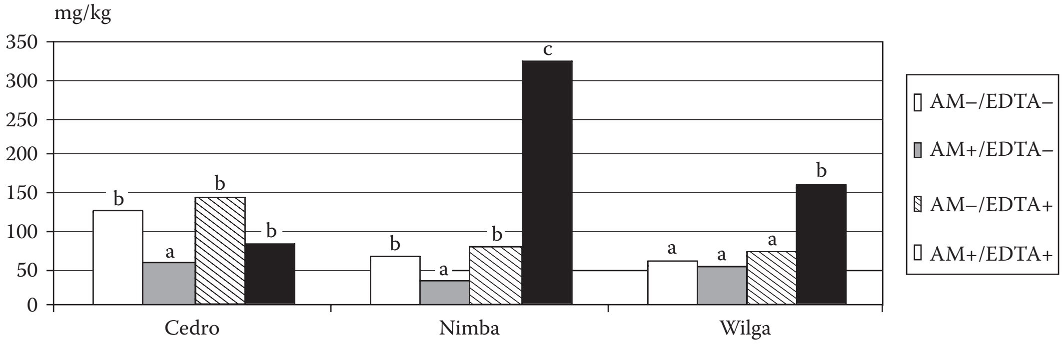

FIGURE 13.1 Pb content in shoots of three maize varieties, mycorrhizal/nonmycorrhizal and with/without EDTA treatment, as examples of different behavior patterns. Different letters above bars indicate statistically significant differences at $p < 0.05$. (Modified from Jurkiewicz, A. et al., *Acta Biol. Cracov. Bot.*, 46, 2004.)

and characterization of the strains, aimed at the selection of the most effective ones. The selected strains should also effectively compete with other fungi that might occur on the given site.

The inoculation of agricultural fields with mycorrhizal fungi in nonpolluted areas often does not improve the situation due to the presence of native strains, which are much better adapted to the given soil conditions, and therefore seems to be ineffective; however, in the case of polluted places that suffer from reduced propagule number and decreased range of fungal species, inoculation is far more effective. The recently developed molecular methods enable scientists to track the fate of the introduced strain in pot cultures as well as in field-collected material. Specific primers exist for a range of species and strains and allow detection of the presence of the introduced fungi in root samples stained to reveal the intraradical mycelium, using the nested PCR method [63–65]. In addition to analysis of the parameters of the fungal partner, it is also necessary to investigate the mechanisms that enable the plant to tolerate metals transferred from the mycelium. Plants show a range of reactions to heavy metals [66]; they can also regulate the degree of mycorrhizal colonization [67].

When selecting plant species for phytostabilization, special attention is usually given to grasses. Although C3 grasses are, under natural conditions, medium or poorly mycorrhizal, they become strongly colonized by AMF on industrial wastes [68–71] and in areas seriously polluted by heavy metals [72–74]. Introduction of the AMF inoculum simultaneously with mixtures of grasses adapted to given conditions is important for the grasses and may be the source of propagules for the establishment of trees such as *Acer* and *Populus* [75]. Similarly, the establishment of ectomycorrhizal tree plantations such as pine or birch (a common practice on industrial wastes) allows improvement of the structure of the soil and increases the soil organic matter content; this in turn creates better growth conditions for herbaceous plants and their symbiotic organisms [76].

Similarly to arbuscular mycorrhizae, ectomycorrhizae stimulate the growth of trees, protect them against pathogens, and may alleviate soil toxicity [77]. Ectomycorrhizal fungi appear on heavily polluted sites faster than arbuscular ones. This is because they form small spores in fruitbodies that usually expose the reproductive layer above the ground level, enabling easy, long-distance dispersion by wind. Individual species and strains of ectomycorrhizal fungi also show diversity in the effectiveness of protecting trees on polluted soils [53,78,79]. This phenomenon is clearly visible when comparing strains isolated from polluted and nonpolluted sites in laboratory conditions [80]. The mycelium can immobilize heavy metals and reduce their transfer to plant tissues, which is regarded as an important protection mechanism allowing trees to survive in polluted areas [53,74].

The phenomenon is explained by the binding of elements by pigments deposited on the surface of extraradical mycelium [60,81], within the hyphal wall [82,83], and in phosphate-rich vacuolar granules [81]. The biofiltration effect of the extraradical mycelium is the most pronounced. The fungal mantle can sometimes also play this role. Such a situation was described in *Rhizopogon roseolus* and *Suillus luteus* from zinc wastes in southern Poland [84,85]. In mycorrhizae of pines with the mentioned fungal species, a typical gradient of heavy metals, decreasing towards the inside of the mantle, was observed. The selection of mycorrhizal strains for the inoculation of tree seedlings planted in polluted areas is important for their establishment.

13.5 MYCORRHIZA IN PHYTODEGRADATION AND PHYTOEXTRACTION

Phytodegradation consists of accelerated degradation of polluting organic compounds, such as hydrocarbons, pesticides, or explosives, in the presence of plants. Existing technologies involve mostly saprobic bacteria and fungi [86–88]. Plants with an abundant root system also have a favorable effect on the degradation of polyaromatic hydrocarbons (PAH) [89,90]. The introduction of rhizosphere microorganisms into such cultures is an alternative to chemical compounds that increase the availability of toxic substances [89,91].

Arbuscular and ectomycorrhizal fungi can enhance phytodegradation [92]. Although the number of propagules of arbuscular fungi decreases with increasing concentration of xenobiotics [93], they can still stimulate plant growth by decreasing the stress related to low phosphorus availability [94] and water deficiency [95]; they can also boost the production of oxidation enzymes [96]. Ectomycorrhizal fungi can additionally produce enzymes taking part in preliminary or intermediate stages of xenobiotics' decomposition [52,97], which enables their further decomposition by other rhizosphere organisms [98–100]. In addition, soil polluted with organic compounds is usually rich in heavy metals. Although these metals cannot be degraded, development of the mycorrhizal mycelium can efficiently alter their availability and plant growth conditions.

Recently, attention has been paid to *Phragmites australis*, which is commonly used for constructed wetlands designed to treat organic effluents [101–103]. *P. australis* was so far believed to be nonmycorrhizal, but again it was recently found to form the symbiosis with enhanced frequency when the water level was reduced and during the flowering time [104]. The presence of potentially mycorrhizal plants in constructed wetlands might be important to enhance phytostabilization and improve the restoration of biodiversity in areas where the processes had ceased.

The least attention was paid to the use of mycorrhiza in phytoextraction. Recent years have brought an increase of interest in the hyperaccumulation of heavy metals by plants, due to the commercial potential of phytoremediation in cleaning up contaminated soil [19,105,106], and as a method to mine metals from low-grade ore bodies [107–110]. Although several reports have been issued on arbuscular mycorrhiza of plants occurring on heavy metal-rich soils such as serpentines [111,112] or strip mines [70,76], arbuscular mycorrhiza was only recently reported in a few hyperaccumulating species belonging to the Asteraceae family growing on nickel-enriched ultramafic soils in South Africa. All plants were found to be consistently colonized by AM fungi, including an abundant formation of arbuscules.

Among them, the most important for phytomining is *Berkheya coddii*, which is capable of accumulating up to 3.8% of Ni in dry biomass of leaves under natural conditions and produces a high yield exceeding that for most hyperaccumulators [113]. The species can also provide an excellent model for laboratory studies on mechanisms mediated by AMF fungi that allow for the phytoextraction process. It has been also shown to form well-developed mycorrhiza under greenhouse conditions. Preliminary results have shown that *B. coddii* inoculated with native fungi (*Gigaspora* sp. and *Glomus tenue*) had not only higher shoot biomass but also significantly increased Ni content (over two times) in comparison to noninoculated plants. This finding greatly contrasts with the conventional opinion that the presence of AMF reduces the uptake of trace elements if they occur in excessive amounts [53].

Mycorrhizal colonization was also reported in Zn and Pb hyperaccumulating *Thlaspi praecox* from the Alps; still, the colonization level of this plant is rather low and decreases with increasing content of heavy metals in the soil [114]. In addition, it is thus far not possible to obtain the formation of mycorrhiza of this group of plants under laboratory conditions; this makes the interpretation of field data hard to confirm.

Although mycorrhiza does not necessarily stimulate phytoextraction, its potential to increase the biomass of the plants, improve soil conditions, and protect the plants from pathogens offers important reasons to include this phenomenon in further research. Three possibilities to increase phytoextraction are being proposed: (1) transgenic plants; (2) hyperaccumulators or high biomass producing crops such as maize, especially for soils relatively less polluted [115,116], treated with chemical chelating substances such as EDTA or sulphur; and (3) stimulating the development of or introducing rhizosphere organisms that will increase the uptake of metals by the plants.

The biotechnological approach aims at producing genetically modified plants characterized by increased tolerance to toxic compounds, higher biomass, and high uptake of heavy metals. A number of transgenic plants have already been obtained by transferring appropriate genes from bacteria or yeasts (see, for example, references 117 through 119) or by generated somatic hybridization between plants such as *Brassica napus* and *Thlaspi caerulescens* [120]. Most transgenic plants have, thus far, only been tested under artificial conditions [121] and they still need further research before the application phase will start. Also, the transformation of AM fungi has been approached. The identification of genes with similar functions can be very important for understanding of the mechanisms of resistance and tolerance to heavy metals and for selection of the fungal strains most suitable for phytostabilization and phytoremediation.

However, although the transformation of many animal, plant, and fungal (e.g., *Saccharomyces*) species is now a relatively easy practice, standard protocols for the transformation of AM fungi are not available yet [122]. Beyond technical problems, the huge diversity of the fungal genome inside one single isolate [123] and the lack of knowledge about the factors controlling the expression of this diversity represent a major problem for an effective exploitation of this kind of approach. In addition, the below-ground environment in which the fungi live and their vegetative reproduction make control of the spread of the transformed fungi very difficult, suggesting a very cautious and careful introduction.

The use of synthetic chelates has been proposed because the amount of metals extracted from the soil by plants depends largely on the availability of the metals. In most soils, even highly polluted ones, only a relatively small percentage of the total metal pool is available to plants. These compounds mobilize metal ions and displace them into the soil solution. Among a variety of chelates tested by Huang et al. [116], EDTA was demonstrated to be the most effective in mobilizing Pb [124], showing that it also increased the availability of other metals such as Cd, Cu, Ni, and Zn.

Experiments carried out on maize show that this common crop can take up as much as 3000 mg kg^{-1} Pb in shoots when grown in laboratory conditions with EDTA (0.5 g kg^{-1} of soil) [116]. A study carried out on 15 commercially available Polish maize varieties inoculated with an AMF strain and treated with EDTA showed that most maize varieties cultivated on metal-rich substratum had higher shoot biomass; this clearly confirmed the role of mycorrhizal fungi in phytoremediation practices, although large differences between varieties have been observed [125].

The data show a large diversity in the effectiveness of phytoextraction among different varieties of the same species. This finding stresses the necessity to screen a large number of cultivars in order to select the best ones for further use. Moreover, the effect of individual varieties might vary when chelators such as EDTA are used (Figure 13.2). Although the use of chemical amendments should be considered carefully, heavy metal release into water flowing out from EDTA-treated pots was found to be substantially lower in the case of mycorrhizal plants than in nonmycorrhizal plants.

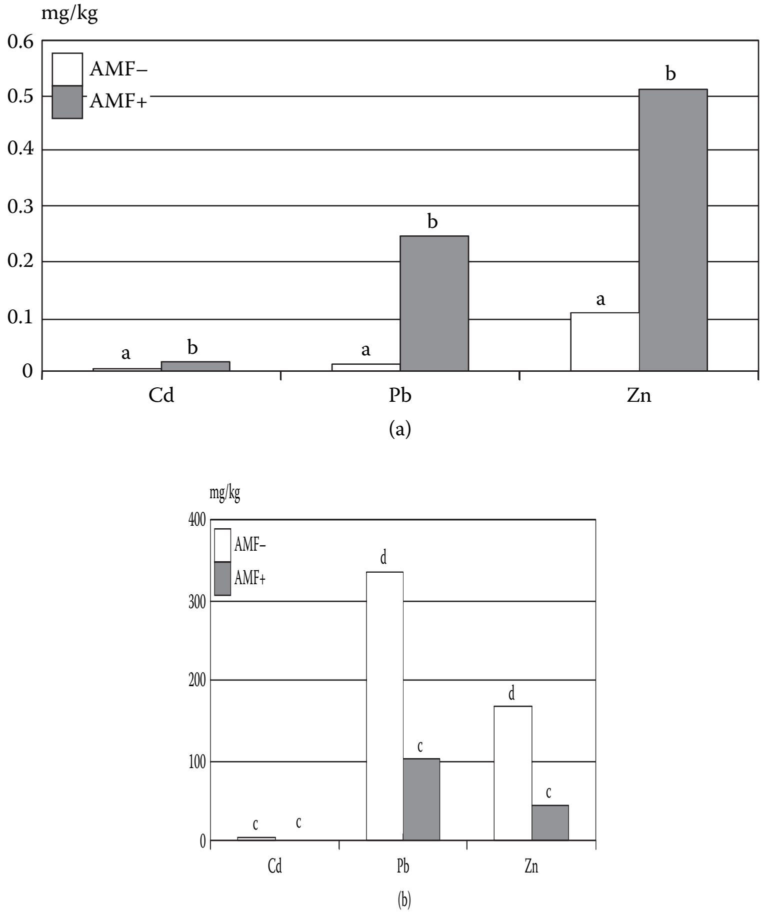

FIGURE 13.2 Heavy metal (HM) release into soil solution: (a) without EDTA treatment; (b) after EDTA treatment. Different letters above bars indicate statistically significant differences at $p < 0.05$. (Modified from Jurkiewicz, A. et al., *Acta Biol. Cracov. Bot.*, 46, 2004.)

This would suggest that mycorrhizal fungi increase the availability of metals to the plants but, at the same time, may decrease pollutant run-off into the ground water (Figure 13.3).

Supplementing soil with sulphur can reduce the soil pH and the application of amendments and fertilizers can cause variations in the abundance of species [14] and modifications in the colonization of roots, thus increasing the amount of vesicles [15]. Also, the use of chelating chemicals, like EDTA, should be applied with much care: it mobilizes the toxic substances, but with the risk of leaching deeper into the soil profile.

In the general literature, not much attention has been paid to the fact that a large diversity in the effectiveness of phytoextraction might exist among different varieties of the same species, as shown by the previously mentioned study. These findings stress the necessity to screen a large number of cultivars in order to select the best ones for further use.

13.6 INFLUENCE OF SOIL BACTERIA ON MYCORRHIZA EFFICIENCY IN POLLUTED ENVIRONMENTS

Rhizosphere bacteria are known to improve mycorrhiza formation and activity by means of a number of so-called mycorrhizosphere activities, which benefit plant growth and health [126].

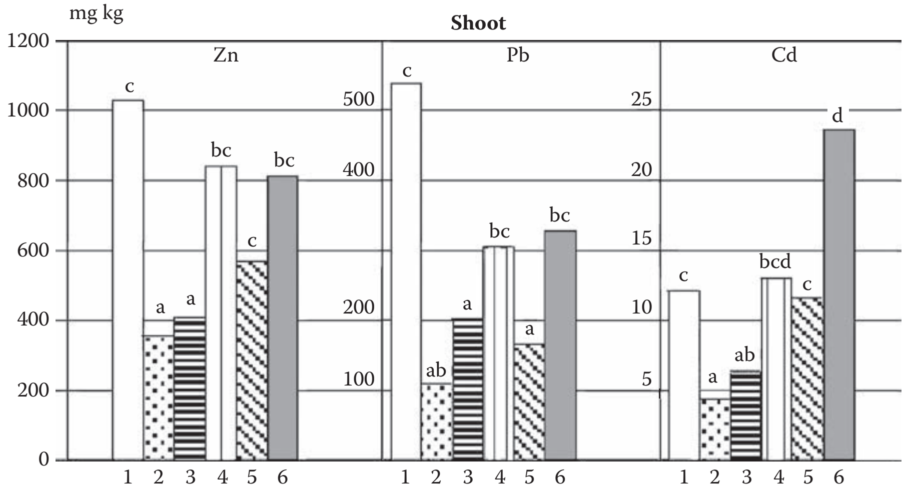

FIGURE 13.3 Heavy metal concentration in shoots of nonmycorrhizal and mycorrhizal *Plantago lanceolata* cultivated in rhizoboxes filled with zinc industrial wastes: 1: control plants (noninoculated); 2–6 — inoculated plants: 2: *Glomus clarum* isolated from zinc wastes; 3: *G. geosporum* from metal-polluted site; 4: *G. geosporum* from salt enriched site; 5: *G. claroideum* from zinc wastes; 6: *G. intraradices* from nonpolluted site. (From Orlowska, E. et al., *Polish Botanical Studies*, Polish Academy of Science [publisher] 2005.)

Therefore, it is to be expected that, with an appropriate selection of target bacteria, these could benefit the role of mycorrhiza in phytoremediation [127,128]. Selection procedures involve:

- Isolation of adapted bacteria from HM-contaminated soils
- Ecological compatibility with mycorrhizal fungi also adapted to HM contamination
- Functional compatibility of both types of microorganisms in terms of promoting phytoextraction and/or phytostabilization of metals from the polluted soil

Soil microbial diversity and activity are negatively affected by excessive concentration of HM [129]. However, indigenous bacterial populations must have adapted in a similar way to mycorrhizal fungi [14] and metal toxicity and evolved abilities that enable the bacteria to survive in polluted soils [127]. Adaptation of mycorrhizal fungi and associated bacteria to HM is considered a prerequisite for exploiting their potential role in phytoremediation [49].

The role of a tailored mycorrhizosphere in phytoremediation was investigated in a series of studies [130–134]. These studies consisted of:

- Isolation and characterization of microorganisms from a target HM contaminated site
- Development of several phytoremediation experiments
- Analysis of the mechanisms involved to account for the demonstrated phytoextraction and/or phytostabilization activities found

Under natural conditions, soil becomes contaminated with more than one metal; thus, it is difficult to determine which metals are responsible for the toxic effects observed [135]. Therefore, only long-term experiments using soils supplemented with a single metal salt can give the opportunity to study the individual toxic effects of each heavy metal on the beneficial microbes for a given time period [136]. In this context, a number of experiments are summarized in the present chapter, all of them using agricultural soil from Nagyhörcsök Experimental Station (Hungary). This soil was contaminated in 1991 with suspensions of 13 microelement salts applied separately. Each salt was applied at four levels (0, 30, 90, and 270 mg kg^{-1}) as described by Biró et al. [136].

Indigenous mycorrhizal fungi and bacterial strains were isolated from this HM-polluted soil 10 years after contamination. They were tested for their influence on plant growth and on the functioning of native mycorrhizal fungi in the face of Cd, Pb, or Ni toxicity.

The most efficient bacterial isolates were identified by means of 16S rDNA sequence analysis and confirmed to belong to the genus *Brevibacillus*. Particularly, *B. brevis* was the most abundant species [130,132]. *Glomus mosseae* was present in all the HM-polluted soil samples, so it was the target mycorrhizal fungus used for phytoremediation inoculation experiments. *G. mosseae* and *Brevibacillus* sp. strains from nonpolluted environments were also used as reference inocula. *Trifolium repens* L. was used as test plant and inoculated with a suspension of *Rhizobium leguminosarum* bv *trifoli*, also an HM-tolerant strain.

In the Cd-contaminated soil [132,133], a high level functional compatibility between both types of autochthonous microorganisms was demonstrated; this resulted in a biomass increase of 545% (shoots) and 456% (roots) and in the N and P content compared to nonmycorrhizal plants. Coinoculation of both microorganisms increased root biomass and symbiotic structures (nodules and AM colonization) to a highest extent, which may be responsible for the beneficial effect observed. The results suggest that bacterial inoculation improved the mycorrhizal benefit in phytostabilization.

Dual inoculation of the Cd-adapted autochthonous *Brevibacillus* sp. and the AM fungus lowered the Cd concentration in *Trifolium* plants. This effect can be due to the ability of the bacteria to accumulate great amounts of Cd. In spite of that, the total Cd content accumulated in plant shoots was higher in dually inoculated plants. This indicates a phytoextraction activity resulting from such a dual inoculation. Further studies [134] demonstrated that the inoculated Cd-adapted bacteria increased dehydrogenase, phosphatase, and β-gluconase activities in the mycorrhizosphere, indicating an improvement of microbial activities concerning plant development in the polluted test soil.

With respect to Pb-spiked soil, experiments following the same methodological approaches [130] showed that *B. agri* at all Pb-spiking levels assayed consistently enhanced plant growth and nutrient accumulation in mycorrhizal plants, as well as nodule numbers and mycorrhizal colonization. This suggests a phytostabilization activity. Auxin production by the test bacteria can account for the beneficial role of these bacteria on mycorrhizal plant development [126]. Dual inoculation increased Pb concentration in plant shoots at the highest level of Pb applied. However, the total content of this metal in plants was consistently enhanced at all levels of Pb, showing that bacterial inoculation enhanced phytoextraction activities in plants inoculated with mycorrhizal fungi.

Dual inoculation of an indigenous Ni-adapted mycorrhizal strain of *G. mosseae* and a Ni-adapted bacterium (*Brevibacillus* sp.) isolated from Ni-contaminated soil was also assayed with *Trifolium* plants growing in Ni-polluted soil [131]. Dual inoculation increased total plant content of this metal at all levels of Ni assayed. This indicates that the tailored mycorrhizosphere carries out phytoextraction of Ni from polluted soils.

The mechanisms by which the bacterial isolates tested enhanced phytoremediation activities in mycorrhizal plants can be therefore summarized as follows:

- Improving rooting, mycorrhiza formation, and functioning
- Enhancing microbial activities in the mycorrhizosphere
- Accumulating metals, thus avoiding their transfer to the aquifers

In conclusion, the dual inoculation of suitable symbiotic and saprophytic rhizosphere microorganisms isolated from HM-polluted soils seems to play an important role in the development and HM tolerance by plants and in bioremediation of HM-contaminated soils.

13.7 MYCORRHIZA AS INDICATOR OF SOIL TOXICITY AND REMEDIATION RATE

Mycorrhizal fungi can also be useful as indicators of soil toxicity [71,137] and the effectiveness of remediation [76]. The toxicity of heavy metals and other pollutants (xenobiotics, PAH) has been monitored using AMF spore germination [13], mycorrhizal colonization of roots analyzed by PCR technique [138], and mycorrhizal infectivity [61]. Recently, the toxicity of zinc wastes of different ages and resulting from different extraction technologies has been compared using various AMF strains and species. The activity of the alkaline phosphatase [139], a vital staining of mycorrhizal colonization, has been found to provide a more sensitive test than the estimation of the total mycorrhizal development [57,62]. Similarly to other indicator organisms such as plants, earthworms, algae, and fish, the disadvantage is that all of them, including AMF, are not specific to pollutants and also react to soil properties (P and N content, pH, etc.).

The appropriate selection of the control soil seems to be problematic. The examples studied thus far prove that they are sensitive indicators of the changes that occur during phytoremediation or during spontaneous succession. Concerning a wider range of soils, the most useful is the germination test, especially that the technique is presently standardized and may rely on the fungal strains supplied commercially (Leyval, C., personal communication, 2004). Further approaches have been recently done to establish new methods considering a wide range of features, such as abundance of intraradical and extraradical mycelium; formation of vesicles; distribution of lipid droplets; etc. They seem to react sensitively to pollutants, at least in the case of the most widely used *Glomus mosseae* strain, BEG12.

Some AMF can be more sensitive to pollutants than plants [72], although some species, especially those originating from strongly polluted places, are well adapted to survival under extremely harsh conditions and their disappearance might be caused only by the lack of the symbiotic plants. The selection of an appropriate fungal strain and plant varieties is therefore of utmost importance [76]. Among the plant species analyzed thus far, English plantain (*Plantago lanceolata* L.), strongly colonized by arbuscular fungi, deserves special attention as an indicator used for bioassays. It occurs in diverse habitats and is resistant to a wide range of stress factors; it is also easy to obtain clones of one plant, which eliminates genetic variability among individuals in their response to toxic compounds [140–142].

13.8 CONCLUSIONS

Central and Eastern Europe are regions where large industrial wastes, deposits of various kinds of waste materials, and places polluted by insufficiently secured unused plant protection products — as well as sites subjected to intense motorization and industrialization — are especially common. Despite the usually well-designed actions aiming at explaining the problem of pollution to the local community, one can still see the production of plants destined for human consumption on heavily polluted soils. Cheap and fast monitoring methods are needed here, followed by low-cost and effective phytoremediation techniques; mycorrhizal fungi should play the key role. It should be emphasized that research on this group of fungi should be conducted in a complex way and include other mycorhizosphere organisms with which the fungi interact [143].

The previously mentioned questions illustrate how broad and diverse are the possibilities to use natural phenomena in solving difficult problems of today's civilization. This will certainly stimulate the dynamic development of a range of scientific fields aimed at explaining the mechanisms of the mentioned phenomena and at optimizing their practical applications.

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