Chapter 14
Fungi and Their Role in Phytoremediation of Heavy Metal-Contaminated Soils

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14.1 Introduction

Contamination of soil and water with heavy metals (HM) and metalloids is an increasing environmental problem worldwide that has accelerated dramatically since the beginning of industrial revolution and represents an important environmental problem due to their toxicity, and accumulation throughout the food chain leads to serious ecological and health problems. The primary source of this pollution includes the industrial operations such as mining, smelting, metal forging, combustion of fossil fuels, and sewage sludge application in agronomic practices. The metals released from these sources accumulate in soil and, in turn, adversely affect the microbial composition and their metabolic activities. In addition, the elevated concentration of metals in soils and their uptake by plants adversely affect the growth, symbiosis, and consequently the yields of crops (Moftah 2000; Wani et al. 2007a) by disintegrating cell organelles and disrupting the membranes (Stresty and Madhava Rao 1999), acting as genotoxic substance (Sharma and Talukdar 1987) disrupting the physiological process such as photosynthesis (Van Assche and Clijsters 1990; Wani et al. 2007b), or inactivating the respiration, protein synthesis, and carbohydrate metabolism (Shakolnik 1984). The remediation of metal-contaminated soils thus becomes important as these soils usually cover large areas that are rendered unsuitable for sustainable agriculture. Therefore,

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increasing attention has been paid in recent years to the remediation of polluted soils, among which the use of plants and microbes to remove hazardous metal ions is particularly emphasized (Winge et al. 1985; Mehra and Winge 1991).

The HMs in general cannot be biologically degraded to more or less toxic products and, hence, persist in the environment indefinitely. Conventional methods through common physicochemical techniques that include excavation and land fill, thermal treatment, acid leaching, and electro-reclamation are ineffective for metal detoxification because of the high cost, low efficiency, large destruction of soil structure and fertility, and also production of large quantities of toxic products. The advent of bioremediation technology which is the use of microbial metabolic potential has provided a safe and economic alternative to conventional methods for remediating the metal-poisoned soils. The other effective and promising approach is phytoremediation, which is the use of plants to extract, sequester, and detoxify pollutants to clean up the contaminated soils (Brooks 1998).

Phytoremediation involves the use of metal-accumulating plants to remove, transfer, or stabilize the contaminants from soils, but this technique is time consuming (Wenzel et al. 1999). The success of phytoremediation depends on the extent of soil contamination, bioavailability of the metal, and the ability of the plant to absorb and accumulate metals in shoots. However, plants with exceptionally high metal-accumulating capacity often have a slow growth rate and produce limited amounts of biomass when the concentration of metal in the contaminated soil is very high and toxic. To maximize the chance of success of phytoremediation, plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhiza fungi (AMF), soil microbes that inhabit the rhizosphere, are utilized in the nutrient poor agricultural soils. They increase HM sequestration capacity of plants by recycling nutrients, maintaining soil structure, detoxifying chemicals, and controlling pests while decreasing toxicity of metals by changing their bioavailability. Meanwhile, plants provide the microorganisms with root exudates such as free amino acids, proteins, carbohydrates, alcohols, vitamins, or hormones which are important sources of nutrient (Winge et al. 1985).

The aim of this chapter is phytoremediation of HM-contaminated soils by using the fungi with the emphasis of arbuscular mycorrhizal fungi.

14.2 HM Pollutants

HM pollution is a global concern. The levels of metals in all environments, including air, water, and soil, are increasing in some cases to toxic levels with contributions from a wide variety of industrial and domestic sources. Metal pollution results when human activity disrupts normal biogeochemical activities or results in disposal of concentrated metal wastes. Mining, ore refinement, nuclear processing, the industrial manufacture of batteries, metal alloys, paints, preservatives, and insecticides are examples of processes that produce metal by-products. Thus, while metals are ubiquitous in nature, human activities have caused

metals to accumulate in soil. Such contaminated soils provide a metal sink from which surface waters and groundwaters can become contaminated. Contaminated soil contributes to high metal concentrations in the air through metal volatilization. In addition, industrial emissions and smelting activities cause release of substantial amounts of metals to the atmosphere. Naturally, high metal concentrations can also occur as a result of weathering of parent materials containing high levels of metals.

Although some HMs are essential plant micronutrients since they are required for plant growth and development (Zn, Cu, Fe, Mn, Ni, Mo, Co), high contents of HMs, as well as the long-term presence of potentially toxic metals (Cd, Pb) and metalloids (As) in surface horizon of agricultural soils, are generally considered a matter of concern, as they may adversely affect the quality of soils and surface water and compromise sustainable food production (Pandolfini et al. 1997; Kabata-Pendias 2001; Keller et al. 2002; Voegelin et al. 2003; Kabata-Pendias and Mukherjee 2007). HMs exert their toxicity in a number of ways including the displacement of essential metals from their normal binding sites on biological molecules (e.g., arsenic and cadmium compete with phosphate and zinc, respectively), inhibition of enzymatic functioning, and disruption of nucleic acid structure. It is important to note that the toxicity of a metal depends to a large extent on its speciation which in turn influences metal bioavailability. The chemical nature and, thus, bioavailability of a metal can be changed through oxidation or reduction; however, the elemental nature remains the same because metals are neither thermally decomposable nor microbiologically degradable. Consequently, metals are difficult to remove from the environment. In addition, total metal concentrations in the environment do not necessarily reflect the degree of biological metal toxicity or bioavailability, making it difficult to assess accurately the extent of risk posed by metals.

14.2.1 Detrimental Effects of HMs on Soil Biota

Microbial communities play important roles in soil because of the many functions they perform in nutrient cycling, plant symbioses, decomposition, and other ecosystem processes (Nannipieri et al. 2003). Large HM contents in soil are of concern because of their toxicity to soil microorganisms and impairment of ecosystem functions (Giller et al. 1997). First observations of the effects of HMs on soil microbial processes date back to the beginning of this century (Lipman and Burgess 1914; Brown and Minges 1916). But only when the large adverse effects of HMs emissions from smelters on surrounding ecosystems were observed in the 1960s–1970s was it realized how severely soil microorganisms and soil microbial processes can become disrupted by elevated metal concentrations, sometimes resulting in severe ecosystem disturbance.

Short-term responses of microbial communities to HM contamination are well known (Shi et al. 2002; Ranjard et al. 2000; Gremion et al. 2004; Rajapaksha et al. 2004), but medium- and long-term effects of HM in the field have been less

frequently investigated (Pennanen et al. 1996; Kandeler et al. 2000; Sandaa et al. 1999; Renella et al. 2004). However, a considerable body of information has now been accumulated on the effects of HMs on soil microorganisms and microbially mediated soil processes from both laboratory studies and field experiments (Bååth 1989). HMs exert toxic effects on soil microorganism (Pawlowska and Charvat 2004), hence results in the change of the diversity, population size, and overall activity of the soil microbial communities (Smejkalova et al. 2003; Gupta 1992; Hattori 1996; Kelly et al. 2003).

Gasper et al. (2005) reported that the aftereffect of the observed HMs (Cr, Zn, and Cd) pollution influenced the metabolism of soil microbes in all cases. In general, an increase of metal concentration adversely affects soil microbial activities, for example, soil microbial biomass (Fritze et al. 1996), weak enzyme activity (Kandeler et al. 1996), and increasing microbial respiration rate (Bogomolov and Chen 1996), which appears to be very useful indicators of soil pollutions (Brookes 1995; Szili-Kovács et al. 1999). Given a sufficiently high rate of addition, HMs added to soil in laboratory ecotoxicological studies result in a decrease in the amount of microbial biomass and a change in community structure (Maliszewska et al. 1985; Ohya et al. 1985; Naidu and Reddy 1988; Aoyama et al. 1993; Leita et al. 1995; Speir et al. 1995; Kandeler et al. 1996; Knight et al. 1997). This is not surprising; microorganisms differ in their sensitivity to metal toxicity, and sufficient metal exposure will result in immediate death of cells due to disruption of essential functions, and to more gradual changes in population sizes due to changes in viability or competitive ability. What is perhaps more surprising is that soil microorganisms subject to long-term metal stress, even at modest levels of exposure, are not able to maintain the same overall biomass as in unpolluted soils.

Development of tolerance and shifts in community structure could be expected to compensate for less of more sensitive populations. Instead, results from laboratory ecotoxicological studies suggest that changes in community structure go hand in hand with a decrease in the soil microbial biomass (Frostegård et al. 1993, 1996). There is now a considerable amount of evidence documenting a decrease in the soil microbial biomass as a result of long-term exposure to HM contamination from past application of sewage sludge (McGrath 1994; McGrath et al. 1995). Analysis of soil contaminated with HMs from other sources such as Cu and Zn in animal manures (Christie and Beattie 1989), runoff from timber treatment plants (Bardgett et al. 1994; Yeats et al. 1994), past application of Cu-containing fungicides (Zelles et al. 1994; Filser et al. 1995), and analysis of soils in the vicinity of metal-contaminated army disposal sites (Kuperman and Carreiro 1997) confirms that a decrease in the microbial biomass occurs at a relatively modest and sometimes even at a surprisingly low (Dahlin et al. 1997) metal loading. The widespread occurrence of this effect of metal toxicity suggests that there may be a common physiological explanation.

Enzyme activity is a soil property that is chemical in nature but has a direct biological origin. This activity arises from the presence of many types of enzymes that are present in the soil and within soil microorganisms. From an assortment of enzymes present and active in soil, phosphatases are interesting groups of enzymes

that catalyze the hydrolysis of phosphate from organic monoester linkages (Dmitri and Begonia 2008). Phosphates released from such phosphatase action are very important to the plants and microorganisms that depend on soil for their phosphorus requirements. Indications of specific inhibitory action of HMs have been produced in microbes as well (Fulladosa et al. 2005a, b). Such selective targeting of specific enzymatic systems and pathways suggests that certain members of the microbial community would be more sensitive to HM exposure than others, depending on the sensitivity of their critical metabolic pathways. Thus, while toxicity of HMs to microbes is a well-established phenomenon, the effects of those metals upon specific enzymatic systems at lower ("subacute") concentrations are not well known. Denitrification is a natural microbial process converting nitrate to dinitrogen gas during anaerobic respiration. Such reduction occurs sequentially, with nitrate converted to nitrite, nitric oxide, nitrous oxide, and, finally, nitrogen gas. A number of enzyme classes, mostly located in the periplasmic space, are involved in denitrification (Dmitri and Begonia 2008), with a number of corresponding genes that can be used as genetic markers for presence and expression of such enzymes in the soil metagenome. As denitrification-related enzymes are generally located within the cell membrane or periplasmic space, expelling HM ions out of the cell would place them in the immediate contact with denitrification-related enzymes, thus limiting utility of such a resistance strategy. The fact that denitrification enzymes are located on or near the outer cell surfaces further increases the vulnerability of the entire denitrification pathway to chemical disruption. Recent work has suggested a direct effect of HMs upon extracellular enzyme activities (Begonia et al. 2004; Hinojosa et al. 2004). Combined with the fact that scavenging/pumping systems are unlikely to protect the denitrification pathway from HM effects (and may, in fact, exacerbate the situation), it is expected that denitrification pathway would be uniquely sensitive to HMs. The notion of selective inhibition of denitrification steps by HMs has been supported by work of Holtan-Hartwig et al. (2002), suggesting the potential for production of undesirable by-products, such as nitrous oxide.

The second mechanism of microbial resistance to metals is evolution of enzyme forms resistant to metals. This resistance pathway is expected to be the predominant in the denitrifying bacteria, due to inability to use metal pumps for the reasons described above. The metal-resistant forms of enzymes present in metal-stressed denitrifying community are expected to be readily identifiable by their gene sequence and therefore their genetic signature. Disruption of denitrification by HMs could lead to a number of undesirable consequences, influencing the human health at both global and local levels. Suppressed denitrification in the soil could lead to enhanced nitrogen retention and flushing, resulting in nonpoint nutrient pollution in waterways receiving overland or subsurface flow from impacted locations. Nutrient pollution, in turn, leads to eutrophication and massive algal blooms, including those of toxic algae and cyanobacteria (e.g., *Microcystis*), affecting human populations relying on surface waters for municipal, recreational, or agricultural needs. Specific inhibition of nitrous oxide reductase by metal has been observed recently (Holtan-Hartwig et al. 2002), resulting in incomplete denitrification leading

to emission of nitrous (and possibly nitric) oxides. As nitrous oxide is a potent greenhouse gas that also damages ozone layer (Crutzen 1970; Dickinson and Cicerone 1986), denitrification disruption via metal contamination could act as a link between local metal contamination and global climate change phenomena.

Another features of HM pollutes soils are impeded litter decomposition and soil respiration (Marschner and Kalbitz 2003; Illmer and Schinner 1991). The degree of impedance, however, is determined by the rate of carbon and nitrogen mineralization. Thus, under HM pollution, the rates of such activities are impaired and carbon and nitrogen accumulate in the soil. Assay of soil respiration also helps to quantify the effects of metals on the total biological activity of soils. Addition of HM salts to soils usually causes an immediate decrease in respiration rates, but responses are determined by the properties of both the metal and the soil. The response of base respiration to metals is dependent on the nature of the substrates mineralized at the time of measurement. The response of base respiration to increasing doses of Cu, Cr, Ni, and Zn can be inconsistent, with increases in base respiration sometimes occurring even though both higher and lower doses of the same metal resulted in a decrease in base respiration (Doelman and Haanstra 1984). These bizarre and inexplicable responses probably result from strong interactive effects between both abiotic and biotic factors. A potential difficulty is that it is not possible to distinguish a metal toxicity effect from an effect of metal addition on substrate availability. Some metals such as Pb may decrease the amount of substrate available for respiration through the formation of complexes and thus decrease respiration, whereas death of microbial cells as a result of metal addition may explain the increase of the base respiration in response to metal addition (Leita et al. 1995). The initial response in soil respiration due to metal addition may therefore bear little relation to long-term effects, and possibly even less relation to the typical field situation where there often is an increasing amount of metal contamination over a period of many years.

When metal toxicity data to soil microbial processes and populations from the literature is summarized, an enormous variability in the data becomes apparent. In principle there are only two factors which may contribute to the discrepancies between studies: (1) factors which modify the toxicity of the metals and (2) differences in sensitivity of the microorganism(s) or microbial process(es). It is extremely difficult to separate these factors when metal toxicity is studied in soils, both because of the difficulties in determining the "bioavailability" of metals in soils and because of the complexity of soil microbial communities.

In microbial investigations, the term "bioavailability" is usually ill defined and is rarely quantified. Bioavailability is dependent on soil characteristics such as mineralogy, pH, texture, organic matter, iron oxide, and HM content as well as plants and microorganisms and can be assessed by the growth of the organism of interest and an evaluation of the uptake or toxicity of a metal after the fact (Wolt 1994). Plant root exudates both directly (e.g., Fe^{3+}) or through the effects exudates have on microbial activity and resulting rhizosphere chemistry. As bacteria are present within colonies in soil (Harris 1994) or protected by clays (Van veen et al. 1985; Ladd et al. 1995), they may often not be exposed to the equilibrium solution activity

of HMs. Metals may become bound to bacterial or fungal cell walls or on extracellular polysaccharides of bacteria, and the ingestion of such bacteria by protozoa or nematodes will result in vastly different exposures to metals in the predators than would result simply from exposure to the metals present in the soil solution. Microorganisms may also alter metal availability in their vicinity due to localized acidification on the environment or production of compounds which complex metals. Species of microorganisms (e.g., Berdicevsky et al. 1993), strains of the same species (e.g., Romandini et al. 1992), and also activities of the same microbial species (e.g., Balsalobre et al. 1993; Torslov 1993) can all show considerable differences in their sensitivity to metal toxicity.

14.3 Remediation Techniques of HM-Contaminated Soils

Since the industrial revolution, anthropogenic impacts have caused more and more hazardous HMs releasing to environment. Soils, being the basic and most essential part of the ecological system, are heavily contaminated, too. Compared to organic pollutants, the remediation of toxic metals in porous matrices (soil and sediment) requires a specific approach since hazardous HMs are indestructible, as they cannot be chemically or biologically degraded, hence require appropriate methods for their removal. Treatments make necessary metal extraction (e.g., by solubilization or complexation) to avoid their dissemination in the environment and/or the food chain contamination. Therefore, increasing attention has been paid in recent years to the remediation of polluted soils. To date, main four methods, chemical or physical remediation, animal remediation such as earthworm, phytoremediation, and microremediation, were proposed by researchers. Because of the obvious disadvantages and deficiency in feasibility, wide application of the former two methods is restricted. The latter two, namely, the use of plants and microbes, are preferred because of their cost-effectiveness, environmental friendliness, and fewer side effects. Using transgenic technology is a tendency in the future to create an ideal species purposely. In the future crop hyperaccumulators will be a better choice due to its feasibility, in the field of which current emphasis is scarce. Microbes, in many cases, are more efficient in accumulating and absorbing HMs because of their astronomical amount and specific surface area. Furthermore, technique of genetic engineering in microbes is easier and more mature than in plant cells. Therefore, using transgenic technology to create an optimum plant + soil + microbes combination would be a promising way in the future development (Gang et al. 2010).

14.3.1 Conventional Methods

Chemical or physical method which is named “conventional method” is early used and even endemically commercialized in America. The in situ or ex situ remediation of these methods is more often based on (1) improvement of the solubility and

bioavailability of HMs by synthetic chelators such as ethylenediaminetetraacetic acid (EDTA); (2) solidification/stabilization by either physical inclusion or chemical interactions between the stabilizing agent and the pollutant; (3) vitrification using thermal energy for soil fusion, allowing physical or chemical stabilization; (4) electrokinetical treatment which ionic species of the pollutant migrate to electrodes inserted into the soil; (5) chemical oxidation or reduction of the pollutant to attain chemical species with lower toxicity that are more stable and less mobile; and (6) excavation and off-site treatment or storage at a more appropriate site (Saxena et al. 1999). Most of these conventional remediation technologies are expensive and labor intensive, are technically limited to relatively small areas, and cause further disturbance to the already damaged environment (Alloway and Jackson 1991; Mench et al. 1994). These techniques for soil remediation may render the land useless for plant growth as they remove all biological activities, including useful microbes such as nitrogen-fixing bacteria, mycorrhiza, fungi, as well as fauna in the process of decontamination. Furthermore, natural soil, structure, texture, and fertility can be impaired by the method itself and by the regent added. Additionally, excessive use of chelators like EDTA which is both toxic and nonbiodegradable would poison both plants and microbes (Gang et al. 2010). Therefore, due to improved knowledge of the mechanisms of uptake, transport, tolerance, and exclusion of contaminants in microorganisms and plants, development of alternative technologies, named bioremediation and phytoremediation which respectively refer to the use of microbes and plants, has been promoted.

14.3.2 Biological Methods

Bioremediation is based on the potential of living organisms, mainly microorganisms and plants, to detoxify the environment (Anderson and Coats 1994). Bioremediation technologies could be classified under two main categories, namely, “microbial-based” and “plant-based” remediation methods. For organic pollutants, the goal of phytoremediation is to completely mineralize them into relatively nontoxic constituents, such as carbon dioxide, nitrate, chlorine, and ammonia (Cunningham et al. 1997). However, HMs are essentially immutable by any biological or physical process short of nuclear fission and fusion, and thus their remediation presents special scientific and technical problems. Furthermore, in the case of uptake of HMs by microbes, there is no cost-effective method to collect the microbes from soil body. Plant-based bioremediation technologies have been collectively termed as “phytoremediation” that refers to the use of green plants and their associated microbiota for the in situ treatment of contaminated soil and groundwater. While the use of plants for remediation of contaminated soils has been developed much more recently, it was not until the 1990s that the concept of phytoremediation emerged as a promising technology that uses plants for decontamination of polluted sites (Barceló and Poschenrieder 2003). With a few notable exceptions, the best scenarios for the phytoremediation of HMs involve plants

extracting and translocating a toxic cation or oxyanion to aboveground tissues for later harvest, converting the element to a less toxic chemical species (i.e., transformation), or at the very least sequestering the element in roots to prevent leaching from the site (Meagher 2000).

Although phytoremediation offers cost advantages and is comparable to in situ bioremediation and natural attenuation (Cunningham et al. 1997), it has its own limitations, for example, the difficulty with treating wastes greater than three meters deep, possible uptake of contaminants into leaves and release during litter fall, inability to assure cleanup below action levels in a short period of time, difficulty in establishing the vegetation due to toxicity at the site, and possible migration of contaminants off-site by preferential flow or by binding with soluble plant exudates (Schnoor 1997). Therefore, and most likely due low bioavailability of PTEs and/or low biomass of hyperaccumulators, phytoremediation method usually remains as a time-consuming process (Cunningham et al. 1997; Khodaverdiloo and Homaee 2008). However, numerous studies have indicated that soil microbial community such as arbuscular mycorrhizal (AM) fungi could help to overcome these limitations, for example, by enhancing uptake of nutrient elements as well as water by host plants through their extraradical mycelial networks (Marschner and Dell 1994) and protecting the host plants against HM toxicity (Leyval et al. 1997). Therefore, inoculation of plants with AM fungi can be a potential biotechnological tool for successful restoration of degraded ecosystems (Dodd and Thompson 1994; Mathur et al. 2007).

14.3.2.1 Phytoremediation

Phytoremediation is a solar-driven remediation technology with greatly reduced costs and minimum adverse side effects (Cunningham and Ow 1996; Cunningham et al. 1997; Garbisu et al. 2002; Glick 2003). Within the field of phytoremediation, different categories have been defined such as phytofiltration, phytostabilization, phytovolatilization, phytodegradation, phytostimulation, and phytoextraction; among them phytoextraction and phytostabilization are of great concern for remediation of HM-contaminated soils. Phytoextraction is the use of hyperaccumulating/high-biomass plants to uptake the contaminants in their aboveground tissues with subsequent harvest, recovery, and disposal or recycling of the metals (Geiger et al. 1993; Kayser et al. 2000; Hammer et al. 2003). Hyperaccumulators are wild species that can accumulate large amounts of specific metals in their shoots, but they are often with low biomass. The fast-growing, high-biomass plants are usually not metal specific and have low to average HM concentrations (Hammer et al. 2003). Phytoextraction has been proposed as a suitable alternative to destructive techniques used so far to clean up soils contaminated with HMs. Indeed, the use of plants to remove metals from soils is environmental friendly, and its cost is much lower compared to engineering-based techniques (Cunningham and Ow 1996; Cunningham et al. 1997; Garbisu et al. 2002; Glick 2003). Although phytoextraction is a promising option to remediate contaminated soils, so far, no suitable method is yet available to

remove metals in a reasonably short time. Indeed, the potential for phytoextraction depends not only on bioaccumulation factor but also on plant biomass. However, the hyperaccumulator and high-biomass plant species fulfill only one of these conditions.

In phytostabilization, plants are used for immobilizing contaminant metals in soils or sediments by root uptake, adsorption onto roots, or precipitation in the rhizosphere. By decreasing metal mobility, these processes prevent leaching and groundwater pollution, and bioavailability is reduced and fewer metals enter the food chain (Barceló and Poschenrieder 2003).

Metal Hyperaccumulators

Selection of plants for phytoremediation of metals depends on the type of application (Schnoor 1997). Plants show several response patterns to the presence of potentially toxic concentrations of HMs. Most are sensitive even to very low concentrations, others have developed resistance, and a reduced number behave as hyperaccumulators of HMs (e.g., Brooks 1998; Salt et al. 1998). Hyperaccumulators have opened up the possibility to use phytoextraction for remediation of HM-contaminated environments (Barceló and Poschenrieder 2003) and provide valuable tools for reclamation of polluted soils, enhancement of soil quality, and recovery and reestablishment of biotic. Plants with metal resistance mechanisms based on exclusion can be efficient for phytostabilization technologies. Hyperaccumulator plants, in contrast, may become useful for extracting toxic elements from the soil and thus decontaminate and restore fertility in polluted areas. In recent years, improved knowledge of the mechanisms of uptake, transport, and tolerance of high metal concentrations in these plants (e.g., Assunçao et al. 2001; Hall 2002) has opened up new avenues for remediation by phytoextraction.

At least 400 species distributed in 45 botanical families are considered metal hyperaccumulators (Brooks 1998). By definition, hyperaccumulators are herbaceous or woody metallophytes, belong to the natural vegetation of metal-enriched soils, and accumulate and tolerate without visible symptoms a hundred times or greater metal concentrations in shoots than those usually found in non-accumulators. Baker and Brooks established 0.1 % as the minimum threshold tissue concentrations for plants considered Co, Cu, Cr, Pb, or Ni hyperaccumulators, while for Zn or Mn the threshold is 1 % (Baker and Brooks 1989). As discussed in the next section, these species have evolved internal mechanisms that allow them to take up and tolerate large metal concentrations that would be exceedingly toxic to other organisms.

An ideal plant species for remediation purposes should grow easily and produce high biomass quickly on HM-contaminated soils, have high root-to-shoot translocation and high bioconcentration factors, and tolerate high shoot metal concentrations (Barceló and Poschenrieder 2003). Unfortunately, most metal hyperaccumulator plants grow quite slowly and have a low biomass, while plants that produce a high biomass quickly are usually sensitive to high metal concentrations.

HM complexes in hyperaccumulators plants are mainly associated with carboxylic acids like citric, malic, and malonic acids. These organic acids are implicated in the storage of HMs in leaf vacuoles. Amino acids like cysteine, histidine glutamic acids, and glycine also form HM complexes in hyperaccumulators (Homer et al. 1997). These complexes are more stable than those with carboxylic acids. They are mostly involved in HM transport through xylem. Moreover, hyperaccumulator plants can increase availability of metals like Fe and also Zn, Cu, and Mn by releasing chelating phytosiderophores. Hyperaccumulation mechanisms may then be related to rhizosphere processes such as to the release of chelating agents (phytosiderophores and organic acids) and/or to differences in the number or affinity of metal root transporters (Lombi et al. 2001).

Although hyperaccumulator plants are widely used in phytoextraction, they are generally of low biomass, inconvenient for phytoremediation. However, arbuscular mycorrhizae fungi (AMF), especially *Glomus intraradices*, and colonized *Festuca* and *Agropyron* species have shown higher HM (Zn, Cd, As, and Se) content than non-colonized controls (Giasson et al. 2006). As for hyperaccumulators, fungi can synthesize cysteine-rich metal-binding proteins called metallothioneins (Gadd and White 1989). AMF might therefore be directly implicated in HM hyperaccumulation in plants.

Cellular Mechanisms of Plant Metal Detoxification and Tolerance

Although many metals are essential, all metals are toxic at higher concentrations, because they cause oxidative stress by formation of free radicals and/or they can replace essential metals in pigments or enzymes disrupting their function. Thus, metals render the land unsuitable for plant growth and destroy the biodiversity. However, as discussed earlier, some specific plant species preferentially grow on metalliferous soils and are capable to accumulate very high levels of specific metals.

These plants are perfectly adapted to the particular environmental conditions of their habitat, and high metal accumulation may contribute to their defense against herbivores and fungal infections (Barceló and Poschenrieder 2003). However, usually, the metabolic and energetic costs of their adaptation mechanisms do not allow them to compete efficiently on uncontaminated soil with non-metallophytes. Metal hyperaccumulators are highly specialized models of plant mineral nutrition. As it has been discussed by Barceló and Poschenrieder (2003), several hypotheses have been proposed to explain the mechanisms of metal hyperaccumulation including (1) complex formation and compartmentation, (2) deposition hypothesis, (3) inadverted uptake, and (4) hyperaccumulation as a defense mechanism against abiotic or biotic stress conditions (Barceló and Poschenrieder 2003).

Plants may use several potential cellular/molecular mechanisms for detoxification of and tolerance to excess concentrations of specific HMs in the environment (Hall 2002). Generally, the strategy adopted by plants aims to avoid the buildup of excess metal levels in the cytosol and thus to prevent the onset of toxicity

symptoms. This is achieved by the use of various mechanisms that are present and likely to be employed in general metal homeostasis in all plants. It appears likely that specific mechanisms are employed for specific metals in particular species. Potential cellular mechanisms for metal detoxification and tolerance in higher plants include (but not limited to) (a) restriction of metal movement to roots by mycorrhizas, (b) binding to cell wall and root exudates, (c) reduced influx across plasma membrane, (d) active efflux into apoplast, (e) chelation in cytosol by various ligands, (f) repair and protection of plasma membrane under stress conditions, (g) transport of PC–Cd complex into the vacuole, and (h) transport and accumulation of metals in vacuole (Hall 2002). It is also possible that more than one mechanism may be involved in reducing the toxicity of a particular metal (Hartley-Whitaker et al. 2001; Hall 2002). These processes involved in reducing toxicity are of considerable current interest because an understanding of the means of manipulating metal tolerance could be important in the development of crops for phytoremediation of, for example, HM-contaminated soils (Salt et al. 1998). However, as discussed by others (e.g., Hall 2002), there is no single mechanism that can account for tolerance to a wide range of metals (Macnair et al. 2000).

Although not always considered in general reviews of plant metal tolerance mechanisms, mycorrhizas, and particularly ectomycorrhizas that are characteristic of trees and shrubs, can be effective in ameliorating the effects of metal toxicity on the host plant (e.g., Hüttermann et al. 1999; Jentschke and Godbold 2000). However, the mechanisms involved in conferring this increase in tolerance have proved difficult to resolve; they may be quite diverse and show considerable species and metal specificity since large differences in response to metals have been observed, both between fungal species and to different metals within a species (e.g., Hüttermann et al. 1999; Rahmanian et al. 2011).

The mechanisms employed by the fungi at the cellular level to tolerate HMs are probably similar to some of the strategies employed by higher plants, namely, binding to extracellular materials or sequestration in the vacuolar compartment. Regarding the role of ectomycorrhizas in metal tolerance by the host plant, most mechanisms that have been proposed involve various exclusion processes that restrict metal movement to the host roots. These have been extensively reviewed and assessed (Jentschke and Godbold 2000) and include absorption of metals by the hyphal sheath, reduced access to the apoplast due to the hydrophobicity of the fungal sheath, chelation by fungal exudates, and adsorption onto the external mycelium.

14.3.2.2 Bioremediation

Gadd (2001) defined bioremediation as an area of environmental biotechnology and as the application of biological processes to the treatment of pollution. Applications of fungi in environmental protection and recovery of metals have received more attention in recent years. Biosorbent fungi are engaged microorganisms for the process of biosorption of metal ions on their surface. Biosorption to *Rhizopus*,

Mucor, *Penicillium*, and *Aspergillus* genera is well documented. Biosorption is the non-metabolic sorption process. Many potential binding sites are present in fungal cell walls, including chitin, chitosan, amino, carboxyl, phosphate, sulfhydryl, and other functional groups (Volesky and Holan 1995; Gadd 2001). Fungal solubilization of insoluble metal compounds occurs by several mechanisms such as protonation of the anion of the metal compound, the production of organic acids, siderophores (it is also as extracellular metal-binding molecules), and chelating agents (Morley et al. 1996; Singh 2006). Metal sequestration in the cytosol by induced metal-binding molecules such as metallothioneins and phytochelatins is an intracellular detoxification in fungi (Cobbett 2000). Metal(loid)s may be transformed by fungal reduction, methylation, and dealkylation, so through this mobility and toxicity of metals modified (Gadd 2001). Mechanisms of fungal biosorption, solubilization, transformation, and immobilization of metal(loid)s are of potential for bioremediation.

14.3.2.3 Mycoremediation: Fungal Bioremediation

Mycoremediation is a form of bioremediation, which more broadly refers to degrading or removing organic and inorganic toxicants in the environment using biological processes. Mycoremediation went from the theoretical to the practical just over a decade ago. The term "mycoremediation" was coined by the American mycologist Paul Stamets, who has studied many potential uses of mushrooms.

Mycoremediation is the process of using fungi to return an environment contaminated by pollutants to a less or without contaminated state. It can apply to contaminated soil, oil spills, industrial chemicals, contaminated surface water, and farm waste. It is not widely used at present, but the below-noted applications suggest its broader potential. Some examples of used fungi included the following: *Lentinus edodes* can degrade pentachlorophenol (PCP), *Pleurotus pulmonarius* can degrade atrazine, *Phanerochaete chrysosporium* can degrade biphenyl and triphenylmethane, and some fungi have also proven useful in remediation of HMs that are not degraded further but fungi can extract them from soil or water and accumulate them in their or host tissues (Singh 2006). Some of them are hyperaccumulators, capable of absorbing and concentrating HMs in the mushroom fruit bodies. The mushrooms can be used to remediate the metal-polluted soil. Many studies carried out to evaluate the possible danger to human health from the ingestion of mushrooms containing HMs (Gast et al. 1998; Ouzouni et al. 2007; Elekes et al. 2010). Numerous data on metal contents in fungal fruiting bodies were published previously (Alonso et al. 2003; Soylak et al. 2005; Svoboda et al. 2006; Elekes et al. 2010), and the reported metal concentrations in the fruiting body of mushrooms vary from one species to another, because of many factors affecting the accumulation rate (Elekes et al. 2010). Elekes et al. (2010) indicated that HM concentrations in the fruiting body of mushrooms were mean values of 11.94 mg kg^{-1} for Ti, 1.07 mg kg^{-1} for Sr, 1,163.86 mg kg^{-1} for Bi, and 17.49 mg kg^{-1} for Mn. The bioconversion factor of HMs represented the level of metals concentration in the

mushrooms body correlated with the metallic element in the soil on which the fungus grow and had the highest values in *Marasmius oreades* species for bismuth and titanium. Totally, fungi perform a wide variety of ecosystem functions such as the important role in mycoremediation and may be a simple and relatively cheap method of environmental remediation, especially if indigenous species of each site are isolated, identified, and used.

Mycorrhizoremediation with the Emphasis on Arbuscular Mycorrhizal Fungi

Mycorrhizas are mutualistic associations of plant roots and fungi. The symbiotic fungi are provided with carbon by the photobionts, while the fungi may protect the symbiosis from harsh environmental conditions, increase the absorptive area, and provide increased access to inorganic nutrients and water (Gadd 2010). The mycelium of mycorrhizal fungi is more resistant to abiotic agents than the root itself, and this may compensate for reduced root growth. They increase tolerance to extreme conditions. They are crucial in the ecology and physiology of terrestrial plants and are the rule in nature, not the exception (Khan 2006). Mycorrhizal associations vary widely in structure and function and included arbuscular mycorrhiza, ectomycorrhiza, ectendomycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, ericoid, and orchid mycorrhiza. Mycorrhizal fungi act on ecosystems in widely different ways. Amongst them the arbuscular mycorrhizal fungi (AMF) are of ecological and economical importance. AMF are universal and ubiquitous rhizosphere microflora forming symbiosis with plant roots of Bryophyta, Pteridophyta, Gymnospermae, and Angiospermae in nature (Smith and Read 2008). The AMF are as biofertilizers and bioprotectants. They cannot be cultured in the absence of their host, and the extracellular hyphal network is not as extensive as ectomycorrhiza associations. These fungi belong to Glomeromycota (Schubler et al. 2001).

Occurrence of AMF has been reported in relation to plants growing on HM-polluted soils (Leyval et al. 1995; Göhre and Paszkowski 2006; Khade and Adholeya 2007; Zarei et al. 2008a, b). Many of plants are highly dependent on arbuscular mycorrhiza. Use of arbuscular mycorrhizal symbiosis has multidirectional effects such as excretion of chelating agents, producing of plant growth-promoting factors and increasing of plant biomass, extending of soil rhizosphere (mycorrhizosphere), and increasing of uptake per unit surface area. AMF can help in ecosystem remediation (Gaur and Adholeya 2004). Rhizoremediation by mycorrhiza symbiosis, that is, mycorrhizoremediation, is an enhanced form of phytoremediation (Khan 2006). In some cases, AMF have generally such a strong influence on plant biomass and can increase HMs uptake and root-to-shoot transport (phytoextraction), while in other cases AMF contribute to HM stabilization within the soil/root and reduce their uptake (phytostabilization) (Zarei and Sheikhi 2010).

It was proved that the AMF are effective in immobilization of metals in the plant rhizosphere and help in HM stabilization by their accumulation in a nontoxic form in plant roots and extracellular mycelia (Zarei and Sheikhi 2010). There are similar

strategies in decreasing of the toxic effects of HMs for fungi and host plants that include immobilization of these elements by the fungal exudations, their deposit in polyphosphate granular, adsorption of elements on the cell wall, and chelation in the fungal organs (Göhre and Paszkowski 2006). Glomalin is a glycoprotein produced abundantly on hyphae and spores of AMF in soil and in roots and is able to link with HMs and extract them from the soil. Therefore, it can be said the fungal strains that secrete more glomalin are more suitable for biological stabilization (Göhre and Paszkowski 2006). Binding HMs with chitin in the cell wall of fungal organs reduces their concentration in the soil solution, and broad absorption surface of extraradical mycelia is considered an important source of discharged HMs from the soil solution. The vesicles of fungi also have a role in accumulation of toxic compounds and in this way can help in the detoxification of metals. High concentration of HMs in mycorrhizal roots than non-mycorrhizal ones showed that the fungus could maintain HMs in surface and/or within mycelia, for example, zinc concentration in fungal mycelia in comparison with plant tissues was reported more than ten times (Chen et al. 2001). Kaldorf et al. (1999) showed that most of zinc was accumulated within the fungal tissue, such as vesicles inside the cells of root cortex. It seems that the immobilization of HMs in fungal tissue is one of the mechanisms of reducing HMs toxicity in mycorrhizal plants. The results of Rufyikiri et al. (2004) indicated the accumulation of uranium in the plant root of mycorrhizal plant that was exposed to the high levels of uranium and the supportive effect of this fungus for the host plant. High uptake of HMs by mycorrhizal roots and the possible role of AM fungus in phytostabilization were demonstrated by Wang et al. (2007b) and Wang et al. (2007c).

Plant colonized by AMF can also increase the uptake and accumulate of HMs in plant shoots or phytoextraction (Leung et al. 2006; Wang et al. 2007a). AMF increased the uptake and accumulation of arsenic in hyperaccumulator plant of *Pteris vittata* (Leung et al. 2006). It was shown dynamic and mobilization of zinc and transferring to shoots of corn and clover colonized by AMF (Chen et al. 2003). In a pot experiment, Zarei and Sheikhi (2010) illustrated that for corn and *Festuca* plants and under the high soil pollution (500 mg Zn kg^{-1}), *Glomus mosseae* (a noticeable indigenous fungus in HM-contaminated soil) was the most effective fungal species in Zn extraction and translocation.

Overall, it is possible to enhance and improve the capabilities of plants in different types of phytoremediation processes by inoculating with appropriate arbuscular AMF (i.e., mycorrhizoremediation).

The potential role of mycorrhizoremediation in HM-contaminated soils is becoming an interest, and it needs to completely understand the ecological complexities of the plant–microbe–soil interactions, mechanisms for how AMF are involved in HM absorption and transportation in plants and the tolerance to HM. Multidisciplinary investigations using molecular, biochemical, and physiological techniques and employment of appropriate combination of plant–fungus in remediation strategies for HM-contaminated soils may be helpful.

Diversity of AMF in Contaminated Soils

The presence of AMF propagules in the HM-polluted soils was abundantly reported (Bohn and Liberta 1982; Diaz and Honrubia 1994; Pawlowska et al. 1996; Gaur and Adholeya 2004). Weissenhorn et al. (1993) measured root colonization in the polluted soils with 1,220 and 895 mg cadmium and lead per kg, respectively, up to 40 %. Root colonization rate in the dominant native plants of an HM-contaminated site was measured 35–85 % and the spore numbers 80–1306 per 200 g dry soil along the transect (Zarei et al. 2008a). Many plant species, such as *Fragaria vesca*, *Viola calaminaria*, *Veronica rechingeri*, *Solidago gigantea*, *Thymus polytrichus*, *Holcus lanatus*, and *Thlaspi praecox*, growing well at natural HM-polluted areas were colonized by diverse AMF. *Acaulospora*, *Entrophospora*, *Gigaspora*, and *Glomus* genera in symbiosis with plant species grown in the HM-contaminated soils were identified (Tonin et al. 2001; Turnau et al. 2001; Gonzalez-Chavez et al. 2002; Whitfield et al. 2004; Vallino et al. 2006; Vogel-Mikus et al. 2006; Zarei et al. 2008a, b; Long et al. 2010; Zarei et al. 2010). Some AMF species and sequences types may be exclusively found in the high HM pollution levels (Zarei et al. 2010). Long et al. (2010) studied the diversity of AMF communities associated with five selected plant species (*Phytolacca americana*, *Rehmannia glutinosa*, *Perilla frutescens*, *Litsea cubeba*, and *Dysphania ambrosioides*) from severely HM-polluted soils in Dabaoshan Mine region, China, using molecular methods. DGGE and sequence analysis revealed that *Glomus* dominated all of the samples except for the roots of *D. ambrosioides*, while *Kuklospora* and *Ambispora* dominated the roots of *D. ambrosioides* and the rhizosphere of *P. americana*. The studies indicated that diverse AMF are associated with plants grown in HM-polluted soils.

Arbuscular Mycorrhiza and HMs

High amounts of HMs can delay, reduce, or even completely eliminate AMF spore germination and AM colonization (Gildon and Tinker 1981; Del Val et al. 1999). Similarly, Boyle and Paul (1988) reported a negative correlation between Zn concentrations in a soil treated with urban-industrial sludge and AM colonization in barley. In other studies, however, the addition of metal containing sludge did not affect AM development under field conditions (Arnold and Kaputska 1987). These contrasting results may be explained due to the fact that different AMF ecotypes can exhibit varying degrees of tolerance to metals (Haselwandter et al. 1994). A higher tolerance to Cu, Zn, Cd, and Pb of indigenous fungi from sludge-polluted sites, in comparison to reference isolates from unpolluted soils, has been reported (del Val et al. 1999). AMF species isolated from the HM-polluted soil could be more adapted and tolerated to HM pollution (Gildon and Tinker 1983; Weissenhorn et al. 1993; Diaz et al. 1996). Gildon and Tinker (1981) isolated a strain of *Glomus mosseae* from HM-contaminated soils that could tolerate 100 mg Zn kg^{-1}. Dueck et al. (1986) reported the presence of some strains of *Glomus fasciculatum* as

tolerant strains to HMs in the several HM-contaminated areas in the Netherlands. Weissenhorn et al. (1993) isolated the spores belonging to *Glomus mosseae* group from contaminated soils with HMs in France. They showed that the two *Glomus mosseae* strains isolated from the polluted soils with cadmium had the ability to tolerate the cadmium concentrations from 50 to 70 and 200 to 500 mg l^{-1}, respectively. Sensitivity of different AM species or isolates and even propagules to different HMs may be varied. This can be dependent on phenotypes or genotypes characteristics of fungal species and HM type. Zarei (2008) demonstrated spore numbers were more affected by Zn and Pb concentrations than root colonization. The variations of AM fungi propagules were more related to available than total concentration of both metals. Metal-adapted AMF have a more efficient protecting effect on metal tolerance of host.

Mechanisms for HM Tolerance in AMF

Because of being compulsive of AMF symbiosis with plants and lack of its growth in conventional culture media, less information is available about the tolerance mechanisms of these fungi to HMs. The more information was based on plant response to HMs and observation of fungal structures within colonized roots, which were difficult to separate of fungal and plant responses. Different mechanisms were proposed for explanation of plant responses to the high concentrations of HMs. Primary effects can be diagnosed in the molecular, biochemical, and cellular levels, and next effects are visible in the physiological and organelle levels. Generally, suggested tolerance mechanisms of AMF to HMs are reviewed by Göhre and Paszkowski (2006), Gonzalez-Chavez et al. (2006), and Hildebrandt et al. (2007). In these fungi, the tolerance does not have a general pattern and may be different among species in the response to a particular metal. There are also large changes in the rate of tolerance to HMs among different populations of a species or ecotype. The mechanisms included extracellular chelation, binding of HM to the cell wall components of fungi, control of HMs transferring to the cell by metals' specific and nonspecific carriers in their plasma membrane, chelation in the cytoplasm as an intracellular buffer system, HM export via specific or nonspecific active or passive transport from cells and metal sequestration in the vacuoles, transport of HMs in the hyphae of the fungus, and active and passive transport of metals from the arbuscules to plant cells. Effects of AMF on the plant nutrition, root exudations, rhizosphere microbial communities, soil structure, and protection against environmental stresses can be considered indirect mechanisms in the increasing tolerance to HMs (Chen et al. 2003; Turnau et al. 2006; Vivas et al. 2006).

AMF colonization of the roots has a significant impact on the expression of several plant genes coding for proteins presumably involved in HM tolerance/detoxification. A novel metallothioneins (MT)-like polypeptide designated *GmarMT1* that is modulated in a metal and life cycle stage-dependent manner and may afford protection against HMs (and other types of stress) to both partners of the AM symbiosis (Lanfranco et al. 2002). Hildebrandt et al. (2007) described

genes expression in extraradical mycelia (ERM) of in vitro cultured *Glomus intraradices* Sy167 supplemented with different HMs (Cd, Cu, or Zn). The expression of several genes encoding proteins potentially involved in HM tolerance varied in their response to different HMs. Such proteins included a Zn transporter, a metallothionein, a 90-kDa heat shock protein, and a glutathione S-transferase (all assignments of protein function are putative). Studies on the expression of the selected genes were also performed with roots of *Medicago truncatula* grown in either a natural, Zn-rich HM soil or in a non-polluted soil supplemented with 100 μM $ZnSO_4$. The transcript levels of the genes analyzed were enhanced up to eightfold in roots grown in the HM-containing soils. The data obtained demonstrate the HM-dependent expression of different AMF genes in the intra- and extraradical mycelium. The HM-dependent induction of genes encoding a heat shock protein and a glutathione S-transferase in the mycelium of the AMF *G. intraradices* Sy167 suggests that alleviating the HM-induced oxidative stress might be of primary concern for AMF exposed to elevated HM. Other strategies possibly contributing to HM tolerance appear to be involved as well, which is indicated by the significantly enhanced expression of the metallothioneins and the Zn transporter gene, particularly under Cu stress. Molecular bases of HM tolerance in AM symbiotic system may also help the selection of the most effective AMF isolates (Tonin et al. 2001; Turnau et al. 2001) and plant–fungus combinations for bioremediation and soil protection purposes.

14.4 Biotechnological Approaches to Improve Phyto- and Bioremediation Efficiencies

With advances in biotechnology, biological remediation techniques, including phyto- and bioremediation, have become one of the most rapidly developing fields of environmental restoration and have been commercially applied for the treatment of hazardous wastes and contaminated sites. HM-hyperaccumulating plant species which possess a unique ability to accumulate metals to extremely high concentrations without suffering any toxic effects are unsuitable for phytoextraction purposes, due to their slow growth rate and low biomass. Genetic modification of fast-growing plants might be a viable alternative and provides a powerful method of improving the capacity of these plants to remediate various contaminants including HMs. Additionally, hyperaccumulators can provide an important resource of genes which are responsible for trace element hyperaccumulation and detoxification through unique biochemical and genetic mechanisms (Glazer and Nikaido 2007).

One of the important approaches using genetic engineering to enhance phytoremediation potential is to transform fast-growing host plants with unique genes from natural hyperaccumulators. One such gene encodes the enzyme selenocysteine methyltransferase (SMT), which has been cloned from the Se

hyperaccumulator, *Astragalus bisulcatus* (Arshad et al. 2007). SMT converts the amino acid, selenocysteine, to the nonprotein amino acid, methylselenocysteine (MetSeCys). By doing so, it diverts the flow of Se from the Se-amino acids that may otherwise be incorporated into protein, leading to alterations in enzyme structure and function and possible toxicity. Additionally, Se-Cys may also cause oxidative damage. Transgenic plants overexpressing SMT show enhanced tolerance to Se, particularly selenite, and produced three- to sevenfold more biomass than the wild type plants.

Metallothioneins (MTs) and phytochelatins (PCs) are well-known HM-chelating proteins and peptides that play important roles in the detoxification of toxic HMs and the regulation of intracellular concentrations of essential metals in various organisms. Therefore, the expression of MTs and PCs in higher plants in order to enhance tolerance to HMs and their accumulation has great potential for phytoremediation of toxic HMs from contaminated soil and water (Meagher 2000; Mejare and Bülow 2001). Researchers expected that increasing the concentrations of metal-binding proteins or peptides in plant cells would increase metal-binding capacity and tolerance. In higher plants, PCs mainly function for detoxification of toxic HMs rather than MTs. Moreover, PCs have a higher metal-binding capacity rather than do MTs (Mehra and Mulchandani 1995). Therefore, modification or overexpression of PC synthase for accumulation of high levels of PCs seems to be a more practical approach to enhance HM accumulation in plants (Kazumasa et al. 2005). Overexpression of genes involved in PC synthesis, such as GSH1, GSH2, and PCS, encoding gamma-glutamylcysteine synthetase, glutathione synthetase, and PC synthase, respectively, has been shown to increase Cd tolerance in various heterologous expression systems. Likewise, heterologous MT overexpression often leads to increased tolerance to Cu and, occasionally, Cd and Zn. In general, overexpression of metal sequestration traits is associated with marginally to moderately increased accumulation of the metals concerned, presumably due to a delayed downregulation of the transporters involved in their uptake.

The approach of overexpressing genes that catalyze rate-limiting steps can also be used for the phytoremediation of HMs. GSH (Glu-Cys-Gly) plays an essential role in HM detoxification by plants. GSH is the direct precursor of PCs, which are metal-binding peptides involved in HM tolerance and sequestration (Steffens 1990). Additionally, GSH is a major component of the active oxygen scavenging system of the cell (Thomas 2008) and can protect the plant cell from Cd-induced oxidative stress (Gallego et al. 1996; Wecks and Clisjsters 1997). It is also possible that GSH detoxifies Cd by directly forming a GSH–Cd complex such as that reported for yeast (Litz and Lavi 1997). The role of GSH and PCs in HM tolerance is illustrated by the Cd hypersensitivity of Arabidopsis mutants defective in GSH and PC biosynthesis (Howden et al. 1995). γ-glutamylcysteine synthetase (γ-ECS) catalyzes the first step in the ATP-dependent synthesis of GSH. This is considered to be the rate-limiting step in the biosynthesis of GSH since the activity of this enzyme is subject to feedback regulation by GSH and is dependent upon the availability of cysteine (Steffens 1990). Zhu et al. (1999) studied the effect of overexpression of *E. coli*-γ-ECS, targeted to the chloroplasts of Indian mustard. The transgenic plants had

two- to threefold higher levels of γ-EC as well as GSH and PC when subjected to Cd. Their increased Cd tolerance was almost certainly due to their higher production of PCs or GSH. In addition to conferring tolerance to Cd, overexpression of γ-ECS led to an increase in total shoot S suggesting an added advantage of enhanced S assimilation (Zhu et al. 1999). Similar results were also obtained in the case of poplar plants overexpressing γ-ECS (Arisi et al. 2003; Noctor et al. 1998). Overexpression of glutathione synthetase in Indian mustard also led to enhanced levels of GSH and PC2 in the presence of HMs (Zhu et al. 1999).

In the field of bioremediation, advances in genetic and protein engineering techniques have opened up new avenues to move towards the goal of genetically engineered microorganisms (GEMs) to function as "designer biocatalysts," in which certain desirable biodegradation pathways or enzymes from different organisms are brought together in a single host with the aim of performing specific reactions. A number of opportunities for improving degradation performance using GEMs have been described (Timmis and Piper 1999). Genetic engineering also permits the combination of several degradative activities within a single host organism. If a single strain is constructed to perform several related or unrelated metabolic activities, the efficiency and predictability of the process may be significantly enhanced. Such recombinant strains may be useful for the bioremediation of recalcitrant compounds (Brenner et al. 1994). Requirements for the design of bacteria with multiple pathways for use in bioremediation have been described (Lau and Lorenzo 1999; Gibson and Parales 2000). Timmis and Piper (1999) suggested a strategy for designing organisms with novel pathways and the creation of a bank of genetic modules encoding broad specificity enzymes or pathway segments that can be combined at will to generate new or improved activities. The use of appropriate regulatory circuits can enhance substrate flux through these designed pathways, and rationally engineering the pathway branch points can avoid or reduce substrate misrouting (Timmis and Piper 1999).

The diversity and adaptability of microorganisms allows them to thrive in harsh, toxic environments that prevent the growth of higher plants. For example, solar evaporation ponds, which are used to collect Se-contaminated agricultural drainage water, have extremely high concentrations of salt, Se, and other toxic trace elements. The specific composition of the microbial communities present in these ponds may themselves be useful for the bioremediation of Se since bacteria are able to produce volatile Se (Danika et al. 2005). Additionally, they may serve as reservoirs of unique genes involved in tolerance and volatilization of Se. Identification of the genes involved in these processes could pave the way for generating highly efficient plants by transferring these genes to the plants (McIntyre 2003).

Nowadays, developing methods to accelerate natural processes used in bioremediation of contaminated environments and also scientific understanding needed to harness these processes is necessary. Except few limiting factors, this technology has the ability to rejuvenate the contaminated environments effectively. However, rapid advances in the last few years have helped us in the understanding of process of bioremediation. The use of culture-independent molecular techniques has definitely helped us to understand the microbial community dynamics and structure and

assisted in providing the insight in to details of bioremediation which has surely facilitated to make the technology safer and reliable. Bioremediation in relation to process optimization, validation, and its impact on the ecosystem can be performed, and by judicious use of the models that can predict the activity of microorganisms that are involved in bioremediation with existing geochemical and hydrological models, transformation of bioremediation from a mere practice into a science is now a reality. With the exciting new development in this field and focus on interdisciplinary research and using it on gaining the fundamental knowledge necessary to overcome the obstacles facing current technologies and also with respect to ethical, legal, and social issues involved, this technology will go a long way in cleaning the environment in near future (Keshav et al. 2010).

14.4.1 Assessment of Remediation Efficiency by Microbial Indicators of Soil Health

Despite the current great interest in improving the HM extraction capacity of hyperaccumulating plants, their influence on soil microorganisms has been rarely investigated (Delorme et al. 2001; Gremion et al. 2004). In fact, up to date, when evaluating the success of a phytoextraction process, emphasis has mostly been placed on metal removal. But it is most important to emphasize that the ultimate goal of any soil remediation process (physicochemical or biological processes) must be not only to remove the contaminant(s) from the polluted site or to render their harmless but also, most importantly, to restore the capacity of the soil to perform or function according to its potential as well (i.e., its health) (Hernandez-Allica et al. 2006). After all, some traditional methods of soil "remediation" irreversibly alter the functionality of the soil ecosystem while removing the contaminants, which is clearly not desirable and must be avoided at all costs. Soil quality or soil health (both terms are often used interchangeably) can be defined as the capacity of a given soil to successfully and sustainably perform its functions and ecosystem services from both an anthropocentric and ecocentric point of view and, most importantly, to properly recover its functionality after a disturbance. Regarding the recovery of soil health/functioning derived from the phytoextraction process, an ideal target should be to return to the conditions of a valid control soil (i.e., a vegetated, unpolluted soil of similar physicochemical properties and subjected to the same edaphoclimatic conditions). In this respect, indicators of soil health are needed to properly assess the efficiency of a phytoextraction process (Alkorta et al. 2003). Although to date, much more emphasis has been placed on physicochemical indicators of soil health, particularly, when evaluating the impact of agricultural practices on soil fertility and quality. Nonetheless, in the last years biological indicators such as enzyme activities, microbial biomass, basal- and substrate-induced respiration, potentially mineralizable N, and structural and functional biodiversity are most promising due to their being more sensitive to changes in

the soil as well as to their capacity to provide information that integrates many environmental factors (Alkorta et al. 2003). Moreover, biological monitoring has special relevance to human health because it evaluates the effects of environmental changes on key elements of the food chain (Pandolfini et al. 1997). From all of the above, it is concluded that the success of metal phytoremediation procedures (phytoextraction and phytostabilization) must be evaluated not only in relation to the reduction of the concentration of total and bioavailable HMs but, most importantly, through the careful monitoring of the recovery of soil health using, among others, soil microbial properties as bioindicators, as microorganisms have a vital role in the functioning of the soil ecosystem.

Dehydrogenase activity, an intracellular process that occurs in every viable microbial cell, is used to determine overall microbiological activity of soil (Nannipieri et al. 2002). Soil microbial activity can also be measured through the determination of soil basal respiration (ISO 16072 Norm). In turn, soil microbial functional diversity can be determined through the utilization of community level physiological profiles (CLPPs) which reflect the potential of the cultivable portion of the heterotrophic microbial community to respond to carbon substrates (Bending et al. 2004). Substrate (glucose)-induced respiration (SIR) is a suitable indicator of potentially active microbial biomass (ISO 17155 Norm). The addition of carbon sources, other than glucose, commonly reported as constituents of root exudates might convert SIR into an ecologically more relevant parameter for testing rhizospheric microbial communities (Dedourge et al. 2004). The microbial respiration quotient (QR ¼ basal soil respiration to SIR ratio) has been used to assess the effects of various perturbations in soil ecosystems (Insam and Domsch 1988).

Evaluating soil microbial biomass through numerous methods is another bioindicator to access the success of remediation processes. Traditional enrichment culture-based techniques, such as heterotrophic plate counts, are frequently used; however, biases may be introduced by media type and richness, presence or absence of oxygen, and numerous other factors. Such techniques are thought to reveal as little as 10 % of the total microbial diversity in soil. For this reason, innovative methods have been developed to more completely describe soil microbial diversity. Recent scientific advances have made it possible to use molecular biological techniques for assessment of microbial communities in complex environmental systems. Several molecular biological techniques, such as PCR amplification, cloning, and sequencing of ribosomal RNA genes, denaturing gradient gel electrophoresis (DGGE), phospholipid fatty acid (PLFA) analysis, and thermal-GGE (TGGE), have recently been embraced by the environmental science community as important tools for predicting soil and water remediation success. Molecular methods, such as denaturing gradient gel electrophoresis (DGGE), rely on genetic differences to draw distinctions between microbes and microbial populations. Chemical extraction of phospholipid-fatty acids from soil can provide both a description of the diversity in that soil and an estimate of the microbial biomass present. Finally, most probable number (MPN), a specialized enrichment technique utilizing substrates of interest, gives an estimate of the number of organisms in an environment capable of degrading specific contaminants. Taken individually,

DGGE, phospholipid-fatty acid analysis, and MPN are all useful tools for understanding microbial communities. In combination, however, they are likely to yield extensive information on microbial biomass and community diversity. Furthermore, they provide the capability to pinpoint dominant groups of organisms and to assess the microbial community's ability to degrade contaminants. Integration of these diverse methods represents a potentially powerful tool for characterization—and, ultimately, optimization—of bioremediation systems.

Well-characterized techniques such as DGGE and TGGE separate amplification products by sequence-dependent helix denaturation and the accompanying change in electrophoretic mobility. Another approach, single-strand conformation polymorphism (SSCP), takes advantage of sequence-dependent conformational differences between reannealed single-stranded products, which also results in electrophoretic mobility changes. The T-RFLP was developed most recently and has three clear advantages. First, direct reference to the sequence database is possible. Second, the nucleic acid sequencing technology has considerably greater resolution than the electrophoretic systems of either DGGE or SSCP. Third, the T-RFLP gel analysis is instantaneous and the output is digital. So, T-RFLP is a molecular approach that can assess subtle genetic differences between strains as well as provide insight into the structure and function of microbial communities. The technique has both high sensitivity and throughput; it is an ideal tool for comparative analyses.

Amplified ribosomal DNA restriction analysis (ARDRA) is another community analysis technique that provides a representation of the microbial community through restriction analysis of clones in an rDNA library. Although ARDRA was effective for identifying phylogenetic groups in a highly diverse community, it is prohibitively expensive and time consuming for this research project, because of the construction of clones and the identification of environmental clones by sequence analysis. In conclusion, T-RFLP provides a sensitive and rapid technique for assessing amplification-produced diversity within a community as well as comparative distribution across communities.

14.5 Conclusions and Perspective

The soil will become more and more valuable as a commodity. The preservation of soil quality, including its restoration in case the latter has been lost, certainly is a business opportunity, which will keep growing in all developed and industrialized countries. Phytoremediation is the processes of cleaning contaminated soils by making use of the metabolic properties of plants. During the last two decades, this field of soil remediation holds great potential as an environmental cleanup technology which has been extensively researched and developed. The search for hyperaccumulating plants has recently involved the identification of metal-excluding plants to learn more about plants that ensure that at least the edible part of the plant will be free of toxic metals. Scientists expect that the mechanisms

responsible for storing or excluding metals in plants are interrelated and that knowledge of one will lead to an understanding of the other. Genetic engineering approaches are currently being used to optimize the metabolic and physiological processes that enable hyperaccumulating plants to phytoremediate sites contaminated with HMs and metalloids. Someday, genetically modified plants will be developed to extract all types of contamination from soil and water and, at the same time, eliminate the largest drawback to current phytoremediation technology—the time required for plants to remediate contaminated sites. Microbes isolated from highly contaminated environments represent another potentially huge reservoir of new genes and unique metabolic capabilities that could be transferred to plants to enhance their phytoremediation potential. Microbes, in many cases, are more efficient in accumulating and absorbing HMs because of their astronomical amount and specific surface area. Furthermore, technique of genetic engineering in microbes is easier and more mature than in plant cells. Just as in pristine sites, there is always a close interaction between the microorganisms in the rhizosphere like plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) and host plants which can lead to an increased activity related to soil remediation (Compant et al. 2010). Thus, understanding and controlling the combination of soil, a beneficial rhizo- and/or endospheric microbial community and plants systems, and, even more important, their interactions provides a great opportunity for various innovative approaches to improve soil cleaning and production processes. Recent research on PGPR and AMF combined with genetic engineering illustrates a promising vision for future research. While advances in remediation have increased the effectiveness of HM degradation, still little is known about the interactions between PGPR, AMF, plant roots, and other microorganisms. Also, the mechanism of mobilization and transfer of metals is not fully understood. Additionally, most phytoremediation studies with PGPR and AMF have been conducted in the lab or greenhouse, overlooking the more complicated natural ecosystem. A more comprehensive understanding of these microbes in their natural environment is needed for this technology to reach its full potential.

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